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# Impact of Oxidative Stress on Female Fertility.

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IMPACT OF OXIDATIVE STRESS ON FEMALE FERTILITY

Thesis submitted in partial fulfillment of Honors

By

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Midway Honors Program  
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### Abstract

This study examined nutritional status and fecundity of women and was approved for patient enrollment by the ETSU Institutional Review Board (IRB). Once enrolled, participants filled out two food frequency questionnaires (FFQ, fruit/vegetable intake, fat intake), self-reported anthropometric data, and had one 5 mL vacutainer of whole blood (EDTA) collected for measures of oxidative stress (OS), coenzyme Q<sub>10</sub> level, and fatty acid profile of the red blood cell (RBC) membrane. Participants were either designated to the case group (13 women experiencing fertility problems and an anti-mullerian hormone level of 1 ng/ml or less (AMH, <1 ng/mL)) or the control group (13 women experiencing normal ovulation cycles and no evidence of polycystic disease). This study hypothesized that the case group would have poorer plasma antioxidant status, consume more dietary saturated fat and have lower levels of omega-3 and higher levels of omega-6 fatty acids in the RBC membrane. Results showed that case participants had significantly higher body mass index (BMI) when graduating from high school, BMI upon enrollment in the study, and trended to gain more weight since high school. Antioxidant status of the plasma tended to be higher for the case group. This result was surprising as case participants consumed significantly fewer servings of fruits and vegetables (sources of antioxidants) than control subjects. FFQ results indicated that case participants consumed significantly higher amounts of total fat (g), saturated fat (g), unsaturated fat (g), and their diets had a higher dietary fat percent. No significant differences were noted in the individual fatty acids isolated from the RBC membrane or in calculated ratios of fatty acid between case and control groups. The fatty acid profile from RBC membranes are in agreement with self-reported fat intake from FFQ data and may provide a rationale for no differences observed between the two groups.

## **I. Review of Literature**

### **Introduction**

Infertility is a problem affecting approximately 11 percent of the women of reproductive age in the United States (1). Generally speaking, infertility can be described as the inability to conceive after 12 or more months of unprotected sex. After that time period, investigation of issues regarding fertility is typically conducted. Assisted reproductive technology (ART) has become a viable treatment option for those trying to conceive and has been helping women become pregnant in the United States since 1981, most commonly through *in vitro* fertilization (1). The use of ART is still relatively rare; however over 1% of all infants born in the United States every year are conceived using this process based on the CDC's 2011 ART Fertility Clinic Success Rates Report (1). The literature suggests that environmental and lifestyle factors play an important role in the effects of fertility including body weight. Although body weight is not a reliable predictor of conception among infertile women, there is evidence to suggest a negative correlation (2, 3, 4, 5, 6, 7). An increasing amount of research suggests that excessive amounts of oxidative stress may also play a significant role in fertility. Oxidative stress (OS) is caused by an imbalance between the formation of reactive oxygen species (ROS), such as free radicals and oxidants, and the body's ability to neutralize them (8). OS, in turn, affects spermatozoa quality, endometriosis, fertilization rates, early embryo development and implantation and ultimately, pregnancy rates (8, 9, 10, 11, 12). Although ROS are naturally formed during many metabolic pathways, determining ways to counteract the build up of these reactive oxygen species has the potential to provide advantageous outcomes. Antioxidant intake and supplementation could provide

the answer in alleviating some of the problems regarding infertility through normalization of oxidative stress status (8, 13).

## **ROS and OS**

Oxidative stress can occur when oxidants outnumber antioxidants, when products of peroxidation develop, and the pathological effects caused by these phenomena (10). OS is the consequence of the excessive production of reactive oxygen species and impaired defense by antioxidants (10). ROS are produced mainly by mitochondria and must be constantly inactivated to avoid excess formation in order to maintain normal cell function (10, 14). The main ROS molecules are generated as natural by-products of aerobic respiration and metabolism including the superoxide molecule ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical (OH), and peroxynitrite ( $ONOO^-$ ) (15). Oxygen radicals target mitochondrial DNA due to its location near the inner mitochondrial membrane, which is where the majority of oxidants are formed (9). Another factor could be due to the lack of protective histone proteins within mitochondrial DNA (16). The ovaries, testes, and uterus are particularly affected by ROS because they contain the highest amount of mitochondria in the body due to the need for ATP, or energy, in those processes (17). Mitochondrial dysfunction can lead to a number of cellular consequences, which includes increased production of reactive oxygen species (18). Overproduction of ROS induces oxidative stress and can damage the cellular membrane, structure, and functions within mitochondria (14). Numerous clinical studies have shown that ROS may have roles in the female reproductive tract that are both physiological and pathological (8, 10, 11, 13). Evidence suggests that ROS production is increased when an upturn in mitochondrial ROS generation inhibits the release of calcium ( $Ca^{2+}$ ) and  $Ca^{2+}$ -

loading (14). Protecting the mitochondria against the oxidative damage of ROS is crucial for continued ATP production and prevention of cell death, or apoptosis (14). Disruption of the mitochondrial membranes from high levels of ROS can result in the release of cytochrome-C protein and thus activate apoptosis (10). Oxidative stress and endoplasmic reticulum (ER) stress can both result in cell death if left unresolved (19). While OS can affect several functions, ER stress essentially disturbs the folding of proteins on the ER (20).

Furthermore, OS plays a critical role in the process of ovulation by inducing apoptosis of luteal cells within the ovary, which perturbs the area in and around ova and granulosa cells that could potentially decrease oocyte and embryo viability (21). However, repeated exposure of oxidative stress in the ova and other cells during ovulation suggests this might trigger ovarian aging (21). Polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic (DHA), are important in biological membranes; however, they are the most vulnerable to attack from free radicals (14, 22). PUFAs also play important roles in mitochondrial  $\text{Ca}^{2+}$  homeostasis and function (14). PUFAs have a high degree of renewal in mitochondrial membranes and are a direct target of ROS, which makes them highly susceptible to peroxidation reaction (14). Peroxidation reactions that occur in mitochondrial membrane phospholipids can alter fluidity, permeability, and membrane function (14). ROS are generated by lipid peroxidation, which can alter mitochondrial function and increase reactive oxygen species (23). Lipid peroxidation can produce a considerable amount of ROS, however certain antioxidants are known to inhibit these events, such as catalase, superoxide dismutase, and glutathione peroxidase (24). Although originally regarded as cytotoxic, ROS molecules are considered important signaling molecules in several cell pathways (23). The peroxidation of omega-3 PUFAs by ROS can induce the formation of toxic products; therefore, dietary intake of these fatty acids could be

detrimental to cellular function (14). However, the cardiovascular and hemodynamic effects from consuming omega-3 PUFAs may counteract these effects. More research needs to be conducted on omega-3 fatty acid intake and supplementation in order to determine more conclusive results.

Healthy individuals are able to maintain balance of ROS and antioxidants, however an imbalance of these reactive oxygen species can cause many problems. The generation of ROS and other oxidants are formed consequently through normal biological reactions (8). ROS can also be formed through external factors such as alcohol consumption, smoking, and environmental pollutants (8). Based on previous research, it is possible to infer that endometriosis and poor oocyte quality, among other problems affecting female reproduction, can occur due to excessive levels of these reactive oxygen species (25, 26, 27). Although more studies need to be conducted, some investigations have shown a correlation in the role of the imbalance of OS and the adverse effects of fertility including decreased oocyte quality and subsequent viability of embryos (9, 11, 21, 28, 29).

Low levels of ROS play an important role in many processes including: normal reproductive function, intracellular messaging, apoptosis, immunity, and microorganism defense; however, the level at which ROS becomes degenerative requires further research (10, 30). It is interesting to note that pregnancy itself may produce OS as a result of increasing tissue growth and catabolic activities (8). Increases in OS have been indicated in the formation of acute pregnancy complications or spontaneous abortion (8). Infertility can occur due to multiple reasons since successful pregnancies require numerous steps to occur such as ovulation of mature oocytes, proximity of sperm and oocyte in the reproductive tract, fertilization of the oocyte, and embryo development. The reasons for infertility may vary,

however, a significant correlation regarding the oxidative stress and fertility has been established and gained considerable interest in recent years.

### **Problems surrounding OS and ROS**

An imbalance of OS in the follicular (FF) fluid environment of ovaries can cause detrimental issues, such as poor oocyte development, embryo development, and the overall outcome of pregnancy (9, 11, 12, 31). ROS can be produced in the FF environment, which contain oocytes, granulosa cells and surrounding cells, leukocytes, cytokines, and macrophages (11). Poor oocyte quality or increased risks of poor oocyte quality can be attributed to excessive OS production (9). Antioxidant enzymes, such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPX), may inactivate ROS and help protect embryos and oocytes (19). Again, ROS and OS may occur due to an insufficient amount of these enzymes.

Some researchers hypothesize that ovulated oocyte reduction and function could be the result of mitochondrial abnormality from repeated ovulation (21). One study analyzed the intracellular localization of mitochondria, which revealed that “the number of oocytes having heterogeneously localized mitochondria increased” considerably during repeated ovulation (21). Moreover, one of the biomarkers for OS, 8-OHdG (8-hydroxydeoxyguanosine), increased by repeated ovulation was inhibited by the administration of L-carnitine even though it lacks the ability to scavenge free radicals directly (21). L-carnitine has been shown to share a close resemblance to the properties of antioxidants, which can protect cells from oxidative damage and apoptosis (21).

L-carnitine plays a crucial role in human metabolism, more specifically in the mitochondrial transport of long-chain free fatty acids (32). L-carnitine actively shuttles long-chain fatty acids across the mitochondrial membrane for production of ATP and  $\beta$ -oxidation in tissues (32). ROS, as previously stated, are continuously produced under normal physiological conditions. Furthermore, they can easily initiate the peroxidation of membrane lipids and lead to the accumulation of lipid peroxides (32). Antioxidants can protect the human body from the effects of ROS and impede the progression of lipid peroxidation (32). The main enzymes in the antioxidant defense system include: superoxide dismutase, glutathione peroxidases, and catalase (32). L-carnitine may be able to protect these enzymes from further damage (32). According to a study comparing the antioxidant capacity of L-carnitine with  $\alpha$ -tocopherol, a natural antioxidant, L-carnitine was proven an effective antioxidant (32).

### *Preeclampsia*

Oxidative stress is considered a key factor in the disease process of preeclampsia, which is the major cause of maternal morbidity and death afflicting approximately 3% to 5% of pregnancies (33, 34). Preeclampsia can be defined as the “new onset of hypertension and proteinuria (protein in urine) during the second half of pregnancy” (34). Overproduction of reactive oxygen species and synthesis of lipid peroxides may lead to maternal endothelial cell activation, which causes a proinflammatory response and is a hallmark of the disease (33). Antioxidant supplementation may be able to assist in the prevention or improvement of this disease (33). One randomized trial assessed the potential supplemental benefit of vitamin C and E in the prevention of preeclampsia, which was associated with reduced incidence of the disease and a reduction in the measure of endothelial and placental dysfunction (33). This

study's results confirmed that antioxidant supplementation of vitamins C and E improved indicators of placental function oxidative stress in a group of low-risk women resulting in similar values as a group of healthy women (33).

Increased levels of circulating lipid peroxides is one of the consequences of decreased antioxidant activity (35). The synthesis of the endothelial cell-derived vasodilator prostacyclin is inhibited when lipid peroxide levels are elevated (35, 36). The disruption of platelet cell membranes is another action of peroxides resulting in the elevation of the prostaglandin, thromboxane (35, 36). An imbalance between thromboxane and prostacyclin is associated with this disease (36). The abnormal action of prostaglandins is one of the triggering factors of endothelial cell damage of certain diseases of pregnancy, such as preeclampsia (35).

### *Endometriosis*

Endometriosis is a common disorder characterized by the growth of endometrial cells outside the uterus (27). Patients with endometriosis may have worse outcomes with assisted reproduction techniques, such as *in vitro* fertilization (IVF) due to the lower quality of oocytes seen in these women (25). Lower oocyte quality is one of the many problems effecting women with infertility. The imbalance between ROS and the antioxidant defense system could be the possible cause of the resulting lower egg quality (25). ROS may "increase growth and adhesion of endometrial cells in the peritoneal cavity", which could promote endometriosis and infertility (27). Two studies have found a positive correlation between oxidative stress and endometriosis (27, 37, 38). One study found that SOD, was significantly lower in infertile women with endometriosis suggesting that ROS could potentially play a role in the cause of infertility in these women (37). Another study viewed the autoantibodies of oxidative stress in

women and found that increased amounts of these autoantibodies were found in women with endometriosis, suggesting OS is occurring in women with endometriosis at an increasing rate (38). More research is required in order to find a significant correlation between endometriosis and infertility.

Achieving greater antioxidant capacity in patients with endometriosis has shown promising results (25). In one experimental study, patients with endometriosis revealed lower levels of vitamin C in follicular fluid, which was most likely due to excessive consumption of antioxidants attempting to scavenge excess ROS (25). Moreover, researchers found significant correlations between “vitamin C levels and the number of oocytes retrieved, number of mature oocytes, and the number of fertilized oocytes” (25). Overall, these findings suggest that infertile women with endometriosis could have promising results if enhanced higher antioxidant capacity is achieved.

#### *In vitro fertilization (IVF)*

Despite advances in reproductive technology, the national success rate of IVF remains around 40% in most cases (39). Characteristics of *in vitro* fertilization success have been associated with the antioxidant capacity of FF during oocyte retrieval. A recent study of oxidative stress and oocyte quality in women undergoing IVF, using 8-OHdG as a biomarker for OS, indicated that 8-OHdG was considerably higher in the follicular fluid of women undergoing IVF and embryo transfer who showed high rate of degenerative oocytes compared to women with low rates of degenerative oocytes (9). Results from this study suggest that OS negatively impacts oocyte maturation and that supplementation of melatonin, vitamin E, or a combination of the two could improve oocyte quality and provide protection from OS (9).

Melatonin is a free radical scavenger that reduces oocyte DNA damage and  $\alpha$ -tocopherol, the most abundant form of supplemented vitamin E, helps by maintaining homeostatic levels of ROS with evidence to suggest that it improves oocyte quality (9). The results also suggest a close relationship between oxidative stress and poor oocyte quality.

During IVF, the follicular fluid removed from the ovary has provided insight in understanding the environment of the mature oocyte in infertility (8). One prospective study evaluated total antioxidant capacity (TAC) using follicular fluid collected from women undergoing oocyte retrieval for IVF (8, 13). Within the study, a lower baseline TAC was observed in the follicular fluid of the embryos that survived to the day of transfer (8, 13). There was an association found between successful fertilization and the higher FF total antioxidant capacity (8, 13). This study suggests that higher antioxidant status increases the probability of a successful conception using IVF. In contrast, another study measuring ROS and TAC in women undergoing IVF, found that of women who became pregnant, showed significantly higher FF ROS levels rather than those who did not become pregnant (8). Also, the TAC did not differ by pregnancy status in these women (8). This information was surprising; however, lack of reference values for healthy women was excluded in the comparison of this study population (8). These studies suggest that follicular fluid ROS may be a potential marker of IVF success and that higher TAC levels may predict higher fertilization potential (8). Follicular fluid containing high levels of antioxidants may help to control the generation of ROS and lipid peroxidation, resulting in improved fertilization rates (11).

## Antioxidants

Oxidative stress can form through increased production of ROS or due to a deficiency of antioxidants (35). Antioxidants quench free radicals by donating an electron to the unpaired electron and becoming reduced (16). A deficiency of antioxidants could be due to decreased antioxidant intake, synthesis of antioxidant enzymes, or increased antioxidant utilization (35). In order to prevent the overproduction of reactive oxygen species, antioxidant enzymes provide a defense against ROS-induced oxidative damage (15). The main enzymes in the antioxidant defense system include: copper-zinc containing superoxide dismutase (Cu, Zn-SOD or SOD1), manganese-containing SOD (Mn-SOD or SOD2), glutathione peroxidases (GPX) and catalase (CAT) (15). One of the central elements of cell signaling and maintenance of oxidation-reduction (redox) homeostasis is through the control of ROS by antioxidant enzymes (15). These enzymatic pathways are vital in processes of reproduction and development (15). Evidence suggests that the critical stages of gestation are implantation and early post-implantation since oxidative damage can have the most impact on the developing organism during those times (15). Therefore, antioxidant systems are essential against the protection of oxidative damage and cope with highly reactive and toxic free radicals (15).

The naturally occurring antioxidants protect cells from oxidation, which is essential as lipid peroxidation can cause DNA damage and directly inhibit certain proteins (30). Decreased antioxidant protection and increased lipid peroxidation generate epoxides, which may ultimately lead to cytotoxicity, allergy, mutagenicity, or carcinogenicity (30). Superoxide dismutase (SOD) is the antioxidant enzyme that catalyzes  $O_2$  into the less reactive  $H_2O_2$ , which can then be destroyed by CAT or GPX (30). Catalase (CAT) and glutathione (GPX) each play an important role in protecting cells against oxidative damage (30). Moreover, one of the most

essential antioxidant defense mechanisms is glutathione metabolism (30). The control of OS can be obtained by supplying known nutrients with antioxidant capabilities and minimizing substances that stimulate ROS (30).

### *Antioxidants from the Diet*

In addition to antioxidant enzymes present in the human body, there are several beneficial dietary antioxidants including but not limited to: vitamin E, vitamin C, beta-carotene, leutin,  $\alpha$ -lipoic acid, coenzyme Q<sub>10</sub>, lycopene, and selenium (16). There is sufficient evidence to suggest that dietary antioxidants, including certain nutrients and cofactors, may impact oxidative stress and thus play a major role in female fertility (8, 40, 41). There are many proposed beneficial effects of antioxidants, folate, and flavonoids, including scavenging free radicals and ROS, which could potentially reduce inflammation and oxidative stress (40). Antioxidants such as vitamins C and E have shown to be helpful in reducing stress to ova, sperm, and reproductive organs (16, 41). Other nutrients shown to improve female reproduction and fertility include: vitamin B<sub>12</sub>, folic acid, magnesium, iron, and zinc (16, 41, 42, 43, 44). Antioxidants scavenge molecules and work to convert ROS to water in order to prevent overproduction under normal conditions (29). One trial provided preconception multivitamins to females in couples trying to conceive, which indicated higher rates of conception among women receiving the multivitamin in comparison to women receiving trace elements or no supplementation (42). Another study reported that reduced antioxidant status increased the risk of spontaneous abortion (35). In these women, an imbalance was found between thromboxane and prostacyclin, which is associated with preeclampsia (35, 36). Wang et al. demonstrated that an imbalance between these prostaglandins was similarly

found in women with recurrent abortion (35, 36). Overall, sufficient nutrient supplementation should be a prerequisite of fertility for women trying to conceive and supplementation of vitamins and minerals may play an important role in optimizing fertility health.

Several studies established that increased consumption of antioxidant-rich fruits and vegetables showed a negative correlation to oxidative stress, meaning that higher dietary antioxidant intake produced a lower occurrence of oxidative stress (24, 40). A diet that includes fruits and vegetables, which provides flavonoids and other antioxidants, has been associated with lower risk of oxidative stress in adults (40). One study involving adolescents showed that the beneficial effects of fruit and vegetable intake on oxidative stress are present by early adolescence and provide support for individuals to consume five or more servings per day to promote health (40). These results showed that increased consumption of fruits and vegetables have an effect on markers of inflammation and oxidative stress (40). For instance, lower concentrations of IL-6 (interleukin-6; cytokine that functions in inflammation) appeared when a greater amount of legumes and vegetables were consumed, while the vegetable group and TNF- $\alpha$  (tumor necrosis factor; inflammatory cytokine) were inversely related (40). These findings suggest that an ongoing pattern of increased consumption of fruits and vegetables could be beneficial throughout life in relation to oxidative stress (40). Another study focusing on the effects of increased vegetable and fruit consumption indicated significant reductions in markers of oxidative cellular damage to DNA and lipids by adherence to this type of diet (24). These studies help to confirm that a continual diet with high amounts of vegetables and fruits could result in several beneficial effects including decreased levels of ROS and free radicals, thus lowered oxidative stress.

**Vitamin D**

Vitamin D has become a focus in recent years in regards to its roles in biological responses. The predominant source of vitamin D in humans occurs after the conversion of 7-dehydrocholesterol by UV-B ray component of sunlight to produce pre-vitamin D<sub>3</sub> (45). Nutritional forms of vitamin D consist of cholecalciferol (D<sub>3</sub>) and ergocalciferol (D<sub>2</sub>) (45). Vitamin D<sub>3</sub> can be found in fatty fish and eggs whereas D<sub>2</sub> is synthesized through the UV irradiation from yeast and fungi (45). A recent finding showed that, vitamin D receptor (VDR), is present in the ovary, uterus, placenta, and testis, which suggests that vitamin D may have a regulatory role in reproductive physiology; however, the vitamin may not be critical for successful reproduction (46). A recent study shows a reduction in anti-mullerian hormone with decreasing vitamin D levels, which indicates a possible role in ovarian reserve (47). More research is needed on the role of vitamin D in reproductive status and to determine if individuals with low serum levels (< 32 ng/mL, 25-OH-D<sub>3</sub>) have a higher frequency of reproductive difficulties (either male or female).

**Coenzyme Q<sub>10</sub> (Ubiquinol)**

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>), or ubiquinol, consists of a benzoquinone and a hydrophobic tail that contains isoprenoid units (16). CoQ<sub>10</sub> can be reduced to ubisemiquinone (QH) and further reduced to ubiquinol (QH<sub>2</sub>). These reducing properties allow CoQ<sub>10</sub> to function as an antioxidant or pro-oxidant depending on a number of factors, such as pH, or the presence of other redox couples including: vitamin E, vitamin C, and  $\alpha$ -lipoic acid (16). It is possible that CoQ<sub>10</sub> may become pro-oxidant (induce oxidative stress) if not given in conjunction with a redox couple previously stated (16). CoQ<sub>10</sub> appears to play multiple roles in cells. The

functional roles of CoQ<sub>10</sub> include: transferring electrons in the mitochondrial electron transport chain and functioning as an antioxidant in the inner mitochondrial membrane by inhibiting lipid peroxidation thus scavenging free radicals (48). CoQ<sub>10</sub> is also the major source of superoxide and hydrogen peroxide generation (48). Coenzyme Q<sub>10</sub> is mainly carried by lipoproteins in circulation where it is largely present in its reduced form as CoQ<sub>10</sub>H<sub>2</sub> (49). CoQ<sub>10</sub>H<sub>2</sub> has been shown as the first antioxidant depleted when low-density lipoprotein (LDL) undergoes *in vitro* oxidative stress (49). This also shows the possibility that CoQ<sub>10</sub> could be a marker of oxidative stress or produce beneficial results if combined with other nutrient compounds. The antioxidant function of this enzyme may be significant to protect LDL from lipid peroxidation (49). The main role is considered the transfer of electrons through the electron transport chain and subsequent production of ATP, or energy (16).

Age is an important factor to consider when looking at reproductive potential. The peak age range of female fertility is 25 to 35 (50). Older patients trying to conceive may run into considerable difficulties. There are two main reasons for poor reproductive performance of older patients: reduced ovarian reserve and increased risk of miscarriages and aneuploidy due to a higher rate of chromosomal aberrations (50). Reproductive changes in aging can lead to decreased ovarian reserve (50). One of the theories associated with decline in oocyte quality due to age is that highest-quality oocytes are selected first, which leaves unfavorable oocytes for later reproductive years (50). The second theory is that the process of aging produces unfavorable oocytes that remain in the ovary until possible selection in later years (50). The effects of aging are still widely unknown, however, one of the hallmarks of aging is the accumulation of mutations and deletions within mitochondrial DNA (50). Oocytes largely rely on ATP, or energy, produced by the mitochondria by oxidative phosphorylation (50). The

production of ROS is one of the byproducts of mitochondrial respiration, of which 90% is produced by the mitochondria (50). Increased amount of ROS has been correlated with the following factors: fatty acid oxidation, apoptosis, enzyme deficiencies, and reduced action of antioxidants (50). CoQ<sub>10</sub> has shown promising results in the ability to normalize the bioenergetics status and oxidative balance in CoQ<sub>10</sub> deficient patients *in vitro* (50). Therefore, there a possible correlation is present between CoQ<sub>10</sub> status and decreased energy production seen in aging oocytes (50).

Several studies involving the oral administration of CoQ<sub>10</sub> show that it can act as a greater antioxidant against oxidative damage, especially in the mitochondria. In the rat, long-term administration of CoQ<sub>10</sub> caused a higher concentration CoQ<sub>10</sub> in various tissues and their mitochondrial fraction, which suggests that supplementation of CoQ<sub>10</sub> may be beneficial in lowering *in vivo* oxidative stress (48). Another study looking at the plasma concentration in patients with mitochondrial diseases revealed that the plasma concentration of CoQ<sub>10</sub> was significantly higher after combination therapy of creatine, CoQ<sub>10</sub>, and lipoic acid (18). The results also discovered that lactate levels and measures of oxidative stress were reduced in these patients (16). In another study involving aged mice, treatment with CoQ<sub>10</sub> increased the number of ovulated oocytes (51). Conversely, oocyte mitochondrial membrane potential (MMP) was reduced by CoQ<sub>10</sub> supplementation while ROS levels increased as compared to similar levels in young oocytes (51). Overall, supplementation of CoQ<sub>10</sub> in old mice appears to improve ovarian response and oocyte mitochondrial function (51). Although more studies need to be conducted, lone supplementation of CoQ<sub>10</sub> may produce unexpected or inadequate results. However, combined with other compounds, increased plasma concentrations of CoQ<sub>10</sub> could be beneficial in mitochondrial function (16, 18). It is possible to surmise that the

production of ATP is crucial to the reproductive tract since the process of ovulation, developing an embryo, etc. are expensive in regards to energy. Therefore, the higher amount of plasma concentration of CoQ<sub>10</sub> could produce positive outcomes in fertility and reproduction.

### **Fatty Acid Intake and Fertility**

Fertility can be adversely affected in many ways, however the effects of underweight and obesity have been shown to correlate negatively (2, 3, 4, 5, 6, 7, 19). Obesity is a major prevalence around the world and is a major risk factor in a number of chronic diseases, such as diabetes and heart disease (19). Polycystic ovarian syndrome (PCOS), which is one of the most common metabolic aberrations in women of reproductive age (52), can negatively affect fertility. PCOS and obesity have also shown a significant correlation to one another with obesity affecting 38-88% of women with PCOS (52). Several studies indicate that weight loss can improve or restore fertility among these women (52).

Obesity is associated with “lipid accumulation in non-adipose tissue cells and the induction of oxidative stress”, which can be linked with systemic inflammation (19). Oxidative stress is one of the hallmark responses to intracellular lipid overload and is known to have detrimental effects in many tissues including the female reproductive tract (19). As adipose tissue expands in the body during obesity, its physiology drastically changes and initiates a chronic inflammatory response that influences the entire body (19). Oxidative stress is exhibited as “adipocytes undergo excessive hypertrophy” in order to store fat, and in turn, produce increased levels of cytokines or adipokines (19).

Female infertility is one of the neglected complications of obesity (19). The risk of infertility in obese women is almost three times as likely in comparison to nonobese women (3). Several factors are affected due to obesity besides infertility including: decreased conception rates and oocyte quality. Conception rates are considerably decreased by obesity even if the woman is experiencing normal menstrual cycles and in otherwise good health (19). With the rate of obesity at epidemic proportions, understanding the effects of obesity on the female reproductive system has become essential. Increased knowledge of obesity will help young women to alleviate the detrimental consequences on fertility (19).

Anovulation, or a menstrual cycle without release of an oocyte, which is increased with obesity, may account for the delayed time to pregnancy (4, 19). Further studies show that obese women experiencing anovulation are less likely to be improved by treatment, such as clomiphene and gonadotropin hormones (53). Oocyte quality may be affected by obesity. Oocyte maturity was shown to be decreased in women with obesity in one of the studies with these evaluations (5, 19). Studies using mouse models were able to establish clearer results that concluded diet-induced obesity is harmful to oocytes (19). These mice models exhibited delayed maturation of oocytes, reduced rates of *in vivo* fertilization, and decreased oocyte development (6, 53). Thus, it can be concluded that obesity negatively impacts oocyte development and ovulation (6, 53). As previously stated, obesity is the “lipid accumulation in non-adipose tissue cells” and the ovary is not excluded from this response (19). In women, obesity can cause an increase in triglyceride levels in the follicular fluid that correlate with increased levels of free fatty acids and thus ROS (19).

Lipotoxicity, or metabolic syndrome, results in oxidative stress and the production of ROS, which are damaging to cells because they can oxidize other proteins, such as LDL (19).

Oxidized LDL has recently become a marker of oxidative stress and has shown to be elevated in the serum and follicular fluid of obese women (19). Inflammatory ROS production can lead to the generation of  $H_2O_2$ , however, catalase and glutathione peroxidase enzymes can purge these oxidants (19). Increased levels of catalase and glutathione peroxidase enzymes have been seen in obese women suggesting an up-regulation of these antioxidant systems (19). One prospective study using mouse oocytes indicate that  $H_2O_2$  is significantly harmful on the maturation of oocytes (9). However, another study observed a significant decrease in  $H_2O_2$  and the production of reactive oxygen species through supplementation with 6 g EPA (eicosapentaenoic acid) plus DHA per day (54).

### **Omega-3 and Omega-6 Fatty Acids**

Polyunsaturated fatty acids (PUFAs), which are fatty acids that contain more than one double bond, can be divided into various subcategories including: omega-3, omega-6, omega-7, and omega-9 fatty acids, depending on the location of the first double bond (55). Several sources suggest that primitive human beings survived on a diet with a ratio of 1:1 omega-6 to omega-3 essential fatty acids (EFA) (56). The typical Western diet of the United States has shown a steady decline in the consumption of omega-3 EFA, with a ratio of omega-6 and omega-3 EFA as high as 20:1 (56). The significant increase in omega-6 fatty acid consumption along with a high omega-6 to omega-3 ratio in the Western diet has been shown to promote the pathogenesis of several diseases (e.g. cardiovascular disease, cancer, and certain inflammatory and autoimmune diseases) (56). The increased ratio of omega-6 to omega-3 EFA may be, in part, due to decreased consumption of fish and the change in modern agriculture, which increased reliance on cereal grains. Common foods, such as fish, green leafy

vegetables, fruits, nuts, and berries, shaped pre-agricultural humans (56). The greater portion of our current food supply is highly dependent upon grains, which are high in carbohydrates and omega-6 EFA, but low in antioxidants and omega-3 EFA (56). Omega-3 polyunsaturated fatty acids are typically found in vegetable oils in the form of  $\alpha$ -linolenic (ALA), or fatty fishes and marine food in the form of EPA and DHA (14, 57). Industrialized societies have reported an increase in energy intake, saturated fat, omega-6 EFA, trans-fatty acids, and cereal grains (56). In addition, these societies have reported a decrease in energy expenditure, omega-3 EFA, complex carbohydrates, fiber, protein, antioxidants, and calcium intake (56). Modern agriculture may also serve as a factor in the increasing ratio of omega-6 to omega-3 EFA. Omega-3 fatty acids have decreased in animals, such as cattle, due to being fed a diet of grains as compared to their normal diet of plant life (56). The same reduction in omega-3 EFA can be seen in eggs, vegetable oil, cultivated vegetables, and cultivated fish (56). To ensure good health and normal development, one should strive for the balance of these essential fatty acids (56). Inflammation is influenced by the dietary intakes of both omega-3 and omega-6 PUFAs (58). Consumption of essential fatty acids, especially omega-3 EFA, are generally found to have favorable effects such as reduced risk of inflammation and chronic diseases.

The predominant omega-6 PUFA in humans is linoleic acid (LA), which can be elongated and desaturated to arachidonic acid (AA) (55). AA is usually the major substrate for eicosanoid synthesis, which is a process involved in modulating the intensity and duration of inflammatory responses, and increases proinflammatory cytokine production (54, 58). Eicosanoids derived from AA are considered pro-inflammatory in comparison to those derived from omega-3 PUFAs, EPA, which curb the production of AA-derived eicosanoids (55, 56, 58). Increased availability of long chain omega-3 fatty acids could potentially provide

eicosanoid synthesis with more EPA and DHA than AA, thus reducing inflammation and certain diseases. The typical Western diet high in omega-6 EFA shifts the physiological state to pro-inflammatory and pro-thrombotic, which may increase the risk of chronic diseases such as coronary heart disease, atherosclerosis, and stroke (56). Research has shown that omega-3 fatty acids could modify inflammatory and immune reactions and provide therapeutic effects against these inflammatory diseases (56). Therefore, higher levels of omega-3 PUFAs and lower omega-6 to omega-3 PUFA ratios have been associated with lower proinflammatory cytokine production (58).

The increased ratio of omega-6 to omega-3 EFA in individuals who consume a Western diet also may potentially contribute to fertility problems (59). Recent research has emphasized the importance of consumption and supplementation of these essential fatty acids in pre- and postnatal outcomes in development (14). On the other hand, some researchers found that the dietary intake and increased content of omega-3 fatty acids may be detrimental to health due to the formation of toxic products caused by ROS-induced oxidative peroxidation of omega-3 EFA within membrane phospholipids (14). Exposure to dietary omega-3 fatty acids has been shown to disturb mitochondrial metabolism and reduce embryonic development in women in early pregnancy (14). This statement is consistent with the findings of a study involving the oocytes of adult female mice in order to determine how omega-3 PUFA supplementation might influence oocyte and pre-implantation embryo development (23). Evidence suggests that dietary omega-3 PUFA levels may alter the fatty acid composition of the oocyte, thus potentially impacting oocyte maturation and development (23). Several intracellular effects may occur due to high omega-3 consumption including: increased oxidative stress level and impaired embryo development (23). Overall,

additional research is needed in order to properly state the impact of omega-3 EFA intake and supplementation, especially in regards to pregnancy outcomes (14).

## **Conclusion**

Infertility is a significant health problem that may be due, in part, to the adverse effects of oxidative stress and inflammation. Although ROS are naturally formed by mitochondria and during metabolic pathways, determining ways to counteract the build up of these free radicals could provide advantageous outcomes. The ovaries, testes, and uterus are particularly affected by ROS because they contain the highest amount of mitochondria in the body due to the need for ATP in those processes (17). Antioxidant intake and supplementation could propose alleviation of problems regarding infertility by normalizing oxidative stress status (8, 13). Significant evidence has been compiled regarding the positive effects that the following nutrients have on multiple aspects of overall reproductive health: vitamin E, vitamin C, vitamin B<sub>12</sub>, beta-carotene, leutin,  $\alpha$ -lipoic acid, coenzyme Q<sub>10</sub>, lycopene, selenium, folic acid, magnesium, iron, and zinc (16, 41, 42, 43, 44). Several factors are known to decrease fertility in women including body weight and decreased function of antioxidant enzymes. Intake of antioxidant nutrients, such as multivitamins or other therapeutic combinations, impacts the generation of ROS and may also play a beneficial role in female fertility. The assessment of OS through the use of laboratory techniques has helped to gain insight. The impact of the dietary fatty acid profile still needs further investigation; however, maintaining a diet that includes a lower ratio of omega-6 to omega-3 FA and more PUFAs should improve hemodynamics in patients. Ultimately, results from this research project could be helpful in developing

supplementation therapies to optimize probabilities related to successful pregnancy outcomes for at risk patients.

## **II. Materials and Methods**

### *Participant selection*

The protocol for this study was reviewed and approved by the Institutional Review Board of East Tennessee State University's Office of Research and Sponsored Programs. Participants in the case group were recruited from the Quillen Fertility and Women's Services clinic and are the patients of Dr. Norman A. Assad, Associate Professor, OB/GYN. Females between the ages of 25 and 40 experiencing fertility difficulties, and with a low anti-mullerian hormone level (AMH, <1 ng/mL), served as the case group for this investigation. Exclusion criteria for the case group included women with polycystic ovarian syndrome. The control group also consisted of females between the ages of 25 and 40. Exclusion criteria for the control group included women experiencing irregular menstrual cycles and polycystic ovarian syndrome. A total of 60 patients will be recruited for this study with 30 women in each treatment group. The subject of this thesis included 26 patients; 13 women were recruited for the case group and 13 women for the control group. Participants filled out two food frequency questionnaires (FFQ, fruit and vegetable intake, fat intake), anthropometric data, and had one 5 mL of whole blood (EDTA) collected for analysis of measures of oxidative stress, coenzyme Q<sub>10</sub> level, and fatty acid profile of the red cell membrane. Participants understood they were under no obligation to participate in this trial. Participation in this research experiment was completely voluntary and participants could discontinue at any time.

The following laboratory methods sections (Red blood cell (RBC) fatty acid profile, Isolation of RBC and lipid extraction, Analysis of fatty acids, and FRAP assay) were taken from an ETSU graduate thesis study (60). Due to the similarity of the laboratory methods used in both studies, permission was obtained to use the same procedures. Additionally, Ms. Litwin assisted in performing some of the blood sample analyses used for this research study.

#### *Self-reported patient anthropometric data*

Patients filled out information related to their anthropometric measurement including current body weight, body weight when graduating from high school, height, and current age. This data was used to calculate measures of body mass index (BMI) and weight gain since high school.

#### *Food Frequency Questionnaire*

Each participant was prompted to fill out two food frequency questionnaires (FFQ) upon enrollment in this study. Block et al. (61) designed these instruments that have been validated through research and licensing was received for intended survey use. These food frequency surveys took approximately 5 minutes to complete and helped to provide a snapshot of the patient's diet and offer a simple, inexpensive tool for researchers to assess (61). The meat/snacks survey, described as a brief fat screener, consisted of 15 items designed to assess dietary fat consumption in each patient (61). The fruit/vegetable/fiber survey consisted of 10 items designed to assess fruit and vegetable, fiber, and micronutrient intake in much less time than traditional food records or extensive FFQs (61). The food screener surveys were designed in comparison to the "gold standard" 1995 Block 100-item

Food Frequency Questionnaire (61). The gender and age of each patient were used as potential covariates in order to construct prediction equations using the food screener scores obtained from each patient (61). These equations were developed by Block et al. in order to compute point estimates of selected nutrients such as: total fat, saturated fat, percent fat, fruit/vegetable servings, vitamin C, magnesium, and potassium (61). The equations were used to predict intake of the above-mentioned nutrient classes in this study.

*Prediction equations for Block Dietary Fat Screener (61):*

Total fat (gm) =  $32.7 + [2.4 * (\text{Meat/snack score})] + [11.2 * \text{Sex}]$

Saturated fat (gm) =  $9.4 + [0.88 * (\text{Meat/snack score})] - [3.5 * \text{Sex}]$

Percent fat =  $19.8 + [0.6 * (\text{Meat/snack score})] + [2.3 * \text{Sex}]$

*Prediction equations for Block Fruit-Vegetable-Fiber Screener (61):*

Fruit/vegetable servings (Pyramid definition of servings per day) =  $-0.23 + [0.37 * (\text{Fruit/vegetable score})] - [0.55 * \text{Sex}]$

Vitamin C (mg) =  $56.5 + [6.6 * (\text{Fruit/Veg/Beans score})] - [26.7 * \text{Sex}] - [0.45 * \text{Age}]$

Magnesium (mg) =  $272 + [11.6 * (\text{Fruit/Veg/Beans score})] - [92.3 * \text{Sex}] - [1.7 * \text{Age}]$

Potassium (mg) =  $2348 + [114.8 * (\text{Fruit/Veg/Beans score})] - [759 * \text{Sex}] - [13.8 * \text{Age}]$

Dietary fiber (gm) =  $12.6 + [0.77 * (\text{Fruit/Veg/Beans score})] - [5.12 * \text{Sex}] - [0.16 * \text{Age}]$

## **Laboratory Methods**

*Red blood cell (RBC) fatty acid profile (60)*

Blood samples were collected from trial participants into a 5-mL EDTA vacutainer and transported to the Human Nutrition and Dietetics Research Laboratory at the ETSU Valleybrook campus. Blood samples were processed within 48 hours after withdrawal to ensure validity. To analyze the blood, each sample was centrifuged at room temperature for

10 minutes at 4000 RPM. Once centrifuged, the resulting supernatant composed of plasma was decanted using a Pasteur pipette and transferred to a 3 mL glass amber vial. These vials were stored at -30° C until further analysis for coenzyme Q<sub>10</sub>, vitamin D<sub>3</sub>, and antioxidant potential of the plasma (FRAP). The remaining sample was modified with equal parts normal saline (0.9% NaCl), vortexed briefly to disrupt the RBC pellet, and centrifuged for 5 minutes at 4000 RPM. The clear supernatant layer was removed and discarded. This process was repeated two more times with the clear supernatant layer removed and discarded each time.

#### *Isolation of RBC and lipid extraction (60)*

Kang and Wang (62) developed the procedure for lipid extraction and methylation of RBC, which was modified for use in this experiment. The final RBC pellet was put back into suspension in 25 µL of saline solution (0.9% NaCl) and vortexed. One-hundred µL of the RBC were extracted and transferred to clear, 150 mm screw-top test tubes with Teflon line caps. This step was performed twice due to duplicate samples run. Hexane (2 mL) (Fisher Scientific) and boron trifluoride-methanol solution (2 mL) (BF<sub>3</sub>; Sigma-Aldrich) were added and vortexed in order to methylate the RBC samples. In a heating block, the mixture was heated for one hour at 100°C in order to generate fatty acid methyl esters (FAME). The solution was allowed to cool to room temperature. Deionized distilled water (1.5 mL) (DDW) was added, vortexed briefly, and centrifuged for 1 minute at 4000 RPM. The hexane layer was removed and added to a new, 150 mm screw-top test tube, and dried in a heated water bath at 50° C. Once evaporated, FAME were put back into suspension with 275 µL of hexane and transferred to a 1.5 mL amber glass autosampler vial. These vials were equipped with a low volume insert (300 µL) (Fisher Scientific) and with an internal standard (5 µL, C-17 fatty acid,

Nu-Chek Prep, Inc.) added to validate FAME time signatures for peak integration. Samples were stored at -30° C until analysis by gas chromatography.

#### *Analysis of fatty acids (60)*

Flame ionization gas chromatography (GC) (Shimadzu) was used for analyzing FAME using a capillary column (Phenomenex, Zebron ZB-WAX). The column conditions necessary for analysis included the following: helium as a carrier gas with a flow rate of 30 mL/min and a temperature program of a constant temperature ramp (2° C/minute) between hold temperatures. The temperature was held for 5 minutes at an initial temperature of 160° C; then held for 8 minutes, 10 minutes, 15 minutes, 15 minutes, and 20 minutes at increasing increments of 10° C per hold time from the initial temperature. The total run time for each sample in the GC was 100 minutes. Additional instrument conditions included: autosampler injection of 1 µL; flame ionization detector (FID) temperature of 255° C; injector port temperature of 250° C; hydrogen flow rate of 40 mL/min; and air flow rate of 400 mL/min. Using comparison of known standards, individual peaks were identified and individual fatty acids were measured as a percent of total area under the peak.

#### *FRAP Assay (60)*

The plasma was evaluated for antioxidant capacity using a spectrophotometric technique: ferric reducing ability of plasma (FRAP) assay as described and modified by Benzie and Strain (63). FRAP assay is used to determine total antioxidant capacity in serum samples. The FRAP reagent was prepared fresh before each series of measurements to ensure validity. The reagent contained the following materials: 200 mL of acetate buffer (300mM, pH

3.6), 20 mL TPTZ solution (0.062 g 2,4,6-tripyridyl-s-triazine, or TPTZ, in 20 mL 40mM HCl), 20 mL FeCl<sub>3</sub> solution (0.2748 g FeCl<sub>3</sub>-6H<sub>2</sub>O in 50 mL DDW), and 24 mL DDW. Samples were run in triplicate with 20 µL of serum added to 60 µL of DDW and 1000 µL of FRAP reagent, briefly vortexed, and incubated for 4 minutes at room temperature. The absorbance (592 nm) was determined against a blank vial consisting of 1000 µL of FRAP reagent and 80 µL of DDW. The standard curve was calibrated using a series of standards from diluting 1 mM ferrous sulphate solution. The concentration for the series of dilutions included: 0.1, 0.2, 0.4 at increasing increments until the concentration reached 1.0 mM. FRAP values were determined by UV-visible spectrophotometer.

#### *Coenzyme Q<sub>10</sub>*

The plasma will be evaluated for total Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) using an assay developed by Mosca et al. (64) and modified by W. A. Clark and R. M. Mohseni (personnel communication). Plasma (200 µL) was mixed with 50-µL of 1, 4-benzoquinone solution (2 mg/mL ethanol) and vortexed for 10 seconds. The solution was allowed to incubate for 10 minutes at room temperature. Normal propanol (1 mL, Fisher Scientific) was added to the mixture, briefly vortexed and centrifuged for 9 minutes at 4000 RPM. The supernatant was decanted with a Pasteur pipette, transferred to a 3 mL amber vial and stored at -30°C until further analysis. Analysis of plasma CoQ<sub>10</sub> levels will be completed via high-pressure liquid chromatography with mass spectroscopy using a technique developed by R. M. Mohseni (personnel communication). This analysis will not be completed until all 60 samples have been collected and only the methodology for sample preparation is the subject of this thesis.

### *Statistical analysis*

Mean values and standard deviations were calculated for FRAP, RBC fatty acid profile, RBC fatty acid ratios, and data derived from FFQ surveys and self-reported anthropometric data. Data was analyzed using general linear models program in SPSS version 20 (IBM Corp., Armonk, NY, USA). Case status was identified as the independent variable (case vs. control patients) while data from laboratory analysis and surveys were used in a univariate model as dependent variables. No covariates were used in any analysis. Differences between the values were considered significant if the *P*-values were  $\leq 0.05$ .

## **III. Results**

### *Participants*

Twenty-six participants fulfilling the inclusion criteria were included in this study. Participants wanting to be included in the study signed informed consent documents developed through the IRB approval process. Once enrolled in the trial, all 26 participants filled out a questionnaire related to anthropometric measures, two food frequency questionnaires (FFQ, fruit and vegetable intake, fat intake) and consented to a blood draw. Two groups were formed according to whether the participants' were fertility patients of Dr. Norman Assad (Quillen Fertility and Women's Services) or control patients otherwise experiencing normal ovulation cycles and no evidence of polycystic disease. The case group consisted of 13 women experiencing problems with fertility and having an anti-mullerian hormone level of 1 ng/ml or less (AMH, <1 ng/mL). The mean age of the participants was  $28.7 \pm 1.1$  years for the control group and  $34.5 \pm 0.9$  years for the case group as shown in *Table 1*.

All of the participants were women and were not experiencing issues with polycystic ovarian syndrome. Race and ethnicity were not determining factors for this thesis.

Descriptive statistics pertaining to the participants from basic information acquired from each participant is shown in *Table 1*. Participants in the case group (fertility patients) had significantly higher age at enrollment in the study, body mass index (BMI) when graduating from high school (self-reported), BMI at enrollment in the study (self-reported), and weight gain since high school was approaching significance ( $P=0.081$ ). The average BMI (current) of the control group was  $22.9 \pm 3.89$  and  $29.9 \pm 10.34$  for the case group. Based on BMI value, the case group was borderline obese, but still considered to be overweight (Overweight: 25.0-29.9) (65). The control group, on the other hand, was considered to be at a healthy weight (65). As noted in *Table 1*, participants from the control and case group were considered to be at a healthy weight in high school, however the control group was significantly ( $P=0.03$ ) lighter.

*Table 1: Self-reported anthropometric data of participants reported as mean  $\pm$  std and classified as case or control with the corresponding value of significance. Where (n) equals the number of participants per group.*

<b>Variable</b>	<b>Control mean <math>\pm</math> std, n=13</b>	<b>Case mean <math>\pm</math> std, n=13</b>	<b>P-value</b>
Age (years)	28.7 $\pm$ 4.13	34.5 $\pm$ 3.48	0.001
Wt. gain since high school (lbs.)	16.5 $\pm$ 17.11	37.38 $\pm$ 37.60	0.081
High School BMI (kg/m <sup>2</sup> )	20.2 $\pm$ 2.88	23.5 $\pm$ 4.43	0.030
Current BMI (kg/m <sup>2</sup> )	22.9 $\pm$ 3.89	29.9 $\pm$ 10.34	0.030

*Measures of Oxidative Stress*

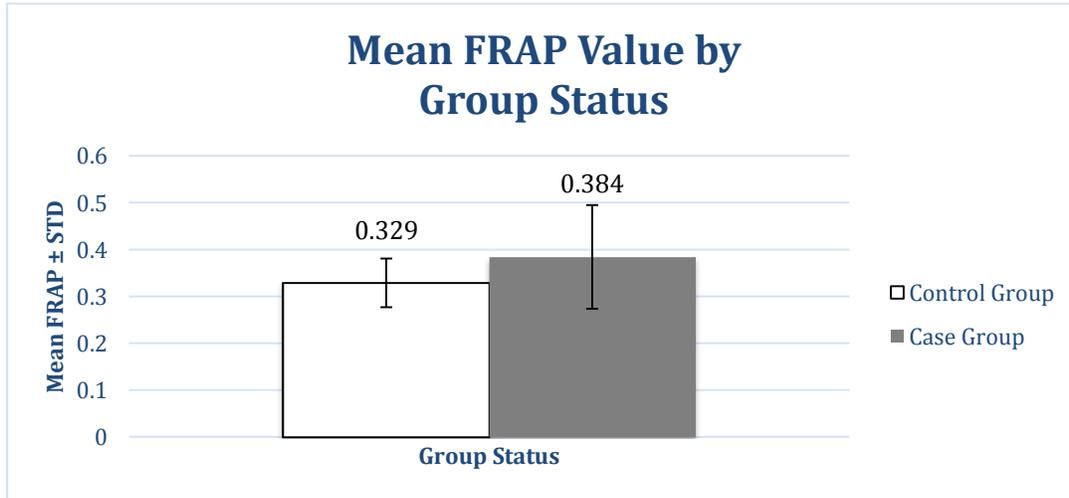
A total of 26 FRAP assays (13 per group) were conducted and included in the analysis for this study as noted in *Table 2* and *Graph 1*. The average concentration of FRAP values for the case group (n=13) was  $0.384 \pm 0.111 \mu\text{mol/L}$ , while the average FRAP value for the control group (n=13) was  $0.329 \pm 0.052 \mu\text{mol/L}$  (mean  $\pm$  std). Although the difference between the two groups was not considered significant, the level was approaching significance ( $P=0.12$ ). Most of the participants in the case group had higher mean FRAP concentrations when compared to control participants. The higher antioxidant assay of the plasma was surprising especially since the control group participants were shown to consume more antioxidants as seen in the results of the food frequency questionnaires.

Plasma from this study will be evaluated for CoQ<sub>10</sub> concentration and this information, in addition to the FRAP analysis, may be useful in assessing the true antioxidant status of the participants in this study. However, analysis of CoQ<sub>10</sub> levels in the plasma will not be completed until all 60 samples have been collected and only the methodology for sample preparation is the subject of this thesis. Once all samples have been collected, analysis of plasma CoQ<sub>10</sub> levels will be evaluated via high-pressure liquid chromatography with mass spectroscopy using a technique developed by R. M. Mohseni (personnel communication).

*Table 2: Analysis from FRAP Assays reported as mean  $\pm$  std and classified as case or control with the corresponding value of significance. Where (n) equals the number of participants per group.*

	<b>Control mean <math>\pm</math> std, n=13</b>	<b>Case mean <math>\pm</math> std, n=13</b>	<b>P-value</b>
<b>FRAP Assay</b>	$0.329 \pm 0.052 \mu\text{mol/L}$	$0.384 \pm 0.111 \mu\text{mol/L}$	0.120

Graph 1: Average FRAP value by case and control status. Values are reported as mean  $\pm$  std.



#### Responses to Food Frequency Questionnaire

Descriptive statistics pertaining to the food frequency questionnaires are found in Table 3. The data is derived from prediction equations gleaned from the answers reported in the FFQs. Prediction equations were developed by Block et al. and are validated as reported in the rapid food screener study (61). Two separate FFQs were used in this study: dietary fat survey and dietary fruit-vegetable-fiber survey. The mean results of the FFQs revealed that case participants consumed significantly higher nutrients in each of the following categories: total fat (g), saturated fat (g), unsaturated fat (g), and percent fat. The average total fat consumption reported for the control group was  $85.8 \pm 12.80$  g and  $96.9 \pm 16.67$  g for the case group; these values were approaching significance ( $P=0.069$ ). The average saturated fat consumption reported for the control group was  $22.0 \pm 6.61$  g and  $25.4 \pm 6.11$  g for the case group; however, these values were considered insignificant ( $P=0.197$ ). The average unsaturated fat consumption reported for the control group was  $63.8 \pm 7.02$  g and  $71.5 \pm 10.56$  g for the case group; these values were considered significant ( $P=0.037$ ). Unsaturated

fat consumption was calculated by subtracting the saturated fat (g) from the total fat (g) as determined by the prediction equations in the FFQ. The average percent fat reported for the control group diet was  $32.6 \pm 3.19\%$  and  $35.3 \pm 4.17\%$  for the case group; these values were approaching significance ( $P=0.071$ ).

The responses from the fruit and vegetable questionnaire revealed that control participants consumed approximately 68% more servings of fruits and vegetables than the case group, which was  $4.2 \pm 1.55$  and  $2.9 \pm 1.04$  servings per day, respectively ( $P=0.019$ ). These results were consistent with the higher amount of consumed nutrients calculated by the fruit and vegetable FFQ as seen in control participants noted in *Table 3*. The average vitamin C (mg) reported for the control group was  $143.4 \pm 51.15$  mg and  $99.6 \pm 27.45$  mg for the case group; these values were considered significant ( $P=0.012$ ). The average magnesium (mg) reported for the control group was  $353.0 \pm 90.40$  mg and  $270.9 \pm 48.27$  mg for the case group; these values were considered significant ( $P=0.008$ ). The average dietary fiber (g) reported for the control group was  $17.7 \pm 6.06$  g and  $11.9 \pm 3.21$  g for the case group; these values were also considered significant ( $P=0.006$ ). Finally, the average potassium (mg) reported for the control group was  $3113.9 \pm 1195.39$  mg and  $2595.9 \pm 477.41$  mg for the case group; however, these values were considered insignificant ( $P=0.160$ ).

Data was also developed for saturated fat (g) and unsaturated fat (g) as a percent of total fat (g) and the ratio between the two. The average saturated fat as a percent of total fat was  $25.3 \pm 3.72$  for the control group and  $25.9 \pm 1.91$  for the case group ( $P=0.601$ ). The average unsaturated fat as a percent of total fat was  $74.4 \pm 3.72$  for the control group and  $74.1 \pm 1.91$  for the case group ( $P=0.601$ ). Finally, the ratio of saturated to unsaturated fat, as a

percent of the total fats, was reported for the control group as  $0.34 \pm 0.74$  and  $0.35 \pm 0.36$  for the case group ( $P=0.641$ ).

*Table 3: Data from Food Frequency Questionnaires with the corresponding value of significance for each variable. Where (n) equals the number of participants per group.*

Variable	Control mean $\pm$ std, n=13	Case mean $\pm$ std, n=13	P-value
Total fat (g/day)	85.8 $\pm$ 12.80	96.9 $\pm$ 16.67	0.069
Saturated fat (g/day)	22.0 $\pm$ 6.61	25.4 $\pm$ 6.11	0.197
Unsaturated fat (g/day)	63.8 $\pm$ 7.02	71.5 $\pm$ 10.56	0.037
Percent Dietary fat (%)	32.6 $\pm$ 3.19	35.3 $\pm$ 4.17	0.071
Saturated fat (% of total fat)	25.3 $\pm$ 3.72	25.9 $\pm$ 1.91	0.601
Unsaturated fat (% of total fat)	74.4 $\pm$ 3.72	74.1 $\pm$ 1.91	0.601
Saturated: Unsaturated calculated ratio	0.34 $\pm$ 0.74	0.35 $\pm$ 0.36	0.641
Fruit/Vegetable (servings/day)	4.2 $\pm$ 1.55	2.9 $\pm$ 1.04	0.019
Vitamin C (mg/day)	143.3 $\pm$ 51.15	99.6 $\pm$ 27.45	0.012
Magnesium (mg/day)	353.0 $\pm$ 90.40	270.9 $\pm$ 48.27	0.008
Potassium (mg/day)	3113.9 $\pm$ 1195.39	2595.9 $\pm$ 477.41	0.160
Dietary Fiber (g/day)	17.7 $\pm$ 6.06	11.9 $\pm$ 3.21	0.006

#### *Analysis of RBC Fatty Acid Composition*

Fatty acid composition of red blood cells for case and control patients is displayed in *Table 4* and *Table 5* regarding the results of individual fatty acid profiles as well as correlated ratios of average saturated FA, average unsaturated FA, monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). This research study hypothesized that the case group would consume more saturated fat, have lower levels of omega-3 fatty acids, higher levels of omega-6 fatty acids, and have a higher saturated to unsaturated fatty acid ratio. Results from the analysis of the red blood cell membrane fatty acid profile do not support this hypothesis. The average omega-6 fatty acids reported from the control group was  $13.67 \pm 2.42$  and  $14.20 \pm 2.65$  for the case group ( $P=0.600$ ) while the average omega-3 fatty

acids reported from the control group was  $2.28 \pm 0.67$  and  $1.52 \pm 1.35$  for the case group ( $P=0.426$ ). The average fatty acids of total saturated fat reported from the control group were  $18.77 \pm 4.17$  and  $18.81 \pm 4.19$  for the case group ( $P=0.979$ ). However, the data did show some numerical trends consistent with expected results. The control group consumed less omega-6 fatty acids, higher amounts of omega-3 fatty acids, and lower amounts of total saturated fatty acids. The patient population included in this study is less than half of the complete study population and final results still need to be evaluated once the research trial completes patient enrollment.

*Table 4: Analysis of Individual Fatty acid profiles of RBC membranes for cases and controls. Fatty acids are expressed by average % area under the peak and reported as mean  $\pm$  std; n. Where (n) equals the number of participants.*

<b>Fatty Acid, omega type, carbon:double bonds</b>	<b>Control mean <math>\pm</math> std, n</b>	<b>Case mean <math>\pm</math> std, n</b>	<b>P-value</b>
Myristic, 16:0	$1.43 \pm 0.52, 8$	$1.32 \pm 0.83, 8$	0.764
Palmitic, 16:0	$26.56 \pm 2.24, 13$	$25.75 \pm 2.44, 13$	0.385
Palmitoleic (n-7), 16:1	$2.08 \pm 0.65, 12$	$2.49 \pm 0.90, 11$	0.224
Stearic, 18:0	$21.34 \pm 1.75, 13$	$21.65 \pm 1.13, 13$	0.588
Oleic (n-9), 18:1	$13.72 \pm 1.36, 13$	$13.76 \pm 0.92, 13$	0.928
Linoleic (n-6), 18:2	$13.79 \pm 1.51, 13$	$13.51 \pm 1.63, 13$	0.655
Arachidonic (n-6), 20:4	$19.62 \pm 1.65, 13$	$19.99 \pm 2.47, 13$	0.655
EPA (n-3), 20:5	n=0	1.05, 1	n/a
Adrenic(n-6), 22:4	$2.09 \pm 1.13, 8$	$2.94 \pm 1.58, 7$	0.252
DPA (n-3), 22:5	n=0	0.94, 1	n/a
Lignoceric, 24:0	n=0	1.76, 1	n/a
DHA (n-3), 22:6	$2.28 \pm 0.67, 3$	$3.55 \pm 0.95, 2$	0.171

*Table 5: Analysis of Fatty Acid Ratios. Fatty acids are expressed by average % area under the peak and reported as mean  $\pm$  std; n. Where (n) equals the number of participants.*

<b>Fatty Acid Types &amp; Ratios (% area under chromatogram curve)</b>	<b>Control mean <math>\pm</math> std, n</b>	<b>Case mean <math>\pm</math> std, n</b>	<b>P-value</b>
Average Omega-6	13.67 $\pm$ 2.42, 13	14.20 $\pm$ 2.65, 13	0.600
Average Omega-3	2.28 $\pm$ 0.67, 3	1.52 $\pm$ 1.35, 3	0.426
Omega-6:Omega-3 Ratio	5.35 $\pm$ 1.71, 3	5.43 $\pm$ 1.31, 2	0.959
MUFA: PUFA	0.45 $\pm$ 0.06, 13	0.45 $\pm$ 0.06, 13	0.984
Total Saturated FA	18.77 $\pm$ 4.17, 13	18.81 $\pm$ 4.19, 13	0.979
Total Unsaturated FA	10.99 $\pm$ 1.89, 13	11.33 $\pm$ 1.85, 13	0.643
Saturated: Unsaturated Ratio	1.72 $\pm$ 0.32, 13	1.67 $\pm$ 0.32, 13	0.715

#### **IV. Discussion**

##### *Participants*

The aim of our study was to explore the possible relationship between oxidative stress and the fecundity of women. Therefore, in order to form an accurate comparison between oxidative stress (OS) and fertility, case participants consisted of fertility patients of Dr. Norman Assad between the ages of 25 and 40 with a low anti-mullerian hormone level (AMH, <1 ng/mL) experiencing fertility difficulties. Control patients were compared to the case participants, which consisted of women ages 25 to 40. Exclusion criteria for controls were women experiencing irregular menstrual cycles and the presence of polycystic ovarian syndrome. The subject for this thesis includes 26 patients; 13 women were recruited for the case group and 13 women for the control group. Participants understood that this research was completely voluntary and could discontinue at any time. No participants opted out of the trial or were excluded from the data. The mean age of the participants was  $28.7 \pm 4.13$  years for the control group and  $34.5 \pm 3.48$  years for the case group as shown in *Table 1*.

Participants in the case group (fertility patients) had significantly higher age upon enrollment in the study, which could be an overall factor in fertility. The peak age range for

female fertility is 25 to 35 years (50). One of the theories that explains why older patients experience poor reproductive performance is the decline in oocyte quality (50). This theory goes on to explain that the highest-quality oocytes are selected first, leaving less favorable oocytes to be selected in later reproductive years (50). The effects of aging are still widely unknown, however the accumulation of cellular mutations and deletions within mitochondrial DNA has been noted (50). Our results indicate that the volunteers who met the age restriction criteria and enrolled in the research trial as control patients are significantly younger than the case group experiencing infertility. Age in our test population may be a factor in the case groups' reduced reproductive potential and explain why the women undergoing fertility treatments are older.

Participants in the case group reported significantly higher body mass index (BMI) when graduating from high school ( $P=0.03$ ), BMI upon enrollment in the study ( $P=0.03$ ), and weight gain since high school approached significance ( $P=0.081$ ). The average BMI (current) of the control group was  $22.9 \pm 3.89$  and  $29.9 \pm 10.34$  for the case group. Based on BMI value, the case group was borderline obese, but still considered to be overweight (Overweight: 25.0-29.9) (65). As noted in *Table 1*, participants from the control and case group were considered to be at a healthy weight in high school; however, the control group was significantly lighter ( $p=0.03$ ). Weight may be considered another significant factor in infertility. The effects of obesity have been shown to correlate adversely with fertility and may be one of the neglected complications of infertility (2, 3, 4, 5, 6, 7, 19). Anovulation, or a menstrual cycle without release of an oocyte, which is increased with obesity, may account for the delayed time in pregnancy (4, 19). Studies using mouse models were able to establish clearer results that concluded in showing that diet-induced obesity is harmful to oocytes (19). These mice models

exhibited delayed maturation, reduced rates of *in vivo* fertilization, and decreased oocyte development (6, 53). Thus, it can be concluded that obesity negatively impacts oocyte development and ovulation (6, 53). The risk of infertility in obese women is almost three times as likely in comparison to non-obese women (3), which is consistent with the participant results seen in this study.

### *Measures of Oxidative Stress*

Ferric reducing antioxidant power (FRAP) assays were conducted on each of the participants and included in the analysis of this study. Results from the FRAP assays are noted in *Table 2* and *Graph 1*. Surprisingly, the ferric reducing ability of the plasma in case group participants was found to have a numerically higher antioxidant capacity ( $P=0.12$ ) as compared to participants in the control group. The average concentration of FRAP values for the case group ( $n=13$ ) was  $0.384 \pm 0.111 \mu\text{mol/L}$ , while the average FRAP value for the control group ( $n=13$ ) was  $0.329 \pm 0.052 \mu\text{mol/L}$  (mean  $\pm$  std). A possible explanation to this result could be due to the case group participants' antioxidant defense system being in a hyperactive state in order to compensate for the amount of oxidative stress within the body. This result is consistent with other models comparing the antioxidant capacity of dogs with cardiac disorders and that of controls (66, 67). Researchers from these studies found increased levels of plasma antioxidants (FRAP) in dogs with heart disease and other cardiac problems (66, 67). The researchers from these studies found a possible explanation to be increased uric acid levels, which could increase FRAP levels (66). Uric acid or other compensatory mechanisms may be contributing to increased antioxidant level exhibited by the case group. Levels of uric acid were not measured in this research trial. The differences

between the antioxidant levels of the groups in this study did not reach significance ( $P=0.12$ ), however, these results may become more significant once all participants are recruited for this study. Additional assays of antioxidant capacity should be considered in order to determine if uric acid was a factor in this measure of antioxidant potential.

### *Food Frequency Questionnaires*

Within the data self-reported by participants, which includes the responses from the food frequency questionnaires, several variables were evaluated and showed a significant difference between control and case groups. These variables can be found in *Table 3*. Results of the FFQs revealed that case participants consumed significantly higher amounts of: total fat (g), saturated fat (g), unsaturated fat (g), and their diets had a higher dietary fat percent. The average total fat consumption reported for the control group was  $85.8 \pm 12.80$  g and  $96.9 \pm 16.67$ g for the case group; these values were approaching significance ( $P=0.069$ ). Data was also developed for saturated fat and unsaturated fat as a percent of total fat consumed and the ratio between the two (Saturated:Unsaturated). The average saturated fat as a percent of total fat was  $25.3 \pm 3.72$  % for the control group and  $25.9 \pm 1.91$ % for the case group ( $P=0.601$ ). The average unsaturated fat as a percent of total fat was  $74.4 \pm 3.72$  % for the control group and  $74.1 \pm 1.91$  % for the case group ( $P=0.601$ ). Finally, the ratio of saturated to unsaturated fat, as a percent of the total fats, was reported for the control group as  $0.34 \pm 0.74$  and  $0.35 \pm 0.36$  for the case group ( $P=0.641$ ). These results were consistent with the red blood cell membrane fatty acid, which can be found in *Table 5*.

The fruit and vegetable FFQ revealed that control participants consumed approximately 68% more servings of fruits and vegetables than the case group, which was 4.2

$\pm 1.55$  and  $2.9 \pm 1.04$  servings per day, respectively ( $P=0.027$ ). The higher fruit and vegetable consumption by the control group is reflected in the nutrient analysis. There is sufficient evidence to suggest that dietary antioxidants, including vitamins, minerals, and specific cofactors may impact oxidative stress and thus play a major role female fertility (8, 40, 41). There are many proposed beneficial effects of antioxidants, folate, and flavonoids, including scavenging free radicals and ROS, which could potentially reduce inflammation and oxidative stress (40). Antioxidants such as vitamins C and E have shown to be helpful in reducing stress to ova, sperm, and reproductive organs (16, 41). Other nutrients shown to improve female reproduction and fertility include: vitamin B<sub>12</sub>, folic acid, vitamin E, magnesium, selenium, iron, and zinc (16, 41, 42, 43, 44).

#### *Analysis of RBC Fatty Acid Profile*

Fatty acid composition of red blood cell membranes for case and control patients is found in *Table 4* and *Table 5*. Results of individual fatty acid profiles as well as correlated ratios of average saturated FA, average unsaturated FA, MUFAs, and PUFAs are indicated in these tables. This research study hypothesized that the case group would consume more saturated fat, have lower levels of omega-3 fatty acids, higher levels of omega-6 fatty acids, and have a higher saturated to unsaturated fatty acid ratio. However, results from the analysis of the red blood cell membrane fatty acid profile did not support this hypothesis. The average omega-6 fatty acids reported from the control group was  $13.67 \pm 2.42$  and  $14.20 \pm 2.65$  for the case group ( $P=0.600$ ) while the average omega-3 fatty acids reported from the control group was  $2.28 \pm 0.67$  and  $1.52 \pm 1.35$  for the case group ( $P=0.426$ ). The ratio of omega-6 to omega-3

fatty acids was calculated at  $5.35 \pm 1.71$  for the control group and  $5.43 \pm 1.31$  for the case group ( $P=0.959$ ).

The predominant omega-6 PUFA in humans is linoleic acid (LA), which can be elongated and desaturated to arachidonic acid (AA) (55). AA is usually the major substrate for eicosanoid synthesis, which is a process involved in modulating the intensity and duration of inflammatory responses, and increases proinflammatory cytokine production (54, 58). Eicosanoids derived from AA are considered pro-inflammatory in comparison to those derived from omega-3 PUFAs, EPA, which curb the production of AA-derived eicosanoids (55, 58). Increased availability of long chain omega-3 fatty acids could potentially provide eicosanoid synthesis derived from EPA and DHA than AA, thus reducing inflammation and certain diseases. Therefore, higher levels of omega-3 PUFAs and lower omega-6 to omega-3 PUFA ratios have been associated with lower proinflammatory cytokine production (58). The increased ratio of omega-6 to omega-3 EFA in individuals who consume a Western diet also may potentially contribute to fertility problems (59). Recent research has emphasized the importance of consumption and supplementation of these essential fatty acids in pre- and postnatal outcomes in development (14).

On the other hand, some researchers found that the dietary intake and increased content of omega-3 fatty acids may be detrimental to health due to the formation of toxic products caused by ROS-induced oxidative peroxidation of omega-3 EFA within membrane phospholipids (14). Exposure to dietary omega-3 fatty acids has been shown to disturb mitochondrial metabolism and reduce embryonic development in women in early pregnancy (14). This statement is consistent with the findings of a study involving the oocytes of adult female mice in order to determine how omega-3 PUFA supplementation might influence

oocyte and pre-implantation embryo development (23). Evidence suggests that dietary omega-3 PUFA levels may alter the fatty acid composition of the oocyte, thus potentially impacting oocyte maturation and development (23).

Although the ratio of omega-6 to omega-3 was found to be higher in the case group versus the control group, the data was not statistically significant ( $P=0.959$ ). The data was able to show some numerical trends consistent with expected results, however. The control group consumed higher amounts of omega-3 fatty acids (FA), lower amounts of omega-6 FA, and lower total saturated fatty acids. The statistical insignificance of omega-6 to omega-3 FA shown could be attributed to the insignificant amount of omega-3 FA analyzed from each participant's fatty acid profile; only 5 participants had omega-3 FA in the RBC. Further interpretation may be possible once all 60 participants have been recruited and complete the study.

### *Conclusion*

In the summer of 2014, all sixty trial participants will have been evaluated and analyzed for their antioxidant capacity, complete fatty acid profile including ratios of omega-6 to omega-3 and saturated to unsaturated fatty acids, levels of coenzyme Q<sub>10</sub>, along with the FFQs for each subject. This thesis consists of only preliminary data, as the study will not conclude until a total of 60 patients have been recruited and meet the inclusion requirements. The complete trial should supply more conclusive and significant results regarding each variable. This project has been designed to test the hypothesis that women undergoing more oxidative stress are more likely to have problems with fertility. The results of the conclusion of the trial may be able to indicate whether the proposed hypothesis is correct; however, the initial results are still inconclusive at this time.

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Appendix

Permission Letter for Use of Material in Thesis

Tasha Harris  
2108 Welch Road,  
Kingsport, TN 37660

December 11, 2013.

Dear Ms. Nicole Litwin:

I am completing an undergraduate thesis at East Tennessee State University, Johnson City, Tennessee. The thesis title is: *The Impact of Oxidative Stress on Female Fertility*. Due to the similarities of the laboratory methods used in both studies, I would like your permission to reprint in my thesis excerpts from the following:

Assessment of red blood cell membrane fatty acid composition in relation to dietary intake in patients undergoing cardiac catheterization.

The following sections include information developed by you and the above mentioned: Red blood cell (RBC) fatty acid profile, Isolation of RBC and lipid extraction, Analysis of fatty acids, and FRAP assay.

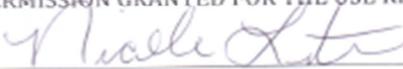
The requested permission extends to any future revisions of my thesis, to the public circulation of my thesis by East Tennessee State University, and to the prospective reproduction of the thesis by East Tennessee State University.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed envelope. Thank you very much.

Yours truly,

Tasha Harris

PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

  
\_\_\_\_\_  
[Nicole Litwin]

Date: 12/11/13