COMBINED ORAL CONTRACEPTIVE PILLS IN OBESE WOMEN: EFFECTIVENESS, SAFETY, AND THE ROLE OF BIOMARKERS IN RISK ASSESSMENT

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A THESIS

Presented to the Department of Public Health & Preventive Medicine
and the Oregon Health & Science University School of Medicine
in partial fulfillment of the requirements for the degree of

Master of Public Health

May 19, 2015

School of Medicine

Oregon Health & Science University

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LIST OF ABBREVIATIONS

AUC - Area under the curve

APC - Activated protein C

BL - Baseline

BMI - Body mass index (kg/m^2)

CC - Continuous cycling group

Cmax - Maximum serum concentration

COC - Combined oral contraceptive pill

CDC - Centers for Disease Control and Prevention

CHC - Combined hormonal contraception

EE - Ethinyl-estradiol

ELISA - Enzyme - linked immunosorbent assay

FDA - Food and Drug Administration

GI - Gastrointestinal

ID - Increased dosing group

IUD - Intrauterine device

HRT - Hormone replacement therapy

LNG - Levonorgestrel, a second-generation progestin

OC - Oral contraceptive

OHSU – Oregon Health & Science University

OR - Odds ratio

PD - Pharmacodynamics

PK - Pharmacokinetics

RNA - Ribonucleic acid

SHBG - Sex hormone binding globulin

VTE - Venous thromboembolism

WHO - World Health Organization

ACKNOWLEDGEMENTS

I am grateful to my thesis committee, Drs. Alison Edelman, Jeffrey Jensen, Rochelle Fu, William Lambert and Paula Bednarek, for their continued support, encouragement and guidance during this project. I would like to thank Dr. Ganesh Cherala for his assistance with pharmacokinetic concepts, and the endocrinology lab at the Oregon National Primate Research Center for their assistance in interpretation of laboratory results. I am also grateful for the Fellowship in Family Planning and the anonymous funder for giving me the opportunity to complete the Master of Public Health degree. This project was supported by grants from the National Institutes of Health (RO1 HD061582-01 NICHD; 2K 12HD043488 NICHD), the OHSU Oregon Clinical & Translational Research Institute (NIH NCRR 1 ULI RR024120) and the Bioanalytical Shared Resource/Pharmacokinetics Core at OHSU.

ABSTRACT

Background: Obesity alters pharmacokinetics (PK) of contraceptive hormones and has the potential to contribute to contraceptive failure. It is also a risk factor for venous thromboembolism (VTE). The current project aims to study the influence of the binding protein sex hormone binding globulin (SHBG) on levonorgestrel (LNG) pharmacokinetics, and to study changes in anticoagulation parameters protein C activity and free protein S, in a population of obese women initiating combined oral contraceptive pills (COCs).

Methods: This project is a secondary analysis of data from a clinical trial conducted by Dr. Alison Edelman at Oregon Health & Science University. The original study evaluated three dosing regimens of COCs in a population of obese women. In this study, SHBG, protein C activity and protein S were measured at baseline at during use of COCs, and results were compared using mixed effects models.

Results: SHBG rose differently depending on COC dose, altering the amount of pharmacologically active LNG. Body mass index was not related to SHBG or total LNG serum levels. Thrombotic biomarkers protein C and protein S did not demonstrate prothrombotic changes in obese women initiating COCs.

Conclusions: The effect of SHBG on contraceptive pharmacokinetics remains incompletely understood, but SHBG's strong binding of LNG has the potential to alter contraceptive effect in women of all BMIs. Protein C activity and Protein S remain unvalidated biomarkers and should not be used in assessing VTE risk for women initiating COCs.

Keywords: Obesity, oral contraceptive pills, pharmacokinetics, thromboembolism, biomarkers

CHAPTER 1 – BACKGROUND

Part 1

A. Obesity and overweight

The rising rate of obesity and overweight is a public health crisis in the United States and increasingly around the globe. Worldwide, obesity has more than doubled in the past 30 years, now affecting 13% of the world's population (over 600 million people). An additional 1.9 billion adults are overweight, representing 39% of the world's population. Obesity is defined by the World Health Organization (WHO) as a body mass index (BMI) over 30kg/m^2, whereas overweight is a BMI between 25-29.9kg/m^2.

Worldwide, more deaths are now attributed to obesity and overweight than underweight or starvation. This is attributed to an increased intake of calorie-dense foods, and a decrease in physical activity resulting from sedentary work and increasing urbanization (1). Less developed nations are increasingly affected, with obesity rising especially in urban areas (1,2). In developing countries, rates of childhood obesity are rising faster than in developed countries.

People affected by obesity are more likely to experience cardiovascular disease, stroke, type 2 diabetes, osteoarthritis, thromboembolic disease, and cancer, and obesity is the fifth leading cause of mortality worldwide(3). Women of reproductive age face additional complications of obesity, particularly during pregnancy. Obesity now affects 34% of reproductive age women in the United States and 12% in Western Europe, and continues to rise, with nearly 300 million women affected by obesity as of 2008 (3,4) (Figure 1).

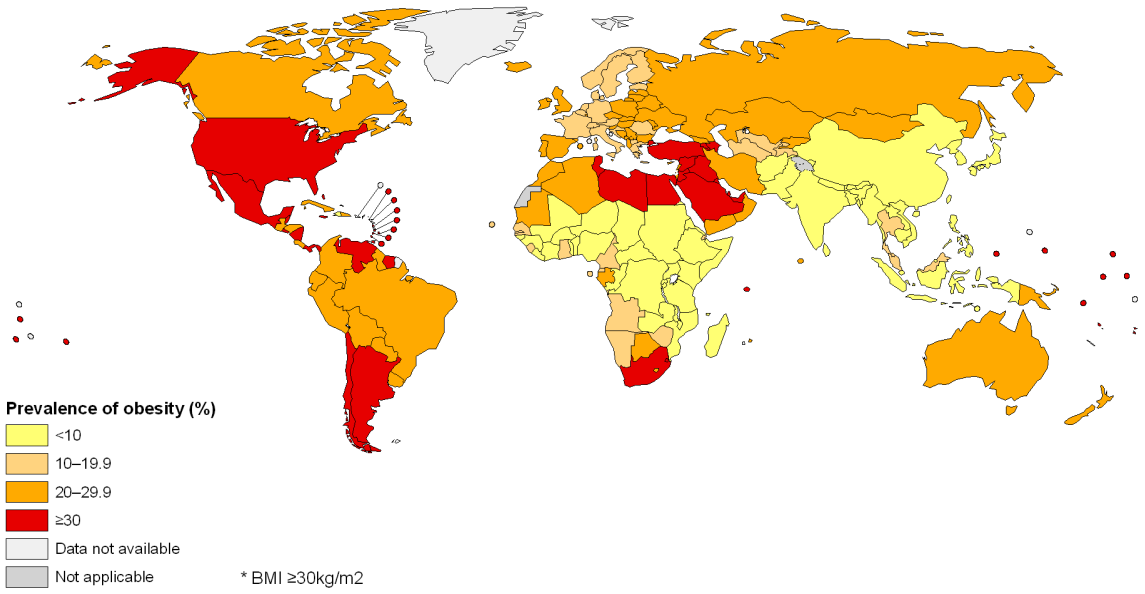

Figure 1. Prevalence of obesity in females over age 20, age standardized, as of 2008. Figure reproduced from World Health Organization, Public Health Information and Geographic Information Systems 2011.

B. Female reproductive health and obesity

Obesity is associated with a hyperestrogenic state due to the peripheral conversion of androstenedione to estrone and estradiol within adipose tissue. This aromatase reaction increases directly with BMI (3). Obese women have a higher incidence of oligoovulation and anovulation, conditions that exacerbate this hyperestrogenic state. Combined, these factors increase the risk of abnormal uterine bleeding, endometrial hyperplasia and endometrial cancer (5).

In obese women who become pregnant, the large prospective multicenter FASTER trial (First and Second Trimester Evaluation of Risk) demonstrated increased risk of gestational hypertension, diabetes, pre-eclampsia, anesthesia complications, and an

increased rate of Cesarean delivery (33.8% for obese and 47.4% for morbidly obese, compared to 20.7% for normal weight) (6). Fetal complications are also increased, including a higher and otherwise unexplainable risk of stillbirth, fetal growth restriction, neural tube defects, and an increase in childhood obesity among the children of obese mothers (6-8). Finally, obese women are less likely to return to pre-pregnancy weight following a pregnancy, adding to their weight-associated problems (8,9).

C. Contraceptive use and obesity

Women are at risk of unintended pregnancy if they are able to become pregnant, sexually active with a male partner, and not currently desiring conception. Rates of contraceptive use by overweight and obese women do not appear to differ from normal weight women. The 2002 National Survey of Family Growth (NSFG) demonstrated that the odds of contraceptive nonuse were not significantly different for obese and normal weight women after adjusting for age, ethnicity, education, and pregnancy desire, with 28.0% of normal weight women, 25.2% of overweight women, and 25.3-33.0% of obese women reporting use of no method of contraception (10). Likewise, in a secondary analysis of the 2006 Behavioral Risk Factor Surveillance System (BRFSS) family planning module, which specifically surveys women at risk of pregnancy, use of contraception was not associated with BMI after controlling for age, race, education, income, parity, and marital status (11).

There is a wide range of contraceptive efficacy between methods, with the most effective methods (intrauterine devices, contraceptive implants, sterilization, and hormonal methods) requiring placement or prescription by a medical provider. Due to concerns about possible differences in efficacy or safety in obesity, obese women may have

more difficulty accessing these most effective methods. However, most population level studies of contraceptive use do not demonstrate an association between contraceptive method choice and BMI. For example, using data from the 2002 NSFG, Kaneshiro et al (12) grouped contraceptive methods into most effective (permanent, IUDs, implants), effective (short acting hormonal methods such as the injection, pills, ring or patch), and least effective (non-prescription such as condoms, foam, withdrawal). There was no statistically significant association between BMI and level of contraceptive method effectiveness.

These studies provide some evidence that overweight and obese women are using effective methods of contraception at similar rates to normal weight women. But are these methods as effective in this overweight and obese population? Contraceptive development studies have historically excluded women over 130% of ideal body weight, leaving patients and providers with a gap in understanding of contraceptive efficacy in this population.

Part 2

A. Contraceptive effectiveness in obese women

Combined estrogen/progestin oral contraceptive pills (COCs) are the most commonly utilized form of contraception in the United States, among women of all BMIs (11). In COCs, contraceptive effect is provided by both a synthetic progestin and ethinyl-estradiol (EE). The progestin component provides ovulation suppression through suppression of luteinizing hormone at the level of the pituitary. Additional ovulation suppression is provided by the EE component, which suppresses follicle-stimulating hormone release to prevent formation of a dominant follicle.

Evidence on effectiveness of COCs in obese women is conflicting, with most studies showing high effectiveness similar to that of normal weight women (13-15). However, those studies demonstrating an association between increasing BMI and COC failure were not designed to differentiate between pharmacokinetic factors and behavioral factors such as pill compliance (16,17). As an example, a large prospective cohort study of over 52,000 women studied contraceptive failure for four regimens of COCs in obese and normal weight women (17). Adjusting for age, parity and education, there was an increase in failure rates as BMI increased, specifically a hazard ratio of 1.5 (95% CI 1.3 - 1.8) for contraceptive failure for women with a BMI over 35kg/m^2. This study was unable to account for compliance with pill regimens.

Effectiveness of COCs is dependent on consistent daily use. Social factors such as economic status, housing stability, employment, and education may influence a woman's ability to consistently access and utilize contraception such as a daily pill. Westhoff et al found that non-adherence with OCPs was associated with residential poverty, obesity, and race/ethnicity (18). Because obesity is associated with female poverty in the United States, an inability to fully differentiate these variables makes it difficult to conclude whether obesity alone is a risk factor for pill non-compliance.

Effectiveness of OCPs in obese women may also be affected by pharmacokinetic alterations of drug metabolism. The next section will review basic pharmacokinetic (PK) terminology, pharmacokinetics of contraceptive steroid hormones in normal weight women, and how these parameters change in obese women.

B. Pharmacokinetic alterations in obesity

There are four primary processes involved in the passage of a drug, including an oral contraceptive, through the body. These include absorption, distribution, metabolism and excretion. Factors such as sex, age, nutritional status, co-administered drugs, body weight, pregnancy, and disease can alter one or more of these pharmacokinetic processes. Despite a large increase in obesity rates, the effect of obesity on pharmacokinetics is incompletely understood.

Absorption of drugs may be increased in obesity due to increased cardiac output leading to increased blood flow to the GI tract, as well as faster gastric emptying (19). This could cause a shorter time to maximum plasma concentration of a drug. Distribution of a drug is altered by changes in lean body mass, adipose tissue, and circulating plasma proteins. Obesity results in a higher volume of distribution for hydrophobic drugs (such as steroids), while the volume of distribution for hydrophilic drugs tends to correlate with lean body mass and may be less affected by obesity (19). The degree of plasma protein binding also contributes to volume of distribution. Albumin is a major drug-binding protein, and appears unchanged in obese women (19). However, higher levels of lipoproteins in the obese may compete with drugs for albumin binding sites, potentially leading to higher concentrations of unbound drugs. Plasma alpha-1-acid glycoprotein (AAG) is elevated in obese subjects, which may increase binding of basic drugs (19). Finally, some studies demonstrate an association between obesity and lower levels of circulating sex hormone binding globulin (SHBG) (20). SHBG is a plasma protein that binds endogenous estrogens and androgens, as well as synthetic progestins. Alterations in SBHG would therefore have the potential to alter distribution of hormonal contraceptives.

Hepatic metabolism of drugs may be altered in obesity. Metabolism occurs in two phases. Phase I includes oxidation, reduction and hydrolysis, and phase 2 includes conjugation reactions (21). Alterations in metabolic enzymes have been noted in obese human subjects, specifically a decrease in CYP3A and CYP2E1 activity during phase I metabolism. These enzymes are regulated by cytokines, many of which are elevated in the chronic low-grade inflammatory state of obesity. Depending on the drug, phase I metabolism may be increased, decreased, or unaffected by obesity. Likewise, increased activity of phase 2 enzymes such as uridine disphosphate glucuronosyltransferase (UGT) may lead to increased total body clearance in obesity (19).

Finally, drug excretion may be altered by obesity. Most drug excretion occurs in the kidney. Renal clearance of drugs increases with BMI, potentially related to higher mean estimated glomerular filtration rates (19). Biliary excretion of hepatically cleared drugs may also be altered by changes to bile salt secretion and transporters in obesity.

C. Pharmacokinetics of orally administered steroid hormones

Orally administered contraceptive hormones rely upon systemic levels to provide contraceptive effect. Orally administered steroids are first subject to dissolution in the stomach and metabolic transformation by bacterial enzymes in the small intestine. These metabolized and unmetabolized steroids are absorbed from intestinal mucosa into the portal vein blood supply, and are then delivered to the liver. Once in the liver, metabolizing enzymes transform both unmetabolized and metabolized steroids (first pass metabolism). After this first pass through the liver, some of the original steroid hormone remains unmetabolized, and is released into the systemic circulation along with steroid metabolites.

Bioavailability is the proportion of the originally ingested steroid hormone that reaches the systemic circulation after first pass metabolism. Bioavailability for EE (the estrogen component of oral contraceptive pills) ranges from 25-65%, and for most synthetic progestins is between 70-90% (21).

In normal weight women, 90% of oral EE is absorbed from the stomach and small intestine during the first hour after ingestion. Peak blood levels are achieved in many women by 1-2 hours, though in some women it can take up to six hours to reach maximum circulating levels (21). As it undergoes first pass hepatic metabolism, some EE molecules undergo 2-hydroxylation, mediated by cytochromes P450 CYP3A4 and CYP2C9. EE and its hydroxlyated metabolites are then conjugated to sulfates, which circulate systemically and undergo enterohepatic recirculation, and glucuronides, which are renally excreted. Both unmetabolized EE and EE sulfates circulate and undergo additional hepatic passes, where the steps are repeated. Elimination half-life for EE ranges from 6 to 27 hours for normal weight women. Of note, there is significant variation in systemic EE exposure both within and between individuals because of differences in CYP enzyme activity, and ethnic differences in metabolite composition have also been observed (22).

There are many synthetic progestins in use for contraception or hormonal therapy, and these differ in their metabolism and pharmacokinetics. Some progestins are "prodrugs," meaning they become systemically active only after metabolism to an active form. Oral progestins are well absorbed and undergo hepatic first-pass metabolism like EE. Time to maximum concentration in systemic circulation is 1-3 hours (23). Norethindrone is a progestin structurally related to testosterone that is used in progestin-only

contraceptive pills. This progestin has a half-life of 8-12 hours, whereas most others have half-lives between 12-24 hours (22).

D. Sex hormone binding globulin

SHBG is a glycoprotein produced in the liver that serves as a carrier protein for steroid hormones in the blood. Steroid hormones can circulate in "free" form (not protein bound), or they may bind to albumin or SHBG. Pharmacologic activity of steroid hormones is thought to be determined primarily by free and albumin bound forms, whereas hormone bound to SHBG is not biologically active (24). Therefore, levels of SHBG affect the relative amounts of free and bound estrogens and progestins that are available to hormonally sensitive tissues (25). Most synthetic progestins bind with high affinity to SHBG, particularly levonorgestrel. In women taking oral levonorgestrel, up to 87% of the circulating hormone is bound to SHBG (24).

Contraceptive hormones alter production of several hepatic proteins, including SHBG, as they pass through the liver. Estrogens are the most potent inducing factors for SHBG. Increases in SHBG levels have been reported in all formulations of COCs and some non-oral formulations (26,27). During the hyperestrogenic state of pregnancy, there is a 10-fold increase in SHBG levels (25). By contrast, progestins have a varying effect on SHBG, usually decreasing SHBG levels. Importantly, levonorgestrel (LNG) alone reduces serum SHBG levels, and when co-administered with estrogen it diminishes the estrogenic increase of SHBG (24). The total change in SHBG observed with a COC is determined by the sum of the estrogen and progestin effects. The contraceptive effect of COCs relies on sufficient free progestin to provide ovarian suppression even in the face of rising SHBG.

Morbidly obese women demonstrate baseline lower levels of SHBG, and overall SHBG levels appear inversely related to BMI (25). However, obese women using COCs demonstrate normalization of SHBG levels, with no difference in SHBG levels observed between obese and non-obese women on the same pill (28,29).

E. Pharmacokinetics of COCs in obese women

The interaction of oral contraceptives, SHBG, and obesity with contraceptive pharmacokinetics (PK) is complex and incompletely understood. Given the range of PK alterations in obesity, there is certainly biologic plausibility for PK-based changes in COC effectiveness in obese women. An early theory proposed that because contraceptive hormones are hydrophobic steroids, the increased volume of distribution in obese women may lead to lower effectiveness. However, pharmacokinetic studies have demonstrated instead that the primary differences in contraceptive hormone metabolism in obese women relate to drug clearance and half-life rather than distribution.

Changes to PK parameters in obese women taking a 20mcg EE/100mcg LNG pill include a longer half life, lower clearance and longer time to reach study state than normal weight controls (30). An increase in half-life translates to a longer time to steady state, which may alter the time to reach levels sufficient for ovulatory suppression. In fact, in a recent cohort of obese women, mean time to reach LNG steady state was 13.6 days (SD 8.4), compared to a mean of 5.3 days for normal weight controls (SD 1.9)(30). Given that ovulation generally occurs on cycle day 14, this could indicate that serum levels of LNG may not reach a threshold to successfully prevent ovulation in obese users initiating COCs, or following the seven-day hormone-free interval in typical cyclic COCs.

Area under the curve ($AUC_{0-\infty}$) is another important indicator of drug exposure, and higher AUC of LNG has been associated with better end organ suppression in obese women using COC (30). However, AUC of total LNG may not be fully representative of available drug, since much of the progestin will be bound to SHBG and therefore pharmacologically inactive. These PK changes are not linearly related to BMI, and it is unknown what degree of obesity begins to affect PK processes. The first aim of this study is to examine changes to SHBG and LNG with three dosing regimens of COCs in obese women.

Part 3. Contraceptive safety in obese women

A. Venous thromboembolism and obesity

Most contraindications to estrogen-containing contraceptives relate to the increased thromboembolism risk. Venous thromboembolism (VTE) has an annual incidence around 2 per 1000 individuals, with a risk that rises with age to nearly 1% annually in the very old (31). Two thirds of VTE cases occur in the leg, with one third presenting as pulmonary embolism, which can be fatal. VTE is caused by both acquired and genetic factors, with inherited thrombophilias, malignancy, estrogen exposure, and obesity as commonly cited risk factors(31).

The Multiple Environmental and Genetic Assessment of risk factors for VTE study (MEGA) is a population based case-control study conducted in the Netherlands that aimed to identify risk factors contributing to VTE (32). This study included consecutive cases with a first diagnosis of VTE selected from two anticoagulation clinics, with imaging confirmation of the diagnosis. In a sub-analysis of this study, 3834 first VTE cases and 4683 controls, all non-pregnant without active malignancy, were assessed for weight,

height, BMI, and factor V Leiden mutation (31). The odds of VTE increased with BMI after adjusting for age and sex. For overweight subjects (BMI 25-30kg/m^2), the odds ratio for VTE was 1.7 (95% CI 1.55-1.87) compared to the normal weight reference group (BMI < 25kg/m^2). Obesity (BMI over 30kg/m^2) was associated with a 2.44 higher odds of VTE (95% CI 2.15-2.78) compared to normal weight individuals.

Several other large epidemiologic studies have confirmed the increased risk of VTE among the obese. In the Nurses' Health Study, which included over 1.5 million person-years of prospective follow up of women, obesity (BMI > 29kg/m^2) was associated with a relative risk of pulmonary embolism of 2.9 (95% CI 1.5 - 5.4) compared to normal weight women, after adjusting for age, cigarette smoking, OCP or HRT use, hypertension and hyperlipidemia (33). Overall, most studies demonstrate about a two to threefold increase in VTE risk with a BMI over 30 compared to people with a BMI under 25.

B. Venous thromboembolism and estrogen

It is well known that pregnancy, the postpartum period, and use of estrogen-containing hormonal contraceptives increase the risk of VTE. The MEGA case control study discussed previously reported a nearly five-fold increase in the odds of VTE during pregnancy (OR 4.6, 95% CI 2.7 - 7.8) compared to non-pregnant state, adjusting for age (34). The immediate postpartum period had an even higher odds of VTE; the OR was 60.1, (95% CI 26.5 - 135.9) during the first three months after delivery. These risks were highest during the third trimester of pregnancy and the first six weeks postpartum (OR 8.8, 95% CI 4.5 - 17.3, and OR 84.0, 95% CI 31.7 - 222.6, respectively). Despite these impressive odds

ratios, absolute risk of VTE during pregnancy and postpartum is overall low, ranging from 8 to 32/10,000 in most studies (34-38).

Oral contraceptive use is also associated with an increased risk of VTE. Both EE dose and progestin type can contribute to the thrombotic risk profile of oral contraceptives (39). COCs containing 50mcg of EE have a higher risk of thrombosis than "low dose" COCs containing 20-30mcg of EE. However, the relative and absolute risk of VTE in low dose COC users is overall lower than that of pregnancy and the postpartum period when adjusting for age and other risk factors.

Estimations of VTE risk in COC users come from several retrospective and prospective studies. Nightingale et al performed a meta-analysis of two case-control studies in British populations (40). Women included as cases were age 15-49 with an idiopathic VTE treated with oral anticoagulants, who were exposed to oral contraceptives at the time of the event. VTE events were not included if they were related to trauma, surgery, cancer, pregnancy or postpartum. This study identified 395 qualifying cases of idiopathic VTE over 5 years, or 1.003 million exposed woman years. The crude incidence of VTE among OC users was 3.9 per 10,000, and increased with age from a low of 3 per 10,000 in women 15-19 years, to 17.5/10,000 in women 45-49 years. These rates are about 2-3 times the incidence of VTE in non-pregnant women in the general population.

Likewise, in 2011, the FDA published a retrospective analysis of insurance claims from four US sites with over 800,000 woman years of exposure to COCs (41). The incidence of all VTE in users of estrogen-containing contraceptives was 6.96 per 10,000 woman years.

Some studies suggest a higher thrombotic risk with third and fourth generation progestins such as desogestrel or drosperinone compared to second generation levonorgestrel, though prospective studies controlling for other VTE risk factors have not demonstrated this effect (42-44). Overall, women and providers must recognize that the absolute risk of VTE with hormonal contraception is lower than that in pregnancy and postpartum. The relative risk of VTE in CHC users increases with age over 39, BMI over 35kg/m^2, and smoking (40).

C. Obesity and hormonal contraceptive use: what is the risk?

As previously noted, COCs are the most commonly used form of contraception in the US, and this includes obese women. The interaction between obesity and estrogen on VTE risk is debated, with some studies demonstrating an additive risk and others a multiplicative risk (45). Overall, the WHO and CDC Medical Eligibility Criteria rates COCs as a category 2 for obese women - concluding that benefits of use generally will outweigh the risks (46,47).

The MEGA study is one that demonstrated a multiplicative risk; women with a BMI 25-30 without COC use had an OR of 2.52 (95% CI 1.38 - 4.57), whereas women in the same BMI category using COCs had an OR of 11.63 (95% CI 7.46 - 18.14) for VTE (31). For the over 30kg/m^2 BMI group this was even stronger - an odds of 3.04 (95% CI 1.66 - 5.57) for non-COC users and 23.78 (13.35-42.34) for COC users.

Nightingale performed two nested case control studies to study possible risk factors for VTE among COC users, with up to four controls matched to each case by age (40). Confounders included were BMI, smoking status, duration of COC use, hypertension, and

chronic disease. BMI over 25 kg/m^2 was positively associated with risk of VTE in OC users. Women with a BMI over 35kg/m^2 had over three times the risk of VTE compared to users with a BMI between 20-24.9kg/m^2 (OR 3.1, 95% CI 1.6 - 5.8).

These findings are consistent with several other studies, including the Boston Collaborative Drug Surveillance Program, using the general Practice Research Database. In this study, the relative risk for COC users over 25kg/m^2 was 2.7 (1-7.6) times that of women with a BMI under 20kg/m^2 (44).

Trussel et al reviewed these and other studies, and estimated that among women with a BMI over 35kg/m^2, the absolute risk of death from COC use is between 0.9 to 2.4 per 100,000 COC users, and the attributable risk of death 2.1 per 100,000 women per year (45). By comparison, the attributable risk of death for smokers over age 35 using COCs was almost 10 times as high: 19.4 per 100,000.

The outcome of VTE in obese COC users remains rare, but it can be devastating. In an attempt to better understand the interaction of obesity and estrogen on VTE risk, and to potentially predict women at the highest risk, some have turned to studying possible biomarkers for VTE risk, which will be reviewed here.

D. Mechanisms of hypercoagulability

The mechanisms for the increased risk of VTE with COCs and pregnancy are incompletely understood. One mechanism may be a direct effect of estrogens on vascular walls, leading to endothelial dysfunction (48). However most studies focus on coagulation factors, as both oral contraceptives and pregnancy are known to alter the levels of some hepatic coagulation factors. After oral administration of estradiol or the more potent EE

contained in COCs, concentrations of steroid hormones in the portal vein are very high. This affects production of liver proteins including SHBG, angiotensin, and coagulation and fibrinolytic factors. EE is highly potent and resistant to metabolism, undergoing many passes through the liver before it is fully metabolized. Each hepatic pass by EE or its metabolites continues to affect protein production in the liver. As a result, even non-oral administration of EE (e.g. transdermal patch, vaginal ring) that bypasses the first pass hepatic metabolism will alter hepatic synthesis of coagulation factors on recurrent hepatic passes (49).

BMI is thought to influence VTE risk in several ways. Obesity is associated with venous stasis, as well as an increase in procoagulant hepatic proteins (31,48). Given the similarities of these presumed causal pathways, some have argued that measurement of coagulation biomarkers can be used to predict the risk of VTE among COC users (50). Prior to discussing these biomarkers in more detail, I will briefly review mechanisms of the hemostatic system.

E. Coagulation and fibrinolysis

The hemostatic system provides a rapid repair system for damaged vessels by forming clots to block defects in vascular walls. Clot formation (coagulation) and later dissolution (fibrinolysis) allow the body to maintain the cardiovascular system. In some circumstances, due to either over activity of coagulation or under activity of fibrinolysis, clots can form inappropriately, leading to VTE. VTE is incompletely understood, but the three factors of Virchow's triad (venous stasis, hypercoagulability, and endothelial damage) remain central tenants.

A simplified portion of the clotting cascade is depicted in figure 2. The last steps leading to clot formation involve the activation of prothrombin (II) to thrombin (IIa), with the assistance of factors Xa and Va. Thrombin acts as a cofactor for the activation of fibrinogen to fibrin, which allows clot formation.

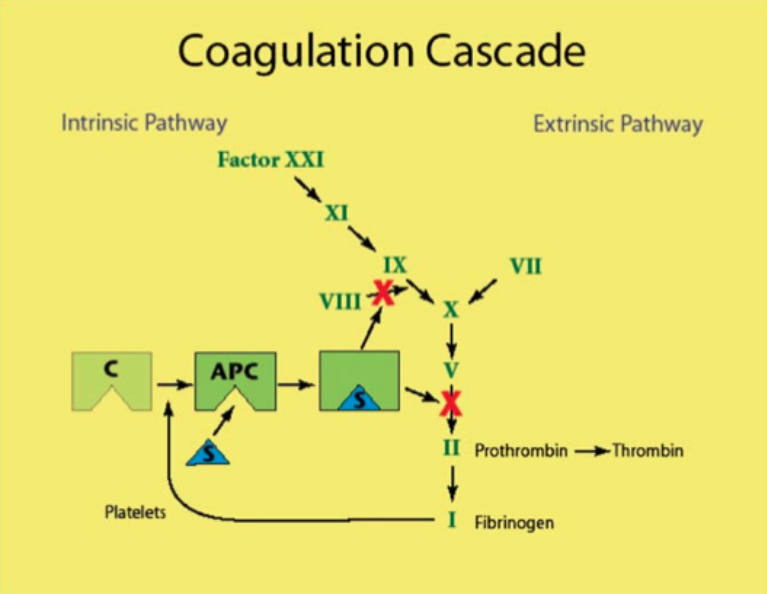

Figure 2. A simplified depiction of the coagulation cascade. Protein S is a cofactor for activation of protein C, which is critical to anticoagulation. Reproduced from Jensen et al, 2008.

The balance of this process is anticoagulation. The anticoagulant pathway is based upon protein C, which is located on the surface of endothelial cells. Once a clot forms, thrombin activates both thrombomodulin and protein C. Protein C and its cofactor protein S accelerate anticoagulant activity through formation of activated Protein C (aPC). aPC acts on factors VIII and V, blocking their roles in the activation of Xa and Va. This disrupts the activation of thrombin, and modulates the clotting system.

Thrombophilic states occur when procoagulant activity is increased or anticoagulant activity is decreased. This can occur through inherited or acquired deficiencies in the anticoagulation system, such as deficiencies of antithrombin, protein C and protein S, all of which are strong risk factors for venous thrombosis (48).

F. Oral contraceptives and coagulation parameters

OC use leads to changes in procoagulant as well as anticoagulant parameters, and the net effect is prothrombotic (51).

Protein C:

Protein C is a vitamin K dependent protein produced in the liver. It circulates as an inactive enzyme precursor, and primarily exerts functionality as an anticoagulant when it is activated to its protease form, activated protein C. Protein S (discussed separately) is a critical cofactor for this activation process. The average circulating concentration of protein C is 4mcg/ml. The logarithms of protein C concentrations are normally distributed, with a 95% CI falling between 70-140% of the mean value. Levels of protein C are reported as a percentage of this normalized mean value. Mean levels of protein C increase with age, about 4% per decade (52).

When screening for protein C deficiency, levels less than 55% of the normalized value (100%) are consistent with a probable type 1 genetic deficiency. Type II deficiency is associated with normal circulating levels of protein C, but with decreased functional activity, measured by functional clotting-based or chromogenic assays. Individuals with an inherited protein C deficiency are 7.3 times more likely to experience VTE in their lifetime

than an individual without a thrombophilic defect (53). In a normal population, the frequency of protein C deficiency is between 1/200 and 1/500 (52).

Alterations in protein C levels have been reported during COC use, with larger doses of estrogen being associated with larger changes in these levels. One recent study showed a decrease in protein C levels for users of a 30mcg EE/150mcg LNG pill compared to control ($p<0.01$) (54). However, many other studies have reported an increase in protein C levels during COC use (48). Activity of protein C may also be altered in COC use. This is sometimes measured by a percentage of activity, or by measurements of acquired resistance to activated protein C.

Protein S

Protein S is another vitamin K dependent glycoprotein. It functions as a non-enzymatic cofactor for APC. Protein S is produced in the liver, in endothelial cells and megakaryocytes. It circulates in two forms. About 40% is the free form, and the remaining 60% is bound to complement C4b binding protein. It was originally thought that only the free form has cofactor activity for activated protein C, though some recent reports suggest the bound form also has some cofactor activity (55). Average plasma concentration of total protein S is 23mcg/ml, which is equivalent to 100% or 1 unit/ml (100units/dl). Levels of total protein S but not free protein S increase with age.

Inherited protein S deficiencies include quantitative defects with lower free and total protein S levels (type 1), and a qualitative defect with decreased function despite normal circulating levels of protein S (type II). Inherited protein S deficiency is rare, with an estimated prevalence of 0.03 - 0.13% (56). The lifetime excess risk of VTE in carriers of protein S deficiency is 20% compared to the general population (53).

Acquired deficiency of protein S can occur during pregnancy, use of oral contraceptives, HIV infection, and liver disease. It is unknown how the clinical significance or VTE risk of these acquired deficits differs from the inherited deficiencies. In an early study of women taking a 35mcg EE COC, total but not free protein S levels were significantly lower in COC users compared to non users (mean total protein S nonusers 28mcg/ml (SD 3.9) vs 24.3mcg/ml (SD 3.6) in COC users ($p<0.005$), mean free protein S 86% (SD 17%) in COC users compared to 90% (SD 20%) non-users ($p=0.1$) (57). Multiple other studies have reported a decrease in free protein S levels during OC use (48,54,58). Importantly, protein S decreases as much as 70% during pregnancy, suggesting that the relatively smaller 10-20% decreases of protein S in COC use may pose a smaller theoretical risk (59).

G. Obesity and anticoagulation

Obesity affects both procoagulant and anticoagulant factors. In a study of 32 obese women with BMIs ranging from 28-50kg/m^2, prothrombotic factors fibrinogen, factor VII and others were significantly higher in obese women compared to normal weight controls ($p<0.001$) (60). In that same study, anticoagulant factor protein C was also elevated in the obese group ($p<0.001$). The authors proposed that the increase in protein C might be a protective response to counteract the increase in pro-thrombotic factors. In another study of 150 adults, protein C was also significantly positively correlated with BMI, while protein S was unrelated (61). However, activated protein C ratio, a more specific marker of protein C activity, was slightly negatively correlated with BMI.

Despite the alterations of the coagulation and anticoagulation systems reported in obesity and COC use, there is little information on how these changes interact when obese women consume estrogen. Also unknown is how these changes might relate to the increased risk of VTE in obese users of COCs. The second aim of this study is to examine changes in protein C activity and free protein S levels in obese women using different doses of COCs.

Specific Hypotheses

We hypothesize that:

1. SHBG will increase in a dose-dependent fashion in obese women using COCs.
2. Systemic levels of total levonorgestrel as measured by maximum concentration and AUC will be inversely related to BMI and SHBG.
3. Compared to baseline, both protein C activity and free protein S levels will decrease in a dose-dependent fashion upon initiation of a COC.

CHAPTER 2 - MATERIALS AND METHODS

Study procedures

These aims will be addressed through analysis of data from a previously published study that aimed to optimize PK parameters in obese women using COCs. The original study was a randomized controlled trial conducted by Dr. Alison Edelman at Oregon Health & Science University between January 2010 and June 2011 (62). The study received IRB approval by OHSU and all subjects underwent informed consent.

In this study, 32 otherwise healthy obese women (BMI > 30kg/m^2) enrolled for a total of four 28-day cycles (figure 3). Inclusion and exclusion criteria and detailed methods have been previously described. Qualifying women took an oral contraceptive pill containing 20mcg of EE and 100mcg LNG (Aviane, Teva, Israel), dosed with 21 days of active pills followed by a seven-day hormone-free interval, for two consecutive cycles (cycle 1 and 2). After two cycles, women were randomized to one of two dosing arms: continuous cycling (CC), which consisted of the same dose COC taken continuously without a hormone break for 8 weeks, and increased dose (ID), in which women switched to an oral contraceptive pill containing 30mcg EE/150mcg LNG taken in a 21/7 day cyclic fashion.

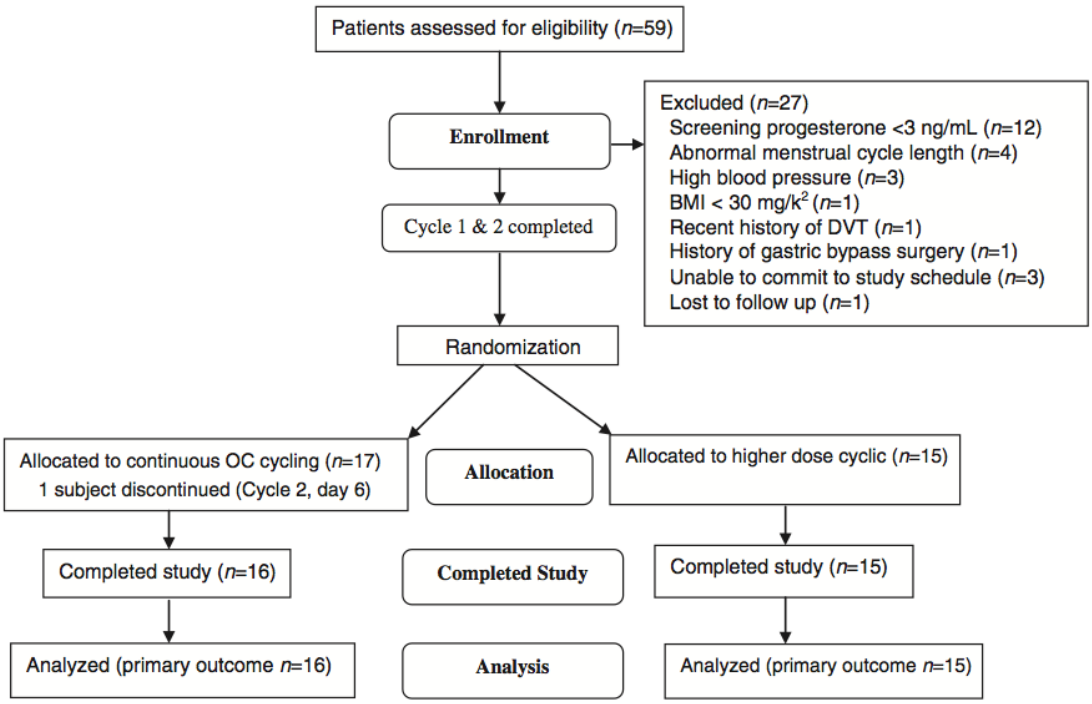

Figure 3. Flow of subjects through the original CONSORT study by Edelman et al (2014).

Pharmacokinetic parameters

Levonorgestrel pharmacokinetic data was analyzed by noncompartmental methods using WinNonLin as described in Edelman et al, 2014. During cycle 1, day 21 (last day of active 20mcg pills), serum LNG was recorded at time 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12h. Additional samples were obtained on days 22, 23, 24, 27. These measurements were used to calculate AUC from time 0 to 168 hours ($AUC_{0\text{-}t}$) using the linear trapezoidal rule. Maximum serum concentration (C_{max}) was also obtained. Sex hormone binding globulin (SHBG) was measured in serum at baseline prior to starting COCs, and on days 21 of cycles 1 and 4. SHBG samples were analyzed by a Roche Cobas e411 at the Oregon National Primate Research Center (ONPRC, Beaverton, OR, USA), with 4 samples over the curve high.

These four were reanalyzed with 1:5 dilution by PCMA. There was less than 10% intra and inter-assay variation. Original units were mcg/ml, which were converted to nmol/L.

Coagulation parameters

Protein C and Protein S were collected at baseline prior to COC initiation and on days 21 of cycles 1 and 4. Total protein C activity was measured in plasma with the Coamatic Protein C chromogenic assay from DiaPharma. Interassay variation was 9.89%, with averaged intraassay variation of 2.27%. Results are reported in protein C activity percent, with a normal reference range 70-149%. Free protein S was analyzed in plasma with ELISA kits from Helena Lab. The ELISA had 8.98% intraassay variation, and 14.97% interassay variation. Free protein S is reported as relative % of normal. The reference range for free protein S is 60-150%. Both analyses were performed at the ONPRC.

Sample size

Power calculations were based on the primary outcome of the original clinical trial, LNG AUC. 15 subjects per group were needed to provide 99% power to detect a difference of 112pg*h/ml in LNG AUC between the two treatment arms (CC and ID), with an alpha of 0.05. Planned enrollment was 32 to allow for drop out.

Of note, this study was not designed to detect changes in free protein S and protein C activity. Prior studies have reported small shifts in free protein S (7%) and protein C levels (17%) (54,63) with a 30mcg EE/150mcg LNG COC. Shifts of this size have no known clinical implications, so powering based on these small effect sizes may not be necessary. The planned sample size of 32 participants would have 99% power to detect a change from

100% (baseline) to values outside of the normal reference range for both biomarkers (less than 70% for protein C activity, and less than 60% for free protein S). Likewise, the planned sample size would have over 99% power to detect previously reported shifts in SHBG (25nmol/L) (69).

Statistical methods

Statistical analysis was performed in STATA®, version 13. A p-value of less than 0.05 was considered statistically significant unless otherwise noted.

Descriptive statistics were generated for baseline demographics including age, race/ethnicity, parity, and BMI between the two randomization groups, as well as baseline biomarkers (SHBG, protein C, protein S) (Table 1). Additional descriptive statistics were generated to describe biomarkers during each dosing arm (baseline, 20mcg cyclic, 20mcg continuous (CC), and 30mcg cyclic (ID)) (Table 2).

To assess the association between drug dosing regimen and each biomarker, mixed effects models were used. For each biomarker, a linear mixed effects model was created using dosing arm, age, BMI, race, and parity as fixed effects, and time (cycle 0, 1, 4) and individual subject as random effects (equivalent to a random intercept and random slope model). For these models, age and BMI were continuous variables, race was dichotomous (white/non-white), and parity was dichotomous (parous, not parous). The random effects adjust for the correlation among repeated measures within the same subjects. For each model, interaction terms were examined and found to be insignificant, and were thus excluded. Linear combinations were performed for sub-group comparisons between ID and CC groups. Bonferonni correction for multiple comparisons was used for

interpretation of the biomarker models. Assuming six comparisons, the adjusted p value was 0.0083 for each of these models.

Model diagnostics were performed to check the adequacy of normality using Q-Q plots based on residuals. When appropriate, multicollinearity among predictor variables was assessed with variance inflation factor, and Cook's distance was used to check for influential points.

Finally, multiple linear regression was performed to assess the relationship between drug exposure to LNG (as measured by Cmax and AUC) and predictor variables SHBG, age, and BMI. Model diagnostics were performed to assure that normality and homogeneity of variance of the models were adequately satisfied. Log transformed AUC was modeled to better approximate normality, and other variables were analyzed on their original scale.

CHAPTER 3 - Results

In the original study, 32 women signed informed consent. One subject withdrew from the study during cycle 2 and was excluded from analysis. Women were randomized to two groups: ID (n=15) and CC (n=16). Baseline demographic characteristics were similar between the groups, except that more women in the continuous dosing group were parous (table 1). Baseline biomarkers (SHBG, total protein C, free protein S) were also similar between the randomization groups. BMIs over both groups ranged from 31 to 66.7kg/m^2.

	ID group n=15	CC group n=16
Age (years), mean (SD)	27.4 (4.9)	30.12 (4.1)
BMI (kg/m^2), mean (SD)	41.5 (7.5)	38.3 (5.3)
Parous (yes), n (%)	2 (13)	9 (56.3)
Race		
Caucasian, non-Hispanic, n (column %)	13 (87)	13 (81)
Other, n (%)	2 (13)	3 (19)
SHBG (nmol/L), mean (SD)	30.7 (9.7)	36.5 (15.5)
Total protein C activity (%), mean (SD)	100.47 (4.4)	99.41 (4.1)
Free protein S (%), mean (SD)	97.43 (8.1)	107.42 (6.5)

Table 1. Demographics and baseline biomarkers for the two randomization groups.

	N	Mean	SD
SHBG (nmol/L)			
Baseline	31	33.7	13.2
Cycle 1 20mcg	31	79.0	27.8
Cycle 4 CC	16	95.6	33.9
Cycle 4 ID	15	49.3	27.8
Free Protein S (relative % of normal)			
Baseline	31	102.6	28.7
Cycle 1 20mcg	31	110.2	47.2
Cycle 4 CC	16	115.9	44.9
Cycle 4 ID	15	96.5	27.4
Total Protein C (% activity)			
Baseline	31	100.4	16.4
Cycle 1 20mcg	31	106.9	17.0
Cycle 4 CC	16	110.2	24.1
Cycle 4 ID	15	103.9	16.7

Table 2. Descriptive statistics for biomarkers in each of the four dosing arms. For baseline and cycle 1, data from the two randomization groups are combined.

Sex hormone binding globulin

Normality was examined for baseline SHBG values (Appendix A1) and for the mixed effects model. Though log transformed SHBG values were slightly better normally distributed (appendix A.2-3), the untransformed SHBG values were adequately normally distributed (appendix A. 4-6) and used for analysis to facilitate interpretation.

Treatment dosing arm was significantly related to SHBG ($p<0.001$) after adjusting for age, BMI, race, parity and time (cycle) (Figure 4, Table 3, Appendix A.4-6). All three treatment doses (20mcg cyclic, 20mcg continuous, 30mcg cyclic) led to statistically significant elevations in SHBG compared to baseline (values transformed from mcg/ml to

nmol/L). Continuous dosing of 20mcg pills led to significantly higher SHBG than cyclic dosing of 20mcg pills (adjusted mean difference 13.1 nmol/L, unadjusted difference by paired t test 12.2nmol/L, p<0.001) (Appendix A.7). Subjects transitioning from a 20mcg EE cyclic pill to the 30mcg EE cyclic pill experienced a mean decrease in SHBG of 26.1 nmol/L (p<0.001). Parity was significantly related to SHBG, with parous women having a higher mean SHBG level than nulliparous women (p=0.017), after adjusting for age, BMI, race, and treatment dose.

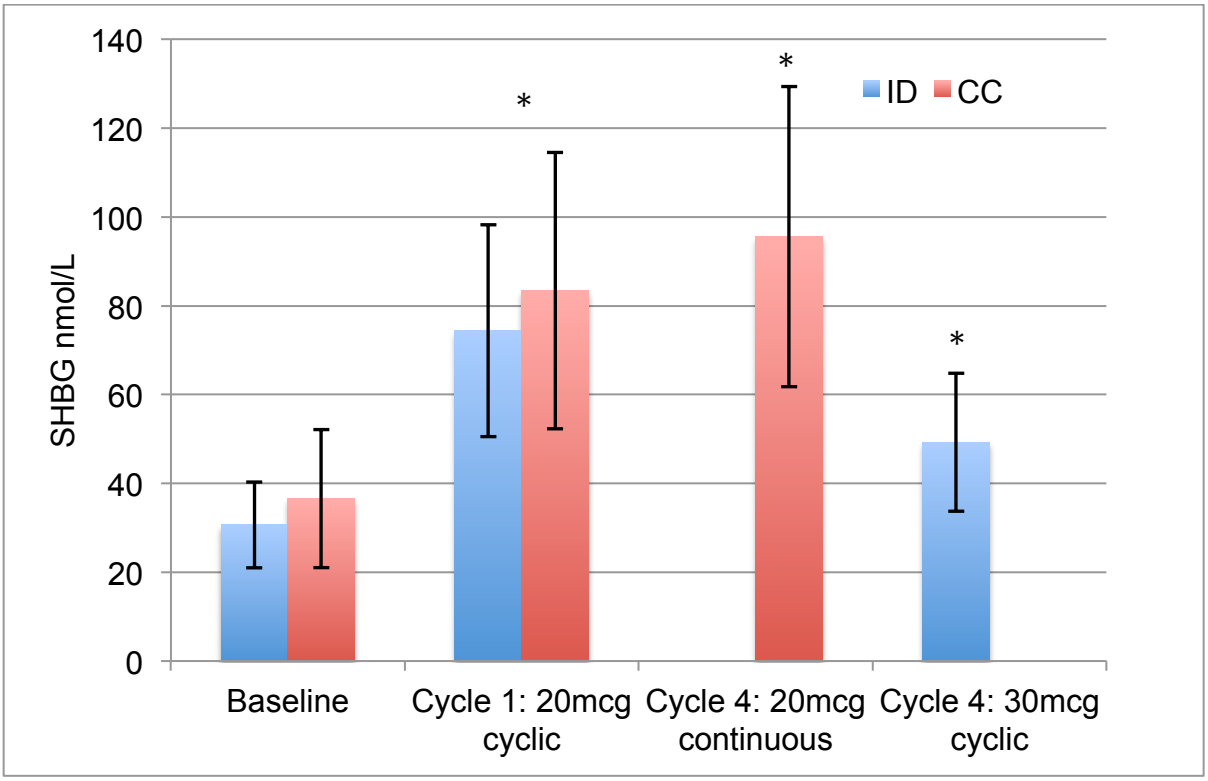

Figure 4. SHBG levels in each cohort at baseline, cycle 1 and cycle 4. * indicates significant difference from baseline at <0.001 after adjusting for age, BMI, race and parity. Error bars represent standard deviation.

	Adjusted mean difference (nmol/L)	95% Confidence interval	P value
Dose groups			<0.001
20mcg cyclic vs BL	45.4	39.6 – 51.1	<0.001
20mcg continuous vs BL	58.4	49.5 – 67.4	<0.001
30mcg cyclic vs BL	19.3	10.0 – 28.4	<0.001
20mcg continuous vs 20mcg cyclic	13.1	5.1 – 21.6	0.003
30mcg cyclic vs 20mcg continuous	-39.2	-51.0 - -27.4	<0.001
30mcg cyclic vs 20mcg cyclic	-26.1	-35.0 - -17.3	<0.001
Age (year)	0.9	-0.1 – 2.1	0.084
BMI (kg/m^2)	-0.7	-0.1 - 0.00	0.063
Race	-6.8	-21.7 – 8.0	0.369
Parity	14.2	2.5 – 25.9	0.017

Table 3. Results for SHBG based on the linear mixed model.

BL = baseline values prior to COC initiation

	Mean (SD)	Range
LNG AUC (h*ng/ml)	191.62 (83.67)	84.72 - 450.8
LNG log AUC	5.17 (0.43)	4.44 - 6.11
LNG Cmax (ng/ml)	3.52 (1.12)	1.07 - 7.21

Table 4. Descriptive statistics - Levonorgestrel exposure during cycle 1, with a 20mcg EE/100mcg LNG pill.

Levonorgestrel exposure - Area under the curve

Examination of the AUC for LNG demonstrated that log transformation was necessary to achieve normal distribution (appendix B.1). Scatterplots of logAUC vs continuous predictor variables BMI, age, and SHBG demonstrated overall linear

relationships. Based on the final model, log AUC is significantly associated with SHBG ($p<0.001$) after adjusting for BMI and age, representing a 1.11 times increase in the geometric mean of LNG AUC for every mcg/ml unit increase in SHBG (appendix B2-3). Neither BMI nor age were found to be significantly associated with the log AUC ($p=0.336$, $p=0.881$, respectively).

Levonorgestrel exposure - Maximum serum concentration

Examination of maximum serum concentration (Cmax) during cycle 1 revealed that the data are close to normally distributed (appendix C.1). Scatterplots of Cmax vs continuous predictor variables BMI, age, and SHBG demonstrated overall linear relationships. Interpretation of the final model indicates that Cmax was also significantly associated with SHBG ($p<0.001$) after adjusting for BMI and age (appendix C.2-3). For every mcg/ml increase in SHBG, Cmax increased by 0.276 ng/ml (95% CI 0.16 – 0.39ng/ml). Neither BMI nor age were found to be significantly associated with Cmax ($p=0.146$, $p=0.178$, respectively).

Protein C

Investigation of the dependent variable protein C activity percent revealed normal distribution (appendix D.1, D3). Protein C activity appeared linearly related to both age and BMI. The final model for Protein C activity included dosing arm, age, BMI, race, and parity as fixed effects, with random effects accounting for correlation from repeated measurements from each subject over three time points (appendix D.2).

Overall, there was a significant effect of treatment dose on Protein C activity (p=0.0002) (Appendix D.2-4, Table 5, Figure 5) after controlling for age, BMI, parity, race and time (cycle). Total protein C activity was significantly higher for both 20mcg pill formulations compared to baseline. When taken cyclically, the mean increase in protein C activity was 6.5% (95% CI 3.2 - 9.7%, p<0.001), and when used continuously the mean increase was 9.4% (95% CI 3.9 - 14.9, p=0.001). However, total protein C activity did not change from baseline for women taking the higher 30mcg EE pills. Direct comparisons of protein C activity between the three treatment formulations showed no statistically significant differences.

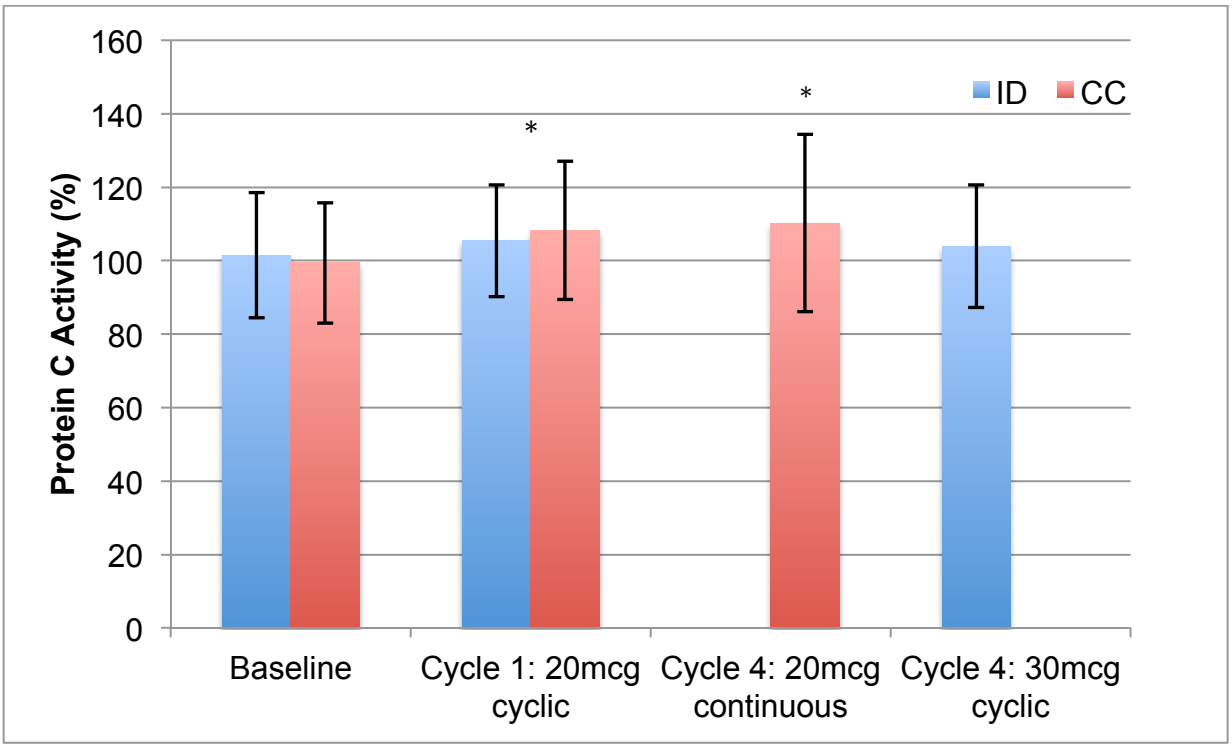

Figure 5. Protein C Activity levels (%) in each cohort at baseline, cycle 1 and cycle 4. * indicates significant difference from baseline at <0.001 after adjusting for age, BMI, race and parity. Error bars represent standard deviation.

	Adjusted mean difference in proportions	95% Confidence interval	P value
Dose groups			
20mcg cyclic vs BL	6.477	3.245 - 9.708	<0.001
20mcg continuous vs BL	9.437	3.914 - 14.960	0.001
30mcg cyclic vs BL	3.962	-1.712 - 9.637	0.171
20mcg continuous vs 20mcg cyclic	2.960	-2.228 - 8.149	0.263
30mcg cyclic vs 20mcg continuous	5.474	-1.796 - 12.745	0.140
30mcg cyclic vs 20mcg cyclic	2.514	-2.836 - 7.863	0.357
Age (year)	0.704	-0.464 - 1.872	0.237
BMI (kg/m^2)	0.381	-0.407 - 1.169	0.343
Race	-3.319	-18.233 - 12.217	0.675
Parity	3.989	-8.233 - 16.2111	0.522

Table 5. Results for Protein C activity (%) based on the linear mixed model.

Protein S

Initial evaluation of baseline free protein S values revealed normal distribution and linear relationships with age and BMI (appendix E.1). A linear mixed effect model was run using dosing arm, age, BMI, race, and parity as fixed effects, with random effects accounting for correlation from repeated measurements from each subject over three time points (appendix E.2). Examination of residual plots and Q Q plots revealed several extreme outlier points affecting the fit of the model and the normality of residuals (appendix E.3). These points were traced to a single subject in the CC group with a BMI of $41.5kg/m^2$ who had normal baseline protein S values, but an extreme rise in protein S during cycles 1 and 4. The model was rerun excluding this outlier subject (appendix E.4) with great improvement in the residual and Q Q plots. The second model excluding the outlier subject was used for interpretation.

There was no overall effect of treatment dose on free protein S levels (p=0.67, appendix E.5). Free protein S did not significantly change between baseline and COC use, nor were there differences in free protein S between dosing regimens, after adjusting for age, BMI, race, parity and time (cycle). Age, BMI, race and parity also were not associated with free protein S levels (table 6) after adjusting for treatment dose and time.

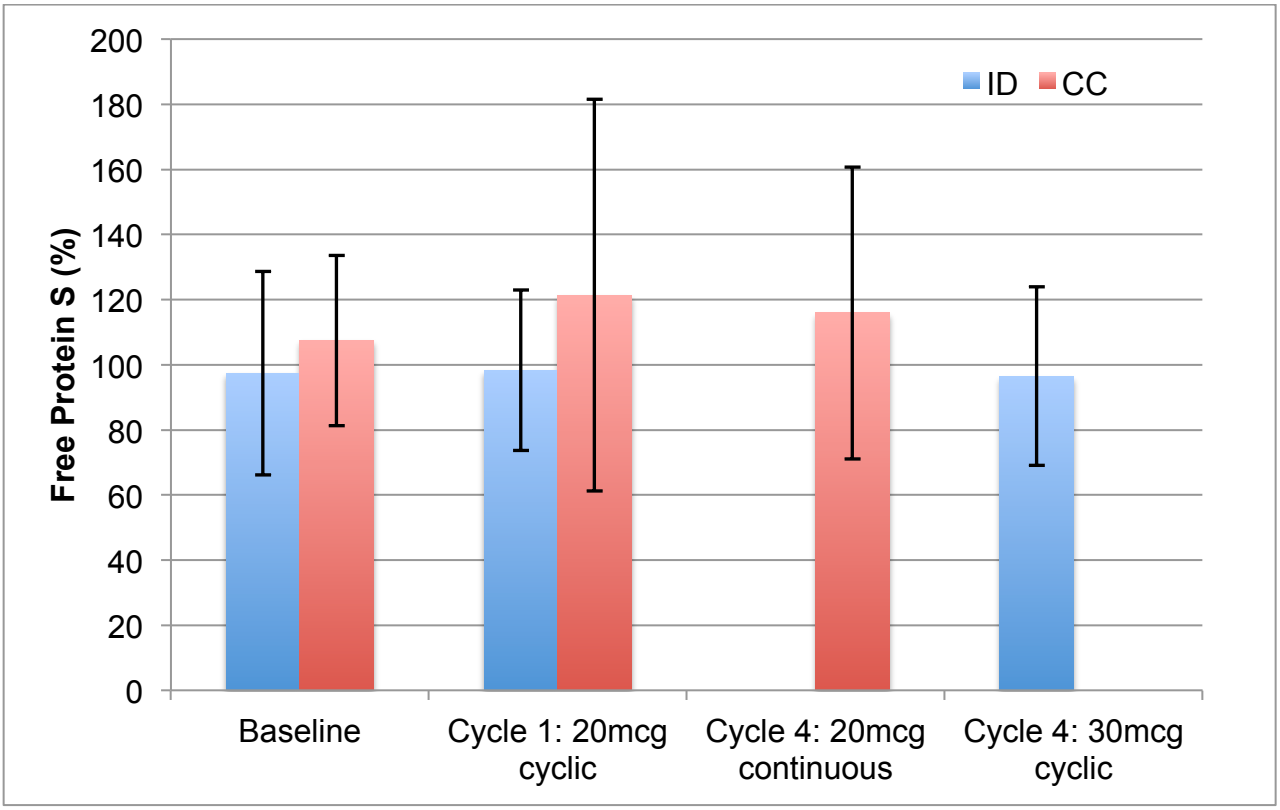

Figure 6. Free Protein S levels (%) in each cohort at baseline, cycle 1 and cycle 4. No treatment group varied from baseline after adjusting for age, BMI, race and parity. Error bars represent standard deviation.

	Adjusted mean difference in proportions	95% Confidence interval	P value
Dose groups			
20mcg cyclic vs BL	1.10	-2.78 - 4.97	0.579
20mcg continuous vs BL	1.54	-4.36 - 7.43	0.609
30mcg cyclic vs BL	-2.29	-8.16 - 3.58	0.445
20mcg continuous vs 20mcg cyclic	0.44	-5.22 - 6.11	0.879
30mcg cyclic vs 20mcg continuous	-3.83	-3.67 - 11.32	0.317
30mcg cyclic vs 20mcg cyclic	-3.38	-2.27 - 9.04	0.241
Age (year)	0.25	-1.61 - 2.12	0.787
BMI (kg/m^2)	1.03	-0.13 - 2.20	0.081
Race	-6.34	-32.50 - 19.82	0.635
Parity	-9.49	-28.95 - 9.96	0.339

Table 6. Results for protein S based on the linear mixed model.

CHAPTER 4 - Discussion:

A. SHBG and contraceptive pharmacokinetics in obese women

Consistent with our hypothesis, mean SHBG increased in obese women initiating a LNG-based COC. This finding is consistent with studies of normal weight COC users (25, 26). The mean rise of SHBG was significantly different for each dosing strategy, and was not dose dependent, which is a new finding.

SHBG increased the most with a lower dose COC (20mcg EE/100mcg LNG) taken continuously or cyclically. The higher dose COC (30mcg EE/150mcgLNG) resulted in a smaller increase from baseline. Subjects who switched from the cyclic lower dose to the cyclic higher dose COC displayed a decrease in SHBG at the higher dose. This was unexpected, as SHBG is thought to increase in a dose-dependent fashion with administration of EE (64). One potential explanation is that the higher dose of LNG in this COC counteracted EE's effect on hepatic production of SHBG. An alternative possibility is that cyclic dosing allowed SHBG to fall during hormone-free intervals, curbing the overall rise with sequential cycles. A fall in SHBG during hormone-free intervals has been previously reported in normal weight and obese women (28). SHBG did not have this temporary fall with continuous dosing and may reach higher levels in subsequent months as a result. More frequent monitoring of SHBG during continuous dosing could elucidate this pattern in SHBG rise.

An increase in SHBG in obese women using COCs has a few possible clinical implications, including potential non-contraceptive benefits. Higher levels of SHBG bind free androgens, reducing hyperandrogenic symptoms that can occur with obesity or polycystic ovarian syndrome. This study demonstrated that continuous dosing resulted in

a greater rise in SHBG than cyclic dosing, which might offer greater anti-androgenic benefits.

Prior studies with cyclic dosing of a 30mcg EE/150mcg LNG pill demonstrated a rise of about 25nmol/L from baseline (63,65). Although this study was not originally powered to detect changes in SHBG, the sample size in this study would have provided greater than 99% power to differences of this magnitude. Importantly, there are no known clinical outcomes based on this magnitude of rise. The normal reference range for SHBG is between 23-165nmol/L, and all SHBG values in this study remained in that range. The variations in SHBG levels between dosing strategies observed in this study could be statistically but not clinically significant. As such caution should be used in making clinical conclusions based on these findings without replication and correlation to androgenic symptoms.

Another consideration for obese women using COCs is the effect of obesity on serum levels of active contraceptive hormones. We demonstrated that total systemic LNG exposure (AUC and Cmax) was not related to BMI within a population of obese women using the same low dose COC. This finding is consistent with prior studies (21, 29). However, this does not necessarily imply that contraceptive effects will be the same over diverse BMIs. SHBG complicates interpretation of total LNG exposure when inferring contraceptive effect, as the majority of LNG circulates bound to SHBG (and is therefore inactive). Relating SHBG and total LNG levels to pharmacodynamic observations can help to assess whether a dosing regimen has adequate free serum levels for contraception.

In the previously published results from this trial, the steady state level of total LNG for the ID group (3.58 +/- 0.35ng/mL) was similar to the steady state level of historical

normal weight controls using a lower dose 20mcg EE/100mcg LNG pill (28,62). Women in the CC group maintained a lower steady state of total LNG (3.01 +/- 0.19ng/ml, $p<0.001$). Given that SHBG levels were higher in the CC group, one can infer that the CC group would have less circulating free LNG than the ID group. However, end organ suppression was good in both of these dosing strategies with no confirmed ovulation in either group. This suggests that both ID and CC dosing strategies led to an increase in pharmacologically active LNG that outpaced the rise in SHBG. It is reassuring that despite rising SHBG, effective contraceptive thresholds of free LNG were met, based on pharmacodynamic observations.

By contrast, over half of obese women using a cyclically dosed 20mcg EE/100mcg LNG pill during cycle 2 of this study had evidence of reduced end organ suppression including formation of dominant follicles or ovulation (62). Among these women, greater total LNG exposure by AUC and Cmax was associated with higher SHBG levels, which was inconsistent with our hypothesis. Women with the highest LNG exposure had a parallel rise in SHBG, potentially resulting in more binding of LNG and less free (pharmacologically active) LNG for end organs. It is possible that lower steady state LNG levels during cyclic dosing of the 20mcg EE/150mcg LNG pill were compromised enough by this rise in SHBG that levels of free LNG were under the contraceptive threshold.

The interplay of EE, total and free LNG, and SHBG is complex, and is probably altered in obesity due to lower baseline levels of SHBG and alterations in the pharmacokinetics of steroid hormones. All of these factors can affect the level of free circulating LNG available for contraceptive effect in obese women. The commonly cited therapeutic concentration of total LNG is 0.4ng/ml, derived from normal weight women

using a progestin-only LNG contraceptive implant, Norplant (66). However, SHBG is negatively correlated to total LNG levels in Norplant users, resulting in a greater proportion of free LNG for a given level of total LNG (67). Therefore, the actual contraceptive threshold of total LNG probably differs when LNG is co-administered with EE, when SHBG levels are significantly higher and binding to a greater proportion of LNG.

Due to the complex role of SHBG, the concentration of total LNG required for contraceptive effect during co-administration with EE is unknown. Differences in SHBG by dosing regimen observed in this study suggest that therapeutic thresholds of total LNG might differ for each COC formulation. Concurrent measurement of SHBG could theoretically have some utility in interpreting relative total LNG levels. However, there is not a known predictable relationship between SHBG and the proportion of free/bound LNG, and free LNG levels cannot currently be inferred from total levels. Future research should elucidate whether there is a reliable contraceptive threshold concentration of LNG in users of COCs, either through direct measurements of free LNG, or by observing a predictable relationship between SHBG and total LNG with pharmacodynamics observations.

B. Coagulation biomarkers in obese users of COCs: Observations and implications

Some authors have proposed using thrombosis biomarkers to predict VTE risk for women using COCs (68,69). Given the known increased VTE risk among obese women and COC users, we measured two common thrombotic markers in obese women using three dosing regimens of EE/LNG COCs to assess whether dosing strategy affected these biomarkers. We found minimal to no changes to the coagulation parameters protein C

activity and free protein S levels in obese women after initiation of low dose cyclic, higher dose cyclic, and low dose continuous COCs. This finding was inconsistent with our hypothesis.

Protein S deficiency is a known genetic risk factor for VTE, and an acquired deficiency in protein S occurs during pregnancy. Normal weight women using COCs demonstrate statistically significant decreases in free protein S in some studies, though the deficiency appears to be less than occurs during pregnancy (53). It is unknown whether these acquired protein S deficiencies are causally related to the increased VTE risk occurring during pregnancy and COC use. Importantly, the absolute changes to free protein S levels during COC exposure are small, with historical values remaining completely in the normal range (54). In our data set, only one subject had a value outside the lower reference range (54.2%), which occurred at baseline prior to COC exposure. It is therefore highly unlikely that the slight alterations sometimes observed during COC use have clinical relevance.

The second biomarker addressed in this study is total protein C activity. Protein C activity increased significantly from baseline after initiation of a 20mcg EE/100mcg LNG COC, taken both continuously and cyclically. Protein C activity did not change from baseline with use of a 30mcg EE/150mcg LNG pill. Even when shifts were statistically significant, they remained within normal reference ranges. Overall, changes to protein C activity were minimal, and actually favored anticoagulation, in obese women initiating an LNG-based COC. These findings are consistent with prior reports that protein C levels and activity do not change in a predictable fashion during COC use (48).

The results of this study add to an extensive literature on clotting factors as potential surrogate end points for VTE risk. Some have theorized that a decrease in free protein S, total protein C, or activated protein C resistance could contribute to a prothrombotic state, increasing risk for VTE. This could be particularly concerning among obese women, who are known to be at higher baseline risk. However, most studies attempting to elucidate the mechanism and degree of VTE risk with COCs have used these biomarkers incorrectly, drawing conclusions to clinical outcomes that have not been demonstrated.

Protein S levels are a poor predictor of VTE risk even among high-risk populations. Lijfering et alperformed a retrospective cohort study of 1143 relatives in a thrombophilic family cohort (55). Among this family with a genetic risk of Protein S deficiency, individuals with free protein S less than the 2.5%ile (<33%), were at an elevated risk of thrombosis compared to family members with upper quartile protein S levels (>91%), with an annual incidence of VTE of 1.81% (95% CI 1.01 - 2.99), and an adjusted hazard ratio of 11.3 (95% CI 5.4 - 23.6) (adjusted for age and sex). The authors noted that a protein S cutoff of 41% (equivalent to the 5th %ile) increased the absolute risk of first VTE from 0.2% per year to 1.2% per year in this high-risk family.

Importantly, this cutoff level is far below the lower limit of normal among healthy, asymptomatic individuals. The MEGA study demonstrated that among individuals with no family history of VTE, low protein S levels are not associated with an increased risk of VTE; both free and total protein S levels below the 0.10th percentile (<33% for this study) were not associated with a significant increased risk of VTE (58). Monitoring protein S levels in

individuals without a family history has no clinical utility, as no threshold of protein S is known to be predictive of VTE risk in a low risk population.

Clinical research studies can have three tiers of end points (70). The first tier includes clinical end points, such as venous thromboembolism. When clinical end points are rare, use of a validated surrogate endpoint (second tier) can be used as a substitute for the outcome of interest. Validation of a surrogate biomarker requires a prospective trial in which both the surrogate marker and the clinical outcome are measured, to determine whether the surrogate truly represents the effect of the intervention on the clinical outcome of interest. Correlation with the outcome alone is insufficient to substitute for a clinical outcome of interest. A successful example of a validated surrogate biomarker is RNA viral load, which has been prospectively validated to predict survival with AIDS, while CD4 counts do not (71).

Attempts to validate coagulation markers as surrogate endpoints for VTE have been based in retrospective or case-control studies such as the MEGA trial (55,58), and even these were unable to determine a clear association between coagulation markers and VTE risk. Many studies of coagulation biomarkers in COC users have not used any clinical outcome at all, drawing inappropriate conclusions about VTE risk based only on observed changes in clotting factors (63,72). Coagulation biomarkers such as protein S and protein C have never been shown to capture or predict the risk of VTE, and cannot therefore be used as surrogate end points.

Coagulation biomarkers in COC use therefore fall into the category of clinical correlates (third tier endpoints), which may be associated with the outcome, but do not predict the outcome. Biomarkers are laboratory tests that are thought to be in the causal

pathway between an intervention and a clinical outcome. Though they can be useful in research, they do not predict clinical illness and should not be used to guide clinical care (70).

It is a strength of this study that coagulation lab draws were completed both three weeks and three months after COC initiation. Prior studies have measured these proteins after at least two to three months of COC use, and therefore miss the opportunity to study rapid shifts in coagulation factors occurring during the first cycle (54, 69, 72). Risk of thrombosis is greatest during the first month of COC use, so measuring these supposed biomarkers during the period of greatest risk would allow the best potential for correlation with the outcome (39). It is therefore additionally reassuring that no changes in these anti-coagulant factors were observed during the first cycle of COC use, when VTE risk would be expected to be the highest.

In conclusion, thrombotic biomarkers protein C activity and free protein S levels did not demonstrate a trend towards procoagulation in a population of obese women using LNG-based COCs. These markers do not correlate with the expected population-level outcome of elevated VTE risk in this population. Biomarkers such as coagulation factor levels cannot be used to assess thrombotic risk from hormonal contraceptives, as no prospective study has determined a level that predicts a higher VTE risk in people without other genetic risk factors. Obese women wishing to initiate COCs should be counseled based on population level risks. Interpretation of individual thrombotic biomarkers has no role in risk assessment.

C. Conclusions

Combined oral contraceptive pills remain the most commonly used method of reversible contraception in the US. As the proportion of obese women increases, it is critical to ensure that contraceptive methods remain effective and safe.

Prior studies have confirmed altered PK profiles in obese women using COCs, particularly alterations in clearance, time to steady state and area under the curve (28). These alterations have the potential to reduce effectiveness of COCs in obese women. The original study demonstrated improvements to total LNG PK parameters and ovulation suppression with both CC and ID dosing strategies (62). The current study demonstrated that SHBG levels rise differently depending on dosing strategy, altering the amount of free (pharmacologically active) LNG. BMI was not related to SHBG levels. The interplay of EE, LNG, and SHBG with other PK parameters remains incompletely understood, but SHBG's strong binding of LNG has the potential to alter contraceptive effect in women of all BMIs. Future PK studies should monitor SHBG along with total LNG to better elucidate the relationship between LNG PK and ovulation suppression. A better understanding of this metabolism could lead to tailored COC dosing based on BMI to maximize contraceptive effect, rather than our current "one size fits all" approach to COC dosing.

A second finding from this study is that thrombotic biomarkers protein C and protein S did not demonstrate prothrombotic changes in obese women initiating COCs. These biomarkers remain unvalidated and should not be used in assessing VTE risk for women initiating COCs. Due to the rarity of the VTE outcome, it would be challenging to conduct a prospective study to validate a surrogate marker. A phase four post-marketing surveillance study would potentially have the power to achieve this goal, if measurement of

coagulation markers was made at baseline and after initiation of COCs for all women initiating a new product.

Population level studies show a higher risk of VTE in obese COC users than non-obese COC users. However, the magnitude of this risk is less than that with aging or smoking (45). COCs remain category 2 for obese women according to the CDC and WHO, recognizing that the relative risk of pregnancy and postpartum outweighs that of COCs. Obese women initiating COCs should be informed of their VTE risk, but thrombotic markers cannot be used to individualize this risk.

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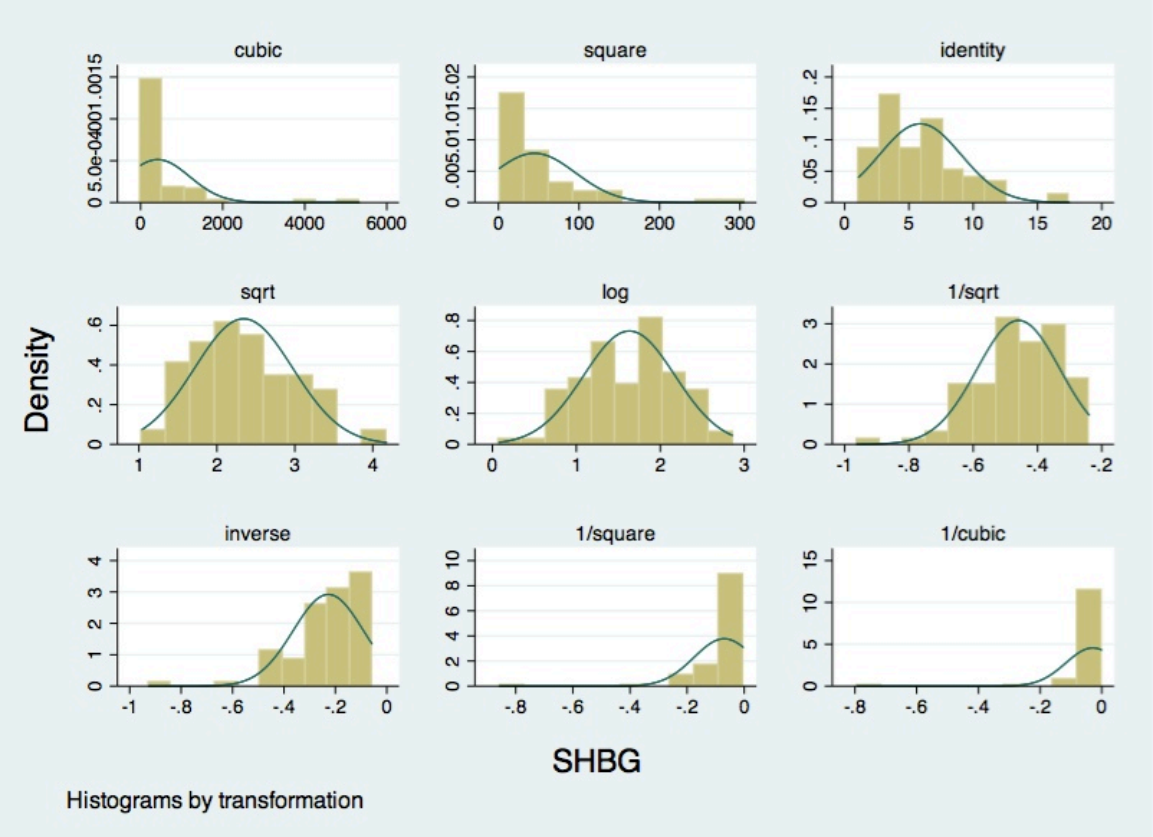
Appendix A.1 – Sex hormone binding globulin models

Analysis of potential transformations for SHBG

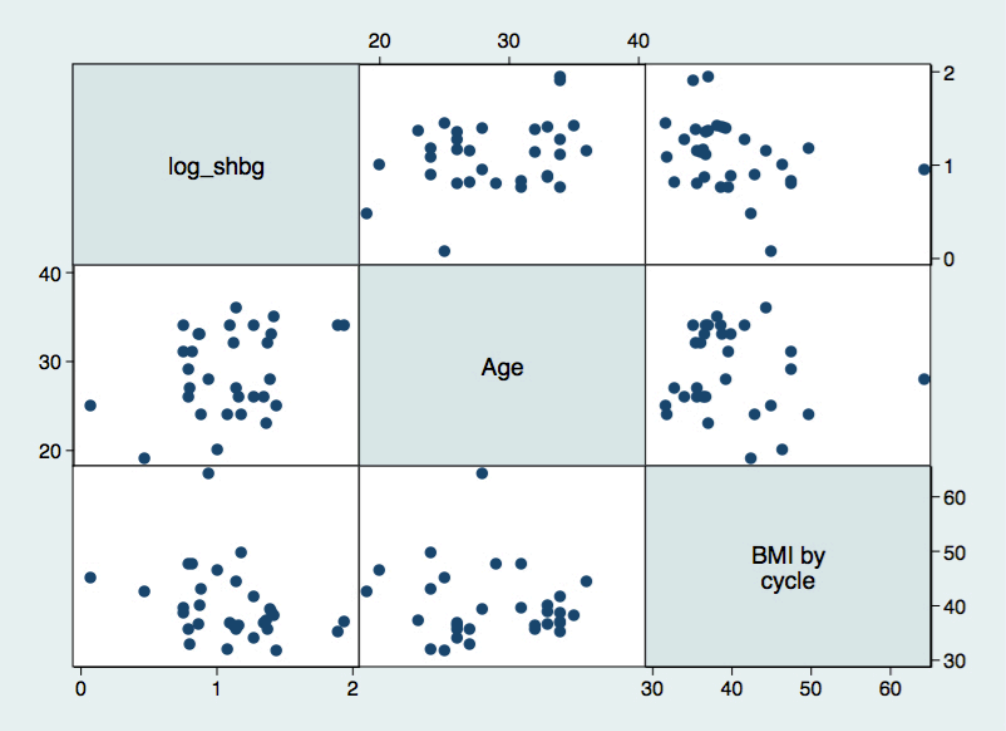


Assessment of normality for dependent variable SHBG

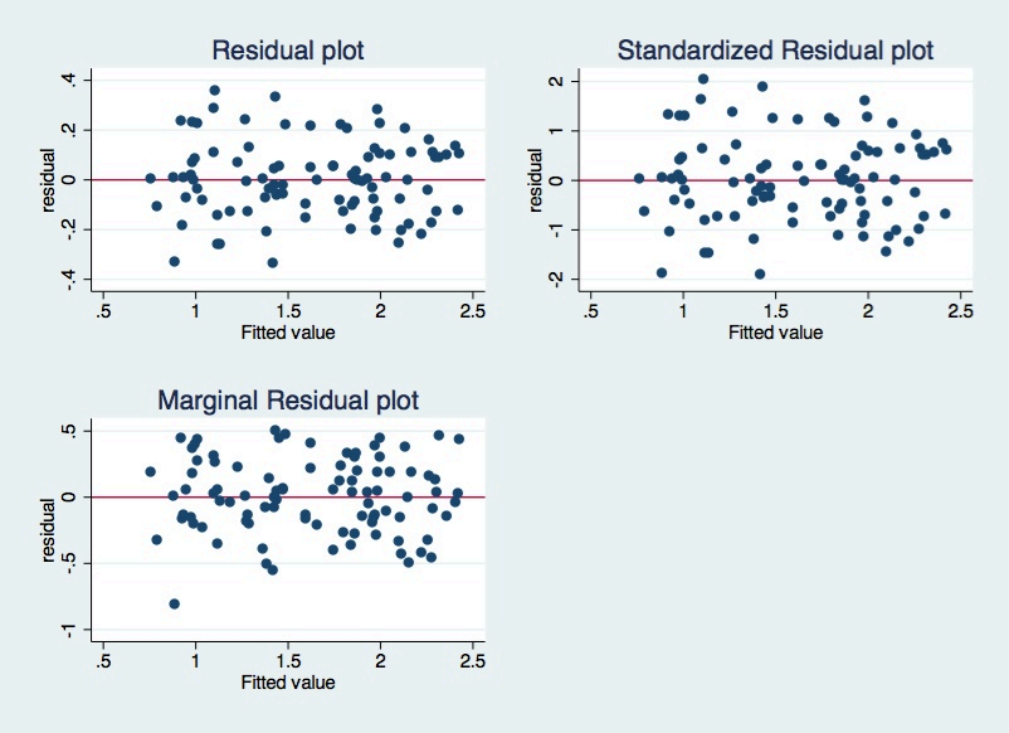
```
. swilk shbg log_shbg if cycle==0

                    Shapiro-Wilk W test for normal data

    Variable |   Obs        W           V         z       Prob>z
-------------+--------------------------------------------------
        shbg |    31    0.87717      4.001     2.873     0.00203
     log_shbg |    31    0.95780      1.375     0.659     0.25485
```

Appendix A.2
Model diagnostics - log SHBG

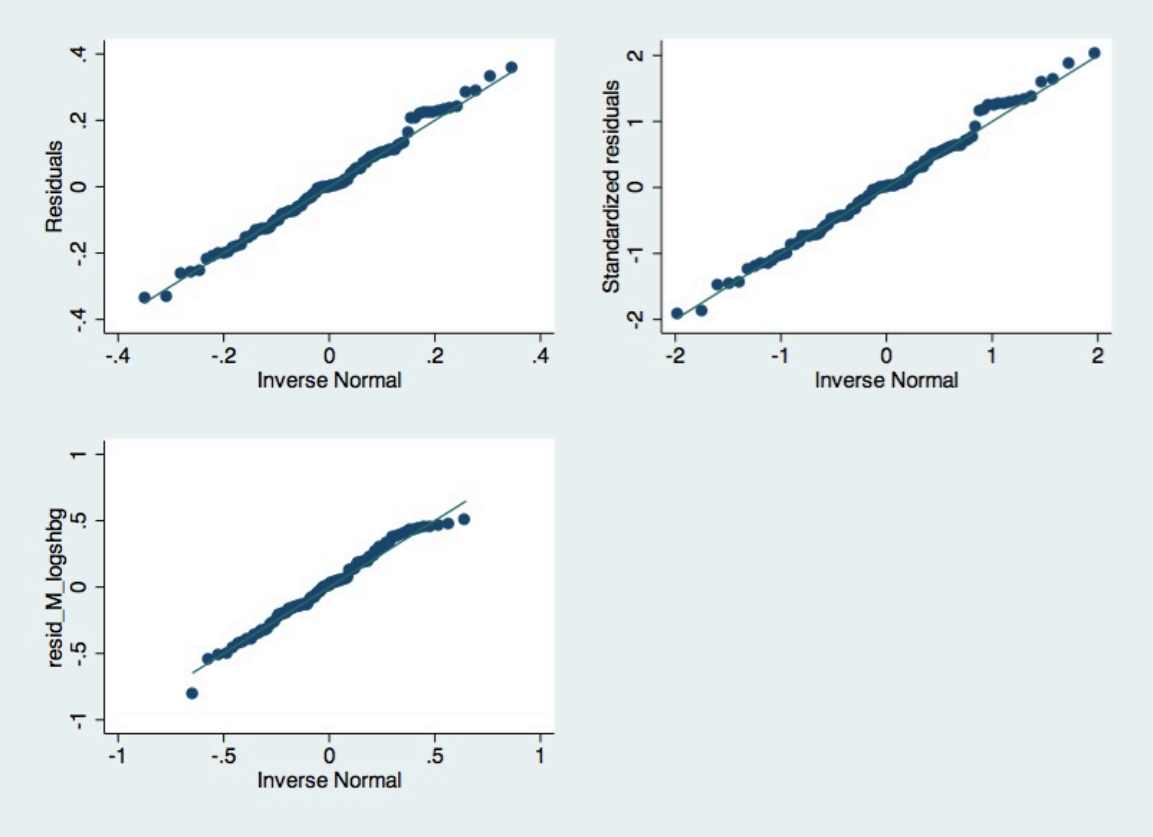
Linearity:



Homogeneity of variance:



Normality of variance:




```
. swilk res_logshbg standard_res_logshbg resid_M_logshbg

                    Shapiro-Wilk W test for normal data

    Variable |  Obs       W           V         z       Prob>z
-------------+--------------------------------------------------
 res_logshbg |   93    0.98978       0.795    -0.508    0.69432
standa~gshbg |   93    0.98978       0.795    -0.508    0.69432
resid_M_lo~g |   93    0.98148       1.439     0.805    0.21048
```

Appendix A.3 – Model utilizing log SHBG

```
. xi:mixed log_shbg i.dose age bmibycycle i.race i.parity || id: cycle, cov(unst
> r)
i.dose            _Idose_0-3          (naturally coded; _Idose_0 omitted)
i.race            _Irace_0-1          (naturally coded; _Irace_0 omitted)
i.parity          _Iparity_0-1        (naturally coded; _Iparity_0 omitted)

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0:   log likelihood =  2.5064355
Iteration 1:   log likelihood =  2.9018418
Iteration 2:   log likelihood =  2.9454086
Iteration 3:   log likelihood =   2.947429
Iteration 4:   log likelihood =  2.9474311
Iteration 5:   log likelihood =  2.9474316
Iteration 6:   log likelihood =  2.9474317

Computing standard errors:

Mixed-effects ML regression                     Number of obs      =        93
Group variable: id                              Number of groups   =        31

                                                Obs per group: min =         3
                                                               avg =       3.0
                                                               max =         3


                                                Wald chi2(7)       =    513.56
Log likelihood =  2.9474317                     Prob > chi2        =    0.0000
```

log_shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
_Idose_1	.4887086	.0592365	8.25	0.000	.3726071	.6048101
_Idose_2	.978142	.0577457	16.94	0.000	.8649625	1.091322
_Idose_3	.8657453	.0448833	19.29	0.000	.7777756	.9537149
age	.0192815	.0095825	2.01	0.044	.0005002	.0380628
bmibycycle	-.0094201	.0064043	-1.47	0.141	-.0219723	.0031322
_Irace_1	-.1221345	.1275392	-0.96	0.338	-.3721068	.1278378
_Iparity_1	.2999302	.1014037	2.96	0.003	.1011825	.4986778
_cons	.8286173	.3880774	2.14	0.033	.0679995	1.589235

Random-effects Parameters	Estimate	Std. Err.	[95% Conf.	Interval]
id: Unstructured				
var(cycle)	.000093	.	.	.
var(_cons)	.0534167	.	.	.
cov(cycle,_cons)	-.0022283	.	.	.
var(Residual)	.031178	.	.	.

LR test vs. linear regression: chi2(3) = 31.86 Prob > chi2 = 0.0000

```
. estat ic

Akaike's information criterion and Bayesian information criterion
```

Model	Obs	ll(null)	ll(model)	df	AIC	BIC
.	93	.	2.947432	8	10.10514	30.36593

Note: N=Obs used in calculating BIC; see [R] BIC note

```
. lincom  _Idose_2 -  _Idose_3

 ( 1)  [shbg]_Idose_2 - [shbg]_Idose_3 = 0
```

shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	1.241136	.4148957	2.99	0.003	.427955	2.054316

```
. lincom  _Idose_2 - _Idose_1

 ( 1)  - [shbg]_Idose_1 + [shbg]_Idose_2 = 0
```

shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	3.721244	.5701492	6.53	0.000	2.603772	4.838716

```
. lincom _Idose_3 - _Idose_1

 ( 1)  - [shbg]_Idose_1 + [shbg]_Idose_3 = 0
```

shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	2.480108	.4273892	5.80	0.000	1.642441	3.317776

```
. testparm _Idose_*

 ( 1)  [shbg]_Idose_1 = 0
 ( 2)  [shbg]_Idose_2 = 0
 ( 3)  [shbg]_Idose_3 = 0

           chi2(  3) =  297.32
         Prob > chi2 =    0.0000
```

Appendix A.4 – Model utilizing untransformed SHBG

```
. xi:mixed shbg i.dose age bmibycycle i.race i.parity || id: cycle, cov(unstr)
i.dose            _Idose_0-3          (naturally coded; _Idose_0 omitted)
i.race            _Irace_0-1          (naturally coded; _Irace_0 omitted)
i.parity          _Iparity_0-1        (naturally coded; _Iparity_0 omitted)

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0:   log likelihood = -169.95242
Iteration 1:   log likelihood = -169.27702
Iteration 2:   log likelihood = -169.26212
Iteration 3:   log likelihood = -169.26212

Computing standard errors:

Mixed-effects ML regression                     Number of obs      =        93
Group variable: id                              Number of groups   =        31

                                                Obs per group: min =         3
                                                               avg =       3.0
                                                               max =         3


                                                Wald chi2(7)       =    308.62
Log likelihood = -169.26212                     Prob > chi2        =    0.0000
```

shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
_Idose_1	1.826982	.4458596	4.10	0.000	.9531136	2.700851
_Idose_2	5.548226	.4339288	12.79	0.000	4.697742	6.398711
_Idose_3	4.307091	.2785589	15.46	0.000	3.761125	4.853056
age	.0935703	.054111	1.73	0.084	-.0124853	.1996258
bmibycycle	-.0695439	.0374069	-1.86	0.063	-.14286	.0037723
_Irace_1	-.6465001	.7199302	-0.90	0.369	-2.057537	.7645372
_Iparity_1	1.352149	.5670141	2.38	0.017	.2408221	2.463476
_cons	2.900352	2.231866	1.30	0.194	-1.474025	7.27473

Random-effects Parameters	Estimate	Std. Err.	[95% Conf.	Interval]
id: Unstructured				
var(cycle)	.0714986	.0420969	.022549	.2267085
var(_cons)	1.02104	.428593	.4484782	2.324578
cov(cycle,_cons)	.2701904	.0892037	.0953544	.4450264
var(Residual)	1.166957	.213889	.8147796	1.671359

LR test vs. linear regression: chi2(3) = 37.84 Prob > chi2 = 0.0000

Note: LR test is conservative and provided only for reference.

```
. estat ic
```

Akaike's information criterion and Bayesian information criterion

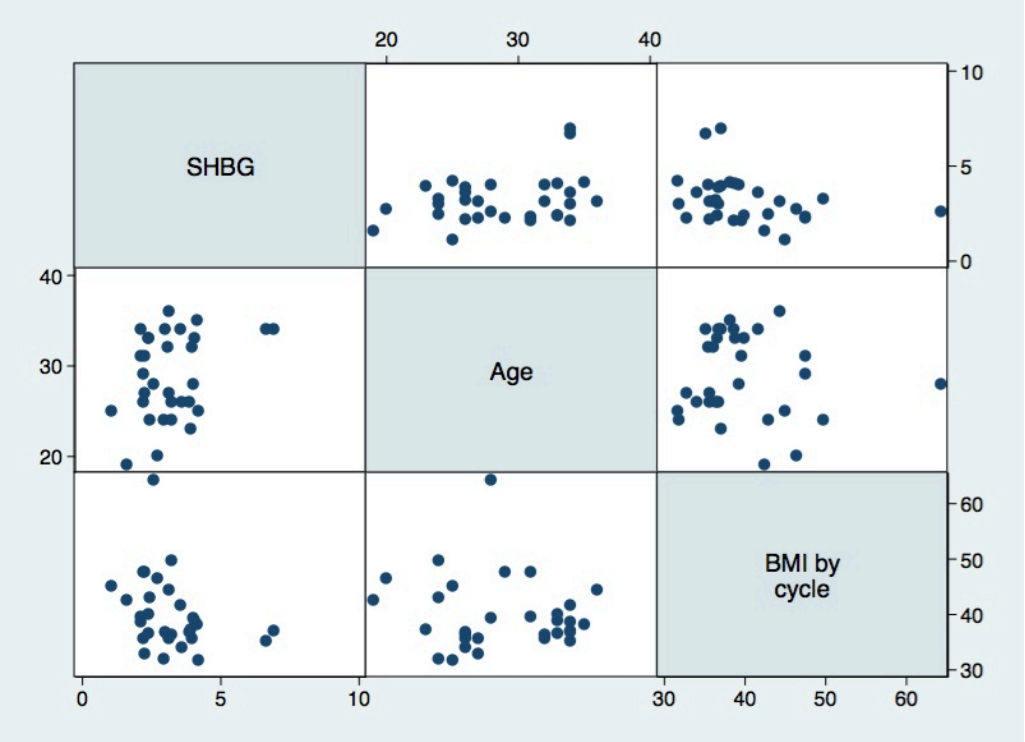
Model	Obs	ll(null)	ll(model)	df	AIC	BIC
.	93	.	-169.2621	12	362.5242	392.9154

Note: N=Obs used in calculating BIC; see [R] BIC note

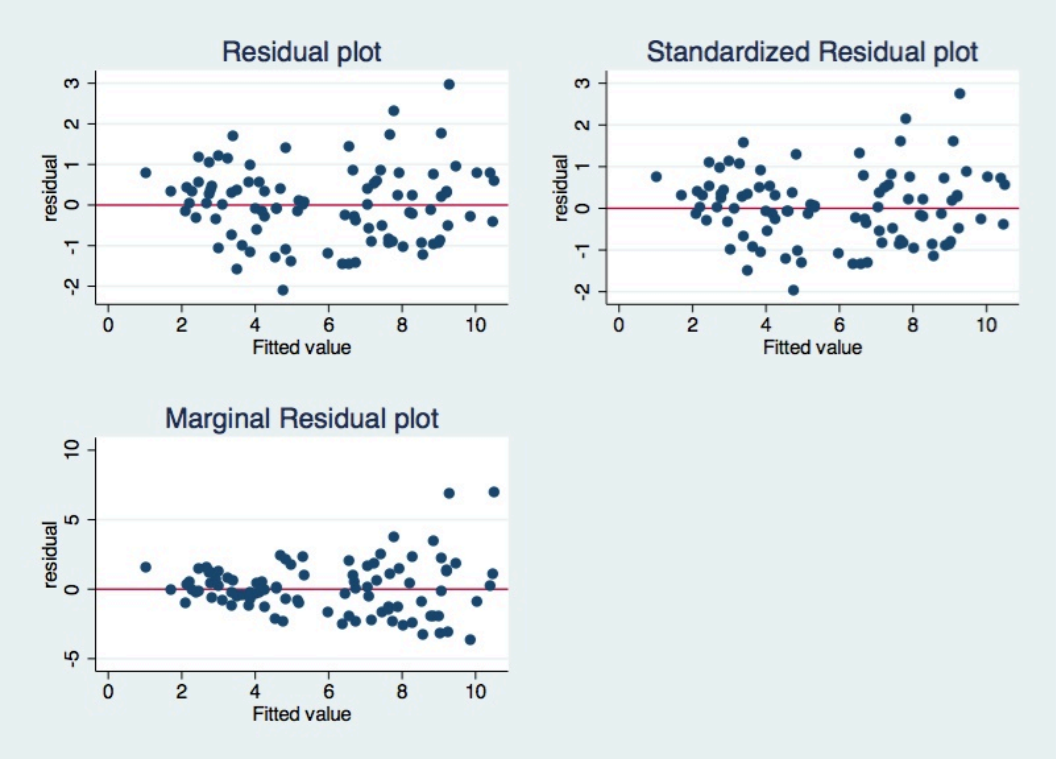
Appendix A.5

Model diagnostics – untransformed SHBG:

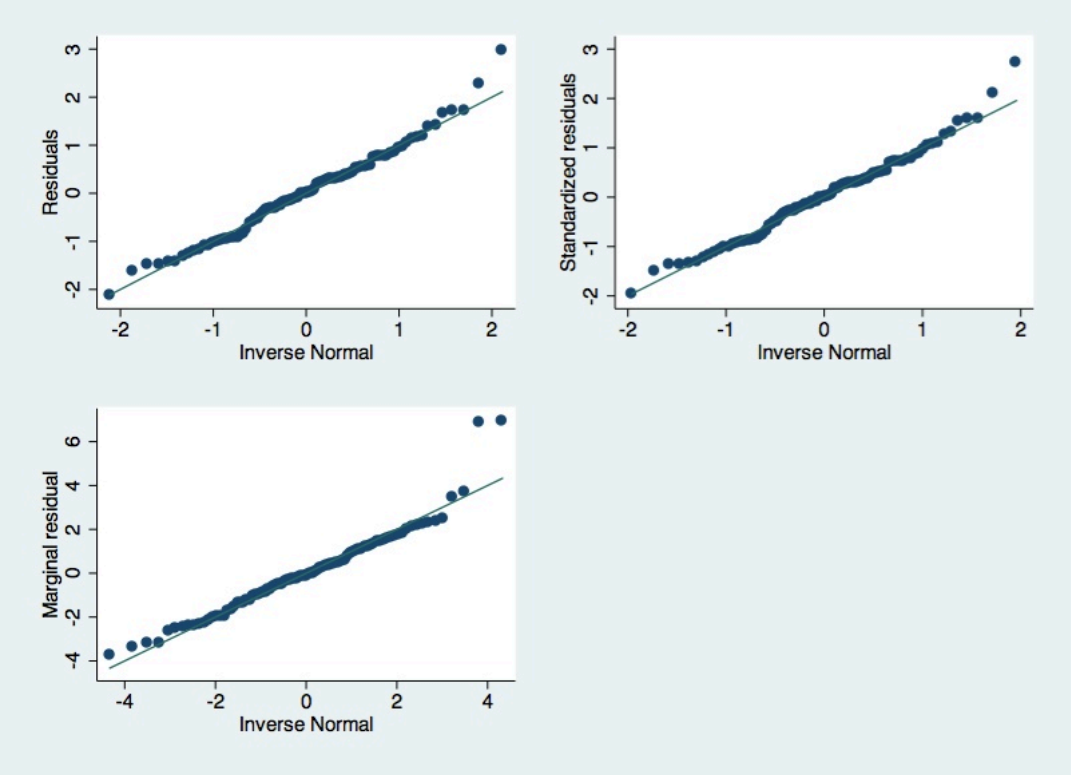
1. Linearity of continuous variables



2. Residual analysis



3. Normality analysis




```
. swilk res_shbg resid_M_shbg standard_res_shbg

                    Shapiro-Wilk W test for normal data

    Variable |      Obs       W           V         z       Prob>z
-------------+--------------------------------------------------
     res_shbg |       93    0.98625      1.069     0.147    0.44147
resid_M_shbg |       93    0.94784      4.054     3.093    0.00099
standard_r~g |       93    0.98625      1.069     0.147    0.44147
```

Appendix A.6 – Linear contrasts using untransformed SHBG

```
. lincom  _Idose_1 -  _Idose_3

 ( 1)  [shbg]_Idose_1 - [shbg]_Idose_3 = 0
```

shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	**-2.480108**	**.4273892**	**-5.80**	**0.000**	**-3.317776**	**-1.642441**

```
. lincom  _Idose_2 - _Idose_3

 ( 1)  [shbg]_Idose_2 - [shbg]_Idose_3 = 0
```

shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	**1.241136**	**.4148957**	**2.99**	**0.003**	**.427955**	**2.054316**

```
. lincom  _Idose_1 - _Idose_2

 ( 1)  [shbg]_Idose_1 - [shbg]_Idose_2 = 0
```

shbg	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	**-3.721244**	**.5701492**	**-6.53**	**0.000**	**-4.838716**	**-2.603772**

```
. testparm  _Idose_*

 ( 1)  [shbg]_Idose_1 = 0
 ( 2)  [shbg]_Idose_2 = 0
 ( 3)  [shbg]_Idose_3 = 0

           chi2(  3) =  297.32
         Prob > chi2 =    0.0000
```

Appendix A.7 - Paired analysis

```
. by grouppaired, sort : ttest cycle1 == cycle4
```

```
-------------------------------------------------------------------------------
-> grouppaired = 1

Paired t test
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
cycle1	16	83.40418	7.777906	31.11163	66.82597	99.9824
cycle4	16	95.57291	8.463821	33.85528	77.5327	113.6131
diff	16	-12.16873	2.923489	11.69396	-18.4	-5.937461

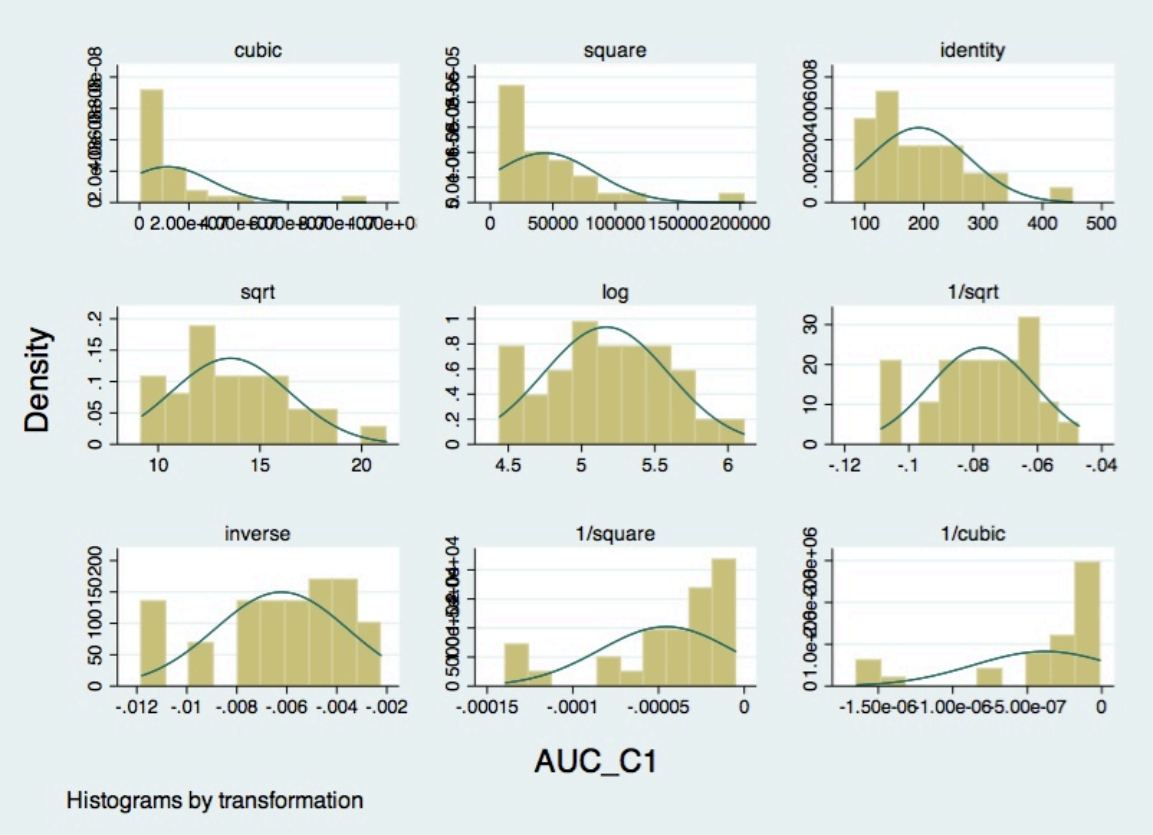
```
    mean(diff) = mean(cycle1 - cycle4)                            t =  -4.1624
Ho: mean(diff) = 0                             degrees of freedom =       15

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.0004         Pr(|T| > |t|) = 0.0008          Pr(T > t) = 0.9996

-------------------------------------------------------------------------------
-> grouppaired = 2

Paired t test
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
cycle1	15	74.36286	6.158524	23.85186	61.15414	87.57158
cycle4	15	49.26636	4.010783	15.53369	40.66409	57.86863
diff	15	25.0965	2.8922	11.20144	18.89335	31.29965

```
    mean(diff) = mean(cycle1 - cycle4)                            t =   8.6773
Ho: mean(diff) = 0                             degrees of freedom =       14

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 1.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.0000

-------------------------------------------------------------------------------
```

Appendix B.1 – Levonorgestrel Area Under the Curve Models

AUC variable assessment

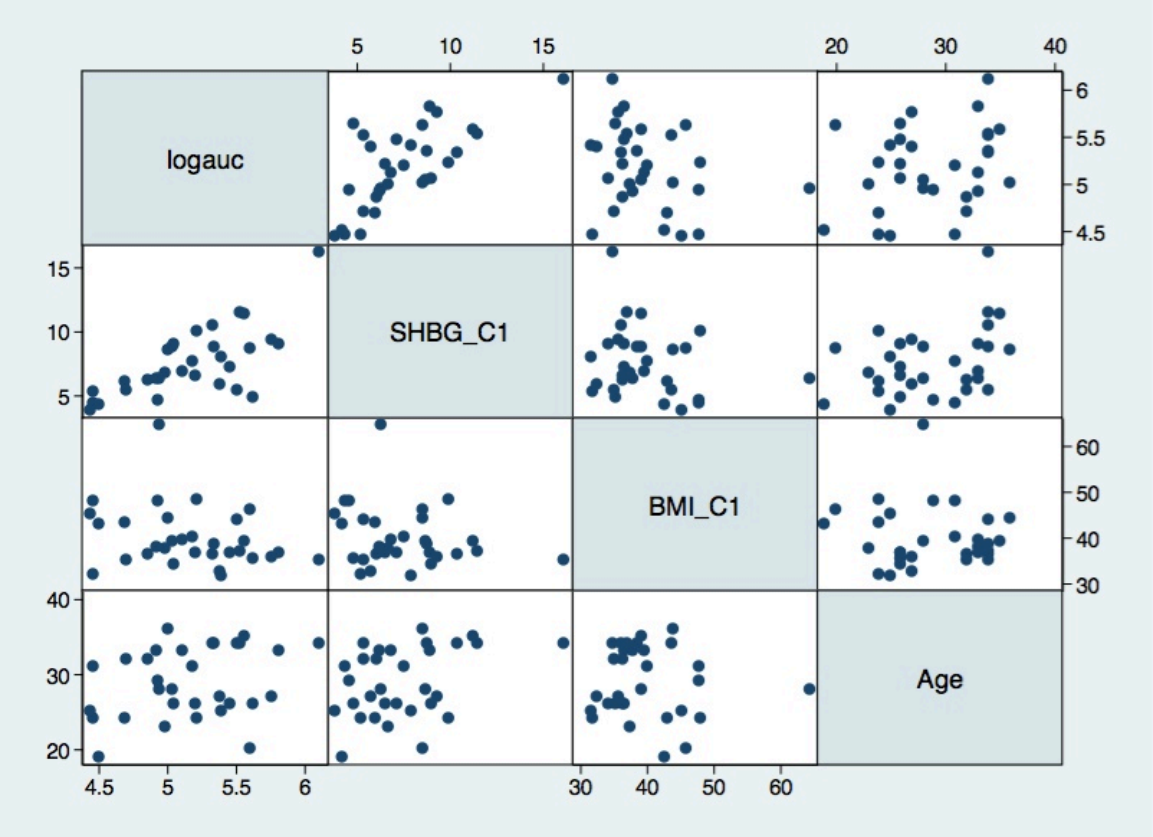



```
. swilk auc logauc
```

Shapiro–Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
auc	**31**	**0.92518**	**2.437**	**1.846**	**0.03247**
logauc	**31**	**0.97701**	**0.749**	**−0.599**	**0.72554**

2. Assessment of predictor variables




```
. pwcorr logauc shbg_c1 bmi_c1 age1
```

	logauc	shbg_c1	bmi_c1	age1
logauc	**1.0000**			
shbg_c1	**0.7028**	**1.0000**		
bmi_c1	**-0.2855**	**-0.2237**	**1.0000**	
age1	**0.2841**	**0.3778**	**-0.0828**	**1.0000**

Appendix B.2

Interaction terms

```
. regress logauc shbg_c1 bmi_c1 age shbgbmi
```

Source	SS	df	MS	Number of obs =	31
Model	2.81067581	4	.702668953	F(4, 26) =	6.86
Residual	2.66126714	26	.102356428	Prob > F =	0.0007
				R-squared =	0.5137
				Adj R-squared =	0.4388
Total	5.47194295	30	.182398098	Root MSE =	.31993

logauc	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.0457372	.1935782	0.24	0.815	-.3521685	.4436429
bmi_c1	-.0193715	.0340936	-0.57	0.575	-.089452	.050709
age	.0027992	.0137476	0.20	0.840	-.0254593	.0310577
shbgbmi	.0016105	.0049966	0.32	0.750	-.0086602	.0118812
_cons	5.04034	1.320427	3.82	0.001	2.326165	7.754516

```
. regress logauc shbg_c1 bmi_c1 age agebmi
```

Source	SS	df	MS	Number of obs =	31
Model	2.80241585	4	.700603962	F(4, 26) =	6.82
Residual	2.6695271	26	.102674119	Prob > F =	0.0007
				R-squared =	0.5121
				Adj R-squared =	0.4371
Total	5.47194295	30	.182398098	Root MSE =	.32043

logauc	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.1096141	.0277623	3.95	0.001	.0525478	.1666804
bmi_c1	-.0230573	.0943189	-0.24	0.809	-.2169326	.170818
age	-.0194455	.1417087	-0.14	0.892	-.3107319	.2718408
agebmi	.000522	.0034332	0.15	0.880	-.006535	.0075791
_cons	5.22629	3.812744	1.37	0.182	-2.610919	13.0635

Appendix B.3

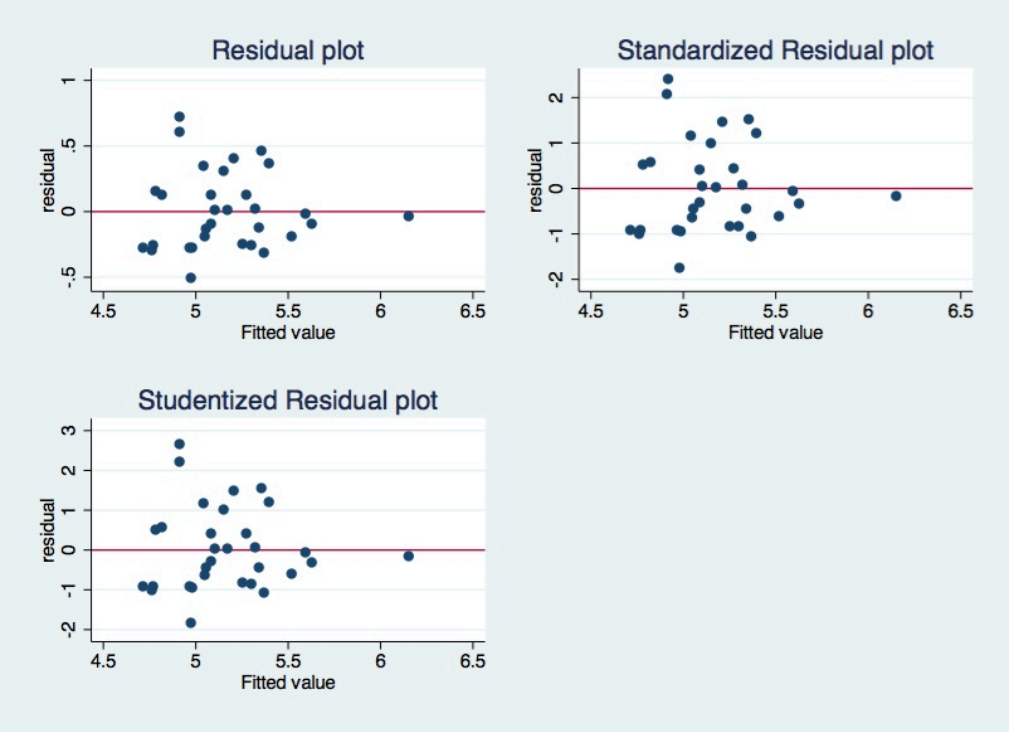
Model and diagnostics:

```
. regress logauc shbg_c1 bmi_c1 age
```

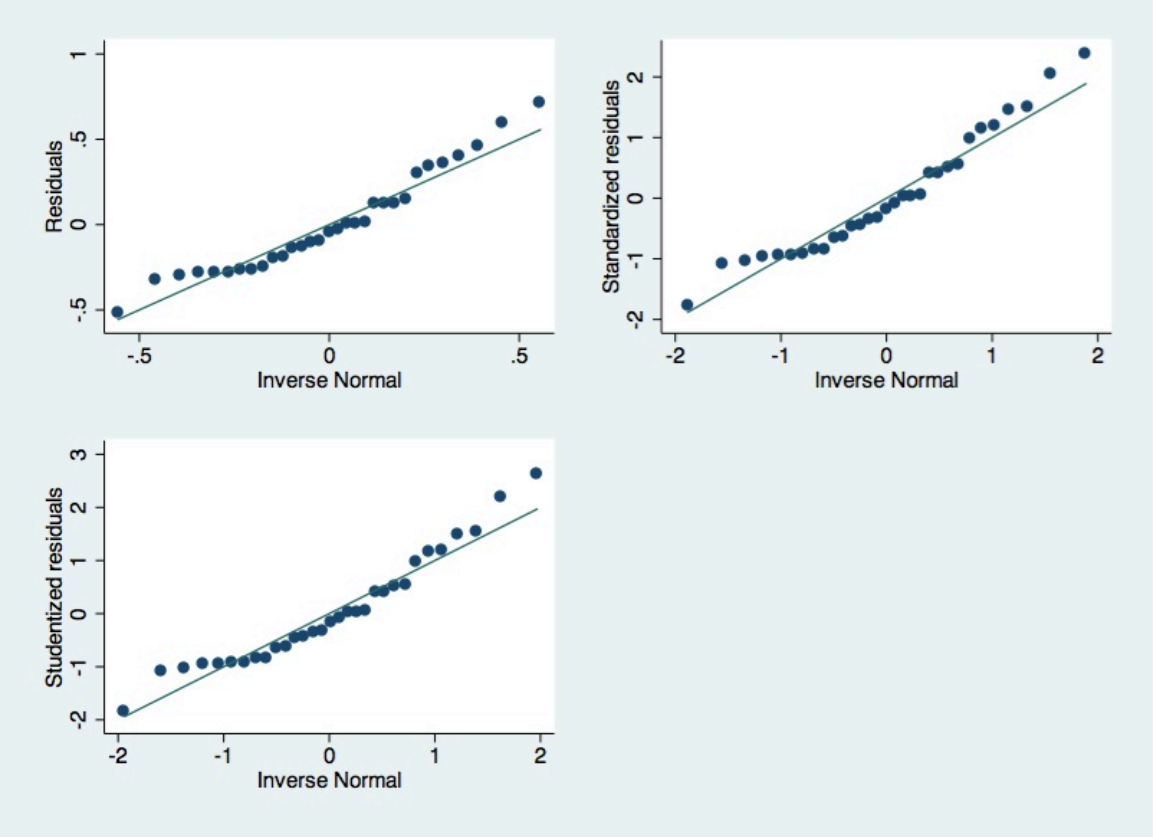
Source	SS	df	MS		
Model	2.80004203	3	.933347344	Number of obs =	31
Residual	2.67190092	27	.098959293	F(3, 27) =	9.43
				Prob > F =	0.0002
				R-squared =	0.5117
				Adj R-squared =	0.4575
Total	5.47194295	30	.182398098	Root MSE =	.31458

logauc	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.1076307	.0240597	4.47	0.000	.0582642	.1569972
bmi_c1	-.0087833	.0089722	-0.98	0.336	-.0271927	.009626
age	.0020029	.0132975	0.15	0.881	-.0252812	.0292871
_cons	4.652496	.5346057	8.70	0.000	3.555576	5.749417

Residual plots



Q-Q plots




```
. swilk  res_auc standard_auc student_auc
```

Shapiro-Wilk W test for normal data

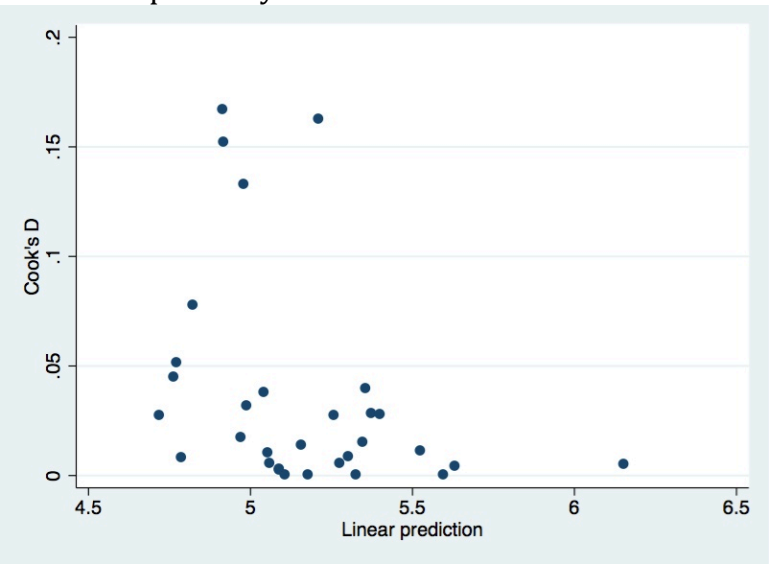
Variable	Obs	W	V	z	Prob>z
res_auc	**31**	**0.94358**	**1.838**	**1.261**	**0.10367**
standard_auc	**31**	**0.94498**	**1.792**	**1.209**	**0.11337**
student_auc	**31**	**0.94307**	**1.854**	**1.280**	**0.10034**

Multicollinearity:

```
. estat vif
```

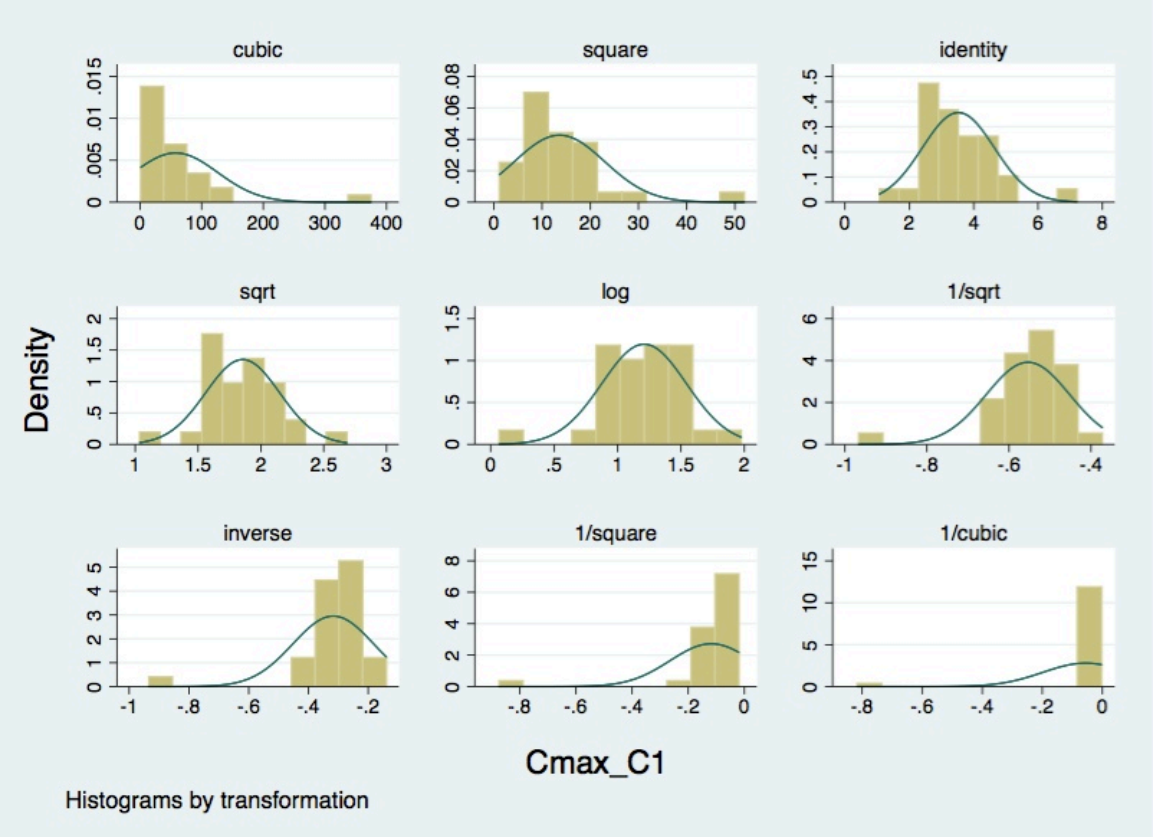
Variable	VIF	1/VIF
shbg_c1	**1.22**	**0.819988**
age	**1.17**	**0.857261**
bmi_c1	**1.05**	**0.949955**
Mean VIF	**1.15**	

Influential points by Cook's distance



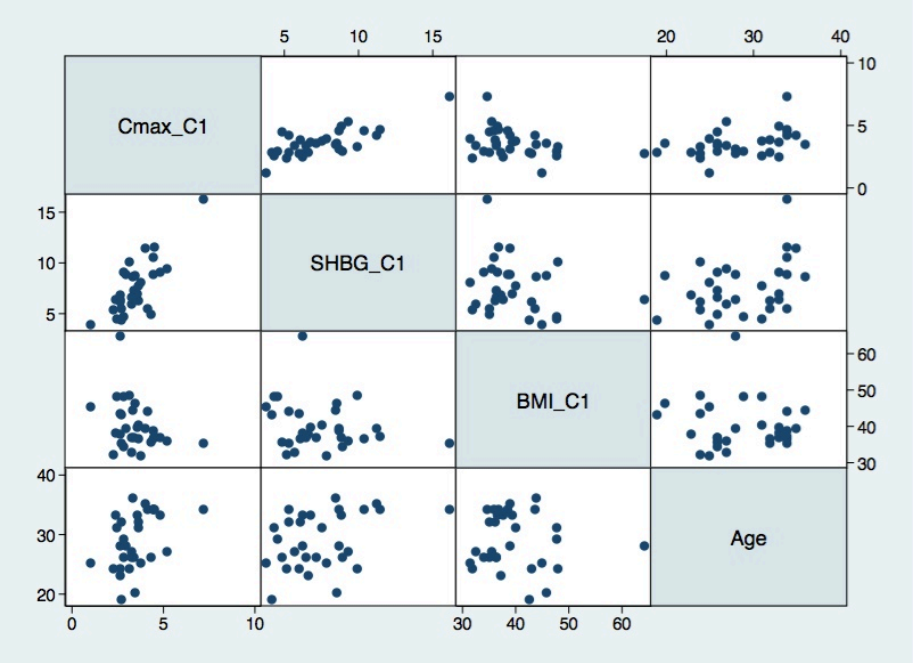
Appendix C.1 – Levonorgestrel Cmax Models

1. Outcome variable assessment




```
. swilk cmax logcmax sqrtcmax

                    Shapiro-Wilk W test for normal data

    Variable |   Obs        W           V       z       Prob>z
-------------+--------------------------------------------------
        cmax |    31    0.93051      2.263   1.692    0.04528
     logcmax |    31    0.92352      2.491   1.891    0.02929
    sqrtcmax |    31    0.95538      1.453   0.774    0.21932
```

Assessment of predictor variables




```
. pwcorr cmax shbg_c1 bmi_c1 age1
```

	cmax	shbg_c1	bmi_c1	age1
cmax	**1.0000**			
shbg_c1	**0.7555**	**1.0000**		
bmi_c1	**-0.3400**	**-0.2237**	**1.0000**	
age1	**0.4354**	**0.3778**	**-0.0828**	**1.0000**

Appendix C.2

```
. regress cmax shbg_c1
```

Source	SS	df	MS		
Model	21.4890188	1	21.4890188	Number of obs	31
Residual	16.1551191	29	.557073073	F(1, 29)	38.57
				Prob > F	0.0000
Total	37.6441379	30	1.2548046	R-squared	0.5708
				Adj R-squared	0.5560
				Root MSE	.74637

cmax	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.3210513	.0516919	6.21	0.000	.2153296	.4267731
_cons	1.114329	.4104629	2.71	0.011	.2748382	1.95382

```
. regress cmax bmi_c1
```

Source	SS	df	MS		
Model	4.35106015	1	4.35106015	Number of obs	31
Residual	33.2930778	29	1.14803716	F(1, 29)	3.79
				Prob > F	0.0613
Total	37.6441379	30	1.2548046	R-squared	0.1156
				Adj R-squared	0.0851
				Root MSE	1.0715

cmax	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
bmi_c1	-.0579853	.0297851	-1.95	0.061	-.1189026	.002932
_cons	5.836175	1.20324	4.85	0.000	3.375274	8.297077

```
. regress cmax age1
```

Source	SS	df	MS		
Model	7.13785558	1	7.13785558	Number of obs	31
Residual	30.5062823	29	1.05194077	F(1, 29)	6.79
				Prob > F	0.0143
Total	37.6441379	30	1.2548046	R-squared	0.1896
				Adj R-squared	0.1617
				Root MSE	1.0256

cmax	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
age1	.1045637	.0401414	2.60	0.014	.0224653	.1866621
_cons	.5117619	1.170913	0.44	0.665	-1.883024	2.906547

Interaction terms:

```
. regress cmax shbg_c1 bmi_c1 age1
```

Source	SS	df	MS	Number of obs =	31
				F(3, 27) =	15.19
Model	23.6392281	3	7.87974271	Prob > F =	0.0000
Residual	14.0049098	27	.518700362	R-squared =	0.6280
				Adj R-squared =	0.5866
Total	37.6441379	30	1.2548046	Root MSE =	.72021

cmax	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.2757677	.0550834	5.01	0.000	.1627459	.3887895
bmi_c1	-.0307475	.0205413	-1.50	0.146	-.0728947	.0113997
age1	.0421015	.0304438	1.38	0.178	-.020364	.104567
_cons	1.467525	1.22395	1.20	0.241	-1.043813	3.978862

```
. regress cmax shbg_c1 bmi_c1 age1 bmishbg
```

Source	SS	df	MS	Number of obs =	31
				F(4, 26) =	11.84
Model	24.3047989	4	6.07619973	Prob > F =	0.0000
Residual	13.339339	26	.5130515	R-squared =	0.6456
				Adj R-squared =	0.5911
Total	37.6441379	30	1.2548046	Root MSE =	.71628

cmax	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.765432	.4333905	1.77	0.089	-.125415	1.656279
bmi_c1	.0530195	.0763302	0.69	0.493	-.1038794	.2099185
age1	.0358021	.0307786	1.16	0.255	-.0274642	.0990683
bmishbg	-.0127414	.0111866	-1.14	0.265	-.0357359	.0102531
_cons	-1.600866	2.956223	-0.54	0.593	-7.677469	4.475737

```
. regress cmax shbg_c1 bmi_c1 age1 agebmi
```

Source	SS	df	MS	Number of obs =	31
				F(4, 26) =	10.99
Model	23.6563104	4	5.91407761	Prob > F =	0.0000
Residual	13.9878275	26	.537993365	R-squared =	0.6284
				Adj R-squared =	0.5713
Total	37.6441379	30	1.2548046	Root MSE =	.73348

cmax	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.2704472	.0635498	4.26	0.000	.1398187	.4010756
bmi_c1	.0075432	.2159021	0.03	0.972	-.4362498	.4513362
age1	.0996383	.3243802	0.31	0.761	-.5671347	.7664114
agebmi	-.0014004	.0078588	-0.18	0.860	-.0175544	.0147537
_cons	-.07171	8.727616	-0.01	0.994	-18.01158	17.86816

Appendix C.3

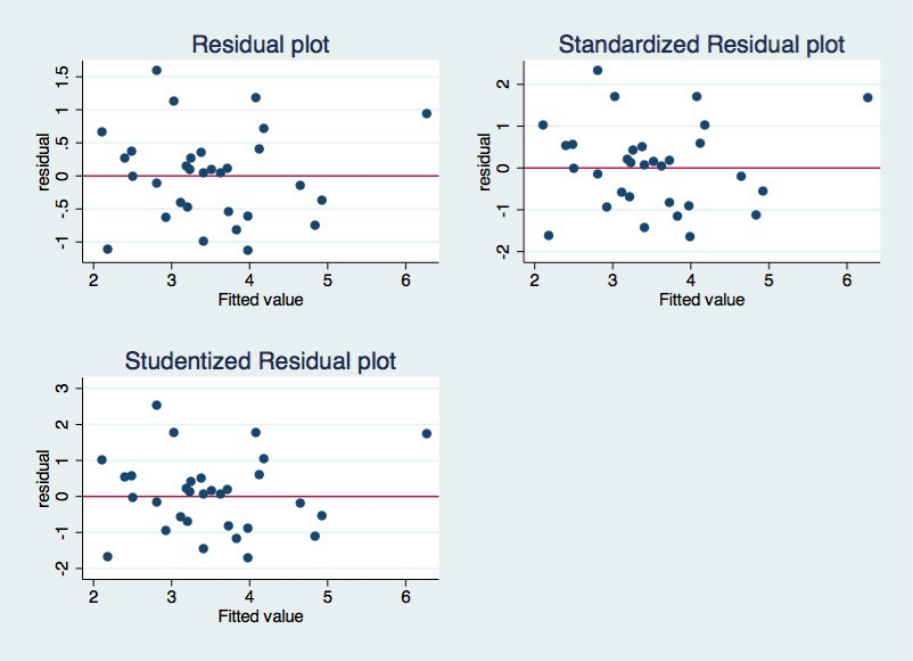
Model diagnostics:

```
. regress cmax shbg_c1 bmi_c1 age1
```

Source	SS	df	MS		
Model	23.6392281	3	7.87974271	Number of obs =	31
Residual	14.0049098	27	.518700362	F(3, 27) =	15.19
				Prob > F =	0.0000
				R-squared =	0.6280
				Adj R-squared =	0.5866
Total	37.6441379	30	1.2548046	Root MSE =	.72021

cmax	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
shbg_c1	.2757677	.0550834	5.01	0.000	.1627459	.3887895
bmi_c1	-.0307475	.0205413	-1.50	0.146	-.0728947	.0113997
age1	.0421015	.0304438	1.38	0.178	-.020364	.104567
_cons	1.467525	1.22395	1.20	0.241	-1.043813	3.978862

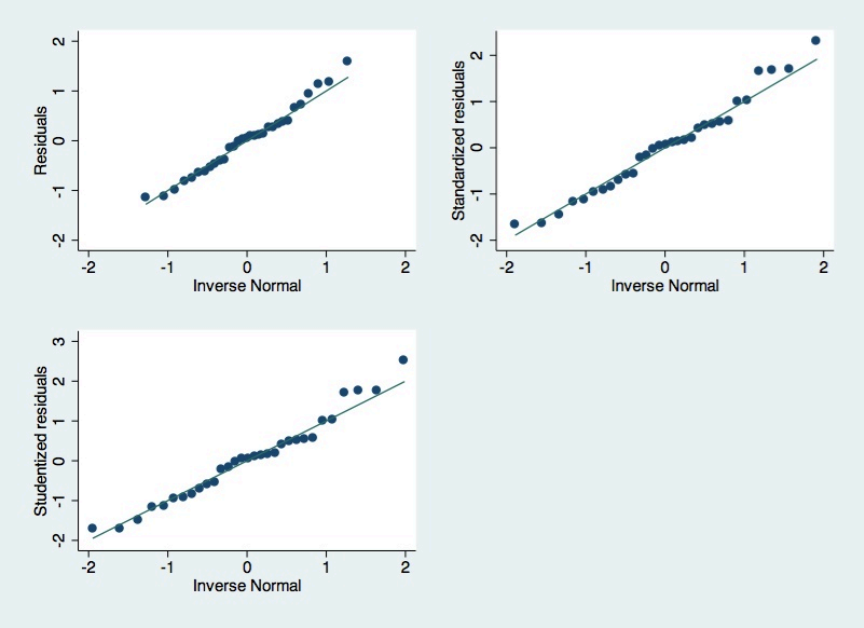
Residual plots:




```
. swilk  res_cmax standard_res_cmax student_res_cmax
```

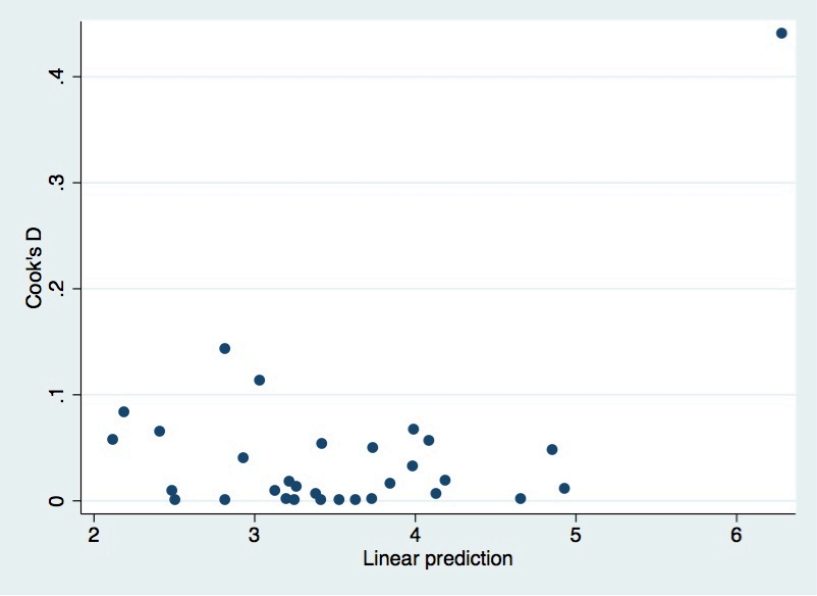
Shapiro-Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
res_cmax	31	0.97641	0.768	-0.546	0.70744
standard_r~x	31	0.96986	0.982	-0.038	0.51523
student_re~x	31	0.96946	0.995	-0.011	0.50431




```
. estat vif
```

Variable	VIF	1/VIF
shbg_c1	**1.22**	**0.819988**
age1	**1.17**	**0.857261**
bmi_c1	**1.05**	**0.949955**
Mean VIF	**1.15**	



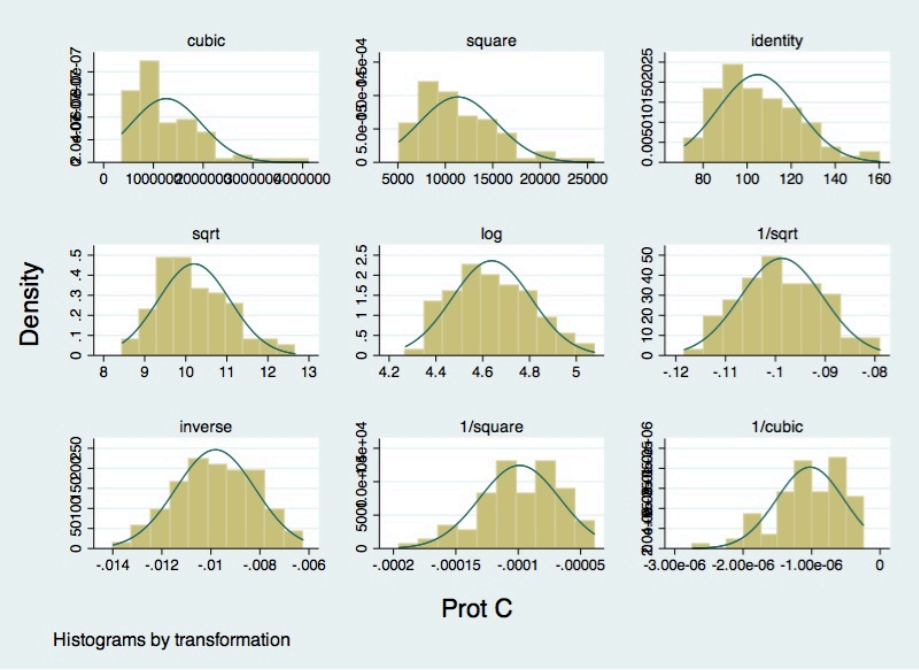
Appendix D.1 – Protein C models

Normality of outcome variable Protein C:

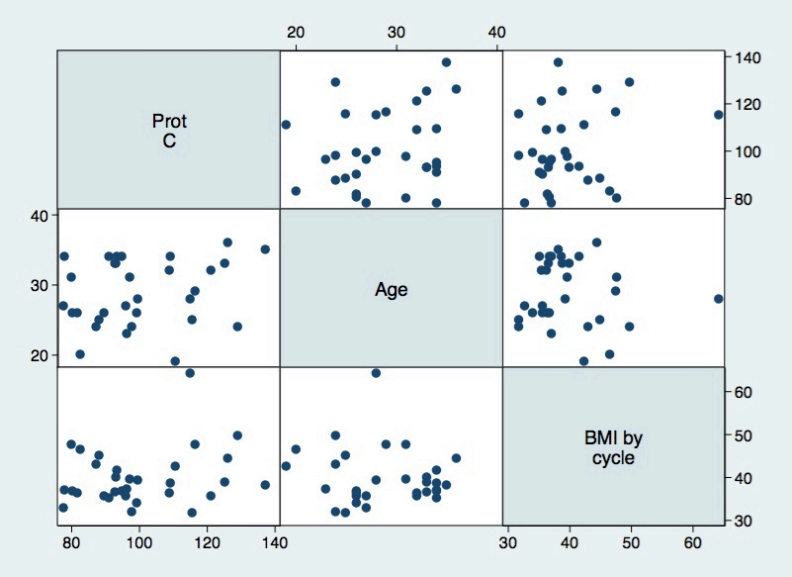
```
. swilk protc if cycle==0
```

Shapiro–Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
protc	31	0.94169	1.899	1.329	0.09189



Linearity of continuous variables:



Appendix D.2

```
. xi:mixed protc i.dose age bmibycycle i.race i.parity || id: cycle, cov(unstr)
i.dose            _Idose_0-3          (naturally coded; _Idose_0 omitted)
i.race            _Irace_0-1          (naturally coded; _Irace_0 omitted)
i.parity          _Iparity_0-1        (naturally coded; _Iparity_0 omitted)

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0:   log likelihood = -356.56935
Iteration 1:   log likelihood = -356.56074
Iteration 2:   log likelihood = -356.56074

Computing standard errors:

Mixed-effects ML regression                    Number of obs      =        93
Group variable: id                             Number of groups   =        31

                                               Obs per group: min =         3
                                                             avg =       3.0
                                                             max =         3


                                               Wald chi2(7)       =     22.01
Log likelihood = -356.56074                    Prob > chi2        =    0.0025
```

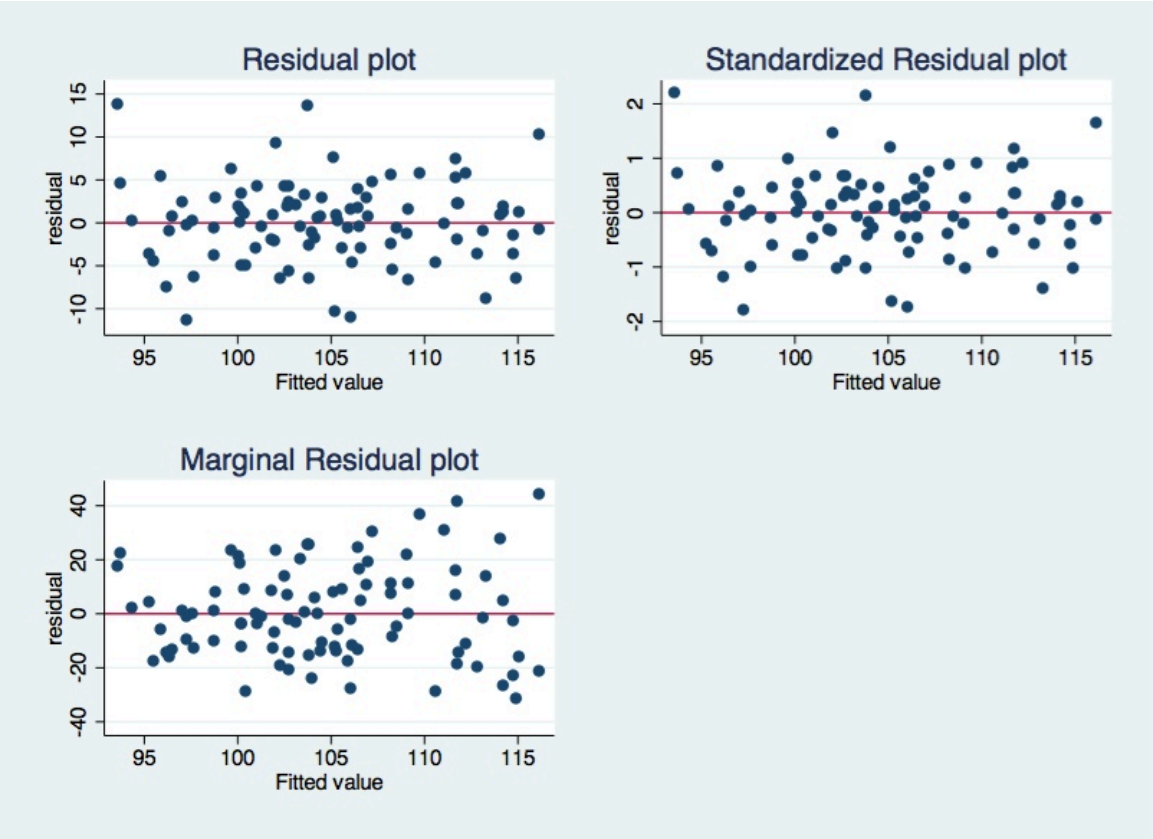
protc	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
_Idose_1	3.962565	2.895401	1.37	0.171	-1.712316	9.637446
_Idose_2	9.436952	2.817729	3.35	0.001	3.914305	14.9596
_Idose_3	6.476514	1.648597	3.93	0.000	3.245322	9.707706
age	.7040674	.5959125	1.18	0.237	-.4638996	1.872034
bmibycycle	.3814784	.4020573	0.95	0.343	-.4065393	1.169496
_Irace_1	-3.319451	7.927186	-0.42	0.675	-18.85645	12.21755
_Iparity_1	3.989255	6.235984	0.64	0.522	-8.233049	16.21156
_cons	64.04605	24.26087	2.64	0.008	16.49562	111.5965

Random-effects Parameters	Estimate	Std. Err.	[95% Conf.	Interval]
id: Unstructured				
var(cycle)	4.132695	2.557857	1.228563	13.90174
var(_cons)	190.1257	55.51833	107.2711	336.9759
cov(cycle,_cons)	10.62447	8.405164	-5.849348	27.09829
var(Residual)	40.05877	10.25001	24.26034	66.14519

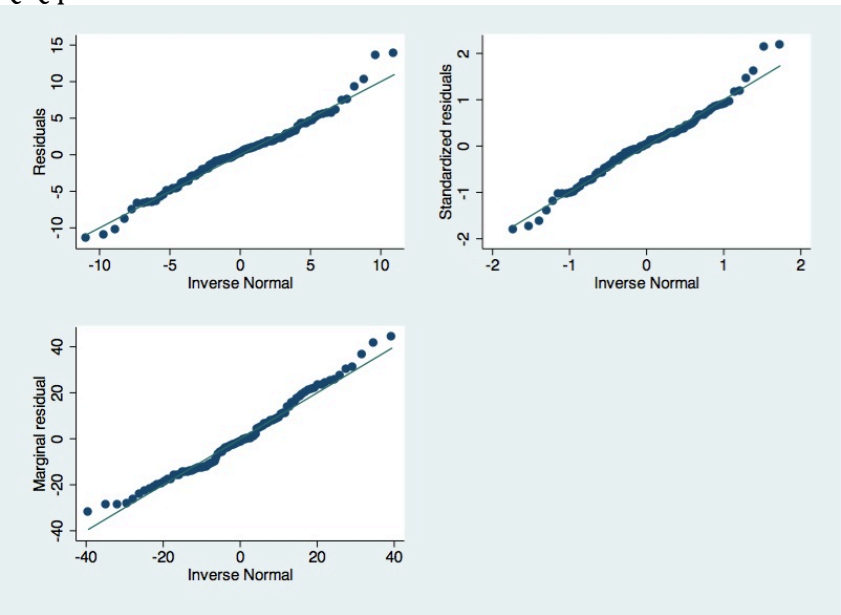
LR test vs. linear regression: chi2(3) = 77.10 Prob > chi2 = 0.0000

Note: LR test is conservative and provided only for reference.

Appendix D.3
Model Diagnostics:
Residual plots



Q Q plots



```
. swilk pred_protc res_protc standard_res_protc marg_res_protc
```

Shapiro–Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
pred_protc	**93**	**0.97511**	**1.934**	**1.458**	**0.07244**
res_protc	**93**	**0.98354**	**1.279**	**0.544**	**0.29321**
standard_r~c	**93**	**0.98354**	**1.279**	**0.544**	**0.29321**
marg_res_p~c	**93**	**0.97794**	**1.714**	**1.191**	**0.11683**

```
. estat ic
```

Akaike's information criterion and Bayesian information criterion

Model	Obs	ll(null)	ll(model)	df	AIC	BIC
.	**93**	.	**−356.5607**	**12**	**737.1215**	**767.5127**

Note: N=Obs used in calculating BIC; see **[R] BIC note**

Appendix D.4

```
. testparm _Idose_*

 ( 1)  [protc]_Idose_1 = 0
 ( 2)  [protc]_Idose_2 = 0
 ( 3)  [protc]_Idose_3 = 0

           chi2(  3) =   19.31
         Prob > chi2 =    0.0002

. lincom _Idose_2 - _Idose_3

 ( 1)  [protc]_Idose_2 - [protc]_Idose_3 = 0
```

protc	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	2.960437	2.647045	1.12	0.263	-2.227675	8.14855

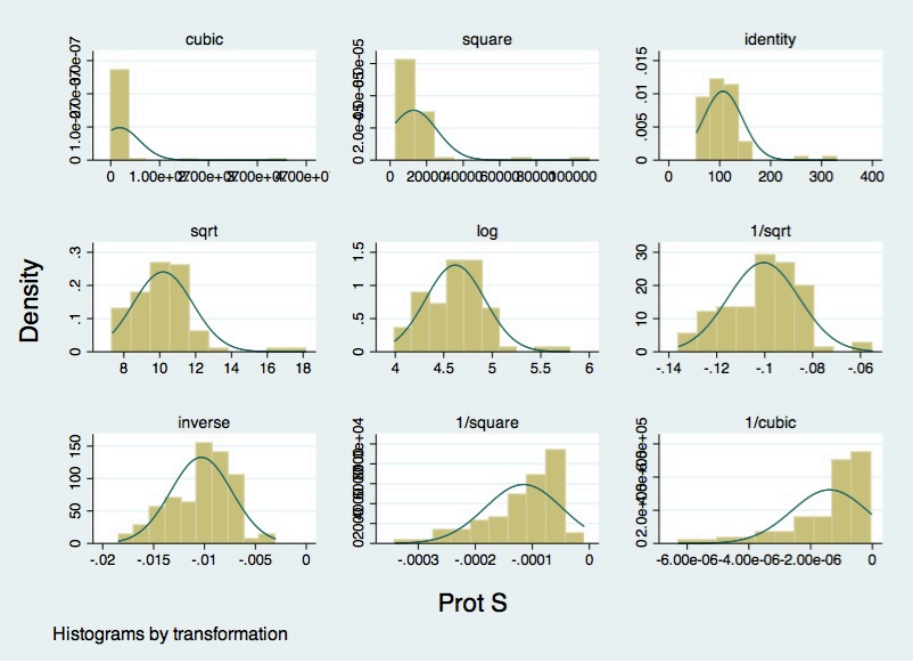
```
. lincom  _Idose_2 - _Idose_1

 ( 1)  - [protc]_Idose_1 + [protc]_Idose_2 = 0
```

protc	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	5.474387	3.709681	1.48	0.140	-1.796454	12.74523

```
. lincom _Idose_3 - _Idose_1

 ( 1)  - [protc]_Idose_1 + [protc]_Idose_3 = 0
```

protc	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	2.513949	2.729496	0.92	0.357	-2.835764	7.863663

Appendix E. 1 – Protein S models

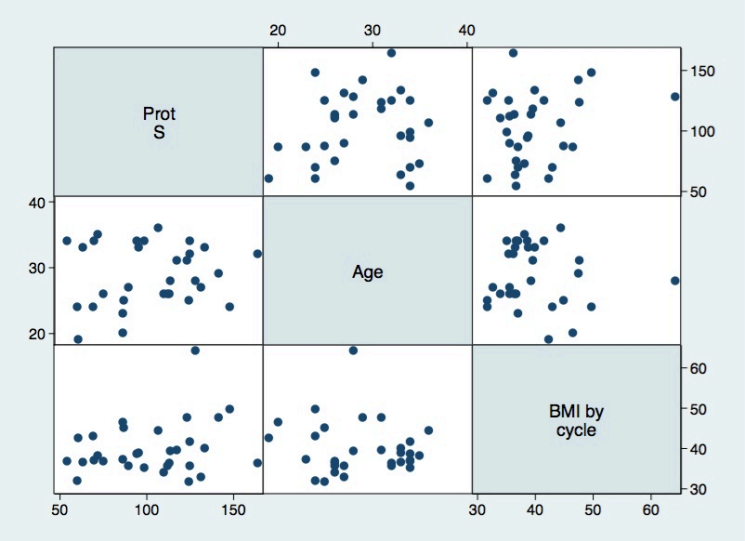



```
. swilk prots if cycle==0
```

Shapiro–Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
prots	**31**	**0.97057**	**0.959**	**−0.087**	**0.53479**

Linearity with continuous variables:



Appendix E.2

```
. . xi:mixed prots i.dose age bmibycycle i.race i.parity || id: cycle, cov(unstr
> )
i.dose            _Idose_0-3          (naturally coded; _Idose_0 omitted)
i.race            _Irace_0-1          (naturally coded; _Irace_0 omitted)
i.parity          _Iparity_0-1        (naturally coded; _Iparity_0 omitted)

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0:   log likelihood = -441.01599
Iteration 1:   log likelihood = -440.30123
numerical derivatives are approximate
nearby values are missing
Iteration 2:   log likelihood =  -440.2959
numerical derivatives are approximate
nearby values are missing
Iteration 3:   log likelihood =  -440.2959

Computing standard errors:

Mixed-effects ML regression                     Number of obs      =        93
Group variable: id                              Number of groups   =        31

                                                Obs per group: min =         3
                                                               avg =       3.0
                                                               max =         3


                                                Wald chi2(7)       =      8.85
Log likelihood =  -440.2959                     Prob > chi2        =    0.2638
```

prots	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
_Idose_1	2.333661	6.856625	0.34	0.734	-11.10508	15.7724
_Idose_2	5.467091	6.669728	0.82	0.412	-7.605335	18.53952
_Idose_3	7.593227	4.921138	1.54	0.123	-2.052026	17.23848
age	1.651034	1.254865	1.32	0.188	-.8084562	4.110525
bmibycycle	1.406943	.8490814	1.66	0.098	-.2572263	3.071112
_Irace_1	21.18928	16.70874	1.27	0.205	-11.55925	53.93782
_Iparity_1	-11.38248	13.12198	-0.87	0.386	-37.10109	14.33613
_cons	-.4150077	51.19909	-0.01	0.994	-100.7634	99.93337

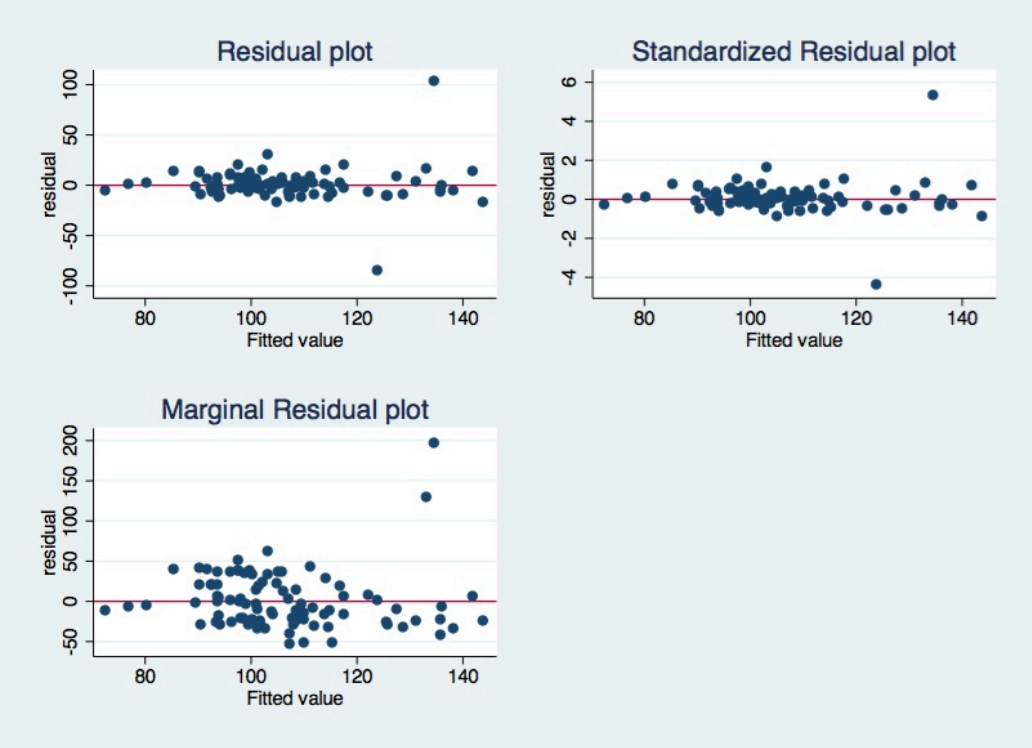
Random-effects Parameters	Estimate	Std. Err.	[95% Conf.	Interval]
id: Unstructured				
var(cycle)	3.87689	5.192003	.2808916	53.50918
var(_cons)	719.1415	243.9195	369.9155	1398.061
cov(cycle,_cons)	52.80182	33.89012	-13.6216	119.2252
var(Residual)	373.4257	67.7082	261.7384	532.7715

LR test vs. linear regression: chi2(3) = 46.51 Prob > chi2 = 0.0000

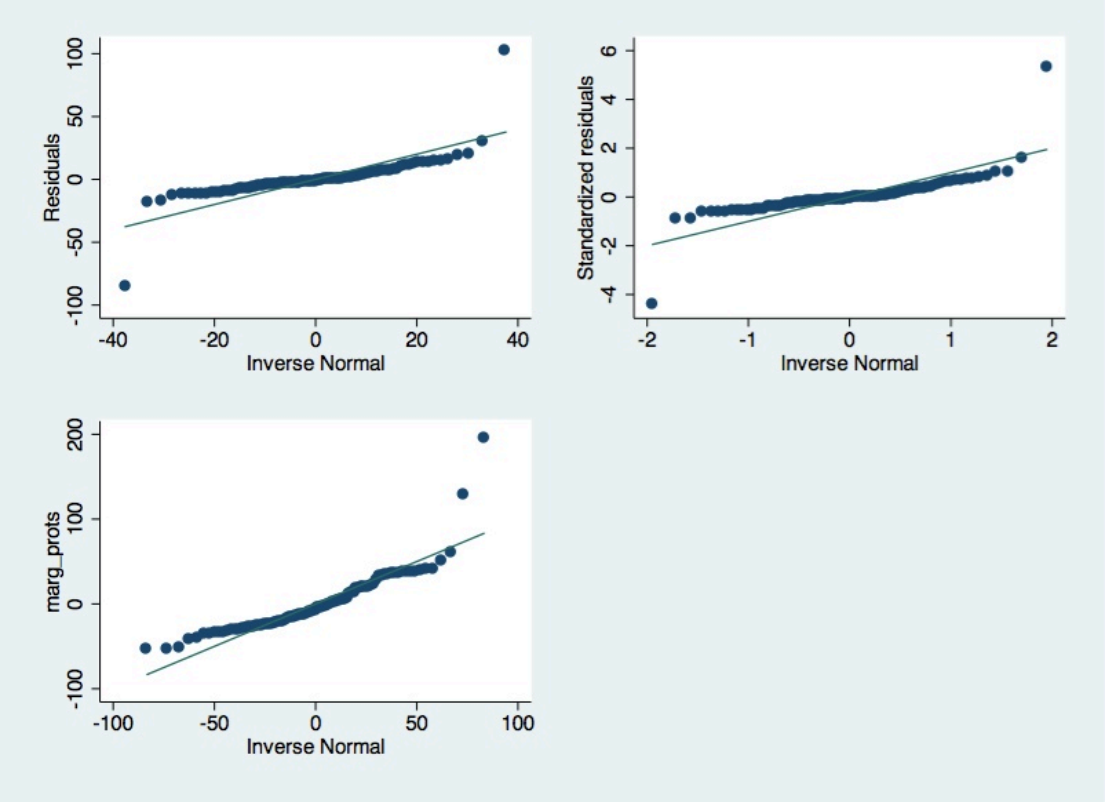
Note: LR test is conservative and provided only for reference.

Appendix E. 3

Residual plots:



Q-Q plots:



```
. swilk  res_prots standard_prots marg_prots
```

Shapiro–Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
res_prots	**93**	**0.65018**	**27.189**	**7.298**	**0.00000**
standard_p~s	**93**	**0.65018**	**27.189**	**7.298**	**0.00000**
marg_prots	**93**	**0.83194**	**13.062**	**5.678**	**0.00000**

```
. estat ic
```

Akaike's information criterion and Bayesian information criterion

Model	Obs	ll(null)	ll(model)	df	AIC	BIC
.	**93**	.	**−440.2959**	**12**	**904.5918**	**934.983**

Note: N=Obs used in calculating BIC; see **[R] BIC note**

Appendix E.4

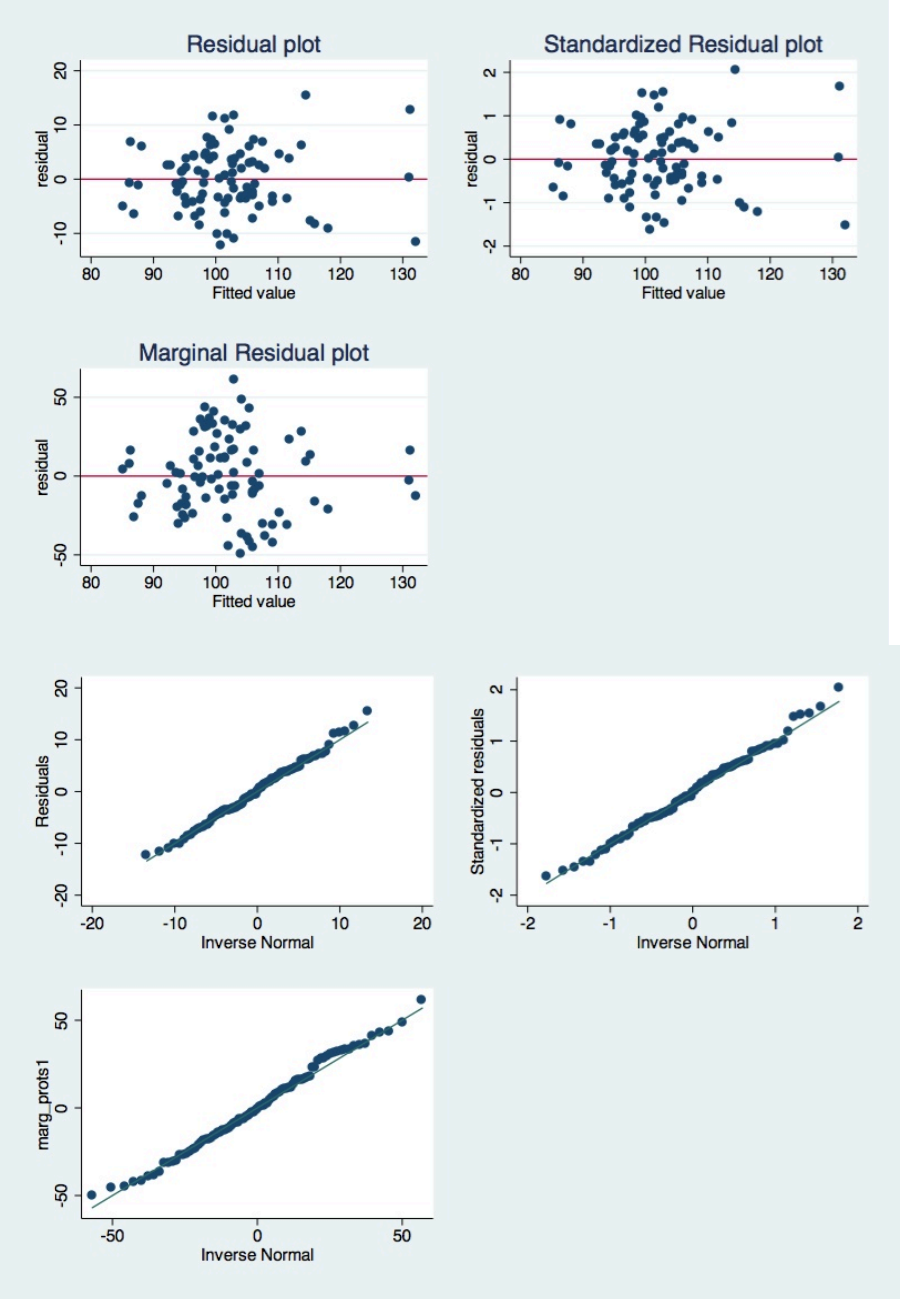
```
. xi:mixed prots i.dose age bmibycycle i.race i.parity || id: cycle, cov(unstr)
i.dose            _Idose_0-3          (naturally coded; _Idose_0 omitted)
i.race            _Irace_0-1          (naturally coded; _Irace_0 omitted)
i.parity          _Iparity_0-1        (naturally coded; _Iparity_0 omitted)

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0:   log likelihood = -365.18703
Iteration 1:   log likelihood = -365.16294
Iteration 2:   log likelihood = -365.16288
Iteration 3:   log likelihood = -365.16288

Computing standard errors:

Mixed-effects ML regression                     Number of obs      =        90
Group variable: id                              Number of groups   =        30

                                                Obs per group: min =         3
                                                               avg =       3.0
                                                               max =         3


                                                Wald chi2(7)       =      5.75
Log likelihood = -365.16288                     Prob > chi2        =    0.5692
```

prots	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
_Idose_1	-2.288399	2.997053	-0.76	0.445	-8.162515	3.585717
_Idose_2	1.537769	3.007532	0.51	0.609	-4.356886	7.432423
_Idose_3	1.096194	1.976136	0.55	0.579	-2.776961	4.969349
age	.2571413	.95334	0.27	0.787	-1.611371	2.125653
bmibycycle	1.034193	.593068	1.74	0.081	-.1281987	2.196585
_Irace_1	-6.341327	13.34784	-0.48	0.635	-32.50261	19.81996
_Iparity_1	-9.490828	9.926344	-0.96	0.339	-28.94611	9.964449
_cons	57.33438	37.60287	1.52	0.127	-16.36589	131.0347

Random-effects Parameters	Estimate	Std. Err.	[95% Conf.	Interval]
id: Unstructured				
var(cycle)	2.903531	3.000856	.382995	22.01202
var(_cons)	612.5977	173.6566	351.4636	1067.752
cov(cycle,_cons)	-22.46771	17.47856	-56.72506	11.78963
var(Residual)	57.1144	14.75942	34.4175	94.77894

```
LR test vs. linear regression:       chi2(3) =    99.01   Prob > chi2 = 0.0000

Note: LR test is conservative and provided only for reference.
```

Model diagnostics:




```
. swilk  res_prots1 standard_prots1 marg_prots1

                    Shapiro-Wilk W test for normal data

    Variable |   Obs       W           V         z       Prob>z
-------------+--------------------------------------------------
  res_prots1 |    90    0.99150       0.643    -0.973    0.83476
standard_p~1 |    90    0.99150       0.643    -0.973    0.83476
 marg_prots1 |    90    0.98718       0.970    -0.068    0.52717
```

Appendix E.5

```
. estat ic

Akaike's information criterion and Bayesian information criterion
```

Model	Obs	ll(null)	ll(model)	df	AIC	BIC
.	90	.	-365.1629	12	754.3258	784.3235

Note: N=Obs used in calculating BIC; see [R] BIC note

```
. lincom  _Idose_2  -  _Idose_3

 ( 1)  [prots]_Idose_2 - [prots]_Idose_3 = 0
```

prots	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	.4415746	2.889817	0.15	0.879	-5.222362	6.105511

```
. lincom  _Idose_2 - _Idose_1

 ( 1)  - [prots]_Idose_1 + [prots]_Idose_2 = 0
```

prots	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	3.826168	3.823209	1.00	0.317	-3.667185	11.31952

```
. lincom _Idose_3 - _Idose_1

 ( 1)  - [prots]_Idose_1 + [prots]_Idose_3 = 0
```

prots	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	3.384593	2.883886	1.17	0.241	-2.26772	9.036906

```
. testparm _Idose_*

 ( 1)  [prots]_Idose_1 = 0
 ( 2)  [prots]_Idose_2 = 0
 ( 3)  [prots]_Idose_3 = 0

           chi2(  3) =    1.57
         Prob > chi2 =    0.6665
```