EFFECTS OF CAFFEINE, STRESS, AND FAMILY HISTORY OF HYPERTENSION ON BLOOD PRESSURE AND BLOOD MARKERS OF CARDIOVASCULAR DISEASE RISK

A Thesis in
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by

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ABSTRACT

Cardiovascular disease has been the leading cause of death in the United States since 1918. A number of studies have investigated the connection between caffeine, primarily through coffee, and its potentially detrimental effects on cardiovascular health. The vast majority of such investigations have been focused on blood cholesterol, while other blood markers of cardiovascular disease such as fibrinogen and C-reactive protein and their relationship with caffeine has been vastly understudied. A large number of studies have reported that both fibrinogen and C-reactive protein can be useful predictors of future cardiovascular disease, but their relationship with caffeine remains unknown. Furthermore, to this date, no published studies have reported on the effects of psychological stress on these blood markers, either alone, or in combination with caffeine, and no gender differences have been reported.

The current study examined the effects of caffeine and psychological stress on a population particularly vulnerable to future development of cardiovascular disease, those with a parental history of hypertension. This study used a 2 (gender; 26 men, 26 women) X 2 (drug condition; 26 no caffeine, 26 3.3mg/kg caffeine) experimental design. Participants came to a initial screening session where blood was drawn for a cholesterol assessment and extensive health screening to ensure that all participants were healthy. Qualified participants then completed a 3.5 hour experimental session to examine stress reactivity to caffeine or no caffeine exposure. Blood pressure and heart rate were collected during the experimental session, as well as 3 blood samples at baseline, stress, and recovery for later assessment of cortisol, C-reactive protein (CRP) and fibrinogen levels, and, for females, progesterone and estradiol to confirm luteal phase cycle status.

Findings from this study revealed increases in fibrinogen levels in response to caffeine, but no significant CRP changes. However, significant increase in CRP levels were observed when post-hoc analyses were conducted that involved the grouping of participants into low, moderate, and high CRP risk group based on baseline CRP levels. Systolic blood pressure significantly increased in males and females in response to both the stressor and caffeine. Diastolic blood pressure and heart rate significantly increased in males and females in response to the stressor, but not in response to caffeine. Implications of these findings are discussed, as well as methodological differences between this dissertation project and previous research. Suggestions for future studies and the contribution of the current study to the literature are presented.
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CHAPTER 1: INTRODUCTION

Scope of the Problem

Caffeine is the most widely consumed psychostimulant drug in the world, with about 75% of its consumption taken in the form of coffee (Chou, 1992). Other sources of caffeine include tea, caffeinated cola drinks, and chocolate, as well as herbal supplements and prescription and over-the counter diet drugs (James, 1997). In the United States, of those individuals who drink coffee, the average consumption is three cups per day (James, 1997). Higher caffeine levels are consumed in Central and South America and Western Europe, compared to the United States, and the highest level of consumption is found in Scandinavia, especially Finland and Sweden (Chou & Benowitz, 1994).

While some investigators have found that coffee consumption may be related to decreased risk of certain conditions, such as type II diabetes (van Dam & Hu, 2005), a large number of researchers have reported adverse effects of caffeine on different aspects of health, such as cardiovascular disease (Kawachi et al., 1994), fibrocystic breast disease (Curatolo & Robertson, 1983; Garattini & Tognoni, 1993), several types of cancer (Chou, 1992), and reproductive problems (Hinds et al., 1996; Klesges et al., 1994; Patton et al., 1995). Since the 1900s, cardiovascular disease (CVD) has been the number 1 killer in the United States every year except 1918. Indeed, nearly 2,600 Americans die of CVD each day, an average of 1 death every 33 seconds. The purpose of this section is to review the current knowledge on the relationship between caffeine (coffee being its major
source) and cardiovascular disease risk. The importance of better understanding how caffeine and coffee can affect the development of cardiovascular disease could have a tremendous impact on improving health outcomes. Not only is caffeine the most widely consumed psychoactive drug, but for the majority of caffeine consumers, exposure to this drug is effectively lifelong (James, 1997). Level of exposure is a key factor in assessing the cumulative biobehavioral impact of any drug-induced effects. Considering current levels of caffeine use, even small effects, when accumulated across entire populations, may have very important health consequences.

Cardiovascular disease risk markers include elevated resting systolic and/or diastolic blood pressure, low density lipoproteins (i.e., cholesterol), triglycerides, blood clotting factors (e.g., fibrinogen), and the acute-phase protein C-reactive protein (CRP). To the extent that changes in these markers are associated with exposure to caffeine, usually in the form of coffee, then a biological mechanism(s) that underlies the relationship between caffeine consumption and CVD can be better understood. The relationship between acute caffeine consumption and elevated blood pressure levels has been well-established, along with vascular mechanisms for this effect (Hartley et al., 2001). What is less understood is the role that caffeine exposure and coffee intake play in influencing the development of cardiovascular disease via blood markers of CVD.

The purpose of this section is to outline the literature that examines the relationship between caffeine intake, primarily via coffee consumption, and blood makers of CVD risk. More specifically, this review will focus on four blood markers that have been shown to be related to the development of cardiovascular disease, namely, blood lipids, which include cholesterol and triglycerides, fibrinogen, and C-reactive protein.
With respect to these blood markers, blood lipids (especially cholesterol), and their relationship to coffee has been the most studied, while very few studies have examined the effects of either coffee or caffeine by itself on fibrinogen and C-reactive protein. This section also will highlight key methodological problems (e.g., controlling for diet, sample size, tobacco use) and gaps (e.g., effects of caffeine on these markers among women) that still exist in our understanding. Coffee is the main source of caffeine. However, very little work has been done examining the effects of caffeine per se in humans. Therefore, the primary focus will be on studies examining coffee, although caffeine-specific studies are included when they are available.

**Blood Markers of Cardiovascular Health**

**Cholesterol**

Cholesterol, a waxy, fatty substance found in the bloodstream is necessary for the normal functioning of various body systems, such as the production of cell membranes and a number of hormones (AHA, 2003a). A link between high levels of total cholesterol and a higher incidence of cardiovascular disease (CVD) was first reported in 1955 (Bronte-Stewart et al., 1955). Although a high level of total serum cholesterol appears to be associated with the development of cardiac disease (Heiss et al., 1980; Manninen et al., 1988), it is important to examine the two main types of cholesterol, high-density lipoproteins (HDL) and low-density lipoproteins (LDL), which are better predictors of CVD than is total serum cholesterol alone. HDL cholesterol is known as the beneficial kind because it seems to protect against cardiovascular disease, while LDL cholesterol is
considered to be the detrimental kind as it appears to increase one’s risk of developing CVD (Gordon et al., 1977; Heiss et al., 1980; Manninen et al., 1988; Miller et al., 1977; Rhoads et al., 1976) by transporting cholesterol from the liver to the tissues of the body. In contrast, HDL carries cholesterol molecules away from arteries and back to the liver, where these molecules are metabolized and excreted. It also is believed that HDL removes excess cholesterol from plaques that have already formed in arteries, therefore slowing down the buildup process that leads to atherosclerosis and the development of CVD (AHA, 2003a).

There are some important caveats to keep in mind when reviewing the literature on changes in blood cholesterol levels. First, triglycerides also can be used as predictors of serum cholesterol levels, as individuals with high levels of triglycerides typically also have a high total cholesterol level, high levels of LDL cholesterol, and low levels of HDL (AHA, 2003a). Triglycerides are also a form of fat, which primarily come from dietary fat, but also are endogenously produced (AHA, 2003a). Second, dietary fat intake, age, body mass index [BMI; weight (kg)/height (meters)$^2$], and cigarette smoking have been found to be positively associated with total cholesterol levels and LDL cholesterol levels (Jacobsen & Thelle, 1987). In contrast, physical activity and moderate doses of alcohol have been found to be negatively associated with serum total and LDL cholesterol levels (Mukamal, 2003). Therefore, these variables need to be controlled for or acknowledged when interpreting the effects of caffeine on these blood markers.
**Fibrinogen**

Fibrinogen, a clotting protein produced in the liver, is now starting to receive much research attention as investigators believe that it may be the missing link among tobacco smoking, elevated LDL cholesterol, and CVD (American Heart Association, 2003a). This soluble, acute-phase protein circulates in the blood and provides the materials from which the insoluble fibrin clot is formed during blood coagulation. Fibrinogen, therefore, helps to stop bleeding by helping blood clots to form in a process that involves the breakdown of fibrinogen by thrombin into short fragments of fibrin, which activate the release of Factor XIII. Factor XIII, in turn, weaves the fibrin segments together, closing off the injured blood vessel walls; blood platelets then attach to the fibrin segments and clump together to form blood clots and stop bleeding (Meade et al., 1993). Fibrinogen levels increase in response to a number of factors, such as infection and other short-term inflammatory stressors, such as cigarette smoking. Elevated fibrinogen levels are also seen with advancing age, obesity, diabetes, physical inactivity, and high levels of blood LDL and total cholesterol (Stec et al., 2000).

Increases in CRP in response to cardiovascular reactivity have been found to be mediated through the activation of acute-phase proteins (i.e. inflammatory cytokines). Both CRP and fibrinogen are part of this group of proteins and released via the activation of the inflammatory cytokines IL-6 and IL-1 beta (Black, 2002, 2003; Black & Garbutt, 2002; Ganter et al., 1989; Owen et al., 2003; Steptoe et al., 2001). Therefore, changes in the level of these blood markers are not expected to be mediated through cholesterol, but through induction of inflammatory cytokines.
A number of studies have found that a single measure of plasma fibrinogen can be used as an allostatic load marker [cumulative negative effects of stress on one’s body due to less efficient physiological functioning or by being physiologically overwhelmed by too many challenges (McEwen, 1998)], with higher levels of fibrinogen indicating a higher allostatic load and poorer adaptation of the haemostatic system to physical, psychological, and environmental stressors (Bosma et al., 1997; E. Brunner et al., 1996; E. J. Brunner et al., 1993; Heinrich et al., 1994). High plasma fibrinogen levels in adulthood have been associated with a statistically significant increase in the risk of CVD and stroke. For example, prospective studies in both men (Heinrich et al., 1994; Kannel et al., 1987; Meade et al., 1993; Wilhelmsen et al., 1984; Yarnell et al., 1991) and women (Kannel et al., 1987) have shown that a single fibrinogen measurement predicts fatal and non-fatal cardiovascular events as much as 16 years later (Meade et al., 1993). Along the same lines, other investigators have found that fibrinogen levels are higher among participants with cardiovascular disease compared with those without cardiovascular disease (Obradovic et al., 2003; Stec et al., 2000). At this point researchers are not entirely sure as to whether a rise in fibrinogen is a cause or a consequence of CVD. Fibrinogen may promote atherosclerotic changes and thrombosis through increases shown in vitro on platelet aggregability and blood viscosity (Yarnell et al., 1991). This increased aggregation and change in blood viscosity increases the risk of stroke and heart attack. As a result, this process seems indicative of a causal role for fibrinogen in CVD. An alternative view is that the prospective association between fibrinogen and CVD may be a consequence, rather than a cause, of the disease process, perhaps due to an inflammatory response to progressive endothelial damage (Heinrich et al., 1994; Kannel
et al., 1987). Both of these perspectives, which are not mutually exclusive, support the hypothesis that fibrinogen may play a role in the development of CVD (E. Brunner et al., 1996).

In the laboratory setting, investigators have found that psychological stress may also play a role in raising fibrinogen levels. For example, Muldoon and colleagues (1995) found that a frustrating cognitive task considered to be stressful led to significant increases in plasma viscosity, compared to rest. Fibrinogen levels also were found to increase in the frustrating task condition compared to rest. Furthermore, a recent review discusses a number of findings indicating increases of fibrin-D (small fibrinogen fibers that have been broken down) in response to chronic and acute psychological distress (von Kanel &Dimsdale, 2003). With respect to measurement of this blood marker, fibrinogen (as indicated by fibrin-D dimer) has not been found to show diurnal variation (Jafri et al., 1992).

C-Reactive Protein (CRP)

C-reactive protein (CRP) is an immune maker of inflammation and another acute-phase protein that also appears to be correlated with increased risk for CVD (Benzaquen et al., 2002; Ridker et al., 2003b; Rifai & Ridker, 2002; Roberts et al., 2001). What makes this marker an exciting advancement in our understanding of the development of CVD is that it appears to make an independent contribution to CVD that goes beyond currently available disease risk markers such as fibrinogen, LDL cholesterol, and arterial wall thickness (Cao et al., 2003; Ridker et al., 2002), (see Danesh et al., 2004 for an alternative view). For example, CRP elevations independently increase the risk of stroke, above the level of risk that would be predicted by the increased thickness of carotid artery
walls (typically an indicator of atherosclerotic plaque in the arteries leading to the brain) (Cao et al., 2003). In addition, individuals with elevated CRP levels do not receive the same cardiovascular benefits of cholesterol reduction while on a low-fat, low-cholesterol diet as do those with lower CRP levels (Erlinger et al., 2003). Further, elevated CRP levels, combined with high blood pressure levels, appears to place women at a significantly greater risk for heart attacks and strokes compared to women with hypertension who do not have elevated CRP levels (Ridker et al., 2003b).

In a recent review of the connection between CRP and the development of cardiovascular disease, Ridker (2003d) discusses the role of CRP as an acute-phase protein and how it plays an important role in the body’s immune response, which appears to be mediated by changes in the cytokines IL-6 and IL-1 beta (Ganter et al., 1989). For men and women, a large number of studies indicate that CRP levels are an independent predictor of future cardiovascular disease risk (Arici & Walls, 2001; Backes et al., 2004; Blake & Ridker, 2003a, 2003b; Blake et al., 2003; Cesari et al., 2003; Harb et al., 2003; Hashimoto et al., 2001; Pradhan et al., 2003; Ridker, 2003a, 2003d; Ridker et al., 2003a; Ridker et al., 2003b; Ridker & Morrow, 2003; Sesso et al., 2003; Torres & Ridker, 2003). For example, the question of whether CRP levels could be used as a future cardiovascular disease risk predictor independently of cholesterol levels was tested in a large sample in the Women’s Health Study. The population of this study was composed of 27,939 women with no history of cardiovascular disease, who were followed for a period of 8 years. After controlling for age, smoking status, diabetes, blood pressure, and use of hormone replacement therapy, the findings of this study indicate that CRP is a better predictor of future cardiovascular disease than LDL cholesterol (Ridker et al., 2002).
Recently, however, a study conducted by Danesh and colleagues (2004) indicated that perhaps CRP may only be a moderate predictor of cardiovascular disease. In this study, CRP levels were measured in about 2400 participants with cardiovascular disease diagnosed since their enrollment in the study, and about 4000 controls that did not develop cardiovascular disease. These investigators found that the long-term prediction of cardiovascular disease by CRP was lower than the value previously reported by other investigators, and the value of CRP was relatively moderate, adding only marginally to the predictive value of other established cardiovascular disease predictors such as increased total cholesterol and cigarette smoking (Danesh et al., 2004).

It is important to note that prior studies such as al’Absi and colleagues (1998) have indicated that caffeine leads to increased cardiovascular reactivity, and other studies, such as Burker and colleagues (1994) also reveal that cardiovascular reactivity is associated with higher levels of total and LDL cholesterol. CRP appears to be a predictor of cardiovascular disease that is independent of cholesterol (e.g. Ridker, 2003b; e.g. Ridker et al., 2002), therefore, changes in CRP in response to cardiovascular reactivity are not mediated through changes in cholesterol levels. The role of CRP in coronary artery disease remains unclear; is not known at this point whether it is merely a marker of disease or whether it actually plays a role in the development of atherosclerotic disease (Benzaquen et al., 2002).

In the laboratory setting, CRP increases have been seen in response to acute psychological stressors. For example, Black and colleagues (2003, 2002) have shown that the inflammatory response is an integral part of the stress response. These investigators found significant increases in the inflammatory cytokines IL-1 and IL-6 in response to
acute stressors in the laboratory, and suggest that these inflammatory events which are caused by stress may account for about 40% of atherosclerotic patients who have no other known risk factors (Black & Garbutt, 2002). With respect to measurement of CRP, this blood marker has not been found to show diurnal variations in healthy participants (Meier-Ewert et al., 2001).

The timing of sample collection with respect to CRP has not been well studied. After close examination of the literature in this area, I was able to locate a single article that employed several measurements of CRP, finding significant increases in this blood marker following 1-hour after the administration of a high-fat meal (Carroll & Schade, 2003). Although this study did not examine caffeine administration or cardiovascular reactivity, this study demonstrates that the CRP response system is labile, and that significant rises can be seen in as little as one hour following a physiological stressor (these changes may even be seen in less than one hour, however studies have not been published to demonstrate this effect). Following this literature search, I also contacted Dr. David Scheade, leading expert in the measurement of CRP and one the authors in the previously mentioned study, about the timing of CRP measurement for stress protocols. Dr. Schade’s reply indicated that he is not aware of any data that would suggest how fast CRP levels can change in response to a psychological stressor. Therefore, it was important to determine whether cytokines mediating the inflammatory response had been measured in response to a psychological stressor.

A number of investigators have, however, found that acute and chronic psychological stress leads to significant increases in CRP levels, as well as CRP’s mediating cytokines IL-6 and IL1 beta (Black, 2002, 2003; Black & Garbutt, 2002; Owen
et al., 2003; Steptoe et al., 2001). For example, investigators have found that cardiovascular reactivity to two stressor tasks (mirror tracing and color-word interference) led to significant elevations in CRP and its mediating cytokines (Steptoe et al., 2001). IL-6 also was significantly elevated 45 minutes following the stressor, while IL-1 was significantly elevated at 2 hours following the stressor. The same investigators also found that the magnitude of blood pressure reactivity was significantly positively correlated with the level of cytokine concentration. These studies provide us with some indication as to the when to sample CRP following administration of a stressor, or any given substance to be studied.

**Summary**

Taken together, the literature suggests that lipoproteins (e.g., LDL cholesterol), fibrinogen, and CRP are important blood markers of CVD risk that are sensitive to biobehavioral influences such as age, body weight, gender, and diet, including, perhaps, caffeine and coffee intake. To the extent that changes in these makers are associated with different levels of caffeine intake, a better understanding of the effects of caffeine intake on the development of CVD could be developed. The rest of this review will focus on the effects of caffeine and coffee consumption on these blood markers.

**Caffeine, cholesterol, and triglycerides**

**Early Findings**

Early laboratory-based findings on the relationship between caffeine and its potential to elevate blood lipids (i.e., cholesterol and triglycerides) are inconsistent, with
some studies reporting a significant rise in cholesterol levels following ingestion of caffeine through coffee in rats and rabbits (Fears, 1978), and also in humans (Bellet et al., 1968), and others showed no effect in rhesus monkeys (Callahan et al., 1979) or humans (Steinke, 1973). Eventually, however, epidemiological studies began to report a strong positive association between coffee consumption and elevated blood lipid levels, which attracted much attention to this area of study due to the known positive connection between serum total and LDL cholesterol and the development of CVD. The major findings of these epidemiological studies examining the relationship between consumption of coffee and its effect on blood lipids are discussed below, as well as key methodological flaws.

**Epidemiological studies examining the relationship between coffee and blood lipids**

One of the first epidemiological studies to report a strong association between consumption of coffee and blood lipid levels was the Tromso heart study (Thelle et al., 1983). This study was conducted in Tromso, Norway, and included 7,213 women and 7,368 men between the ages of 20 and 54 years, following a screening in 1979-1980. The major finding of this study was that coffee consumption was strongly positively associated with levels of total cholesterol and triglycerides in both men and women, and was negatively associated with HDL cholesterol levels in women. This relationship remained very strong and statistically significant (p < 0.0001) even after adjustment for age, body-mass index (BMI), physical activity, alcohol consumption, and cigarette smoking (Thelle et al., 1983). Interestingly, the predominant type of coffee consumed in the Tromso region is prepared by boiling, while other European regions drink predominantly filtered and French-press coffee. This difference in preparation methods
led the investigators to question whether the brewing method could be the explanation to the rise in blood lipids.

As a follow-up to the initial Tromso study (Thelle et al., 1983), the investigators designed a study intended to assess the effects of coffee consumption and coffee brewing methods on serum cholesterol concentrations in men. Thirty-three men diagnosed with hypercholesterolemia (i.e., elevated cholesterol levels) were randomized into one of the three following conditions: (a) participants were told to continue their usual coffee intake, (b) participants were told to stop drinking coffee, or (c) participants were told to stop drinking coffee for five weeks, followed by consumption of either boiled or filtered coffee. Cholesterol concentration significantly dropped in all of the participants abstaining from coffee for 5 weeks, and continued to drop in those who abstained for 10 weeks, compared to participants who continued to drink coffee. Cholesterol concentrations significantly rose in those participants who began drinking boiled coffee but remained the same in those drinking filtered coffee following abstinence.

Unfortunately, this study did not include women, even though a negative association between boiled coffee and HDL cholesterol was only found for women in the initial study. As a result, the results only apply to men, which leaves out important information regarding what the effects of coffee consumption and brewing method may be for women. This information is especially important in light of their early findings indicating that women may be at higher risk for the development of CVD due to a decrease in HDL cholesterol levels in response to coffee. This study also did not include elderly individuals, who might display an even more pronounced increase in serum lipid levels, and are more prone to the development of CVD than a younger population.
Although this study seemed to provide some evidence that brewing method may be an explanation for the cholesterol raising effects of coffee, it was still early to say conclusively whether this was the case, because this was the first study to examine this relationship. Brewing methods will be further explored in the next section of this chapter, examining the rest of the experimental evidence of a relationship between coffee and blood lipids.

In the early 1990s, other epidemiological studies began to emerge such as the Heidelberg-Michelstadt-Berlin study (Kohlmeier et al., 1991), and the Olivetti Heart Study (Jossa et al., 1993; Jossa et al., 1991). These studies started to account for a number of variables that had not been previously examined, such as age, sex, alcohol consumption, and cigarette smoking. Kohlmeier and colleagues (1991), in the Heidelberg-Michelstadt-Berlin study, investigated the relationship between coffee consumption and serum cholesterol levels in a population of 395 young (ages 18-24) and 385 (65-74) elderly German men and women. Some of the methodological highlights of this study were that the investigators separately analyzed the data from men and women in order to determine whether any sex differences existed, as well controlling for BMI, total calorie intake, fat, fish, tea, and milk intake, physical activity, alcohol consumption, smoking, and the use of oral contraceptives. Elevated levels of total serum and LDL cholesterol were positively correlated with increased coffee intake for the group of young men only. This study showed no trend between the consumption of coffee and rises in total or LDL cholesterol in the elderly.

The next epidemiological study to report a positive relationship between coffee consumption and serum lipids was the Olivetti Heart Study (Jossa et al., 1993), which
was carried out at the Olivetti factory in Naples, Italy. After 12 years of follow-up, the investigators obtained questionnaires from 942 males and 48 females. This study took into account important variables such as BMI, smoking, and age. Due to the very small number of women, they were excluded from statistical analyses. In this study, 900 males were included in the statistical analyses and it was revealed that increased coffee consumption (measured in cups per day) was associated with lower levels of HDL cholesterol, as well as positively related to serum triglyceride levels. Interestingly, however, after accounting for tobacco smoking status, a significant positive linear trend between coffee consumption and total serum cholesterol only was observed in smokers. No significant trend was found among smokers for LDL cholesterol and triglycerides (Jossa et al., 1993). Although this study did not examine the effects of coffee consumption in women and did not include other important variables such as physical activity and dietary intake, as well as coffee preparation method, it does show that cigarette smoking may change the relationship between coffee consumption and cholesterol levels. Therefore, investigators should further examine this variable.

Taken together, the findings of these epidemiological studies illustrate a positive relationship between coffee consumption and serum cholesterol levels. With the exception of Kohlmeier et al. (1991) who did not report a significant increase in total serum and LDL cholesterol in the elderly, but did find a significant relationship among young participants, all of the other investigators (Jossa et al., 1993; Jossa et al., 1991; Thelle, 1991; Thelle et al., 1983) found a significant increase in serum total cholesterol in response to coffee, in addition to a significant increase in LDL (Kohlmeier et al., 1991), and a significant decrease in HDL cholesterol (Jossa et al., 1993; Thelle et al., 1983).
Interestingly, Jossa and others (1993) found that after accounting for smoking status, a significant increase in serum total cholesterol was only seen in smokers. Therefore, it appears that cigarette smoking increases the risk of the negative effects of coffee on serum total cholesterol. Other studies did not account for tobacco smoking, so this variable should be further examined in future studies. Although not all of the studies included important variables such as the inclusion of females, careful monitoring of dietary intake, smoking habits, BMI, and alcohol consumption, the common variable in all of the studies is a correlation between coffee consumption and higher levels of serum total cholesterol. At this time, no epidemiological studies have investigated the effects of coffee or caffeine on CRP or fibrinogen.

Despite the fact that these epidemiological studies do not elucidate the potential mechanisms through which coffee may be leading to rises in cholesterol levels and therefore an increased risk for CVD, these studies do set the ground for true experimental studies, where different hypotheses can be explored such as whether the preparation method may be the missing link between coffee consumption and rises in serum cholesterol. Throughout the next section of this chapter, a review of experimental studies will be presented and potential mechanisms of action for the coffee/cholesterol connection will be discussed.

**Potential mechanisms that contribute to the relationship between coffee consumption and cholesterol levels: Human studies**

Because epidemiological findings brought about the question of whether the brewing method played a significant role in the coffee/cholesterol connection, experimental studies were designed to test that hypothesis and also to determine a
mechanism that would explain the connection between coffee intake and a rise in cholesterol levels. This section will describe the research that has led to our current understanding of how coffee consumption may lead to an increase in cholesterol.

**Boiled versus filtered coffee effects on lipids.** Although epidemiological studies led to the questioning of whether preparation method could be the cause leading to an increase in cholesterol following exposure to coffee, only carefully controlled experimental designs would truly answer this question. These types of studies began to emerge in the late 1980’s and the findings indeed seemed to support the hypothesis that preparation method may be a factor explaining the coffee/cholesterol connection (Bak & Grobbee, 1989; Hryniewiecki et al., 1992; Strandhagen & Thelle, 2003; van Dusseldorp et al., 1990; Zock et al., 1990). The reason for this is that coffee contains a lipid component that may be responsible for its cholesterol raising effects. Unfiltered coffee brews contain approximately 1 to 2 grams of lipids per liter, while lipid levels found in filtered coffee are nearly negligible (Ratnayake et al., 1993).

In 1989, Bak and colleagues conducted a study examining the effects of filtered and boiled coffee on total serum cholesterol and cholesterol fractions (Bak & Grobbee, 1989). This 12-week randomized trial included 107 young adult male and female participants with normal serum cholesterol levels. After a three-week run-in period during which they all consumed filtered coffee, the participants were randomly assigned to one of three groups receiving four to six cups of boiled coffee a day, four to six cups of filtered coffee a day, or no coffee, for a period of nine weeks. Compared to baseline serum total cholesterol levels, there was a 10% increase in cholesterol levels among participants who consumed boiled coffee for nine weeks. There was no significant
difference in the change of LDL cholesterol levels between the filtered-coffee group and
the group that drank no coffee (Bak & Grobbee, 1989). The findings of this study indicate
that boiled coffee consumption increases serum total cholesterol levels, whereas filtered
coffee does not. These findings suggest that the lipid component of boiled coffee may be
the causative agent responsible for the cholesterol-raising effects of coffee.

Following these findings, other investigators sought to test the hypothesis of
whether the lipid part of boiled coffee was the responsible component for the cholesterol-
raising effects seen with coffee consumption (Zock et al., 1990). Ten volunteers (5
males, 5 females) consumed a lipid-enriched fraction from boiled coffee for six weeks.
Serum total cholesterol rose in every subject, mainly due to LDL cholesterol (29 percent
increase), as well as a 55 percent average triglyceride increase. HDL cholesterol was
unchanged. After supplementation ended, lipid levels returned to baseline (Zock et al.,
1990). Therefore, this study shows that boiled coffee contains a lipid that powerfully
raises serum cholesterol. Although the rises seen in total and LDL cholesterol levels, as
well as triglycerides, were reversible, it is important to keep in mind that for the vast
majority of coffee consumers, the exposure to coffee is life long. Furthermore, an
increase of 29 percent in LDL cholesterol, as well as 55 percent rise in triglycerides can
be of clinical significance. For example, such an increase may push someone with
borderline high levels into a category where they are considered to be abnormally high
and may require medical attention.

Van Dusseldorp and colleagues (van Dusseldorp et al., 1991) investigated
whether boiled coffee that is filtered still results in elevated cholesterol levels by
examining cholesterol changes among participants who drank boiled, filtered coffee to
those who drank boiled, unfiltered coffee or no coffee at all. Serum cholesterol increased only among participants who drank boiled, unfiltered coffee, a finding that was confirmed in subsequent studies with participants who had elevated blood lipid profiles (i.e., hyperlipidemia) (Hryniewiecki et al., 1992).

Although studies examining the lipid component of boiled coffee appeared to consistently find that the lipid component was the responsible variable for significant rises in cholesterol and that filtering would remove (or at least decrease it enough so that rises in cholesterol would not be clinically significant) the cholesterol-raising component in coffee, a small number of studies suggested that filtered coffee may also increase cholesterol levels, and began to cast some doubt into what appeared to be a fairly clear picture. Fried and colleagues (Fried et al., 1992), designed a randomized controlled trial with an 8-week washout period followed by an 8-week intervention period during which men were randomly assigned to drink one of the following filtered coffee conditions: (a) 720 ml of caffeinated coffee, (b) 360 ml of caffeinated coffee, (c) 720 ml of decaffeinated coffee, or (d) no coffee at all. All coffee consumed in this study was filtered. Participants were 100 healthy males. A significant increase in plasma total cholesterol (0.25 mmol/L, P = .02), LDL cholesterol (0.15 mmol/L, P = .17), and HDL cholesterol (0.09 mmol/L, P = .12) was observed after the consumption of five cups of filtered coffee for eight weeks (Fried et al., 1992). Christensen and colleagues also observed a decrease of 0.28 mmols/l in plasma total cholesterol levels following cessation of approximately 4 cups per day of filtered coffee for 6 weeks in 191 healthy, nonsmoking, coffee-drinking volunteers aged 24-69 years (Christensen et al., 2001).
To assess the effects of intake and abstention of filtered coffee on blood lipids, Strandhagen and Thelle (Strandhagen & Thelle, 2003) designed a prospective, controlled study involving 121 healthy, nonsmoking men and women aged 29-65. The study consisted of four consecutive trial periods: the first and third periods were 3 weeks of total coffee abstention, the second and fourth periods consisted of 4 weeks with participants consuming 600 ml of filter-brewed coffee per day. Coffee abstention for 3 weeks decreased total serum cholesterol by 0.22-0.36 mmol/l (approximately 9 to 14 mg/dl). A volume of 600 ml (about four cups) of filtered coffee per day during 4 weeks raised total serum cholesterol by 0.15-0.25 mmol/l (approximately 6 to 10 mg/dl). Although these numbers do not appear to be very high, even a change of 6 to 10 mg/dl may increase one’s risk for developing CVD. For example, an increase of 40 mg/dl total cholesterol in those who have a total cholesterol level near 200mg/dl places these individuals at twice the risk for CVD as those who have total cholesterol levels that are lower than 200mg/dl.

Taken together, these findings indicate that, although the cholesterol raising effects brought about by the consumption of filtered coffee may not be as strong as that of the boiled coffee, it is important not to discard the possibility that filtered coffee may also play a small but important role in explaining the cholesterol raising effects of coffee. Although these findings do not appear to be clinically significant, at least in healthy populations, these findings may be of particular importance for those individuals already suffering from hyperlipidemia. While studies have not yet been carried out to test this hypothesis, previous studies do suggest that these small changes may play a greater and more significant role in a hyperlipidemic population (Hryniewiecki et al., 1992).
Therefore, continuation of research in this area, especially including those who suffer from high levels of blood lipids, continues to be necessary.

**Caffeine versus coffee effects on lipids.** Investigators then began to question whether the cholesterol-raising factor found more predominantly in boiled coffee and less predominantly in filtered coffee was related to caffeine content. In a 12-week double-blind trial, van Dusseldorp et al. (van Dusseldorp et al., 1990) examined the effect of decaffeinated versus regular coffee on the serum lipid levels of 45 healthy volunteers (23 women and 22 men aged 25-45 years) with a habitual intake of 4-6 cups of regular coffee per day. Participants received five cups of regular coffee each day for 6 weeks and five cups of decaffeinated coffee for the next 6 weeks, or vice versa. Their background diet was kept constant and was low in caffeine. Differences between the effects of decaffeinated and regular coffee on total serum cholesterol, HDL cholesterol, and serum triglycerides were essentially zero, leading to the conclusion that, in healthy adults, replacement of regular coffee by decaffeinated coffee does not affect serum cholesterol and lipoproteins and that caffeine does not appear to play a role in the cholesterol-raising effects of coffee (van Dusseldorp et al., 1990).

Other trials studying caffeine effects on lipid levels have reported similar results. Bak and Grobbee (Bak & Grobbee, 1991) used a double-blind, randomized trial with two parallel groups in 69 young, male and female healthy participants. After a 3-wk run-in period, participants were randomly assigned to one of two groups receiving either 4-6,140-mL cups of filtered decaffeinated coffee per day and an equal number of pills containing 75 mg caffeine or 4-6 140-mL cups filtered decaffeinated coffee per day and an equal number of placebo pills for 9 weeks. In both groups caffeine intake from other
sources was not allowed. At the end of the study, abstinence from caffeine for a period of 9 weeks showed no effect on either serum lipids (total serum and LDL cholesterol and triglycerides) or blood pressure, and caffeine consumers did not show significantly elevated blood lipids or blood pressure (Bak & Grobbee, 1991). Other investigators examining the effects of two kinds of decaffeinated coffee on serum lipid profiles also describe similar findings. Wahrburg and colleagues (Wahrburg et al., 1994) found that consumption of two different types of decaffeinated coffee did not lead to any significant changes in serum total and LDL cholesterol or triglycerides compared to filtered caffeinated coffee in a sample of 119 healthy students (60 male, 59 female). All of the participants in this study consumed 750-1000 ml of caffeinated filtered coffee per day for a 2 week wash-in period, followed by a 6 week test period where one group continued drinking the caffeinated coffee, while the two other groups consumed different kinds of decaffeinated coffee (Wahrburg et al., 1994).

The literature examining the effects of caffeine on serum blood lipids consistently demonstrates that caffeine does not appear to play a role in the cholesterol raising effects of coffee. However, to our knowledge, only one study (Bak & Grobbee, 1991) has specifically studied caffeine by itself (in the form of caffeine pills) while other studies have studied the effects of caffeine by comparing caffeinated and decaffeinated coffee. It is important to note that, to date, all of the studies examining the effects of caffeine on blood lipids have used healthy volunteers. Based on previous research by Hryniewiecki and colleagues (1992), it is possible that those populations with hyperlipidemia (i.e., high blood lipid levels) may respond differently. For example, those with hyperlipidemia may show a greater response to the effects of caffeine on blood lipids compared to those with
normal lipid levels. This area of research should be further explored with more studies that include caffeine consumption alone.

**Potential mechanisms that contribute to the relationship between coffee consumption and cholesterol levels: Animal studies**

Animal studies have examined the relationship between coffee and cholesterol in gerbils, hamsters, monkeys, and rats. Mensink and colleagues (1992) designed a study in which three groups of 20 male gerbils and three groups of 6 male hamsters were fed either a control diet or a control diet supplemented with either freeze-dried boiled coffee or freeze-dried filtered coffee. The hamsters were 4 to 6 weeks old. At the end of this study, it was reported that following the 5-week feeding period serum cholesterol levels were not different in either species fed the different diets (R. P. Mensink et al., 1992). Although these results suggest that perhaps these animal species are not sensitive to the cholesterol raising effects of boiled coffee, it is difficult to make this statement with any kind of certainty, as there is a possibility that the cholesterol-raising factor in boiled coffee may be lost during the process of freeze-drying. Furthermore, although the experimenters did control for body-weight and used a standard diet, once again, the effect of coffee on females was either not explored or not reported.

Results of a study using rhesus and cebus monkeys showed similar findings to the previous study (Terpstra et al., 1995). Terpstra and colleagues (Terpstra et al., 1995), tested a solution of coffee bean oil containing the diterpenes cafestol and kahweol, which had been previously tested in their laboratory and shown to greatly elevate cholesterol in humans. These investigators then tested the coffee bean oil from the same batch by feeding it to male and female cebus and rhesus monkeys. Two groups of eight cebus
monkeys were fed a purified diet containing 0.5% coffee oil or placebo oil (sunflower plus palm oil, 3:2, wt/wt) for seven and a half weeks in a crossover design. The daily intake of the coffee oil was approximately six times higher than that in the human study, where strong elevations in cholesterol were seen as result of ingestion of coffee bean oil. Coffee oil did not affect plasma cholesterol or triglyceride concentrations compared with the placebo oil. Two groups of three rhesus monkeys were fed a commercial diet containing either 0.5% coffee oil or 0.5% placebo oil for 6 weeks in a crossover design. The daily intake of coffee oil was more than six times that of human consumption shown to greatly elevated serum cholesterol and, once again, there was no effect of coffee oil on plasma cholesterol or triglyceride concentrations in monkeys. The findings from this study also suggest no effect of the cholesterol-raising diterpenes from coffee oil present in boiled coffee on cholesterol. Therefore it is suggested that these effects may be specific for human primates, because coffee oils have been shown to consistently raise cholesterol in humans, but not in other primates.

Other research in hamsters and rats further confirms what the previous two studies found: no significant rise in cholesterol due to consumption of boiled coffee in hamsters and rats (Beynen et al., 1996). In a continuous search for an animal model for the effect of coffee lipids on serum cholesterol concentrations, these investigators fed male and female hamsters and rats diets consisting of a purified base diet and either boiled water, unfiltered boiled coffee or filtered boiled coffee. After a feeding period of 8 weeks there was no statistically significant effect of unfiltered boiled coffee on serum total cholesterol concentrations in either the hamsters or the rats. The initial level of serum cholesterol did respond predictably by rising in response to the addition of cholesterol and/or saturated
fatty acids to the diet. The investigation also showed that the lack of effect of unfiltered boiled coffee in the hamsters and the rats, when compared with the previously reported activity in humans, could not be explained by dosage, duration of treatment, mode of administration or by insufficient statistical power. Therefore, this study is consistent with the previous literature (R. P. Mensink et al., 1992) showing that hamsters are insensitive to unfiltered boiled coffee and therefore are not suitable models for investigating its hypercholesterolemic effect. This study also contributes to the previous literature by showing that the same is true for rats in that they do not show the hypercholesterolemic effect in response to coffee that humans show.

Last, in a review of the literature dealing with the validity of animal models for the cholesterol raising effects of coffee diterpenes in human participants (de Roos et al., 1999), investigators searched for an animal species in which cafestol and kahweol would increase cholesterol similarly to humans (by greatly increasing LDL-cholesterol concentration). In the primate species, where a rise in cholesterol was observed, the rise in total cholesterol was less pronounced than that observed in human participants. In contrast to what is found in humans, the increase in total cholesterol was predominantly due to a rise in HDL-cholesterol rather than LDL-cholesterol. In other animal species, cafestol and kahweol did not raise cholesterol consistently. Most studies found no significant rise in cholesterol due to kahweol and cafestol. The variability in effects on serum lipids could not be explained by the mode of administration or dose of diterpenes, nor by the amount of cholesterol in the diet (de Roos et al., 1999). Therefore, when searching the animal literature for a valid model in which cafestol and kahweol elevate plasma lipoproteins to the same extent as in human participants, one could not be found.
Therefore, at least at this time, the scientific evidence leads us to believe that studies on coffee and caffeine’s mechanism of action should be done in human participants.

**Coffee oil diterpenes’ effects on cholesterol: Cafestol and kahweol**

Analyses of the substances present in coffee oil responsible for raising cholesterol levels led to two components: cafestol and kahweol, classified as diterpenes, which are found in the lipid component of coffee and mostly removed through filtering. Unfiltered coffee brews contain approximately 1 to 2 grams of lipids per liter, of which approximately 10 percent are diterpenes (Ratnayake et al., 1993). The coffee diterpene cafestol occurs in both robusta and arabica beans, while the related compound kahweol occurs only in arabica beans (Urgert *et al.*, 1997a). Through controlled experiments, investigators were able to determine that diterpenes also are responsible for the cholesterol-raising effects of unfiltered coffee (Weusten-Van der Wouw *et al.*, 1994; Zock *et al.*, 1990). In a study using coffee brews containing the diterpene cafestol, serum triglycerides and alanine amino-transferase, as well as decreased levels of serum creatinine and gamma-glutamyl-transferase (GGT) were observed (Weusten-Van der Wouw *et al.*, 1994).

Urgert *et al.* (1996a) studied the effects of prolonged intake of cafeteria coffee, which is rich in the diterpenes cafestol and kahweol, versus filtered coffee, on serum aminotransferase and lipid concentrations in healthy men and women aged 19 to 69. Cafeteria coffee raised alanine aminotransferase (a liver enzyme) concentration by up to 80% above baseline values compared to filtered coffee and also raised LDL cholesterol concentrations by 9-14%. All increases were reversible following cessation of coffee consumption. These two studies indicated that consumption of coffee diterpenes may
affect liver cells as suggested by increases in serum alanine aminotransferase concentration. These results indicate that ingestion of cafestol is accompanied by alterations in liver function enzymes, however the mechanisms underlying these changes are not fully understood.

As the previous studies showed, coffee diterpenes appear to target the liver, so investigators began to search for other molecules found in the liver that may be associated with a rise in cholesterol and thus an increased risk for CVD. One of these molecules is lipoprotein (a), which consists of a large glycoprotein attached to an LDL molecule (Dahlen, 1994). The main source of apolipoprotein (a) is the liver, and indeed, serum levels of lipoprotein (a) are determined by the rate of this molecule’s production in the liver (Rader et al., 1993). Urgert and colleagues (1996b) investigated the association between intake of boiled coffee and serum levels of lipoprotein(a) in healthy male and female Norwegians aged 40 to 42 years. Participants who habitually consumed five or more cups of boiled coffee per day were compared with filtered coffee consumers who ingested the same amount. Participants consuming nine or more cups of coffee per day had higher lipoprotein(a) levels than did those drinking five to eight cups per day, although the elevations did not quite reach statistical significance. However, in a subsequent study of four randomized, controlled trials using healthy male and female participants, Urgert and colleagues (1997b) reported a 4 % decrease in lipoprotein (a) in response to coffee diterpenes ingestion after four weeks. Although these findings may initially appear indicative of a protective health effect against CVD, a meta-analysis of eleven trials with coffee preparations rich in cafestol and kahweol indicated that each 10 mg of cafestol ingested per day raises serum total cholesterol by 0.15 mmol/l, which was
mostly due to an increase in LDL cholesterol (Urgert & Katan, 1996). The hypercholesterolemic property of coffee diterpenes therefore undermines any potential benefits brought about by a small decrease in lipoprotein (a).

In a continuous search for a mechanism explaining the effects of diterpenes on cholesterol, van Tol and others (1997) investigated whether the coffee diterpenes may affect lipoprotein metabolism via effects on cholesteryl ester transfer protein (CETP), phospholipid transfer proteins (PLTP) and lecithin:cholesterol acyltransferase (LCAT). CETP catalyzes the transfer of cholesterol esters, synthesized by LCAT, from HDL to LDL and VLDL (Tall, 1995). PLTP can affect the net mass transfer of phospholipids between lipoproteins, and also converts small HDL molecules into larger and smaller HDL molecules (Lusa et al., 1996). Both CEPT and PLTP appear to play a major role in determining the size and quantity of HDL particles in plasma (Tall, 1995). Therefore to study these proteins, the current study used of a randomized, double-blind cross-over design with 10 healthy male volunteers. Either cafestol or a mixture of cafestol and kahweol was given to participants for 28 days. Compared to baseline values, cafestol significantly raised the activity of CETP and PLTP (Ps < 0.001), LCAT activity was significantly reduced (P = 0.02). This study provides a mechanism by which coffee diterpenes could influence serum lipoprotein metabolism. For example, since CETP catalyzes the transfer of cholesterol esters, synthesized by LCAT, from HDL to LDL and VLDL, the increase in CETP levels seen here may explain the rises in LDL cholesterol in response to coffee diterpenes. Furthermore, as the liver is the major organ for LCAT synthesis, the small decrease in LCAT activity levels by the mixture of cafestol and
kahweol could be caused by a slightly impaired integrity of liver cells at high diterpene intake.

In an attempt to determine whether cafestol and kahweol had different effects on serum lipid aminotransferases, Urgert and colleagues (1997a), designed a randomized, double-blind crossover study, where 10 healthy male volunteers were given either pure cafestol or a mixture of cafestol and kahweol for 28 days. Relative to baseline values, cafestol significantly raised total serum cholesterol concentrations, LDL cholesterol, fasting triacylglycerols, and alanine aminotransferase (all $P < 0.01$). Relative to cafestol alone, the mixture of cafestol plus kahweol increased did not significantly add to the increases seen in total cholesterol ($P = 0.08$), LDL cholesterol ($P = 0.09$) or triacylglycerols ($P = 0.20$), but did significantly increase alanine aminotransferase ($P = 0.004$). This study shows that the effect of cafestol on serum lipid concentrations was much larger than the additional effect of kahweol, which indicates that the hyperlipidemic effects of unfiltered coffee mainly depends on the cafestol content.

As van Tol and others (van Tol et al., 1997) had previously shown, the consumption of coffee diterpenes is associated with increased serum activity levels of the lipid transfer proteins CETP and PLTP, as well as decreased activity of LCAT. Due to these findings, the investigators designed a study examining the long term effects of French-press coffee (rich in diterpenes) on serum lipid transfer proteins and LCAT. Additionally, these investigators compared the initial changes in CEPT activity with the initial changes in LDL and HDL cholesterol as well as triglycerides (De Roos et al., 2000). Forty-six healthy participants ingested 0.9 liters per day of either French-press or filtered coffee for 24 weeks. Compared to baseline levels, French-press coffee
significantly increased average CETP activity by 18% after 12 weeks, and by 9% after 24 weeks. PLTP activity was significantly increased by 10% after 12 and 24 weeks. LCAT activity was significantly decreased by 6% after 12 weeks and by 7% after 24 weeks. The increase in CETP preceded the increase in LDL cholesterol, but not the increase in total triglycerides, demonstrating that the consumption of coffee diterpenes cause a long-term increase in CETP as well as PLTP activity; the increase in CETP activity may contribute to the rise in LDL cholesterol.

**Additional biobehavioral factors that may contribute to the effects of caffeine on cholesterol and cardiovascular disease**

The review of the literature indicates direct effects of coffee preparation and caffeine on changes in lipid markers of CVD risk. However, it is important to understand the role that biobehavioral factors may play in the caffeine-CVD risk relationship because they can moderate the effects that changes in blood markers can have on health and disease outcomes. For example, individuals who have a family history of hypertension (i.e., parent is diagnosed with hypertension) appear to be particularly sensitive to the blood pressure effects of caffeine (Boekschoten et al., 2003). The following section focuses on biobehavioral factors that may influence the cardiovascular health outcomes of caffeine and coffee.

**Genetic influences**

Although genetic factors play a role in the development of cardiovascular disease, we now know that genotype alone does not singly determine whether an individual will develop a given health disorder (Plomin, 2003). The only study to date that we have
found which examines a genetic component of cholesterol responses to caffeine recently was by Boekschoten and colleagues (Boekschoten et al., 2003). These investigators noticed that both animals and humans show some consistency in the response of their serum lipids to fat-modified diets, which may be indicative of a genetic basis underlying this response. The investigators sought out to determine to what extent the effect of coffee oil on serum lipid concentrations was reproducible within participants. The serum lipid response of 32 healthy volunteers was measured twice in two separate five-week periods in which coffee oil was administered (69 mg cafestol/day). Total cholesterol levels increased by 24% in period 1 and 18% in period 2, LDL cholesterol by 29 % and 20%, triglycerides by 66% and 58%, while HDL cholesterol did not change significantly. An increase in total and LDL cholesterol of even 20% may significantly increase one’s risk for CVD. For example, for an individual with total cholesterol levels of 170 mg/dl, a 20% increase means an increase of 34 mg/dl, elevating his/her cholesterol to 204 mg/dl, and almost doubling his/her chances of developing CVD. The correlation between the two responses was 0.20 for total cholesterol, 0.16 for LDL, 0.67 for HDL, and 0.77 for triglycerides. Therefore, the responses of total and LDL cholesterol to coffee oil were poorly reproducible within subjects. However, the responses of HDL and triglycerides appeared to be highly reproducible, which is suggestive of a genetic basis. It appears, therefore, that examining the genetic sources of the variation in the serum-lipid response to coffee oil is more promising for determining HDL and triglyceride levels.

An important limitation of this study is that although cafestol is known to potently increase serum lipid levels, scientists do not know at this time whether this oil is a good candidate for the study of variation in genes regulating the serum-lipid response.
(Boekschoten et al., 2003). Additionally, participants in this study did not follow a controlled diet. Therefore, the response seen in serum lipids to coffee oil may have been due to other dietary factors not accounted for in the study. While this study shows a promising area to be explored, at least with respect to changes in HDL and triglycerides in response to coffee oil, this is the first study to date reporting on potential genetic underlying factors related to a coffee/serum lipid connection. Other studies are certainly needed that would continue to examine these variables, as well as use other populations that include non-healthy participants, and make use of better controlled dietary intake.

With respect to race differences in caffeine metabolism, there have been no published data indicating race differences in the metabolism of caffeine, although a 1999 report on food group behavior of a mixed ethnic population did report that white elderly participants consumed more caffeine than elderly African American participants (Prothro & Rosenbloom, 1999). Because this study did not measure caffeine metabolism, it is difficult to say whether their findings of greater consumption of coffee by elderly white participants compared to African Americans is due to genetic differences in the metabolism of caffeine.

Another important genetic component that needs to be further explored with respect to the connection between coffee, caffeine, and CVD is family history of hypertension (FH). Family history of hypertension illustrates the importance of genetic influence on future development of CVD. Different studies have found that normotensive offspring of hypertensive parents are at a significantly higher risk for developing essential hypertension (Cook et al., 2001; Lauer & Clarke, 1989) and increased left ventricular mass (LVM) (Cook et al., 2001; Galderisi et al., 1993). Children with a positive FH (F+)
of hypertension show higher systolic blood pressure (SBP) levels than those children with a negative FH (F-) for hypertension (Cook et al., 2001; Munger et al., 1988). Similar findings have been observed in adolescents (Cook et al., 2001; Munger et al., 1988) and young adults (Lauer & Clarke, 1989), with adolescents and young adults with a family history of hypertension displaying higher SBP than those without a family history of hypertension. In addition, a higher LVM adjusted for body surface area has been observed in adolescents (Cook et al., 2001; Radice et al., 1986) and young adults (Celentano et al., 1988; Galderisi et al., 1993) from hypertensive parents compared with those from normotensive parents. To date, the studies that have examined the connection between caffeine and family history of hypertension have found that those participants with a family history of hypertension display higher SBP and heart rate (HR) in response to caffeine and stress than do those participants without a family history of hypertension (al'Absi et al., 1998; al'Absi & Wittmers, 2003). No studies to date have examined whether this increased reactivity in participants with a family history of hypertension in response to stress and caffeine alters blood markers of CVD, such as CRP and fibrinogen.

**Sex differences**

The only study to date that specifically examined sex differences in the response of serum cholesterol to unfiltered coffee was published in 1999. This study was designed to study the effects of dietary changes, which included responses to the coffee diterpene cafestol, in nine trials including 133 participants (72 men and 61 women) (Weggemans et al., 1999). All participants included in this study were lean and healthy. The reduction of total cholesterol in response to a decrease in the intake of cafestol was significantly larger
in men than in women. Similar findings were reported by el Shabrawy Ali and Felimban (1993) showing that in a trial of 252 adult drinkers or non-drinkers of Arabic coffee, both male and female coffee drinkers showed significantly higher levels of total cholesterol than non-coffee drinkers, but this rise was significantly greater in females compared to males. These results indicate that cafestol is a greater risk factor for rises in cholesterol in women compared to men. These findings are particularly troubling considering that a number of studies have not included females in their sample population, or have not separately analyzed data from men and women (Hertog et al., 1993; Jossa et al., 1993; Jossa et al., 1991; Urgert et al., 1996b). More studies including both men and women in their sample population are clearly needed.

**Tobacco smoking**

Early studies on the connection between coffee consumption and tobacco smoking indicate that nicotine increases the speed of caffeine metabolism (Parsons & Neims, 1978), and that caffeine consumption is positively associated with cigarette smoking (Kozlowski et al., 1993). Therefore, it is important to explore the possibility that smoking may lead to greater consumption of coffee, which could lead to greater increases in cholesterol, especially if the coffee is unfiltered. It is also important to determine whether smoking in the presence of coffee leads to more of an increase in cholesterol levels through mechanisms other than increased coffee consumption. Furthermore, a previous epidemiological study found a significant positive linear trend between coffee consumption and total serum cholesterol that was only seen in smokers (Jossa et al., 1993). In 1993, Mensink and other investigators examined the relationship between coffee consumption and serum cholesterol while taking smoking history into account (G.
B. Mensink et al., 1993). A large German population sample of 6,820 men and 7,258 women was used to investigate the relationship between coffee consumption, total serum cholesterol, and HDL cholesterol. Analyses were conducted on men and women separately. This study controlled for a number of variables, such as BMI, diastolic blood pressure, smoking habits, alcohol, fish, milk and tea consumption, physical activity and medication use. For men, a positive relationship between coffee consumption and total serum cholesterol was found among smokers and life-long abstainers. Interestingly, however, this positive relationship was not found in the group of non-smokers. The lack of relationship between coffee consumption and cholesterol elevations in the group of non-smokers is not understood. In women, a relationship between coffee consumption and total serum cholesterol was also present, but very weak. Similar results were seen from previously described findings of the Olivetti Heart Study (Jossa et al., 1993), demonstrating a positive relationship between serum cholesterol levels and coffee consumption in smokers. However, the Olivetti Heart Study did not include women, therefore comparisons between these two studies with respect to sex cannot be made. At the current time, studies examining the effects of smoking on the coffee/cholesterol connection are scarce, and more studies in this area are clearly needed. It is also important to note that, as indicated in previous studies, smoking does appear to speed up caffeine metabolism, therefore, it is important that smoking status be controlled when studying the relationship between caffeine and cholesterol.
Caffeine and coffee effects on fibrinogen

At this time, only three studies have examined the effects of coffee or caffeine on fibrinogen (Bak et al., 1990; Happonen, 1987; Naismith et al., 1970). The findings at this time are conflicting, with some investigators finding that coffee consumption was associated with increased fibrinogen levels (Happonen, 1987), while others found no increase in plasma fibrinogen levels in response to coffee or caffeine consumption (Bak et al., 1990; Naismith et al., 1970). For example, Happonen and others (Happonen, 1987) reported that in a large cross-sectional study, middle-aged males who consumed more than four cups of coffee per day had significantly higher fibrinogen levels than did those who did not drink coffee. Conversely, Bak and colleagues (Bak & Grobbee, 1990) found no effect of coffee or caffeine consumption on fibrinogen plasma levels among young, healthy, males and females in two randomized trials. In the first trial, 107 participants either drank filtered coffee, boiled coffee, or no coffee for 9 weeks. In the second trial, 69 participants either received 4 to 6 tablets of 75 mg of caffeine, or the same amount of placebo tablets. No significant differences were seen between baseline levels of fibrinogen and levels obtained after 9 weeks of either boiled coffee, filtered coffee, or caffeine consumption. Similarly, Naismith and others (Naismith et al., 1970), reported no change in fibrinogen levels among 14 participants aged 21 to 49, regular coffee drinkers, in response to coffee abstinence for 2 weeks.

Due to great differences in the methodology of these studies, comparisons between these findings are difficult to make. For example, Bak and others (Bak & Grobbee, 1990) used a sample of young and healthy participants, while Happonen and
colleagues (Happonen, 1987) used a sample of middle-aged participants. It is possible that increasing age may be a factor in the response of fibrinogen to coffee and caffeine, therefore this may explain why increased levels in response to coffee were only seen in Happonen (Happonen, 1987), but not Bak’s (Bak & Grobbee, 1990) study. Although Naismith (Naismith et al., 1970) included a broader age range, this study had such a small sample size (n=14) that significant changes in fibrinogen levels may not have been captured. Overall, very little data are currently available, making definite conclusions hard to be drawn at this point. More studies in this area are certainly needed, especially those examining coffee and caffeine in quantities that are similar to those commonly consumed by millions of people.

Caffeine, coffee, and CRP levels

To this date, there are no published reports examining the relationship between coffee or caffeine by itself on levels of C-reactive protein in vivo, and only one study exploring this relationship in vitro (Ganapathi et al., 1990). In the only study reported to date, the investigators evaluated the effects of caffeine on acute-phase proteins (CRP being one of these proteins). Changes in the concentration of a group of plasma proteins called acute-phase proteins represent an important biochemical response to tissue injury or infection (Kushner & Mackiewicz, 1987). Within the first few hours after an inflammatory stimulus, the hepatic rate of synthesis of a number of plasma proteins increases, while that of some others decrease. C-reactive protein is one of the two major acute-phase proteins that increase in plasma concentrations as much as several-thousand-
fold (Ganapathi et al., 1990). The synthesis of acute-phase proteins is regulated by cytokines, and this study explored the signal-transduction mechanisms by which cytokines regulate the synthesis of acute-phase proteins in human hepatoma cells and how this process is affected in the presence of caffeine. Two mM of caffeine led to a significant potentiation of CRP induction, ranging from 40 to 180-fold increase above that seen in control cultures. Although the exact mechanisms by which caffeine exerts its biological effects are not fully understood, they are believed to be mediated through increases in intracellular concentrations of cyclic AMP or calcium (Ganapathi et al., 1990). In a different study, 100 mg of caffeine (approximately 1 cup of coffee), led to highly significant increase of 150% on platelet reactivity (Ammaturo et al., 1988).

At this time, more research is still need to elucidate the effects of caffeine on CRP levels. Although an increase in CRP was seen in hepatoma cells in the presence of caffeine, it is nearly impossible to predict whether similar findings would occur in the plasma levels of living human population as a response to caffeine. Only further studies in this area will help to elucidate this relationship.

**Conclusions**

Boiled, unfiltered coffee consumption appears to increase the risk of developing cardiovascular disease as indexed by changes in lipoproteins (i.e., cholesterol, triglycerides). The cause of this cholesterol increase seems to be the ingestion of coffee diterpenes, which are removed through filtering. The effects of boiled or filtered coffee on fibrinogen levels are equivocal probably because of the paucity of data collected in
this area. One study suggests that coffee consumption does increase fibrinogen, but the coffee preparation methods were not reported. Studies that did control for coffee preparation methods reported no effect of boiled, unfiltered coffee on fibrinogen levels, but this lack of an effect could be the result of large differences in the age of participants among studies, as well as health status or lack of control over basal cholesterol levels. Further studies clearly are needed in this area before any definite conclusions can be drawn regarding the health effects of coffee – boiled or not – on fibrinogen levels in men and women. This final statement also can be made with regard to C-reactive protein (CRP) in that there are no published reports on the influence that coffee intake can have on CRP levels. This is a critical gap in the research area given the independent role that CRP may play in the development of CVD.

With regard to caffeine’s effects on CVD risk markers, the research findings are very limited in determining the causal relationship between caffeine alone and blood markers of CVD risk. Cholesterol levels do not appear to change in response to caffeine consumption among healthy adults. Observed cholesterol changes in response to caffeine administration seem to be influenced by biobehavioral factors such as stress, smoking status, lipid status, age, diet, and family history of hypertension. More specifically, some studies do not detect a measurable cholesterol change to caffeine consumption, but this could be a result of inadequate or incomplete dietary histories, difficulty with statistical analyses due to inappropriate sample size, failure to adjust for covariates that may have been confounders, lack of non-healthy populations, inclusion of elderly participants whose serum cholesterol levels are markedly higher than a younger cohort, daily/personal stress levels, and failure to include women as well as other minority populations.
Overall, the data suggest that individuals already at risk for developing CVD (e.g., elevated cholesterol or blood pressure, family history of hypertension) should avoid high levels of caffeine intake. Recommendations for healthy men and women are difficult to make at this time until further, better controlled studies are done.

With respect to fibrinogen, one study reported no effects of low-dose daily caffeine consumption in the form of a tablet. (Bak & Grobbee, 1989; Forde et al., 1985; Fried et al., 1992; Jacobsen & Thelle, 1987; Thelle et al., 1983; Zock et al., 1990) Given that this is the only published report found in this area, a consistent effect of caffeine on fibrinogen remains elusive. Caffeine effects on CRP in humans have not been reported to the best of my knowledge, but in vitro results do suggest a direct potentiating effect of caffeine on CRP induction from the liver (Bak & Grobbee, 1989; Forde et al., 1985; Fried et al., 1992; Jacobsen & Thelle, 1987; Thelle et al., 1983; Zock et al., 1990), the result of which would be elevated CRP levels in vivo. Given the small number of published reports, it is difficult to make any definitive conclusions about the effects of caffeine on fibrinogen and CRP levels in humans. There clearly is a need for further laboratory, controlled studies as well as epidemiological explorations of these caffeine-CVD relationships.

In evaluating the statistical analyses used by the authors cited in this review, several main issues become apparent and need to be considered in future investigations. First, in some instances, sample sizes were either so large (e.g. Berndt et al., 1993; Jossa et al., 1991; e.g. Stec et al., 2000; Urgert et al., 1995) or so small (Berndt et al., 1993; Forde et al., 1985; Jossa et al., 1991; Urgert et al., 1996b; van Tol et al., 1997; Zock et al., 1990) that statistical evaluation of the clinical outcome was difficult and potentially
misleading. Very large sample sizes may lead to statistically significant effects that may not be clinically relevant. Conversely, the randomized clinical trial conducted by Forde et al. (1985) was composed of a total of 33 participants divided into four groups, with 8 or 9 participants in each group. While the randomized clinical trial is a strong design, the opportunity for bias in using such a small sample is self-evident. Findings from such studies provide much less generalizability and little opportunity (due to less power) to find significant relationships that may exist.

Second, the vast majority of studies used multivariate statistical analyses to consider covariates and to assess any additional relationships that may have occurred. Given the complexity of choices involved in such analyses, it is extremely difficult to comment on the appropriateness of the choice of statistical models used. If multiple variables are believed to be involved, the choice of multivariate analyses appears to be appropriate. However, a number of studies neglected to analyze any factors as covariates (e.g. Bak & Grobbee, 1989; e.g. Naismith et al., 1970; van Dusseldorp et al., 1990; Zock et al., 1990). Third, it is important to report overall caffeine intake as indexed by coffee consumption for between-study comparisons. More specifically, it was difficult to assess the actual amount of coffee consumed by participants across the studies in the present review, as some studies (e.g. Bak & Grobbee, 1989; Forde et al., 1985; Fried et al., 1992; Jacobsen & Thelle, 1987; e.g. Naismith et al., 1970; Thelle et al., 1983) neglected to report coffee cup size.

For several reasons, it also is important to consider the health status, age, sex, and ethnic status of participants in future investigations. To date, only a few studies examined the effects of diterpenes on non-healthy (hypercholesterolemic) populations
(e.g. Hryniewiecki et al., 1992; Thelle et al., 1983). While it is understandable that investigators may want to exclude participants falling into this category, because it is still uncertain whether these participants respond to changes in coffee diterpenes in the same way that healthy participants respond -- for example, hypercholesterolemic participants may be hyper or hypo-responders -- this is an important population to be studied, because they appear to be in greater risk for CVD and may, therefore, benefit even more from this information than will healthy participants. With regard to age, The Lipids Research Clinics Program made an extensive survey of the distribution of lipoproteins in the American population as a function of age and found that cholesterol levels rise substantially with aging. For example, total cholesterol concentrations of 151 mg/dl in a 20 year-old individual are comparable to 200 mg/dL in a 50-year old person. As a result, lack of even age distribution across studies may affect observed cholesterol changes in a particular study. Finally, elderly participants metabolize coffee more rapidly than those under the age of 65 (Blanchard & Sawers, 1983), and may respond differently to coffee consumption than do younger participants. With regard to gender, caffeine metabolism among women also changes throughout the menstrual cycle (e.g., 25% decrease in caffeine elimination during the luteal phase (Benzaquen et al., 2002; Ridker et al., 2003b; Rifai & Ridker, 2002; Roberts et al., 2001; Stec et al., 2000), which may also be a source of bias across studies. Further investigations that control for these variables very much are needed to help the field move forward.

The issue surrounding gender is not inconsequential: a common misconception with respect to cardiovascular disease is that men suffer from this condition more than do women. However, according to the American Heart Association (2003b), in terms of total
deaths, in every year since 1984, CVD has claimed the lives of more women than for men. Furthermore, the gap between male and female deaths has increased dramatically over the years. These staggering statistics stress the importance of including females in studies investigating the connections between blood markers, coffee and caffeine, and risk factors for CVD.

Last, a very discouraging observation is that none of the reviewed studies reported any attempts to investigate the potential effects of race on the relationship between coffee and caffeine consumption and increased risk for CVD. The American Heart Association (2003b) has reported that the overall death rate in 2000 from CVD was significantly greater among black men versus white men and also significantly greater among black women compared to white women. Furthermore, Black and Mexican-American women have higher cardiovascular risk factors than white women of comparable socioeconomic status (AHA, 2003b). Therefore, there is a great need for studies that include minority populations in their examination of risk factors for CVD in respect to coffee and caffeine.

In conclusion, according to the American Heart Association (AHA, 2003b), heart disease continues to be the number one killer in America, and claims more lives each year than the next 5 leading causes of death combined. Therefore, it is extremely important to conduct research that will lead to further understanding of this field and, perhaps, contribute to decrease the number of deaths brought about by CVD. Studies examining blood markers for CVD such as CRP and fibrinogen in relationship to coffee and caffeine would certainly advance this area.

The purpose of this dissertation study was to address some of these unstudied areas. The current study examined the effects of caffeine and psychological stress on
blood markers or cardiovascular disease risk in healthy males and females with a family
history of hypertension.
CHAPTER 2: EXPERIMENT

Overview

The current study examined the effects of caffeine and stress on blood markers of cardiovascular disease in men and women with a family history of hypertension (FH+) using a 2 (gender; 26 men, 26 women) X 2 (drug condition; 26 no caffeine, 26 3.3 mg/kg caffeine) design. Participants came to the Penn State University General Clinical Research Center for 2 laboratory sessions, an initial screening session where blood was drawn for a cholesterol assessment and where potential participants underwent an extensive health screening to ensure they met inclusion criteria, and a 3.5 hour experimental session to examine stress reactivity to caffeine or no caffeine administration. Participants were randomly assigned to receive caffeine or no caffeine during the experimental session, and all were asked to perform a stressful task based on the Trier Social Stress Task (Kirshbaum, 1993). Blood pressure and heart rate were collected during the experimental session, as well as 3 blood samples at baseline, stress, and recovery for later assessment of cortisol, C-reactive protein (CRP) and fibrinogen levels, and for females, progesterone and estradiol.

Hypotheses

Hypotheses, set One: Caffeine and Cardiovascular Reactivity

It was hypothesized that for both males and females, caffeine would lead to elevations in SBP and DBP compared to no caffeine, but HR would not be different between groups. Sex differences with respect to caffeine and blood pressure and
heart rate were explored; however, no directional hypotheses were made as sex
differences with respect to these variables had not been reported in the literature.

**Rationale:** Previous studies conducted by Hartley and colleagues (2000) have
demonstrated that caffeine, when administered in a dose of 3.3 mg/kg, leads to significant
elevation in both systolic and diastolic blood pressure. These experimenters also found
that the elevations in blood pressure due to caffeine ingestion increased as severity of risk
for hypertension also increased. More specifically, their results revealed that the strongest
blood pressure response to caffeine was observed in those diagnosed with hypertension,
followed by those with stage 1 hypertension and high-normal groups and then by normal
and optimal groups (Hartley et al., 2000).

In a different study that examined the effects of caffeine on blood pressure
responses in a male hypertensive population, Goldstein and Shapiro (1987) found that
200 mg of caffeine significantly increased both systolic and diastolic blood pressure,
compared to a placebo; however, caffeine had no effect on heart rate (Goldstein &
Shapiro, 1987). Similar findings were also reported by Goldstein, Shapiro, Hui, & Yu
(1990) in a population of normotensive white males who ingested 150 mg of caffeine
(approximately 2.1 mg/kg). In this study, a significant increase in systolic and diastolic
blood pressure was seen when participants ingested 150 mg of caffeine, while no
significant changes were seen in response to a placebo (Goldstein et al., 1990).

**Hypotheses, set Two: Stress and Cardiovascular Reactivity**

It was hypothesized that stress would lead to elevations in SBP, DBP, and HR,
compared to baseline for both males and females. It was further hypothesized that
males would display significantly higher SBP and DBP in response to stress than would females, and that there would be no sex differences with respect to HR.

Rationale: A number of investigators have consistently found that activation of the HPA-axis by stress leads to significant elevations in systolic and diastolic blood pressure, as well as heart rate (al'Absi et al., 1998; Goldstein & Shapiro, 1987; Goldstein et al., 1990; Greenberg & Shapiro, 1987; Greenstadt et al., 1988; Hartley et al., 2001; Hartley et al., 2000; Lovallo et al., 1996; Lovallo et al., 1991; Lovallo et al., 1989; H. F. Myers et al., 1989; Myers, 1988, 1995). In fact, the elevations seen in blood pressure and heart rate in response to stress are seen with a wide variety of stress task, such as public speaking challenges (al'Absi & Wittmers, 2003; Bardwell et al., 2000; Clark, 2003), mental arithmetic (al'Absi et al., 1998; Goldstein & Shapiro, 1987; Greenberg & Shapiro, 1987; Greenstadt et al., 1988; Marrero et al., 1997), reaction time (al'Absi et al., 1998; Lovallo & al'Absi, 1998; Lovallo et al., 1996; Lovallo et al., 1991; Lovallo et al., 1989; Marrero et al., 1997), and handgrip tests (A. C. King et al., 1994).

al'Absi and Wittmers (2003) examined the effects of stress (public speaking challenges) on both males and females with or without a high risk for developing hypertension. Participants were considered to be at low risk if they did not have a parental history of hypertension and had a resting systolic blood pressure bellow median (median systolic blood pressure was 114 mmHg for males and 106 mmHg for females), or at high risk if they had above median resting SBP regardless of parental history for hypertension. These investigators found that although all participants displayed a significant elevation of SBP, DBP, and HR in response to stress, participants in the high risk group displayed a significantly greater elevation in SBP and HR than did those in the
low risk group (al'Absi & Wittmers, 2003). Another study conducted by al'Absi and colleagues (al'Absi et al., 1998) also demonstrated that males at risk for hypertension showed a significantly greater elevation in ACTH and cortisol, which are indicators of HPA-axis activation, compared to those participants at low risk for developing hypertension. In this study, high risk was defined as having a baseline resting BP of between 125/80 and 140/90 mmHg and diagnosis of hypertension in one or both parents, while low risk was defined as having a baseline resting BP of less than 125/78 mmHg and no parental history of hypertension (al'Absi et al., 1998). Similarly, Greenberg & Shapiro (1987), found that among a sample of young males, those who had at least one parent diagnosed with hypertension display significantly higher SBP in response to a mental arithmetic stressor than those who did not have a parental history of hypertension (Greenberg & Shapiro, 1987). With respect to sex differences, previous reports have shown that males display a significantly greater cardiovascular reactivity in response to psychological stressor than do females (Traustadottir et al., 2003), while other studies have shown that males display significantly higher cortisol levels in response to psychological stressors than do females (Kirschbaum et al., 1999) which indicates greater HPA-axis activation in males than females, and could translate into an elevated risk for CVD more so for males than females.

**Hypotheses, set Three: Caffeine and Blood Markers of Cardiovascular Disease**

It was hypothesized that participants in the caffeine group would show increased levels of CRP and fibrinogen compared to those in the no-caffeine group. It was
further hypothesized that males would display higher levels of these blood markers in response to caffeine than would females.

**Rationale:** Although no previous published report has examined the direct relationship between caffeine and CRP or fibrinogen, all of these markers are associated with an increased risk for the development of cardiovascular disease, as is blood pressure, and may mark the initial stages in the development of cardiovascular problems (AHA, 2003b; Benzaquen et al., 2002; Cao et al., 2003; Ganapathi et al., 1990; Ganter et al., 1989; Obradovic et al., 2003; Ridker et al., 2003b; Ridker et al., 1998). In the only study reported to date investigating the effects of caffeine on CRP, the investigators evaluated the effects of caffeine on acute-phase proteins (CRP being one of these proteins). Two mM of caffeine led to a significant potentiation of CRP induction in human liver cells, ranging from 40 to 180-fold increase above that seen in control cultures.

With respect to caffeine and its effects on fibrinogen, at this time, only three studies have been published examining this relationship (Bak et al., 1990; Happonen, 1987; Naismith et al., 1970). The findings at this time are conflicting, with some investigators finding that coffee consumption was associated with increased fibrinogen levels (Happonen, 1987), while others found no increase in plasma fibrinogen levels in response to coffee or caffeine consumption (Bak et al., 1990; Naismith et al., 1970). For example, Happonen and others (1987) reported that in a large cross-sectional study, middle-aged males who consumed more than four cups of coffee per day had significantly higher fibrinogen levels than did those who did not drink coffee. Conversely, Bak and colleagues (1990) found no effect of coffee or caffeine consumption on fibrinogen plasma levels among young, healthy, males and females in two randomized
trials. Similarly, Naismith and others (1970), reported no change in fibrinogen levels among 14 participants aged 21 to 49, regular coffee drinkers, in response to coffee abstinence for 2 weeks.

With respect to family history of hypertension, because those with a parental history of hypertension are found to show an increased blood pressure reactivity in response to caffeine than those with no parental history of hypertension (al'Absi et al., 1998; Greenberg & Shapiro, 1987), and are also at a greater risk for developing hypertension and other cardiovascular diseases (Dekkers et al., 2003), it is hypothesized that caffeine would elicit the presence of these blood markers of cardiovascular disease in a population at risk for the development of hypertension and future development of other cardiovascular diseases.

**Hypotheses, set Four: Stress and Blood Markers of Cardiovascular Disease**

**It was hypothesized that stress would lead to increases in CRP and fibrinogen compared to baseline for both males and females, with males displaying higher levels than females in response to stress.**

**Rationale:** Although no previous published report has examined the direct relationship between psychological stress and the blood markers CRP or fibrinogen, stress has been shown to be associated with an increased risk for the development of cardiovascular disease (D. Carroll et al., 2003; Din-Dzietham et al., 2004; Lee et al., 2004). Indeed, recent findings by Carroll and colleagues (2003) revealed that systolic and diastolic blood pressure reactivity to psychological stress significantly predicted a rise in systolic and diastolic blood pressure at a 5-year follow-up for both male and female healthy
volunteers. However, these investigators also found that the predictive value of reactivity was greater for males than females. Similarly, Lee and colleagues (2004) have recently reported that for a sample of healthy females, the stress of job insecurity significantly increased the risk of non-fatal myocardial infarction two years later. Because stress has been shown to increase one’s risk for cardiovascular disease (D. Carroll et al., 2003; Din-Dzietham et al., 2004; Lee et al., 2004), and because the presence of CRP and fibrinogen indicate an increased risk or the beginning stages of cardiovascular disease (AHA, 2003b; Benzaquen et al., 2002; Cao et al., 2003; Ganapathi et al., 1990; Ganter et al., 1989; Obradovic et al., 2003; Ridker et al., 2003b; Ridker et al., 1998), it is predicted that stress will lead to significant increases in the presence of these blood markers, as compared to baseline. It is also important to note that psychological stress alters the immune system, for example, by leading to increases in the cytokine interleukin-6 (Brydon et al., 2004). Because CRP is part of this immune response, it too should be altered in response to stress.

With respect to family history of hypertension, a number of studies have demonstrated that those participants with a family history of hypertension display significantly higher reactivity to stress than those without a familial history of hypertension (al'Absi et al., 1998; Greenberg & Shapiro, 1987), and are also at a greater risk for developing hypertension and other cardiovascular diseases (Dekkers et al., 2003). Therefore, it was hypothesized that those participants with a family history of hypertension would display greater increases of CRP and fibrinogen in response to stress than those without a familial history of hypertension.
Methods

Participants. Participants in this study were 52 healthy males (N=26) and females (N=26), between the ages of 18 and 31 years (mean 21.63 ± 0.36) (see Table 1). Sixty-nine percent of participants were Caucasian (N=36), 14% were African American (N=7), 11% were Asian (N=6), 4% were Hispanic or Latino (N=2), and 2% were self-reported “other” (N=1). All participants were high school graduates who had at least some college education, and 15% had more than a college education. A trained researcher initially interviewed potential participants over the telephone to review their health history and to determine eligibility for the initial screening lab session (see Appendix A).

All participants had a family history of hypertension (i.e. parent) that was confirmed by a questionnaire completed by the potential participant’s parents (see Appendix B) (Page & France, 2001). Family history of hypertension was considered present if the participant had at least one parent who was (1) diagnosed with hypertension and, (2) currently taking prescription medication or had taken prescription medication in the past to treat his/her blood pressure (Greenberg & Shapiro, 1987). Participant’s level of caffeine intake was initially assessed during the telephone interview and later confirmed through the use of a caffeine questionnaire (Lane, 2002) (see Appendix C) administered during the experimental session. All procedures were reviewed and approved by the Penn State University Institutional Review Board (IRB) and the General Clinical Research Center Advisory Committee prior to the initiation of the project.

Recruitment. Participants were recruited through the use of flyers posted around the Penn State campus and local community. Additionally, potential participants were recruited in Penn State classrooms following permission from the course instructor.
Interested individuals were asked to contact the Biobehavioral Studies Laboratory to receive further information about the study and to provide their name, telephone number, and the best time to be contacted from one our laboratory members. Potential participants then were contacted by a trained interviewer who asked questions about significant health problems and use of medication that may interfere with the interpretation of the blood pressure and blood marker data.

Based on the telephone screening, eligible participants were contacted through a follow-up telephone call and a cholesterol screening session was scheduled at the General Clinical Research Center (see “Cholesterol Screening” section for details about screening session). These participants also received an email confirming the time and location of their appointment and were asked to fast for 12 hours for a proper cholesterol reading (Craig et al., 2000). Once the participant’s blood test results were received and it was confirmed that cholesterol levels were normal (total cholesterol less than 240 mg/dl, high-density lipoprotein greater or equal to 40 mg/dl, low-density lipoprotein less than 130 mg/dl), and after at least one parent’s hypertension information questionnaire was received, s/he was contacted and a date was scheduled for a 3.5 hour laboratory session. All participants received an email confirmation of time and date of the session.

**Exclusionary Criteria.** Potential participants were not allowed to participate in the study if there was a presence of significant health problems or use of medication that would interfere with the interpretation of the blood pressure and blood marker data. This included conditions such as diabetes and hypertension; neurological disorders such as stroke; history of depression, anxiety, or other mental illnesses; cognitive or attentional disorders such as attention deficit/hyperactivity disorder; current use of medications that
would interfere with normal hormonal, cognitive, metabolic, and cardiovascular functioning, such as oral and injected corticosteroids, psychostimulants, aspirin, antioxidant vitamins (Carroll & Schade, 2003); use of tobacco or other nicotine products; or severe obesity as indicated by a body mass index (kg/m$^2$) greater than 30. Height and weight recorded at the beginning of each session was used to calculate body mass index (BMI). Mean body indices did not differ across experimental groups (see Table 1).

Participants were daily consumers of at least 100 mg of caffeine per day (equivalent to one 8 oz. cup of coffee or four 12 oz. cans of soda), and, at the most, daily 500 mg of caffeine per day. Previous studies in this area suggest that participants who consume 25 to 500 mg of caffeine consumption per day can tolerate the level of caffeine administered in this protocol without significant differences in their reactivity to the caffeine administration and to stress (M. al'Absi, personal communication; al'Absi et al., 1998; al'Absi & Wittmers, 2003). The choice of 100 mg of caffeine as the lower end of the inclusionary criteria for this study ensured that the participants would tolerate caffeine well and would not ‘over’ react to the caffeine administration. The upper end of 500 mg ensured that the participant tolerated caffeine well, but, at the same time, still responded to the dosage of caffeine administered in this study (al'Absi et al., 1998). Additionally, participants were asked to abstain from caffeine use for 4 hours prior to the study, which allowed enough time for proper caffeine elimination before initiation of the study, and to eat a low-fat lunch as a high fat meal has been found to significantly increase levels of C-reactive protein (CRP) (Carroll & Schade, 2003).

Due to the effects of oral, transdermal, and transmucosal contraceptive steroids and hormone replacement therapy on neuroendocrine hormones (in this case, cortisol)
women volunteers who reported the use of any of these medication were not allowed to participate in the study (Kirschbaum et al., 1999). Women who were undergoing menopause also were excluded. Due to the large hormonal changes that takes place during pregnancy, and potentially teratogenic effects of caffeine on the fetus (Christian & Brent, 2001), women volunteers were not included if they reported current pregnancy, current attempts to become pregnant, or a pregnancy within the previous year. Laboratory sessions for women were scheduled during the luteal phase of the menstrual cycle, when estrogen levels are highest, in order to maximize any potential sex differences (Elhadd, 2003). Furthermore, a 25 % reduction in caffeine metabolism has been reported in females during the luteal phase of the menstrual cycle (e.g. Benzaquen et al., 2002), compared to follicular phase, which can also help to emphasize the effects of caffeine in the current study. Out of 216 potential participants who were screened over the telephone, 106 were disqualified due to the presence of one or more of the previously described exclusionary criteria.

**Family History of Hypertension Selection.** Family history status was determined through the use of a questionnaire specifically designed to capture this variable (Page & France, 2001) (see Appendix B). This questionnaire has been used successfully to screen for family history status by a number of different investigators (al'Absi & Wittmers, 2003; Lovallo et al., 1996; Lovallo et al., 1989). This questionnaire was mailed to the participant’s parents following the participant’s permission during the initial cholesterol screening. Alternatively, participants who indicated that he/she would be going home in the near future received directions to have the questionnaire completed by his/her biological parents and to return the sealed envelope containing the
questionnaire to the investigator. Because this study was concerned with the effects of a genetic history of hypertension, only participants from whom data could be obtained from biological parents were included in the sample. This request was clearly stated in bold letters at the top of the blood pressure questionnaire that was completed by the parents. If the potential participants did not have a family history of hypertension or his/her parents had not been prescribed hypertension medication, the potential participant was not allowed to participate in the study. The questionnaire, as well as permission to use it in this research, was obtained through personal communication with Dr. al’Absi and Dr. France (May, 2004). Out of 59 questionnaires returned, one participant was excluded based on lack of parental history of hypertension.

**Protocol**

**Cholesterol Screening Session.** Because studies consistently have found that a high level of total cholesterol, low levels of high density lipoproteins (HDL cholesterol), and high levels of low density lipoprotein (LDL cholesterol) are a risk factor in the development of cardiovascular disease (Gordon et al., 1977; Heiss et al., 1980; Manninen et al., 1988; Miller et al., 1977), and because they can be associated with elevated blood levels of fibrinogen and CRP (Fong & Chia, 1990; Nissen et al., 2005), participants were screened for their total cholesterol and fractions to ensure that their total cholesterol was within normal limits (total cholesterol less than 240 mg/dl, high-density lipoprotein greater or equal to 40 mg/dl, low-density lipoprotein less than 130 mg/dl).

Following completion of a telephone screening form (see Appendix A) potential participants came in to a screening session at the General Clinical Research Center (GCRC) on the Penn State University campus, where 15 ml of blood was collected via
antecubital venipuncture for assessment of total cholesterol and fractions to ensure that the participant’s total cholesterol fell within normal limits. Females were not scheduled at a specific phase of their menstrual cycle as other investigators did not find menstrual cycle variations in the levels of lipoproteins (e.g., Elhadd et al., 2003).

During the initial screening session the experimenter went over the procedures to be followed during the course of the study and the consent form (Appendix D) was signed by both the participant and experimenter. At this time, the experimenter also asked the participant to address an envelope to his/her parents that contained the parental history of hypertension survey. This survey was returned by the parents to the experimenter in a self-addressed, pre-stamped envelope.

Once the consent form was signed, the fasting blood draw took place by a trained phlebotomist. In addition, in order to enhance participant involvement in the study and perhaps increase the likelihood of compliance with caffeine abstinence on the day of the experimental session, participants were asked to measure his/her heart rate 25 minutes following their first cup of coffee or caffeinated beverage on the day of the laboratory session. A GCRC nurse instructed the potential participants on how to take their own heart rate when he/she came for the cholesterol screening. On the day of the study, participants took their heart rate before taking the first caffeinated beverage of the day, and 25 minutes following the ingestion of this beverage; this information was recorded on a form that was given to the participant at the screening session when she/he had learned the heart rate taking procedures from the nurse.

If the cholesterol screening revealed that a potential participant had a total cholesterol level that was greater than 240 mg/dl (American Heart Association, 2004),
he/she was not allowed to participate in the study. As indicated earlier, family history of hypertension status was assessed during the telephone-screening interview, and reassessed by the investigator once the family history of hypertension questionnaire completed by the parent was received. Four potential participants were excluded for having total levels of cholesterol that exceeded 240 mg/dl.

**Drug.** Anhydrous caffeine (3.3 mg/kg) (Spectrum Chemical Corporation, Gardena, CA) was administered to participants mixed with refrigerated white grapefruit juice (Unsweetened White Grapefruit Juice, Giant® brand, Landover, MD). Caffeine dosage was calculated based on the participant’s body weight at the second laboratory session (i.e. experimental session). The placebo consisted of grapefruit juice without the addition of caffeine. Grapefruit juice has consistently been used in the administration of caffeine in other studies (al'Absi et al., 1998; Hartley et al., 2000; Lovallo et al., 1996; Lovallo et al., 1991; Lovallo et al., 1989; Pincomb et al., 1996; Pincomb et al., 1987; Pincomb et al., 1985), and has been found to have no effect on caffeine’s pharmacokinetics and no hemodynamic effects (Maish et al., 1996). This caffeine dosage was based on previous studies that have found significant effects of caffeine and stress when participants were administered 3.3 mg/kg of caffeine mixed in grapefruit juice (al'Absi et al., 1998; Hartley et al., 2000; Lovallo et al., 1996; Lovallo et al., 1991; Lovallo et al., 1989; Pincomb et al., 1996). In order to keep the experimenter blind to the drug condition, the participant’s drink was prepared by a GCRC nurse who was given a sealed envelope indicating the given participant’s drug condition.

**Laboratory Session.** Table 2 presents a timeline of the experiment; Appendix E contains the laboratory session protocol and all questionnaires administered during the
laboratory session. Following their cholesterol screening, eligible participants arrived at the GCRC for a 3.5 hour laboratory session. All sessions were held in the afternoon, starting at 1:00 PM to control for neuroendocrine hormone fluctuations with respect to cortisol measurement (Van Cauter, 1990). Upon arrival at the GCRC, the participant’s height and weight was measured and recorded to allow proper calculation of caffeine dosage for those receiving caffeine and a medical history check was performed by a nurse to confirm eligibility. In order to ensure that female participants were not pregnant, they were asked to provide a urine sample before the medical history check.

Participants then completed a demographic information and caffeine consumption questionnaire to gather information on demographics and daily caffeine consumption. In order to ensure that the participants’ blood pressure was within the normal range, a Dinamap (Model 1847-SX; Critikon, Tampa, FL) blood pressure cuff then was placed on the participant’s dominant arm and a sample blood pressure was taken to ensure that the participant’s blood pressure was below 140 mmHg systolic or 90 mmHg diastolic as this would indicate clinical hypertension (AHA, 2003b) and, therefore, could result in potentially increased levels of CRP and fibrinogen. No participants were excluded as a result of this blood pressure criterion. The participant was then asked to lie on a GCRC bed and an indwelling catheter (IV) was inserted in the non-dominant arm by a trained nurse. Once the IV was properly started and the participant indicated feeling well, he/she was asked to get up from the bed and sit quietly in a chair for 30 minutes (baseline).

**Baseline.** Throughout the 30 minutes of rest, blood pressure and heart rate data were collected fifteen times at 2-minute intervals. Following rest, participants were administered a symptom report questionnaire to ensure participant’s well-being (see
Appendix E). Participants then were administered either caffeine (3.3 mg/kg, mixed in grapefruit juice) or a placebo (grapefruit juice alone). Whether the participant received caffeine or placebo was determined by chance. The drink prepared was administered by a GCRC nurse and the experimenter (IR) was blind to the drug condition.

Participants then were asked to sit quietly for 20 minutes following drug administration to allow enough time for the caffeine to take effect. Blood pressure and heart rate readings were collected every two minutes during this 20-minute period. Once the 20-minute period ended, 30 ml of blood were collected. Blood was drawn into chilled ethylenediaminetetraacetic acid (EDTA) “purple top” and anticoagulant-free tubes “red top” tubes. Blood samples were used to determine baseline levels of C-reactive protein, fibrinogen, and cortisol, as well as progesterone and estradiol for females, which were used to confirm the luteal phase of their menstrual cycle.

**Stress Protocol.** Participants then received instructions about the next part of the study, which involved a preparation and delivery of a brief speech, and a mental arithmetic task consisting of serial subtraction (see Appendix F for stress protocol). Both of these tasks are based on the Trier Social Stress Task and have consistently been show to elicit significant increases in cardiovascular reactivity and cortisol levels (al'Absi et al., 1998; al'Absi & Wittmers, 2003). This portion of the study was conducted by a different experimenter (LCK or MMS), who the participant believed to be a psychologist who would evaluate his/her performance on the tasks. The speech task consisted of 10 minutes of speech preparation and 3.5 minutes of speech delivery, with the topic of the speech dealing with an experience of personal failure in front of a videocamera. Participants
were told that their speech would be videotaped for later evaluation by a panel of psychologists. However, the session was not videotaped and would not be shown later to a panel of psychologists. The mental arithmetic task involved counting backwards from a four-digit number by 7’s and 13’s as accurately and as quickly as possible and lasted 15 minutes. Blood pressure and heart rate were monitored at 2-minute intervals throughout the stressor tasks. Pre and Post-task Appraisal questionnaires were administered to evaluate the participant’s pre and post evaluation of the task (see Appendix E). The stressor tasks were followed by a 15-minute recovery period, where the participant was asked to sit quietly while their blood pressure was monitored at 2-minute intervals. Participants also were asked to complete questionnaires that dealt with his/her impressions of the tasks, as well as a mood assessment (see Appendix E).

After this 15 minute recovery period to allow stress hormone levels to rise, 30 ml blood sample was then collected to allow for later assessment of neuroendocrine and cardiovascular reactivity to the stress challenges. Blood sample collection timing is based on the fact that a number of investigators have found that acute and chronic stress leads to significant increases in CRP levels, as well as CRP’s mediating cytokines IL-6 and IL1 beta (Black, 2002, 2003; Black & Garbutt, 2002; Owen et al., 2003; Steptoe et al., 2001).

**Recovery.** Following the second blood draw participants then continued to sit quietly for 45 minutes (recovery), which allowed enough time for rises in CRP and fibrinogen to be captured by the third blood draw (Carroll & Schade, 2003; Steptoe et al., 2001), where 30 ml of blood was collected. During this 45-minute recovery period, participants were asked to complete another series of questionnaires including state and
trait anxiety questions, symptom report, mood assessment, and caffeine recognition (See Appendix E). Following collection of the third blood sample and confirmation that vital signs had returned to baseline, the participant was debriefed, received compensation of 50 dollars, and was discharged from the study. Out of 55 sessions scheduled, 3 were not run because an I.V. could not be started.

**Blood Collection and Storage.** Blood was collected three times during the experimental session (30 ml each draw); once at the beginning of the session (baseline), and 15 and 60 minutes following the stress tasks. After each draw, chilled ethylenediamine tetra-acetic acid (EDTA) “purple top” tubes were immediately centrifuged for 15 minutes at 4 degrees C at 1500 X g. Plasma samples then were aliquotted into separate 1 ml cryovials and stored at -70 degrees C for later assessment of fibrinogen (Appendix G). Serum samples were allowed to sit at room temperature for 15 minutes and then were centrifuged for 15 minutes at 4 degrees C at 1500 X g, and later used for assessment of CRP (Appendix H), cortisol (Appendix I), estrogen (Appendix J), and progesterone (Appendix K). CRP (DakoCytomation, Glostrup, Denmark), fibrinogen (Affinity Biologicals, Inc., Ancaster, Ontario, Canada), as well as cortisol, estradiol, and progesterone (Diagnostic Systems Laboratories, Inc., Webster, Texas) levels were evaluated by enzyme linked immunosorbant assay. All assays were run from a single assay batch and assay kits came from the same lot.

**Statistical Analyses**

All data were entered into SPSS statistical software package for statistical analyses. Repeated-measures analyses of variance (ANOVAs) with sex and drug as the
independent variables and time (baseline, stress, and recovery) as the within-subjects variable were conducted to test the effects of sex and caffeine on blood pressure, heart rate, and blood markers of cardiovascular disease, including CRP and fibrinogen.

Repeated-measures analyses of variance (ANOVAs), with sex and drug as the independent variables and time (baseline, stress, and recovery) as the within-subjects variable also were conducted to test the effects of sex and caffeine on self-reported mood measures which included stress, nervousness, frustration, and anger. Separate repeated-measures ANOVAs were used to test the effects of stress on blood pressure and blood markers of cardiovascular disease with sex and drug as the independent variables and time (pre-stress and post-stress) as the within-subjects variable. Separate ANOVAs were used to test any group X time interactions.

Cortisol, C-reactive protein, and fibrinogen data were positively skewed. Therefore, separate log transformations were performed on these data to reduce variance and normalize the distribution so that parametric tests could be used. These transformations resulted in normal distribution of the data. All cortisol, C-reactive protein, and fibrinogen data analyses were conducted on log transformed data. However, mean values (± standard error of the mean) are reported in the text for clarity.

Average baseline systolic and diastolic blood pressure values, as well as heart rate, were calculated by averaging the 10 measurements that were taken every 2 minutes during a 20-minute baseline period immediately following caffeine administration. Average stress heart rate, systolic and diastolic blood pressure were calculated by averaging the 6 readings collected during speech preparation, 2 readings collected during the speech, and the 6 readings collected during the math task. Readings during the stress
task were taken every two minutes. Average recovery systolic and diastolic blood pressure values, and heart rate, were calculated by averaging the 10 readings taken every 5 minutes during a 45-minute recovery period.

All tests were two-tailed and criterion for statistical significance was set at alpha = 0.05. All tables and graphs in this study represent means (± SEM) based on untransformed (raw) data values.
CHAPTER 3: RESULTS

Stressor and Caffeine Administration Confirmation

Self-reported measures of stress on a Lickert Scale of 1 to 7 (1 = not at all, 7 = very much), serum cortisol levels, systolic and diastolic blood pressure and heart rate were collected at baseline, stress, and recovery. These measures were used to confirm the validity of the stressor and caffeine administration.

Self-Report Measures

Stress. Figure 1 presents self-reported stress levels by all participants during baseline, immediately following stress, and at the end of recovery. A repeated-measures ANOVA revealed a significant effect of time \( F(2,94) = 28.59, p < 0.05 \) that followed a quadratic function (i.e., inverted U-shaped curve) \( F(1,47) = 66.70, p < 0.05 \). Specifically, self-reported stress levels were higher under stress compared to baseline and recovery \( F(1,47) = 38.91, p < 0.05 \) and \( F(1,47) = 32.34, p < 0.05 \), respectively], but baseline and recovery levels were similar. Time did not interact with gender or caffeine conditions and there were no main effects of gender or caffeine treatment on self-reported levels of stress.

Nervousness. Figure 2 presents self-reported nervousness levels by all participants during baseline, stress, and recovery. A repeated-measures ANOVA revealed a significant effect of time \( F(2,94) = 26.87, p < 0.05 \) that followed a quadratic
function (i.e., inverted U-shaped curve) \([F(1,47)=56.94, \ p<0.05]\). Specifically, self-reported nervousness levels were higher under stress compared to baseline and recovery \([F(1,47)=34.68, \ p<0.05\) and \(F(1,48)= 28.58, \ p<0.05\), respectively\], but baseline and recovery levels were similar. Similar to self-reported stress levels, time did not interact with gender or caffeine conditions and there were no main effects of gender or caffeine treatment on self-reported levels of nervousness.

**Anger.** Figure 3 presents self-reported anger levels by all participants during baseline, stress, and recovery. Similar to the other mood reports, a repeated-measures ANOVA revealed a significant effect of time \([F(2,94)=16.65, \ p<0.05]\) that followed a quadratic (i.e., inverted U-shaped curve) function \([F(1,47)=17.93, \ p<0.05]\). Specifically, self-reported feelings of anger were higher under stress compared to baseline and recovery \([F(1,47)=17.85, \ p<0.05\) and \(F(1,48)=18.51, \ p<0.05\), respectively\]. In contrast, baseline and recovery levels of anger did not differ from one another. Interestingly, time interacted with gender \([F(2,94)=3.83, \ p<0.05]\). Separate repeated-measures ANOVAs were conducted by gender to determine the nature of this interaction. Self-reported anger levels among males increased in response to stress compared to baseline and recovery \([F(1,47)=17.85, \ p<0.05\) and \(F(1,48)=18.51, \ p<0.05\), respectively\] and anger levels during recovery were similar to baseline levels. Among women, however, self-reported levels of anger did not differ significantly among the 3 time points, which is consistent with previous findings (Tomaka et al., 1999). Time did not interact with caffeine condition and there were no main effects of gender or caffeine treatment on self-reported levels of anger for any of the analyses reported above.
Serum Cortisol

Figure 4 presents serum levels of cortisol for men and women exposed to caffeine or to no caffeine during baseline, stress, and recovery. A repeated-measures ANOVA with gender and caffeine group as the independent variables looking at the 3 experimental time points (baseline, stress, and recovery) revealed a significant effect of time $[F(2, 94) = 19.62, p < 0.05]$ that followed a quadratic (i.e., inverted U-shaped curve) function $[F(1, 47) = 34.16, p < 0.05]$. As expected, levels of serum cortisol were significantly higher following stress than during baseline and recovery $[F(1, 48) = 26.20, p < 0.05$ and $F(1, 47) = 27.13, p < 0.05$, respectively]. However, baseline and recovery serum cortisol levels did not differ significantly. A time by gender interaction also was found $[F(1, 94) = 4.20, p < 0.05]$, as well as a time by caffeine interaction $[F(1, 94) = 4.01, p < 0.05]$. No 3-way interaction was found among time, gender, and caffeine $[F(1, 94) = 0.12, \text{n.s.}]$.

In order to determine the nature of the time by gender interaction, separate repeated-measures ANOVAs were conducted for men and for women. Serum levels of cortisol increased significantly from baseline to stress among males $[F(1, 24) = 36.38, p < .05]$, but only for those in the caffeine group. Females did not show this effect. This finding is not unexpected, as other investigators have shown that some individuals do not show an increase in cortisol following stress, but rather a decrease (Dickerson & Kemeny, 2004; Kunz-Ebrecht et al., 2003; Susman et al., 1988). As expected, both males and females showed a statistically significant decrease in serum levels of cortisol from stress to recovery $[F(1, 24) = 14.43, p < 0.05$ and $F(1, 23) = 12.80, p < 0.05$, respectively] in the caffeine and no caffeine groups. Interestingly, however, when comparing serum levels of
cortisol at baseline and recovery, males showed a statistically significant increase
\[F(1,24)=11.44, p<0.05\], but significant increases were only seen in those participants in
the caffeine condition. Serum cortisol recovery levels returned to baseline for all women.

Separate repeated-measures ANOVAs also were conducted by caffeine group to
determine the nature of the overall time by caffeine interaction. Both the no-caffeine and
caffeine groups showed a statistically significant increase in serum cortisol levels
between baseline and stress \[F(1, 24)=7.50, p<0.05\] and \[F(1,24)=18.79, p<0.05,\]
respectively], however, in the no caffeine condition this effect was only seen in males.
Interestingly, between baseline and recovery, participants in the no-caffeine group
displayed a statistically significant decrease in serum cortisol levels as expected
\[F(1,24)=1.32, p<0.05\], however, this effect was only present for females. In the caffeine
group, serum cortisol levels were significantly greater at recovery than at baseline
\[F(1,23)=6.35, p<0.05\] for males, but not for females.

**Blood Markers of Cardiovascular Disease Risk**

**Fibrinogen**

Figure 5 presents plasma levels of fibrinogen for men and women exposed to
caffeine or to no caffeine during baseline, stress, and recovery. A univariate analysis with
fibrinogen levels at baseline as the dependent variable and caffeine group as the
independent variable revealed that fibrinogen levels were significantly different at
baseline \[F(1,52)= 4.51, p<0.05\], with participants in the no caffeine condition displaying
significantly higher fibrinogen levels than those in the caffeine condition. Therefore,
baseline fibrinogen levels were used as a covariate in the following analyses. A univariate ANCOVA with fibrinogen levels at stress as the dependent variable, and gender and caffeine group as the independent variables, revealed a main effect of gender \[F(1, 47)=12.45, p<0.05\], as well as a main effect of caffeine \[F(1, 47)=11.86, p<.05\]. Females displayed increased levels of fibrinogen compared to males, and participants in the caffeine condition displayed significantly greater fibrinogen levels than did those in the no caffeine condition. No interaction effects of gender and caffeine were present \[F(1,47)=0.03, \text{n.s.}\]. A univariate analysis using a fibrinogen change score from baseline to stress also was performed. In order to normalize the data and adhere to the assumptions of the statistical test, fibrinogen change score was calculated by subtracting the log-transformed fibrinogen levels at stress from the log-transformed fibrinogen levels at baseline. This analysis also revealed a main effect of gender \[F(1,47)=12.45, p<0.05\] and caffeine \[F(1, 47)=11.86, p<.05\], with females displaying higher levels of fibrinogen than males, and those in the caffeine condition displaying higher levels than those who did not receive caffeine.

**C-Reactive Protein**

One male participant was removed from the C-reactive protein (CRP) analyses because his CRP levels were identified as outliers (5 standard deviations above the overall sample mean at all 3 timepoints). All CRP figures and analyses presented in this dissertation study include CRP values for all participants, but exclude the previously described male outlier. Figure 6 presents serum CRP levels during baseline, stress, and
recovery. A repeated-measures ANOVA performed with caffeine and gender as the independent variables revealed no significant effect of time on CRP levels among baseline, stress, and recovery \(F(2,94)=0.55, \text{n.s.}\). No interaction effects of time and gender or time and caffeine were present \(F(2,94)=0.51, \text{n.s.; } F(2,94)=0.67, \text{n.s.}\). Similarly, no 3-way interactions were found among time, gender, and caffeine \(F(2,94)=2.52, \text{n.s.}\).

**Blood pressure and Heart Rate**

Average baseline systolic and diastolic blood pressure values, as well as heart rate, were calculated by averaging the 10 measurements that were taken every 2 minutes during a 20-minute baseline period, immediately following caffeine administration while caffeine was being absorbed.

In order to ensure that there were no differences between caffeine groups at baseline, separate univariate ANOVAs on caffeine group using systolic blood pressure, diastolic blood pressure, and heart rate as the dependent variables revealed no group differences with respect to systolic and diastolic blood pressure, or heart rate \(F(1,48)=1.08, \text{n.s.; } F(1,48)=0.53, \text{n.s.; } \text{and } F(1,48)=0.09, \text{n.s.}, \text{respectively}\).

Figures 7 and 8 present systolic and diastolic blood pressure values at one minute and twenty minutes following caffeine administration, respectively. A repeated-measures ANOVA examining the effects of caffeine administration on systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate was conducted in order to confirm caffeine exposure. A time by caffeine group effect was found for both systolic and
diastolic blood pressure \( F(9,423)=3.69, p<0.05 \) and \( F(9,423)=3.28, p<0.05 \), respectively, such that those who received caffeine displayed a statistically significant increase from time 1 to time 10 while resting for 20 minutes following the drink administration. No time by caffeine effect was found for heart rate \( F(9,423)=0.44, \text{n.s.} \).

**Systolic Blood Pressure**

Figure 9 presents average systolic blood pressure values for men and women exposed to caffeine or no caffeine during baseline, stress, and recovery. A repeated-measures ANOVA with gender and caffeine group as the independent variables revealed a main effect of time \( F(2,96)=198.02, p<0.05 \). More specifically, there was a significant increase in SBP from baseline to stress, and from baseline to recovery \( F(1,48)=234.82, p<0.05; F(1,48)=48.22, p<0.05 \). As expected, a statistically significant decrease was observed from stress to recovery \( F(1,48)=202.01, p<0.05 \). Additionally, a time by caffeine group interaction also was found \( F(2,96)=3.52, p<0.05 \), with participants who received caffeine displaying a significantly greater increase in SBP from baseline to stressor and from baseline to recovery than those who did not receive caffeine \( F(1,48)=8.52, p<0.05; F(1,48)=4.29, p<0.05, \text{respectively} \). Time did not interact with gender and there were no 3-way interactions among time, gender, and caffeine.

**Diastolic Blood Pressure**

Figure 10 presents average diastolic blood pressure values for men and women exposed to caffeine or no caffeine during baseline, stress, and recovery. A repeated measures ANOVA revealed a main effect of time \( F(2,96)=248.18, p<0.05 \). More specifically, a statistically significant increase in DBP was revealed when comparing
baseline to stress, and baseline to recovery \(F(1,48)=317.79, p<0.05; F(1,48)=91.43, p<0.05\), respectively]. Conversely, DBP levels significantly decreased from stress to recovery \(F(1,48)=237.79, p<0.05\]. Time did not interact with gender or caffeine and no 3-way interactions were found among time, caffeine, and gender.

**Heart Rate**

Figure 11 presents average heart rate values for men and women exposed to caffeine or no caffeine at baseline, stress, and recovery. A main effect of time was revealed when a repeated measures ANOVA was performed, and, more specifically, a statistically significant increase in HR was seen from baseline to stress \(F(1,48)=88.64, p<0.05\], while a decrease was observed from stress to recovery \(F(1,48)=113.7, p<0.05\]. No significant change was observed from baseline to recovery \(F(1,48)=2.48, n.s.\]. Time did not interact with caffeine or gender, and there were no 3-way interactions among time, gender, and caffeine.

**Post-Hoc CRP Analyses: CRP Risk Group**

Because levels of CRP did not follow the predicted increase during stress and no caffeine effect was found, further analyses were conducted to evaluate potential individual differences in CRP’s response to stress and caffeine. One male participant was excluded from the following CRP analyses because his CRP levels were identified as outliers. Following the American Heart Association guidelines for clinical CRP levels (AHA, 2003; Pearson et al., 2003), participants were divided into one of three groups, which indicates their risk for developing cardiovascular disease: 1) Those whose
baseline CRP levels were less than 1 mg/L (low risk), 2) those whose baseline CRP levels were between 1 and 3 mg/L (average risk), and 3) participants who had a baseline CRP level greater than 3 mg/L (high risk). Separate chi-square analyses confirmed that there were equivalent numbers of (1) men and women and (2) caffeine condition in each of the three CRP groups [$\chi^2 (2,51)=0.92$, n.s; $\chi^2 (2,51)=0.76$, n.s, respectively]. Within each CRP group were men (Low-risk: N=10; Average-risk: N =9; High-risk: N =6) and women (Low-risk: N=11; Average-risk: N=8; High-risk: N=7) who received either 3.3 mg/kg of caffeine (Low-risk: N=9; Average-risk: N=9; High-risk: N=7), or no caffeine (Low-risk: N=12; Average-risk: N=8; High-risk: N=6).

Figures 12 presents CRP levels at baseline, stress, and recovery for all participants. Figures 13 and 14 present CRP levels at baseline, stress, and recovery for males and females, respectively. Repeated-measures ANOVA using CRP risk group, gender, and caffeine as the independent variables, and CRP at baseline, stress, and recovery as the dependent variables showed a caffeine group by CRP risk group interaction [$F(2,38)=3.69$, $p<0.05$]. More specifically, caffeine only had an effect on CRP levels for those participants who were grouped into the ‘average-risk’ category; for participants in this group 3.3 mg/kg of caffeine led to significant increases in CRP levels over time. No main effect of time was found and time did not interact with gender, caffeine group, or CRP risk group. No interactions were found among gender and caffeine, or gender and CRP risk group.

Fibrinogen levels also were re-examined after splitting CRP into the 3 risk groups. Figure 15 presents fibrinogen levels at baseline, stress, and recovery for all participants. Figures 16 and 17 present fibrinogen levels at baseline, stress, and recovery
for males and females, respectively. Baseline levels of fibrinogen were once again used as a covariate due to baseline differences between those in the caffeine and no caffeine condition. A univariate ANOVA with fibrinogen levels at stress as the dependent variable, and gender and caffeine group as the independent variables revealed a main effect of gender \( F(1, 39) = 17.23, p < 0.05 \), as well as a main effect of caffeine \( F(1, 39) = 11.42, p < 0.05 \). Females displayed significantly higher levels of fibrinogen at stress than did males, and those in the caffeine condition also displayed significantly higher fibrinogen levels at stress than did those in the no caffeine condition. Additionally, a caffeine by CRP risk group was found \( F(2, 39) = 4.71, p < 0.05 \), such that significantly higher levels in fibrinogen levels were found for those who received caffeine, but only for those in the low CRP risk group. A univariate ANOVA with fibrinogen levels at recovery as the dependent variable, and gender and caffeine group as the independent variables revealed no main effects of gender or caffeine, and no gender by caffeine interaction. A univariate analysis using a fibrinogen change score from baseline to stress also was performed. Fibrinogen change score was calculated by subtracting the log-transformed fibrinogen values at stress from the log-transformed fibrinogen values at baseline. This analysis revealed no main effect of gender or caffeine, and no gender by caffeine interaction.

Additional heart rate and blood pressure analyses also were performed once CRP levels were divided into the 3 risk categories. Figures 18 and 19 present heart rate levels at baseline, stress, and recovery for males and females, respectively. A repeated-measures ANOVA using CRP risk group, gender, and caffeine as the independent variables, and heart rate (HR) at baseline stress, and recovery as the dependent variables
revealed a main effect of time \( F(1,39)=122.68, p<0.05 \), such that HR during stress was significantly higher than at baseline and recovery. Time did not interact with gender, caffeine group, or CRP risk group, nor were any interactions observed among gender, caffeine, or CRP risk group.

Figure 20 presents systolic blood pressure levels at baseline, stress, and recovery for all participants. Figures 21 and 22 present systolic blood pressure levels at baseline, stress, and recovery for males and females, respectively. A repeated-measures ANOVA using SBP as the dependent variable and CRP risk group, gender, and caffeine as the independent variables revealed a main effect of time \( F(1,39)=266.62, p<0.05 \), such that SBP was higher at stress than at baseline and recovery. A main effect of gender also was found \( F(1,39)=26.42, p<0.05 \), with males displaying higher overall levels of SBP than females. A gender by CRP risk group interaction, as well as a gender by caffeine group interaction also was found \( F(2,39)=4.76, p<0.05; F(1,39)=9.48, p<0.05 \), respectively]. More specifically, males displayed higher overall SBP in the ‘low’ and ‘high’ risk CRP risk group then did females, while there was no difference between males and females in the average CRP risk group. While males had significantly higher overall levels of SBP than did females, this difference was significantly greater in the caffeine group, compared to no caffeine.

Figures 23 and 24 present diastolic blood pressure levels at baseline, stress, and recovery for males and females, respectively. When re-examining DBP with a repeated-measures ANOVA using CRP risk group, gender, and caffeine as the independent variables and DBP as the dependent variable, a main effect of time was found \( F(1,39)=240.86, p<0.05 \), such that DBP was higher at stress than at baseline and
recovery. Time did not interact with gender, caffeine, or CRP risk group, and there were no main effects of gender or caffeine group.

Summary of Hypotheses

Hypothesis 1: Caffeine and Cardiovascular Reactivity

It was hypothesized that caffeine would lead to elevations in SBP and DBP in response to stress, while no changes would be seen in HR compared to no caffeine in both males and females. This hypothesis was supported for SBP and HR, but not supported for DBP. Systolic blood pressure was significantly higher in response to stress in the caffeine group, while DBP and HR did not differ in response to stress between caffeine and no caffeine groups.

Hypothesis 2: Stress and Cardiovascular Reactivity

The hypothesis that stress would lead to elevations in SBP, DBP, and HR compared to baseline for both males and females was supported. It was further hypothesized that males would display higher SBP and DBP in response to stress than would females, but there would be no sex differences with respect to HR. This hypothesis was supported with respect to HR, but not supported with respect to SBP and DBP.

Hypothesis 3: Caffeine and Blood Markers of Cardiovascular Disease

The hypothesis that participants in the caffeine group would show increased levels of CRP and fibrinogen compared to those in the no-caffeine group was supported with respect to fibrinogen, and was not supported with respect to CRP. However, once
participants were separated into CRP risk groups, it was found that caffeine led to a significant increase in CRP levels in those participants in the ‘average-risk’ category.

It was further hypothesized that males would display higher levels of these blood markers in response to caffeine than would females. This hypothesis was not supported. While a sex difference in levels of fibrinogen was found, this difference was in the opposite direction of our prediction, with females displaying significantly higher levels than males, and it was not in response to caffeine. No sex differences were found with respect to CRP and caffeine.

**Hypothesis Four: Stress and Blood Markers of Cardiovascular Disease**

The hypothesis that stress would lead to increases in CRP and fibrinogen compared to baseline for both males and females was supported with respect to fibrinogen and not supported with respect to CRP. C-reactive protein levels did not change from baseline to stress. However, once participants were separated into 3 CRP risk groups (Low, Average, and High), it was found that for those participants who were grouped into the Average-risk category 3.3 mg/kg of caffeine led to a significant increase in CRP levels from baseline to stress. No significant changes were found for those participants grouped into the Low and High categories.
**Table**: Age and body mass indices (means ± SEM) of men and women in each experimental group and baseline progesterone and estradiol (means ± SEM) of females in the luteal phase of the menstrual cycle in each experimental group.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>3.3 mg/kg Caffeine</th>
<th>No Caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Age (years)</td>
<td>22.85 ± 0.93</td>
<td>22.31 ± 0.87</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>25.52 ± 0.60</td>
<td>23.14 ± 0.94</td>
</tr>
<tr>
<td>Progesterone (ng/mL)</td>
<td>N/A</td>
<td>26.44 ± 6.12</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>N/A</td>
<td>190.37 ± 39.80</td>
</tr>
<tr>
<td>Experimental Events</td>
<td>Purpose</td>
<td>Duration</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Study introduction, Height, weight, Health History</td>
<td>Collect participant’s height and weight; ensure participant health qualification for study</td>
<td>15 min.</td>
</tr>
<tr>
<td>Questionnaires</td>
<td>caffeine consumption assessment, demographic information collection</td>
<td>10 min.</td>
</tr>
<tr>
<td>I.V. Insertion</td>
<td>Insert I.V. catheter for collection of blood samples</td>
<td>10 min.</td>
</tr>
<tr>
<td>Baseline</td>
<td>Collection of baseline levels of blood pressure and heart rate; gather information on potential symptoms</td>
<td>30 min.</td>
</tr>
<tr>
<td>Drug Administration</td>
<td>Administration of caffeine or placebo</td>
<td>5 min.</td>
</tr>
<tr>
<td>Resting Period # 1</td>
<td>Allow time for caffeine to take effect; gather information on mood, anxiety, and pre-task appraisal</td>
<td>20 min.</td>
</tr>
<tr>
<td>Sample Collection # 1</td>
<td>Determine resting levels of cortisol and cardiovascular risk blood markers</td>
<td>10 min.</td>
</tr>
<tr>
<td>Laboratory Challenges</td>
<td>Evaluate cortisol and cardiovascular reactivity to challenges</td>
<td>30 min.</td>
</tr>
<tr>
<td>Recovery</td>
<td>Allow time for participant to recover from stress task</td>
<td>15 min.</td>
</tr>
<tr>
<td>Sample Collection # 2</td>
<td>Determine reactivity levels of cortisol and cardiovascular risk blood markers</td>
<td>5 min.</td>
</tr>
<tr>
<td>Resting Period # 3</td>
<td>Allow time for blood markers to change</td>
<td>45 min.</td>
</tr>
<tr>
<td>Sample collection # 3</td>
<td>Determine levels of cortisol, CRP and fibrinogen in response to caffeine and stress</td>
<td>5 min.</td>
</tr>
<tr>
<td>Debriefing</td>
<td>Answer participant’s questions about the study, participant payment</td>
<td>10 min.</td>
</tr>
</tbody>
</table>
Figure: Average level of self-reported stress levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average level of self-reported nervousness levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average level of self-reported anger levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average serum cortisol (ng/mL) levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average plasma fibrinogen (mg/dL) levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average serum C-reactive protein levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Systolic blood pressure (mmHg) levels (± SEM) at one minute and twenty minutes following caffeine administration.
Figure 8: Diastolic blood pressure (mmHg) levels (± SEM) at one minute and twenty minutes following caffeine administration.
Figure 9: Average systolic blood pressure (mmHg) levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average diastolic blood pressure (mmHg) levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average heart rate (beats/minute) levels (± SEM) by males and females exposed to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average serum C-reactive protein (mg/L) levels (± SEM) by CRP risk group for males and females in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average serum C-reactive protein (mg/L) levels (+ SEM) by CRP risk group for males in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure 14: Average serum C-reactive protein (mg/L) levels (± SEM) by CRP risk group for females in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure 15: Average plasma fibrinogen (mg/dl) levels ($\pm$ SEM) by CRP risk group for males and females in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average plasma fibrinogen (mg/dl) levels (± SEM) by CRP risk group for males in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure 17: Average plasma fibrinogen (mg/dl) levels (+ SEM) by CRP risk group for females in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure 18: Average heart rate (beats/minute) levels (± SEM) by CRP risk group for males in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average heart rate (beats/minute) levels (± SEM) by CRP risk group for females in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average systolic blood pressure (mmHg) levels (± SEM) by CRP risk group for males and females in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure 21: Average systolic blood pressure (mmHg) levels (± SEM) by CRP risk group for males in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average systolic blood pressure (mmHg) levels (± SEM) by CRP risk group for females in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average diastolic blood pressure (mmHg) levels (± SEM) by CRP risk group for males in response to caffeine or no caffeine during baseline, stress, and recovery.
Figure: Average diastolic blood pressure (mmHg) levels (± SEM) by CRP risk group for females in response to caffeine or no caffeine during baseline, stress, and recovery.
CHAPTER 4: GENERAL DISCUSSION

Overview

The overall goal of this dissertation was to examine the effects of caffeine and stress on blood pressure and the blood markers of cardiovascular disease, fibrinogen and C-reactive protein, in healthy participants with a parental history of hypertension. Cardiovascular disease has been the leading cause of death in the United States since 1918, and kills nearly 2,600 people each day, therefore it is extremely important that factors associated with the development and progression of cardiovascular disease be better understood.

A large number of investigators have raised the question as to whether caffeine (coffee being its major source, and the most widely consumed drug in world) consumption can be a contributor to cardiovascular disease. In order to address this question, several blood markers of cardiovascular disease have been investigated in association with caffeine, such as triglycerides and low and high density lipoproteins (cholesterol). The most well studied of these makers has been cholesterol, with studies finding that as coffee consumption increases, so do levels of LDL and total cholesterol (i.e., Jossa et al., 1993; Kohlmeier et al., 1991). Other markers of cardiovascular disease that have received more recent attention are fibrinogen and C-reactive protein (CRP). Fibrinogen and CRP are two acute-phase proteins, which are proteins whose plasma concentrations vary by 25% or more during inflammatory processes. While a large
number of studies have found that elevated levels of both fibrinogen and CRP are associated with an increased risk of cardiovascular disease (e.g. Benzaquen et al., 2002; e.g. Heinrich et al., 1994; Obradovic et al., 2003; Ridker, 2003b, 2003c; Stec et al., 2000), very few studies have investigated the relationship between caffeine and these blood markers. No studies have investigated the effects of caffeine on CRP \textit{in vivo} (Ganapathi et al., 1990), however, the only published in vitro study reported a significant CRP potentiation in response to caffeine in human liver cells. Very few studies have examined the potential connection between caffeine and fibrinogen, and the ones that have, reported conflicting results, with some investigators finding that coffee consumption was associated with increased fibrinogen levels (Happonen, 1987), while others found no increase in plasma fibrinogen levels in response to coffee or caffeine consumption (Bak et al., 1990; Naismith et al., 1970).

Genetics is another important factor that plays a role in the development of cardiovascular disease. Studies examining the role of genetic predisposition have demonstrated that participants who have a parental history of hypertension display significantly greater blood pressure reactivity in the presence of caffeine in response to a psychological stressor than do those without a family history of hypertension (i.e., al'Absi \textit{et al.}, 1998; Greenberg & Shapiro, 1987). While it is certainly a step in the right direction that some studies have investigated the relationship of caffeine and cardiovascular stress reactivity in participants with a family history of hypertension, to date, none of these studies have included females. According to the American Heart Association (2003b), in every year since 1984, cardiovascular disease has claimed the lives of more women than men. Furthermore, the gap between male and female deaths has increased dramatically
over the years, which further emphasizes the importance of including females in such studies.

Blood pressure reactivity, however, is not the only cardiovascular risk that is elevated by psychological stress. A number of studies have demonstrated that levels of fibrinogen and CRP can also be elevated by physiological stress, such as the consumption of a high fat meal (Black & Garbutt, 2002; Blake & Ridker, 2003a; Muldoon et al., 1995; von Kanel &Dimsdale, 2003). To date, no studies have examined the connection between caffeine, psychological stress, blood pressure changes, and blood markers of cardiovascular disease. To the extent that cardiovascular disease is so widespread, and that caffeine is so widely consumed in our society, a better understanding of the connection between these factors can have a tremendous impact in the prevention and potential treatment of cardiovascular disease.

The goal of this dissertation research was to address some of these unstudied areas in the relationship between caffeine, stress, blood pressure reactivity, and blood markers of cardiovascular disease. Healthy, young, male and female adults with a parental history of hypertension were used to evaluate the role of caffeine and stress on blood pressure reactivity and blood markers of cardiovascular disease (i.e., CRP and fibrinogen). There were two main points of investigation for this study: 1) to examine the effects of caffeine and stress on cardiovascular reactivity and blood markers of cardiovascular disease, and 2) to examine sex differences in these effects.
Cardiovascular Reactivity to Caffeine and Stress

To test the efficacy of the stressor used in the current study and to confirm the findings of previous studies (Al'Absi et al., 1997; al'Absi et al., 1995; al'Absi & Wittmers, 2003), it was hypothesized that participants would display a significant elevation in systolic and diastolic blood pressure, as well as heart rate, in response to the stressor. This hypothesis was confirmed. It was also expected that those who received caffeine would display significantly higher elevations in SBP and DBP, but that HR would not differ between those who did and those who did not receive caffeine. The current results confirmed a significant elevation in SBP in response to the stressor in the caffeine condition compared to no-caffeine, as well as no differences in HR between the two groups. This study differed from previous reports, however, in that no differences were found in DBP in response to stress between the caffeine and no-caffeine groups. A possible explanation for these findings may be a habituation process that takes place in response to repeated caffeine exposure. While caffeine-naïve subjects display significant changes in heart rate and blood pressure, the heart rate changes quickly adapt to the repeated administration of caffeine (Sudano et al., 2005). Diastolic blood pressure also has been shown to adapt to repeated caffeine exposure, and systolic blood pressure appears to be the only consistent blood pressure measure to become elevated in response to caffeine (Corti et al., 2002; Sudano et al., 2005). Because the participants in the current study were daily consumers of caffeine, it is likely that their cardiovascular system habituated to the diastolic blood pressure effects of caffeine prior to their lab session, and
therefore, no changes were observed in response to the experimental caffeine administration.

**Caffeine, Stress, and Blood Markers of Cardiovascular Disease**

The major goal of this dissertation was to investigate the effects of caffeine and stress on fibrinogen and C-reactive protein, two blood markers of cardiovascular disease risk. From a biobehavioral health perspective, understanding the effects of caffeine and stress on these blood markers of cardiovascular disease may be an important aspect of disease diagnosis and intervention that so far has been overlooked. Based on previous research, it was hypothesized that levels of plasma fibrinogen (Happonen, 1987) and serum levels of C-reactive protein (Bak et al., 1990; Naismith et al., 1970) would increase in response to caffeine following a stressor. However, only plasma levels of fibrinogen were significantly elevated in response to caffeine. At first glance, the data from C-reactive protein were not clear. In fact, it appeared that caffeine had no effect on levels of this circulating blood marker of cardiovascular disease. However, more detailed analyses revealed the presence of specific individual differences in the response of C-reactive protein to stress and caffeine, which was independent of sex. These differences appeared to be related to levels of baseline C-reactive protein, and the risk level of cardiovascular disease based on such baseline levels.

Because no previous research has examined the effects of caffeine on C-reactive protein, the hypotheses for the current study with respect to CRP were based on an *in vitro* study, which found significant potentiation of CRP induction in human liver cells in
the presence of caffeine (Ganapathi et al., 1990). Caffeine increased CRP induction 3-6 times compared to a control medium, although the mechanism by which caffeine modifies the induction of CRP is still poorly understood. This study also showed that caffeine not only potentiated synthesis and secretion of CRP into culture medium, but it also led to increased accumulation of CRP messenger RNA, which indicated that caffeine acts at a pretranslational level, and not only by facilitating the release of newly synthesized CRP or by decreasing CRP degradation (Ganapathi et al., 1990).

When data from the current study initially revealed no changes in CRP as a result of receiving caffeine, it initially appeared that there were no significant effects of caffeine and stress on CRP, or that the timing of measurement was not optimized to capture changes in this blood marker. However, more detailed analyses examining individual differences helped to clarify these findings. The American Heart Association (AHA) recently added a recommendation that individuals at risk for developing cardiovascular disease be screened for CRP levels (Pearson et al., 2003). According to the AHA guidelines, CRP levels and risk for cardiovascular disease should be interpreted according to three different categories: low risk (CRP < 1mg/L), average risk (CRP between 1 and 3 mg/L), and high risk (CRP >3mg/L). These different risk categories are based on research showing that for each doubling in CRP levels, there is a 1.5 times increased risk for future development of cardiovascular disease (Pearson et al., 2003). Because there is a lack of data in the literature regarding the effects of stress and caffeine on CRP levels, post-hoc analyses examining individuals differences based on CRP risk group is one possible way of expanding our knowledge of the relationship among these three variables.
Once participants were divided into risk categories according to baseline CRP levels (low risk = CRP < 1 mg/L; average risk = CRP between 1 and 3 mg/L; high risk = CRP > 3 mg/L), a CRP by caffeine interaction was indeed found and it became apparent that CRP levels did become significantly elevated, but only in those participants whose baseline CRP levels were between 1 and 3 mg/L (average-risk). While it is not entirely clear why this effect was only observed in participants in the ‘average’ CRP risk group, it is possible that those participants in the ‘low-risk’ CRP group were protected by the low levels of this blood marker, which prevented their CRP levels from changing significantly in response to stress or caffeine. The average CRP level for participants in the ‘high-risk’ group was 6.3 mg/L, more than twice than moderate risk individuals. Therefore, it is possible that those participants in the ‘high-risk’ CRP group may have had such high basal CRP levels that they demonstrated a ceiling effect, and the addition of caffeine or stress could not increase CRP levels further.

Examination of such individual differences was not only important to clarify findings with respect to CRP, but also revealed additional interesting findings regarding fibrinogen. Without the inclusion of CRP risk group, fibrinogen levels at baseline were significantly different, with those in the no caffeine group having higher fibrinogen levels than those in the caffeine condition. Sampling error may have contributed to such baseline differences, and therefore baseline values were used as a covariate in the initial fibrinogen analyses. These initial analyses (without the inclusion of CRP risk group as an independent variable) revealed a main effect of caffeine, with participants who received caffeine displaying significantly higher levels of fibrinogen 15 minutes following the stressor than those who did not receive caffeine. The caffeine effect found in the current
study supported previous findings (Happonen, 1987) reporting an increase in fibrinogen levels in response to coffee consumption, but did not support other studies that found no change in plasma fibrinogen levels in response to coffee or caffeine consumption (Bak et al., 1990; Naismith et al., 1970). One reason why our study did not support previous studies reporting no effects of coffee or caffeine on fibrinogen levels may be due to the amount of caffeine administered. In Bak’s (1990) study, caffeine dose was the same for all individuals (200mg), while in the current study caffeine dose was adjusted for body weight. Tailoring the amount of caffeine to each individual may be a reason why significant increases in fibrinogen level were found in the current study, but not in others (Bak et al., 1990; Naismith et al., 1970). Furthermore, previous studies did not examine the effects of caffeine on fibrinogen levels in combination with stress, nor have they examined the effects of caffeine on fibrinogen levels in a population with a parental history of hypertension. These possible reasons may help to explain why the findings from the current study differ from previous reports not finding significant changes in fibrinogen level in response to caffeine. Only future studies addressing these specific areas will be able to answer these currently unanswerable questions. Additionally, a main effect of gender was found; however, this effect was in the opposite direction of that predicted, with females displaying significantly higher levels of fibrinogen than males 15 min. following the stressor. Because previous studies either did not include females or did not examine sex differences within their sample only future studies can help to shed some light into this unexpected finding.

Once CRP risk levels were included in the fibrinogen analyses, a caffeine by CRP risk group interaction was revealed. More specifically, for those in the ‘low-risk’ CRP
group, caffeine led to significant increases in fibrinogen levels in response to a stressor. These findings strongly emphasize the importance of examining individual differences. As it is demonstrated by these results, caffeine does not seem to affect all individuals equally, and in this particular case, it only appears to have a significant effect among those whose baseline CRP levels are in the low risk group. Because the current study is the first one to report such findings, more studies are necessary until we can thoroughly understand these results.

**Future Directions**

This dissertation study provided an essential examination into the relationship between the effects of caffeine on blood pressure stress reactivity and two blood markers of cardiovascular disease risk, fibrinogen and C-reactive protein. This study was the first to examine changes in C-reactive protein and fibrinogen in response to caffeine and stress, and also included women, a sample population that had been previously overlooked. Results from this study revealed important individual differences in the relationship between stress, caffeine and blood markers of cardiovascular disease. The inclusion criteria for the current study were strict and well-controlled. Future studies, however, may consider providing additional controls, such as dietary intake, exercise, and sleep, variables that were either not controlled for in the current study (sleep and exercise level), or not controlled strictly enough (dietary intake).

Previous research has found that a high fat meal leads to elevations in CRP and plasminogen activator inhibitor or PAI-1, a measure of fibrinolytic activity. For example,
elevations in CRP and PAI-1 were observed even 4 hours following a high fat meal (Carroll & Schade, 2003). Other studies (Liu et al., 2002; Liu & Willett, 2002) have found that a high fat meal is not the only dietary culprit in a typical western diet leading to elevations in CRP. Liu and colleagues (2002) found that a high glycemic load also is positively associated with rises in CRP levels in healthy middle-aged women. Those women who consumed the highest amount of high glycemic index foods (e.g. potatoes, white bread, muffins, white rice) displayed CRP levels that were nearly twice as high as those who consumed the lowest amounts. While the current study attempted to control for dietary intake by asking participants to consume a light, low-fat meal 2 hours prior to the start of the experimental session, this attempt was probably insufficient to ensure proper control of dietary intake. No attempt was made to confirm that participants followed the dietary instructions, and no data were collected on the composition of the meal. Furthermore, because participants were not asked to abstain from high glycemic index foods, this uncontrolled variable may also have contributed to confounded results. In the future, studies should not only instruct participants to abstain from high fat and high glycemic index foods, but also make use of a previously validated dietary intake measure, such as the semiquantitative food-frequency questionnaire (SFFQ) (Liu & Willett, 2002).

Another dietary component that should be examined by future studies is alcohol consumption. A growing body of literature suggests that moderate levels of alcohol consumption exert a protective effect against cardiovascular disease (e.g. Gronbaek et al., 1995; Rehm & Sembros, 1995; Simons et al., 2000). While there are several biological mechanisms proposed by which a protective effect of alcohol could be mediated (e.g. Svardsudd, 1998), the most widely accepted idea is that alcohol positively affects lipid
profile by increasing HDL cholesterol. It is known that lower HDL levels, as well higher levels of LDL and total cholesterol, are associated with increased levels of fibrinogen and C-reactive protein (Danesh et al., 2004). Therefore, it is important to closely examine this variable, as it may play a role in how fibrinogen and C-reactive protein change in response to stress and caffeine.

The relationship between physical exercise, caffeine consumption, CRP, and fibrinogen levels is another area that should be further examined in future studies. A study conducted by Okita and colleagues (2004) found that a two-month weight-loss program consisting of supervised aerobic exercises led to significant decreases in CRP levels in healthy, middle-aged women. Other investigators (D. E. King et al., 2003) examined the relationship between the type of exercise and CRP and fibrinogen levels in 4072 healthy adult males and females. Compared with those who did not exercise, significantly lower levels of inflammatory markers were found among those who engaged in jogging, swimming, cycling, aerobic dancing, calisthenics, and weight lifting, but not gardening. However, control of confounding factors such as age, race, sex, and body-mass index revealed that only participants who regularly jogged or participated in aerobic dancing remained significantly less likely to have elevated markers of cardiovascular disease risk (D. E. King et al., 2003). While a decrease in inflammatory markers of CVD appears to be a consistent finding in response to certain types of physical activity, future studies should also investigate potential connections between caffeine-related CRP and fibrinogen elevations and physical exercise. For example, future studies could use a within-subjects design and specifically screen for participants with average CRP risk levels. In order to investigate the short term effects of exercise
(which at this point have not yet been examined) on CVD risk markers in response to caffeine, blood could be collected at baseline, and every hour for 12 hours following administration of caffeine. On a different day, participants would receive caffeine but would not exercise. Other conditions could also be created by using different types of exercise and different exercise durations. Such studies could provide valuable information about lifestyle choices that may be more (or less) conducive to better cardiovascular health.

A number of studies have also reported on the effects of sleep on CRP. More specifically, reports show that individuals who suffer from sleep apnea have significantly greater CRP levels than do those who do not suffer from this condition (Shamsuzzaman et al., 2002; Yokoe et al., 2003). For example, Shamsuzzaman and colleagues (2003) studied males and females with a recent diagnosis of obstructive sleep apnea (OSA), who were otherwise healthy and had never received treatment for OSA. The OSA group was compared to a group of control participants who did not have OSA and were matched for age and BMI. C-reactive protein levels were significantly greater among those with OSA, compared to those without. While such studies indicate that disturbances in sleep are linked to elevations in CRP, a recently published study provides even stronger evidence of the relationship between sleep deprivation and elevation in CRP. Meier-Ewert and colleagues (2004) directly investigated the effects of sleep loss on CRP levels. Two experiments were performed in this study. The first one was comprised of 10 healthy adults who stayed awake for 88 consecutive hours. Blood samples were collected for CRP assessment every 90 minutes for 5 consecutive days. In the second experiment, 10 participants were randomly assigned to either 8.2 hours of sleep (control) or 4.2 hours
(partial sleep deprivation) for 10 consecutive days. Blood samples were collected every hour for the 10 day-period. Both acute total (no sleep for 88 consecutive hours) and short-term partial sleep deprivation (4.5 hours of sleep per night for 10 consecutive days) resulted in significant elevations of CRP, highlighting the importance of acquiring enough sleep. No studies to date have directly investigated whether lack of sleep plays a role in changing fibrinogen levels, although evidence based on elevations in IL-6, and activated coagulation factors XIIa, VIIa, which are fibrinogen precursors, in response to sleep deprivations would indicate that such changes would be seen (Robinson, et al., 2004). Future studies should investigate not only the direct effects of sleep deprivation on fibrinogen levels, but also whether caffeine consumption further adds to (or subtracts from) the elevations in fibrinogen and CRP levels due to lack of sleep.

Another potentially important area of investigation is that of stress duration. While the current study made use of an acute psychological stressor, the study of chronic stress would certainly add valuable information to this field of research. Given that the many individuals in today’s society experience chronic levels of stress, this area certainly deserves further assessment. Yet another important area to be further explored is how interpretation of the stressor can affect changes in blood markers of CVD in response to a stressor and caffeine. Previous research has shown that cognitive appraisal of a given stress is related to how a participant will physiologically respond to the task (Tomaka & Blascovich, 1994). Therefore, it may be the case that variations in CRP and fibrinogen will be encountered in response to caffeine and stress when this variable is considered.

The age range of participants who completed the study was 18-31 years. While it was important to conduct this research on a healthy population, it is also important that
older populations be studied, as fibrinogen and CRP levels increase with age, as well as the risk of developing cardiovascular disease. It is possible that exaggerated responses compared to the findings of this study would be found when older participants are exposed to the same research protocol. It is important to keep in mind, however, that individuals over the age of 65 years have a slightly shorter plasma caffeine half-life than individuals under the age of 25; therefore, caffeine administration should be carefully tailored to the group of interest in order to avoid confounded results. Along the same lines, it would also be important to conduct this research in participants with disease conditions, as these participants may experience short-term benefits from potential diagnostic and intervention measures that may become apparent.

Studies that target interventions also need to be examined. The current study revealed that caffeine leads to a significant increase in CRP levels in those individuals whose baseline CRP levels puts them into an average risk category. Although the mean elevation in CRP level in response to caffeine was 0.1 g/L, and, on average, this elevation was not enough to place participants on a higher CRP risk category. Consistent elevations in CRP levels in response to caffeine, over time, may be of clinical significance. For example, if individuals are engaging in activities that are known to elevate CRP levels, such as eating a high fat diet, and not engaging in other activities that are known to prevent increases in CRP, such as exercise, the addition of caffeine to this equation may further increase one’s CRP levels and, consequently, increase the risk for development of cardiovascular disease. Future studies should try to address different variables that could prevent this rise from occurring. For example, it has been found that ingestion of vitamin E or vitamin C can lead to decreases in the CRP levels. In a study conducted by Carroll
and Schade (2003), participants received a high fat test dinner, equivalent to a McDonald’s Big Mac meal. Blood was drawn before dinner and during 4 hours following the meal for assessment of CRP. Participants received the meal and 800 IU vitamin E, in addition to 1g ascorbic acid (vitamin C). Control studies were performed with no vitamins and no test meal. During the no test meal arm of the study, participants fasted from 6 to 10 PM, which is the equivalent time used for the postprandial period during the test meal arms. A significant rise in CRP levels was observed in response to the meal. However, the ingestion of vitamin C and E prevented the rises seen in CRP levels as a response to the meal (Carroll & Schade, 2003). It is important to note, however, that the current study is the first to report changes in CRP levels in response to caffeine \textit{in vivo}, therefore, it is very important that these findings be replicated before any definitive conclusions can be made.

Because the current study only found caffeine-related elevations in CRP levels in those participants whose baseline CRP levels fell between 1 and 3 mg/L, future studies could use a similar protocol as the one used in the current study, and specifically screen for participants in the ‘average-risk’ CRP category. On a different day (experimental day 2), participants would come in for an experimental session where they would all receive caffeine. Participants would be randomly assigned to receive the intervention (e.g. vitamin C, vitamin E), and blood would be drawn at the same time points as they were during the current study, in addition to extra time points extending beyond the times used in this study. Previous studies did not include measurements of CRP over an extended amount of time. Therefore, it is difficult to say with certainty that the findings from the current study accurately reflect all potential changes in these markers in response to
caffeine and stress. Therefore, it would be necessary to include additional time points which would help to ensure that changes that may take place over a prolonged period of time will be captured appropriately.

Because certain medications can affect the metabolism of caffeine, the present study did not include females who were currently taking oral contraceptives. However, the large majority of young females do make use of oral contraceptives. Previous reports indicate that the use of oral contraceptives can double caffeine half-life (Abernethy & Todd, 1985; Patwardhan et al., 1980). Future studies examining women currently taking oral contraceptives can provide important information with regard to vulnerabilities that were perhaps not captured by the current study. Furthermore, because caffeine is more slowly metabolized in women who are taking oral contraceptives, such women would be specially vulnerable to the effects of caffeine. Findings from such future studies would also have a greater generalizibility to the young female population than the current study can provide.

While the use of oral contraceptives is a variable that should be considered in studies examining the effects of caffeine on blood markers of CVD in young women, hormone replacement therapy is another variable that should be studied in women who have undergone menopause. For instance, future studies should examine differences in blood markers of CVD in response to stress and caffeine in women of a similar age group who would fall within one of two categories (1) Those taking hormone replacement therapy and (2) Those not currently taking hormone replacement therapy. Given the protective effects of estrogen against CVD (e.g. Dubey et al., 2005), such studies would be able to address whether increases in blood markers of CVD would be seen in response
to caffeine and stress once the protective effects of estrogen were no longer present. Further comparisons could be made by adding a group of young females, who could be compared to those in the HRT and no HRT groups. Such studies could provide great insight into the vulnerability of women for developing cardiovascular disease and would provide great potential for the development of better diagnostic measures and treatment of cardiovascular disease. Given that a significantly greater number of older women die of cardiovascular disease compared to men, studies that can provide us with insight into this relationship can be a tremendous asset to the field.

**Summary and Conclusion**

In summary, this dissertation study contributes to the literature on the effects of caffeine, cardiovascular reactivity to stress, and blood markers of cardiovascular disease. The current study was the first to examine the effects of caffeine and on fibrinogen and C-reactive protein. This study was also the first one to exam effects of caffeine on females with a family history of hypertension. The current study illustrated the importance of examining individual differences in response to stress and caffeine. This study also showed that while an acute stressor consistently leads to elevations in SBP, DBP, and HR, caffeine does not lead to such consistent changes. In addition, it was revealed that, based on individual differences regarding levels of baseline CRP levels, that: 1) Caffeine does not always increase fibrinogen levels, 2) changes in fibrinogen in response to an acute stressor appear to be gender-specific, and 3) caffeine significantly
increases CRP levels, but only in those whose baseline CRP levels are within 1 and 3 mg/L.

The data from this study are important and add to the literature in a number of ways: 1) the current study was the first to examine the effects of caffeine and a psychological stressor on blood levels of cardiovascular disease, 2) this study was the first to examine changes in cardiovascular disease blood markers in a population with a parental history of hypertension, and 3) was the first study to examine cardiovascular reactivity to a stressor and caffeine in females with a family history of hypertension. Finally, the relationship between caffeine, stress, and blood markers of cardiovascular disease suggests that this line of research may have implications in the prevention, diagnosis and treatment of cardiovascular disease. While the necessity for replication of the current findings is unarguable, the current study suggests that the combination of stress and caffeine may be particularly detrimental to certain individuals, depending on their baseline CRP levels. Future investigation is certainly needed.
REFERENCES


Appendix A. Telephone Screening Form
Hello, this is __________ calling from Penn State University. I am calling regarding an ongoing research project on biological and behavioral effects of caffeine that you expressed an interest in participating. Do you have a few minutes for me to tell you about the study?

YES - continue

NO. Okay. Is there a better time that I may call you to tell you more about the study?

YES. Time: __________ Date: __________

NO. Well, thank you for your time.

To help maintain confidentiality of this conversation, you may wish to use a hardwired telephone (in other words, not cordless OR A CELL PHONE) if it is available.

The purpose of this study is to understand how caffeine consumption influences blood pressure, neuroendocrine (hormone) levels, and blood markers of cardiovascular disease. The investigators will use the blood pressure, hormone, and blood marker measures to determine how people respond to challenges and caffeine. Because having a family history of hypertension (high blood pressure) is an important component of our research, whether you have a family history of hypertension is an important factor for participation in this study.

The study involves one screening session and one laboratory session. For the screening session, you will be asked to come to Penn State’s Clinical Research Center at the Noll Laboratory on campus where a blood sample will be taken to make sure that your cholesterol (fatty substance found in your blood) levels are normal. In addition, we will ask for your permission to contact your parents through a letter to verify their hypertension status. If we receive the letter back from you parents that confirms a hypertension status, and if your blood levels are normal, you will be asked to come to the General Clinical Research Center for a 3-hour laboratory session in the afternoon, Monday through Friday. The appointment will be scheduled on a day that is convenient for you.

Upon arriving at the Penn State Clinical Research Center for the study session, you will be seen by a nurse practitioner who will briefly review your health history. A research assistant will go over informed consent for your participation in the study. In addition, your height and weight will be measured.

If you agree to participate, you will be asked to fill out some questionnaires that will ask you about your moods, and daily activities. Next, a research assistant will place a blood pressure cuff on your arm and a small needle will be secured in a vein in your arm by a trained nurse in order to draw blood during
the laboratory session. You will then be asked to drink 6 ounces of grapefruit juice that contains either caffeine or no caffeine.

After a rest period, you will be asked to perform two tasks. The first task will involve preparation and delivery of a brief speech and the second task will consist of a series of arithmetic problems. Following the tasks you will be given another rest break, and then asked to complete several questionnaires. These questionnaires will ask you about your mood, your responses to challenging situations, and your social relationships.

During your time at the Research Center, you will be asked to provide 3 blood samples that we will use to measure your biochemical responses to the tasks in the study. In addition to the blood samples, we will measure your blood pressure and heart rate responses during the tasks and rest periods.

You will receive $50 for completing the laboratory session.

Do you have any questions so far?
FOR WOMEN ONLY: Before you come to the lab for the study session, we will need to determine your menstrual cycle phase. We will determine your menstrual cycle phase based on the date of your last period. We will use this information to determine the length of your menstrual cycle. Then, your laboratory session will be scheduled around your ovulation dates. Do you have any questions?

Do you think that you would like to participate in this study?

NO. Okay. Thank you for your time.

YES – continue:
I now would like to ask you a series of questions about your medical and social history to determine your eligibility for participating in the study. It should take approximately 5-10 minutes to complete this screening, and you may choose not to answer any specific questions.

IF PARTICIPANT DOES NOT HAVE TIME TO FINISH TELEPHONE QUESTIONNAIRE:

What is a good time that I may call you to determine your eligibility for participating in our study?

Time:___________ Date:___________

Thank you for your time and I’ll speak with you soon.

INTERVIEWER: Begin Health History Questionnaire on next page.
DEMOGRAPHIC INFORMATION

1. What is your date of birth?  
   Month    Day    Year  
   At least 1986  AGE=18+

2. What is your ethnic background?  
   ______ White/Caucasian  
   ______ African American  
   ______ Chinese  
   ______ Japanese  
   ______ Latino  
   ______ Other (SPECIFY)

3. Is English your primary language?  
   _____ Yes  _____ No

4. What is the highest level of education that you have attained? CIRCLE LAST GRADE COMPLETED  

   NONE  
   GRADE SCHOOL……. 1 2 3 4 5 6 7 8  
   HIGH SCHOOL………. 9 10 11 12  
   COLLEGE……………. 13 14 15 16+ (ADVANCED DEGREE)  
   9+
HEALTH

I AM GOING TO READ YOU A LIST OF HEALTH CONDITIONS. PLEASE LET ME KNOW IF A DOCTOR HAS EVER TOLD YOU THAT YOU HAVE ANY OF THEM. IT IS NOT NECESSARY FOR YOU TO INDICATE WHICH DIAGNOSIS YOU HAVE RECEIVED.

LIST ALL DISORDERS FOR CALLER -- DO NOT PROBE FOR A SPECIFIC DISORDER

1. Has a doctor ever told you that you have or had any of the following disorders:

   a. Endocrine disorders?
   b. Thyroid problems?
   c. Hypertension/High BP?
   d. Diabetes?
   e. Stroke?
   f. Heart attack?
   g. Irregular heart beat?
   h. Angina (chest pain)?
   i. Congestive heart failure?
   j. Other heart condition? (e.g., murmur, rheumatic heart disease)
   k. Shortness of breath?
   l. Pulmonary/lung disease?
   m. Asthma?
   n. Kidney/renal problems?
   o. Liver disease?
   p. Cancer?
   q. Anemia or other blood conditions?
   r. Problems with bleeding?
   s. Problems with memory?
   t. Problems with seizures?

   DID PARTICIPANT SAY YES TO ANY ITEMS? ______NO _____YES

2. a. Do you have any other chronic medical conditions? If so, please describe:

   ______________________________________________________________

   b. Do you or anyone in your family suffer from panic disorders? If so, please tell us who that person is/are

   ______________________________________________________________

3. Do you take any regular medication? ______No _____Yes

   Could you please tell me the names? ______

   ______________________________________________________________
4. What about stimulant medications used to treat Attention Deficit/Hyperactivity Disorder (ADHD) such as Adderall, Ritalin or Concerta?

   ____ No   ____ Yes

   Could you please tell me the name(s)? ____________________________________________

5. Are you sensitive to caffeine or do you try to avoid eating or drinking foods that contain caffeine?

   ____ No   ____ Yes

6. Are you taking any over-the-counter stimulant medications or supplements such as ephedrine (Sudafed, Herbal XTC, Energel) or caffeine supplements like Vivarin?

   ____ No   ____ Yes

   Could you please tell me the name(s)? ____________________________________________

7. Do you regularly drink any energy or herbal drinks during the day that contain ephedra (Ma Huang), guarana, or caffeine such as allergy or sinus teas, Red Bull beverages?

   ____ No   ____ Yes

   Could you please tell me the name(s)? ____________________________________________

8. I am going to read you a list of caffeinated beverages. Please let me know how many servings of each beverage you consume in a typical day, on average.

   a. Coffee cups: _______ if >0  # 6-8 oz mugs (ordinary mugs)______
      # 10 oz or more______
   b. Cups of tea (other than decaf or herbal):_______
   c. Cans of caffeinated cola/diet cola:_______
   d. Cans of high caffeine soda (Mt. Dew, etc.):_______
   e. Bottles of Caffeine Water/Juice (Water Joe, etc.):_______
   f. Bottles of Iced Coffee or Tea Drinks:_______
9. Would you be ok consuming grapefruit juice during a laboratory session?

   _____No  _____Yes

   NO

10. Are you taking antidepressants or medications for mood or anxiety/nervousness?

   _____No  _____Yes

   NO

   Could you please tell me the name(s)? ____________________________________________

11. What about oral or parenteral (injected) corticosteroids?

   _____No  _____Yes

   NO

   Could you please tell me the name(s)? ____________________________________________

WOMEN ONLY:

12. Are you currently pregnant or have you been pregnant within the last year?

   _____No  _____Yes

   NO

   How long ago was that? ____________

13. Are you attempting to get pregnant or do you plan to become pregnant within the next year?

   _____No  _____Yes

   NO
14. Are you currently lactating or have you breast-fed within the last year?

   _____ No  _____ Yes  NO

15. Are you a smoker or have you quit smoking within the last year?

   _____ No  _____ Yes  NO

16. Do you use other forms of tobacco such as cigars or chewing tobacco?

   _____ No  _____ Yes  NO

17. Do you use over-the-counter nicotine products such as nicotine gum or nasal spray?

   _____ No  _____ Yes  NO

18. Are you: right-handed:_______  left-handed:________ (Check one)

19. What is your present weight (in pounds):____________

20. What is your height: _______ feet _______ inches → _______ total inches

INTERVIEWER: calculate Body Mass Index from chart  BMI:_______
DEPRESSION
Now I have some questions about your feelings during the past week. For each of the following statement, please tell me if you felt that way: Rarely or none of the time; some of the time; much of the time; most or all of the time.

<table>
<thead>
<tr>
<th></th>
<th>Rarely or none of the time</th>
<th>Some of the time</th>
<th>Much of the time</th>
<th>Most or all of the time</th>
<th>R</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>I was bothered by things that usually don’t bother me.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>I did not feel like eating; my appetite was poor.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>3.</td>
<td>I felt that I could not shake off the blues even with help from my family and friends.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>4.</td>
<td>I felt that I was just as good as other people.</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>5.</td>
<td>I had trouble keeping my mind on what I was doing.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>6.</td>
<td>I felt depressed.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>7.</td>
<td>I felt that everything I did was an effort.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>8.</td>
<td>I felt hopeful about the future.</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>9.</td>
<td>I thought my life had been a failure.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
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<tr>
<td>10.</td>
<td>I felt fearful.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
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<td>11.</td>
<td>My sleep was restless.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>12.</td>
<td>I was happy.</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>13.</td>
<td>It seemed that I talked less than usual.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
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<td>14.</td>
<td>I felt lonely.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>15.</td>
<td>People were unfriendly.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
16. I enjoyed life.  
17. I had crying spells.  
18. I felt sad.  
19. I felt that people disliked me.  
20. I could not get going. 

<table>
<thead>
<tr>
<th></th>
<th>3</th>
<th>2</th>
<th>1</th>
<th>0</th>
<th>6</th>
<th>7</th>
</tr>
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<tbody>
<tr>
<td>16</td>
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<td>17</td>
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<td>20</td>
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</tbody>
</table>

TOTAL SCORE: <16
CONCLUSION OF TELEPHONE HEALTH HISTORY

For this current study, we cannot include people who have certain health conditions or are taking certain medications. Therefore, I will have one of our medical staff review the information you gave me during this telephone call. After that, someone from our research staff will get back in touch with you. In order to contact you, I will need some additional information:

What is your full name?_________________________________________________

Home Address

Street:_______________________________________________________________

City, State:_______________________________________________________

Zip Code:________________________________________________________

Email address:_______________________

Home phone:_______________________

Day/work phone:____________________

Best time and place to reach you:

Morning (8-12):_________________________

Afternoon (1-5):_______________________

Evening (7-9):_________________________

Thank you for your time.

Experimenter: Be sure to remove this page after subject is scheduled for lab session(s).
Appendix B. Parental History of Hypertension Survey (Mother and Father)
Because this study is concerned with the effects of a genetic history of hypertension (high blood pressure), this form should be completed by the biological mother only.

1. What is your age? _______

2. How long has it been since you last had your blood pressure checked by your doctor?
   - ___ 0 to 6 months
   - ___ 6 to 12 months
   - ___ 1 to 5 years
   - ___ more than 5 years
   - ___ never

3. If you know, what is your typical blood pressure now?
   systolic  _____  diastolic  _____

4. Have you *ever* been told by a doctor that you had hypertension (high blood pressure)?
   Yes  No  Don't Know
   - If yes, how old were you when you received this diagnosis? _____
   - If yes, was your high blood pressure related to pregnancy? _____

5. Has a doctor *ever* prescribed medication for you to treat hypertension (high blood pressure)?
   Yes  No  Don't Know
   - If yes, please list the medication(s):

6. Do you suffer from diabetes or kidney disease?  Yes  No  Don't Know
   - If yes, please describe: ________________________________

7. Do you suffer from any other significant health problems?  Yes  No  Don't Know
   - If yes, please describe: ________________________________

8. From the list below, please circle any of **your biological** relatives who were told by a doctor that they had hypertension (high blood pressure) before age 55:
   - Your Mother
   - Your Father
   - Your Sister(s)
   - Your Brother(s)
FATHER’S FORM

Because this study is concerned with the effects of a genetic history of hypertension (high blood pressure), this form should be completed by the biological father only.

1. What is your age? _______

2. How long has it been since you last had your blood pressure checked by your doctor?
   ____ 0 to 6 months  ____ 6 to 12 months  ____ 1 to 5 years  ____ more than 5 years  ____ never

3. If you know, what is your typical blood pressure now? ______ _______  
   systolic  diastolic

4. Have you ever been told by a doctor that you had hypertension (high blood pressure)?  
   Yes  No  Don't Know
   If yes, how old were you when you received this diagnosis? ______

5. Has a doctor ever prescribed medication for you to treat hypertension (high blood pressure)?  
   Yes  No  Don't Know
   If yes, please list the medication(s): _______________________________________________________

6. Do you suffer from diabetes or kidney disease?  
   Yes  No  Don't Know
   If yes, please describe: ________________________________________________________________

7. Do you suffer from any other significant health problems?  
   Yes  No  Don't Know
   If yes, please describe: ________________________________________________________________

8. From the list below, please circle any of your biological relatives who were told by a doctor that they had hypertension (high blood pressure) before age 55:
   Your Mother  Your Father  Your Sister(s)  Your Brother(s)
This survey was based on Household Adult Questionnaire (ages 17+ years) from the National Health and Nutrition Examination Survey (NHANES) III Data Collections Forms, US Department of Health and Human Services, Public Health Service, Centers for Disease Control, National Center for Health Statistics, Hyatville, Maryland, March 1990.
Appendix C. Caffeine Use Questionnaire
Caffeine Use Questionnaire

1. Can you tell me how much of the following caffeinated beverages you typically drink during the day?
   - Coffee__________
   - How many are 6-8 oz mugs (ordinary mugs)_______
   - How many are larger (10 oz or more)_______
   - Tea (not decaf or herbal)_______
   - Cola/Diet Cola_______
   - High Caffeine Soda (Mt. Dew, Jolt, etc.)_______
   - Caffeine Water/Juice (Water Joe, etc.)_______
   - Bottled Coffee or Iced Tea Drinks_______

2. Please indicate in the table below how many of each type of caffeinated beverage you drink at each time period throughout the day. Mark 0 if you do not consume a particular type of beverage at a particular time of day. (For example, mark 0 if you do not drink coffee in the mid afternoon.)

<table>
<thead>
<tr>
<th>Beverage</th>
<th>First thing after waking up</th>
<th>Mid morning</th>
<th>With Lunch</th>
<th>Mid Afternoon</th>
<th>With Dinner</th>
<th>Evening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Caffeine Soda</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cola/Diet Cola</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iced Tea/Coffee</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeine Water/Juice</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

3. Are you taking any over-the-counter stimulant medications or cold medications or supplements such as ephedrine (Sudafed, Herbal XTC, Energel) or caffeine supplements like Vivarin?

4. Do you regularly drink any energy or herbal drinks during the day that contain ephedra (Ma Huang), guarana, or caffeine such as allergy or sinus teas, Red Bull beverages?
Appendix D. Informed Consent Form
INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY

Title of Project: The Effects of Caffeine on Cardiovascular Risk Markers

Principal Investigator: Isabella M. Rodrigues
315 East Health and Human Development
Penn State University
University Park, PA 16802
Telephone: (814) 863-5845
Email: imr104@psu.edu

Other Investigator: Laura Cousino Klein, Ph.D.
315 East Health and Human Development
Penn State University
University Park, PA 16802
Telephone: (814)-865-8813
Email: Lklein@psu.edu

This is to certify that you, ____________________________________________, have been given the following information regarding your participation as a volunteer in a program of investigation under the supervision of Isabella M. Rodrigues and Laura C. Klein in the Department of Biobehavioral Health.

Purpose of the study:

The purpose of this study is to see how stress and caffeine affect blood markers of heart disease. Changes in hormones and blood markers of heart disease will be measured in blood samples collected during the study. We also are examining these changes in men and women to see how they compare in their responses to challenge and caffeine. These changes will provide important information about men and women’s vulnerability to stress and caffeine.

You understand that you are being asked to participate in this study because you are a healthy adult (at least 18 years old) who does not have any of the following health conditions or problems:

1. high blood pressure;
2. a history of angina (chest pain due to heart problems) or arrhythmia (fast, slow, or irregular heart beats that require medication);
3. diabetes (excessive amounts of sugar in your blood);
4. any neurological disorder (e.g., stroke), seizures, or head trauma;

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The Pennsylvania State University
Office for Research Protections
Approval Date: 11/18/04 M. Becker
Expiration Date: 06/16/05 M. Becker
Biomedical Institutional Review Board
IRB#19000

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5. smoke cigarettes;
6. depression or psychiatric problem (e.g., panic attacks, severe anxiety);
7. and sensitivity to caffeine

and you are NOT taking any if the following medications:

1. birth control medication (if you are a woman);
2. inhaled β-agonists (a specific type of asthma medication, e.g. Albuterol, Nasacort, Serevent, Foradil, Beclovent, Advair, Combivent);
3. aspirin;
4. oral or injected corticosteroids (steroid medications) within the past 3 months (e.g., Flovent, Azmacort);
5. over-the-counter stimulant medications, over-the-counter cold or flu medications, or supplements that contain ephedrine or caffeine (e.g., Vivarin, pseudophedrine);
6. cimetidine (i.e., Tagament);
7. quinolones (i.e., Cipro);
8. theophylline (a specific type of asthma medication);
9. verapamil (a specific type of medication for heart beat irregularities; e.g., Calan, Isoptin)
10. any benzodiazepine (a specific medication used to treat anxiety or as a muscle relaxant; e.g., Xanax, Ativan);

and you have not been hospitalized within the past year for psychiatric or heart problems.

**Procedures to be followed:**

You understand that if you agree to participate in this study, you will be asked to do the following things:

**Screening Laboratory Session**

You will be asked to fast for 12 hours and to come to the Penn State General Clinical Research center (GCRC) on the Penn State University campus for an initial screening prior to the laboratory session; this screening session will take approximately 30 minutes. If you decide to participate in the study, and you are eligible after the telephone screening, you will be screened during your first visit to the GCRC at Penn State to determine your cholesterol levels. During this visit, you will have your blood drawn after you have not eaten for 12 hours and the sample will be sent to a laboratory. A cholesterol and blood chemistry test (CHEM 24 blood test) will be conducted to determine your cholesterol levels, and to ensure that you are healthy. At this time, we will also ask for your permission to send a questionnaire to your parents that will indicate that you have volunteered to participate in our study, and will ask them questions about their blood pressure. You also will be instructed by a nurse on how to take your pulse. You will then be asked to take your pulse before and after your first cup of coffee or caffeinated beverage during the day of your laboratory session.

If it is found that:

- Your CHEM 24 test results are within a normal range for your age;
- Your total cholesterol levels are within a normal range for your age;
- Your LDL and HDL cholesterol levels are within a normal range for your age; and
- We have received the blood pressure questionnaire back from at least one of your parents indicating a clinical diagnosis of hypertension,

then you will be asked to participate in the laboratory session, which is explained below.
Experimental Laboratory Session

1. You will be asked to come to the Penn State General Clinical Research Center (GCRC) at Noll Laboratory for a 3-hour and 15-minute laboratory session. You will be asked to arrive at 1:00 pm and the session will end by 4:15 pm.

2. During the GCRC visit, you will be asked to do the following:
   a. You will complete a brief interview with a health care provider. During this time, the nurse practitioner will review your telephone health screening form, and will measure your weight and height. If you are a woman, you will be asked to provide us with a urine sample, which will be used to ensure that you are not pregnant.
   b. A trained nurse from the General Clinical Research Facility will place a catheter in a blood vessel in your other (non-dominant) arm in order to draw blood during the study. The first blood sample will consist of 30 ml (about 2 tablespoons), and the next two samples will consist of 30 ml each (about 4 tablespoons total). The first blood sample will be drawn before you begin the laboratory tasks, the second sample will be taken immediately after you have completed the laboratory tasks, and the last sample will be taken 60 minutes after you have completed the tasks. A total of 100 ml of blood (about 7 tablespoons) will be drawn during the study, including your screening blood draw sample.
   c. You will be asked to complete questionnaires that ask you about your mood, emotional states, and the ways that you deal with stress.
   d. You will have a standard blood pressure cuff placed on your dominant arm (the arm that you use to write). After the blood pressure cuff is placed on your arm, you will be asked to sit quietly for 30 minutes while your blood pressure and heart rate are measured.
   e. You will be asked to ingest 6 ounces of grapefruit juice that will contain either 3.3 mg/kg of caffeine or a placebo (no caffeine). You understand that whether you receive a placebo or caffeine will be by chance. Consuming 3.3 mg/kg of caffeine is equivalent to consuming approximately 2-3, 8-ounce cups of coffee.
   f. You will be asked to complete a speech task and a math task for approximately 30 minutes. You will be video-recorded during the speech task so that your responses can be coded later. You understand that this tape will be erased once it has been rated. Each task will be explained to you in more detail before you do them. Also, you will be asked to answer brief questions about your mood before and after each task. You understand that if you have any questions you may ask them at any time, and that you may stop the tasks at any time during the session.
   g. You will be provided with a 15-minute rest break after completing the tasks.
   h. The second blood sample will be drawn after this rest break.
   i. You then will be asked to answer questions about how you felt about doing the tasks and what you thought about the tasks. It will take about 60 minutes to answer these questions. Your blood pressure and heart rate will be measured during this time.
   j. The third blood sample will be drawn after you answer these questions.
k. The blood pressure cuff and needle will be removed after the last blood sample is collected.

l. You will be asked not to exercise for the rest of the day following the end of your laboratory session because if you consumed caffeine, it can elevate your blood pressure too much.

Because the validity of the results of the study could be affected if the caffeine content of the drink you consume is fully divulged to you or the investigator prior to your participation, you understand that neither you nor the investigator will know whether the drink you consume contains caffeine or not. You understand that you will have the opportunity to receive complete information about the caffeine content of the drink you swallowed following your participation in the study.

**Discomforts and risks:**

1. **Blood Drawing.** You understand that in some cases, blood drawing may produce some pain, mild bruising, dizziness or faintness, and/or swelling at the site. Very rarely, an infection or a small clot at the site will develop. An individual who is trained to draw blood will use standard clinical techniques to insert the needle and draw the blood samples. This standard procedure will reduce the risks associated with blood draws.

2. **Blood Pressure Monitoring.** You understand that there may be some discomfort from the cuff inflation of the blood pressure device on your arm.

3. **Tasks.** Potential risks and discomfort are limited to possible tiredness or stress from having to complete the requested tasks. A research assistant will be specifically trained to explain the tasks to you and will administer them in an effort to minimize any discomfort. The two tasks represent a small part of your laboratory session and for much of the rest of the time you will be free to listen to music or watch videotapes that will be made available to you.

4. **Caffeine Consumption.** You understand that caffeine is a naturally occurring substance found in coffee, sodas, and some foods like chocolate. There may be some discomfort from the caffeine including irritability, nervousness, an irregular heart beat, and an increased heart rate. To reduce potential discomfort, the highest amount of caffeine that you will be asked to consume is 3.3 mg/kg, which is equivalent to drinking 2-3, 8-ounce cups of coffee. In addition you will be asked to limit your caffeine consumption prior to your visit to the laboratory and you will be provided a snack at the end of the session.

You understand that medical care is available in the event of an injury resulting from research but that neither financial compensation nor free medical treatment is provided. You also understand that you are not waiving any rights that you might have against the University for injury resulting from negligence of the University or investigators. You understand that you can contact the Office for Research Protections, 212 Kern Graduate Building, University Park, PA 16802 (814-865-1775) if you have additional questions concerning your rights as a participant.
In the event that you experience adverse psychological reactions, you understand that you can call one of the following phone numbers for counseling: Penn State Center for Counseling & Psychological Services (221 Ritenour Building, University Park, PA 16802; 814-863-0395) or Penn State Psychological Clinic (314 Moore Building, University Park, PA 16802; 814-865-2191).

**Potential benefits:**
There is no immediate benefit to you from participating in this study, other than knowing that you have helped a research study that may lead to identifying causes of disease and illness in women and men. You will be informed of any information collected in this study that may affect your health (medical and psychological). The research that is being conducted is the first to be done with women and men in your age group.

There may be benefits to society from the research being conducted. It is hoped that results from this study will increase understanding of the effects caffeine and stress on blood markers of heart disease. The procedures that are used in this study are the only known way to collect the information that we need to answer our research questions. If we find that any of your screening laboratory lab blood test results are abnormal, you will be contacted by a nurse who will go over the results with you and recommend that you discuss them with your family physician.

**Alternative Procedures:**

A. Different sources of caffeine exist and could potentially be used in this study (i.e. Vivarin, NoDoz). Because it is important in this study to calculate specific amounts of caffeine based on your body weight, we have chosen to administer food-grade caffeine in the form of a powder which will be mixed with 6 ounces of grapefruit juice.

B. Different types of stress tasks can be used to elicit changes in blood pressure, heart rate, and hormone levels. The tasks described here and chosen for this study have been successfully used in previous study in our laboratory.

**Statement of confidentiality:**
You understand that your participation in this research is confidential. Only the investigators and their assistants will have access to your identity and to information that can be associated with your identity. In the event of publication of this research, no personally identifying information will be disclosed. All data collected from this study will be stored without identifiers, code links to your name will be kept in a separate locked cabinet. All blood samples are stored without names. The research records may be inspected by the Institutional Review Board of Penn State and/or the Office for Research Protections.

**Right to ask questions:**
You have been given an opportunity to ask any questions you may have, and all such questions or inquiries have been answered to your satisfaction.
You understand that Ms. Rodrigues and Dr. Klein, the investigators, are available to answer any questions that you may have at the time of your participation in this study or if you have questions in the future.
**Compensation:**

You understand that you will receive $50.00 for completing the laboratory session. You understand that circumstances may arise that may cause the investigator to terminate your participation before completion of the study. In the event that your participation in the study is terminated by the investigator, or you, you understand that you will be entitled to payment of $10.00 per hour for the hours that you have participated on a pro-rated basis, up to the maximum amount of $50.00 for the laboratory session.

If you are an employee of Penn State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If are not employed by Penn State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

**Voluntary participation:**

You understand that your participation in this study is voluntary, and that you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or your refusal to participate will in no way affect your care or access to medical services. You understand that you can refuse to answer any specific question during your participation in this study.

This is to certify that you consent to and give permission for your participation as a volunteer in this program of investigation. You understand that you will receive a signed copy of this consent form. You have read this form, and understand the content of this consent form.

_________________________________________________________________________  _____________________
Participant’s signature  Date

I, the undersigned, have defined and explained the studies involved to the above volunteer.

_________________________________________________________________________
Investigator’s signature  Date

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Appendix E. Questionnaires Administered During Experimental Session
1. Symptom Report Questionnaire
**Symptom Report Questionnaire 1**

For each of the following statements, please circle the number that indicates to what degree you feel these symptoms at this moment.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Not at all</th>
<th>A little bit</th>
<th>Moderately</th>
<th>Quite a bit</th>
<th>Extremely</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. My heart is pounding</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>2. I feel drowsy or sleepy</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>3. I feel restless</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4. I feel lethargic</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5. I feel hungry</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>6. I feel tense or edgy</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>7. I feel dizzy</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>8. My skin feels itchy or irritated</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>9. My fingers or hands feel numb</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>10. I feel anxious</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>11. My head aches</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>12. I feel nauseous/my stomach is upset</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>13. I am thirsty</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>14. I feel faint</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>15. I am having trouble concentrating</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
2. Pre Task Appraisal
Pre Task Appraisal

Directions: For each of the following questions, please circle the answer on the scale below the question which best describes how you feel about the upcoming task.

1. How stressful do you expect the upcoming task to be?

   1  2  3  4  5
   Not at all Moderately Very
   Stressful Stressful Stressful

2. How well do you think you will be able to cope with the upcoming task?

   1  2  3  4  5
   I cannot cope at I can cope very
   all with the task. well with the task.

3. How demanding do you expect the upcoming task to be?

   1  2  3  4  5
   Not at all Moderately Very
   Demanding Demanding Demanding

4. How threatening do you expect the upcoming task to be?

   1  2  3  4  5
   Not at all Moderately Very
   Threatening Threatening Threatening

5. How well do you think you will perform in the upcoming task?

   1  2  3  4  5
   I will not I will perform very
   perform well in well in the
   the task. task.
3. Post Task Appraisal
**Post Task Appraisal**

**Directions:** For each of the following questions, please circle the answer on the scale below the question which best describes how you feel about the task you just completed.

1. How stressful did you find the task to be?

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not at all</td>
<td>Stressful</td>
<td>Moderately Stressful</td>
<td>Very Stressful</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. How well do you think you were able to cope with the task?

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>I did not cope well with the task.</td>
<td>I coped very well with the task.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. How demanding did you find the task to be?

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not at all</td>
<td>Demanding</td>
<td>Moderately Demanding</td>
<td>Very Demanding</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4. How threatening did you find the task to be?

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not at all</td>
<td>Threatening</td>
<td>Moderately Threatening</td>
<td>Very Threatening</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. How well did you perform the task?

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>I did not perform well at all.</td>
<td>I performed very well.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Challenge Impression Questionnaire
Challenge Impressions Questionnaire

**Instructions:** For each of the following questions, please indicate your impressions of the session of tasks you just completed. Please circle the number that you think best indicates how you felt about the set of tasks as a whole. We are interested in your overall impression of the past 2-hour session.

1. **This session was:**
   
   1  
   2  
   3  
   4  
   5  

   Not very  
   difficult  
   Very  
   difficult

2. **During the session, I felt:**

   1  
   2  
   3  
   4  
   5  

   Not confident  
   at all  
   Very  
   confident

3. **During the session I:**

   1  
   2  
   3  
   4  
   5  

   Tried hard  
   Didn’t try hard

4. **During the session, I performed:**

   1  
   2  
   3  
   4  
   5  

   Very well  
   Poorly

5. **During the session, I felt:**

   1  
   2  
   3  
   4  
   5  

   Out of control  
   In control

6. **During the session, I think the experimenter’s evaluation of my performance was that I was doing:**

   1  
   2  
   3  
   4  
   5  

   Very well  
   Poorly
7. To do well on the task, it is important to be:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not at all emotional</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Very emotional</td>
</tr>
<tr>
<td>Not at all self-confident</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Very self-confident</td>
</tr>
<tr>
<td>Very submissive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Very dominant</td>
</tr>
<tr>
<td>Not at all competitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Very competitive</td>
</tr>
</tbody>
</table>

8. During the task (as compared to the rest period), I felt that: (circle best response)

<table>
<thead>
<tr>
<th></th>
<th>Decreases a lot</th>
<th>Remained the same</th>
<th>Increases a lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. My heart rate:</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>B. My blood pressure:</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>C. My hand temperature:</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>D. My face flushing:</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>E. My palms sweating:</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>F. Other bodily sensations: (Please name)</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
5. Mood Assessment Scale
MOOD ASSESSMENT SCALE

ID # ____________       Time__________
Session #_______       Date__________

Please indicate below how you are feeling at this moment. Answer quickly & honestly.

<table>
<thead>
<tr>
<th>Feeling</th>
<th>Not at all</th>
<th>Very Much</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nervous, tense, worried</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>2. Happy</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>3. Thirsty</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>4. Frustrated</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>5. Impatient</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>6. Tired, low energy</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>7. Nauseous or queasy</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>8. Angry</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>9. Sad</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>10. Hungry</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>11. Restless</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>12. Focused, attentive</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>13. Irritable</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>14. Curious, interested</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>15. Disorganized</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>16. Relaxed, comfortable</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>17. Stressed</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>18. Light-headed</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
</tbody>
</table>
6. Self-Evaluation Questionnaire (Speilberger State Anxiety Scale)
Directions: A number of statements which people have used to describe themselves are given below. Read each statement and then circle the number to the right of the statement to indicate how you feel right now, that is, at this moment. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best.

<table>
<thead>
<tr>
<th></th>
<th>1= Not At All</th>
<th>2= Somewhat</th>
<th>3= Moderately So</th>
<th>4= Very Much So</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>I feel calm.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>I feel secure.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>I am tense.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>I feel strained.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>I feel at ease.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>I feel upset.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>I am presently worrying over possible misfortunes.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>I feel satisfied.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>I feel frightened.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>I feel comfortable.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>I feel self-confident.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>I am nervous.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>I feel jittery.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>I feel indecisive.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>I am relaxed.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>I feel content.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>I am worried.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>I feel confused.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>I feel steady.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>I feel pleasant.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7. Self-Evaluation Questionnaire (Speilberger Trait Anxiety Scale)
**Self-Evaluation TRAIT Questionnaire**  
Developed by Charles D. Spielberger  
STAI Form Y-2

**Directions:** A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you *generally* feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you *generally* feel.

<table>
<thead>
<tr>
<th></th>
<th>1= Not At All</th>
<th>2= Somewhat</th>
<th>3= Moderately So</th>
<th>4= Very Much So</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>I feel pleasant.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>I feel nervous and restless.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>I feel satisfied with myself.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>I wish I could be as happy as others seem to be.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>I feel like a failure.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>I feel rested.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>I am “calm, cool, and collected.”</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>I feel that difficulties are piling up so that I cannot overcome them.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>I worry too much over something that really doesn’t matter.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>I am happy.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>I have disturbing thoughts.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>I lack self confidence.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>I feel secure.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>I make decisions easily.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>I feel inadequate.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>I am content.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>Some unimportant thought runs through my mind and bothers me.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>I take disappointments so keenly that I can’t put them out of my mind.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>I am a steady person.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>I get in a state of tension or turmoil as I think over my recent concerns and interests.</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
8. Caffeine Recognition Questionnaire
Caffeine Recognition Questionnaire

IDNUM:____________________
DATE:____________________

I believe I was given (Please circle):

1. Caffeine
2. No caffeine

I believe this because (please outline your reasons below):

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
Appendix F. Stress Protocol
“Thank you, Isabella. I’ll take it from here.”

“My name is Dr. __________. I am a psychologist and will be administering the tasks to you today. You now are going to participate in a series of challenges that include a speech task and a math task. It is important that we videotape you during these challenges so that a panel of psychologists can review the tape and evaluate your performance. I’m going to set up the camera now and make sure that is captures your face in the middle.”

(Check to make sure that participant is visible from the chest up and centered in monitor. Turn recording indicator light towards subject. Put camera back on “pause”)

**Speech Task Introduction**

The first task we would like to you to do is to prepare and deliver a 3 and ½ minute speech about a personal failure that had negative consequences on your life. You will have 10 minutes to prepare the speech. Then, you will be asked to deliver that speech in front of this camera. We will videotape your speech for later observation by a group of psychologists including myself. Do you have any questions?

➔ **ADMINISTER PRE-TASK APPRAISAL**

You now have 10 minutes to prepare your speech. Throughout this time, I will collect your blood pressure and heart rate through the cuff on your arm. I am going to leave the room to let you prepare your speech. Please press the pink button if you need me to come back in. Are you ready to begin?

You may begin preparing your speech now.

<Turn on BP monitor to collect readings every 2 minutes; mark BP printout sheet from monitor – “Speech Prep”>

<Start Timer for 10 minutes>

<Leave room>

After ten 10 minutes <continue monitoring BP>

**Speech Delivery**

Ok, Mr./Ms. _______________, it is now time for you to give your speech for the panel of psychologists. Be sure to look into the camera while you speak. You must continue speaking for the entire 3 and ½ minutes, and I will let you know when you can stop. Do you have any questions?

Ok, now I am going to turn on the camera.

Ok, begin you may begin your speech.

<sit with BP machine>

<set timer for 3 and ½ minutes>

<Mark BP printout sheet from monitor – “Speech”>
Arithmetic/Counting, Part I

"OK, Mr./Mrs. _________ the next task we want you to complete is a diagnostic test of mental ability and general intelligence. It is extremely important for you to do well, because we are going to compare your ability to the ability of other people your ages.

First, I’d like you to start with the number 9095 and then count backwards by 7's until I tell you to stop. Speed is very important. To do well, you must give the correct answer as quickly as possible. Your responses, accuracy, and timing will be recorded. I will let you know when you are not going fast enough.

To clarify, I will tell you when to start counting. If you make an error, I will correct you and you will need to begin counting again from that point. Also, you are not allowed to close your eyes during this test. Are you ready to begin?" (Begin when participant is ready.)

START TIMING

When participant makes a mistake, say: “No, that is incorrect. The last correct number was X.”

BE SURE TO CHECK OFF EACH CORRECT RESPONSE & MARK THE NUMBER SHEET WITH EACH INCORRECT RESPONSE

(After 2 minutes of counting) “You’re running out of time; you really need to speed it up.”

If participant closes eyes or looks away, say “Don’t close your eyes.”

(After 4 minutes of counting) “OK, stop now.”

Arithmetic/Counting, Part 2

“OK, Mr./Ms. _________ now let’s go on to the next task. The next test I’m going to give you is also a test of mental ability, more specifically, an IQ test. As before, it is extremely important for you to do well, because we are going to compare your ability to the ability of other people your age. Specifically, I’m going to ask you some questions involving mental arithmetic. I’ll ask the questions, and you give the answers. To do well, you must give the correct answer as quickly as possible. Also, please be sure to give a numeric answer for each question, not just “I don’t know.” Remember that speed and accuracy are important, and that I am timing and recording your responses.

Are you ready? Okay, Begin (START TIMING -- Try to average about 10 seconds per question; pause if necessary.)

(If more than 1 minute elapses for a question, say: “You are being timed; you need give an answer.”)
If answer is wrong, say “No, that is incorrect. Let’s move on to the next question.”

1. If a man buys 6 cents worth of stamps and he gives the clerk 10 cents, how much change should he get back?
   
   (4 cents) _______

2. A newsman collected 25 cents from each of 2 customers. What is the total amount he collected?
   
   (50 cents) _______

3. If you buy seven 2-cent stamps and give the clerk a half dollar, how much change should you get back?
   
   (36 cents) _______

4. A family drove 275 miles in 5 hours. What is the average speed in Miles Per Hour?
   
   (55 mph) _______

5. A family bought some second hand furniture for 2/3 of what it cost new. They paid $400 for it. How much did it cost new?
   
   ($600) _______

6. A coat that normally sells for $60 is reduced by 15% during a sale. What is the price of the coat during the sale?
   
   ($51) _______

7. If 8 machines are needed to finish a job in 6 days, how many machines would be needed to finish the job in ½ day?
   
   (96 machines) _______

STOP TIMING
Record amount of time elapsed:_____________

Arithmetic/Counting, Part 3
“Ok, it’s time to do another test of mental ability. As before, it is extremely important for you to do well. In fact, you should try to do better on this counting task than the one before because you have already had practice at it. This time I’d like you to start with the number 6,233 and then count backwards by 13’s until I tell you to stop. As you know, speed is very important. Give the correct answer as quickly as possible. Remember that I am recording your responses for accuracy, and timing. Are you ready to begin?” (Begin when participant is ready.)

START TIMING
When participant makes a mistake, say: “No, that is incorrect. The last correct number was X.”

BE SURE TO CHECK OFF EACH CORRECT RESPONSE & MARK THE NUMBER SHEET WITH EACH INCORRECT RESPONSE

(After 2 minutes of counting) “You’re running out of time; you really need to speed it up.”
If participant closes eyes or looks away, say “Don’t close your eyes.”
(After 4 minutes of counting) “OK, stop now.”

➔ ADMINISTER POST-TASK APPRAISAL

Hand participant Mood Questionnaire #2

“Thank you for your time. Please fill out this questionnaire while I get Isabella to come back into the room.”

<Mark BP printout sheet with a line>
Appendix G. Fibrinogen Assay Protocol
Matched-Pair Antibody Set
for ELISA of human Fibrinogen antigen (Fg)
Sufficient reagent for 5 x 96 well plates

Product #: FG-EIA
Lot #: XXXX
Expiry Date: XXXX

1395 Sandhill Drive, Ancaster, Ontario, Canada L9G 4V5
905-304-5696 • 800-905-5696 • fax 905-304-9897

Store at -10 to -20°C
For Research Use Only
Not for use in diagnostic procedures.

Description of Fibrinogen (Fg)
Fibrinogen is an abundant plasma protein (8-10 uM) produced in the liver. The intact protein has a molecular weight of 340 kDa and is composed of 3 pairs of disulfide-bound polypeptide chains named Aα, Bβ, and γ. Fibrinogen is a triglobular protein consisting of a central E domain and terminal D domains. Proteolysis by thrombin results in release of Fibrinopeptide A (FPA, Aα18-19) followed by Fibrinopeptide B (FPB, Bβ1-24) and the fibrin monomers that result polymerize in a half-overlap fashion to form insoluble fibrin filaments. The chains of fibrin are referred to as α, β, and γ, due to the removal of FPA and FPB. The polymernised fibrin is subsequently stabilized by the transglutaminase activated Factor XIII that forms amide linkages between γ chains and, to a lesser extent, α chains of the fibrin molecules. Proteolysis of fibrinogen by plasmin initially liberates C-terminal residues from the Aα chain to produce fragment X (intact D-D, which is still clotable). Fragment X is further degraded to non-clotable fragments Y (D-E) and D. Fragment Y can be digested into its constituent D and E fragments. Digestion of non-crosslinked fibrin with plasmin is very similar to the digestion of fibrinogen, which results in production of fragments D and E. Degradation of crosslinked fibrin by plasmin results in fragment DD (D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer consisting of D-Dimer 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Assay Procedure:

1. Coating of plates:
   Dilute the capture antibody 1/100 in coating buffer (preferably in a polypropylene tube) and immediately add 100 μl to every well in the plate. Incubate 2 hours at 22°C or overnight at 2-8°C.

2. Blocking:
   Empty contents of plate and add 150 μl of blocking buffer to every well and incubate for 60 minutes at 22°C. Wash plate X 3 with wash buffer.

3. Samples:
   Reference plasma is diluted 1/10,000 (100%) then serial 1/2 dilutions down to 1/320,000 (3.13%). Sample plasmas are diluted 1/20,000, 1/40,000 & 1/80,000. All dilutions are made in HBS-BSA-120 sample diluent. Apply 100 μl/well and incubate plate at 22°C for 60 minutes. Wash plate X 3 with wash buffer.

4. Detaching Antibody:
   Dilute the detecting antibody 1/100 in HBS-BSA-120 sample diluent and apply 100 μl to each well. Incubate plate at 22°C for 60 minutes. Wash plate X 3 with wash buffer.

5. OPD Substrate:
   Apply 100 μl of freshly prepared OPD substrate to every well. Allow colour to develop for 10-15 minutes then stop colour reaction with the addition of 50 μl/well of 2.5 M H₂SO₄. The plate can be read at a wavelength of 490 nm.

Calculation of Results:

The construction of a proper reference curve is of no less importance than any other aspect of the assay. A reference curve should be constructed by plotting the known concentration of standards versus absorbance. This can be done manually using graph paper, or by using curve-fitting computer software. In our experience, the dose response curves of most immunoassays tend to be sigmoid in shape. Although linear regions can be identified within the curve, the best overall fit is often obtained using an algorithm that provides a weighted theoretical model of fit throughout the entire curve, such as a 4-parameter or 5-parameter logistic curve fit. In general, the simplest model that defines the concentration-response relationship should be used. The backfit test is a simple and reliable method to determine if a curve-fitting method is appropriate. In this test, the apparent concentrations for the absorbance values of each standard point are read from the reference curve. The derived values are compared to the assigned values. An appropriate curve-fitting method will produce derived values that closely match assigned values throughout the range of the curve, within user-defined limits. The coefficient of determination (R²) is a valuable indicator of the overall fit, but should not be used by itself in the selection of a curve fitting method, as a poor fit in a particular region of the curve may not be evident from this value alone.

In the quality control of this product we have determined that under the conditions described above, a reference curve that is constructed using serial dilutions of normal pooled plasma, will produce a correlation coefficient (R²) of at least 0.980 using a log-log fit, and an R² of at least 0.980 using a 4-parameter logistic curve fit algorithm. However, the performance characteristics of in-house assays developed using this product in other laboratories may vary slightly from ours. Different curve fitting methods may be employed but we recommend that the backfit test be applied as evidence that the fitting method is appropriate.

Technical Notes:

- This paired antibody product is intended to facilitate the end user in establishing an inhouse immunoassay for research purposes only. It must not be used for diagnostic applications. Assay validation is the responsibility of the end user and should be done according to user-defined protocols.
- Reference calibrators should be of the same matrix and anticoagulant as the samples to be tested (example serum or plasma, citrate or EDTA).
- DO NOT use samples diluted less than 1:500, as falsely high readings may result.
- The optimal optimal development time should be determined empirically as the time required to obtain an absorbance of at least 1.200 at 490 nm for the 100% reference point, not to exceed 20 minutes.
- Rheumatoid factor in samples may interfere in ELISA by binding to the capture and/or detecting antibody.
- The wells should not be allowed to become dry. Keep plate covered or in a humid chamber during incubations.
- Antibodies are supplied in a 50% glycerol solution and can be centrifuged briefly in a micro-centrifuge to gather residual reagent from the cap and walls of the tube.

References:


Related Products:

Cat # SAFD-010 Sheep anti-human fibrinogen, whole IgG from antisera
Cat # SAFD-AP Sheep anti-human fibrinogen, affinity-purified IgG
Cat # SAFD-HRP Sheep anti-human fibrinogen, peroxidase-labelled IgG
Cat # SAFD-BSI Sheep anti-bovine fibrinogen, whole IgG from antisera
Cat # SAFD-BSAP Sheep anti-bovine fibrinogen, affinity-purified IgG
Cat # SAFD-BSHRP Sheep anti-bovine fibrinogen, peroxidase-labelled IgG
Cat # SAFD-LR Pair anti-human fibrinogen labeled with 125I
Cat # SAFD-G Sheep anti-human fibrinogen E, whole IgG from antisera
Cat # SAFD-E Sheep anti-human fibrinogen E, affinity-purified IgG
Cat # SAFD-F Sheep anti-human fibrinogen F, peroxidase-labelled IgG
Cat # SAFD-A Sheep anti-human fibrinogen A, whole IgG from antisera
Cat # SAFD-AP Sheep anti-human fibrinogen A, affinity-purified IgG
Cat # SAFD-HRP Sheep anti-human fibrinogen A, peroxidase-labelled IgG

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Appendix H: C-Reactive Protein Assay Protocol
CRP ELISA Protocol

1. Dilute 10 µL rabbit anti-human CRP Ab (DakoCytomation #A0073) in 5 mL coating buffer, and add 50 µL to each well of the ELISA plate.

2. Seal plate, place inside a humidified container, and incubate overnight at 4°C.

3. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

4. Block plate with 200 µL per well of 1% BSA/PBS.

5. Seal plate, place inside a humidified container, and incubate for at least 2 hours at room temperature.

6. Wash plate 3x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

7. Prepare standards (Calbiochem #236603) ranging from 4,000 to 3.9 ng/mL (1:1 dil.), plus blanks, by serially diluting the stock aliquot in a matrix similar to samples or in 1% BSA/PBS. Stock standards are 80 µL of 4.0 µg/mL, so dilute 40 µl from the stock vial in 40 µl of the 1% BSA/PBS solution to start the standard dilution series. Samples should be diluted 20 times.

8. Add samples and standards at 10 µL per well.

9. Seal plate, place inside a humidified container, and incubate overnight at 4°C.

10. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

11. Dilute the HRP-conjugated rabbit anti-human CRP detection Ab (DakoCytomation #P0227) 1:4000 times by adding 2.5 µL of the Ab to 10.0 mL of 1% BSA/PBS and apply 100 µL to each well of the plate.

12. Seal plate, place inside a humidified container, and incubate for 2 hours at room temperature.

13. Wash plate 6x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

14. Add 100 µl per well of the ABTS/peroxide substrate solution. (See solutions.)

15. Incubate the plate for 60 – 90 minutes at room temperature in the dark, unsealed.

16. Set the microtiter plate reader to read at a wavelength of 405 nm and read the plate 60 to 90 minutes after substrate addition.
SOLUTIONS:

PBS: 8g NaCl, 1.16g Na₂HPO₄, 0.2g KH₂PO₄, 0.2g KCl; q.s. to 1L with ddH₂O, pH 7.0

COATING BUFFER: (Ngai): 15 mM Na₂CO₃ (0.0318 g/20 mL ddH₂O), 34.8 mM NaHCO₃ (0.0585 g/20 mL ddH₂O), ph 9.6

WASH BUFFER: 0.5mL TWEEN-20 in 1L PBS

BLOCKING BUFFER: 1% BSA/PBS

SUBSTRATE: Add 150 mg 2,2’-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, Sigma) to 500 mL of 0.1 M citric acid (10.505 g per 500 mL ddH₂O, Sigma monohydrate citric acid), pH to 4.35 with NaOH. Aliquot 10 mL per vial and store at –20°C to –70°C. For the assay, thaw an aliquot of ABTS substrate about 15 minutes before use. Just before use add 10μL of 30% H₂O₂ per 10 mL of substrate and vortex.
Appendix I: Cortisol Assay Protocol
This Package Insert (IFU) is intended for Professional Use and must be read completely before product use.

I. INTENDED USE
The DSL-10-2000 ACTIVE® Cortisol Enzyme Immunoassay (EIA) Kit provides materials for the quantitative measurement of cortisol in serum or plasma. This assay is intended for in vitro diagnostic use.

II. SUMMARY AND EXPLANATION OF THE TEST
Cortisol (hydrocortisone, compound F) is the most potent glucocorticoid produced by the human adrenal cortex. As with other adrenal steroids, cortisol is synthesized from cholesterol through a series of enzymatically mediated steps [reviewed in 1,2]. The first and rate-limiting step in adrenal steroidogenesis, conversion of cholesterol to pregnenolone, is stimulated by pituitary adrenocorticotropic hormone (ACTH) which is, in turn, regulated by hypothalamic corticotropin releasing factor (CRF). ACTH and CRF secretion are inhibited by high cortisol levels. In plasma, the major portion of cortisol is bound with high affinity to corticosteroid-binding globulin (CBG, transcortin), with most of the remainder loosely bound to albumin. Cortisol acts through specific intracellular receptors and has effects in numerous physiologic systems, including immune function, glucose-counterregulation, vascular tone, substrate utilization and bone metabolism [1-3]. Cortisol is excreted primarily in urine in an unbound (free) form.

Cortisol production has an ACTH-dependent circadian rhythm with peak levels in the early morning and a nadir at night. The factors controlling this circadian rhythm are not completely defined. The circadian rhythm of ACTH/cortisol secretion matures gradually during early infancy, and is disrupted in a number of physical and psychological conditions [4]. Furthermore, increased amounts of ACTH and cortisol are secreted independently of the circadian rhythm in response to physical and psychological stress.
Elevated cortisol levels and lack of diurnal variation have been identified in patients with Cushing's disease (ACTH hypersecretion) [2,6]. Elevated circulating cortisol levels have also been identified in patients with adrenal tumors [7]. Low cortisol levels are found in primary adrenal insufficiency (e.g. adrenal hypoplasia, congenital adrenal hyperplasia, Addison's disease) and in ACTH deficiency [1,2,8,9]. Due to the normal circadian variation of cortisol levels, distinguishing normal and abnormally low cortisol levels can be difficult. Therefore, various tests to evaluate the pituitary-adrenal (ACTH-cortisol) axis, including insulin-induced hypoglycemia, short- and long-term ACTH stimulation, CRF stimulation and artificial blockage of cortisol synthesis with metyrapone have been performed [8-13]. Cortisol response characteristics for each of these procedures have been reported.

The DSL-10-2000 ACTIVE® Cortisol EIA Kit uses a specific rabbit anti-cortisol antibody, and does not require prior sample extraction of serum or plasma. Cross-reactivity to other naturally-occurring steroids is low.

III. PRINCIPLE OF THE TEST
The procedure follows the basic principle of enzyme immunoassay where there is competition between an unlabeled antigen and an enzyme-labeled antigen for a fixed number of antibody binding sites. The amount of enzyme-labeled antigen bound to the antibody is inversely proportional to the concentration of the unlabeled analyte present. Unbound materials are removed by decanting and washing the wells.

IV. REAGENTS
The DSL-10-2000 ACTIVE® Cortisol EIA Kit contains sufficient reagents for 96 wells. Each kit contains the following reagents:
A. GARG-Coated Microtitration Strips:
One stripholder, containing 96 polystyrene microtiter wells with goat anti-rabbit globulin serum. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.
B. Cortisol Antiserum: (BLUE)
One vial, 11 mL, containing rabbit anti-cortisol serum in a protein-based (BSA) buffer with a non-mercury preservative. Store at 2-8 °C until expiration date.
C. Cortisol Standards:
One vial, 0.5 mL, labeled A, containing 0 µg/dL cortisol and 7 vials, 0.5 mL each, labeled B-H, containing concentrations of approximately 0.5, 1.5, 4.0, 10.0, 20.0, 40.0, and 60.0 µg/dL cortisol in protein-based (BSA) buffer with a non-mercury preservative. Refer to vial labels for exact concentrations. Store unopened at 2-8°C until kit expiration date. Store opened vials at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiration date.
STANDARDIZATION NOTE: Due to the lack of universally accepted material, the reference preparation of the Cortisol Standards & Controls was obtained from Sigma, USA, purified by HPLC, purity verified (single spot) by Thin Layer Chromotography and performance verified by immunoassay.
D. Cortisol Controls:
Two vials, 0.5 mL each, Levels I and II, containing low and high concentrations of
cortisol in protein-based (BSA) buffer with a non-mercury preservative. Store unopened at 2-8°C until kit expiration date. Store opened vials at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiration date.

E. Cortisol Enzyme Conjugate Concentrate:
One vial, containing 0.3 mL of a solution of cortisol conjugated to horseradish peroxidase in a protein-based buffer (BSA) with a non-mercury preservative. Dilute prior to use in Conjugate Diluent. Store at 2-8°C until expiration date.

F. Conjugate Diluent:
One bottle, 11 mL, containing a protein-based buffer (BSA) with a non-mercury preservative. Store at 2-8°C until expiration date.

G. TMB Chromogen Solution:
One bottle, 11 mL, containing a solution of tetramethylbenzidine (TMB) in citrate buffer with hydrogen peroxide. Store at 2-8°C until expiration date.

H. Wash Concentrate:
One bottle, 100 mL, containing buffered saline with a nonionic detergent. Store at room temperature until expiration date. Dilute 10-fold with deionized water prior to use.

I. Stopping Solution:
One vial, 11 mL, containing 0.2M sulfuric acid. Store at 2-8°C until expiration date.

NOTE: All reagents and samples must be allowed to reach room temperature (~25°C) and mixed thoroughly by gentle inversion before use.

V. PRECAUTIONS
For in vitro use only
Not for Internal or External Use in Humans or Animals

The following Good Laboratory Practices should be observed:
• Do not eat, drink, smoke, or apply cosmetics where immunodiagnostic materials are being handled.
• Do not pipet by mouth.
• Wear lab coats and disposable gloves when handling immunodiagnostic materials.
• Wash hands thoroughly afterwards.
• Cover working area with disposable absorbent paper.
• Wipe up spills immediately and decontaminate affected surfaces.
• Avoid generation of aerosols.
• Provide adequate ventilation.
• Handle and dispose of all reagents and material in compliance with applicable regulations

WARNING: POTENTIAL BIOHAZARDOUS MATERIAL This kit may contain some reagents made with human source material (e.g. serum or plasma) or used in conjunction with human source materials. The material in this kit has been tested by FDA recommended methods and found to be non-reactive for HIV-1/2 Antibodies, HCV and HBsAg. No available test method can offer complete assurance of eliminating potential biohazardous risk. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 4th Edition, April 1999.

WARNING: POTENTIAL CHEMICAL HAZARD Some reagents in this kit contain
ProClin® as a preservative. ProClin®, TMB, hydrogen peroxide, and sulfuric acid, in concentrated amounts are irritants to skin and mucous membranes. These substances are in diluted form and therefore may minimize exposure risks significantly but not completely. Avoid contact with skin, eyes, and clothing. In case of contact with any of these reagents, wash area thoroughly with water and seek medical advice. Dispose of all nonradioactive reagents by flushing with large volumes of water to prevent buildup of chemical hazards in the plumbing system. For further information regarding hazardous substances in the kit, please refer to the component specific MSDS, either at DSLabs.com or by request.

VI. SPECIMEN COLLECTION AND PREPARATION
Serum or plasma should be used and the usual precautions for venipuncture should be observed. Serum or plasma may be stored at 2-8°C for up to 24 hours. For longer periods, store at -20°C or lower [14]. Avoid repeated freezing and thawing of samples. Because of the diurnal variation in cortisol levels, the time of collection of the specimen should be noted. Do not use grossly hemolyzed or grossly lipemic samples.

VII. PROCEDURAL NOTES
A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert. A standard curve must be included with each assay. Bring all kit reagents and specimens to room temperature (~25°C) before use. Thoroughly mix the reagents and samples before use by gentle inversion. Do not mix various lots of any kit component within an individual assay. Do not use any component beyond the expiration date shown on its label. Incomplete washing will adversely affect the outcome and assay precision. To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stopping solution into the wells in the same order and speed used to add the TMB Chromogen Solution. Avoid microbial contamination of reagents, especially of the conjugate concentrate and the conjugate diluent. Avoid contamination of the TMB Chromogen Solution with the Enzyme Conjugate. Use a clean disposable pipette tip for each reagent, Standard, Control or specimen. For dispensing sulfuric acid and TMB Chromogen Solution, avoid pipettes with metal parts. Containers and semi-automatic pipette tips used for the Enzyme Conjugate Solution and the TMB Chromogen Solution can be reused provided they are thoroughly rinsed with distilled water and dried prior to and after each usage. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use high quality water. Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.
### VIII. TEST PROCEDURE

#### A. Materials Supplied:

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>CATALOG NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSL ACTIVE® Cortisol EIA Kit, Catalog No. DSL-10-2000</td>
<td></td>
</tr>
<tr>
<td>Cortisol Standard A</td>
<td>10-2001</td>
</tr>
<tr>
<td>Cortisol Standard B</td>
<td>10-2002</td>
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<tr>
<td>Cortisol Standard C</td>
<td>10-2003</td>
</tr>
<tr>
<td>Cortisol Standard D</td>
<td>10-2004</td>
</tr>
<tr>
<td>Cortisol Standard E</td>
<td>10-2005</td>
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<tr>
<td>Cortisol Standard F</td>
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</tr>
<tr>
<td>Cortisol Standard G</td>
<td>10-2007</td>
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<tr>
<td>Cortisol Standard H</td>
<td>10-2008</td>
</tr>
<tr>
<td>GARG-Coated Microtitration Strips</td>
<td>10-3731</td>
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<tr>
<td>Control Level I</td>
<td>10-2010</td>
</tr>
<tr>
<td>Control Level II</td>
<td>10-2020</td>
</tr>
<tr>
<td>Cortisol Antiserum</td>
<td>10-2045</td>
</tr>
<tr>
<td>Enzyme Conjugate Concentrate</td>
<td>10-9755</td>
</tr>
<tr>
<td>Enzyme Conjugate Diluent</td>
<td>10-4030</td>
</tr>
<tr>
<td>Chromogen Solution</td>
<td>10-9780</td>
</tr>
<tr>
<td>Wash Concentrate</td>
<td></td>
</tr>
<tr>
<td>Stopping Solution A</td>
<td></td>
</tr>
</tbody>
</table>

#### B. Materials Required But Not Supplied:

- Microtitration plate reader capable of absorbance measurement at 450 nm and preferentially capable of dual wavelength correction at 600 or 620 nm
- Deionized water
- Precision pipette to deliver 25 µL
- Semi-automatic pipette to deliver 100 µL
- Microtitration plate shaker capable of 500-700 orbital revolutions per minute (rpm)
- Automatic microtitration plate washer
- Vortex mixer
- Absorbent materials for blotting the strips
  - Linear-log graph paper for manual data reduction

#### C. Preparation of Reagents:

1. **Wash Solution**
   - Dilute 100 mL of Wash Concentrate with 900 mL of distilled or deionized water in a suitable storage container. Store at room temperature until expiration date printed on concentrate label.

2. **Enzyme Conjugate Solution**
   - The Enzyme Conjugate Concentrate should be diluted at a ratio of 1 part Enzyme Conjugate Concentrate into 50 parts Conjugate Diluent, according to the number of
wells used. If an entire plate is to be used, pipet exactly 200 µL of the Enzyme
Conjugate Concentrate into 10 mL of the Conjugate Diluent. The Enzyme Conjugate
Solution should be prepared just prior to use.

3. Microtitration Wells
Select the number of coated wells required for the assay. The remaining unused wells
should be placed in the resealable pouch with a desiccant. The pouch must be resealed to
protect from moisture.

D. Assay Procedure:
Allow all specimens and reagents to reach room temperature (~25°C) and mix liquid
reagents thoroughly by gentle inversion before use. Standards, Controls and
unknowns should be assayed in duplicate.

1. Mark the microtitration strips to be used.
2. Prepare the Enzyme Conjugate Solution by diluting the Conjugate in the Conjugate
   Diluent as described under the Preparation of the Reagents section of this package
   insert.
3. Pipet 25 µL of each Standard, Control and unknown into the appropriate wells.
4. Add 100 µL of the Enzyme Conjugate Solution to each well using a semi-automatic
dispenser. Gently tap the well holder for 5-10 seconds.
5. Add 100 µL of the Cortisol Antiserum to each well using a semi-automatic dispenser.
6. Incubate the wells at room temperature (~25°C) on a shaker set at 500-700 rpm for
   45 minutes.
7. Aspirate and wash each well 5 times with the Wash Solution using an automatic
   microplate washer. Blot dry by inverting plate on absorbent material.
   NOTE: Use of an automatic microplate washer is strongly recommended. Incomplete
   washing will adversely affect assay precision. If a microplate washer is not available,
   (a) completely aspirate the liquid from each well, (b) dispense 0.35 mL of the Wash
   Solution into each well, and (c) repeat steps (a) and (b) five times.
8. Add 100 µL of the TMB Chromogen Solution to each well using a semi-automatic
dispenser.
9. Incubate the wells at room temperature (~25°C) for 10-15 minutes on a shaker set at
   500-700 rpm.
10. Add 100 µL of the Stopping Solution to each well using a semi-automatic dispenser.
11. Shake the plate by hand for 5-10 seconds.
12. Read the absorbance of the solution in the wells within 30 minutes, using a
    microplate reader set to 450 nm.
   NOTE: If wavelength correction is available, set the instrument to dual wavelength
   measurement at 450 nm with background wavelength correction set at 600 or 620 nm.

IX. CALCULATION OF RESULTS
A. Calculate the mean absorbance for each Standard, Control or unknown.
B. Using a linear-log graph paper, plot the mean absorbance readings for each of the
   standards along the y-axis versus the cortisol concentrations in µg/dL along the x-
   axis. Alternatively, any data reduction software designed for immunoassays could be
   used. Four-parameter curve-fit is recommended.
C. Draw the best fitting curve through the mean of the duplicate points.
D. Determine the cortisol concentrations of the Controls and unknowns from the standard
curve by matching their mean absorbance readings with the corresponding cortisol concentrations.

E. Any sample reading higher than the highest Standard should be appropriately diluted with the 0 µg/dL Standard and reassayed.

F. Multiply the value by the dilution factor if required.

If the microplate reader is not capable of reading absorbance greater than 2 or greater than the absorbance of the last Standard, a second reading at 490 or 492 nm is needed (reference filter 600 or 620 if available). In this case, proceed to construct a second standard curve as above with the absorbance readings of all standards at 490 nm. The concentration of the off-scale samples at 450 nm are then read from the new standard curve. The readings at 490 nm should not replace the on-scale readings at 450 nm.

X. LIMITATIONS

• The reagents supplied in this kit are optimized to measure cortisol levels in serum or plasma.

• Avoid repeated freezing and thawing of reagents and specimens.

• Grossly hemolyzed, icteric or lipemic specimens should be avoided.

• The results of this assay should be used in conjunction with other pertinent clinical information.

XI. QUALITY CONTROL

• The DSL Controls or other commercial controls should fall within established confidence limits. The confidence limits for the DSL Controls are lot specific and are printed on the Control vial labels.

<table>
<thead>
<tr>
<th>CONC. (µg/dL)</th>
<th>TYPICAL ACTIVE CORTISOL EIA STANDARD CURVE DATA</th>
<th>WELL NO.</th>
<th>WELL CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1,B1</td>
<td></td>
<td>STANDARDS</td>
</tr>
<tr>
<td></td>
<td>C1,D1</td>
<td></td>
<td>A B C D E F G H</td>
</tr>
<tr>
<td></td>
<td>E1,F1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G1,H1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2,B2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C2,D2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2,F2</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>G2,H2</td>
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<td></td>
</tr>
</tbody>
</table>

CAUTION: The above data must not be employed in lieu of data obtained by the user in the laboratory.
XII. EXPECTED VALUES

It is recommended that each laboratory establish its own expected ranges for cortisol. The following data were collected from 62 presumed normal adults using the DSL-10-2000 ACTIVE® Cortisol EIA Kit:

<table>
<thead>
<tr>
<th>SPECIMENS</th>
<th>N</th>
</tr>
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<tbody>
<tr>
<td>Normal Sera</td>
<td>62</td>
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</table>

<table>
<thead>
<tr>
<th>ABSOLUTE RANGE (µg/dL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Sera</td>
<td>62</td>
</tr>
</tbody>
</table>

Mean ± 1 SD (µg/dL) 12.3 ± 5.4  4.0 - 27.0

XIII. PERFORMANCE CHARACTERISTICS

All performance characteristics are stated in µg/dL. To convert to nmol/L: µg/dL x 27.6 = nmol/L

A. Sensitivity:
The theoretical sensitivity or minimum detection limit, calculated by the interpolation of the mean minus two standard deviations of ten replicates of the 0 µg/dL Cortisol Standard, is 0.1 µg/dL.

B. Precision:
The intra-assay precision was determined from the mean of 12 replicates each.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>N</th>
<th>MEAN (µg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III</td>
<td>12</td>
<td>8.4 15.9 29.2</td>
</tr>
</tbody>
</table>

The inter-assay precision was determined from the mean of average duplicates for 12 separate runs.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>N</th>
<th>MEAN (µg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III</td>
<td>12</td>
<td>5.0 11.4 15.0</td>
</tr>
</tbody>
</table>

Standard Deviation (µg/dL) 0.2 0.8 3.0

2.4 5.0 10.3

The inter-assay precision was determined from the mean of average duplicates for 12 separate runs.
### C. Recovery:

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ENDOGENOUS (µg/dL)</th>
<th>ADDED (µg/dL)</th>
<th>EXPECTED (µg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5.1</td>
<td>5.5</td>
<td>10.6</td>
</tr>
<tr>
<td>II</td>
<td>13.3</td>
<td>1.8</td>
<td>15.1</td>
</tr>
<tr>
<td>III</td>
<td>20.4</td>
<td>3.6</td>
<td>24.0</td>
</tr>
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</table>

#### OBSERVED (µg/dL)

<table>
<thead>
<tr>
<th></th>
<th>9.8</th>
<th>18.7</th>
<th>24.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>RECOVERY (%)</td>
<td>93</td>
<td>124</td>
<td>104</td>
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</table>

### D. Linearity:

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>DILUTION FACTOR</th>
<th>EXPECTED (µg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>--- 1:2 1:4</td>
<td>13.0 6.9 3.8 1.8</td>
</tr>
<tr>
<td>II</td>
<td>--- 1:2 1:4 1:8</td>
<td>27.2 12.4 5.5 3.4</td>
</tr>
</tbody>
</table>

#### OBSERVED (µg/dL)

<table>
<thead>
<tr>
<th></th>
<th>106</th>
<th>119</th>
<th>113</th>
</tr>
</thead>
<tbody>
<tr>
<td>RECOVERY (%)</td>
<td>91</td>
<td>81</td>
<td>100</td>
</tr>
</tbody>
</table>
E. Specificity: The cross-reactivity of the Cortisol Antiserum has been measured against various compounds. The percent cross-reactivity is expressed as the ratio of the cortisol concentration to the concentration of the reacting compound at 50% binding of the 0 µg/dL Cortisol Standard.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>% CROS-</th>
<th>SS-REA</th>
<th>CTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>100</td>
<td>58.3</td>
<td>10.9</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>10.9</td>
<td>7.0</td>
<td>5.7</td>
</tr>
<tr>
<td>Prednisone</td>
<td>7.0</td>
<td>1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.9</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>21-Deoxycortisol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>Progesterone</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>DHEA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ND = non-detectable (&lt;0.004%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F. Method Comparison: The DSL ACTIVE® Cortisol EIA was compared to a commercially available cortisol RIA kit (Method X) by assaying 42 serum samples ranging from 4.10 to 30.16 µg/dL (EIA). The regression analysis yielded the following equation:

\[
[\text{DSL-10-2000}] = 0.83 \times [\text{Method X}] + 3.01
\]

\[r = 0.92\]

XIV. REFERENCES
8. Leisti S, Ahonen P, Perheentupa J: The diagnosis and staging of hypocortisolism in
Appendix J: Estradiol Assay Protocol
This Package Insert (IFU) is intended for Professional Use and must be read completely before product use.

I. INTENDED USE
The DSL-10-4300 ACTIVE® Estradiol Enzyme Immunoassay (EIA) Kit provides materials for the quantitative measurement of estradiol in serum. This assay is intended for in vitro diagnostic use.

II. PRINCIPLE OF THE TEST
Estradiol [1,3,5(10)-Estratrien-3,17β-diol; 17β-estradiol; E2], a C18 steroid, is the most potent naturally secreted estrogen and is the major estrogen produced by the ovary [1]. In the ovary, estradiol is produced by demethylation and aromatization of testosterone [1,2]. Ovarian estradiol is also produced from estrone [3-Hydroxy-1,3,5(10)-estratrien-17-one; E1], a less potent estrogen derived from androstenedione. Estrone and estradiol are interconverted in many body tissues. In men, small amounts of estradiol are produced in the testes and from peripheral conversion of androgens. Circulating levels of estradiol increase during fetal life, are relatively high at term in both sexes, decrease rapidly postnatally, show a small increase in early infancy, and are low in prepubertal children [3]. Circulating estradiol levels increase gradually during puberty in both sexes, although the absolute levels are higher in females [3]. In adult premenopausal women, ovarian estradiol production is stimulated by the interactions of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) throughout the menstrual cycle. The increasing estradiol levels during the follicular phase of the menstrual cycle appear to enhance gonadotropin stimulation, leading to a midcycle gonadotropin surge and subsequent ovulation [1]. Increased estradiol (and progesterone) levels during the luteal phase inhibit gonadotropin secretion. In adult men and postmenopausal women, estradiol production is low and estrone is the major estrogen in the circulation. Most of the circulating estradiol is bound to either sex hormone-binding globulin (SHBG) [4] or, with lower affinity, to albumin. The small amounts of free and
dissociable estradiol have diverse biological actions mediated by binding to specific intracellular receptors. The biological actions of estradiol include stimulation of linear bone growth, acceleration of epiphyseal closure, stimulation of mammary development, and maturation of the vaginal mucosa and uterine endometrium. Estradiol may also contribute to the development of the female (gynoid) body habitus, and may have metabolic and behavioral effects [1]. In female isosexual precocious puberty, high estradiol levels with low gonadotropin levels are observed in cases of ovarian cysts and tumors, and in gonadotropin-independent precocious puberty (McCune-Albright syndrome) [5], while elevated estradiol and gonadotropin levels are consistent with central precocious puberty. In males, increased estradiol production may be observed in certain tumors, especially of the testes, although circulating estradiol levels may be only minimally elevated [6]. In adult women, estradiol levels are measured in the evaluation of fertility and menstrual irregularities, and to monitor ovarian follicular function during induction of ovulation (e.g. as part of an in vitro fertilization procedure) [7]. Many immunoassay and bioassay methods have been used to quantify estrogens. The DSL Estradiol EIA Kit uses an antibody with high affinity for estradiol and low cross-reactivity to other naturally-occurring estrogens.

III. PRINCIPLE OF THE TEST

The DSL-10-4300 ACTIVE Estradiol EIA Kit uses the competitive binding enzyme immunoassay format. In the assay, Standards, Controls and unknowns containing estradiol are incubated with biotin-labeled estradiol and rabbit anti-estradiol antiserum in microtitration wells where the unlabeled and biotin-labeled antigens compete for a limited number of anti-estradiol binding sites. After incubation and washing, the wells are incubated with streptavidin-horseradish peroxidase (HRPO), which binds to the biotinylated estradiol. The unbound streptavidin-HRPO is washed, followed by incubation with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm.

IV. REAGENTS

The DSL-10-4300 ACTIVE Estradiol EIA Kit contains sufficient reagents for 96 wells. Each kit contains the following reagents:
A. Anti-Estradiol-Coated Microtitration Strips:
One strip holder containing 96 microtitration wells coated with rabbit anti-estradiol IgG immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.
B. Estradiol Standards:
One vial, 3.0 mL, labeled A, containing 0 pg/mL, and six vials, 1.0 mL each, labeled B-G, containing concentrations of approximately 20, 50, 250, 750, 2000 and 6000 pg/mL (0.07 - 22 nmol/L) estradiol in human serum with a non-mercury preservative. Refer to vial labels for exact concentrations. Store unopened at 2-8°C until expiration date. Store opened vials at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiration date.

Note: The reference preparation used for preparing the DSL Estradiol Standards was obtained from Steraloids, Inc., USA, and was quantified by Thin Layer Chromatography.
C. Estradiol Controls:
Two vials, 1.0 mL each, Levels I and II, containing low and high concentrations of estradiol in human serum with a non-mercury preservative. Refer to Control vial labels for ranges. Store unopened at 2-8°C until expiration date. Store opened vials at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiration date.

D. Estradiol-Biotin Conjugate Concentrate:
One vial, 0.3 mL, containing biotinylated estradiol in a protein-based (BSA) buffer with a non-mercury preservative. Dilute prior to use with the Estradiol-Biotin Conjugate Diluent. Store at 2-8°C until expiration date.

NOTE: The dilution of this reagent should be made just prior to use.

E. Estradiol-Biotin Conjugate Diluent:
One bottle, 11 mL, containing a protein-based (BSA) buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

F. Streptavidin-Enzyme Conjugate Concentrate:
One amber vial, 0.6 mL, containing streptavidin-HRPO concentrate in a protein-based (BSA) buffer and a non-mercury preservative. Dilute 5 - 10 minutes prior to use with the Streptavidin-Enzyme Conjugate Diluent. Store at 2-8°C until expiration date.

G. Streptavidin-Enzyme Conjugate Diluent:
One bottle, 22 mL, containing protein-based (BSA) buffer with EDTA. Store unopened or opened and tightly covered at 2-8°C until expiration date.

H. TMB Chromogen Solution:
One amber bottle, 11 mL, containing a solution of tetramethylbenzidine (TMB) in citrate buffer with hydrogen peroxide. Store at 2-8°C until expiration date.

I. Wash Concentrate:
One bottle, 60 mL, containing buffered saline with a nonionic detergent. Dilute 25-fold with deionized water prior to use. Store room temperature (~25°C) or at 2-8°C until expiration date.

J. Stopping Solution:
One bottle, 11 mL, containing 0.2M sulfuric acid. Store at 2-8°C until expiration date.

NOTE: All reagents and samples must be allowed to reach room temperature (~25°C) and mixed thoroughly by gentle inversion before use.

V. PRECAUTIONS
For in vitro use only
Not for Internal or External Use in Humans or Animals
The following Good Laboratory Practices should be observed:
• Do not eat, drink, smoke, or apply cosmetics where immunodiagnostic materials are being handled.
• Do not pipet by mouth.
• Wear lab coats and disposable gloves when handling immunodiagnostic materials.
• Wash hands thoroughly afterwards.
• Cover working area with disposable absorbent paper.
• Wipe up spills immediately and decontaminate affected surfaces.
• Avoid generation of aerosols.
• Provide adequate ventilation.
• Handle and dispose of all reagents and material in compliance with applicable regulations.

**WARNING: POTENTIAL BIOHAZARDOUS MATERIAL** This kit may contain some reagents made with human source material (e.g. serum or plasma) or used in conjunction with human source materials. The material in this kit has been tested by FDA recommended methods and found to be non-reactive for HIV-1/2 Antibodies, HCV and HBsAg. No available test method can offer complete assurance of eliminating potential biohazardous risk. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 4th Edition, April 1999.

**WARNING: POTENTIAL CHEMICAL HAZARD** Some reagents in this kit contain ProClin® as a preservative. ProClin®, TMB, hydrogen peroxide, and sulfuric acid, in concentrated amounts are irritants to skin and mucous membranes. These substances are in diluted form and therefore may minimize exposure risks significantly but not completely. Provide adequate ventilation. Avoid contact with skin, eyes, and clothing. In case of contact with any of these reagents, wash area thoroughly with water and seek medical advice.

Dispose of all nonradioactive reagents by flushing with large volumes of water to prevent buildup of chemical hazards in the plumbing system. For further information regarding hazardous substances in the kit, please refer to the component specific MSDS, either at DSLabs.com or by request.

**VI. SPECIMEN COLLECTION AND PREPARATION**

Serum should be used and the usual precautions for venipuncture should be observed. Specimens may be stored in glass tubes at 2-8°C for up to 24 hours and may be frozen at -20°C or lower for up to two months [8]. Do not use hemolyzed or lipemic specimens. Avoid repeated freezing and thawing of samples.

**VII. PROCEDURAL NOTES**

A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert. A standard curve must be included with each assay. Bring all kit reagents and specimens to room temperature (~25°C) before use. Thoroughly mix the reagents and samples before use by gentle Inversion. Do not mix various lots of any kit component within an individual assay. Do not use any component beyond the expiration date shown on its label. Incomplete washing will adversely affect the outcome and assay precision. To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stopping solution into the wells in the same order and speed used to add the TMB Chromogen Solution. Avoid microbial contamination of reagents, especially of the conjugate concentrate and the conjugate diluent. Avoid contamination of the TMB Chromogen Solution with the Enzyme Conjugate. Use a clean disposable pipette tip for each reagent, Standard, Control or specimen. For dispensing sulfuric acid and TMB Chromogen Solution, avoid pipettes with metal parts. Containers and semi-
automatic pipette tips used for the Enzyme Conjugate Solution and the TMB Chromogen Solution can be reused provided they are thoroughly rinsed with distilled water and dried prior to and after each usage.

The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use high quality water. Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.

VIII. TEST PROCEDURE

<table>
<thead>
<tr>
<th>A. Materials Supplied:</th>
<th>Materials supplied in the DSL ACTIVE Estradiol EIA Kit, Catalog No. DSL-10-4300:</th>
<th>MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATALOG NO.</td>
<td></td>
<td>Estradiol Standard A</td>
</tr>
<tr>
<td>4401</td>
<td></td>
<td>Estradiol Standard B</td>
</tr>
<tr>
<td>4402</td>
<td></td>
<td>Estradiol Standard C</td>
</tr>
<tr>
<td>4403</td>
<td></td>
<td>Estradiol Standard D</td>
</tr>
<tr>
<td>4404</td>
<td></td>
<td>Estradiol Standard E</td>
</tr>
<tr>
<td>4405</td>
<td></td>
<td>Estradiol Standard F</td>
</tr>
<tr>
<td>4406</td>
<td></td>
<td>Estradiol Standard G</td>
</tr>
<tr>
<td>4407</td>
<td></td>
<td>Anti-Estradiol-Coated Microtitration Strips</td>
</tr>
<tr>
<td>10-4310</td>
<td></td>
<td>Estradiol Biotin Conjugate Concentrate</td>
</tr>
<tr>
<td>10-4320</td>
<td></td>
<td>Streptavidin-Enzyme Conjugate Concentrate</td>
</tr>
<tr>
<td>10-4325</td>
<td></td>
<td>Estradiol-Biotin Conjugate Diluent</td>
</tr>
<tr>
<td>10-4340</td>
<td></td>
<td>Streptavidin-Enzyme Conjugate Diluent</td>
</tr>
</tbody>
</table>
B. Materials Required but not Supplied:
• Microtitration plate reader capable of absorbance measurement at 450 nm and preferentially capable of dual wavelength correction at 600 or 620 nm
• Deionized water
• Precision pipette to deliver 50 μL
• Semi-automatic pipette to deliver 100 μL and 200 μL
• Microtitration plate shaker capable of 500 - 700 orbital revolutions per minute (rpm)
• Automatic microtitration plate washer
• Vortex mixer
• Absorbent materials for blotting the strips
• Linear-log graph paper for manual data reduction

C. Preparation of Reagents:
1. Wash Solution
Pour 60 mL of the Wash Concentrate into a clean container and dilute by adding 1500 mL of deionized water. The Wash Solution is stable for one month at room temperature (~25 °C) when stored in a tightly sealed bottle.

2. Estradiol-Biotin Conjugate Solution
The Estradiol-Biotin Conjugate Concentrate should be diluted at a ratio of 1 part into 50 parts of the Estradiol-Biotin Conjugate Diluent according to the number of wells used. If an entire plate is to be used, pipet exactly 220 μL of the Estradiol-Biotin Conjugate Concentrate in 11 mL of the Estradiol-Biotin Conjugate Diluent.

3. Streptavidin-Enzyme Solution
The Streptavidin-Enzyme Conjugate Concentrate should be diluted at a ratio of 1 part into 50 parts of the Streptavidin-Enzyme Conjugate Diluent, according to the number of wells used. If an entire plate is to be used, pipet exactly 420 μL of the Streptavidin-Enzyme Conjugate Concentrate in 21 mL of the Streptavidin-Enzyme Conjugate Diluent.

NOTE: The Streptavidin-Enzyme Conjugate Concentrate must be diluted immediately prior to use in the assay.

4. Microtitration Wells
Select the number of coated wells required for the assay. The remaining unused wells
should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

D. Assay Procedure:
Allow all specimens and reagents to reach room temperature (~25°C) before use.

Standards, Controls and unknowns should be assayed in duplicate.

1. Mark the microtitration strips to be used.
2. Pipet 50 µL of each Standard, Control and unknown to the appropriate wells.
3. Prepare the Estradiol-Biotin Conjugate Solution as described in the Preparation of the Reagents section of this package insert.
4. Add 100 µL of the Estradiol-Biotin Conjugate Solution to each well using a semi-automatic dispenser.
5. Incubate the wells, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, for 1 hour at room temperature (~25°C).
6. Aspirate and wash each well five times with the Wash Solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
   NOTE: Use of an automatic microplate washer is strongly recommended. Incomplete washing will adversely affect assay precision. If a microplate washer is not available, (a) completely aspirate the liquid from each well, (b) dispense 0.35 mL of the Wash Solution into each well, and (c) repeat steps (a) and (b) five times.
7. Dilute the Streptavidin-Enzyme Conjugate Solution as directed in the Preparation of the Reagents section of this package insert.
8. Add 200 µL of the Streptavidin-Enzyme Conjugate Solution (prepared in Step 7) to each well using a semi-automatic dispenser.
9. Incubate the well, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, for 30 minutes at room temperature (~25°C).
10. Aspirate and wash each well five times with the Wash Solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
   NOTE: Use of an automatic microplate washer is strongly recommended. Incomplete washing will adversely affect assay precision. If a microplate washer is not available, (a) completely aspirate the liquid from each well, (b) dispense 0.35 mL of the Wash Solution into each well, and (c) repeat steps (a) and (b) five times.
11. Add 100 µL of the TMB Chromogen Solution to each well using a semi-automatic dispenser.
12. Incubate the wells, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, for 30 minutes at room temperature (~25°C). Avoid exposure to direct sunlight.
   NOTE: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.
13. Add 100 µL of the Stopping Solution to each well using a semi-automatic dispenser.
14. Read the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450 nm.
   NOTE: If wavelength correction is available, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 600 or 620 nm.

IX. RESULTS
A. Calculate the mean absorbance for each Standard, Control or unknown.
B. Using a linear-log graph paper, plot the estradiol concentration in pg/mL along the x-axis (log) versus the mean absorbance readings for each of the standards along the y-axis (linear). Alternatively, any data reduction software designed for immunoassays could be used. Four-parameter curve-fit is recommended.
C. Draw the best fitting curve through the mean of the duplicate points.
D. Determine the estradiol concentrations of the Controls and unknowns from the standard curve by matching their mean absorbance readings with the corresponding estradiol concentrations.

If the absorbance readings exceed the limitation of the plate reader, a second reading at 405 nm is needed (reference filter 600 or 620 if available). In this case, proceed to construct a second standard curve as above with the absorbance readings of all Standards at 405 nm. The concentrations of the off-scale samples at 450 nm are then read from the new standard curve. The readings at 405 nm should not replace the on-scale readings at 450 nm.

X. LIMITATIONS
• The reagents supplied in this kit are optimized to measure estradiol levels in serum.
• Avoid repeated freezing and thawing of reagents and specimens.
• Hemolyzed, icteric or lipemic specimens should be avoided.

XI. QUALITY CONTROL
• The DSL Controls or other commercial controls should fall within established confidence limits. The confidence limits for the DSL controls are printed on the Control vial labels. Low and high level controls should be run with each assay.

<table>
<thead>
<tr>
<th>WELL NO.</th>
<th>CONC. (pg/mL)</th>
<th>ACTIVE ESTRADIOL EIA STANDARD CURVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1, F2</td>
<td>A1, A2</td>
<td>STANDARDS</td>
</tr>
<tr>
<td>G1, G2</td>
<td>B1, B2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C1, C2</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>D1, D2</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>E1, E2</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WELL CONTENTS</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.179</td>
<td>2.080</td>
</tr>
<tr>
<td></td>
<td>2.080</td>
<td>1.868</td>
</tr>
<tr>
<td></td>
<td>1.868</td>
<td>1.101</td>
</tr>
<tr>
<td></td>
<td>1.101</td>
<td>0.530</td>
</tr>
<tr>
<td></td>
<td>0.530</td>
<td>0.233</td>
</tr>
<tr>
<td></td>
<td>0.233</td>
<td>0.123</td>
</tr>
</tbody>
</table>

MEAN ABSORBANCE
2000
6000
CAUTION: The above data must not be employed in lieu of data obtained by the user in the laboratory.
XII. EXPECTED VALUES

It is recommended that each laboratory establish its own normal ranges for estradiol. An expected range study was performed using the DSL-4400 Estradiol RIA kit. Serum samples from seven normally ovulating women were analyzed during the follicular phase and luteal phase of a menstrual cycle. Thirty-three serum samples from women receiving ovulation induction treatment or in their first trimester of pregnancy were analyzed. Seventeen serum samples from apparently healthy adult males were also analyzed.

<table>
<thead>
<tr>
<th>MEDIAN (pg/mL)</th>
<th>GROUP</th>
<th>N</th>
<th>MEAN ± 1SD (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females: Follicular Phase</td>
<td>7</td>
<td>143.7 ± 92.6</td>
<td></td>
</tr>
<tr>
<td>Luteal Phase</td>
<td>7</td>
<td>173.7 ± 121.1</td>
<td></td>
</tr>
<tr>
<td>Ovulation Induction/ 1st Trimester</td>
<td>33</td>
<td>1068.7 ± 953.2</td>
<td></td>
</tr>
<tr>
<td>Males:</td>
<td>17</td>
<td>40.2 ± 14.5</td>
<td></td>
</tr>
</tbody>
</table>

ABSOLUTE RANGE (pg/mL)
115.0 112.3 763.9 39.3

XIII. PERFORMANCE CHARACTERISTICS
All performance characteristics are stated in pg/mL. To convert to nmol/L: pg/mL x 0.0037 = nmol/L

A. Sensitivity:
The theoretical sensitivity, or minimum detection limit, as calculated by interpolation of the mean minus two standard deviations of 8 replicates of the 0 pg/mL Estradiol EIA Standard, is 7 pg/mL.

<table>
<thead>
<tr>
<th>B. Precision:</th>
<th>SAMPLE</th>
<th>N</th>
<th>MEAN (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The intra-assay precision was determined from the mean of 8 replicates each.</td>
<td>I II III</td>
<td>8 8 8</td>
<td>104.2 282.6 1035.8</td>
</tr>
</tbody>
</table>
The inter-assay precision was determined from the mean of average duplicates for 11 separate runs.

### STANDARD DEVIATION (pg/mL)

<table>
<thead>
<tr>
<th>Mean (pg/mL)</th>
<th>5.0</th>
<th>11.9</th>
<th>33.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.8</td>
<td>4.2</td>
<td>3.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>N</th>
<th>MEAN (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III</td>
<td>11</td>
<td>92.5</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>257.3</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1031.3</td>
</tr>
</tbody>
</table>

### STANDARD DEVIATION (pg/mL)

<table>
<thead>
<tr>
<th>Mean (pg/mL)</th>
<th>6.0</th>
<th>21.0</th>
<th>71.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.5</td>
<td>8.2</td>
<td>7.0</td>
</tr>
</tbody>
</table>

### C. Recovery:

Three serum samples containing different levels of endogenous estradiol were spiked with different amounts of estradiol and assayed.

<table>
<thead>
<tr>
<th>ENDOGENOUS (pg/mL)</th>
<th>ADDED (pg/mL)</th>
<th>EXPECTED (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>307 335.5 362.1</td>
<td>75 200 600</td>
<td>377 502 902</td>
</tr>
<tr>
<td>200 352 477 877</td>
<td>401 526 926</td>
<td>347 462 835</td>
</tr>
<tr>
<td>600</td>
<td>335 440 815</td>
<td>379 475 847</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 92 93</td>
</tr>
</tbody>
</table>

OBSERVED (pg/mL)

| 92 92 93 |
| 95 90 91 |
### D. Linearity:

Three serum samples were diluted with 0 pg/mL ACTIVE® ESTRADIOL EIA Standard and assayed.

<table>
<thead>
<tr>
<th>RECOVERY (%)</th>
<th>SAMPLE DILUTION FACTOR</th>
<th>EXPECTED (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>---</td>
<td>1:2 1:4 1:8 1:16 1:32</td>
</tr>
<tr>
<td>II</td>
<td>---</td>
<td>1:2 1:4 1:8 1:16 1:32</td>
</tr>
</tbody>
</table>

### E. Specificity:

The cross-reactivity of the Estradiol antiserum has been measured against the following compounds:

<table>
<thead>
<tr>
<th>% CROSS-REACTIVITY</th>
<th>Estrone Sulfate</th>
<th>Estrone Glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA-S</td>
<td>ND</td>
<td>2.73</td>
</tr>
<tr>
<td>Equilinin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Equilin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND = Non-detectable = <0.01%

### F. Method Comparison:

The DSL ACTIVE® Estradiol EIA has been compared to another commercially available Estradiol kit [Method x]. Forty-one human serum samples were assayed and linear regression analysis of the results yielded the following:

\[
\text{n} = 41
\]

\[
[\text{DSL-10-4300}] = 0.82 \times [\text{Method x}] + 35.7
\]
R = 0.98

XIV. REFERENCES
Appendix K: Progesterone Assay Protocol
I. INTENDED USE

The DSL-10-3900 ACTIVE® Progesterone Enzyme Immunoassay (EIA) Kit provides materials for the quantitative measurement of progesterone in serum or plasma. This assay is intended for in vitro diagnostic use.

II. SUMMARY AND EXPLANATION OF THE TEST

Progesterone (4-Pregnene-3,20-dione) is a steroid produced in the adrenal glands, gonads and placenta. As with other steroids, progesterone is synthesized from cholesterol via a series of enzyme-mediated steps [1,2]. The first step is mediated by the enzyme P450scc and involves conversion of cholesterol to pregnenolone. This step is stimulated by adrenocorticotropic hormone (ACTH) in the adrenal gland and by gonadotropins in the ovary and placenta [1]. Pregnenolone is then converted to either progesterone (mediated by 3β-hydroxysteroid dehydrogenase) or 17α-hydroxypregnenolone (mediated by 17α-hydroxylase or P450c17). Both progesterone and 17-hydroxypregnenolone can then be converted to 17-hydroxyprogesterone, which serves as a precursor for both glucocorticoid and sex steroid synthesis. Specific sites of progesterone synthesis include the adrenal cortex, the corpus luteum, the testes and the placenta. Adrenal and testicular production of progesterone is almost immediately shunted into other steroidogenic pathways and a small amount is released into the blood. Progesterone produced by the corpus luteum and, during pregnancy, the placenta, serves several independent physiologic functions related to its ability to convert estrogen-primed uterine endometrium into secretory endometrium [3]. These functions include preparation of the uterine endometrium for implantation of the fertilized oocyte and maintenance of the endometrium during pregnancy. Progesterone has multiple other actions in female reproductive physiology, including participation in the feedback regulation of pituitary gonadotropins and ovarian estrogens, stimulation of mammary gland maturation during
lactation, maintenance of the vaginal epithelium, uterine smooth muscle relaxation during embryogenesis and stimulation of basal body temperature. Serum levels of progesterone are relatively high at birth due to placental production, fall rapidly during the first postnatal week and rise during puberty [4]. Serum progesterone levels in adult men and postmenopausal women are similar to levels in women during the follicular phase of the menstrual cycle and reflect adrenal progesterone production. Serum progesterone levels are relatively high in women during the luteal phase of the menstrual cycle and during pregnancy due to gonadotropin stimulation. Measurement of serum progesterone concentrations have been used in evaluating ovarian function [3,5]. The DSL Progesterone EIA kit utilizes an antiserum that is highly specific for progesterone. Cross-reactivity to other naturally-occurring steroids is low and not significant for most biological samples [6-7].

III. PRINCIPLE OF THE TEST
The procedure follows the basic principle of enzyme immunoassay where there is competition between an unlabeled antigen and an enzyme-labeled antigen for a fixed number of antibody binding sites. The amount of enzyme-labeled antigen bound to the antibody is inversely proportional to the concentration of the unlabeled analyte present. Unbound materials are removed by decanting and washing the wells.

IV. REAGENTS SUPPLIED
A. GARG-Coated Microtitration Strips:
One stripholder, containing 96 polystyrene microtitration wells with goat anti-rabbit IgG immobilized to the inside wall of each well. Store at 2-30°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

B. Progesterone Standards:
One vial, 1 mL, labeled A, containing 0 ng/mL, and five vials, 0.5 mL each, labeled B-F, containing concentrations of approximately 0.3, 1.3, 7.5, 30, 80 ng/mL in human serum with a non-mercury preservative. Refer to vial labels for exact concentrations. Store unopened at 2-8°C until kit expiration date. Store opened vials at 2-8°C for up to three weeks. For longer periods, store at -20°C or lower until expiration date.

STANDARDIZATION NOTE: Due to the lack of universally accepted material, the reference preparation of the Progesterone Standards & Controls was obtained from Sigma, USA, purified by HPLC, purity verified (single spot) by Thin Layer Chromatography and performance verified by immunoassay.

C. Progesterone Controls:
Two vials, 0.5 mL each, Levels I and II, containing low and high concentrations of progesterone in human serum with a non-mercury preservative. Refer to vial labels for exact concentrations. Store unopened at 2-8°C until kit expiration date. Store opened vials at 2-8°C for up to three weeks. For longer periods, store at -20°C or lower until expiration date.

D. Progesterone Antiserum:
One vial, 11 mL, containing rabbit anti-progesterone serum in a protein (BSA) based buffer with a non-mercury preservative. Store at 2-8 °C until expiration date.

E. Progesterone Enzyme Conjugate Concentrate:
One vial, containing 0.3 mL of a solution of progesterone conjugated to horseradish peroxidase (HRPO) in buffer with a non-mercury preservative. Dilute 1:50, just prior to use, in Progesterone Conjugate Diluent. Store at 2-8°C until expiration date.

NOTE: The dilution of this reagent should be made prior to each run.

F. Conjugate Diluent D:
One vial, 11 mL, containing a buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

G. TMB Chromogen Solution:
One vial, 11 mL, containing a solution of tetramethylbenzidine (TMB) in citrate buffer with hydrogen peroxide. Store at 2-8°C until expiration date.

H. Wash Concentrate:
One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at room temperature (~25°C). Dilute 25-fold with deionized water prior to use.

I. Stopping Solution:
One vial, 11 mL, containing 0.2 M sulfuric acid. Store at 2-8°C until expiration date.

NOTE: All reagents and samples must be allowed to reach room temperature (~25°C) and mixed thoroughly by gentle inversion before use.

V. PRECAUTIONS

For in vitro use only

Not for Internal or External Use in Humans or Animals

The following Good Laboratory Practices should be observed:

• Do not eat, drink, smoke, or apply cosmetics where immunodiagnostic materials are being handled.
• Do not pipet by mouth.
• Wear lab coats and disposable gloves when handling immunodiagnostic materials.
• Wash hands thoroughly afterwards.
• Cover working area with disposable absorbent paper.
• Wipe up spills immediately and decontaminate affected surfaces.
• Avoid generation of aerosols.
• Provide adequate ventilation.
• Handle and dispose of all reagents and material in compliance with applicable regulations.

WARNING: POTENTIAL BIOHAZARDOUS MATERIAL This kit may contain some reagents made with human source material (e.g. serum or plasma) or used in conjunction with human source materials. The material in this kit has been tested by FDA recommended methods and found to be non-reactive for HIV-1/2 Antibodies, HCV and HBsAg. No available test method can offer complete assurance of eliminating potential biohazardous risk. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 4th Edition, April 1999.

WARNING: POTENTIAL CHEMICAL HAZARD Some reagents in this kit contain
ProClin® as a preservative. ProClin®, TMB, hydrogen peroxide, and sulfuric acid, in concentrated amounts are irritants to skin and mucous membranes. These substances are in diluted form and therefore may minimize exposure risks significantly but not completely. Provide adequate ventilation. Avoid contact with skin, eyes, and clothing. In case of contact with any of these reagents, wash area thoroughly with water and seek medical advice. Dispose of all nonradioactive reagents by flushing with large volumes of water to prevent buildup of chemical hazards in the plumbing system. For further information regarding hazardous substances in the kit, please refer to the component specific MSDS, either at DSLabs.com or by request.

VI. SPECIMEN COLLECTION AND PREPARATION
Serum or plasma should be used and the usual precautions for venipuncture should be observed. The serum or plasma may be stored at 2-8°C for up to 24 hours. For longer periods, store at -20°C or lower for up to 2 years in airtight containers. Avoid repeated freezing and thawing of samples.
Do not use hemolyzed or lipemic samples. Frozen samples should be thawed and mixed thoroughly by gentle swirling or inversion prior to use.

VII. PROCEDURAL NOTES
A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert. A standard curve must be included with each assay.
Bring all kit reagents and specimens to room temperature (~25°C) before use.
Thoroughly mix the reagents and samples before use by gentle inversion.
Do not mix various lots of any kit component within an individual assay. Do not use any component beyond the expiration date shown on its label.
Incomplete washing will adversely affect the outcome and assay precision.
To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stopping solution into the wells in the same order and speed used to add the TMB solution.
Avoid microbial contamination of reagents, especially of the conjugate concentrate and the conjugate diluent. Avoid contamination of the TMB Chromogen Solution with the Enzyme Conjugate. Use a clean disposable pipette tip for each reagent, Standard, Control or specimen. For dispensing sulfuric acid and TMB Chromogen Solution, avoid pipettes with metal parts.
Containers and semi-automatic pipette tips used for the Enzyme Conjugate Solution and the TMB Chromogen Solution can be reused provided they are thoroughly rinsed with distilled water and dried prior to and after each usage.
The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use high quality water.

Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.
### VIII. TEST PROCEDURE

**A. Materials Supplied:**

Materials supplied in the DSL ACTIVE® Progesterone EIA Kit, Catalog No. DSL-10-3900

<table>
<thead>
<tr>
<th>CATALOG NO.</th>
<th>MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-3901</td>
<td>Progesterone Standard A</td>
</tr>
<tr>
<td>10-3902</td>
<td>Progesterone Standard B</td>
</tr>
<tr>
<td>10-3903</td>
<td>Progesterone Standard C</td>
</tr>
<tr>
<td>10-3904</td>
<td>Progesterone Standard D</td>
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<td>10-3905</td>
<td>Progesterone Standard E</td>
</tr>
<tr>
<td>10-3906</td>
<td>Progesterone Standard F</td>
</tr>
<tr>
<td>10-3731</td>
<td>GARG-Coated Microtitration Strips</td>
</tr>
<tr>
<td>10-3910</td>
<td>Progesterone Antiserum</td>
</tr>
<tr>
<td>10-3920</td>
<td>Progesterone Enzyme Conjugate Concentrate</td>
</tr>
<tr>
<td>10-2045</td>
<td>Progesterone Control Level I</td>
</tr>
<tr>
<td>10-3951</td>
<td>Progesterone Control Level II</td>
</tr>
<tr>
<td>10-3952</td>
<td>TMB Chromogen Solution</td>
</tr>
<tr>
<td>10-9755</td>
<td>Wash Concentrate B</td>
</tr>
<tr>
<td>10-9730</td>
<td>Stopping Solution A</td>
</tr>
</tbody>
</table>
B. Materials Required But Not Supplied
• Microtitration plate reader capable of absorbance measurement at 450 nm and preferentially capable of dual wavelength correction at 600 or 620 nm
• Deionized water
• Precision pipette to deliver 50 µL
• Semi-automatic pipette to deliver 100 µL
• Microtitration plate shaker capable of 500 - 700 orbital revolutions per minute (rpm)
• Automatic microtitration plate washer
• Vortex mixer
• Absorbent materials for blotting the strips
  • Linear-log graph paper for manual data reduction

C. Preparation of Reagents:
1. Wash Solution
   Pour 60 mL of the Wash Concentrate into a clean container and dilute by adding up to 1500 mL of deionized water (25X). The Wash Solution is stable for one month at room temperature (~25°C), provided the bottle is kept tightly sealed.
2. Enzyme Conjugate Solution
   The Enzyme Conjugate Solution should be diluted at a ratio of 1 part Enzyme Conjugate Concentrate into 50 parts Conjugate Diluent, according to the number of wells used. If an entire plate is to be used, pipet exactly 220 µL of the Enzyme Conjugate Concentrate into 11 mL of the Conjugate Diluent. The Enzyme Conjugate Solution should be prepared 15-20 minutes prior to performing the assay.
3. Microtitration Wells
   Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

D. Assay Procedure:
Allow all specimens and reagents to reach room temperature (~25°C) and mix thoroughly by gentle inversion before use. Standards, Controls and unknowns should be assayed in duplicate.
1. Mark the microtitration strips to be used.
2. Prepare the Enzyme Conjugate Solution by diluting the Enzyme Conjugate Concentrate in the Conjugate Diluent as described under the Preparation of the Reagents section of this package insert.
3. Pipet 50 µL of the Standards, Controls and unknowns into the appropriate wells.
4. Add 100 µL of the Enzyme Conjugate Solution to each well using a semi-automatic dispenser. Gently tap the well holder for 5-10 seconds.
5. Add 100 µL Progesterone Antiserum to each well using a semi-automatic dispenser. Gently tap the well holder for 5-10 seconds.
6. Incubate the wells shaking at fast speed (500-700 rpm) at room temperature for one hour.
7. Aspirate and wash each well 5 times with the Wash Solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
NOTE: Use of an automatic microplate washer is strongly recommended. Incomplete washing will adversely affect assay precision. If a microplate washer is not available, (a) completely aspirate the liquid from each well, (b) dispense 0.35 mL of the Wash Solution into each well, and (c) repeat steps (a) and (b) five times.

8. Add 100 µL of the TMB Chromogen Solution to each well using a semi-automatic dispenser.

9. Incubate the wells shaking at a fast speed (500-700 rpm) at room temperature (~25°C) for 30 minutes. Avoid exposure to direct sunlight.

NOTE: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

10. Add 100 µL of the Stopping Solution to each well using a semi-automatic dispenser.

11. Read the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450 nm.

NOTE: If wavelength correction is available, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 600 or 620 nm.

IX. RESULTS

A. Calculate the mean absorbance for each Standard, Control or unknown.

B. Using a linear-log graph paper, plot the mean absorbance readings for each of the standards along the y-axis versus the progesterone concentrations in ng/mL along the x-axis. Alternatively, any data reduction software designed for immunoassays could be used. Four-parameter curve-fit is recommended.

C. Draw the best fitting curve through the mean of the duplicate points.

D. Determine the progesterone concentrations of the Controls and unknowns from the standard curve by matching their mean absorbance readings with the corresponding progesterone concentrations.

If the absorbance readings exceed the limitations of the plate reader, a second reading at 405 nm is needed (reference filter 600 or 620 if available). In this case, proceed to construct a second standard curve as above with the absorbance readings of all Standards at 405 nm. The concentration of the off-scale samples at 450 nm are then read from the new standard curve. The readings at 405 nm should not replace the on-scale readings at 450 nm.

X. LIMITATIONS

• The reagents supplied in this kit are optimized to measure progesterone levels in serum or plasma.
• Repeated freezing and thawing of reagents supplied in the kit and of specimens must be avoided.
• Hemolyzed and lipemic specimens may give false values and should not be used.
• The results of this assay should be used in conjunction with other pertinent clinical information.

XI. QUALITY CONTROL

• DSL Controls or other commercial controls should fall within established confidence limits. The confidence limits for DSL Controls are printed on the Control vial labels.
• Low and high level controls should be included in each assay.

• The TMB Solution should be colorless. Development of a blue color may indicate reagent contamination or instability.

<table>
<thead>
<tr>
<th>XII. EXPECTED VALUES</th>
<th>These results are offered as an initial guide only. Each laboratory should determine its own ranges for expected values based on a representative sample population.</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPULATION</td>
<td>MEAN (ng/mL)</td>
</tr>
<tr>
<td>Females</td>
<td>22 18 17 25</td>
</tr>
<tr>
<td>Follicular Phase</td>
<td>1.46 13.44</td>
</tr>
<tr>
<td>Luteal Phase</td>
<td>1.36 1.35</td>
</tr>
<tr>
<td>Oral Contraceptives</td>
<td></td>
</tr>
<tr>
<td>Adult Males</td>
<td>1.39 11.83</td>
</tr>
<tr>
<td></td>
<td>1.19 1.27</td>
</tr>
</tbody>
</table>

• ABSOLUTE RANGE
0.58-2.34 0.00-27.78 0.00-2.56 0.51-2.19
0.75-2.52 3.10-31.50 0.55-2.91 0.82-2.32

XIII. PERFORMANCE CHARACTERISTICS
All performance characteristics are stated in ng/mL. To convert to nmol/L: ng/mL x 3.18= nmol/L
A. Sensitivity:
The theoretical sensitivity, or minimum detection limit, as calculated by interpolation of the mean minus two standard deviations of 14 replicates of the 0 ng/mL progesterone Standard, is 0.13 ng/mL.

<table>
<thead>
<tr>
<th>B. Precision:</th>
<th>The intra-assay precision was determined from the mean of 8 replicates each.</th>
<th>SAMPLE</th>
<th>N</th>
<th>MEAN (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COEFFICIENT OF VARIATION (%)</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>8</td>
</tr>
<tr>
<td>------------------------------</td>
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</table>

<table>
<thead>
<tr>
<th>STANDARD DEVIATION(ng/mL)</th>
<th>0.07</th>
<th>0.30</th>
<th>0.99</th>
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</thead>
<tbody>
<tr>
<td>7.0</td>
<td>3.2</td>
<td>3.4</td>
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</table>

The inter-assay precision was determined from the mean of average duplicates for 12 separate runs.

<table>
<thead>
<tr>
<th>COEFFICIENT OF VARIATION (%)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>12</th>
<th>12</th>
<th>12</th>
<th>0.90</th>
<th>3.54</th>
<th>21.35</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>STANDARD DEVIATION(ng/mL)</th>
<th>0.04</th>
<th>0.15</th>
<th>0.88</th>
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</thead>
<tbody>
<tr>
<td>5.0</td>
<td>4.2</td>
<td>4.1</td>
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</table>

C. Recovery: Three serum samples containing different levels of endogenous progesterone were spiked with different amounts of progesterone and assayed.

<table>
<thead>
<tr>
<th>RECOVERY (%)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>2.80</th>
<th>2.28</th>
<th>2.69</th>
<th>0.6</th>
<th>4.8</th>
<th>16.0</th>
<th>0.6</th>
<th>4.8</th>
<th>16.0</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.4</td>
<td>7.6</td>
<td>18.8</td>
<td>2.88</td>
<td>7.08</td>
<td>18.28</td>
<td>3.3</td>
<td>7.49</td>
<td>18.69</td>
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</table>

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ENDOGENOUS (ng/mL)</th>
<th>ADDED (ng/mL)</th>
<th>EXPECTED (ng/mL)</th>
</tr>
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<tbody>
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</table>

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>N</th>
<th>MEAN (ng/mL)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.90 3.54</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21.35</td>
</tr>
</tbody>
</table>
OBSERVED (ng/mL)
3.8 9.7 20.9 2.89 7.35 16.54 3.98 8.84 20.3
112 128 111 100 104 90 121 118 109

D. Linearity:
Three serum samples were diluted with 0 pg/mL Progesterone Standard and assayed.

<table>
<thead>
<tr>
<th></th>
<th>SAMPLE</th>
<th>DILUTION FACTOR</th>
<th>EXPECTED (ng/mL)</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>--- 1:2 1:4 1:8</td>
<td>--- 16.05 8.03 4.01</td>
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<tr>
<td></td>
<td>III</td>
<td>1:16 --- 1:2 1:4</td>
<td>2.05 --- 12.1 6.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8 --- 1:2 1:4</td>
<td>3.03 --- 4.32 2.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8 1:16</td>
<td>1.08 0.54</td>
</tr>
</tbody>
</table>

E. Method Comparison:
The DSL-10-3900 ACTIVE® progesterone EIA has been compared to another commercially available progesterone kit (Method X). One hundred six (106) samples were assayed and linear regression analysis of the results yielded the following:
n = 106

Regression: \([\text{DSL-10-3900}] = 0.96 \, [\text{Method X}] + 0.57\)

XIV. REFERENCES
CURRICULUM VITAE
ISABELLA M. RODRIGUES

Work Address
Department of Biobehavioral Health
The Pennsylvania State University
311 East Health and Human Development
University Park, PA 16802
Telephone: 814-880-9614
Email: imr104@psu.edu

Home Address
134 Summit Ave.
Lyndhurst, NJ 07071
Telephone: 201-438-2161

Education

<table>
<thead>
<tr>
<th>Education</th>
<th>Degree</th>
<th>Year</th>
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<tr>
<td>The Pennsylvania State University</td>
<td>Ph.D.</td>
<td>2005 (expected)</td>
<td>Biobehavioral Health</td>
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<tr>
<td>Shippensburg University, Pennsylvania</td>
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<td>2000</td>
<td>Biology</td>
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<tr>
<td>Shippensburg University, Pennsylvania</td>
<td>B.A.</td>
<td>2000</td>
<td>Psychology</td>
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</table>

Professional/Research Experience

2001-Present  Research Assistant, Department of Biobehavioral Health, The Pennsylvania State University (Advisor: Dr. Laura Cousino Klein)
2000-2001     Research Assistant, Department of Psychology, The Pennsylvania State University (Advisor: Dr. Karen S. Quigley)
2000-2001     Teaching Assistant, Department of Psychology, The Pennsylvania State University
1998-2000     Research Assistant, Department of Psychology, Shippensburg University

Awards and Honors

2004         Graduate Assistant Outstanding Teaching Award, Penn State Graduate School, Penn State University
2002-Present  Teaching Assistantship, Biobehavioral Health Department, Penn State University
2001         Research Assistantship, Office of Naval Research
2000         Magna Cum Laude, Shippensburg University, Pennsylvania
2000         Who’s Who Among Students in American Universities and Colleges
2000         Senior Outstanding GPA Award, Shippensburg University, Pennsylvania
2000         Undergraduate Research Award, Shippensburg University, Pennsylvania
1999         Psi Chi, Psychology National Honor Society

Complete vita, including list of manuscripts, available upon request