Cardiovascular Risk Biomarkers among Oral and Vaginal Hormonal Contraceptive Users

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Dedication

I would like to dedicate this thesis work to my loving girlfriend, Katherine Krieser.

Abstract

Combined hormonal contraceptives (CHCs) possess an inherent risk of thrombus related events such as stroke, coronary heart disease, venous thrombosis and pulmonary embolism. Recently, *NuvaRing*, a vaginal CHC, has been introduced to the market. The purpose of this study is to elucidate alterations in cardiovascular risk biomarkers among young women using combined oral contraceptives (COCs) or the *NuvaRing* compared to a non-CHC using control group. We observed that *NuvaRing* users were older ($p<0.0001$) and had a greater incidence of family history of cardiovascular disease (CVD)/stroke ($p=0.034$) when compared to the control. COC users reported having a more regular sleeping habit ($p=0.010$). The COC and *NuvaRing* groups had more white participants and they consumed alcohol more often. No significant alterations were observed in complete blood cell count with differentials, plasminogen activator inhibitor-1, thrombin/antithrombin-III, D-dimer, fibrinogen or von Willebrand factor. After adjusting for age, race, alcohol consumption, sleeping habit and family history of CVD/stroke, when compared to control subjects we observed COC and *NuvaRing* users had elevated levels of C-reactive protein and Factor VII while having lower soluble E-selectin, soluble thrombomodulin, and Tissue Factor Pathway Inhibitor concentration. COC users had a significantly elevated soluble CD40 ligand level when compared to both control ($p<0.0001$) and *NuvaRing* ($p<0.0001$) but the *NuvaRing* CD40 ligand level was essentially identical to the control ($p=0.5996$). When compared to the control group, COC users had reduced levels of soluble P-selectin ($p=0.0029$)

while *NuvaRing* users also had reduced levels but failed to reach significance (p=0.011). These results indicate significant alterations in several inflammatory and procoagulant blood biomarkers among CHC users. Larger, long-term longitudinal studies are required to further assess the effect of these biomarker alterations on vascular events.

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Nomenclature

ACT: Activated Clotting Time
BMI: Body Mass Index
BP: Blood Pressure
CBC: Complete Blood Count
CHC: Combined Hormonal Contraceptive
COC: Combined Oral Contraceptive
CRP: C-Reactive Protein
CVD: Cardiovascular Disease
DBP: Diastolic Blood Pressure
ELISA: Enzyme Linked Immunosorbant Assay
FH: Family History
FSH: Follicle Stimulating Hormone
FVII: Factor VII
gb-ACT: glass bead Activated Clotting Time
GnRH: Gonadotropin-Releasing Hormone
HC: Hormonal Contraceptive
HTN: Hypertension
IQR: Interquartile Range
LH: Luteinizing Hormone
MAP: Mean Arterial Pressure
MI: Myocardial Infarction
non-ACT: non Activated Clotting Time
PAI-1: Plasminogen Activator Inhibitor-1
PR: Pulse Rate
SBP: Systolic Blood Pressure
SD: Standard Deviation
sE-selectin: soluble E-selectin
sP-selectin: soluble P-selectin
sTM: soluble Thrombomodulin
TAT-III: Thrombin-Antithrombin III
TF: Tissue Factor
TFPI: Tissue Factor Pathway Inhibitor
UTI: Urinary Tract Infection
US: United States
VT: Venous Thromboembolism
vWF: von Willebrand Factor
WBC: White Blood Cell
WTH: Waist to Hip Ratio

Introduction

Brief History of Hormonal Contraception

In 1960 the first combined hormonal contraceptive (CHC), *Enovid*, was approved for sale in the United States (US) (1). Although this approval was extremely controversial for moral and religious reasons, CHC's effectiveness and ease of use for pregnancy prevention quickly boosted their popularity. However, not long after *Envoid's* introduction, case study reports began being published describing thrombotic complications among CHC users who otherwise had no known thrombotic risk factors (2, 3). From their inception to their current formulation, steps have been taken to reduce the incidence of thrombosis in CHC users by reducing hormone dose, altering the hormone derivatives used, and using different methods of administration (4). However, in spite of these alterations, users of CHCs still possess a 3-5 fold increased thrombotic risk when compared to non-CHC users (5).

Today the CHC pill is used by approximately 10.7 million women in the US alone representing 17.3% (+/-0.8) of sexually active women 15-44 years of age (6). The absolute risk of thrombotic complications related to CHC use among premenopausal women is seemingly quiet low (approximately 3.01 per 10,000 women years for non-users compared to 6.29 per 10,000 women years for CHC users (7)). However due to the widespread and ever increasing use of CHCs, CHC related thrombosis continues to be a significant public health problem. This risk is further exacerbated when CHC use is combined with other thrombotic risk

factors such as smoking, genetic predisposition, obesity, hypertension (HTN), and diabetes (8-10).

CHCs Effect on the Menstrual Cycle

CHCs prevent pregnancy by continually elevating levels of the female steroid hormones estrogen and progestin. Progestin is a generic term for a variety of synthetic derivations of the naturally occurring hormone progesterone. By elevating estrogen and progestin, the hormonal balance is altered to levels similar to a state of pregnancy, thus greatly reducing the chance of actual pregnancy. The progestin component of CHCs acts in a negative feedback loop to decrease the hypothalamus' release of gonadotropin-releasing hormones (GnRH). This decrease in GnRH thereby reduces the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the anterior pituitary. A drop in FSH prevents follicular development and subsequently increases estradiol levels. The progestin negative feedback and reduction of LH release inhibits the LH surge normally seen just prior to ovulation. By inhibiting follicular development and preventing the LH surge, CHC's are able to effectively block ovulation and make fertilization of the ovum essentially impossible (11). Secondary effects of hormonal contraception are perhaps equally important for pregnancy prevention. One such secondary mechanism is the thickening of cervical mucus (12). Progestin is primarily responsible for thickening the cervical mucus to the point where it is impossible for sperm to enter the uterus through the cervix, thus preventing fertilization of the ovum.

Currently the market in the US consists of a wide variety of hormonal contraceptives (HCs) differing in hormone type, concentration and delivery method (4). In spite of these differences, all approved HC methods function in the same primary method of suppressing ovulation along with secondary functions such as increasing cervical mucus viscosity. Many different methods have been developed for hormone delivery such as the oral progestin-only "mini pill", the vaginal ring, hormone injection and trans-dermal patches.

Combined Oral Contraceptives

Combined oral contraceptives (COCs) contain synthetic derivatives of estrogen and progestogen taken orally. Despite their inherent thrombotic risk, COC's remain the most common method of hormonal birth control used today (6). In COC's, it appears that the estrogen component is primarily responsible for the increased risk of thrombosis (7). While not necessarily required for contraceptive benefit, the estrogen component has been retained to reduce the instance of undesired side effects such as breakthrough bleeding, headaches and loss of libido (13,14). The greatest reduction of thrombotic risk was accomplished through the reduction of hormone dose used in COC formulations (4). For instance, *Enovid* contained 9.85 mg norethynodrel (a progestin) and 150 µg mestranol (an estrogen). Current, "low dose" COC formulations contain 20-35 µg estrogen and 0.15-3.0 mg of progestin representing an approximate 5-10 fold reduction in hormone concentration (4). However, despite this effort, a recent large scale study revealed a two-fold increased risk of MI and stroke for COC users when compared to nonusers (15).

Contraceptive Generations

While the estrogen used in CHC composition has remained consistent (ethinyl estradiol), there are many different types of progestin used. Currently marketed CHCs are loosely categorized into three “generations” based on their progestin component. First generation CHCs use estrane progestins such as norethindrone and its derivatives northerindrone acetate, ethycodiol acetate, lynestrenol and norethynodrel. Second generation CHCs use derivatives of the gonane progestins such as dl-norgestrel and levonorgestrel. Third generation CHCs utilize gonane progestins such as desogestrel, gestodene and norgestimate (4). It was assumed that third generation progestins would eventually replace the preceding generations due to their higher biological effectiveness, lower androgenicity and lower risk of adverse metabolic effects (16).

In the mid 1990’s published data suggested an increased risk of thrombotic events among third generation COC users when compared to second generation with odds ratios ranging from 1.5 to 2.2 (17-20). This series of reports resulted in what is referred to as the “pill scare” in which the first time prescription of third generation contraceptives dropped in the decade following these published reports (21). A recent case-control study analyzing venous thromboembolism (VT) events concluded that either confounding variables in the pill scare reports skewed the results or that the difference has somehow disappeared over time (22). Although still somewhat controversial, third generation contraceptives are widely used.

The concept of “total estrogenicity” has been introduced recently to help explain the different risk levels of thrombosis related to the different generations of progestin used in COCs (23, 24). As the term indicates, total estrogenicity refers to the level of estrogen biologically available as opposed to the dose given. This is due to a variable anti-estrogen affect that progestins exhibit. It has been suggested that third generation progestins possess a muted anti-estrogenic activity, and therefore, are less able to counteract the prothrombotic tendency of estrogen (23, 24). This may explain why users of third generation COCs could be predisposed to increased risk of thrombosis.

Vaginal Contraceptive Ring

Relatively new to the marketplace is the vaginal contraceptive ring, which is currently marketed only under the brand name *NuvaRing* (25). The hormone releasing flexible plastic ring is inserted vaginally and left in place for 3 weeks and removed allowing for normal menstruation (26). Because of the more direct method of hormone delivery which largely bypasses hepatic circulation, the *NuvaRing* is able to achieve contraception by continuously delivering low doses of the hormones ethinyl estradiol (15µg/day) and the third generation progestin, etonogestrel (120µg/day) (27). A comparative study between users of the *NuvaRing* and a COC containing 30µg ethinyl estradiol and 150µg desogestrel found that the maximum serum levels of ethinyl estradiol were 30-40% of what was seen in the COC group (27). The *NuvaRing* is believed to lower the risk of thrombotic events due to its lower hormonal dose, and its avoidance of first pass hepatic circulation. The liver is responsible for producing the majority of the

coagulation factor proteins (28). Since first pass hepatic circulation is mostly bypassed, the reduced bioavailability of estrogen and progestin at the liver results in a muted up-regulation of the coagulation factors compared to COCs (29). One recent study has shown a beneficial change in a limited number of biomarkers of thrombosis (sex hormone binding globulin, free protein S and activated protein C resistance) in CHC users who switch from COCs to the *NuvaRing* (30). However, this study has been challenged with evidence suggesting an opposite correlation in biomarkers of thrombosis (31). Due to the relatively recent development of the *NuvaRing* and its limited use (6), large case control studies of clinical thrombotic events have been limited to date. The Transatlantic Active Surveillance on Cardiovascular Safety of *NuvaRing* (TASC) clinical trial is currently ongoing with study completion expected in September 2011. The TASC study is aimed to assess the short- and long-term risks associated with *NuvaRing* use versus COC use.

Cardiovascular Disease

Cardiovascular disease (CVD) possesses the highest rate of morbidity and mortality worldwide and continues to increase in the US population (32). CHC use has been shown to lead to an increased incidence of vascular events such as VT, peripheral arterial disease, pulmonary embolism (PE), myocardial infarction (MI), and stroke. (15, 33-43). Although the absolute risk of HC related thrombosis is low—3.0 per 10,000 women per year among CHC users compared to 0.8 per 10,000 women per year among premenopausal women not using

CHCs (44), it still results in the majority of thrombosis related cardiovascular events in young women due to its widespread use.

Users of low dose COCs have between a 1.4 and 3.4 elevated relative risk of ischemic stroke (IS) when compared to non-users (45-48). Interestingly, the rate of intracerebral hemorrhage is also elevated (approximately by 2.5 fold) in COC users over the age of 35, but is equal to that of non-users in women younger than 35 years old (49). Again, this risk is further exacerbated by smoking in women over the age of 35 resulting in a 5 fold increase in risk when compared to non smokers who did not use COCs (54).

COC users have an approximate 1.7-4.7 risk of MI when compared to non users (42). A meta-analysis of current COC users estimated a 2.48 overall adjusted odds ratio of MI [95% confidence interval: 1.91-3.22] when compared to never-users (43).

Hemostasis

Prior to analyzing the effects of hormones on coagulation, it is important to first review the basic principles related to hemostasis and thrombosis. According to Virchow's Triad (Figure 1), blood coagulation is a result of alterations occurring within three broad physiological constraints; hypercoagulability, hemostasis and endothelial injury.

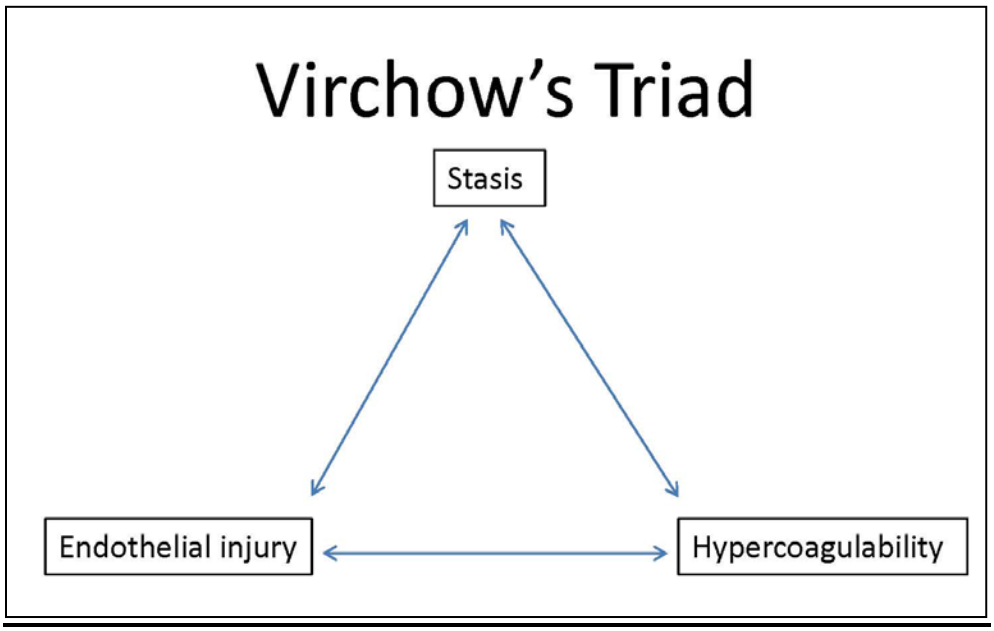

Figure 1: Virchow's Triad describing the three factors related to coagulation: stasis, endothelial injury and hypercoagulability.

Virchow's triad is important in regard to HC related thrombosis because increased thrombotic risk is not simply measured by a change in one protein concentration or inflammatory signal, but it is rather a culmination of several alterations which result in a greater risk for thrombosis. A non-significant hypercoagulable state could then be further exacerbated when other common cardiovascular risk factors are present. Studies in coagulation and thrombosis have yielded better insight into the field of HC and its effects on the coagulation cascade. It has been shown that the estrogen components of CHC's promote the synthesis of coagulation factors (50), decreases fibrinolysis (51) and increases blood pressure (BP) (52, 53)—thus yielding a pro-coagulant phenotype.

Blood Biomarkers

This study focuses on the examination of a pro-coagulant phenotype by measuring biomarkers implicated in the prediction of adverse cardiovascular events. Biomarkers can be objectively measured and interpreted as a marker of normal biological processes and alterations within. Upon planning this study, we sought to identify and quantify biomarkers which had previously been associated with cardiovascular risk and thrombosis representing a broad overview of coagulation and inflammation. Cardiovascular risk assessment was accomplished by measuring a variety of protein levels, inflammatory markers and cellular concentrations which have been implicated as cardiovascular risk markers. Among these are: soluble E-selectin (sE-selectin), soluble P-selectin (sP-selectin), soluble CD40 ligand (sCD40L), Tissue Factor (TF), Plasminogen Activator Inhibitor-1 (PAI-1), soluble Thrombomodulin (sTM), Tissue Factor Pathway Inhibitor (TFPI), Thrombin/Antithrombin III (TAT-III), von Willebrand Factor (vWF), Factor VII (FVII), complete blood count (CBC) with differentials, D-Dimer, fibrinogen, and C-reactive protein (CRP) concentration. This study also assessed the *in vitro* coagulation profile of each research participant using the *Sonoclot® Coagulation and Platelet Function Analyzer (Sienco, Inc., Arvada*, Colorado).

Physiological Measures

Besides the blood biomarkers used, we also collected physical measures on each study participant. These measures include BP, body mass index (BMI)

and waist to hip ratio (WTH). While there isn't any evidence suggesting a correlation between HC related thrombosis due to BMI and WTH, both of these measures closely associated with CVD. A recent study revealed that monitoring BMI and waist circumference in a cohort of adolescent Caucasian females was able to predict those more at risk of developing CVD (54).

Coagulation Proteins

Elevation or reduction in certain protein levels relevant to coagulation have been implemented in propensity for coagulation, or a "pro-coagulant phenotype". Alterations in virtually any protein involved in the coagulation cascade (Figure 2) can have drastic cardiovascular implications. The protein fibrinogen is vital in coagulation. Fibrinogen is proteolytically cleaved by the enzyme thrombin to form fibrin. Many fibrin monomers are polymerized and then cross-linked by Factor XIII to form a thrombus (55). Therefore, elevations in fibrinogen concentration have been associated with increased risk of CVD (56). Fibrinogen level is commonly measured in the clinical laboratory for this purpose. Several published studies have shown significant increases in fibrinogen level and activity due to CHC use (57-59).

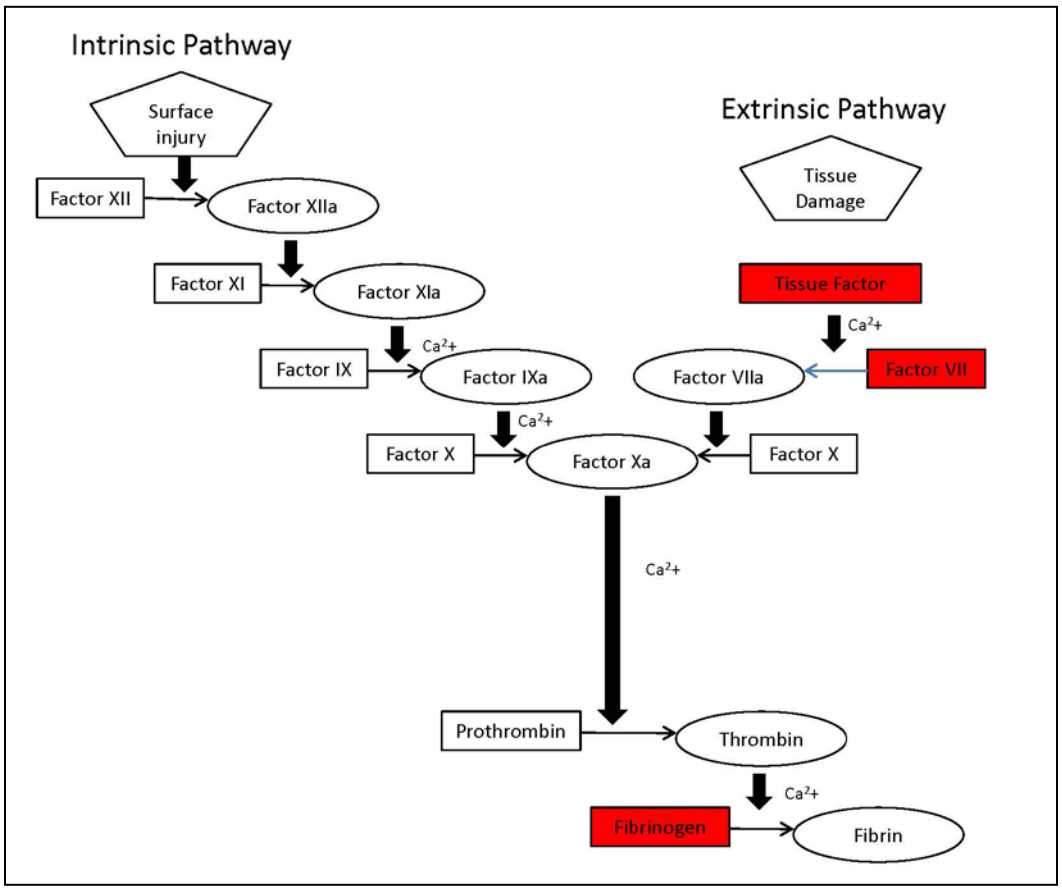

Figure 2: Coagulation Cascade; proteins highlighted in red were measured in our study.

While fibrinogen is one of the most commonly analyzed coagulation proteins, alterations in a variety of proteins involved in coagulation and fibrinolysis can also contribute to cardiovascular problems. For instance, TF also called Factor III or CD142, is the primary initiator of the extrinsic coagulation cascade pathway. TF is a glycoprotein expressed in platelets and in the subendothelium of the vasculature. Upon vessel wall damage or platelet activation, TF binds to FVII, converting it to active FVII, ultimately resulting in thrombin activation and the conversion of fibrinogen to fibrin. Therefore, elevated levels of TF in circulation predispose individuals to coagulation (60).

Elevated levels of FVII also push the extrinsic coagulation cascade toward clot formation in a similar mechanism. Because TF and FVII are so closely related within the extrinsic pathway of coagulation, if one of these proteins is elevated, it is going to push the extrinsic pathway toward coagulation. It has been shown that CHCs increase production of FVII and are perhaps partially responsible for increasing the cardiovascular risk associated with CHC use (61).

Since the extrinsic pathway of coagulation is so vital for cardiovascular risk assessment, regulation of that process is also important to note. For instance, TFPI is the primary regulator of the extrinsic coagulation pathway. TFPI is a single chain polypeptide produced by endothelial cells and released upon heparin stimulation. TFPI acts by binding to and inhibiting the function of activated Factor X (FXa). The TFPI/FXa complex can then further reduce coagulation by inactivating the TF/VIIa complex. So one would expect a decrease in TFPI to lead in deregulation of the extrinsic pathway and increase thrombosis propensity. In has been shown that COCs do in fact reduce concentrations of TFPI in COC users (62).

VWF is a high molecular weight glycoprotein synthesized by endothelial cells and platelet precursor cells (megakaryocytes). VWF plays an important role in the adhesion of platelets to the endothelium as well as transporting Factor VIII and protecting it from degradation (63). Therefore, elevated levels of vWF have been shown to increase platelet/endothelial interaction and activation (64). A limited number of studies have analyzed vWF in CHC users and show either a slight increase (65) or no change (57) in vWF levels among CHC users.

Inflammatory Biomarkers

Several biomarkers of inflammation were tested in this study. Chronic inflammation has been closely associated with atherosclerosis which subsequently increases relative risk of MI and stroke (40). In the case of atherosclerosis, white blood cells (WBCs) called macrophages and neutrophils release lytic substances which damage the surrounding endothelium resulting in an inflamed environment. Inflammatory cytokines released from endothelial cells and immune cells, trigger the liver to produce CRP during the acute phase of inflammation. Normally, CRP goes on to activate the complement system to help facilitate the clearance of microorganisms and damaged cells. Therefore, CRP can be used as a global measure of inflammation. In fact, Kip et al. (66) positively correlated global inflammation and CRP level to cardiovascular risk in women. Furthermore, elevated CRP levels have been linked to elevated chance of future MI and stroke as well as worsening outcomes (67). It has been well established that CRP increases dramatically in users of COC (57, 68, 69). However, the clinical usefulness of CRP measurement for CHC related thrombosis has been controversial due to the argument that systemic inflammation is not occurring despite the rise in serum CRP concentration (69, 70).

Similarly, sCD40L and sE-selectin are markers for chronic inflammation and positively associated with cardiovascular risk and atherosclerosis. CD40L is shed from stimulated lymphocytes and is released from platelets upon activation. Therefore, measurement of sCD40L not only gives insight into the inflammatory

response of leukocytes but also provides an indirect marker of platelet activation—both of which increase cardiovascular risk (71). Little work has been done to evaluate the level of sCD40L in healthy HC users; however one recent study of polycystic ovary syndrome patients, of which COCs are a common method of treatment for, found a relative increase in the plasma CD40L concentration in those patients using COCs (72). E-selectin is expressed by endothelial cells in response to inflammatory cytokines released by activated leukocytes. Normally E-selectin allows for neutrophil binding and extravasation from the vasculature into inflamed tissue. In the case of CVD, however, elevated levels of sE-selectin indicate general endothelial damage and increased risk of CVD (64).

The CBC test is a routine analysis of blood cell count conducted at virtually every medical facility and is one of the first tests ordered for patients with suspected coagulation abnormalities (73). For the objectives of this study, evaluation of CBC lies in analysis of leukocyte and platelet count. Elevated lymphocyte, macrophage and neutrophil concentrations are non-specific markers for inflammation and cardiovascular risk. While platelet count is not specific for inflammation, elevated platelet levels or thrombocytosis have been associated with increased cardiovascular risk (74).

Platelet Activation Biomarkers

Platelet activation markers are also important for deducing a pro-coagulant phenotype. Platelets function to prevent blood loss. In the case of

vascular injury, platelets are recruited to the site of damage, become activated, and aggregate to form a plug. During this process a number of physiological changes occur within the platelet. Surface receptors such as P-selectin are expressed, chemical constituents are secreted from intracellular granules, and platelet morphology is altered to enhance platelet/platelet and platelet/endothelium interactions. These surface molecules are eventually proteolytically cleaved from activated platelets and can be measured in soluble form within plasma. Therefore, sP-selectin concentration may be used as an indirect marker of platelet activation. Moreover, Ridker et al. (75) showed that sP-selectin is a good predictor of future cardiovascular events.

Fibrinolytic Biomarkers

Thrombus formation naturally occurs throughout blood vessels among healthy individuals; however in most instances natural lysing and breakdown processes are able to combat clot formation soon after it has formed, creating a balance between thrombosis and fibrinolysis. This task is effectively accomplished by a group of proteins referred to as plasminogen activators. When activated, plasminogen is converted into plasmin, which then proteolytically cleaves fibrin strands and breaks down the clot (Figure 3). Therefore, inhibitors of plasminogen activators, such as PAI-1, prevent natural process of clot lysis. As a result, decreased levels of PAI-1 have the potential to produce a procoagulant phenotype (76). COCs have previously been shown to decrease PAI-1 levels (59).

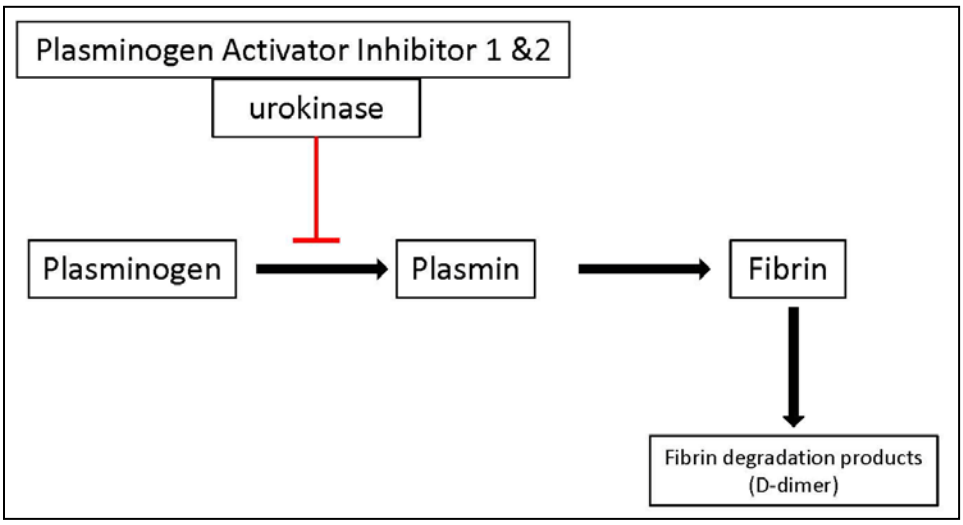

Figure 3: General schematic of fibrinolysis

Additionally, the endothelial surface receptor protein TM acts to help prevent clot formation. This occurs when the enzyme thrombin binds to TM following initiation of clot formation, which subsequently activates the anticoagulant protein, Protein C. While Protein C activation does not initiate clot lysis as does PAI-I, it prevents further coagulation within the vasculature. Therefore, lower levels of sTM result in lower levels of activated Protein C and thus a more procoagulant phenotype (76). However one study showed no significant correlation between CHC use and sTM (77).

Remnants of lysed clots can also serve as a marker for hypercoagulable state and cardiovascular risk assessment. Once clots have formed, they are broken down into small pieces of fibrin, called fibrin degradation products (Figure 3). These changes can be measured in the clinical laboratory setting by analyzing fibrin degradation products such as D-dimer and inactive enzyme constituents of coagulation such as the TAT-III complex. After a stable clot has

formed via fibrin cross-linking provided by Factor XIII, the clot is broken down by plasmin. The fibrin degradation product containing this crosslink is referred to as the D-dimer fragment. Therefore, elevated D-dimer levels are indicative of intravascular coagulation and thus, cardiovascular risk (25). While the fibrinolytic process is often able to combat this condition, prolonged exposure often results in thrombosis, particularity in the deep veins of the leg.

Global Coagulation as a Biomarker

While each of the above described biomarkers for cardiovascular risk assessment provides indirect measurements of pro-coagulant phenotype, perhaps the best *in vitro* method of accessing risk is by studying the coagulation time. Our group conducted whole blood activated clotting time (ACT) and clot rate (CR) using the Sonoclot device. This device operates by measuring the time in which a citrated blood sample clots once re-calcified. Non-activated clotting time (non-ACT) and glass bead activated clotting time (gb-ACT) cuvettes were used for testing. The non-ACT cuvettes allow the re-calcified blood to clot at 37ºC without any other activator of coagulation. The gb-ACT cuvette utilizes the strongly negatively charged silica surface of micro glass beads to activate the intrinsic coagulation cascade. Therefore, we would expect a research participant who possesses a

Figure 4: *Sonoclot Coagulation and Platelet Function Analyzer*

pro-coagulant phenotype to have a non-ACT which is shortened and a CR which is faster. The gb-ACT was done as a negative control to screen for any potential bleeding disorders.

Although many studies have analyzed CHC related adverse vascular clinical outcomes, relatively few studies have investigated the alterations in pro-thrombotic biomarkers among CHC users. Our study aims to access cardiovascular risk by measuring specific biomarkers for cardiovascular risk among a young, healthy female population aged 19-30 years old. We hypothesize that young women who have been actively taking COC or *NuvaRing* for at least 6 months will have biomarker levels indicative of increased cardiovascular risk relative to a non-HC user population. Additionally, we believe these levels will be exacerbated when HC use is combined with other established risk factors such as smoking, high BP, diabetes and obesity. Also, we propose that COCs will posses more dramatic alterations in cardiovascular risk biomarkers than the *NuvaRing* when compared to normal healthy controls.

Methods

We concentrated our study recruitment to healthy young women aged 19-30 years old in three separate categories of HC use in approximately equal proportions; 1) non-HC users, 2) COC users and 3) *NuvaRing* users. The study was approved by the Institutional Review Board at the University of Minnesota, Twin Cities. Our initial recruitment goal was 180 in a 1:1:1 ratio among the three groups however due to the unequal distribution of contraceptive use, this was difficult to accomplish. Participants were recruited at or around the university campus. Multiple recruiting modes were utilized including posting of advertising fliers, physician initiated recruitment at Boynton Health Services Women's Clinic and email advertisement in campus sorority newsletters. All subjects were informed to call a designated telephone number or e-mail the study coordinator to determine eligibility. Research participants were scheduled appropriately for an appointment at the Boynton Health Center, Women's Clinic. The specific inclusion/exclusion criteria are listed below:

Inclusion criteria:

1. Female subjects between 19 and 30 years old
2. Female subjects who have not used any form of HC for at least six (6) months
3. Female subjects who have used COC or *NuvaRing* for at least six (6) months

Exclusion criteria:

1. Male subjects
2. Subjects with recent history of viral or bacterial infection
3. Subjects with recent history of surgery

4. History of antiplatelet/anticoagulant medication use within two weeks of blood sample collections
5. Subjects with chronic inflammatory diseases
6. Subjects with history of malignancies
7. Subjects with known common hereditary coagulation abnormalities such as hemophilia, factor V Leiden, prothrombin mutation, and protein S or C deficiencies
8. Subjects with history of migraine ***with aura***
9. Subjects who are pregnant, or who have been pregnant within the past six (6) months
10. Signed and dated informed consent by the subject cannot be obtained

All participants signed and dated a consent form detailing their specific involvement in the study.

Subject Recruitment:

Once a subject had been properly consented for study participation, they were asked to fill out a questionnaire describing their past medical history, medical conditions since their last clinical exam, medical family history (FH), and lifestyle information such as smoking, alcohol consumption, use of illicit drugs, and sleeping habits. Also, their current and past HC use was recorded. Physical measurements of pulse rate (PR), BP (mmHg), height (cm), weight (kg), body temperature (ºF) as well as hip and waist circumference (cm) were taken by the research staff. Table 1 shows the variety of CHCs used in the study along with their formulation, generation of progestin used and total number of subjects currently using that formulation.

Table 1: Summary of all COCs that subjects were taking during the study.

HC Brand	Generation	Estrogen	Progestin	# of Subjects
Tri-Sprintec (generic version of Ortho Tri-Cyclen)	3rd	ethinyl estradiol (0.035 mg)	norgestimate (0.18-0.25 mg)	8
Microgestin	1st	ethinyl estradiol (0.02mg or 0.03 mg)	norethindrone (1 mg or 1.5 mg)	6
YAZ	3rd	ethinyl estradiol (0.03 mg)	drospirenone (3.0 mg)	5
Apri	3rd	ethinyl estradiol (0.03 mg)	desogestrel (0.15 mg)	4
Ocella	3rd	ethinyl estradiol (0.03 mg)	drospirenone (3.0 mg)	3
Aviane	2nd	ethinyl estradiol (0.02 mg)	levonorgestrel (0.10 mg)	3
Ortho Tri-cyclen	3rd	ethinyl estradiol (0.035 mg)	norgestimate (0.18-0.25 mg)	3
Yasmin	3rd	ethinyl estradiol (0.03 mg)	drospirenone (3.0 mg)	2
Kariva	3rd	ethinyl estradiol (0.02 mg)	desogestrel (0.15 mg)	2
Reclipsen	3rd	ethinyl estradiol (0.03 mg)	desogestrel (0.15 mg)	2
Portia	2nd	ethinyl estradiol (0.03 mg)	levonorgestrel (0.15 mg)	1
Junel	1st	ethinyl estradiol (0.03 mg)	norethindrone acetate (1.5 mg)	1
Enpresse	2nd	ethinyl estradiol (0.02/0.03/0.035 mg)	levonorgestrel (0.05/0.075/0.125 mg)	1
Loestrin FE	1st	ethinyl estradiol (0.02 mg)	norethindrone acetate (1 mg)	1
Lutera	2nd	ethinyl estradiol (0.02 mg)	levonorgestrel (0.1 mg)	1
Necon 1/35	1st	ethinyl estradiol (0.035 mg)	*norethindrone (*1 mg)	1
Cryselle	2nd	ethinyl estradiol (0.03 mg)	norgestrel (0.3 mg)	1
Generic ovcon	1st	ethinyl estradiol	norethindrone	1

		(0.035 mg)	(0.4 mg)	
Sprintec	3rd	ethinyl estradiol (0.035 mg)	norgestimate (0.25 mg)	1
Nuva Ring	3rd	ethinyl estradiol (0.015 mg)	etonogestrel (0.120 mg)	46

Diastolic blood pressure (DBP), systolic blood pressure (SBP), and PR were monitored by an electronic monitor (IntelliSense HEM-907XL, OMRON Corporation) that was calibrated and routinely used in our clinical study. BP was taken following administration of the questionnaire (15 minutes) to ensure measurement of resting BP and PR. Mean arterial pressure (MAP) was estimated by MAP=DBP + 1/3 (SBP-DBP). Body temperature was measured orally using an electronic thermometer (SureTemp plus 690, WelchAllyn, Skaneateles Falls, NY). To measure waist circumference, a measuring tape was placed around the bare abdomen at the level of the hip bone (above the belly button). Hip circumference was measured the widest section of the buttocks, usually at the groin level. BMI is defined as weight/height^2. Overweight condition was defined as having a BMI of 25 to 30 kg/m^2 and obesity as having a BMI of above 30 kg/m^2. We also calculated waist-to-hip ratio (WHR) to study its effect on pro-thrombotic state.

Following the subject examination, a venous blood draw, with minimal stasis at the antecubital vein, was conducted using Vacutainer (Becton Dickenson; Franklin Lakes, NJ) brand blood collection set and tubes. The Boynton Health Center's certified phlebotomists performed venous blood sample collection utilizing 21 gauge vacutainer brand butterfly needle collection sets and

vacutainer blood collection tubes. The first collected tube was discarded to avoid TF contamination and minimize presence of activated platelets. In total 8 vacutainer tubes were collected (2 red top serum 4mL tubes, 4 blue top 3.2% sodium citrate 4.5mL tubes, 1 purple top EDTA 4mL tube and 1 light blue top CTAD 4.5mL tube) totaling 34.5 mL of blood from each participant.

The citrated whole blood samples were centrifuged at 1000 x g for 15 minutes to collect plasma samples. Serum separator tubes were allowed to clot for 30 minutes, and then centrifuged at 1000 x g for 15 minutes. All serum and plasma samples were appropriately aliquoted and labeled for storage at -80°C. Also, Sonoclot testing was completed within 2 hours of blood draw using citrated whole blood. For the Sonoclot testing, a 330µL citrated whole blood sample was added to either a non-ACT or gb-ACT cuvette. The sample was re-calcified using 15µL of 0.25M $CaCl_2$ and the test was initiated by closing the aperture and manually starting the test on the device. Each test was allowed to run for 30 minutes. Each test was printed as well as saved electronically.

Biomarker Tests

Routine tests for CBC, D-Dimer, and fibrinogen concentration were completed by the University of Minnesota-Fairview Clinical Laboratory. Results were faxed to our laboratory within 24 hours of testing. Additional experimental biomarker analysis was conducted using enzyme linked immunosorbant assay (ELISA). ELISA is a common immunological test to measure minute concentrations of specific antigens. Each of the commercially available ELISA assays used in this study utilized the quantitative sandwich enzyme

immunoassay technique. In its simplest terms, a monoclonal antibody which is specific for the antigen of interest (biomarker) is affixed to a 96 well microtiter plate. A fixed volume of each unknown sample is added to individual wells. Additionally, serial dilutions of a known standard concentration of the analyte of interest are made and added to individual wells. The antigen in the unknown samples and control samples will bind to the antibody affixed to the microtiter plate and become immobilized. Next, the plate is washed with buffer to remove residual sample which was not specific to the antibody, followed by addition of a secondary monoclonal antibody coupled to a colorimetric enzyme which binds specifically to the antigen of interest. By adding a substrate solution which activates the colorimetric enzyme attached to the secondary antibody, one is able to differentiate concentrations by comparing absorbance readings for unknown samples to a standard curve generated from the serial dilutions of the control antigen. Biomarkers using ELISA technology measured in this study are: sE-selectin (BD, Franklin Lakes, NJ), sP-selectin (Bender MedSystems, Burlingame, CA), sCD40L (BD, Franklin Lakes, NJ), TF (BD, Franklin Lakes, NJ), PAI-1 (Bender MedSystems, Burlingame, CA), sTM (BD, Franklin Lakes, NJ), TFPI (BD, Franklin Lakes, NJ), TAT III (Siemens, Munich, Germany), vWF (Diagnostica Stago, Asnieres, France), and FVII (Diagnostica Stago, Asnieres, France). Table 2 describes each biomarker measured, its normal function and its predicted level indicating cardiovascular risk.

Table 2: Summary Table of blood biomarkers measured in the study. The arrow in the third column indicates the predicted direction of each biomarker indicating increased cardiovascular risk for HC use when compared to a control group.

Biomarker	Normal Function	Predicted Direction in CV disease
sE-selectin (ng/mL)	E-selectin is a transmembrane glycoprotein expressed on endothelial cells following stimulation by inflammatory cytokines or endotoxins. E-selectin allows for neutrophil extravasation by allowing neutrophil binding to the endothelium. Elevated soluble E-selectin has been associated with a variety of pathological conditions.	▲
CRP (mg/L)	CRP is an acute phase protein produced by the liver in response to inflammatory cytokines. CRP normally activates the complement cascade via the C1Q complex by binding to phosphocholine on the surface of damaged cells or bacteria. Low grade systemic inflammation indicated by elevated CRP has been positively correlated to CVD.	▲
Fibrinogen (mg/dL)	Fibrinogen is a glycoprotein synthesized by the liver. Like CRP, it is considered an acute phase protein and can be elevated in response to inflammatory signals. It is converted into fibrin monomers by the enzyme thrombin and provides the structural integrity of a blood clot. Elevated levels of fibrinogen indicate increased cardiovascular risk.	▲
D-Dimer (ug/mL)	D-dimer is a fibrin degradation product. After a stable clot has formed via fibrin cross-linking provided by Factor XIII, when the clot is broken down by plasmin, the fibrin degradation product containing the crosslink is the D-dimer fragment. Elevated D-dimer levels are indicative of intravascular coagulation.	▲
sCD40L (pg/mL)	CD40L is a glycoprotein member of the TNF super family and upon binding CD40, results in a variety of effects including B lymphocyte proliferation and isotype switch. Increased levels of sCD40L have been associated with chronic inflammatory diseases, cancer, neurodegenerative disorders and atherosclerosis.	▲

TF (pg/mL)	TF, also called factor III or CD142, is the primary initiator of the extrinsic coagulation cascade pathway. TF is a glycoprotein expressed in the subendothelium of the vasculature. Upon vessel wall damage, TF binds to FVII, converting it to active FVIIa, ultimately resulting in the conversion of fibrinogen to fibrin.	▲
PAI-1 (ng/mL)	PAI-1 is a glycoprotein produced by the liver and endothelial cells. PAI-1 is the primary inhibitor of plasminogen activators in plasma, thereby preventing normal clot breakdown or fibrinolysis. Elevated PAI-1 has been associated with increased risk for a variety of CVDs.	▲
sP-selectin (ng/mL)	P-selectin is a glycoprotein receptor on the surface of activated platelets and endothelium mediating the binding of leukocytes. Increased levels of sP-selectin have been associated with inflammatory disorders, acute lung injury, ischemia-reperfusion injury, gram-negative septic shock, thrombotic diseases and rheumatoid arthritis.	▲
sTM (pg/mL)	TM is a transmembrane protein expressed by endothelial cells in the vasculature. sTM is a receptor for thrombin and is important for fibrinolysis and anticoagulation. Thrombin binding results in the activation of protein C which then goes on to degrade factors Va and VIIIa, thereby limiting further thrombin production. Also, sTM activates the thrombin-activatable fibrinolysis inhibitor which then cleaves fibrin in the process of fibrinolysis.	▼
TFPI (ng/mL)	TFPI is a glycoprotein produced by endothelial cells and released by heparin stimulation. TFPI regulates the extrinsic coagulation pathway, inhibiting coagulation through the formation of a complex with Factor X and Factor XII, preventing Factor X activation and thus, clot formation.	▼
TAT-III (ug/mL)	TAT-III is a complex of the proteins thrombin and antithrombin. In normal coagulation, antithrombin binds thrombin, inactivating it and preventing further coagulation. Measurement of TAT-III is useful for the diagnosis of thrombotic events. Elevated TAT-III levels are found in patients predisposed to thrombosis and cardiovascular events.	▲

vWF (% activity)	VWF is a high molecular weight glycoprotein synthesized by endothelial cells and megakaryocytes. VWF plays an important role in the adhesion of platelets to the endothelium as well as transporting factor VIII and protecting it from degradation.	▲
FVII (% activity)	FVII is a vitamin K dependent glycoprotein synthesized by the liver. When FVII is activated by binding TF, it proceeds via the extrinsic coagulation and results in clot formation. Increased activity of FVII has been associated with increased risk of CVD.	▲

Collected data were saved in paper form as well as electronically. Signed consent forms were stored in a secure locked file cabinet. Each subject's survey was collected anonymously using only an abstract study identification number assigned by the research staff for identification. Collected forms were scanned and saved electronically to a secure University of Minnesota server, and the original forms were destroyed. Additionally, a comprehensive database was utilized to collect and centralize data from each patient using Microsoft Access. The Access database was also saved to the University server. Blood test results obtained from Fairview Clinical lab and ELISA results were entered and saved to the database.

Statistical Analysis Methods

All continuous measurements were analyzed for departure of normality. For normally distributed variables, mean and standard deviation (SD) were reported and F-test and t-test were used for 3- and 2-group comparisons, respectively. For non-normally distributed variables, median and interquartile-range (IQR) are reported and Kruskal-Wallis test and Wilcoxon rank sum test

were used. For categorical variables, Chi-square test or Fisher's exact test were used, depending on whether any sparse data cells were present. Demographics, lifestyle, and medical history variables, which showed significant difference among study groups, were adjusted in the multivariate regression models when we study the biomarker difference between groups. The least square means for different groups and the p-values for different contrasts, based on the estimated regression models, were reported. Because multiple biomarkers were analyzed, p-values less than 0.01 were considered as statistically significant. Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). Parametric tests were utilized for analysis due to the fact that there was no evidence that these variables were not normally distributed. For the same reason, mean and SD are reported for these variables. The global test p-value is based on one-way ANOVA F test (the null hypothesis is that the three groups' means are the same). The pair-wise comparisons are based on t-test. All tests are two sided.

Results

In total, 160 participants were used in our statistical analysis. Of the 51 COC users, 10 were classified as using first generation COCs, 7 as second generation and 34 as third generation. By definition, all *NuvaRing* users were third generation. Of the 64 control participants, 40 had never used any HC, while 24 had used HC in the past with a median [range] use of 13 [6-60] months.

Table 3: Enrollment summary

	Control (N=64)		**Users (N=96)**	
	Never user	**Past user**	**COC**	***NuvaRing***
Enrolled Participants (N)	40	24	51	45
Median months last used [range]	n/a	13 [6-60]	n/a	n/a
Generation • First • Second • Third			 10 (20%) 7 (14%) 34 (67%)	 0 0 45 (100%)
Median months using HC [range]	n/a	n/a	40 [6-156]	24 [6-96]

Note: Results are expressed as total number of participants (N), with range in brackets [] and percent of total in parentheses (%).

Summary of collected vital signs for each group (control, COC users and *NuvaRing users)* are shown in Table 4*.* Data are presented as mean with SD in parentheses. The global p-value was accomplished by one way ANOVA. The pairwise comparison of Control vs. COC and Control vs *NuvaRing* utilize the students t-test. A slight elevation in SBP, DBP and MAP was observed for the COC and *NuvaRing* users, when compared to the control group. No difference was seen in pulse or WTH ratio between the groups. The COC user group had a slightly lower BMI when compared to the control (p=0.0451).

Table 4: Physical Measurements

Mean (SD)	Control	COC	*NuvaRing*	Global test p-value	Control vs. COC p-value	Control vs. *NuvaRing* p-value
SBP	114.1 (12.3)	119.3 (11.3)	119.0 (9.8)	0.0243	0.0214	0.0297
DBP	67.4 (10.6)	71.1 (9.7)	72.4 (12.2)	0.0429	0.0576	0.0247
MAP	82.9 (10.0)	87.2 (9.2)	87.9 (10.3)	0.0177	0.0230	0.0136
Pulse	78.8 (13.4)	79.4 (11.6)	74.6 (12.1)	0.1256	0.7958	0.0984
BMI	25.0 (7.5)	22.6 (4.6)	24.2 (4.1)	0.0860	0.0451	0.5273
WTH	0.82 (0.07)	0.82 (0.07)	0.80 (0.06)	0.4117	0.5562	0.4002

As depicted in Table 5, we observed significant differences in age, race, alcohol consumption, sleeping habit and family history of CVD/stroke across our study groups. Therefore, these covariates were adjusted for in our multivariate analysis. All other demographical information was not significantly different.

Table 5: Demographics, Lifestyle and Medical History

	Controls	COC	*NuvaRing*	Global test p-value	Control vs. COC p-value	Control vs. *NuvaRing* p-value
Age mean ±SD [min-max]	21.5±2.3 [19-30]	22.3±2.2 [19-27]	24.2±3.3 [19-30]	**<0.0001**	0.0809	**<0.0001**
Whites	44 (69%)	47 (92%)	40 (91%)	**0.0013**	**0.0024**	**0.0088**
Lifestyle Factors						
Current smoker	2 (3%)	1 (2%)	2 (4%)	0.8590	1.0000	1.0000
Alcohol consumption	35 (55%)	45 (88%)	39 (87%)	**<0.0001**	**0.0001**	**0.0004**
Marijuana	8 (13%)	3 (6%)	4 (9%)	0.5190	0.3413	0.7578
Exercise/ week ≥ 3hrs	44 (69%)	31(61%)	34 (76%)	0.2980	0.3729	0.4381
Regular sleep habit	49 (77%)	48 (94%)	37 (82%)	0.0381	0.0101	0.4758
Regular diet	46 (72%)	40 (78%)	33 (73%)	0.7133	0.4212	0.8667
Medical Conditions						
UTI	3 (4%)	5 (10%)	5 (11%)	0.3830	0.2893	0.2672
Depression	5 (7%)	8 (16%)	4 (9%)	0.3748	0.2349	1.0000
Medical History						
HTN	0	0	0	NA	NA	NA
Heart disease	0	0	1 (2%)	0.2812	NA	0.4128
Thyroid Disease	1 (2%)	1 (2%)	2 (4%)	0.6844	1.0000	0.5679
Migraines	1 (2%)	2 (4%)	3 (7%)	0.3788	0.5836	0.3042
Cancer	0	0	1 (2%)	0.2812	NA	0.4128
Multiple Sclerosis	1 (2%)	0	0	1.0000	1.0000	1.0000
Ever been Pregnant	3 (5%)	1 (2%)	0	0.4637	0.6282	0.2661
Hyperlipidemia	0	0	1 (2%)	0.2812	NA	0.4128
FH of HTN	13 (20%)	13 (25%)	14 (31%)	0.4376	0.5096	0.1985
FH of CVD/ stroke	10 (16%)	14 (27%)	15 (33%)	0.0872	0.1211	**0.0304**

For continuous variables, the global test p-value is based on the one-way ANOVA F test (the null hypothesis is that the three groups' means are the same) and the pair-wise comparisons are based on t-test. For discrete variables, both the global and the pair-wise comparisons are based on Chi-square test (or Fisher's exact test if sparse cells exist). All tests are two sided.

All lab test variables are compared using non-parametric tests: Kruskal-Wallis test for 3-group comparisons and Wilcoxon rank sum test for 2-sample comparisons. Median and IQR are shown in the following tables.

In our univariate analysis, we observed that the *NuvaRing* group had significantly higher hemoglobin levels when compared to the control group (p=0.0090) whereas the COC group did not (p=0.0488). Both COC and *NuvaRing* users had significantly higher CRP levels when compared to the control group (p<0.0001). No differences were observed for any of the other clinical laboratory results.

Table 6: Clinical Laboratory Results, univariate analysis

Median and IQR	Control	COC	*NuvaRing*	Global test p-value	Control vs. COC p-value	Control vs. *NuvaRing* p-value
WBC	6.5 [5.7-7.7]	7.1 [5.8-8.1]	6.8 [5.8-8.0]	0.4825	0.2484	0.8150
Hemoglobin	12.8 [12.1-13.4]	13.0 [12.6-13.8]	13.4 [12.7-13.8]	0.0153	0.0488	**0.0090**
Platelet count	250 [224-293]	276 [251-308]	266 [237-310]	0.1324	0.0577	0.2161
D-Dimer	0.30 [0.20-0.40]	0.30 [0.20-0.30]	0.30 [0.20-0.30]	0.6471	0.2594	0.5973
Fibrinogen	295 [262-327]	301 [283-358]	301 [279-348]	0.1510	0.0861	0.1298
CRP	0.50 [0.20-0.80]	2.00 [1.10-4.50]	1.90 [1.30-.40]	**<0.0001**	**<0.0001**	**<0.0001**
Neutrophils	4.00 [3.10-4.90]	3.80 [3.00-4.75]	3.95 [3.20-.55]	0.9356	0.7410	0.8231
Lymphocytes	0.90 [1.70-2.40]	2.25 [1.90-2.55]	2.20 [1.80-.80]	0.0147	0.0036	0.0728
Monocytes	0.50 [0.40-0.60]	0.50 [0.40-0.50]	0.40 [0.40-.60]	0.3646	0.3589	0.1884
Eosiniphiles	0.10 [0.10-0.20]	0.10 [0.10-0.20]	0.10 [0.10-.20]	0.6088	0.8549	0.4496
Basophils	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-.00]	0.3489	0.8527	0.1608

Results of blood biomarkers tested at Fairview Clinical Laboratory. Results are expressed as Median with IQR in brackets. Note: Neutrophils, lymphocytes, monocytes, eosiniphils, and basophils are absolute counts.

Our univariate analysis of the ELISA results (Table 7) for the COC and *NuvaRing* users yielded significantly lower levels of sE-selectin (p=0.0009 and p=0.0023, respectively), sP-selectin (p<0.0001 and p=0.0091, respectively) and sTM (p=0.0010 and p<0.0001, respectively) when compared to the control. Significantly higher levels of FVII were observed in the COC and *NuvaRing* user groups when compared to the control (p<0.0001). The data revealed higher levels of sCD40L (p<0.0001) and PAI-1 (p=0.0026) in COC users, however sCD40L and PAI-1 were similar in the *NuvaRing* group when compared to the control (p=0.2363 and p=0.2992, respectively). No significant alterations were observed between the groups for TF, TAT-III, TFPI or VWF.

Table 7: ELISA Test Results, univariate analysis

Median and IQR	Control	COC	*NuvaRing*	Global test p-value	Control vs. COC p-value	Control vs. *NuvaRing* p-value
sE-selectin	33.1 [24.0-41.5]	23.0 [17.1-32.7]	24.0 [17.3-33.6]	**0.0005**	**0.0009**	**0.0023**
sCD40L	2069 [1379-3589]	5264 [2740-6963]	2453 [1865-3479]	**<0.0001**	**<0.0001**	0.2363
TF	29.9 [24.3-37.5]	29.6 [25.1-33.7]	27.9 [23.7-33.8]	0.3884	0.7553	0.1815
PAI-1	7.3 [5.1-20.3]	4.8 [2.0-11.2]	6.4 [5.4-9.7]	**0.0049**	**0.0026**	0.2992
sP-selectin	79.6 [48.6-112.6]	43.9 [27.1-61.6]	59.7 [44.7-74.4]	**<0.0001**	**<0.0001**	**0.0091**
sTM	3845 [2930-4722]	3091 [2488-3553]	2793 [2282-3312]	**<0.0001**	**0.0010**	**<0.0001**
TFPI	12.8 [6.0-16.4]	7.9 [5.8-11.3]	8.6 [5.4-12.2]	0.0183	0.0117	0.0340
TAT-III	3.6 [2.1-5.3]	3.1 [2.1-3.9]	4.3 [2.9-8.0]	0.0114	0.1571	0.0928
vWF	76.8 [52.5-101.1]	79.0 [59.1-111.7]	82.0 [55.8-121.3]	0.3919	0.4936	0.1825
F-VII	89.1 [79.1-105.2]	123.6 [108.2-141.1]	115.8 [102.8-121.5]	**<0.0001**	**<0.0001**	**<0.0001**

We did not observe any statistically significant differences across the groups for the Sonoclot Analyzer results (Table 8). However, there was a marginally lower gb-ACT for the two user groups when compared to the control.

Table 8: Sonoclot Analyzer Results, univariate analysis

Median and IQR	Control	COC	*NuvaRing*	Global test p-value	Control vs. COC p-value	Control vs. *NuvaRing* p-value
non-ACT	307 [264-353]	297 [248-351]	271 [232-317]	0.1086	0.6716	0.0458
gb-ACT	154 [137-168]	141 [126-153]	141 [131-159]	0.0492	0.0178	0.1969

Results of Sonoclot testing completed in our laboratory. Results are expressed as Median with IQR in brackets

We sought to identify alterations among the control group of those who had never used HC (Never users) and those who had used HC in the past (past users). These data are presented in Table 9, 10 and 11. No alterations were observed between Never user and Past user, except that sE-selectin was higher in the Past users when compared to Never users (p=0.0066). No discernable differences were observed for the biomarkers when compared to the never user groups.

Table 9: Never users vs. past users, Clinical Laboratory Tests

Median and IQR	Never users	Past users	Never vs. past user P-value	Never user vs. COC P-value	Never user vs. *NuvaRing* P-value
WBC	6.3 [5.5-7.6]	6.8 [6.4-8.4]	0.1540	0.0957	0.3971
Hemoglobin	12.7 [12.0-13.2]	12.9 [12.4-13.7]	0.1319	0.0149	**0.0025**
Platelet count	237 [221-292]	266 [242-297]	0.2294	0.0358	0.1107
D-Dimer	0.30 [0.20-0.40]	0.30 [0.20-0.40]	0.9083	0.3444	0.6765
Fibrinogen	298 [266-324]	294 [261-328]	0.7560	0.1535	0.2098
CRP	0.40 [0.20-0.90]	0.60 [0.30-0.70]	0.4303	**<0.0001**	**<0.0001**
Neutrophils	3.70 [2.90-4.90]	4.35 [3.30-5.40]	0.1481	0.7092	0.6074
Lymphocytes	1.90 [1.50-2.40]	2.25 [1.70-2.50]	0.1150	**0.0028**	0.0430
Monocytes	0.50 [0.40-0.60]	0.50 [0.40-0.60]	0.4146	0.7118	0.4347
Eosiniphiles	0.10 [0.10-0.20]	0.15 [0.10-0.20]	0.0451	0.4166	0.0996
Basophils	0.00 [0.00-0.00]	0.00 [0.00-0.10]	0.2751	0.7418	0.4165

Comparison between Never HC Use and Past HC user for Fairview Clinical Lab Results. Results are expressed as Median with IQR in brackets.

Table 10: Never users vs. past users, ELISA Tests

Median and IQR	Never users	Past users	Never vs. past user P-value	Never user vs. COC P-value	Never user vs. *NuvaRing* P-value
sE-selectin	28.4 [21.6-34.9]	39.7 [33.0-46.3]	**0.0066**	0.0477	0.0754
sCD40L	2206 [1522-3589]	1840 [1338-3945]	0.6242	**<0.0001**	0.4227
TF	30.2 [24.4-37.5]	29.0 [24.1-35.9]	0.5577	0.5449	0.1488
PAI-1	7.1 [5.5-13.9]	11.5 [4.5-30.5]	0.6097	0.0115	0.4177
sP-selectin	79.6 [51.1-109.3]	84.4 [48.6-132.2]	0.4778	**0.0003**	0.0328
sTM	3790 [2935-4941]	3881 [2824-4329]	0.7666	**0.0022**	**0.0002**
TFPI	12.8 [5.3-15.9]	12.9 [7.0-18.1]	0.3894	0.0629	0.1304
TAT-III	3.5 [2.1-5.3]	3.8 [2.2-5.0]	0.6637	0.3479	0.1080
vWF	84.0 [52.5-108.0]	72.6 [53.9-90.2]	0.3320	0.8733	0.4152
F-VII	89.2 [76.4-106.4]	88.7 [82.3-104.0]	0.8037	**<0.0001**	**<0.0001**

Comparison between Never HC Use and Past HC user for ELISA tests. Results are expressed as Median with IQR in brackets.

Table 11: Never users vs. past users, Sonoclot Analyzer

Median and IQR	Never users	Past users	Never vs. past user P-value	Never user vs. COC P-value	Never user vs. *NuvaRing* P-value
non-ACT	306 [279-349]	312 [247-357]	0.7824	0.5662	0.0400
gb-ACT	155 [138-168]	147 [133-167]	0.5318	0.0153	0.1523

Comparison between Never HC Use and Past HC user for Sonoclot tests. Results are expressed as Median with IQR in brackets.

Due to the potentially increased cardiovascular disease risk among third generation COC users, we sought to identify alterations in biomarkers among first, second and third generation COC users (Table 12, 13 and 14). Our results for the third generation COC users agreed with the previous univariate analysis in Table 6, 7 and 8. The limited number of first and second generation COC users in our study potentially limited the statistical analysis.

Table 12: COC Generation Comparison, Clinical Laboratory Tests

Median and IQR	1st gen. COC	2nd gen. COC	3rd gen. COC	Control vs. 1st gen. P-value	Control vs. 2nd gen. P-value	Control vs. 3rd gen. P-value
WBC	8.65 [7.10-9.60]	7.20 [5.10-8.10]	6.85 [5.80-7.60]	0.0224	0.9234	0.6778
Hemoglobin	13.3 [12.6-13.9]	12.9 [12.5-14.7]	13.0 [12.6-13.8]	0.1828	0.5199	**0.0077**
Platelet Count	266 [239-316]	262 [217-304]	281 [259-308]	0.4937	0.6726	0.0542
D-Dimer	0.25 [0.20-0.30]	0.20 [0.20-0.30]	0.30 [0.20-0.30]	0.3403	0.3410	0.4796
Fibrinogen	297 [285-304]	316 [225-365]	304 [266-370]	0.5655	0.5017	0.0516
CRP	1.20 [1.10-3.70]	1.30 [0.70-3.00]	2.90 [1.50-4.60]	**0.0048**	0.0153	**<0.0001**
Neutrophils	4.25 [3.35-6.15]	3.40 [2.80-5.20]	3.80 [3.00-4.30]	0.4282	0.6511	0.6364
Lymphocytes	2.45 [2.20-2.85]	2.10 [1.90-2.20]	2.30 [1.90-2.50]	0.0104	0.7315	0.0123
Monocytes	0.50 [0.40-0.60]	0.50 [0.40-0.60]	0.50 [0.40-0.50]	0.9394	0.7738	0.1452
Eosiniphiles	0.10 [0.10-0.10]	0.20 [0.10-0.30]	0.10 [0.10-0.20]	0.3899	0.3426	0.6958
Basophils	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.00 [0.00-0.00]	0.6396	0.2091	0.5128

Comparison between generations of COC for tests conducted at Fairview clinical laboratory. Results are expressed as Median with IQR in brackets.

Table 13: COC Generation Comparison, ELISA Tests

Median and IQR	1st gen. COC	2nd gen. COC	3rd gen. COC	Control vs. 1st gen. P-value	Control vs. 2nd gen. P-value	Control vs. 3rd gen. P-value
sE-selectin	16.1 [10.9-20.2]	18.3 [16.8-37.5]	26.5 [19.2-34.4]	**0.0001**	0.1297	**0.0019**
sCD40L	4051 [2708-5773]	2740 [2253-6559]	5799 [3588-7310]	0.0199	0.1345	**0.0003**
TF	26.6 [22.9-33.7]	27.4 [25.1-30.6]	31.0 [27.0-34.9]	0.3112	0.4602	0.4899
PAI-1	5.1 [2.1-10.0]	7.9 [4.7-11.6]	2.8 [1.9-11.2]	0.2043	0.4719	0.0167
sP-selectin	61.0 [17.5-92.5]	32.2 [27.1-43.9]	41.8 [28.3-61.1]	0.1962	**0.0016**	**0.0001**
sTM	3021 [2817-3264]	2467 [2178-3425]	3111 [2609-3561]	0.0566	0.0115	**<0.0001**
TFPI	10.0 [5.7-14.6]	10.1 [4.9-13.8]	7.5 [6.1-9.5]	0.4550	0.3057	**0.0050**
TAT-III	3.13 [2.19-3.93]	3.22 [1.85-3.30]	3.00 [2.09-3.99]	0.6089	0.3528	0.6735
vWF	90.0 [79.0-111.7]	69.1 [39.1-123.8]	70.7 [59.1-104.6]	0.1077	0.9617	0.3315
F-VII	116.8 [101.1-130.0]	90.9 [87.3-128.3]	125.5 [115.3-150.6]	**0.0008**	0.2712	**<0.0001**

Comparison between generation of COC for ELISA tests. Results are expressed as Median with IQR in brackets.

Table 14: COC Generation Comparison, Sonoclot Analyzer

Median and IQR	1st gen. COC	2nd gen. COC	3rd gen. COC	Control vs. 1st gen. P-value	Control vs. 2nd gen. P-value	Control vs. 3rd gen. P-value
non-ACT	297 [265-314]	347 [230-383]	296 [248-351]	0.5816	0.5647	0.1094
gb-ACT	140 [122-142]	156 [143-188]	137 [123-151]	0.0822	0.6000	0.0243

Comparison between generations of COCs for the Sonoclot tests. Results are expressed as Median with IQR in brackets.

Multivariate analysis

Since age, race, alcohol consumption, regular sleep habit, and FH of CVD/stroke are found to be significantly different among the 3 study groups, we adjusted for these variables in multivariate regression models when studying the difference in biomarkers among the 3 groups. The least square means, based on the estimated models, for the 3 groups and the p-values for the difference between the control group and the COC users and the *NuvaRing* users, and the global test p-values are reported in the following tables. The regression coefficients for the adjusted covariates are not shown in the table, whereas significant covariates will be indicated in the footnote.

After correcting for the previously mentioned covariates, the Clinical Laboratory Tests multivariate analysis (Table 15) revealed that the *NuvaRing* group no longer had statistically significantly higher hemoglobin levels when compared to the control group ($p=0.1771$). Again, both COC and *NuvaRing* users had significantly higher CRP levels when compared to the control group ($p<0.0001$). No differences were observed for any of the other Clinical laboratory results.

Table 15: Multivariate Analysis, Clinical Laboratory Tests

Least square mean (95% CI)	Control	COC user	*NuvaRing* user	Global test P-value	Control vs. COCP-value	Control vs. *NuvaRing* P-value
WBC	6.82 (6.33, 7.31)	7.08 (6.55, 7.62)	6.92 (6.34, 7.51)	0.7812	0.4868	0.7958
Hemoglobin	12.9 (12.6, 13.1)	13.0 (12.7, 13.3)	13.2 (12.9, 13.5)	0.4010	0.5111	0.1771
Platelet count	259 (243, 275)	281 (264, 299)	279 (260, 299)	0.1559	0.0760	0.1274
D-Dimer	0.32 (0.28, 0.35)	0.32 (0.28, 0.36)	0.31 (0.27, 0.36)	0.9404	0.7929	0.9428
Fibrinogen[1]	297 (281, 313)	318 (300, 335)	319 (300, 338)	0.1644	0.1045	0.0998
CRP[1]	0.82 (0.36, 1.28)	2.83 (2.32, 3.35)	2.56 (1.98, 3.14)	**<0.0001**	**<0.0001**	**<0.0001**
Neutrophils	4.20 (3.78, 4.62)	4.05 (3.58, 4.53)	4.12 (3.63, 4.62)	0.9022	0.6506	0.8245
Lymphocytes	2.00 (1.84, 2.16)	2.31 (2.14, 2.49)	2.30 (2.12, 2.49)	0.0188	0.0117	0.0201
Monocytes	0.58 (0.49, 0.67)	0.47 (0.36, 0.57)	0.50 (0.39, 0.61)	0.2841	0.1240	0.2851
Eosiniphiles	0.16 (0.12, 0.20)	0.14 (0.10, 0.19)	0.15 (0.10, 0.19)	0.8110	0.5365	0.6448
Basophils	0.02 (0.01, 0.03)	0.02 (0.01, 0.03)	0.01 (0.00, 0.02)	0.3796	0.9453	0.2104

Multivariate analysis of Fairview results after correcting for age, race, alcohol assumption, regular sleep habit, and FH of CVD/stroke. Results are expressed as least square mean with the 95% confidence interval in parentheses.

[1] People with a FH of CVD/stroke had a higher Fibrinogen level (mean difference=24.5, p=0.04) and a higher CRP level (mean difference=0.79, p=0.03) than people who didn't.

Our multivariate analysis of the ELISA results (Table 16) for the COC and *NuvaRing* users yielded significantly lower levels of sE-selectin ($p=0.0003$ and $p=0.0010$, respectively) and sTM ($p=0.0007$ and $p<0.0001$, respectively) when compared to the control group. This agreed with the univariate analysis observed in Table 7. Additionally, in contrast to the univariate analysis, multivariate analysis of TFPI revealed statistically significantly lower levels in COC users and *NuvaRing* users when compared to the control group ($p=0.0004$ and $p=0.0026$, respectively). While sP-selectin was still significantly lower in our COC user group ($p=0.0057$) when compared to the control, the *NuvaRing* group was still lower but did not meet our significance cutoff with $p=0.0111$). Again, significantly higher levels of FVII were observed in the COC and *NuvaRing* user groups when compared to the control ($p<0.0001$). In comparison to the univariate analysis, significance was lost in the multivariate analysis of PAI-1 for COC users when compared to control ($p=0.5055$). For sCD40L, the data again showed higher levels in COC users ($p<0.0001$). No significant alterations were observed between the groups for TF, TAT-III or VWF.

Table 16: Multivariate Analysis, ELISA Tests

Least square mean (95% CI)	Control	COC user	*NuvaRing* user	Global test P-value	Control vs. COC P-value	Control vs. *NuvaRing* P-value
sE-selectin	34.2 (30.9, 7.6)	24.7 (21.0, 28.3)	25.1 (21.1, 29.0)	**0.0004**	**0.0003**	**0.0010**
sCD40L	2708 (2086, 3329)	5235 (4561, 5910)	2977 (2243, 3710)	**<0.0001**	**<0.0001**	0.5996
TF	32.4 (29.8, 35.0)	30.9 (28.0, 33.7)	29.1 (26.0, 32.2)	0.3204	0.4605	0.1320
PAI-1[1]	16.1 (11.1, 21.2)	13.5 (8.0, 19.0)	7.3 (1.4, 13.3)	0.0988	0.5055	0.0353
sP-selectin	86.7 (74.5, 98.8)	58.2 (45.0, 71.4)	61.0 (46.6, 75.3)	**0.0057**	**0.0029**	0.0111
sTM	3813 (3556, 4070)	3122 (2843, 3401)	2962 (2659, 3266)	**0.0001**	**0.0007**	**<0.0001**
TFPI[2]	12.8 (11.3, 14.3)	8.5 (6.9, 10.2)	9.0 (7.2, 10.8)	**0.0007**	**0.0004**	**0.0026**
TAT-III[3]	4.72 (3.22, 6.22)	3.81 (2.19, 5.43)	6.33 (4.57, 8.10)	0.1127	0.4338	0.1916
vWF[4]	79.8 (68.5, 91.2)	84.1 (71.7, 96.4)	99.8 (86.3, 113.2)	0.0871	0.6343	0.0350
F-VII	93.2 (87.6, 98.7)	124 (118, 130)	115 (108, 121)	**<0.0001**	**<0.0001**	**<0.0001**

Multivariate analysis of ELISA results after correcting for age, race, alcohol assumption, regular sleep habit, and FH of CVD/stroke. Results are expressed as least square mean with the 95% confidence interval in parentheses.

[1] Subjects with a regular sleep habit had a lower PAI-1 level (mean difference = -11.6, p=0.006) than those who didn't.

[2] White subjects had a higher TFPI level (mean difference = 2.77, p=0.04) then non-White.

[3] Subjects who had alcohol consumption had a lower level of TAT-III (mean difference = -2.91, p=0.01) than those who didn't.

[4] Subjects who had a FH of CVD/stroke had a higher level of vWF (mean difference = 10.3, p=0.01) than those who didn't.

The multivariate analysis of the Sonoclot data (Table 17) did not yield any statistically significant differences across the groups. However, there was a marginally higher gb-ACT for the *NuvaRing* user group when compared to the control (p=0.0993).

Table 17: Multivariate Analysis, Sonoclot Analyzer

Least square mean (95% CI)	**Control**	**COC user**	***NuvaRing* user**	**Global test P-value**	**Control vs. COC P-value**	**Control vs. *NuvaRing* P-value**
non-ACT	302 (276, 328)	302 (274, 330)	307 (276, 339)	0.9637	0.9927	0.8102
gb-ACT	146 (137, 155)	140 (130, 150)	159 (148, 170)	0.0432	0.4016	0.0993

Multivariate analysis of Sonoclot results after correcting for age, race, alcohol assumption, regular sleep habit, and FH of CVD/stroke. Results are expressed as least square mean with the 95% confidence interval in parentheses.

The data presented in Table 18 shows the relationship between COC and *NuvaRing* for our univariate analysis. We observed statistically significantly lower levels of sP-selectin and sCD40L in *NuvaRing* users when compared to COC users (p=0.0042 and p<0.0001, respectively). In addition, we observed significantly higher levels of TAT-III in the *NuvaRing* group when compared to COC users (p=0.0032). No significant alterations were observed in any of the other biomarkers.

Table 18: Univariate analysis of the blood biomarkers

	Median [interquartile range]			p-value			
Blood marker	**Control**	**COC users**	***NuvaRing* users**	**Global test**	**Control vs. COC**	**Control vs. *NuvaRing***	**COC Vs. *NuvaRing***
WBC	6.5 [5.7-7.7]	7.1 [5.8-8.1]	6.8 [5.8-8.0]	0.4825	0.2484	0.8150	0.3968
Hemoglobin	12.8 [12.1-13.4]	13.0 [12.6-13.8]	13.4 [12.7-13.8]	0.0153	0.0488	**0.0090**	0.4888
Platelet count	250 [224-293]	276 [251-308]	266 [237-310]	0.1324	0.0577	0.2161	0.4708
D-Dimer	0.30 [0.20-0.40]	0.30 [0.20-0.30]	0.30 [0.20-0.30]	0.6471	0.2594	0.5973	0.5675
Fibrinogen	295 [262-327]	301 [283-358]	301 [279-348]	0.1510	0.0861	0.1298	0.8778
CRP	0.50 [0.20-0.80]	2.00 [1.10-4.50]	1.90 [1.30-3.40]	**<0.0001**	**<0.0001**	**<0.0001**	0.6835
sE-selectin	33.1 [24.0-41.5]	23.0 [17.1-32.7]	24.0 [17.3-33.6]	**0.0005**	**0.0009**	**0.0023**	0.9242
sP-selectin	79.6 [48.6-112.6]	43.9 [27.1-61.6]	59.7 [44.7-74.4]	**<0.0001**	**<0.0001**	**0.0091**	**0.0042**
sCD40L	2069 [1379-3589]	5264 [2740-6963]	2453 [1865-3479]	**<0.0001**	**<0.0001**	0.2363	**<0.0001**
TF	29.9 [24.3-37.5]	29.6 [25.1-33.7]	27.9 [23.7-33.8]	0.3884	0.7553	0.1815	0.3313
TFPI	12.8 [6.0-16.4]	7.9 [5.8-11.3]	8.6 [5.4-12.2]	0.0183	0.0117	0.0340	0.6712
PAI-1	7.3 [5.1-20.3]	4.8 [2.0-11.2]	6.4 [5.4-9.7]	**0.0049**	**0.0026**	0.2992	0.0255
sTM	3845 [2930-4722]	3091 [2488-3553]	2793 [2282-3312]	**<0.0001**	**0.0010**	**<0.0001**	0.1730
TAT-III	3.6 [2.1-5.3]	3.1 [2.1-3.9]	4.3 [2.9-8.0]	0.0114	0.1571	0.0928	**0.0032**
vWF	76.8 [52.5-101.1]	79.0 [59.1-111.7]	82.0 [55.8-121.3]	0.3919	0.4936	0.1825	0.4872
FVII	89.1 [79.1-105.2]	123.6 [108.2-141.1]	115.8 [102.8-121.5]	**<0.0001**	**<0.0001**	**<0.0001**	0.0209
ACT	307 [264-353]	297 [248-351]	271 [232-317]	0.1086	0.6716	0.0458	0.1144
gb-ACT	154 [137-168]	141 [126-153]	141 [131-159]	0.0492	0.0178	0.1969	0.3034

Note: Data are presented as mean±SD or N(%). A p-value of <0.01 is considered significant

The data presented in Table 19 shows the relationship between COC and *NuvaRing* for our multivariate analysis. We observed statistically significantly lower levels of sCD40L in *NuvaRing* users when compared to COC users

(p=0.0042 and p<0.0001, respectively). Statistical significance was lost in the multivariate analysis compared to the univariate analysis for TAT-III in the *NuvaRing* group when compared to COC users (p=0.0379). Again, no significant alterations were observed in any of the other biomarkers.

Table 19: Multivariate analysis[1] of the blood biomarkers

	Least square mean (95% CI)			p-value			
	Control	**COC users**	***NuvaRing* users**	**Global test**	**Control vs. COC**	**Control vs. *NuvaRing***	**COC Vs. *NuraRing***
WBC	6.82 (6.33, 7.31)	7.08 (6.55, 7.62)	6.92 (6.34, 7.51)	0.7812	0.4868	0.7958	0.6882
Hemo-globin	12.9 (12.6, 13.1)	13.0 (12.7, 13.3)	13.2 (12.9, 13.5)	0.4010	0.5111	0.1771	0.4528
Platelet Count	259 (243, 275)	281 (264, 299)	279 (260, 299)	0.1559	0.0760	0.1274	0.8846
D-Dimer	0.32 (0.28, 0.35)	0.32 (0.28, 0.36)	0.31 (0.27, 0.36)	0.9404	0.7929	0.9428	0.7443
Fibrinogen[2]	297 (281, 313)	318 (300, 335)	319 (300, 338)	0.1644	0.1045	0.0998	0.9168
CRP[2]	0.82 (0.36, 1.28)	2.83 (2.32, 3.35)	2.56 (1.98, 3.14)	**<0.0001**	**<0.0001**	**<0.0001**	0.716
sE-selectin	34.2 (30.9, 37.6)	24.7 (21.0, 28.3)	25.1 (21.1, 29.0)	**0.0004**	**0.0003**	**0.0010**	0.8782
sP-selectin	86.7 (74.5, 98.8)	58.2 (45.0, 71.4)	61.0 (46.6, 75.3)	**0.0057**	**0.0029**	0.0111	0.7765
sCD40L	2708 (2086, 3329)	5235 (4561, 5910)	2977 (2243, 3710)	**<0.0001**	**<0.0001**	0.5996	**<0.0001**
TF	32.4 (29.8, 35.0)	30.9 (28.0, 33.7)	29.1 (26.0, 32.2)	0.3204	0.4605	0.1320	0.4057
TFPI[3]	12.8 (11.3, 14.3)	8.5 (6.9, 10.2)	9.0 (7.2, 10.8)	**0.0007**	**0.0004**	**0.0026**	0.7221
PAI-1[4]	16.1 (11.1, 21.2)	13.5 (8.0, 19.0)	7.3 (1.4, 13.3)	0.0988	0.5055	0.0353	0.1291
sTM	3813 (3556, 4070)	3122 (2843, 3401)	2962 (2659, 3266)	**0.0001**	**0.0007**	**<0.0001**	0.4419
TAT-III[5]	4.72 (3.22, 6.22)	3.81 (2.19, 5.43)	6.33 (4.57, 8.10)	0.1127	0.4338	0.1916	0.0378
vWF[6]	79.8 (68.5, 91.2)	84.1 (71.7, 96.4)	99.8 (86.3, 113.2)	0.0871	0.6343	0.0350	0.0886
FVII	93.2 (87.6, 98.7)	124 (118, 130)	115 (108, 121)	**<0.0001**	**<0.0001**	**<0.0001**	0.0333
non-ACT	302 (276, 328)	302 (274, 330)	307 (276, 339)	0.9637	0.9927	0.8102	0.8107
gb-ACT	146 (137, 155)	140 (130, 150)	159 (148, 170)	0.0432	0.4016	0.0993	0.0129

[1] In addition to study groups, all multivariate regression models were adjusted for covariates, including age, race (white/non-white), alcohol consumption (yes/no), regular sleeping habit (yes/no), and FH of CVD/stroke (yes/no). Their estimated regression coefficient and significance are not shown in the table, however, significant ($p<0.05$) ones are reported in footnote.
[2] Subjects with a FH of CVD/stroke had a higher fibrinogen level (mean difference=24.5, p=0.04) and a higher CRP level (mean difference=0.79, p=0.03) than those without a FH.
[3] White subjects had a higher TFPI level (mean difference = 2.77, p=0.04) then non-white.
[4] Subjects who had a regular sleep habit had a lower PAI-1 level (mean difference = -11.6, p=0.006) than those who didn't.
[5] Subjects who consumed alcohol had a lower level of TAT-III (mean difference = -2.91, p=0.01) than those who didn't.
[6] Subjects who had a FH of CVD/stroke had a higher vWF (mean difference = 10.3, p=0.01) than those who didn't.

Discussion

CHC related thrombosis has been recognized since the early 1960's (3, 78). Despite efforts to reduce the risk, there still exists a 2-3 fold increased risk of thrombosis among CHC users (5). The present study focused on identifying alterations in cardiovascular risk biomarkers among young healthy women using COCs and *NuvaRing* when compared to a non-HC user group. Our non-HC user control group was further classified into two subgroups, those who had never used HCs (never users) and those who had used HCs in the past (past users). Analysis into the relationship between never users and past users showed no significant difference between the two subgroups for the biomarkers studied, except that never users had lower levels of sE-selectin (Table 10).

Despite evidence from published studies (17, 19, 20, 79) that third generation COCs provide an elevated thrombosis risk, they remain prevalent in our study population (67% of total COC users). However, only a true population-based study can reveal the actual utilization of third generation COCs among users in a community. We saw no significant difference in the blood biomarkers analyzed in our study when compared across COC generations (Table 12, 13 and 14). However, the lack of significance could also be due to the limited number of participants in each category with only 10 and 7 in first and second generation, respectively.

In total, 45 *NuvaRing* users were successfully enrolled in the study. The *NuvaRing* is a newer method of birth control, which substantiates our finding that

the duration of use among *NuvaRing* participants was lower. *NuvaRing* users had been using for an average of 24 months, compared to 40 months for COC users (Table 3). Several studies have shown elevated thrombotic risk among first time users or during early (typically with the first 3 months) use of COCs (80-82). Therefore, we designed the study with exclusion criteria requiring participants to be active CHC users for a minimum of 6 months in order to avoid higher transient risk due to early use.

Demographics

Several of the demographic categories associated with CVD differed amongst our groups (Table 5), making it necessary to adjust for these differences in our multivariate statistical analysis. *NuvaRing* users were significantly older than the COC users and control participants. CVD has been shown to be positively associated with increased age in women (83, 84) although due to the relatively young age of our subjects, 19-30 years, this risk is thought to be minimal. The participant's race classification was significantly different among our groups. While only 69% of the control subjects were white, 92% of COC users and 91% of *NuvaRing* users were white. Race has long been recognized as a non-modifiable risk factor for CVD (85-87).

Alcohol consumption differed between CHC users (COC and *NuvaRing*) and control participants. While 88% of COC users and 87% of *NuvaRing* users were alcohol users, the rate among the control group was only 55%. Studies have shown reduced CVD risk resulting from moderate alcohol consumption (88-90) but increased CVD risk from excessive drinking (91). Our study did not

differentiate between moderate drinking and excessive drinking. Interestingly, among all study groups, those who consumed alcohol had significantly lower levels of TAT-III. Alcohol consumption has been linked to creating a hypocoagulable state by various effects on coagulation and fibrinolysis (92-95). An in vitro experiment showed a dose dependent impairment of coagulation and increased fibrinolysis as measured by thromboelastometry (96).

Only 77% of control participants and 82% of *NuvaRing* users reported having regular sleeping habits compared to 94% of COC users. In a recent 2010 National Health Interview Study, it was shown that there is a positive association between CVD and both shorter and longer sleep duration (97). *NuvaRing* users had a higher incidence of FH of CVD/stroke than the control group.

Due to these significant differences among the three study groups, age, race, alcohol consumption, regular sleep habit and FH of CVD/stroke were adjusted for in the multivariate regression model.

Physical Measurements

Physiological variables were affected by the type of CHC used (Table 4). For instance, in Table 4 we see that the SBP, DBP, and thus MAP were elevated in both COC and *NuvaRing* users when compared to non-users. The BP elevation observed in HC users classified them as borderline prehypertensive (SBP of 120 to 139mmHg and DBP of 80 to 89mmHg). Pre-HTN has been associated with a significantly increased risk of MI and coronary artery disease (98). Elevation of BP among COC users has been documented previously (52)

but at least one study has shown no BP change in *NuvaRing* users (99). However, the significance observed in the univariate analysis of BP elevation was negated in the multivariate analysis after adjusting for age, race, alcohol consumption, regular sleep habit and FH of CVD/stroke. As expected, the PR did not differ between the groups.

There was a slight elevation of BMI in the control group, 25.0, when compared to COC and *NuvaRing* users, 22.6 and 24.2 respectively. Several studies have shown that COCs increase BMI (100-102). Additionally, a randomized controlled study showed a similar increase in body weight among COC and *NuvaRing* users (103). It has been well documented that a positive correlation exists between BMI and cardiovascular events (104, 105). We did not observe any significant difference in WTH ratio.

Blood Biomarkers

While we saw no significant alterations in WBC, platelet count, PAI-1, TAT-III, D-dimer, fibrinogen or vWF between the three groups, there was a significant difference for several of the other blood biomarkers (Tables 6, 7, 19 and 20). We observed a decrease in sE-selectin among CHC users (COC and *NuvaRing*) when compared to the control group. E-selectin is a surface endothelial adhesion molecule expressed in response to inflammatory cytokines to facilitate the extravasation of leukocytes from the vasculature into inflamed tissue. Normally elevated levels of sE-selectin are interpreted as an indication of endothelial inflammation and an increased risk of CVD and thrombosis

potential (106). However, in our analysis and several other studies (69, 107, 108) it has been shown that estrogen containing contraceptives and hormone replacement therapies actually decrease the level of sE-selectin. So in terms of cardiovascular risk, it appears that both COCs and *NuvaRing* exhibit some vascular protective features. To further explain this phenomenon, an *in vitro* experiment using human umbilical vein estrogen receptor positive endothelial cells showed that 17β-estradiol inhibited the cytokine mediated transcription activation of endothelial cell adhesion molecules such as E-selectin (109). It is likely that a similar mechanism exists for estrogen containing CHCs.

The fact that the never users cohort in the control group had significantly lower levels of sE-selectin when compared to control participants who were past users, presents a difficult, possible contradictory, explanation as to why past users would have lower sE-selectin levels. However, it is possible that either the observed difference was coincidental, or that a molecular response to the withdrawal of CHC after extended use up regulates E-selectin expression beyond what would have been observed prior to initiation of CHC. We also observed a lower level of sP-selectin among the CHC user groups when compared to the control group. SP-selectin is an indirect marker of endothelial cell and platelet activation in that it is expressed by endothelial cells and platelets in response to a variety of signals. Therefore, normally we would expect elevated levels of sP-selectin to be indicative of increased cardiovascular risk. However, the observation that CHC users had lower levels of sP-selectin shows that CHC could also provide some vascular protection. Several other studies have shown

that the hormones present in contraceptives decrease the amount of sP-selectin found in CHC users (107, 110).

Interestingly, sCD40L levels were greatly increased in the COC user group compared to the control or *NuvaRing* groups. Increased levels of sCD40L serve as a surrogate marker of platelet activation that plays an important role in the pathophysiology of acute coronary syndromes (111). Published studies suggest that both healthy women and patients with a history of atrial fibrillation have a significantly increased risk of developing future MI and stroke when an elevated concentration of sCD40L is observed (112, 113). Changes in levels of sCD40L concentration due to CHC use have not been reported in the literature. The mechanism as to why sCD40L is elevated in the COC user group is unclear. However, it is possible that the observed elevation in sCD40L stems from increased systemic inflammation and the associated proliferation of B and T cells which rely on CD40L for B cell activation and isotype switch. In our study, the lymphocyte level was slightly elevated in our CHC user groups (COC and *NuvaRing*). This could be due to general inflammation as observed from several other markers. It is possible that low grade systemic inflammation could raise the concentration of B and T cells, which also could be a contributing factor to our observed increase in sCD40L. Further work should be done to confirm this finding and propose a suitable mechanism.

Participants with a FH of CVD/stroke had an increased CRP level. The link between an elevated level of CRP and CVD has been established (114) including advanced atheroscrotic disease among young adults (115). After

correcting for FH of CVD/stroke, we still observed that CRP was elevated in the COC and *NuvaRing* user groups compared to the control group. Several other studies have also observed elevations in CRP among COC users (68, 70); however this is the first report of increased CRP levels in *NuvaRing* users. It remains to be determined whether the increase in CRP levels in CHC users is due to a true inflammatory response, or rather, is the result of CHC processing and CRP production in the liver. In support of the latter hypothesis, Silvestri et. al. showed an increase in CRP production in women using hormone replacement therapy while other markers of systemic inflammation such as interleukin-6, sE-selectin and soluble intracellular adhesion molecule-1 remained at normal levels (78). If the CRP increase was solely due to direct induction by the liver, since the *NuvaRing* largely bypasses hepatic circulation, one would expect a lower CRP level among *NuvaRing* users compared to COC users. However, our results show a similar CRP increase in COC and *NuvaRing* users which may support an inflammatory mediated response rather than direct induction of CRP in the liver.

The plasma levels of PAI-1 were relatively unchanged across the groups in our study. However, another protein important in fibrinolysis, sTM was significantly reduced in COC and *NuvaRing* groups when compared to the control. TM is a transmembrane glycoprotein expressed by endothelial cells which acts as a receptor for thrombin. Upon TM binding, thrombin is inactivated preventing further catalysis of the coagulation cascade and the loss of thrombin's procoagulant activity. Increased levels of sTM are considered a surrogate marker

for endothelial injury. The case-cohort Atherosclerosis Risk in Communities Study found that reductions in sTM served as a predictor of future coronary heart disease and asymptomatic atherosclerosis (116). Therefore, the reduced levels of sTM that we observed in CHC users may indicate increased future cardiovascular risk.

While there was little difference between the groups in regard to the protein TF, its regulatory protein, TFPI was negatively impacted by CHC use. TFPI is a glycoprotein produced by endothelial cells to inhibit coagulation by preventing TF from binding to FVII, thus, lower levels of TFPI are indicative of a hypercoagulable state. TFPI inhibits the extrinsic pathway of coagulation, therefore, higher levels are considered beneficial for preventing thrombosis. However, we observed a reduction in TFPI levels in both the COC and *NuvaRing* groups when compared to the control. This observation is supported by a study (117) which showed a strong correlation between reduced TFPI levels and VT, and showed that exogenous female hormones from hormone replacement therapy or HC further exacerbate VT risk.

To further compound the negative effects of TFPI on the extrinsic pathway, we found that FVII was elevated in both groups of CHC users when compared to non users. This increase was less in *NuvaRing* users than in COC users, however, the observed difference in FVII levels between the CHC user groups was not statistically significant at the p=0.01 level. Our finding is in contrast to a study (118) which showed a higher FVII activity among *NuvaRing* users at the 6th cycle of use when compared to second generation COC users at

the same time point. FVII is a coagulation factor vital to the extrinsic pathway of coagulation. Our results indicate that CHC use promotes coagulation by causing increased FVII protein concentration and by compromising the fibrinolytic system by reducing TFPI levels.

We found that CHC use had no effect on the platelet-endothelial linker protein vWF. Although Van Rooijen et. al.(69) described an increase in vWF levels among COC users, in support of our findings, several other studies have shown no correlation between vWF and HC use (57, 77). However, we did observe that vWF was elevated in subjects with a FH of CVD/stroke (mean difference = 10.3, p=0.01) than those who didn't, regardless of CHC use. Levels of the inactivated thrombin complex, TAT-III, were also found to be unchanged due to CHC use. This result is supported by a similar investigation of COC and *NuvaRing* users by Magnusdttir et. al.(118).

Since there are obvious alterations in the inflammatory and coagulation systems of CHC users, it would be advantageous to have a global coagulation screening tool to assess the alterations observed in biomarker levels in an *in vitro* diagnostic test. Our study utilized the Sonoclot device for this purpose. However, despite our observed alterations in proteins of the coagulation and fibrolytic systems, we did not find any significant correlation between CHC use and global *in vitro* coagulation. A slight decrease in non-ACT and gb-ACT was observed for CHC users, but none of the observed differences reached the statistically significant level set for this study. Notably, both COC and *NuvaRing* users exhibited higher hemoglobin levels than controls; however, this increase

was found to be statistically significant only among *NuvaRing* users. This observation is most likely the result of a CHC induced reduction in blood loss during menses. Interestingly, and in agreement with our finding, Westhoff et. al. (119) found that *NuvaRing* users had lighter menstrual bleeding with a shorter duration than COC users. This observation could be an indication of increased *in vivo* coagulation due to the increased cessation of bleeding among CHC users.

Study Caveats

An important consideration for our study is the relatively small number of research participants involved (n=160). However, we view this study as a preliminary query into the relationship between CHC use and cardiovascular risk. Undoubtedly, a large scale, prospective study will be required to truly ascertain the alterations in cardiovascular biomarkers risk associated with COC and *NuvaRing* use.

Ideally, recruitment of control participants who had never used HCs would have been accomplished with random assignment of those participants into treatment groups (COC and *NuvaRing*). By doing this, each participant would essentially serve as their own control prior to CHC treatment. Additionally, a true control group could be kept to observe how the biomarkers change due to other factors such age and lifestyle modifications. However, this was not possible due to cross-sectional nature of this study.

It is possible that the different progestins used in first, second and third generation COCs could introduce a source of interpretive error and limit the

effectiveness and power of our analysis. Perhaps it would have been better to limit enrollment of the COC participants to a specific generation and concentration of progestin. It may have been more advantageous to allow enrollment of subjects only using COCs with the same type of estrogen and progestin that are used in the *NuvaRing* (etonogestrel and ethinyl estradiol). This may have reduced the number of unknown molecular variables affecting our analysis.

Future Work

With the knowledge gathered from this biomarker study, it is necessary to further analyze these biomarkers in a larger population of women. It would be ideal to randomize CHC treatment among a cohort of women who had never used any form of HC. This would greatly reduce study bias due to variability in the participant enrollment. Additional steps such as stricter inclusion criteria of participant age, race, and lifestyle choices should be taken. Determination of an adequate biomarker, biomarker panel or scoring system based on biomarker data is necessary. Additionally, further research could focus more on other populations such as women over the age of 35 or diabetic women. Ideally a large scale prospective study correlating biomarker level to clinical events would be implemented. Future research into HC must focus on determining and reducing the negative effects of HC on coagulatory mechanisms. HC development must concentrate on reducing the negative effects of HC on the cardiovascular system.

References

1. Tyler ET. Oral contraception. JAMA (Chicago, Ill.). 1961;175(3):225-6.

2. Tyler ET. Oral contraception and venous thrombosis. JAMA (Chicago, Ill.). 1963;185(2):131-2.

3. Boyce J, Fawcett JW, Noall EW. Coronary thrombosis and conovide. Lancet. 1963;1(7272):111.

4. Zakharova M, Meyer R, Brandy K, Datta Y, Joseph M, Shreiner P, et al. Risk factors for heart attack, stroke, and venous thrombosis associated with hormonal contraceptive use. Clinical and applied thrombosis/hemostasis. 2010.

5. van Hylckama Vlieg A, Helmerhorst FM, Vandenbroucke JP, Doggen CJM, Rosendaal FR. The venous thrombotic risk of oral contraceptives, effects of oestrogen dose and progestogen type: Results of the MEGA case-control study. BMJ.British medical journal. 2009;339:b2921-.

6. Mosher W, Jones J. Use of contraception in the united states: 1982-2008. Vital and health statistics.Series 23.Data from the national survey of family growth. 2010(29):1-44.

7. Lidegaard j, Løkkegaard E, Svendsen AL, Agger C. Hormonal contraception and risk of venous thromboembolism: National follow-up study. BMJ.British medical journal. 2009;339:b2890-.

8. Farley TM, Meirik O, Chang CL, Poulter NR. Combined oral contraceptives, smoking, and cardiovascular risk. J Epidemiol Community Health. 1998;52(12):775-85.

9. Lidegaard O. Oral contraceptives, pregnancy and the risk of cerebral thromboembolism: The influence of diabetes, hypertension, migraine and previous thrombotic disease. Br J Obstet Gynaecol. 1995;102(2):153-9.

10. Vandenbroucke JP, Koster T, Brit E, Reitsma PH, Bertina RM, Rosendaal FR. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V leiden mutation. Lancet. 1994;344(8935):1453-7.

11. Tyler ET. Antifertility agents. Annu Rev Pharmacol. 1967;7:381-98.

12. Mackay EV, Khoo SK, Adam RR. Contraception with a six-monthly injection of progestogen. 2. effects on cervical mucus secretion and endocrine function. The Australian and New Zealand journal of obstetrics and gynaecology. 1971;11(3):156-63.

13. Pincus G, Rock J, Garcia CR, Ricewray E, Paniagua M, Rodriguez I. Fertility control with oral medication. Obstet Gynecol. 1958;75(6):1333-46.

14. Gambrell RD, Bernard DM, Sanders BI, Vanderburg N, Buxton SJ. Changes in sexual drives of patients on oral contraceptives. J Reprod Med. 1976;17(3):165-71.

15. Kemmeren J, Tanis B, van den Bosch,Maurice A A J., Bollen ELEM, Helmerhorst F, van der Graaf Y, et al. Risk of arterial thrombosis in relation to oral contraceptives (RATIO) study: Oral contraceptives and the risk of ischemic stroke. Stroke. 2002;33(5):1202-8.

16. Skouby SO, Petersen KR. Clinical experience with the recently developed progestogens. Int J Fertil. 1991;36 Suppl 1:32-7.

17. Venous thromboembolic disease and combined oral contraceptives: Results of international multicentre case-control study. world health organization collaborative study of cardiovascular disease and steroid hormone contraception. Lancet. 1995;346(8990):1575-82.

18. Jick H, Jick SS, Gurewich V, Myers MW, Vasilakis C. Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. Lancet. 1995;346(8990):1589-93.

19. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Blller HR, Vandenbroucke JP. Enhancement by factor V leiden mutation of risk of deep-vein thrombosis associated with oral contraceptives containing a third-generation progestagen. Lancet. 1995;346(8990):1593-6.

20. Spitzer WO, Lewis MA, Heinemann LA, Thorogood M, MacRae KD. Third generation oral contraceptives and risk of venous thromboembolic disorders: An international case-control study. transnational research group on oral contraceptives and the health of young women. BMJ.British medical journal. 1996;312(7023):83-8.

21. de Vries CS, van den Berg PB, de Jong-van den Berg,L T. Oral contraceptive use before and after the latest pill scare in the netherlands. changes in oral contraceptive use and how users change. Contraception. 1998;57(4):247-9.

22. Heinemann LAJ, Dinger J, Assmann A, Minh T. Use of oral contraceptives containing gestodene and risk of venous thromboembolism: Outlook 10 years after the third-generation "pill scare". Contraception. 2010;81(5):401-7.

23. Odlind V, Milsom I, Persson I, Victor A. Can changes in sex hormone binding globulin predict the risk of venous thromboembolism with combined oral contraceptive pills? Acta Obstet Gynecol Scand. 2002;81(6):482-90.

24. Kemmeren J, Algra A, Meijers JCM, Tans G, Bouma B, Curvers J, et al. Effect of second- and third-generation oral contraceptives on the protein C system in the absence or presence of the factor VLeiden mutation: A randomized trial. Blood. 2004;103(3):927-33.

25. Koenig W, Rothenbacher D, Hoffmeister A, Griesshammer M, Brenner H. Plasma fibrin D-dimer levels and risk of stable coronary artery disease: Results of a large case-control study. Arterioscler Thromb Vasc Biol. 2001;21(10):1701-5.

26. Kerns J, Darney P. Vaginal ring contraception. Contraception. 2011;83(2):107-15.

27. Timmer CJ, Mulders TM. Pharmacokinetics of etonogestrel and ethinylestradiol released from a combined contraceptive vaginal ring. Clin Pharmacokinet. 2000;39(3):233-42.

28. Mattii R, Ambrus JL, Sokal JE, Mink I. Production of Members of the Blood Coagulation and Fibrinolysin Systems by the Isolated Perfused Liver. Proceedings of the Society for Experimental Biology and Medicine. 1964;116:69-72.

29. Goebelsmann U, Mashchak CA, Mishell DR. Comparison of hepatic impact of oral and vaginal administration of ethinyl estradiol. Obstet Gynecol. 1985;151(7):868-77.

30. Jensen J, Burke A, Barnhart K, Tillotson C, Messerle-Forbes M, Peters D. Effects of switching from oral to transdermal or transvaginal contraception on markers of thrombosis. Contraception. 2008;78(6):451-8.

31. van Vliet,Huib A A M., Rosendaal F, Fleischer K, Rosing J, Helmerhorst F. Effects of the contraceptive vaginal ring, the contraceptive transdermal patch and combined oral contraceptives on markers of hemostasis. Contraception. 2010;81(1):88-9.

32. Divani A, Vazquez G, Asadollahi M, Qureshi A, Pullicino P. Nationwide frequency and association of heart failure on stroke outcomes in the united states. J Card Fail. 2009;15(1):11-6.

33. Baillargeon J, McClish D, Essah P, Nestler J. Association between the current use of low-dose oral contraceptives and cardiovascular arterial disease: A meta-analysis. J Clin Endocrinol Metab. 2005;90(7):3863-70.

34. Curtis K, Mohllajee A, Martins S, Peterson H. Combined oral contraceptive use among women with hypertension: A systematic review. Contraception. 2006;73(2):179-88.

35. Shapiro S. Oral contraceptives, hormone therapy and cardiovascular risk. Climacteric. 2008;11(5):355-63.

36. Shufelt C, Bairey Merz CN. Contraceptive hormone use and cardiovascular disease. J Am Coll Cardiol. 2009;53(3):221-31.

37. Tanis BC. Oral contraceptives and the risk of myocardial infarction. Eur Heart J. 2003;24(5):377-80.

38. Van Den Bosch,M A A J., Kemmeren JM, Tanis BC, Mali WPTM, Helmerhorst FM, Rosendaal FR, et al. The RATIO study: Oral contraceptives and the risk of peripheral arterial disease in young women. Journal of thrombosis and haemostasis. 2003;1(3):439-44.

39. Farley TM, Meirik O, Chang CL, Poulter NR. Combined oral contraceptives, smoking, and cardiovascular risk. J Epidemiol Community Health. 1998;52(12):775-85.

40. Levi M, van der Poll T, Blller H. Bidirectional relation between inflammation and coagulation. Circulation. 2004;109(22):2698-704.

41. Tanis BC. Oral contraceptives and the risk of myocardial infarction. N Engl J Med. 2001;345(25):1787.

42. Acute myocardial infarction and combined oral contraceptives: Results of an international multicentre case-control study. WHO collaborative study of cardiovascular disease and steroid hormone contraception. Lancet. 1997;349(9060):1202-9.

43. Khader Y, Rice J, John L, Abueita O. Oral contraceptives use and the risk of myocardial infarction: A meta-analysis. Contraception. 2003;68(1):11-7.

44. Vandenbroucke JP, Koster T, Brit E, Reitsma PH, Bertina RM, Rosendaal FR. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V leiden mutation. Lancet. 1994;344(8935):1453-7.

45. Schwartz SM, Siscovick DS, Longstreth WT, Psaty BM, Beverly RK, Raghunathan TE, et al. Use of low-dose oral contraceptives and stroke in young women. Ann Intern Med. 1997;127(8):596-603.

46. Haemorrhagic stroke, overall stroke risk, and combined oral contraceptives: Results of an international, multicentre, case-control study. WHO collaborative study of cardiovascular disease and steroid hormone contraception. Lancet. 1996;348(9026):505-10.

47. Heinemann LA, Lewis MA, Thorogood M, Spitzer WO, Guggenmoos-Holzmann I, Bruppacher R. Case-control study of oral contraceptives and risk of thromboembolic stroke: Results from international study on oral contraceptives and health of young women. BMJ.British medical journal. 1997;315(7121):1502-4.

48. Petitti DB, Sidney S, Bernstein A, Wolf S, Quesenberry C, Ziel HK. Stroke in users of low-dose oral contraceptives. N Engl J Med. 1996;335(1):8-15.

49. Farley TM, Collins J, Schlesselman JJ. Hormonal contraception and risk of cardiovascular disease. an international perspective. Contraception. 1998;57(3):211-30.

50. Middeldorp S, Meijers JC, van den Ende AE, van Enk A, Bouma BN, Tans G, et al. Effects on coagulation of levonorgestrel- and desogestrel-containing low dose oral contraceptives: A cross-over study. Thromb Haemost. 2000;84(1):4-8.

51. Tans G, Curvers J, Middeldorp S, Thomassen MC, Meijers JC, Prins MH, et al. A randomized cross-over study on the effects of levonorgestrel- and desogestrel-containing oral contraceptives on the anticoagulant pathways. Thromb Haemost. 2000;84(1):15-21.

52. Atthobari J, Gansevoort R, Visser S, de Jong P, de Jong-van den Berg,Lolkje T W. The impact of hormonal contraceptives on blood pressure, urinary albumin excretion and glomerular filtration rate. Br J Clin Pharmacol. 2007;63(2):224-31.

53. Lubianca JN, Moreira LB, Gus M, Fuchs FD. Stopping oral contraceptives: An effective blood pressure-lowering intervention in women with hypertension. J Hum Hypertens. 2005;19(6):451-5.

54. Tybor D, Lichtenstein A, Dallal G, Daniels S, Must A. Independent effects of age-related changes in waist circumference and BMI z scores in predicting cardiovascular disease risk factors in a prospective cohort of adolescent females. Am J Clin Nutr. 2011;93(2):392-401.

55. Bennett B, Ratnoff OD. The normal coagulation mechanism. Med Clin North Am. 1972;56(1):95-104.

56. Stec JJ, Silbershatz H, Tofler GH, Matheney TH, Sutherland P, Lipinska I, et al. Association of fibrinogen with cardiovascular risk factors and cardiovascular

disease in the framingham offspring population. Circulation. 2000;102(14):1634-8.

57. Johnson J, Lowell J, Badger G, Rosing J, Tchaikovski S, Cushman M. Effects of oral and transdermal hormonal contraception on vascular risk markers: A randomized controlled trial. Obstet Gynecol. 2008;111(2):278-84.

58. Scarabin PY, Plu-Bureau G, Zitoun D, Bara L, Guize L, Samama MM. Changes in haemostatic variables induced by oral contraceptives containing 50 micrograms or 30 micrograms oestrogen: Absence of dose-dependent effect on PAI-1 activity. Thromb Haemost. 1995;74(3):928-32.

59. Wiegratz I, Lee JH, Kutschera E, Winkler UH, Kuhl H. Effect of four oral contraceptives on hemostatic parameters. Contraception. 2004;70(2):97-106.

60. Steffel J, Lscher T, Tanner F. Tissue factor in cardiovascular diseases: Molecular mechanisms and clinical implications. Circulation. 2006;113(5):722-31.

61. Kluft C, Lansink M. Effect of oral contraceptives on haemostasis variables. Thromb Haemost. 1997;78(1):315-26.

62. Harris GM, Stendt CL, Vollenhoven BJ, Gan TE, Tipping PG. Decreased plasma tissue factor pathway inhibitor in women taking combined oral contraceptives. Am J Hematol. 1999;60(3):175-80.

63. Sadler JE. Biochemistry and genetics of von willebrand factor. Annu Rev Biochem. 1998;67:395-424.

64. Blann AD, Miller JP, McCollum CN. Von willebrand factor and soluble E-selectin in the prediction of cardiovascular disease progression in hyperlipidaemia. Atherosclerosis. 1997;132(2):151-6.

65. Roell A, Schueller P, Schultz A, Losel R, Wehling M, Christ M, et al. Effect of oral contraceptives and ovarian cycle on platelet function. Platelets. 2007;18(2):165-70.

66. Kip K, Marroquin O, Shaw L, Arant C, Wessel T, Olson M, et al. Global inflammation predicts cardiovascular risk in women: A report from the women's ischemia syndrome evaluation (WISE) study. Am Heart J. 2005;150(5):900-6.

67. Pearson T, Mensah G, Alexander RW, Anderson J, Cannon R, Criqui M, et al. Markers of inflammation and cardiovascular disease: Application to clinical and public health practice: A statement for healthcare professionals from the centers for disease control and prevention and the american heart association. Circulation. 2003;107(3):499-511.

68. Haarala A, Eklund C, Pessi T, Lehtimki T, Huupponen R, Jula A, et al. Use of combined oral contraceptives alters metabolic determinants and genetic regulation of C-reactive protein. the cardiovascular risk in young finns study. Scandinavian journal of clinical laboratory investigation. 2009;69(2):168-74.

69. van Rooijen M, Hansson LO, Frostegrd J, Silveira A, Hamsten A, Bremme K. Treatment with combined oral contraceptives induces a rise in serum C-reactive protein in the absence of a general inflammatory response. Journal of thrombosis and haemostasis. 2006;4(1):77-82.

70. Silvestri A, Gebara O, Vitale C, Wajngarten M, Leonardo F, Ramires JAF, et al. Increased levels of C-reactive protein after oral hormone replacement therapy may not be related to an increased inflammatory response. Circulation. 2003;107(25):3165-9.

71. Ivandic B, Spanuth E, Haase D, Lestin H, Katus H. Increased plasma concentrations of soluble CD40 ligand in acute coronary syndrome depend on in vitro platelet activation. Clin Chem. 2007;53(7):1231-4.

72. Kebapcilar L, Bilgir O, Taner C, Kebapcilar A, Kozaci D, Alacacioglu A, et al. Oral contraceptives alone and with spironolactone increase sCD40 ligand in PCOS patients. Arch Gynecol Obstet. 2010;281(3):539-43.

73. Anderson J, Ronnow B, Horne B, Carlquist J, May H, Bair T, et al. Usefulness of a complete blood count-derived risk score to predict incident mortality in patients with suspected cardiovascular disease. Am J Cardiol. 2007;99(2):169-74.

74. Vannucchi A, Barbui T. Thrombocytosis and thrombosis. Hematology. 2007:363-70.

75. Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. Circulation. 2001;103(4):491-5.

76. Segarra A, Chacn P, Martinez-Eyarre C, Argelaguer X, Vila J, Ruiz P, et al. Circulating levels of plasminogen activator inhibitor type-1, tissue plasminogen activator, and thrombomodulin in hemodialysis patients: Biochemical correlations and role as independent predictors of coronary artery stenosis. Journal of the American Society of Nephrology. 2001;12(6):1255-63.

77. Tonstad S, Thorsrud H, Torjesen P, Seljeflot I. Do novel risk factors differ between men and women aged 18 to 39 years with a high risk of coronary heart disease? Metabolism, clinical and experimental. 2007;56(2):260-6.

78. Tyler ET. Oral contraception and venous thrombosis. JAMA (Chicago, Ill.). 1963;185(2):131-2.

79. Vasilakis C, Jick H, del Mar Melero-Montes M. Risk of idiopathic venous thromboembolism in users of progestagens alone. Lancet. 1999;354(9190):1610-1.

80. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Vandenbroucke JP. Higher risk of venous thrombosis during early use of oral contraceptives in women with inherited clotting defects. Arch Intern Med. 2000;160(1):49-52.

81. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Bller HR, Vandenbroucke JP. Enhancement by factor V leiden mutation of risk of deep-vein thrombosis associated with oral contraceptives containing a third-generation progestagen. Lancet. 1995;346(8990):1593-6.

82. Vandenbrouke JP, Bloemenkamp KW, Rosendaal FR, Helmerhorst FM. Incidence of venous thromboembolism in users of combined oral contraceptives. risk is particularly high with first use of oral contraceptives. BMJ.British medical journal. 2000;320(7226):57-8.

83. Johansson S, Vedin A, Wilhelmsson C. Myocardial infarction in women. Epidemiol Rev. 1983;5:67-95.

84. Lidegaard O. Thrombotic diseases in young women and the influence of oral contraceptives. Obstet Gynecol. 1998;179(3):S62-7.

85. Jolly S, Vittinghoff E, Chattopadhyay A, Bibbins-Domingo K. Higher cardiovascular disease prevalence and mortality among younger blacks compared to whites. Am J Med. 2010;123(9):811-8.

86. Murray CJL, Kulkarni S, Michaud C, Tomijima N, Bulzacchelli M, Iandiorio T, et al. Eight americas: Investigating mortality disparities across races, counties, and race-counties in the united states. PLoS Medicine. 2006;3(9):e260-.

87. Disparities in premature deaths from heart disease--50 states and the district of columbia, 2001. Morb Mortal Weekly Rep. 2004;53(6):121-5.

88. Padilla H, Gaziano JM, Djouss L. Alcohol consumption and risk of heart failure: A meta-analysis. The Physician and sportsmedicine. 2010;38(3):84-9.

89. Ruidavets J, Ducimetire P, Evans A, Montaye M, Haas B, Bingham A, et al. Patterns of alcohol consumption and ischaemic heart disease in culturally divergent countries: The prospective epidemiological study of myocardial infarction (PRIME). BMJ.British medical journal. 2010;341:c6077-.

90. Suzuki K, Elkind MSV, Boden-Albala B, Jin Z, Berry G, Di Tullio M, et al. Moderate alcohol consumption is associated with better endothelial function: A cross sectional study. BMC cardiovascular disorders. 2009;9:8-.

91. Mukamal K, Chen C, Rao S, Breslow R. Alcohol consumption and cardiovascular mortality among U.S. adults, 1987 to 2002. J Am Coll Cardiol. 2010;55(13):1328-35.

92. van Golde PM, Hart HC, Kraaijenhagen RJ, Bouma BN, van de Wiel A. Regular alcohol intake and fibrinolysis. Neth J Med. 2002;60(7):285-8.

93. Effects of moderate alcohol consumption on platelet aggregation, fibrinolysis, and blood lipids. Metabolism, clinical and experimental. 1988;37(5):502-3.

94. Aikens ML, Grenett HE, Benza RL, Tabengwa EM, Davis GC, Booyse FM. Alcohol-induced upregulation of plasminogen activators and fibrinolytic activity in cultured human endothelial cells. Alcoholism: clinical and experimental research. 1998;22(2):375-81.

95. Dimmitt SB, Rakic V, Puddey IB, Baker R, Oostryck R, Adams MJ, et al. The effects of alcohol on coagulation and fibrinolytic factors: A controlled trial. Blood coagulation fibrinolysis. 1998;9(1):39-45.

96. Engstrm M, Schtt U, Reinstrup P. Ethanol impairs coagulation and fibrinolysis in whole blood: A study performed with rotational thromboelastometry. Blood coagulation fibrinolysis. 2006;17(8):661-5.

97. Sabanayagam C, Shankar A. Sleep duration and cardiovascular disease: Results from the national health interview survey. Sleep. 2010;33(8):1037-42.

98. Qureshi A, Suri MFK, Kirmani J, Divani A, Mohammad Y. Is prehypertension a risk factor for cardiovascular diseases? Stroke. 2005;36(9):1859-63.

99. Roumen F. Contraceptive efficacy and tolerability with a novel combined contraceptive vaginal ring, NuvaRing. European Journal of Contraception Reproductive Health Care. 2002;7 Suppl 2:19-24.

100. Rosenberg M. Weight change with oral contraceptive use and during the menstrual cycle. results of daily measurements. Contraception. 1998;58(6):345-9.

101. Oddens BJ. Women's satisfaction with birth control: A population survey of physical and psychological effects of oral contraceptives, intrauterine devices, condoms, natural family planning, and sterilization among 1466 women. Contraception. 1999;59(5):277-86.

102. Emans SJ, Grace E, Woods ER, Smith DE, Klein K, Merola J. Adolescents' compliance with the use of oral contraceptives. JAMA (Chicago, Ill.). 1987;257(24):3377-81.

103. O'Connell K, Osborne L, Westhoff C. Measured and reported weight change for women using a vaginal contraceptive ring vs. a low-dose oral contraceptive. Contraception. 2005;72(5):323-7.

104. Dyer AR, Stamler J, Berkson DM, Lindberg HA. Relationship of relative weight and body mass index to 14-year mortality in the chicago peoples gas company study. J Chronic Dis. 1975;28(2):109-23.

105. Rabkin SW, Mathewson FA, Hsu PH. Relation of body weight to development of ischemic heart disease in a cohort of young north american men after a 26 year observation period: The manitoba study. Am J Cardiol. 1977;39(3):452-8.

106. Roldn V, Marn F, Lip GYH, Blann A. Soluble E-selectin in cardiovascular disease and its risk factors. A review of the literature. Thromb Haemost. 2003;90(6):1007-20.

107. Ponthieux A, Herbeth B, Droesch S, Haddy N, Lambert D, Visvikis S. Biological determinants of serum ICAM-1, E-selectin, P-selectin and -selectin levels in healthy subjects: The stanislas study. Atherosclerosis. 2004 2;172(2):299-308.

108. Miyamoto N, Mandai M, Suzuma I, Suzuma K, Kobayashi K, Honda Y. Estrogen protects against cellular infiltration by reducing the expressions of E-selectin and IL-6 in endotoxin-induced uveitis. J Immunol. 1999 July 1;163(1):374-9.

109. Caulin-Glaser T, Watson CA, Pardi R, Bender JR. Effects of 17beta-estradiol on cytokine-induced endothelial cell adhesion molecule expression. J Clin Invest. 1996;98(1):36-42.

110. Shaarawy M, Nafea S, Abdel-Aziz O, Rahseed K, Sheiba M. The cardiovascular safety of triphasic contraceptive steroids. Contraception. 1997;56(3):157-63.

111. Heeschen C, Dimmeler S, Hamm C, van den Brand M, Boersma E, Zeiher A, et al. Soluble CD40 ligand in acute coronary syndromes. N Engl J Med. 2003;348(12):1104-11.

112. Schnbeck U, Varo N, Libby P, Buring J, Ridker PM. Soluble CD40L and cardiovascular risk in women. Circulation. 2001;104(19):2266-8.

113. Ferro D, Loffredo L, Polimeni L, Fimognari F, Villari P, Pignatelli P, et al. Soluble CD40 ligand predicts ischemic stroke and myocardial infarction in patients with nonvalvular atrial fibrillation. Arterioscler Thromb Vasc Biol. 2007;27(12):2763-8.

114. Verma S, Kuliszewski M, Li S, Szmitko P, Zucco L, Wang C, et al. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: Further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. Circulation. 2004;109(17):2058-67.

115. Zieske A, Tracy R, McMahan CA, Herderick E, Homma S, Malcom G, et al. Elevated serum C-reactive protein levels and advanced atherosclerosis in youth. Arterioscler Thromb Vasc Biol. 2005;25(6):1237-43.

116. Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom AR, Juneja H, et al. Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the atherosclerosis risk in communities (ARIC) study: A case-cohort study. Lancet. 1999;353(9166):1729-34.

117. Dahm A, Van Hylckama Vlieg A, Bendz B, Rosendaal F, Bertina R, Sandset P. Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. Blood. 2003;101(11):4387-92.

118. Magnusdttir EM, Bjarnadttir RI, Onundarson PT, Gudmundsdttir BR, Geirsson RT, Magnusdttir SD, et al. The contraceptive vaginal ring (NuvaRing) and hemostasis: A comparative study. Contraception. 2004;69(6):461-7.

119. Westhoff C, Osborne L, Schafer J, Morroni C. Bleeding patterns after immediate initiation of an oral compared with a vaginal hormonal contraceptive. Obstet Gynecol. 2005;106(1):89-96.