

M.Sc. Thesis – Jessica Lenchyshyn; McMaster University – McMaster Integrative Neuroscience Discovery & Study Program (MiNDS)

RELATIONSHIP BETWEEN OXIDATIVE STRESS AND COMBINED ORAL
CONTRACEPTIVE USE IN WOMEN WITH BIPOLAR DISORDER

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CONTRACEPTIVE USE IN WOMEN WITH BIPOLAR DISORDER

By: JESSICA LENCHYSHYN, B.A.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the
Requirements for the Degree Masters of Science

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M.Sc. Thesis – Jessica Lenchyshyn; McMaster University – McMaster Integrative
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TITLE: Relationship Between Oxidative Stress and Combined Oral Contraceptive Use In
Women With Bipolar Disorder

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ABSTRACT

(iii)

Background: The objective of this thesis was to measure oxidative stress (OS) in women with Bipolar Disorder (BD) who used combined oral contraceptives (OCU). Based on our literature review, it was predicted that OCU would increase OS levels relative to non-contraceptive users (NCU) in women. Methods: Thirty-five participants (BD n=25; Control n=10) were recruited from an ongoing study based in British Columbia ‘The Systematic Treatment Optimization Program in Early Mania.’ Participants were administered psychological screening tools (Young Mania Rating Scale (YMRS), Montgomery-Åsberg Depression Rating Scale, Mini International Neuropsychiatric Interview and Hamilton Depression Rating Scale) and provided a blood sample for the assays (Lipid Hydroperoxide (LPH), Protein Carbonylation, 4-Hydroxynonenal, 3-Nitrotyrosine (3-NT) and 17-Beta Estradiol). Results: In our primary analysis we did not find differences in OS between BD and controls relative to OCU. Within our remaining analyses, only BD women (n=17) and who gave smoking status were included. We found 3-NT to be increased in OCU compared to NCU ($F(1, 12) = 5.639, p = 0.035$). With respect to mood stabilizer use, 3-NT was increased in OCU relative to NCU ($F(1, 10) = 6.33, p=0.031$). As for atypical antipsychotics, 3-NT was heightened in OCU adjunctive users compared to NCU who did not use atypical antipsychotics ($F(3, 10) = 4.822, p = 0.025$). As for our correlation analyses, YMRS correlated with 3-NT and LPH in OCU BD women ($r(11) = 0.711, p=0.014$ and $r(11) =$

0.676, $p=0.022$, respectively) and 17-Beta Estradiol correlated with LPH ($r(17) = 0.598$, $p = 0.001$). Our results are preliminary and are limited by our small sample size and various other factors (i.e. controls). Conclusion: The association between hormones and oxidative stress still remains controversial. Here we showed, after controlling for smoking, BMI and age the use of a COC significantly increased 3-NT in women with BD. Moreover, hormones may influence the relationship between OS and mood episodes.

DEDICATION

I dedicate this thesis to my parents, who developed my passion, and interest in pursuing a career in research from a young age, thank you.

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LIST OF ABBREVIATIONS AND SYMBOLS

BMI – body mass index

OCU – Oral Contraceptive Use

NCU – No Contraceptive Use

ROS – Reactive Oxygen Species

COC – Combined Oral Contraceptive

OCT – Oral Contraceptive Therapy

3-NT – 3-Nitrotyrosine

4-HNE – 4-Hydroxynonenal

PCC – Protein Carbonyl Content

LPH – Lipid Hydroperoxides

DECLARATION OF ACADEMIC ACHIEVEMENT

I hereby certify that the material presented in this thesis towards completion of a Masters (M.Sc.) degree in the McMaster Integrative Neuroscience Discovery and Study program, at McMaster University, Hamilton, Ontario is exclusively my own work and has not been submitted for any academic assessment other than partial-fulfillment of the degree named above.

Signature of candidate: *Jessica Lenchyshyn*.....

Date: September 25, 2014.....

Abstract

Objectives: Previous clinical studies have suggested an association between endogenous and exogenous hormones as a potential source for pathological diseases and disorders. Presently there are no well-researched biomarkers to explain this relationship, thus the disorders etiology remains largely unknown. One prominent biomarker that is present in these pathological disorders is oxidative stress. The largest source of oxidative stress is the mitochondria. Mitochondria biosynthesize, regulate and are controlled by hormones. Here, we summarize the current literature on combined oral contraceptive use and oxidative stress in women. In addition, our secondary focus will be to critically review and present several hypotheses explaining the relationship between hormones and oxidative stress.

Methods: A search was conducted using the terms *combined oral contraceptive* and *oxidative stress* within the databases: MedLine, PsychINFO, PubMed and Embase. 8 articles were identified and the results were summarized. In addition a review of possible causative links between COC use and oxidative stress were presented and discussed.

Results: The majority of reviewed studies showed women using combined oral contraceptives had increased acute and chronic oxidative stress markers indicative of lipid peroxidation.

Conclusions: The relationship between lipid peroxidation and combined oral contraceptives warrants further investigation, as hormones may be relevant in the etiology of several human pathologies via oxidative stress.

Key Words: combined oral contraceptive– oxidative stress

CHAPTER 1

Introduction

The Centers for Disease Control and Prevention National Center for Health Sciences (NCHS) recently reported that 4 in 5 reproductive aged women use combined oral contraceptives (COC) (Daniels & Mosher, 2013). Contemporary COC formulas contain an ethinyl estradiol (EE) used concomitantly with one of a number of progestational synthetic agents that vary in potency, affinity for steroid receptors, interaction with estrogens and physiological effects (Kaunitz, 2004). Combined oral contraceptives are used to prevent pregnancy by preventing ovulation through inhibition of gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormones (LH) (Borgelt-Hansen, 2001). In addition COCs confer a number of significant non-contraceptive related benefits to users. For example, the use of COC to control menstrual abnormalities (i.e. premenstrual dysphoric disorder) and to decrease blood loss, incidence of iron deficiency anemia, dysmenorrhea (Masimasi, Sivanandy, & Thacker, 2007), and to reduce the incidence of endometrial (Huber, Bentz, Ott, & Tempfer, 2008; Mueck, Seeger, & Rabe, 2010) and ovarian cancers (Bosetti et al., 2002). Although COCs are safe there are concerns regarding adverse biological diseases/disorders. These concerns being COCs association with increased incidence with blood clot formation (Piparva & Buch, 2011; Voelker, 2011), and cardiovascular diseases among women who have a predisposition due to either smoking, age (35 and above), obesity, diabetes or hypertension (Nessa, Latif, & Siddiqui, 2006).

Not surprisingly this increased risk has gained attention amongst the medical and research community. The initial explanation centered on the chemical composition of COC as the source, which led to the improvement of COCs formula and potency, mainly decreasing EE from 50 mcg to < 30 mcg and using new progestin agents (Kaunitz, 2004). These alterations have subsequently reduced the incidence of venous thromboembolism (Gerstman et al., 1991) and cardiovascular related symptoms (Dinger, Heinemann, & Kühl-Habich, 2007) and efforts to further reduce EE to decrease COC linked adverse biological events are an ongoing process. While this is very reassuring, there are disorders and conditions that may be the product of underlining endogenous and exogenous (COC) hormones that have not been reduced following these changes in COCs. These disorders and conditions being movement disorders (and chorea)(Goldzieher, 1989), migraines (Tassorelli, Greco, Allena, Terreno, & Nappi, 2012) and possibly breast cancer (Beaber et al., 2014). Presently the exact relationship between hormones and the above disorders remains elusive.

In 1997 Sies published a paper introducing the concept of oxidative stress, since then their findings have gained significant momentum within the field of pathology as oxidative stress is increased and therefore hypothesized to be associated with the pathophysiology of neurodegenerative diseases and disorders. Oxidative stress is defined as the condition in which the body's antioxidant system is overwhelmed by reactive oxygen species (ROS) (Sies, 1997). Reactive oxygen species are a natural byproduct of the electron transport chain (ETC). The electrons move along the ETC and this flow of electrons generates a proton transfer across the inner mitochondrial membrane, which

creates the electrochemical gradient (Sies, 1997). This electrochemical gradient is utilized to create adenosine triphosphate (ATP) and the entire process is called oxidative phosphorylation (Sies, 1997). As previously mentioned, a natural occurring event is for electrons to escape during this process and react with molecular oxygen creating ROS. These entities have an unpaired electron, which makes them highly reactive. In lieu of this, the body has an endogenous defense system, for example antioxidants and enzymes to turn radicals into non-radicals by donating an electron (Sies, 1997). In situations where ROS increases and overpowers the vital defense system, oxidative stress is produced and can lead to reversible and irreversible damage to proteins, lipids and DNA (Sies, 1997).

Oxidative stress has been linked to several human pathologies (Emiliani, Sedlak, & Sawa, 2014; Hoffer, Osmond, & Smythies, 1954). The exact source(s) that produces excess ROS, which leads to oxidative stress induced pathology (i.e. autoimmune and psychiatric disorders), remains unknown. Reactive oxygen species can be created from both endogenous and exogenous sources (Sies, 1997). Endogenous sources include the mitochondrial electron transport chain, fagocitose, inflammation and catecholamine's degeneration (Halliwell & Whiteman, 2004). While exogenous sources include (but not limited to) smoking, UV radiation, pollution, toxins and pesticides (Halliwell & Whiteman, 2004). Recently acknowledged sources (both endogenous and exogenous) that have been identified are synthetic and non-synthetic hormones. Both 17-beta Estradiol (E2) and progesterone action on oxidative stress have been recently reviewed (Frey & Dias, 2014); as such will not be extensively discussed here. The general consensus is estrogen has antioxidant qualities and ergo reduces oxidative stress, notably lipid

peroxidation (Razmara et al., 2008; Stirone, Duckles, Krause, & Procaccio, 2005).

Progesterone's antioxidant role remains elusive. However, when estrogen and progesterone are co-administered, the beneficial effects of estrogen have been reported to be mitigated (Irwin et al., 2008). Indeed both Kose et al. (1993) and Sissan et al. (1995) noted an increase in oxidative stress following COC administration in rats. In corroboration clinical studies have provided convincing evidence that hormones, specifically progesterone only contraceptives, for example sub-dermal implants of levonorgestrel (Subakir, Abdul Madjid, Sabariah, & Affandi, 2000) or injectable depot medroxy progesterate acetate showed elevated levels of lipid peroxides (Faddah, Al-Rehany, Abdel-Hamid, & Bakeet, 2005). Moreover, clinical studies have revealed that COC use can cause a decrease in serum plasma antioxidant B-carotene (Berg, Kohlmeier, & Brenner, 1997), and lipid soluble antioxidants such as coenzyme Q10 and alpha-tocopherol (Palan, Magneson, Castillo, Dunne, & Mikhail, 2006). As previously mentioned, these antioxidants are important as they provide support to control ROS levels and aid in proper mitochondrial functioning. What influences mitochondrial functioning is of utmost importance as these organelles are the main producers of oxidative stress in the body. The relationship between hormones and mitochondria is intrinsic as mitochondria biosynthesize, regulate and are controlled by the sex hormones, particularly estrogen (Klinge, 2008). This is what gave hormones a prominent role in oxidative stress.

Unfortunately a paucity of studies are available and currently conflict which has made it difficult to establish whether hormones are pro or antioxidants. Thibodeau et al. (2002) postulated that the different results could also be attributed to either the chemical

heterogeneity of the estrogen and progesterone used, or whether or not the dose was administered at the correct physiological level. Albeit controversial, researchers are beginning to acknowledge estrogen has pro-oxidant actions that warrant further investigation.

As reviewed above, clinical studies suggest an association between combined oral contraceptive use and adverse biological events. Based on the following premises (1) oxidative stress is a biomarker for detecting pathology (2) mitochondria are the main producers of oxidative stress (3) mitochondria biosynthesize, regulate and are controlled by sex hormones, we felt this relationship was worthy of investigation. Here we reviewed the literature and summarized the current knowledge concerning oxidative stress and use of COCs in humans.

Methods

Search Strategy

A prospective protocol for this literature review was developed a priori with search terms and inclusion criteria chosen was stringent in an attempt to include only publications that matched our interest. The databases MedLine, PubMed, Embase and PsychINFO were used and searched for the terms *oxidative stress* and *oral contraceptive* (and relevant meSH terms). References cited in the retrieved publications were searched for relevant studies that were not identified in the initial search; no limit on year of publication was used in this review. The endpoint for this literature review was June 2014.

Selection Criteria

One reviewer screened all abstracts of potentially relevant publications. Studies were included if they met the following criteria: (1) measured levels of one or more of the following oxidative stress markers: F2-isoprostanes, lipid hydroperoxides (LPH), malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), protein carbonyl content (PCC), oxidized LDL, d-ROMS, and 8-hydroxy-2'-deoxyguanosine; (2) participants were human; (3) were reported in an original paper in a peer-reviewed journal; (4) participants were taking combined oral contraceptives at the time of blood draw; (5) separated results for oral contraceptive use (OCU) vs. non contraceptive use (NCU) in women; (6) if they adequately described their methodology (e.g. diagnostic criteria, source of samples); and (7) articles were in English. For all included studies, oral contraceptive type, duration use, sample, marker, assay and results were recorded.

A combined total of thirty-six references from the four databases (PubMed, MedLine, PsychINFO and Embase) met our search terms were identified. We did not find any additional relevant research through the identified references. Eight ARTICLES were relevant according to our a priori scripted criteria.

Results

Table 1.

Table summarizing the studies included in this review on Combined Oral Contraceptive Use and Oxidative Stress

Study	N	Age (yrs ± SD)	COC Duration (mos)	Sample	Marker	Assay	Results of OCU compared to NCU
Chen et al. (2012)	OCU n=24 NCU n=62	40 ± 7.9 43 ± 6.6	21 tablets of 0.035 mg of EE, 12 tablets of 0.5 mg NET and 9 tables of 1.0 mg NET 6 months	Serum	Lipid per.	d-ROM	Increased
Massart et al. (2012)	OCU n=12 NCU n=14	22.2 ± 0.5 23.4 ± 1.8	0.03 mg EE and 3 mg DRSP 20+/-10 months	Serum	Lipid per.	LPO & MDA	Increased (lipid peroxides and MDA) in pre- exercise, decreased in post- exercise (MDA)
Zal et al. (2012)	OCU n=80 NCU n=40	30.4 ± 5.1 30.2 ± 4.6 30.53 ± 5	0.03 mg EE and 0.15 mg LNG 33±26 months	Serum	Lipid per.	MDA	Increased
Finco et al. (2012)	OCU n=64	29±4.7	30 mcg ethinyl estradiol and 3 mg	Serum	Lipid per.	d-ROM	Increased

				drospiren one				
				6 months				
Finco et al. (2011)	OCU n=10	27-35	0.05 mg EE	Serum	Lipid per.	d-ROM	Increased	
				0.124 LNG				
				3 months				
Akinloye et al. (2010)	OCU n=10	16-45	N/A	Serum	Lipid per.	TBARS	Increased	
		NCU n=58						
De Groote et al. (2009)	OCU n=32	29.4±4.3 23.0±3.9	0.03 mg EE and 3 mg DRSP	Serum	Lipid per.	LPO Oxi- LDL	Increased Increased	
		NCU n=30	33±26 mos					
Pincemail et al. (2007)	OCU n=49	40-48	N/A	Serum	Lipid per.	LPO Oxi- LDL	Increased NS	
		NCU n=119						

Table 1 OCU = Oral contraceptive Use, NCU = No Contraceptive Use, Lipid Per. = Lipid peroxidation, MDA = Malondialdehyde, Oxi-LDL = Oxidized Low Density Lipoproteins, NS = Not Significant, d-ROMS = Determination of Reactive Oxygen Metabolites, NET = Norethindrone, DRSP = Drospirenone, EE = Ethinyl Estradiol, LNG = Levonorgestrel.

Sample Population

Seven of the eight studies measured oxidative stress markers in healthy eumenorrheic women taking COCs (Akinloye, Oyabiyi, Oguntibeju, & Arowojolu, 2010;

J. T. Chen & Kotani, 2012; De Groote et al., 2009; Finco, Belcaro, & Cesarone, 2011, 2012; Pincemail et al., 2007; Zal, Mostafavi-Pour, Amini, & Heidari, 2012). One study assessed oxidative stress in women who were trained athletes (judiosts) taking COCs (Massart, Portier, Rosado, Toumi, & Filaire, 2012).

Lipid Peroxidation

De Groote et al. (2009a) compared levels of lipid peroxides and oxidized LDL between women OCU (n=30) and NCU (n=32) aged 29.4 ± 4.3 years & 23 ± 3.9 years, respectively. Women enrolled into the study were using oral contraceptives (0.03 mg ethinyl estradiol and 3 mg drospirenone) for 20 ± 12 months (mean \pm SD). Significant increases were observed in mean levels of lipid peroxides (+176%, $p < 0.001$) and oxidized low-density lipoproteins (LDLS) (+145%, $p < 0.002$) for OCU women than NCU women.

Massart, Portier, Rosado, Toumi, & Filaire (2012) evaluated lipid peroxides and MDA in plasma at both resting (20 min before) and post-exercises (10 minutes after) a session of judo training. The sample population consisted of 12 female judoists using oral contraceptives (0.03 mg ethinyl estradiol and 3 mg drospirenone) for 20 ± 12 months (mean \pm SD) and 14 judoist females non-contraceptive users, aged 22.1 ± 0.5 and 23.4 ± 1.8 years old, respectively. Pre-exercise MDA and lipid peroxide levels were significantly increased ($p < 0.001$) (+125.8 and + 165.2%, respectively), for OCU compared to NCU. Following training MDA was increased 60% within NCU ($p < 0.01$ vs. pre-exercise score) with a 40% increase shown in OCU ($p < 0.05$ vs. baseline). The difference between OCU

and NCU was statistically significant ($p < 0.05$). In contrast POOL levels between OCU and NCU post-exercise did not differ (+22% and +36%, respectively).

One article analyzed a subsample ($n=209$) of women (aged 40-48 years old) and quantified their comprehensive oxidative stress status by measuring lipid peroxides (Pincemail et al., 2007). Forty-nine women were OCU, one hundred nineteen were NCU, and forty-one used an IUD. A significant increase in lipid peroxides was observed in OCU women compared to NCU and IUD users ($p < 0.05$). No difference was reported between groups in plasma concentration of oxidized low-density lipoprotein (LDL). The oral contraceptive used was unknown.

Zal, Mostafavi-Pour, Amini, & Heidari (2012) compared the measurement of MDA levels in plasma serum between three diagnostic groups: control ($n=40$), untreated OCU ($n=40$) and treated Vitamin E and C OCU ($n=40$), aged 30.53 ± 5 , 30.42 ± 5.1 , and 30.29 ± 4.6 years old, respectively. The combined oral contraceptive (0.03 mg ethinyl estradiol and 0.15 mg levonorgestrel 21 days) was used for at least 33 ± 26 months. MDA levels (nmol/mL) were significantly elevated ($p < 0.05$) in women untreated OCU, followed by women treated OCU and lastly women NCU (4.17 ± 0.3 , 3.29 ± 0.3 , and 3.04 ± 0.4 , respectively).

Lastly, Akinloye et al. (2010) evaluated TBARS in women aged 15-45 (mean age 33.3 ± 0.9) using COC ($n=10$) and non-contraceptive users ($n=58$). The women using COC had significantly elevated malondialdehyde (as measured by TBARS) in their serums than their non-using counterparts (12.42 ± 1.18 vs. 4.67 ± 0.84 ; $p < 0.01$). The COC used was unknown.

Hydroperoxides

J. T. Chen & Kotani (2012) determined d-ROMS levels in eighty-seven premenopausal healthy women (n=24 OCU and n=63 NCU). OCU subjects received a triphasic contraceptive (ethinyl estradiol and norethisterone). OCU women showed significantly higher d-ROMs levels (median: 380; interquartile range: 328-502 Carr U) than NCU (325 [271-369]; $P < 0.05$). In agreement (Finco et al., 2011) conducted a within subjects analysis to determine d-ROMS values at baseline and following oral contraceptive use every 3 days during a menstrual cycle. 10 healthy controls were recruited and followed consecutively for three cycles (first: oral contraceptive and second/third: oral contraceptive + absence/presence of catechin) with a contraceptive (Microgynon®: ethinyl estradiol 50 mcg plus levonorgestrel 125 mcg). They reported following COCs treatment an increase in d-ROM values was observed from 240 ± 22.3 (mean \pm SD) Carratelli Units to values > 400 Carratelli Units ($p < 0.05$). During the suspension period d-ROMS values returned to baseline. It was revealed that the PM combined with catechins from green tea reduced d-ROMS by 50%.

In further corroboration Finco et al. (2012) repeated their study, this time implementing a placebo group to test the efficacy of the MF Templar®. Sixty-four healthy eumenorrheic women were enrolled in the study after undergoing at least 6 months of COC treatment (30 mcg ethinyl estradiol and 3 mg drospirenone). The experimental design consisted of 3 phases, which were further subdivided into 6 cycles (baseline and 1-V). The phases were as follows; recruitment (n=64) week 4 of COC, phase 1 (n=64) week 5, 6, 7, 8 of COC, phase 2 cycle II was divided into two groups MF

Templar® (n=31) and placebo (n=33) for week 9, phase 2 cycle III was divided into groups MF Templar® (n=31) and placebo (n=33) for weeks 15 and 16 and Phase 3 cycle IV was divided into 3 therapies: MF Templar® (n=11), Green Tea (n=11) and MF Templar® w/o catechins (n=11) for week 17. Phase 3 was repeated for cycle V during the 23rd week of COC treatment, using the placebo only users from Phase 2 (n=33). The treatments for Phase 3 were as follows; MF Templar® group was instructed to consume 1 vial every evening, 90 mg dissolved into 10 mL of solution [solution was a mix of water, fructose and orange flavour); the Green Tea group was instructed to drink 2 cups of tea with catechins in the morning, 150 mL in each cup; and the MF Templar® w/o catechins consumed the same dose of MF Templar® devoid of the green tea catechins (i.e. only lipoic acid, beta-carotene, pyridoxine hydrochloride and selenium yeast). Baseline (placebo week) for each phase (1 and 3) was determined to be week 8 and 16, respectively. During Phase 2 and 3 the experimental procedures were randomized and double blind. Oxidative stress was determined through the quantification of d-ROMS using serum. With respect to results, during baseline the placebo week(s) d-ROMS values were within normal range (W4: 269±27.2 and W8: 268±25.4), while during the active COC use all values were > 300 U.CARR (W5: 406±29.5, W6: 396±23.1, W7: 408±27.4) which is indicative of oxidative stress. During cycles II (W9) and III (W15), treatment with the MF Templar® significantly reduced d-ROMs values relative to placebo users (W9: 302±25 vs. 404±22.3 and W15: 305±23.3 vs. 394±46.9, respectively) (p< 0.05). Lastly during Phase 3 (W17 and W23) treatment with MF Templar® significantly decreased oxidative stress as compared to the green tea group and MF

Templar® w/o catechines group (W17: 319±29.6 vs. 404±32.6 vs. 395±28.5, respectively and W23: 332±28.4 vs. 407±24.7 vs. 398±21.2, respectively) ($p < 0.05$). No differences were observed between baseline d-ROMS values between treatment groups.

Discussion

The main finding from this literature review is that oxidative stress levels are impacted by the use of COCs. All of the papers included in this review measured markers for lipid peroxidation. Lipid peroxidation refers to lipid breakdown and the formation of primary oxidation products for example, conjugated dienes (CD) and LPH (and d-ROMS) and secondary products such as MDA (and TBARS) and F2-Isoprostanes (Sies, 1997). In addition later damage can be detected when early stage LPH forms adducts with proteins which produces lipid peroxide – modified proteins (Oxidized LDL) (Sies, 1997). Within our analysis, we found that COCs significantly increased acute lipid oxidative stress as measured by lipid hydroperoxides (J. T. Chen & Kotani, 2012; De Groote et al., 2009; Finco et al., 2011, 2012; Pincemail et al., 2007). With respect to chronic or late lipid oxidative damage COC use increased MDA (Massart et al., 2012; Zal et al., 2012), TBARS (Akinloye et al., 2010) and oxidized LDL (De Groote et al., 2009). Not all reported an increase in oxidized LDL (Pincemail et al., 2007) and MDA decreased following an intense workout only in COC users (Massart et al., 2012).

Despite a dearth of published research available, we have found promising evidence that COC use may be a component in early and late lipid oxidative damage. The evidence comes from studies, which were well controlled and at times used the same

inclusion criteria, which strengthens the validity of the findings. For example, both Pincemail et al. (2007) and Chen et al. (2012) compared similar aged (40-48 years) participants while the remaining researchers used younger participants (Akinloye et al., 2010; Finco et al., 2011, 2012; Massart et al., 2012; Zal et al., 2012). In addition Massart et al. (2012) and De Groote et al. (2009) used the same COC, while De Groote et al. (2009) and Zal et al. (2012) used the same inclusion criteria for COC use duration. Overall the majority of papers included in this review support COC use can induce oxidative stress. Next we will review existing theories followed by our own hypotheses.

Luteinizing Hormone and Follicle Stimulating Hormone Antioxidant Protection

Reactive oxygen species are a natural byproduct and are required for sustained cellular functioning and signal transduction (Sies, 1997). Within the female menstrual cycle an increase in ROS is required to release the ovum during ovulation and during the proliferation phase (late follicular and early luteal) which requires energy (increase in ATP/COX activity corresponds to higher ROS levels) (Agarwal, Aponte-Mellado, Premkumar, Shaman, & Gupta, 2012). In response to this, it has been theorized that because an increase in ROS is required for ovulation, there must be a safety feedback loop in which the body can neutralize the excess ROS. The feedback loop that has been suggested is another biological event that co-occurs during ovulation which is the increase in follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Finco et al., 2012). Ergo FSH and LH may actually be “protective” and are responsible for combating the excess ROS (Finco et al., 2012). With respect to COC use the main

function is to prevent pregnancy through prevention of ovulation. This is achieved through preventing the surge of FSH and LH. Women using COCs are not afforded this “protection” by FSH and LH, and therefore have increased oxidative stress levels.

However, recent studies that measured oxidative stress throughout the menstrual cycle report conflicting results that refute the hypothesis that FSH and LH are “protective” or have the ability to deplete ROS to controllable levels. Indeed Cornelli et al. (2013) recently published evidence that women who were not using COCs had increased oxidative stress products in their serums, which positively correlated with the onset of late follicular and early luteal phases – which surround the ovulatory phase (when LH and FSH are at their peak). They analyzed d-ROMS every 3rd day of the entire menstrual cycle (t1-t27) and reported that hydroperoxide levels increased concomitantly following the increase in E2 (Umberto Cornelli, Belcaro, Cesarone, & Finco, 2013).

Hydroperoxides remained elevated and returned to baseline at menstruation, which is similar to Finco et al. (2011) who observed women on the pill had baseline hydroperoxide levels during the placebo week. Schisterman et al. (2010) also reported premenopausal women (n=259) with normal menstrual cycles showed variation in the F2-isoprostane levels. Moreover F2-isoprostane levels were highest at the expected time of ovulation and lowest during the follicular phase. Overall, this suggests FSH and LH may not be “protective.”

Oxidant Action Dependent on Dose

It has been suggested that the concentration of estrogen can determine the oxidant stance i.e. pro or anti. Indeed within the above studies it was found that oxidative stress was lowest during the early follicular phase (Umberto Cornelli et al., 2013; Schisterman et al., 2010). The follicular phase has the lowest level of estrogen. Supplementing this hypothesis, Andozia et al. (2010) found E2 protected against oxidative stress for endothelial cells by increasing nitric oxide (NO) synthesis. Interestingly, EE the synthetic form of estrogen and the compound found in COCs did not protect against oxidative stress (Andozia et al., 2010). According to the study, cells co-incubated with H₂O₂ had increased viability when incubated with E2 and not EE (Andozia et al., 2010). Therefore at a certain dose E2 definitely has the potential to be oxidative protective, however not EE. Ethinyl estradiol failed to activate NO synthesis, can it influence (inhibit) the body's other defense mechanisms responsible for decreasing ROS and could this be the link between OCU and oxidative stress?

Antioxidant Defense System

The body's endogenous defense systems against ROS are the antioxidant enzymes: SOD (superoxide dismutase), GSH (glutathione), GPx (glutathione peroxidase), and GR (glutathione reductase). The necessary components required for these enzymes to function properly are: vitamins, trace element ratios and available ATP. Researchers have reported that COCs deplete vitamin bioavailability (Palan et al., 2006; Pincemail et al., 2007), unfavorably alter the trace element ratio (De Groote et al., 2009), and consume

ATP to metabolize hormones which compromises replenishing antioxidant enzymes (Musalmah et al., 2005; Panda, Ramarao, Raju, & Chatterjee, 2008; Vats, Singh, Singh, & Singh, 2008). – see Figure 1 for GSH.

Vitamins

Vitamins work by scavenging ROS and up regulating the activities of antioxidant enzymes. Less vitamin bioavailability increases oxidative stress (Morganti et al., 2002). Both Palan et al. (2006) and Pincemail et al. (2007) reported B-Carotene was decreased in OCU women. This is in agreement with Chen et al. (2012) who demonstrated within a controlled study that exogenous supplementation of vitamin E, C and beta-carotene lowered MDA levels in OCU women. It has been theorized that estrogen induces the activation of retinal binding protein, which leads to the decrease in B-Carotene (Mooij, Thomas, Doesburg, & Eskes, 1991). This natural antioxidant barrier is crucial to preventing lipid peroxidation. Interestingly lipid peroxidation starts only after depletion of these essential antioxidants, notably: vitamin E, C and B-carotene in the body (Warden, Smith, Beecher, Balentine, & Clevidence, 2001). Vitamin E is responsible for inhibiting ROS-induced generation of lipid peroxy radicals and protecting cells from peroxidation of polyunsaturated fatty acid in membrane phospholipids (Morganti et al., 2002). The decrease in Vitamin C is detrimental to returning Vitamin E to a nonreactive compound. In the presence of Vitamin C, Vitamin E is reduced and the newly formed Vitamin C radical is reduced by GSH (Morganti et al., 2002). Palan et al. (2006)

published a study that found combined oral contraceptives depleted vitamins as measured by Coenzyme Q10, vitamin E and total antioxidant activity.

Metal Ions

Estrogens and their metabolites have both pro-oxidant and antioxidant properties depending on the availability of metal ions. Interestingly COCs increase trace elements, specifically copper (Cu) (De Groote et al., 2009). Copper increases via induction by estrogen of the hepatic synthesis of the acute phase protein ceruloplasmin, the main Cu carrier protein (Akhter, Shamsuzzaman, Banarjee, Seema, & Deb, 2006; Benes et al., 2005; Berg, Kohlmeier, & Brenner, 1998). Copper plays an intrinsic role with Zinc (Zn), they act as cofactors responsible for an enzyme, which decreases ROS: CuZn-SOD (Uriu-Adams & Keen, 2005). However the efficiency of this enzyme is contingent upon the intricate balance between Cu and Zn (Ferns, Lamb, & Taylor, 1997; Patel, Svistunenko, Wilson, & Darley-Usmar, 1997). An imbalance between these elements could lead the improper functioning of an enzyme and ultimately fail in eliminating ROS. De Groote et al. (2009) reported that Cu levels were positively correlated with lipid peroxide levels. This leads to a decrease in protection to lipids, which would be in agreement that women taking oral contraceptives have higher lipid peroxides levels.

Antioxidant Enzymes

Zal et al. (2012) showed that basal GPx and GR levels were lower in OCU women. These levels are significant because these enzymes are responsible for

scavenging ROS. For these enzymes to function properly ATP is required. ATP is responsible for GSH regeneration, which is triggered by increased consumption and bioavailability of estrogen/progestin (Valko et al., 2007). In a counterproductive essence ATP is required to metabolize the excess hormones (estrogen and progestin) and this can compromise the regeneration of GSH (Musalmah et al., 2005; Panda et al., 2008; Vats et al., 2008). Thus COC may increase ROS, which GSH (through a series of steps) then reduces, however to replenish and reuse GSH, ATP is required. However because of the excess COC hormones – ATP is re-routed to metabolize hormones thereby compromising the regulation of immune system via glutathione (Musalmah et al., 2005; Panda et al., 2008; Vats et al., 2008). It has been suggested that perhaps the amount of estrogen and progestin to digest might impact oxidative stress levels.

There are different types of combinations for oral contraceptives, which vary in dose for estrogen and progestin. They are referred to as monophasic, biphasic and triphasic. The pills differ in the manner of estrogen and progestin being delivered and amount. Monophasic deliver the same amount of estrogen and progestin every day. Biphasic pills deliver the same estrogen every day for the first 21 days of the cycle in which during the first half the uterus is allowed to thicken (progestin/estrogen ratio lower) and the second half the lining of the uterus is shed (progestin/estrogen ratio higher). Triphasic have constant or changing estrogen concentrations and varying progestin throughout the cycle. To date neither combination of oral contraceptive has been determined to be superior to the other in preventing pregnancy (H. a. a. M. Van Vliet, Grimes, Helmerhorst, & Schulz, 2006; H. A. A. M. Van Vliet, Grimes, Lopez, Schulz, &

Helmerhorst, 2011; H. Van Vliet, Grimes, Helmerhorst, & Schulz, 2001). At present a similar conclusion can be drawn on COC type and oxidative stress production. Within the ELAN female cohort, one study reported it was too diverse to accurately delineate a differential COC induced lipid hydroperoxide increase according to COC type (mono, bi, and triphasic). Interestingly across each study (in this literature review) women taking monophasics, biphasics or triphasics all had higher oxidative stress than their COC nonuser counterparts. Unfortunately, comparative results are currently unavailable for the variables estrogen/progestin release (designated by COC type) and more importantly the required dose to induce oxidative stress.

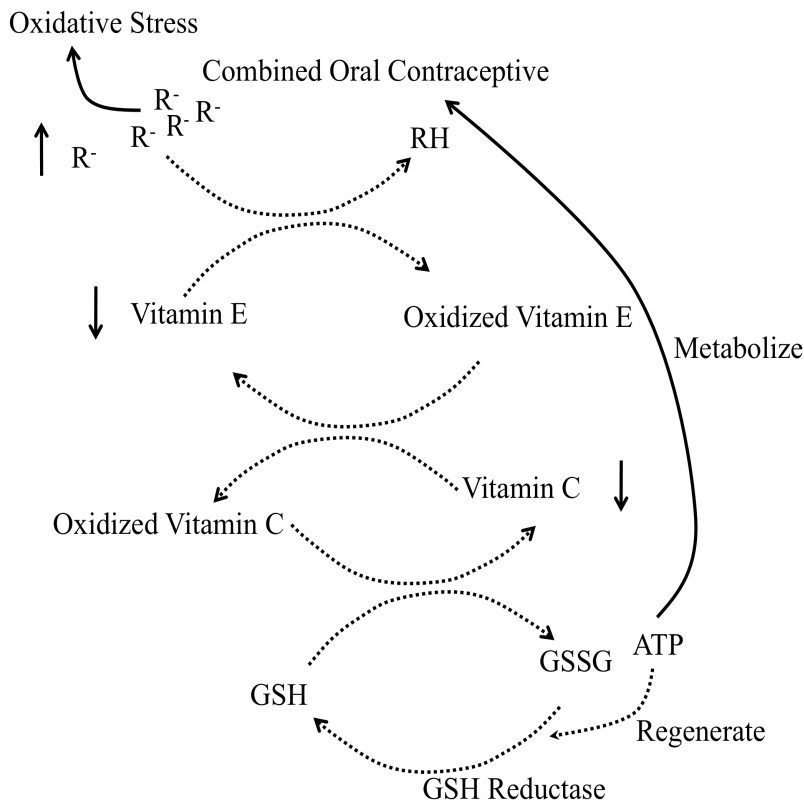


Figure 1.

This figure shows the relationship between combined oral contraceptives and oxidative stress via reducing antioxidant enzymes and re-routing ATP that are required to replenish enzymes to scavenge excess free radicals (R·). Adapted from namrata.co.

Summary

Overall the reduction in antioxidants and vitamins are controversial (Torkzahrani, Heidari, Mostafavi-Pour, Ahmadi, & Zal, 2014) moreover it is debatable that if COC down-regulates vitamins can that significantly alter a woman's oxidant profile (S. Arnold, Victor, & Beyer, 2012; Arsenijevic et al., 2000). The amount of estrogen and progesterone required to compromise the GSH regeneration is unknown, however the results from the reviewed studies revealed even low-dose combined oral contraceptives increases oxidative stress, thus OCU does have the potential to significantly affect oxidative stress profiles. Perhaps the OCU-induced oxidative stress relies not on the disabling the antioxidant and enzyme protection system, but direct impact on mitochondrial functioning.

Novel hypothesis: Impact on Mitochondrial functioning

ROS are a natural byproduct of electron transport chain (ETC) found within the mitochondria, and occurs at a 1-3% rate (Sies, 1997). Electrons moving along the ETC can escape and react with molecular oxygen, creating superoxide anion (Sies, 1997). Recent studies have dedicated resources to researching which mitochondria mechanisms derive ROS and how this occurs. As stated above, ROS occurs when electrons escape, complexes which have received notable attention are complex I and III. Complex I via its iron-sulfur appendages in the hydrophilic arm releases the most unpaired electrons, followed by complex III (S. Arnold et al., 2012). The functioning level of these complexes could hold the answer. In particular the speed in which they operate which is

contingent upon the proton motif and ATP production. In situations where the proton motif depletes, the ETC will increase the flux of electron movement in an effort to build up the proton motif. This will increase the likelihood of electrons escaping and interacting with metal ions and becoming deleterious. On the opposite spectrum, in which the proton motif is high this will slow down the ETC, which will also have negative ramifications, as unused electrons buildup. The complex activity can be altered by many mechanisms, most notably uncoupling proteins and ATPase activity.

ATPase: It has been demonstrated by several studies that estrogen affects ATP production. Zheng & Ramirez (1999) were the first to provide evidence when they demonstrated that radioactive-labeled estrogen coupled with bovine serum albumin binds directly to and inhibits the subunit of the proton F₀/F₁ mitochondrial ATPase in brain tissue. At the time, this was assumed to be a rapid way E₂ acted on oxidative phosphorylation (Moreno, Moreira, Custódio, & Santos, 2013). This is important because the ATPase synthase is the complex, which generates ATP by utilizing the proton motif energy. E₂ exerts its affect on the ATPase synthase by binding to the oligomycin sensitivity-conferring protein, which is a part of the F₀/F₁ stalk (Moreno et al., 2013). When E₂ binds to the F₀/F₁ unit the protons are re-entering the matrix however not via the classical pathway. By slipping the F₀/F₁, protons are bypassing the ATPase creating machinery (Moreno et al., 2013). Moreno et al. (2013) showed in a dose dependent manner, ATP production was inversely related to E₂ concentration. This occurs at physiological conditions and must be in the presence of ADP. With the addition of E₂,

the mitochondrial membrane potential becomes depolarized which is due to the proton leak. Oxidative phosphorylation requires the energy from the proton motif to generate the production of ATP. Typically in the coupled phase for the F₀/F₁, protons enter into the F₀ and facilitate the movement of the stalk, which exerts a conformational change on the chambers L, T, and O. It is the O conformation that turns out ATP. When the F₀/F₁ is in the uncoupled phase, as stated above, the proton motif is not being utilized thus cannot induce a conformational change, hence production of ATP decreases (Moreno et al., 2013). Recent literature has lent to support to the non-genomic actions of estrogen. Given its lipophilic entities, it can diffuse with ease into the mitochondria via the mitochondrial membranes high content of unsaturated fats and low content cholesterol (Moreno et al., 2013). This establishes an intrinsic affinity between estrogens to mitochondria (Moreno et al., 2013).

Mitochondrial Coupling Complexes: The second aforementioned process by which ROS is increased is through uncoupling protein (UCP) activity. Sastre-Serra et al. (2010) showed that E₂ down regulates UCP's in the mitochondrial membrane in MCF-7 ER-positive cell lines, which was not shown in the ER-negative cell line MDA-MB-231 (Sastre-Serra et al., 2010). When the membrane is coupled there is a marked increase in ATP synthesis efficiency, however at the expense of increased ROS production (S. Arnold et al., 2012). This primary mechanistic way of producing ROS starts when the mitochondrial membrane is coupled the proton motif builds up, which slows down the flux of electrons through the ETC. When UCP's are activated they create an alternative

channel for protons to re-enter into the matrix. Currently five UCP's have been identified: UCP1 facilitates thermo while the others (2-5) have been hypothesized to lower mitochondrial potential and thereby mitigate ROS derived from mitochondria (Sastre-Serra et al., 2010). E2 decreased the levels of the five UCP isoforms in the ER-positive MCF-7 cell line (Sastre-Serra et al., 2010). It was found that E2-induced (1nM – the E2 peak) an increase in mitochondrial potential and an increase in ROS: this was derived from the positive correlation that was found between E2 induced mitochondrial coupling and ATPase/Cox ratio (Sastre-Serra et al., 2010). The tight coupling of the mitochondrial membrane decreases reentry of protons into the mitochondrial matrix, builds up a large mitochondrial membrane potential and results in enhanced superoxide production (Sastre-Serra et al., 2010). Sastre-Serra et al. (2010) reported that the results suggest that estrogen may increase mitochondrial-derived ROS production by repressing uncoupling proteins and antioxidant enzymes.

Figure 2

The below figure outlines the relationship between estrogen and hydroperoxides. Figure 2a shows a menstrual cycle unaffected by combined oral contraceptives (COC). Using the results reported by previous studies, hydroperoxides increase at t9, the moment E2 increases and remains above the baseline threshold until E2 starts to decrease in the late luteal phase. Hydroperoxide levels return to normal during late luteal and early follicular. Figure 2b shows a menstrual cycle affected by COCs. We used the results by Finco et al. (2011) who measured hydroperoxides throughout the menstrual cycle. As shown hydroperoxides increase as soon as oral contraceptive use commences (t3). Hydroperoxide levels remain elevated until cessation of the COC during the placebo week.

Figure 2a

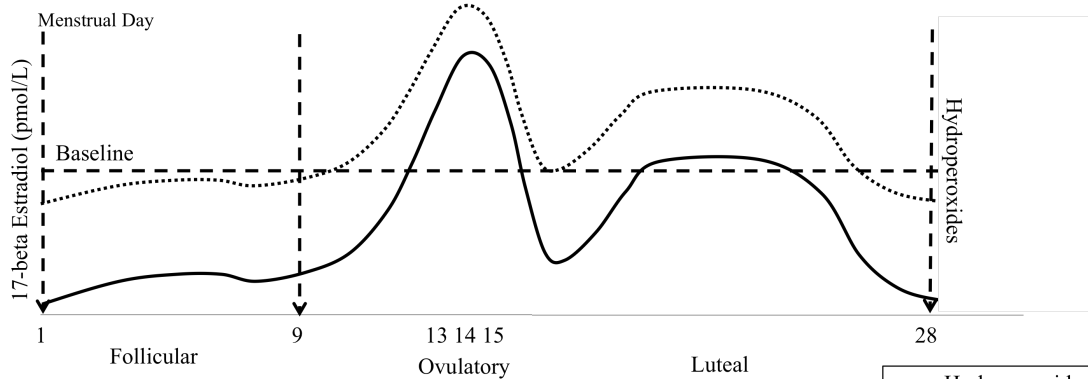
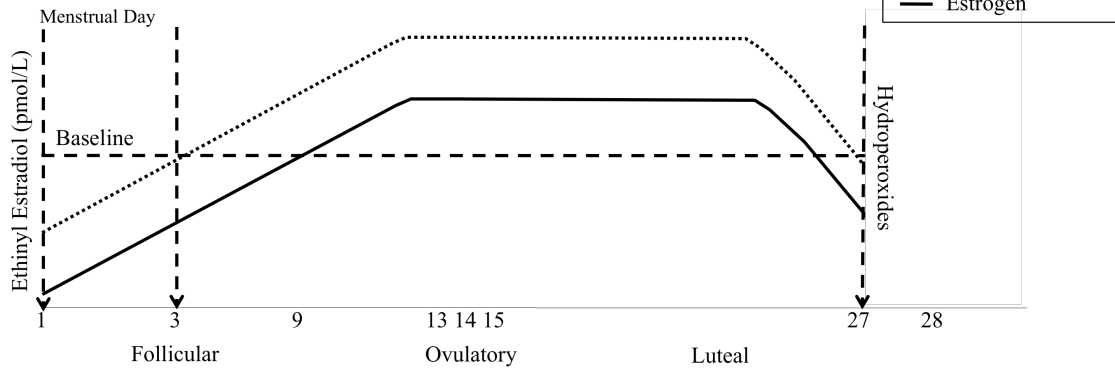


Figure 2b



Stressed System

Massart et al. (2012) was the only study identified that showed OCU providing a protective effect against oxidative stress. In explanation of this phenomena, Massart et al. (2012) suggested that OCU results in the formation of ROS, however the amount generated may not be able to stimulate the number of GPx enzymes required to reduce free radicals. Given that aerobic exercise is associated with an increase in ROS coupled with the ROS increase from OCU, the OCU could afford protection by prompting the body to activate GPx. This suggests that there could be an enzyme defense ROS threshold.

Stressed hypothesis: Reactive oxygen species as aforementioned are important in cellular functioning and respiration. Therefore completely mitigating ROS would be dysfunctional. To maintain this vital ROS functioning level the body could have a feedback system and or similar to action potentials a threshold that must be breached in order to activate antioxidant enzymes. It has been suggested that *in vitro* studies, ROS mediates the activation of antioxidant enzymes which differs between species (Hameed, Qadri, Mahmooduzzafar, Siddiqi, & Iqbal, 2011). Perhaps this mediation is performed through the level of ROS. With relation to OCU, one could argue that OCU increases ROS however the production isn't considered pathological or has the ability to activate antioxidant enzymes responsible for scavenging ROS. The parameters measured in the above studies reported oxidative stress was increased and in the studies measuring d-ROMS reported it was beyond the 300 Carr U threshold. Normal d-ROM values are between 250-300 Carr U. Therefore this additional ROS could be pathological however

isn't the required amount needed to trigger the body's antioxidant defense system. In agreement with this hypothesis, OCU women after exercising actually had lower MDA levels than their NCU exercising counterparts (Massart et al. 2012). Additional support can be found in women with polycystic ovary syndrome (PCOS) had higher asymmetric dimethylarginine (ADMA) circulating levels pre-COC treatment than healthy controls, symbolizing a stressed system. Following COC (for 3 months), plasma ADMA levels were significantly reduced for PCOS women (Ozgurtas et al., 2008). Although the authors observed a decrease in ADMA levels they were unable to directly attribute it to either an improved oxidant profile or decrease in inflammatory markers (Ozgurtas et al., 2008). However, we could postulate that an already stressed system (high free radicals), combined with COC induced ROS, did have the potential to activate antioxidant enzymes thus decreasing oxidative stress levels. Building from this concept each antioxidant enzyme might have a different activation threshold level. What remains unknown is whether or not synthetic estrogens (used in COCs) mimic natural estrogenic actions on the mitochondrial machinery.

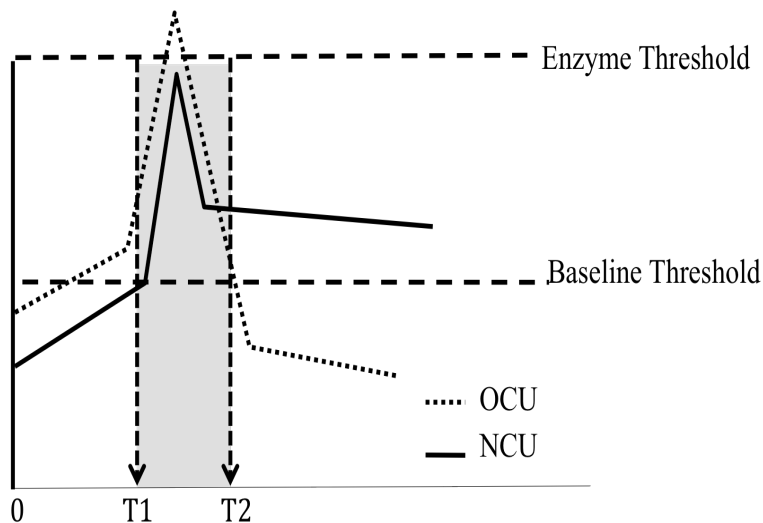


Figure 3.

Figure 3 displays the stressed hypothesis. Each line corresponds with hydroperoxides. The grey area symbolizes the exercise period (T1 to T2).

Using the results reported by Massart et al. (2012), this diagram shows oxidative stress (MDA) at pre-exercise (T1) and post-exercise (T2). Pre-exercise NCU women have less MDA levels than OCU women. Post-exercise, OCU women have less MDA than NCU women. The exercise-induced reactive oxygen species combined with OCU induced reactive oxygen species breached the enzyme threshold and activated enzymes, which reduced free radicals – thus decreased MDA.

Clinical Implications

The brain is one of the most vital organs in the human body. It is responsible for receiving, relaying, organizing and storing information. To perform all of these tasks the brain requires a lot of energy and therefore oxygen. Subsequently this makes the brain vulnerable to oxidative damage (Ng, Berk, Dean, & Bush, 2008). This oxidative damage to the brain can severely impact how the brain functions and performs its role, which is why it has been implicated as a potential source for psychiatric disorders (Hoffer et al., 1954). The other oxidative stress marker oxidized LDL has been implicated in promoting atherosclerosis and increasing the risk of a heart attack or stroke (Holvoet et al., 2001). Oxidized LDL encourages inflammation and the buildup of macrophages and platelets, which can form plaque in the arteries (Holvoet et al., 2001). Plaque buildup restricts the blood flow and this can result in coronary heart disease and peripheral vascular disease (Holvoet et al., 2001). This might explain the increased prevalence of atherosclerosis in women who use COCs. Another concern with COC use is the prevalence of adverse mood in the form of depression, irritability and mood swings (Poromaa & Segebladh, 2012) in a subset of women who use COCs. Interestingly oxidative stress has been linked to specific mood episodes in individuals with Bipolar Disorder (BD), mainly mania and depression (Ana Cristina Andreatza et al., 2007; Kapczinski et al., 2011; Kunz et al.,

2008). While non-psychiatric women using COCs report high levels of satisfaction, 4-10% do complain of adverse mood effects (Ernst, Baumgartner, Bauer, & Janssen, 2002; Kelly et al., 2010). Recently, a randomized, double-blinded, placebo-controlled trial study investigated if COC use would produce more pronounced mood symptoms than placebos in women with a previous history of COC-induced adverse mood (Gingnell et al., 2013). Within the previous history cohort, depressed mood was the most commonly reported symptom (88.2%) followed by mood swings (82.4%), irritability (70.6%), decreased interest in usual activities (44.1%), anxiety (38.2%), disordered sleep (20.6%), feelings of guilty (8.8%) and difficulties concentrating (5.9%) (Gingnell et al., 2013). They found women with a previous history who used COCs experienced more adverse mood episodes compared to placebo users with similar histories (Gingnell et al., 2013). Using functional magnetic resonance imaging (fMRI) Gingnell et al. (2013) further reported that these adverse mood episodes were accompanied by changes in emotional brain reactivity, as reflected by activation of left insula, left middle frontal gyrus, and bilateral inferior frontal gyri. If oxidative stress is related to adverse mood episodes, and hormones can produce oxidative stress, the next step would be to determine if adjunctive supplements can be taken to reduce COC induced oxidative stress.

Treatment

Currently a paucity of studies are available that have researched treatment for COC induced oxidative stress. First was Zal et al. (2012) who reported that vitamins E, C and beta-carotene lowered MDA levels, however not to baseline levels. The second,

Finco et al. (2011) they considered various factors necessary to mitigate oxidative stress, and concluded that to reduce OS to baseline, the exogenous supplied antioxidant formula must require physiological modulators. Their hypothesis was derived from previous evidence that involved the use of an AR Stenovit (membrane, circulatory, system and internal cell antioxidant types). Administration of an AR Stenovit provided healthy controls with antioxidant support, however did little to reduce OS damage from OCU (U Cornelli, Terranova, Luca, Cornelli, & Alberti, 2001). Essentially, this provided the necessary framework and demonstrated the importance of particular characteristics a modulator must include to combat COC induced OS. This essential component was catechins. Catechins work synergistically with the body's endogenous defense system to reduce OS. Indeed Finco et al. (2011) revealed women using the MF Templar® (with catechin) had substantially reduced OS levels. The MF Templar® also contains lipoic acid and Coenzyme Q10, both of which support mitochondrial ATP production. As aforementioned, ATP is required for both breakdown of excess hormones and GSH regeneration to control ROS. Interestingly, OCU women have been reported to have decreased Co-Enzyme Q10 and alpha tocopherol (Palan et al., 2006). Therefore adjunctively supplementing with this MF Templar® could restore ATP levels and prove to be beneficial.

Limitations and Future Directions

Several limitations have been identified in the studies. While the majority of reviewed studies were well controlled and p-values were adjusted for variables known to

influence oxidative stress (BMI, smoking status, and incidence of chronic disease) and or dietary intake (vitamins, alcohol, tea and chocolate). Several did not, for example Akinloye et al. (2010) was the only study that didn't control for diet and Finco et al. (2011) didn't control for smoking or chronic disease status, used a small sample size (n=10) and omitted using a control group. Within their follow-up study, Finco et al. (2012) modified their protocol to include a control group and excluded excess users of cigarette smoking/alcohol/tea and chocolate consumption, however did not address the incidence of chronic disease. Massart et al. (2012) & Pincemail et al. (2007) were the only researchers that controlled for exercise. Both Massart et al. (2012) and De Groote et al. (2009) collected blood samples between the third and fifth day of the menstrual cycle. While Finco et al. (2011) collected blood every third day of the menstrual cycle for each participant. With respect to their follow-up study, blood was collected from weeks 4 – 24 between Days 5 and 7 of the respective week (Finco et al., 2012). However a majority of studies (Akinloye et al., 2010; J. T. Chen & Kotani, 2012; Pincemail et al., 2007; Zal et al., 2012) didn't address or control for was menstrual cycle day. This is important to record and control for as women who are non-COC users, their E2 levels change throughout the menstrual cycle and this could be a potential confounder. Overall the sample sizes were relatively small, most but not all (Finco et al., 2011, 2012; Massart et al., 2012) used study designs that were cross-sectional.

Prospective studies should incorporate larger sample sizes, include additional oxidative stress markers, use biological samples other than plasma (i.e. leukocytes and urine) and control for menstrual cycle day. One other limitation is the questionable

efficacy of d-ROMS ability to realistically detect concentrations of hydroperoxides (Kilk, Meitern, Härmson, Soomets, & Hõrak, 2014), this could question the results of several studies included in this review. Despite the listed shortcomings, the results from the above adequately controlled studies provide powerful insight into the relationship between OCU and oxidative stress markers.

Conclusion

Collectively, the studies included in this review reveal combined oral contraceptives have pro-oxidant actions. Moreover, the results suggest that if oxidative stress are related to the entrainment and or maintenance of the diseases briefly mentioned, women using COCs could benefit by reducing their oxidative stress with adjunctive use of a MF Templar®. Future research should be dedicated to the effectiveness of this formula or developing other pharmaceutical treatments to be used adjunctively with combined oral contraceptives.

CHAPTER 2: *Masters Introduction*

Bipolar disorder (BD) is a severe, recurrent illness that affects 1-2% of the population (Kessler, Chiu, Demler, Merikangas, & Walters, 2005; Regier et al., 1984). The clinical course is defined by the occurrence of one or more manic episodes (hypomanic/mixed or manic episode) with recurrent episodes of major depression disorder (Vahia, 2013). A manic episode is defined by a period of abnormally and persistently elevated, irritable or expansive mood lasting at least one week (or any duration if hospitalization is required) (Vahia, 2013). Major depressive episodes are associated with prolonged periods of depressed mood for at least two weeks (Vahia, 2013). Depending on the initial symptom presentation, e.g. manic or rapid cycling episodes (four or more episodes of mania or depression within one year) and or recurrent depression with subsequent hypomanic (milder state of mania - less severe) episodes determines a diagnosis of Bipolar Disorder Type I or Bipolar Disorder Type II, respectively (American Psychiatric, 2013). The precise etiology remains elusive, however existing literature supports an interplay between a myriad of contributing factors: genetic, personality, biological and environmental. To date treatments include pharmacological (atypical antipsychotics, mood stabilizers, anticonvulsants, and antidepressants) and psychosocial (cognitive behaviour therapy) (Geddes & Miklowitz, 2013). The most successful treatment is the combination of pharmaceuticals with psychotherapy which has been reported to hasten recovery from episodes, delay mood

episode recurrences, reduce residual mood symptoms, and improve psychosocial functioning (Miklowitz, 2008). While efficacious, there are patients who do not respond to pharmaceutical therapy and even when adjunctive with psychotherapy (Miklowitz, 2008). Approximately the BD prognosis is 37% of patients relapse into depression or mania within 1 year and 60% within 2 years (Gitlin, Swendsen, Heller, & Hammen, 1995). Within the STEP-BD cohort ($N=1469$), BD type I and II 58.4% ($n=858$) achieved recovery, with 48.5% ($n=416$) had recurring episodes within a 2-year interval (with depression twice (34.7%, $n=298$) as prevalent than manic episodes (13.8% $n=118$) (Perlis et al., 2006). With respect to pharmaceutical prognosis, 50% of BD patients will respond to lithium, 20-30% will respond to another medication or combinations, 10-20% will have chronic symptoms and 10% will be treatment-resistant (Geddes & Miklowitz, 2013). Living with untreated BD can be a detriment to the emotional and physical health of the patient. Subsequently research has been dedicated to explaining why a subset of patients does not respond to treatment. Evidence from contemporary studies published suggests that the number of episodes (either mania or depression) impacts the rate of recovery, response to treatment and relapse (Berk et al., 2011; Peters et al., 2014). Indeed results from the STEP-BD project demonstrated independent of treatment condition, what determined whether or not a patient was likely to recover and have a faster time to recovery was contingent on the number of depressive and manic episodes (1-9 and > 20 , respectively) (Peters et al., 2014). Overall, number and type (manic or depressive) of episodes influenced whether or not patients responded to intensive psychotherapy and collaborative care, with patients responding to psychotherapy better than collaborative

care if they previously had 10-20 episodes of depression, with individuals responding to both forms of therapy equally if they had only 1-9 depressive episodes (Peters et al., 2014). This trend has been confirmed in pharmacotherapy empirical studies, where response rates for mania and maintenance (with olanzapine use) ranged from 29-59% and 11-40% given the number of previous episodes 1-5 and >5, respectively (Berk et al., 2011). Similar with Peters et al. (2014) response rates were significantly higher for 1-5 prior depressive episodes (Berk et al., 2011). As for relapse, within the maintenance studies, both mania and depression were reduced by 40-60% in both 1-5 or 6-10 prior episodes with >10 pooled episodes increased the rate of relapse (Berk et al., 2011). Thus what has been defined as ‘chronicity’ of the disorder can be divided into two components: number of previous mood episodes and illness duration (Berk et al., 2011). Both of which impact a patient’s response to psychosocial and pharmacotherapy treatments, in addition the rate of recovery and relapse. The question now is what initiates and or exacerbates these manic or depressive episodes?

A general consensus is that the management of BD in women can present additional challenges associated with the reproductive cycle. Hence one likely candidate has emerged and that is the patient’s biological sex. While current epidemiological studies consistently reveal an equal sex ratio in the 12-month and lifetime prevalence and incidence of BD (Mitchell, Slade, & Andrews, 2004; Wells et al., 2006), a review contends diagnostic evidence suggests differences in severity and prevalence of manic and depressive moods between men and women with BD (Diflorio & Jones, 2010). For example two retrospective studies (Angst, 1978; P Roy-Byrne, 1985) found women with

BD have more depressive episodes, however this finding is nonexistent in recent publications (Grant et al., 2005; Suominen et al., 2009). When the diagnostic criteria is modified to include lifetime experience of low mood rather than a discrete episode, women report more depression than men ('depressed mood and dysphoria' 84.9% and 74.6%, respectively) (Morgan, Mitchell, & Jablensky, 2005; Rasgon et al., 2005). This can apply to studies on sex distribution for both mixed episodes, rapid cycling, and BD type II prevalence. For example using the criteria for mixed episode (defined as a state in which the person experiences manic and depressive symptoms simultaneously) under the DSM V and CDC classification "requiring a greater number of depressive symptoms during mania", mixed mania was reported more in women than men (L. M. Arnold, McElroy, & Keck, 2000; Cassidy & Carroll, 2001). Moreover when the duration of an illness is analyzed, Kessing (2008) reported mixed episodes for women increased from 6.7% to 18.2%, whereas rates for the men remained consistent (5-7%). Rapid cycling remains controversial with meta-analyses reporting increases found in women (women 29% and men 16.5%) (Kupka, Luckenbaugh, Post, Leverich, & Nolen, 2003; Tondo & Baldessarini, 1998), with more contemporary findings failing to find an association between sex and rapid cycling (Baldassano et al., 2005; Schneck et al., 2008; Suominen et al., 2009). The impact of sex on the course of BD remains inconclusive, which is in part largely due to study (clinical or epidemiology), design (prospective or retrospective), and diagnostic criteria utilized. While not consistent, the current consensus is women with BD are prone to an increase in rapid cycling episodes (Schneck et al., 2004; A. C. Viguera, Baldessarini, & Tondo, 2001), mixed episodes (Kessing, 2008) and BD type II

(15.3% M vs. 29.0% F)(Baldassano et al., 2005). A number of hypotheses have been postulated to explain the differences in symptom presentation between men and women with BD, one consistent theme between many of them is the role of hormonal factors.

A growing body of literature has emerged which consistently links the onset or worsening of mood illness to hormonal fluctuations (within the menstrual cycle the decrease in E2 and progesterone in the late luteal phase). For example, retrospective studies report worsening mood during the premenstrual phase in BD women (Diamond, Rubinstein, Dunner, & Fieve, 1976; Price & DiMarzio, 1986), however prospective studies reveal inconsistencies (Leibenluft, Ashman, Feldman-Naim, & Yonkers, 1999; Rasgon, Bauer, Glenn, Elman, & Whybrow, 2003; Shivakumar et al., 2008). Case controlled studies provide insight into individual differences and show manic (Conrad & Hamilton, 1986; D’Mello, Pinheiro, & Lalinec-Michaud, 1993; Hsiao & Liu, 2007), hypomanic (Ghadirian & Kamaraju, 1987) and depressive (Becker, Rasgon, Marsh, Glenn, & Ketter, 2004; Ghadirian & Kamaraju, 1987) episodes co-occurring during the premenstrual and menstrual phase (Day 1), with one woman experiencing an episode of mania during ovulation (perioovulatory phase) (Becker et al., 2004). Moreover a recent systematic review found that disorders associated with menstrual syndromes, premenstrual syndrome (PMS) and premenstrual dysphoric disorder (PMDD) have been reported to be comorbid among women with BD (Cirillo, Passos, Bevilacqua, López, & Nardi, 2012). For a diagnosis of PMS, symptoms (affective and somatic) must be present five days before menses and are in remission following four days of menses and do not return until cycle day thirteen, for at least three consecutive menstrual cycles (American

Psychiatric, 2013). As for PMDD (a more severe version of PMS), symptoms must occur seven days before menses, within the several days after menstrual onset symptoms must improve and remit for up to seven days, this must occur within two consecutive menstrual cycles (American Psychiatric, 2013). Retrospective studies show 25-77% of women with BD are comorbid with PMS (Blehar et al., 1998; Choi et al., 2011; Payne et al., 2009). In addition a recent review summarized that the severity of PMS being more common among BD type II patients than BD type I and controls (52%, 23%, and 20% respectively) (Teatero, Mazmanian, & Sharma, 2014). This was not confirmed in all retrospective studies (Diamond et al., 1976; Roy-Byrne et al., 1986). From prospective studies women have reported severe PMS and had (more) rapid-cycling BD than during their menstrual phase when compared to healthy age-matched controls (Leibenluft et al., 1999; Rasgon et al., 2005, 2003; Whybrow et al., 2003), but not all found this difference (Shivakumar et al., 2008; Sit, Seltman, & Wisner, 2011). As for PMDD, from a paucity of studies (Choi et al., 2011; Fornaro & Perugi, 2010) a review by Teatero (2014) indicated 15-27% of women with BD have a history of PMDD. This prevalence is greater than the general population of 3-8% (Choi et al., 2011). With respect to other pivotal reproductive events, where hormonal changes are profound include pregnancy, the postpartum period and menopause. Data from pregnancy shows rates of recurrence are equal between pregnant and non-pregnant women with BD (A. C. Viguera et al., 2001). However, these rates could be dependent on the decision to continue their psychiatric medication during pregnancy (Adele C. Viguera et al., 2007). The postpartum period reveals an upsurge in BD related illness, with Munk (2006) estimating that women with

BD were twenty-three times more likely to be admitted with a mood episode during the first postpartum month. Lastly, during the menopausal transition, women with BD have complained of severe emotional disturbance (Blehar et al., 1998; Marsh, Templeton, Ketter, & Rasgon, 2008).

The rationale ascribed to the differences between studies has been attributed to the incongruent definitions used to describe menstrual cycle-related conditions (Endicott, 1993) and or a subgroup of women with BD have either hypersensitivity to normal hormonal fluctuations or abnormal levels (Rasgon et al., 2003). This could be the potential answer as to why a subset of women with BD may not respond to treatment. In support of this theory, Karadag et al. (2004) observed that healthy women reported PMS more than treatment-responsive (lithium and or/valporate) women with BD. Recently both PMS and PMDD are thought to occur due to the low hormone levels (estrogen and progesterone) which is characteristic of the premenstrual phase, as reported by the American Psychiatric Association DSM IV (1994). However, from case reports it has been determined the differences experienced may not be due to biological levels. Currently all of the laboratory tests within case studies have revealed normal levels (blood count, pelvic ultrasound, ovarian hormone levels, in addition kidney, liver and thyroid function) (Hsiao & Liu 2002; Al-Dabbas, 2001; Al-Habeeb, 2003). With respect to mood episodes, currently estrogen and progesterone levels do not differ between women with BD who have rapid-cycling (Karadag et al., 2004) or postpartum episodes (Wieck et al., 2003) relative to controls. However, there have been case studies, which

report success with estrogen treatment to alleviate manic/psychosis in women with BD following an increase in their estrogen.

Similar to hormone replacement theory to alleviate mood in women with menopause, exogenous estrogen supplementation and tamoxifen have promising implications. With remarkable consistency tamoxifen trials show anti-manic effects (Amrollahi et al., 2011; Kulkarni et al., 2006; Yildiz, Guleryuz, Ankerst, Ongür, & Renshaw, 2008; Zarate et al., 2007) in patients with BD. Tamoxifen is used in breast cancer treatment however has recently been promoted to treat BD. Tamoxifen is labeled an antagonist for estrogen, however in other tissues it can be an agonist (O'Regan & Jordan, 2002). The clinical studies were double-blinded, randomized, placebo-controlled with a trial length of 21 days to 42 days and consisted of both men and women (in total a review calculated n=135 with n=61 men and n=74 women with BD) (Meinhard, Kessing, & Vinberg, 2013). Study participants were prescribed either tamoxifen or a placebo. Within each respective study, participants either: (1) discontinued all psychotropic medications (except benzodiazepines) at least 48 hrs before trials begun (Yildiz et al., 2008; Zarate et al., 2007), (2) prescribed lithium and or/valporate at baseline and then adjunctive with Tamofixen (Amrollahi et al., 2011; Kulkarni et al., 2006), or (3) permitted to use lorazepam during the study which was restricted to doses of 2-5 mg/ 24h for the first 10-12 days of the trial, after this lorazepam was avoided (Amrollahi et al., 2011; Yildiz et al., 2008; Zarate et al., 2007). The studies which used lithium reported adjunctive use of tamoxifen was the most effective form of treatment, even superior to lithium treatment (Amrollahi et al., 2011; Kulkarni et al., 2006). Within one study that

measured estradiol (pmol/l) a significant increase in serum estradiol was found in the tamoxifen treatment which was not found in the placebo group, in fact estradiol level was decreased (Kulkarni et al., 2006). Anti-manic conditions were found in all participants who continued with their respective treatment relative to the placebo group. With respect to the estrogen studies, two studies reported women with postpartum psychosis at baseline had low estrogen levels and responded positively to sublingual 17-beta estradiol (Ahokas & Aito, 1999; Ahokas, Aito, & Rimón, 2000). Both studies were open-label trials and across each participant's baseline serum estradiol levels were lower than the threshold for gonad failure (110 pmol/l) (Ahokas & Aito, 1999; Ahokas et al., 2000). Within the clinical study (n=10) after two weeks of treatment, estradiol levels increased to normal levels found in the follicular phase (Ahokas et al., 2000). One participant was reported to discontinue the treatment, following this her estradiol level dropped from 1000 to 46 pmol/l and she developed florid psychotic symptoms (Ahokas et al., 2000). Participants who continued the trial, their psychotic symptoms significantly decreased (Ahokas et al., 2000). As for the case study (n=2), one woman continued her treatment and her psychotic symptoms gradually reduced, as for another woman she was originally treated chlorpromazine which was ineffective and discontinued (Ahokas & Aito, 1999). She was then put on 17-beta estradiol and within two weeks her symptoms were resolved (Ahokas & Aito, 1999). Following this she discontinued her treatment and her psychotic florid symptoms relapsed (Ahokas & Aito, 1999). Taken together, this suggests there may be a hormonal component to the etiology of the disease. This hormonal component

among a subset of women with BD could be either an increased sensitivity to normal hormone fluctuation or perhaps increased hormonal fluctuations.

Based on prior work linking hormones to BD in at least a subset of women, and the results of work looking at estrogen and tamoxifen treatments for the illness, a natural next step was to determine the role of oral contraceptive therapy (OCT) in BD. Combined oral contraceptives (COC) formula contain two components that are synthetically derived estrogens (ethinyl or mestranol estradiol) and progestins (norethidrone, norethindrone acetate, ethynodiol diacetate, levonorgestrel, norgestrel, lynestrenol, desogestrel, noregestimate and gestodene) (Kaunitz, 2004). COCs are orally administered to prevent pregnancy by preventing the release of gonadotropins (follicle-stimulating hormone and luteinizing hormone), and for non-contraceptive purposes, mainly to control menstrual abnormalities, decrease blood loss, incidence of iron-deficiency anemia and dysmenorrhea (Borgelt-Hansen, 2001; Masimasi et al., 2007). Currently only one study is available that has tested the efficacy of hormonal contraceptive use in women with BD who were comorbid with PMDD. This recently published case study (n=3) reported women with BD responded positively (severe premenstrual symptoms resolved) to adjunctive contraceptive treatment (Frey & Minuzzi, 2013). Ms. X was treated a transdermal patch of norelgestromin 6.0 mg and ethinyl estradiol 0.60 mg continuously for 3 months, within 8 weeks of continuous patch and medication (lamotrigine 200mg/day, aripiprazole 2 mg/day, and bupropion XL 150 mg/day) her symptoms significantly improved (Frey & Minuzzi, 2013). Ms. Y was treated with lithium 1200 mg/day and quetiapine 300 mg at bedtime and the combined

oral contraceptive (3.0 mg of drospirenone and 0.030 mg of ethinyl estradiol) for 3 months, within 9 weeks she reported mild “emotional sensitivity” during the premenstrual phase (Frey & Minuzzi, 2013). Ms. Z was treated with olanzapine 7.5 mg/day and fluoxetine 20 mg/day with a combined oral contraceptive (3.0 mg drospirenone and 0.030 mg of ethinyl estradiol), 4 weeks later her premenstrual symptoms significantly improved (Frey & Minuzzi, 2013).

In addition one case study showed, following oral contraceptive (ethinylestradiol and norethisterone) cessation resulted in the primary onset of BD in the form of rapid cycling (Michael & Pfleiderer, 2009). Interestingly, the role of hormones impacting mood is not unique to women with BD as this phenomena is also apparent in non-psychiatric women. A review by Nyberg (2013) reported women who experienced severe menstrual symptoms who were treated with a COC had improved mood, however this finding was not apparent in their healthy counterparts who had no or mild-moderate symptoms. Taken together, the reviewed studies above suggest a subset of women with BD may have entrainment (onset) or exacerbation of BD symptoms, which correspond with the menstrual cycle, notably hormones. Based on the case study on BD and adjunctive contraceptive use, it would appear COCs can be therapeutically beneficial, however there are some caveats that need to be considered when using OCT’s in this population. It is important to note BD is commonly comorbid with a myriad of other disorders and or diseases. For example, patients with BD are at an elevated risk for cardiovascular disease (Weiner, Warren, & Fiedorowicz, 2011). Contemporary literature has shown that the use of high-dose estrogen-progestin oral contraceptives are associated

with increased incidence of cardiovascular morbidity and mortality in women (Kemmeren et al., 2002). In addition OCT can influence thyroid health, which may increase the risk of endocrine diseases, which are suspected to underlie or exacerbate the pathophysiology of BD. While there are no well-researched physiological markers of menstrual cycle-related BD episodes, one physiological marker has predominately emerged within the majority of persons diagnosed with BD (Brown, Andreazza, & Young, 2014a). This biomarker is increased oxidative stress.

Oxidative stress can cause irreversible cell lesions via oxidative alterations to important structures (lipids, DNA, and proteins) impact effective cellular signaling and have been suspected to be linked to the development or maintenance of several human pathologies (Sies, 1997). Thus the best way to advance treatment options available for mental health is to first understand what is happening centrally with respect to brain functions that are no longer working normally. This has been a focus in the treatment of BD. As aforementioned one possible link that has emerged, which has been implicated in other neurodegenerative disorders and diseases, is oxidative stress. With respect to women, from our literature review we found that COC use can increase oxidative stress in non-psychiatric women (Akinloye et al., 2010; J. T. Chen & Kotani, 2012; De Groote et al., 2009; Finco et al., 2011, 2012; Massart et al., 2012; Pincemail et al., 2007; Zal et al., 2012). Despite the literature, the concept of COC induced oxidative stress relationship remains controversial. However, given the consistent results found between the studies in our literature review, we felt this was worthy to investigate in women with BD. Women with BD are already at an increased risk for oxidative stress, ergo the hypothesized COC

induced oxidative stress could have serious ramifications. Thus the purpose of the following Masters thesis is to analyze oxidative stress profiles in women with BD who are on COCs compared to BD women who are not using COCs. Oxidative stress was determined by measuring 3-Nitrotyrosine, 4-Hydroxynoneal, Protein Carbonyl Content and Lipid Hydroperoxide in serum. The studies in this review that addressed oxidative stress and combined oral contraceptive use in women were drawn from a comprehensive literature review. These studies were selected based on key words from the online databases MedLine, Embase, PsychINFO and PubMed. Articles were searched from no set beginning to June 2014. This literature review helped formulate the rationale for this research study, along with all the other papers included in this thesis.

CHAPTER 3

Defining Oxidative Stress

The main producers of oxidative stress via reactive oxygen species are the mitochondria (Sies, 1997). Mitochondria are membrane-bound organelles housed inside the cell, responsible for producing the cell's energy supply in the form of adenosine triphosphate (ATP) (Figure 1). In addition, mitochondria are involved in cell signaling, cell differentiation and cell: apoptosis, cycle and growth. The mitochondria are compartmentalized into outer membrane, intermembrane space, inner membrane, cristae and matrix. The inner membrane region houses proteins with distinct functions required for producing ATP (Sies, 1997). These proteins are labeled complexes I to IV which perform redox reactions of oxidative phosphorylation (Sies, 1997). The remaining complex V is the "ATPase Synthase" and produces ATP and releases it into the matrix (Sies, 1997). The electron carriers are NADH and FADH₂, which are oxidized when they transfer their electrons to complex I and complex II, respectively (Sies, 1997). From these complexes the electrons are transferred to complex II by ubiquinol (Sies, 1997). Electrons are then transferred from complex III to complex IV by Cytochrome *c* (Sies, 1997). Complex IV is when the electrons are reduced to water by interacting with oxygen. The flow of electrons through the electron transport chain generates a proton (H⁺) transfer across the inner mitochondrial membrane at complexes I, III and IV (Sies, 1997). This creates an electrochemical gradient, which is utilized to create ATP when the protons reenter into the mitochondria matrix, via the ATPase Synthase (complex V). The process by which mitochondria create ATP is termed oxidative phosphorylation. A

common event during oxidative phosphorylation is electrons escaping from the electron transport chain mainly through complexes I and IV (Sies, 1997). These escaped electrons can interact with molecular oxygen and create free radicals. These free radicals called ‘reactive oxygen species’ have an unpaired electron, which makes them highly reactive (i.e. OH, O₂⁻, and H₂O₂) (Sies, 1997). In lieu of this the body has a natural defense antioxidants and enzymes. The enzymes turn free radicals into non-radicals by donating electrons. For example superoxide is dismutated into H₂O₂ by Cu, ZnSOD (intermembrane space) and by magnesium superoxide dismutase (SOD2) in the matrix. H₂O₂ is then reduced to H₂O by glutathione peroxidase (GPx) using GSH. The oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase. ROS are continuously produced (1-3%) and at low concentrations they support cellular functioning, participate in immune defense against infections, and act as secondary messengers capable of regulating cell apoptosis, activating transcription factors and modulating the expression of various genes involved in immune response (Sies, 1997). However if ROS overpowers the body’s antioxidant system either by an increase in ROS or a decrease in functioning antioxidant enzymes the result is oxidative stress. Recently due to the dichotomy role of ROS that is contingent upon the level, ROS can either support ‘redox biology’ or cause pathology ‘oxidative stress’ (Schieber & Chandel, 2014). In a state of oxidative stress these generated free radicals can interact with proteins, lipids, DNA and RNA, which ultimately alters their structure causing them to dysfunction (Sies, 1997). For example consequences of lipid peroxidation are: structural changes in membrane (alter fluidity and channels, alter membrane-bound signaling proteins, increases ion permeability), form

adducts/crosslink's with non-lipids (form covalent adducts with cysteine, lysine, or histidine residues of proteins and induce changes in their function), can cause direct toxicity of lipid peroxidation products (4-hydroxynoneal) (Ana C. Andreazza & Young, 2014; Stark, 2005). Damage to proteins can result in oxidation of catalytic sites on proteins can lead to loss or gain of enzyme activity or protein function, protein aggregation, enhanced or diminished susceptibility to proteolysis, abnormal cellular uptake, modified gene transcription, and modulation of cell signaling (Ana C. Andreazza & Young, 2014; Squier, 2001).

Measuring Oxidative Stress

Oxidative stress can be measured by quantifying the products of oxidative damage to proteins and lipids. The products to assess acute damage are lipid hydroperoxides (LPH) and 3-Nitrotyrosine (3-NT), which quantify reversible damage to lipids and proteins, respectively. As for chronic damage (either cumulative or late) it is determined by measuring 4-Hydroxynonenal (4-HNE) and Protein Carbonyl Content (PCC), these measure damage to lipid and proteins, respectively.

Oxidative Stress and Human Pathology

Oxidative stress has been suspected to be linked to human pathologies. This hypothesis has been supported by human biochemical data and has been confirmed in a plethora of multi-dimensional studies. As for psychiatric disorders, it was first proposed in 1950 that free radicals may be a factor in the pathophysiology of schizophrenia (Hoffer

et al., 1954). Since then subsequent papers have found empirical evidence linking increased oxidative stress in patients with schizophrenia (Emiliani et al., 2014).

Oxidative stress has been thought to be implicated with psychiatric disorders due to the brain's vulnerability to oxidative damage (Ng et al., 2008). For example the brain requires a lot of oxygen and energy, which leads to the subsequent increase in free radicals. It has a modest antioxidant defense, moreover its general makeup which consists of lipid-rich substrates which can easily be oxidized by the increased presence of redox-catalytic metals such as iron and copper (Halliwell, 2006).

Oxidative Stress and Bipolar Disorder

One other psychiatric illness that has been linked to oxidative stress is BD. While the results are conflicting, surmounting evidence points to the role of oxidative stress and the pathophysiology of BD. To summarize, studies using serum found increases in protein carbonyl content within manic and depressive participants relative to controls (Magalhães et al., 2012), however no differences were reported between early or late manic patients compared to controls (Ana Cristina Andreazza et al., 2009). Within that same study, 3-Nitrotyrosine was increased in early and late stage manic patients relative to controls (Ana Cristina Andreazza et al., 2009). As for lipid peroxidation, thiobarbituric acid reactive substances (TBARS) were increased (relative to controls) in BD patients (Banerjee, Dasgupta, Rout, & Singh, 2012; Kunz et al., 2008), in manic untreated BD patients (Machado-Vieira et al., 2007), BD patients who were depressive or manic (Kapczinski et al., 2011), while no differences were reported between control and BD

patients (Magalhães et al., 2012). Studies using red blood cells (RBC) as their sample found similar results with an increase (Kuloglu et al., 2002) in TBARS and no differences (Ranjekar et al., 2003) between BD and controls. Currently one study has measured lipid hydroperoxides with an assay kit using whole blood, and they too found an increase in BD relative to controls (Versace et al., 2014). As for prefrontal cortex samples, protein oxidation measured by protein carbonyl content has been reported to be increased in the bipolar group but not in the depressed or schizophrenia groups compared with controls (Ana C. Andreazza, Shao, Wang, & Young, 2010). Within the same study, 3-Nitrotyrosine was increased in BD and schizophrenia groups compared to depressive and controls (Ana C. Andreazza et al., 2010). While 4-HNE has been reported to be increased in synaptosomal sections of patients with BD compared to controls, with no differences being reported in lipid hydroperoxide measurements (Ana C. Andreazza, Wang, Salmasi, Shao, & Young, 2013). Overall, an updated meta-analysis confirmed that patients with BD relative to controls have increased oxidative stress (LPH and DNA/RNA damage) in their serums, RBC and prefrontal cortexes (Brown et al., 2014a). The majority of studies that have measured oxidative stress in BD are cross-sectional studies, and the authors urge longitudinal analyses are required. Due to the negative actions of uncontrollable ROS and mitochondria being the main producers of ROS, interim consideration has been granted to researching mitochondrial function. Although no sex differences have been reported on oxidative stress in patients with BD, mitochondria biosynthesize, regulate and are controlled by sex hormones.

CHAPTER 4

Hormones and Oxidative Stress

Currently none of the published studies measuring oxidative stress in patients with BD have assessed oral contraceptive use (in women). While only a handful of studies have assessed this relationship in non-psychiatric women. This could largely be attributed to estrogen being presumed to have antioxidant qualities, however recently this viewpoint is changing. Mitochondria contain both estrogen receptors (ER) alpha and beta, which has been confirmed by findings by Chen (2004) in the rabbit reproductive tract, which has ER's in Breast Cancer Cell Lines (MCF-7 cells). This was later corroborated by findings that brain endothelial cells containing intra-mitochondrial ER-alpha (Razmara et al., 2008) and mitochondrial ER-beta in the forebrain and hippocampus (Milner et al., 2005). A recent review confirmed that mitochondria have both types of classical ER's (Levin, 2009). Estrogen can influence mitochondrial functioning through classical and non-classical pathways. The classical pathway involves sex steroid hormones binding to their respective nuclear receptors, estrogen receptor (ERalpha and ERbeta) (Lonard & O'Malley, 2012). This binding enables a steroid-hormone-receptor-co-regulator compound to influence gene expression. Compelling research has been published on the anti-oxidant effects of estrogen. For example following administration, E2 has been reported to, stabilize mitochondrial membranes and reduce lipid peroxidation (Razmara, Duckles, Krause, & Procaccio, 2007), increase mitochondrial energy and decrease ROS (Stirone et al., 2005). Estrogen has been hypothesized to regulate mitochondrial biogenesis functioning through regulation of NRFs and PCG-1 (J.-Q.

Chen, Cammarata, Baines, & Yager, 2009; Ivanova et al., 2011; Mattingly et al., 2008; Tcherepanova, Puigserver, Norris, Spiegelman, & McDonnell, 2000). The non-classical pathway contributes to mitochondria function through non-genomic actions. This pathway is rapid and operates within a short time frame – demonstrating estrogen's impressive global control. The suggested pathway results in intracellular protein-kinase-mediated phosphorylation signaling cascades (Hammes & Levin, 2011). This signaling cascade has thought to be implicated in decreasing mitochondrial oxidative damage. This theory postulates estrogen activates MAPkinase and NF-kB pathways which stimulates the nuclear transcription of mitochondrial antioxidant enzyme superoxide dismutase (SOD2) thereby decreasing cellular levels of H₂O₂ (Borrás et al., 2005). Furthermore estrogen has been found to increase cytochrome c oxidase complex IV activity by activating extracellular-signal-regulated kinases (ERK) (Ronda, Vasconsuelo, & Boland, 2013). In addition estrogen has been reported to inhibit low density lipoprotein oxidization (Harman, 1988) and act as a peroxidation chain-breaking antioxidant for both lipids and DNA *in vitro*. Progesterone's antioxidant pathway remains relatively elusive. When administered separately estrogen and progesterone both exert oxidative protective actions, however when co-administered the beneficial effects can be mitigated (Irwin et al., 2008). In fact progesterone counteracts estrogen's antioxidant action by inhibiting the expression and activity of MnSOD and eSOD (which estrogen promotes) (Itagaki et al., 2005; Wassmann, Wassmann, & Nickenig, 2005). Thibodeau et al. (2002) postulated the differences found between studies could be also attributed to chemical heterogeneity of the estrogen and progesterone used, and whether or not the dose is administered at the

correct physiological level. Despite these controversies however it is now becoming accepted that estrogen has pro-oxidant inducing actions that warrant further investigation.

Estrogen as a Pro-Oxidant

Several theories have been presented to explain the increased production of oxidative stress markers following estrogen administration: (1) unstable compounds (semiquinones) derived from E2 generation (Liehr & Roy, 1990); (2) production of catechol E2 metabolites (Liehr & Roy, 1990); and (3) altering the necessary components required for mitochondrial functioning and controlling ROS production (trace element ratios and vitamins) (Musalmah et al., 2005; Panda et al., 2008; Vats et al., 2008) (*see attached review for further descriptions of these processes*). However, researchers have argued that the reduction in vitamins would be irrelevant in the production of ROS required to cause oxidative stress (S. Arnold et al., 2012). We recently contended in a literature review that E2's direct impact on mitochondrial functioning could be the source, for example E2's effect on the ETC within the mitochondria. It has been confirmed that the complexes, which embody the ETC, could be a potential source of oxidative stress. In particular the speed in which the complexes operate which is contingent upon the proton motif and ATP production. Recently E2 has been reported to slip the ATPase machinery (Moreno et al., 2013), and promote ETC coupling within ER positive cell lines (Sastre-Serra, Nadal-Serrano, Gabriel Pons, Roca, & Oliver, 2013). Coupling the ETC slows down the flux of electrons and builds a healthy proton motif, however at the expense of producing ROS. What remains questionable is whether or not

synthetic estrogen (ethinyl estrogen), the compound found in combined oral contraceptives mimics the action of the non-synthetic form 17-beta estradiol on mitochondrial complexes.

Combined Oral Contraceptive and Oxidative Stress

Based on a recent literature review it would appear that ethinyl estradiol might mimic the actions of E2 on mitochondrial complexes. Several lines of evidence have demonstrated that combined oral contraceptives induce oxidative stress. This was initially established in experimental animal models using the rat, where researchers showed that estrogen has pro-oxidant actions (Gordon, Macrae, & Carswell, 2005; Köse, Doğan, & Ozesmi, 1993; Sissan, Menon, & Leelamma, 1995). Indeed animal studies have provided the platform for investigating this potentially pathological relationship. Both Kose et al. (1993) and Sissan et al. (1995) noted an increase in oxidative stress following COC administration in rats. In agreement with rat studies, we found within our literature search (Table 1) that women using COCs had increased lipid peroxidation products in their serums when compared to women who weren't using COCs. In well-controlled studies, COCs significantly increased MDA and lipid peroxide levels (De Groote et al., 2009; Massart et al., 2012; Pincemail et al., 2007). Lipid peroxides are used to detect acute damage and MDA assess chronic damage. Both Massart et al. (2012) and De Groote et al. (2009) used the same COC (0.03 mg EE and 3 mg drospirenone). With Zal et al. (2012) and De Groote et al. (2009) using the same inclusion criteria for COC duration. All of the studies measured products of lipid peroxidation and damage

was confirmed in both acute and chronic oxidative stress markers. These contemporary studies were conducted on healthy women, however and unfortunately data is unavailable on women with psychiatric disorders. There is an imminent need for prospective studies to address this COC induced oxidative stress relationship in psychiatric patients, specifically those with BD as they are already at a predisposition for increased oxidative stress.

CHAPTER 5

Master’s Project “Evaluating Oxidative Stress Levels in Women with BD who took Oral Contraceptives”

To the best of our knowledge no studies to date have been published comparing oxidative stress levels between oral contraceptive users (OCU) and non oral contraceptive users (NCU) women with BD. The literature on BD and women suggests that hormone fluctuations seem to affect the course of BD. Given that 1) oxidative stress has been linked to pathophysiology of BD and individuals with BD already have increased oxidative stress products in their serum and 2) our recent literature review concluded that healthy women using COCs had increased oxidative stress profiles, we felt this was an important area worthy of exploration. The aim of this study was to measure oxidative stress markers in a BD cohort relative to COC use. In addition, we planned to assess if a relationship existed between OCU and oxidative stress on the following parameters: (1) interaction with medications (mood stabilizers and atypical antipsychotics), and (2) mood symptoms as assessed by the use of standardized rating scales (Young Mania Rating Scale (YMRS), Montgomery-Asberg Depression Rating Scale (MADRS), and Hamilton Rating Scale for Depression (HAM-D)).

Hypotheses

Hypothesis I – It was proposed that women with Bipolar Disorder using combined oral contraceptives will have higher oxidative stress levels (4-HNE, PCC, LPH, and 3-NT) relative to women with BD who are non-contraceptive users (NCU) and OCU/NCU healthy control women.

Hypothesis II – It was proposed women with BD using psychiatric medication will have lower oxidative stress levels than non-psychiatric medication-users, with non-COC psychiatric medication users having less oxidative stress than COC psychiatric adjunctive users.

Hypothesis III – It was proposed that YMRS, HAMD and MADRS scores would correlate positively with oxidative stress markers (4-HNE, LPH, 3-NT and PCC) in both OCU and NCU women with BD.

Methods

Study participants were recruited from University of British Columbia, Vancouver General Hospitals and affiliated sites as well as through referrals from physicians and psychiatrists in British Columbia. Participants were recruited into a prospective study STOP-EM that aims to assess clinical outcomes, health status, brain morphology, neurochemistry, cognitive functioning, quality of life, and functional outcomes in patients who have recently experienced their first bipolar manic episode, according to the DSM-IV-TR criteria. This project is ongoing and initially started in July 2004. Our study population came from baseline participants enrolled between July 2004 to July 2012. People aged between 14 to 35 years were recruited if they were currently or had experienced a first manic episode within the past 3 months.

Our portion of the study was divided into three parts. Part one was the initial agreement to be enrolled for non-invasive procedures (clinical scales); the second part was an agreement to the invasive procedure (e.g. blood draw); and lastly the third part

involved a follow-up email asking whether or not the participant had used an oral contraceptive at the time of their initial blood draw.

First part: At baseline, assessment of all subjects enrolled completed a comprehensive clinical interview by a board-certified psychiatrist. This was to establish the diagnosis of BD-recent manic episode, which was based on all the available clinical information and a MINI International Interview. For the purposes of this study subjects who had a previously undiagnosed or untreated manic symptoms were excluded. Subjects who had a previous hypomanic (diagnosed or undiagnosed) were included. Comorbidities were diagnosed according to the DSM-IV-TR and other criteria available however for the purposes of this study were not included. Psychiatric status was assessed using clinical rating scales, YMRS, MADRS and HAMD (see materials). Psychiatric, medical histories, and information about current medication use, were obtained using all the available sources of information, including patient interview, completion of the Patient Health Survey, and when available, collateral information from family members and health records. In addition to psychiatric information, study participants had a physical examination, including height and weight. Participants were weighed and the formula for BMI = weight (kg)/ height (meters)². Underweight was defined as BMI < 18.50, normal weight as BMI = 18.50-24.99, overweight as BMI = 25.00-29.99, and obesity as BMI ≥ 30.00.

Second Part: At baseline study participants were also given the opportunity to take part in an optional study portion in which they provided blood samples for mitochondrial assay analyses. It is from this cohort that we obtained our samples.

Third Part: For the purposes of our study, participants who agreed to the second portion of the study and were female were re-contacted between July – August 2013 to ask if they had used a contraceptive agent when they had their blood drawn at baseline of the STOP-EM study.

The STOP-EM program and all of the procedures described in this report have received approval from the UBC Clinical Research Ethics Board. Participants were not billed for any clinical visits or for any tests that are required for this study.

Transportation costs were reimbursed (~\$10.00), receipts were not required. No monetary compensation was provided to participants who provided blood. Written consent was obtained from all participants prior to the commencement of any study procedures.

Participants

Inclusion/Exclusion Criteria

Study participants were diagnosed a priori with Bipolar Disorder and enrolled into the STOP-EM program. In order to be enrolled in the study, participants needed to be (1) aged 14 to 35 years old; (2) have experienced their first DSM-IV-TR defined manic episode ≤ 3 months before recruitment; (3) fluently speak English; (4) be able to read and consent. The inclusion criterion was deliberately broad to mimic and capture the full range of participants who routinely approach clinical practice. Participants were enrolled if they had either (5) pure mania or (7) mixed mania with or without psychotic features and (9) with or without co morbidity, which includes substance abuse or dependence.

Patients were excluded from the study if they demonstrated or met the following criteria; (1) if their initial manic episode was primarily due to a medical illness or substance use; (2) if their history indicated they have had a previous manic episode > 3 months before recruitment. Healthy participants were included if they (1) aged 14 to 35 years old; (2) without a previous history of psychiatric illness; (3) and no family history of their first or second degree relatives with a psychiatric illness, applying the same inclusion/exclusion criteria previously described.

Study Sample

Our sample was derived from the STOP-EM cohort 2nd portion, which collected blood from study participants (n=125). For the purposes of this project we eliminated BD male samples (n=42) and male controls (n=11). From the remaining population of women with BD (n=60) we eliminated samples if they did not respond to follow-up email/call asking whether or not they had taken a contraceptive agent at the time of blood draw and the type (n=7) and lastly samples which were collected at different time points (n=28). Samples were eliminated at different time points because they were not collected at baseline and the different time points were for some of the same patients (i.e. baseline, 6 mos, 9 mos, 12 mos and so forth). Using the same study participants more than once would question the integrity of this study. As for the controls, samples were eliminated if they did not respond to the follow-up contraceptive question (n=3) and with one sample being eliminated as it was collected at a different time point and was for the same

participant. Thus our final sample size of unique women was N=35, with BD n=25 (OCU (n=11) and NCU (n=14)) and healthy controls n=10 (OCU (n=5) and NCU (n=5)).

Determining Contraceptive Use

Women were considered OCU if they had taken a hormonal contraceptive (combined oral contraceptives or Depo Provera). Depo Provera (n=1) was included due to a previous study, which found an association between its use and increased lipid peroxides in serum for women (Faddah et al., 2005). Lastly, women were considered NCU if they had not taken a hormonal contraceptive, with the exception of an IUD and NuvaRing at the time of blood draw. Although IUD's can have a hormonal component (participants didn't specify), it was determined that it did not significantly affect oxidative stress (LPH) by a previous study (Pincemail et al., 2007). As for the NuvaRing, while no study has actually measured oxidative stress in NuvaRing users it was found to not impact antioxidant levels relative to COC users (Palan, Strube, Letko, Sadikovic, & Mikhail, 2010). Thus for the purposes of this study COC and Depo Provera will be combined and designated as 'Oral Contraceptive Use' (OCU), with IUD, NuvaRing and non-contraceptive use under the singular term 'Non-Contraceptive-Use' (NCU).

Procedure

Collection and Processing of Blood Samples

The blood samples were drawn by a registered nurse or via a hospital laboratory technician. Participants and controls that agreed and consented to the additional study

portion provided 20-mL blood samples that were collected by venipuncture into two BD (Becton, Dickinson and Company, Rutherford, NJ) 367820 Vacutainer Serum Tubes without anticoagulants. Non-fasting blood were obtained and on average early in the afternoon. We obtained the serum by centrifugation at 3000 *RPM* for 10 minutes which was aliquoted into Eppendorf tubes and kept frozen at -80°C for up to 6 months until we performed the biochemical assays.

Blood Withdrawal Protocol for Serum Collection

Sample Handling

To prevent large clots from forming prior to sample extraction, the vacutainers containing fresh blood were allowed to coagulate naturally before cooling.

Serum Extraction

To isolate the serum 10 mL of blood was collected using a *Serum Tube, Increased Silica Act Clot Activator, and Silicone-Coated Interior*. The blood was centrifuged for 15 minutes at 3000 rpm at 21 degrees Celsius. A pipette was used to extract 500uL of the plasma (supernatant) into appropriately labeled 1.5 mL eppendorf tube and stored at -80 degrees Celsius.

Materials

The following measures were included:

1. *BMI*. Patients' BMI's were calculated and provided from the University of British Columbia.

2. MINI – The Mini International Neuropsychiatric Interview is a structured diagnostic interview used to diagnose psychiatric disorders. The interview is divided into modules and a series of questions in each module are asked and require the responder to give a “yes” or “no” answer . All answers given are rated and the MINI is scored according. The MINI was also used to diagnose any co-morbidities. Most subjects that come into this study have a confirmed diagnosis from a clinician at a hospital or clinic (Sheehan et al., 1998).
3. *Young Mania Rating Scale* – The YMRS is a robust scale used to assess manic symptoms within the past 48 hours . The scale includes 11 items, which represent the core symptoms of mania and rely both on the patient’s subjective report and clinical observations by the interviewer during the clinic interview. Four of the eleven items are graded on a 0-8 scale while the remaining are on a 0-4 scale. The scale can accurately assess baseline severity of manic symptoms (i.e. a manic or hypomanic episode), which is determined by the overall interview score (Young, Biggs, Ziegler, & Meyer, 1978).
4. *Montgomery-Åsberg Depression Scale* – The MADRS is a clinical interview used to assess depression within the past two weeks . This scale relies on the interviewer to ask a series of broadly phrased questions about symptoms to more detailed ones to assess level of severity. Rating is determined either at defined scale steps (0, 2, 4, 6) or between them (1, 3, 5) (Montgomery & Asberg, 1979)
5. *The Hamilton Rating Scale For Depression* – The HAMD is clinical-administered scale used to determine/assess depression (Hamilton, 1967). Similar to the

YMRS, the HAMD incorporates 17 items known to depict the symptoms of depression over the past week. A score of 0-7 is generally considered to be within a normal range while a score of 20 indicates moderate severity.

Assays

17-beta Estradiol

17 beta-Estradiol was measured using an ab108667 by abcam 17 beta Estradiol Human ELISA Kit. Samples used were whole blood and were stored in -80 until prior use. The immunoenzymatic assay was competitive and quantified the concentration of 17 beta-Estradiol in serum. The ELISA was performed by adding 25 µl of samples, controls (positive and negative) as well as the standards to microtiter strip wells, which were precoated with anti-Estradiol 17 beta antibodies. 200 µl of conjugate were added to each well as well, leaving two wells for substrate blanks. Wells were covered with foil and incubated for 2 hours at 37 °C. After incubation the foil was removed and the contents of the wells were aspirated and washed three times with 300 µl of the diluted washing solution. The soak time between each wash cycle was approximately >5 sec. After washing was completed 100 µl of TMB substrate solution was added into the wells. The wells were incubated in the dark at 22-28°C for exactly 30 minutes. 100 µl of Stop Solution was added to all wells in the same order and at the same rate as the TMB substrate Solution. The absorbance values were measured immediately at 450 nm. (> 30

minutes after addition of the Stop Solution). Final concentrations were calculated with a Standard Curve: $y = -0.0062x + 1.8517$ ($R^2 = 0.92482$).

4-Hydroxynonenal

The OxiSelect™ catalogue number: STA-334 Cell Biolabs, INC HNE-His Adduct ELISA kit was used to quantify 4-HNE levels in samples (serum). Samples were diluted to 10 µg/mL in 1X PBS solution. 100 µL of the 10 µg/mL protein samples or reduced HNE-BSA standards were added to the 96-well Protein Binding Plate. It was then incubated at 37°C for at least 2 hours to allow binding to occur. Wells were then washed twice with 250 µL 1X PBS per well. After each wash wells were aspirated and tapped onto an absorbent pad to remove excess solution. Then 200 µL of Assay Diluent was added to each well and incubated for 1-2 hours on an orbital shaker at room temperature. Following incubation wells were washed three times with 250 µL of 1X Wash Buffer with thorough aspiration between and tapped onto an absorbent pad to remove excess 1X Wash Buffer. After washing, 100 µL of the diluted Anti-HNE-His Antibody was added to the wells and incubated for 1 hour at room temperature on an orbital shaker. Wells were then washed three times with 250 µL of 1X Wash Buffer (aspirated and tapped onto absorbent pads). After the second washing with 1X Wash Buffer, the substrate solution was warmed to room temperature and 100 µL was added to each well. Wells were then incubated at room temperature on an orbital shaker. Incubation time varied between 2-30 minutes depending on the rate of enzymatic reaction. When color was detected, 100 µL of Stop Solution was added to each well. Results were read immediately to ensure

accuracy. Absorbance was read at 450 nm. Final concentrations were calculated with a Standard curve: $y=0.2245 + 0.0367 (R^2 = 0.99061)$.

Protein Carbonyl Content

Protein Carbonyl content was quantified using the OxiSelect™ catalogue number: STA-310 Cell Biolabs, INC Protein Carbonylation ELISA kit. Samples were first diluted to 10 µg/mL in 1X PBS. 100 µL of the 10 µg/mL protein samples, including reduced/oxidized BSA standards were added to Protein Binding Plate and incubated for at least 2 hours at 37°C to ensure proper binding occurred. After incubation, wells were washed 3 times with 250 µL of 1X PBS. In-between washes wells were aspirated and tapped onto absorbent pads. Following the last wash (wells empty), 100 µL of the DNPH Working Solution was added to all wells. The wells were incubated for 45 minutes at room temperature in the dark. After incubation wells were washed again with 250 µL of 1X PBS/Ethanol (1:1) with incubation on an orbital shaker for 5 minutes. This was repeated 5 times with thorough aspiration between each washing/incubation period. After last washing with 1X PBS/Ethanol (1:1) wells were emptied and thoroughly aspirated. Wells were then washed twice with 250 µL of 1X PBS. After wells were washed 200 µL of Blocking Solution was added and the wells were incubated for 1-2 hours at room temperature on an orbital shaker. Following incubation wells were washed 3 times with 250 µL of 1X Wash Buffer and thoroughly aspirated between each wash. 100 µL of the diluted anti-DNP antibody was then added to wells and incubated for 1 hour at room temperature on an orbital shaker. Wells were washed again 3 times with 250 µL of 1X

Wash Buffer (with thorough aspiration between each wash). After last wash wells were emptied and 100 μL of the diluted HRP conjugated secondary antibody was added to all wells. Wells were incubated for 1 hour at room temperature on an orbital shaker.

Following incubation wells were washed 5 times with 250 μL (thorough aspiration between each wash). After last wash wells were emptied and 100 μL of the Substrate Solution (room temperature) was added to each well. The wells were incubated at room temperature on an orbital shaker. Incubation time varied between 2-30 minutes. Once color was detected, 100 μL of the Stop Solution was added to wells. Results were read immediately at 450 nm on a spectrophotometer. Final concentrations were calculated with a Standard Curve: $y=1.0647x + 0.1363$ ($R^2 = 0.99592$).

Lipid Hydroperoxides

Lipid Hydroperoxides were quantified using the ab133085 by abcam Lipid Hydroperoxide (LPH) Assay Kit. Samples were prepared the following: first they were aliquoted (500 μL) into a glass test tube, second an equal volume of Extract R saturated methanol (500 μL) was added to each test tube and vortexed. Following vortexing, 1 ml of cold chloroform was added to each test tube and then rigorously vortexed (1,500 x g for 5 minutes at 0°C). Next only the bottom layer (chloroform) was extracted by insertion of a pasteur pipette along the side of the test tube. The assay was then performed by adding ~500 μL of the chloroform extract (where the sample now is) into glass test tubes. 450 μL of chloroform-methanol solvent mixture was added to the sample test tubes. The Chromogen was then prepared at equal ratios between FTS Reagent 1 and

FTS Reagent 2 (50 μ L for each tube). 50 μ L of the Chromogen was added to each assay test tube and vortexed rigorously. The assay test tubes were then incubated at room temperature for five minutes. Following incubation absorbance was measured at 500 nm in glass 1 ml curvettes with spectrophotometer. Final concentrations were calculated with a Standard Curve: $y=0.0325x + 0.1675$ ($R^2 = 0.98026$).

3-Nitrotyrosine

The OxiSelect™ catalogue number: STA-305 Cell Biolabs, INC Nitrotyrosine ELISA kit was used to quantify 3-Nitrotyrosine (3-NT) in serum. 50 μ L of serum or nitrated BSA standard was added to the wells, which were pre-coated with EIA (the ELISA plate). The wells were incubated for 10 minutes on an orbital shaker for 10 minutes. Following incubation, 50 μ L of the diluted anti-nitrotyrosine antibody was added to each well and incubated at room temperature for 1 hour on an orbital shaker. Following this incubation period, wells were washed three times with 250 μ L 1X Wash Buffer with thorough aspiration between each wash. Wells were tapped onto an absorbent pad to remove any excess 1X Buffer Wash. After the last wash 100 μ L of the diluted Secondary Antibody-Enzyme Conjugate was added to the wells. This was then incubated at room temperature for 1 hour on an orbital shaker. After incubation, the wells were washed again 3 times with 1X Buffer Wash with thorough aspiration in between washes. After the last wash, wells were emptied and 100 μ L of Substrate Solution (room temperature) was added to each well. The wells were incubated at room temperature on an orbital shaker. The incubation period varied between 2-30 minutes. When color was detected, 100 μ L of

Stop Solution was added in the same order/rate to each well. Results were read immediately at 450 nm with spectrophotometer. Final concentrations were calculation with a Standard Curve: $y = 0.0545x + 0.3347$ ($R^2 = 0.82435$).

Results

Statistical Analyses

Statistics were calculated using SPSS Student version 20.0. A p value of < 0.05 was determined a priori to confer significance. Shapiro-Wilk and Levene's tests were used to assess normality and variance homogeneity. Samples that were not normally distributed were transformed, if the transformation did not normalize the sample a nonparametric test was used. For our first hypothesis, to determine if the means for oxidative stress markers: LPH, 3-NT, 4-HNE and PCC differed between women with BD (OCU and NCU) and women who were healthy controls (OCU and NCU) the nonparametric test Krustal-Wallis was used because the sample was not normally distributed. Within our second analysis, a MANCOVA was run to assess if a difference in means for oxidative stress markers existed between women with BD who used oral contraceptives and those did not, adjusting for smoking status. To assess psychiatric medication use (lithium, divalproex, olanzapine, risperidone and quetiapine) on oxidative stress markers a series of MANCOVA's were run. Our sample size was small and did not have the power required to run empirical analyses involving multiple factors. Therefore for each psychiatric medication analysis we decided to combine our categorical variables into one variable "group." To ensure human error was limited we used SPSS syntax to

digitally create the levels in “group.” The categorical variables we combined were (1) participant (BD or control), (2) oral contraceptive use (OCU or NCU), and psychiatric medication. Within the variable ‘psychiatric medication’ we combined variables by use (Y/N), type (mood stabilizer or atypical antipsychotic) and for further analyses type of mood stabilizer (lithium or divalproex) and atypical antipsychotic (risperidone, olanzapine, quetiapine). For example, to determine if mood stabilizer use affected oxidative stress in our sample we used SPSS syntax to create the four levels of “group”: BD + OCU + Yes Mood Stabilizer, BD + NCU + Yes Mood Stabilizer, Control + OCU + No Mood Stabilizer, Control + NCU + No Mood Stabilizer. While we cannot determine if an interaction exists between psychiatric medication use, oral contraceptive users and participant, this allowed us to assess the results in the most efficient way, given our small sample size. To determine if medication use impacted oxidative stress levels we looked at the following simulations: mood stabilizer (Y/N), type of mood stabilizer (lithium vs. divalproex vs. none), atypical antipsychotic use (Y/N), type of atypical antipsychotic (risperidone vs. olanzapine vs. quetiapine vs. none) and combined medication users (mood stabilizer + atypical antipsychotic). All analyses were calculated and adjusted for smoking status (n=17), as such are the ones discussed in this thesis. Unfortunately, smoking information was only provided for the BD group. Smoking severely impacts oxidative stress and in order to get a clear analysis of oxidative stress levels between the levels in our factor, it was pertinent to control. For the ANCOVA the covariates used were smoking (Y/N), BMI and age.

Lastly, a Spearman's correlation test was implemented to assess if a correlation existed between clinical scale (YMRS, MADRS and HAMD) scores and oxidative stress markers (LPH, 3-NT, 4-HNE, and protein carbonyl content) within each group (OCU and NCU) for women with BD. The Spearman's correlation was conducted with each clinical scale (YMRS, MADRS and HAMD) and compared separately to the four oxidative stress markers (LPH, 3-NT, 4-HNE and PCC).

Power Analysis

Our power analyses were calculated with the program G*Power. Sample sizes were calculated based on effect size (0.25, 0.50 and 0.75), α err probability (0.05), power (0.80), numerator df, number of groups (4) and number of covariates (2). The calculated sample sizes via different effect sizes were, $n=128$, $n=34$, and $n=17$, respectively. The sample size required for the MANCOVA (number of groups ~ 4) based on the same principles above yielded, $n=179$, $n=48$, $n=24$, respectively.

Within our own sample the power calculated with G*Power was 0.63 with $n=35$, effect size of 0.5, α err probability (0.05) with four groups, and two covariates. Unfortunately our project sample did not confer adequate power to draw a definitive conclusion.

Participant Characteristics

The sample size consisted of thirty-five women, twenty-five with BD (OCU $n=11$ and NCU $n=14$) and ten healthy controls (OCU $n=5$ and NCU $n=5$). Within the BD group, women who used COC's had a mean age of 25.90 SD 5.90 and an average BMI of

25.04 SD 4.30 and those who did not (NCU) had a mean age of 23.07 SD 4.71 and an average BMI of 24.59 SD 2.74. As for the healthy controls (n=10), women who used contraceptives (n=5) had a mean age 23.80 SD 4.27 and an average BMI 23.22 SD 2.62 and those who did not (NCU n=5) had a mean age of 27.20 SD 7.56 and an average BMI of 24.59 SD 2.74 (Table 1).

Descriptive Bipolar Disorder Characteristics

As Table 2 illustrates, a majority of participants (7 in the OCU group and 9 in the NCU group) had a previous hypomanic episode within the past 6 months and were more likely on a mood stabilizer (Lithium or Divalproex) and an atypical antipsychotic (Risperidone, Quetiapine or Olanzapine) than one medication. Of the participants who responded to smoking status (n=17), they were less likely to smoke (6 in the OCU and 8 in the NCU). The HAMD and MADRS mean scores were within normal range (0-7) for study participants in OCU and NCU groups. The YMRS mean score for OCU was within the depression range (YMRS=4), we attributed this to one high score of twenty-eight. When it was removed, the score was 1.6 (euthymia). With regards to the NCU group, the YMRS mean score was within normal range, indicating euthymia (YMRS=1.5) (Table 1).

Hormonal Contraceptive Statistics

The study makeup of COCs used were as follows: three women took Yasmin® (3 mg Drospirenone (DRSP) and 0.03 mg ethinyl estradiol (EE)), three women took Ortho Tricyclen® (0.180, 0.215, 0.250 mg of norgestimate (NG): with 0.035 mg EE during 1-7

days, 8-14 days, and 15-21 days, respectively) while the remaining four women took either: Minastrin® (1.0 mg of Norethindrone Acetate (NEA) and 0.020 mg of EE), Loestrin® (1.0 mg of NEA and 0.020 mg of EE), Cyclen® (0.25 mg of NG and 0.035 mg of EE) or Marvelon® (0.150 mg of desogestrel and 0.030 mg of EE). One patient took Depo Provera®, which is a medroxyprogesterone acetate injection (150 mg 1ml/Vial). Two of the controls used IUD's and one used a NuvaRing. The precise duration of OCU use is unknown. As for study participants who were controls the combined oral contraceptives used were: Brevicon® 28 (0.035 mg of EE and 0.5 mg NE), Depo Provera®, Femcon® FE (0.035 mg of EE and 0.4 mg NE), Alesse Ethinylestradiol and Levonorgesterel (100 mcg of levonorgestrel an 20 mcg of EE). Ortho Tri Cyclen Lo®. 1-7 days of 0.025 mg EE and 0.180 mg NG, 8-14 days 0.025 mg of EE an 0.215 mg of NG, 15-21 days 0.025 mg of EE and 0.250 mg of NG) (Table 22).

Statistic Tests

Kruskal Wallis

A Shapiro-Wilk test was performed to assess normality of the sample. The samples were not normally distributed and thus a nonparametric test was used. No differences were found between groups (BD and control) whether they used oral contraceptives (OCU) or not (NCU) on the oxidative stress parameters: LPH, 3-NT, HNE and PCC (Table 2).

Univariate Analysis of Covariance

A Shapiro-Wilk test was performed to assess the normality of the BD cohort

(n=25). The following oxidative stress markers, LPH, 4-HNE and PCC were not normally distributed. The markers that were not normally distributed were log transformed. LPH and PCC achieved normality however 4-HNE did not. Therefore an ANOVA was employed using 3-NT the transformed values LPH and PCC. No differences were found between BD oral contraceptive users and BD non-contraceptive users on oxidative stress markers LPH, 3-NT and PCC.

Mann-Whitney U

No differences were found between BD oral contraceptive users and BD non-contraceptive users for the biomarker 4-HNE.

Multivariate Analysis of Covariance

A Shapiro-Wilk test was performed to assess the normality of the smoking BD sample (n=17; OCU=8 and NCU=9). Lipid hydroperoxide was the only biomarker that wasn't normally distributed and was log transformed which achieved normality. A MANCOVA was used with the dependent variables being oxidative stress markers 4-HNE, PCC, 3-NT and the log transformed LPH with the factor as OCU or NCU (for BD). Covariates used were age, BMI and smoking status (Y/N). The test was significant with $F(1, 12)=5.639$, $p = 0.035$ (OCU BD 8.95 SD 1.62 vs. NCU BD 6.73 SD 2.22), with p-values adjusted by the Bonferroni correction. All assumptions were met for the ANCOVA including the covariates BMI, age and smoking for homogeneity of regression test, $F = 2.005$, $p > 0.05$ 0.190; $F = 0.003$, $p > 0.05$; $F = 2.90$, $p > 0.05$, respectively. The

partial eta squared was 0.320 and R squared was 0.366. Power was 58.8% with an effect size of 0.62 (Table 4 – 6).

Medication and Oxidative Stress Analysis

Univariate Analysis of Covariance

Mood Stabilizer

A Shapiro-Wilk test was performed to assess the normality of the sample. All of the oxidative stress markers were normally distributed and had equal variances as determined by Levene's Test of Equality of Error Variances. An ANCOVA was used with each individual dependent variable as an oxidative stress marker (4-HNE, 3-NT, LPH, and PCC), the independent variable was women with Bipolar disorder who used mood stabilizers by contraceptive use (OCU or NCU). 3-Nitrotyrosine was the only significant result and was increased in OCU (9.15 SD 1.64) relative to NCU (6.48 SD 2.23) women with BD who used mood stabilizers $F(1, 10) = 6.33, p = 0.031$. Covariates used were BMI, Age and Smoking Status (Y/N) and met homogeneity of regression ANCOVA assumption ($F = 0.420, p > 0.05$; $F=0.431, p > 0.05$; and $F=0.269, p > 0.05$, respectively). The partial eta squared was 0.388 with a R squared value of 0.429. The observed power was 62% and effect size was 0.72 (Table 7 – 9 showing results only for 3-NT).

Univariate Analysis of Covariance

Mood Stabilizer Type

A Shapiro-Wilk test was performed to assess the normality of the sample. All of the oxidative stress markers were normally distributed. Moreover the variances were equal as calculated with Levene's Test of Equality of Error Variances. An ANCOVA was used with each the dependent variable being the oxidative stress marker: 3-NT, 4-HNE, LPH or PCC. The independent factor consisted of 3 groups: women with BD who used oral contraceptives and divalproex (n=7) and women with BD who did not use oral contraceptives and used either lithium (n=5) or divalproex (n=2). Protein carbonyl content was the only significant test ($F(2, 9) = 5.010, p = 0.034$). Covariates used were BMI and Smoking Status (Y/N) and met homogeneity of regression ANCOVA assumption ($F=3.29, p > 0.05$; and $F=0.207, p > 0.05$, respectively). The pairwise comparison with Bonferroni adjusted alpha levels was significant between NCU divalproex and NCU lithium users and non significant between NCU divalproex and OCU divalproex and OCU divalproex and NCU divalproex. Protein carbonyl content was significantly higher in the Lithium NCU group than NCU Divalproex group (0.0978 SD 0.016 vs. 0.047 SD 0.005, respectively). Partial et squared was 0.527, with the R squared value was 0.708. The observed power was 66% with an effect size of 0.91 (Table 10– 12 showing results only for PCC).

Univariate Analysis of Covariance

Atypical Antipsychotic Use

A one-way univariate analysis of covariance was conducted to assess oxidative stress levels in women with BD who used contraceptives and did not and atypical antipsychotic use (Y/N). The dependent variable was each individual oxidative stress markers (3-NT, 4-HNE, LPH, or PCC) and independent variable included 4 levels: OCU and atypical antipsychotic use (n=4), NCU and atypical antipsychotic use (n=5), OCU and no atypical antipsychotic use (n=4) and NCU no atypical antipsychotic use (n=4). The ANCOVA test was significant for 3-Nitrotyrosine ($F(3, 10) = 4.822, p = 0.025$). The covariates used were smoking, BMI and age. The preliminary analysis evaluating regression of homogeneity of variances revealed that there was no relationship between the covariates and factor ($F = 2.86, p > 0.05$; $F=0.129, p > 0.05$; and $F=0.07, p > 0.05$, respectively). The observed power was 75% and the associated effect size was 1.05 (Table 13– 15 showing results only for 3-NT).

The follow-up pairwise comparison analysis for 3-Nitrotyrosine was used to evaluate differences between factor levels. The Bonferroni procedure was used to control for error across the pairwise comparisons. The results showed that 3-Nitrotyrosine was significantly increased in OCU group who used atypical antipsychotics (9.19 SD 1.24) when compared to NCU group who did not use atypical antipsychotics (5.01 SD 0.94), controlling for BMI, age and smoking status. Differences between the following pairs were non-significant: Atypical OCU (9.19 SD 1.24) and Atypical NCU (8.11 SD 1.97), Atypical NCU (8.11 SD 1.97) and No Atypical OCU (8.7 SD 2.11), and No Atypical

NCU (5.01 SD 0.94) and No Atypical OCU (8.70 SD 2.11) (Table 13– 15 showing results only for 3-NT).

Univariate Analysis of Covariance

The analysis of covariance was conducted with each dependent variable as an oxidative stress marker (3-NT, 4-HNE, LPH or PCC) and independent variable type of atypical antipsychotic use (Y/N) with four levels; OCU and Quetiapine (n=2), NCU and Quetiapine (n=3), OCU no atypical antipsychotic use (n=4) and NCU atypical antipsychotic use (n=4). The covariates used were BMI, age and smoking status (Y/N). No differences were shown between groups on oxidative stress markers.

Correlations

The Shapiro-Wilk test revealed my samples for YMRS, MADRS and HAMD for OCU/NCU were not normally distributed. To perform a log transformation, an arbitrarily assigned number '3' was added to each YMRS/MADRS/HAMD score (i.e. 0 became 3 and 1 became 4). The log-transformed data did not confer a normal distribution and thus a nonparametric test was utilized. A Spearman's bivariate analysis was performed on OCU and showed a positive and significant correlation between levels of YMRS and 3-Nitrotyrosine ($r(11) = 0.711$, $p = 0.014$) and lipid hydroperoxides ($r(11) = 0.676$, $p = 0.022$). No significant correlations were found within OCU between clinical scales (MADRS and HAMD) and oxidative stress markers (3-NT, 4-HNE, LPH and protein carbonylation), in addition no relationship was found between YMRS and the oxidative

stress: protein carbonylation and 4-HNE. While not significant there were strong trends between lipid hydroperoxide levels and HAMD ($r(11) = 0.549$, $p = 0.08$) and MADRS ($r(11) = 0.578$, $p = 0.062$) scores for COC users. With regards to NCU clinical scales (YMRS, MADRS and HAMD) did not correlate with oxidative stress markers (3-NT, 4-HNE, LPH and protein carbonylation). Correlations were significant at the 0.05 level (2-tailed). After removing the YMRS score of 28, lipid hydroperoxides ($r(11) = 0.555$, $p = 0.096$) and 3-NT ($r(11) = 0.603$, $p = 0.06$) were no longer significant (Table 16 showing results only for YMRS and 3-NT, 4-HNE, LPH, and PCC).

Additional Analysis: Hormone Correlation

A Spearman's two-tailed correlation was utilized to determine if a relationship existed between 17-beta Estradiol serum level and oxidative stress markers: 3-NT, 4-HNE, LPH and PCC. A significant positive correlation was found between the oxidative stress marker LPH and 17-beta Estradiol ($r(17) = 0.598$, $p = 0.011$) (Figure 5).

Discussion

The primary finding from this cross-sectional study, which focused on BD women was that oxidative stress markers were not influenced by COC use relative to healthy controls. This is incongruent with our recent literature review, which found that women using COC had increased oxidative stress relative to NCU women. These results are surprising yet may be linked in part to data collection. For example both healthy women who used COCs (Finco et al., 2011) and those who did not (Umberto Cornelli et al.,

2013), were shown to have baseline hydroperoxide levels during the placebo week and early follicular phase, respectively. Therefore, with respect to our study it could be argued that women with BD and controls using COCs were on the placebo week and non-contraceptive users might have been premenstrual when the blood was drawn. This is entirely a guess as we did not accurately collect this information. However, after adjusting for smoking (Y/N), BMI and age there were significant differences within the BD group, women using COCs had increased levels of 3-Nitrotyrosine relative to BD COC nonusers. 3-Nitrotyrosine is a product of protein nitrosative damage that occurs when peroxynitrite/carbon dioxide-derived radicals attack the hydroxyl group of tyrosine residues (Tsikas, Mitschke, & Gutzki, 2012). Other studies have found similar results of increased levels of 3-NT in adult patients with BD. For example Andreazza et al. (2009) reported that 3-NT was increased in early and late staged manic patients compared to healthy controls in serum. This has also been confirmed in prefrontal cortex samples, where 3-NT was elevated in patients with BD and schizophrenia relative to patients with depression and healthy controls (Ana C. Andreazza et al., 2010). However, we cannot comment on 3-NT and COC use as there are no published studies available to compare to. While we found differences (after controlling for smoking, BMI and age) between OCU/NCU BD women, a possible explanation as to why we did not see differences between OCU/NCU BD and healthy controls could be the use of psychiatric medications among BD women, which could be influencing oxidative stress.

To date there are no drug-drug interaction studies which have evaluated the use of COC adjunctively with either mood stabilizers or atypical antipsychotics on oxidative

stress markers. Based on preclinical data for BD both *in vitro* (Lai, Zhao, Warsh, & Li, 2006; Shao, Young, & Wang, 2005) and *in vivo* (Ana Cristina Andreazza et al., 2008; Frey et al., 2006) it has been hypothesized that lithium and valproate could potentially decrease oxidative stress by promoting an antioxidant defense. Our secondary finding from this study showed that women with BD using COC in adjunctive use with a mood stabilizer had increased levels of 3-NT relative to NCU BD who used a mood stabilizer. Within our type of mood stabilizer analysis PCC was increased in lithium users relative to divalproex users, in women with BD who were COC nonusers. While not significant it appears divalproex has anti-oxidant properties as OCU BD women on divalproex had lower PCC levels relative to NCU women lithium users. However, while not significant it appears there is a relationship between COC and divalproex, as COC users with adjunctive use of divalproex had increased PCC levels relative to COC nonusers who used divalproex. Our results conflict with previous studies which found lithium to have antioxidant properties, for example de Sousa et al. (2014) reported 6 weeks of lithium treatment corresponding to a decrease in thiobarbituric acid reactive substances (TBARS - a measurement of MDA). Our results do support a recent study which found lithium increases oxidative stress in patients with BD relative to controls, however differed with their secondary analysis which found oxidative stress did not differ between BD lithium and lithium non-users (Ana Cristina Andreazza et al., 2014). This is incongruent with our results as we found lithium users had increased oxidative stress compared to non-lithium users, irrespective of COC use. As for combined medications, Aliyazicioglu et al. (2007) measured TBARS in patients with BD using two treatments, lithium or lithium in

combination with olanzapine. They observed lipid peroxides decreased significantly following combined treatment, and decreased further within lithium only treatment (Aliyazicioğlu et al., 2007). It is not unlikely that patients with BD are prescribed a combination of medications, therefore this needs to be taken into consideration. It was found within our sample that women with BD were prescribed a combination of medications, mainly a mood stabilizer with an atypical antipsychotic. Thus the use of an atypical antipsychotic could affect oxidative stress levels. With respect to our study, atypical antipsychotics were found to affect oxidative stress, with 3-Nitrotyrosine being significantly increased in NCU BD women who did not use atypical antipsychotics relative to women who were COC users with adjunctive use of atypical antipsychotics. Within our atypical antipsychotic type analysis, no significant differences were found between risperidone, olanzapine, quetiapine or non-users in women with BD who were COC users and nonusers of COCs. It has been reported that olanzapine can induce oxidative stress relative to the administered dose (Türkez & Toğar, 2010). Unfortunately we did not record the dose of each medication, therefore we were unable to conclude whether our results differ relative to the current literature. Within schizophrenia there have been reports of oxidative stress changes associated with atypical antipsychotic use which resulted in improved symptoms over 8 (Dakhale et al., 2004) and 12 (Zhang et al., 2003) week treatments. However not all have found significant changes in oxidative stress with atypical antipsychotic treatment (Sarandol et al., 2007). Currently there are no studies published that have evaluated the relationship between oral contraceptive use with psychiatric medications on oxidative stress in women with BD. There however has been

studies on oral contraceptive adjunctive use with anticonvulsants. Combined oral contraceptives with EE can induce the activity of uridine-diphosphate glucuronosyltransferase and increase the rate of drug glucuronidation, which is the suspected mechanism that is responsible for the increase in renal excretion and reduced concentrations of lamotrigine (Reimers, Helde, & Brodtkorb, 2005). If COC can impact anticonvulsant concentrations, and if anticonvulsants have antioxidant actions, we can speculate that the combined use may alter oxidative stress levels. Overall it is difficult to draw conclusions, as we did not confer the necessary power required for significant results.

With regards to our last research question, our results are congruent with the literature. Indeed YRMS scores correlated positively with 3-NT and LPH levels. Which is supported by both Andreazza et al. (2007a) and Kapczinski et al. (2011) as they found increases in TBARS in patients with BD, with the later study finding increased PCC relative to healthy controls. However the relationship within our study was found to only be significant within the COC users with BD. The COC nonuser women's YMRS score corresponded to 'euthymic', in support Andreazza et al. (2007a) found lower levels of TBARS in euthymic patients relative to manic patients. Interestingly when we removed a high YMRS score of 28 from the COC cohort, the positive relationship with LPH and 3-NT was no longer significant. After removing the extreme YMRS score the COC users average YMRS was 'euthymic.' This is in agreement with Andreazza et al. (2007a) who found TBARS were not increased in euthymic patients relative to manic and controls, but not in agreement with Kunz et al. (2008) who reported TBARS were increased in

euthymic patients compared to controls. Within our non-contraceptive using cohort, MADRS, HAMD and YMRS scores were not significantly correlated. All scores were within euthymia range, which is in agreement with Andreazza et al. (2007a), however not in agreement with Kunz et al. (2008). Overall, it is difficult to draw conclusions on the relationship between COCs, BD and oxidative stress. However our additional analysis provides insight on whether a relationship exists between hormones and oxidative stress.

Our additional analysis compared levels of 17-beta estradiol to oxidative stress markers. We found a significant positive relationship between LPH and 17-beta Estradiol across all women (BD and controls). This currently is incongruent with the current consensus on E2 and oxidant ability. However is supported by recent studies conducted by Cornelli et al. (2013) and Schisterman et al. (2010) who analyzed oxidative stress markers d-ROMs and F2-Isoprostanes, respectively across the menstrual cycle in normal menstruating women not using a hormonal contraceptive. They found that oxidative stress markers were heightened during the expected time of ovulation and lowest during the follicular phase (Umberto Cornelli et al., 2013; Schisterman et al., 2010). With respect to the follicular phase E2 levels are generally low, ergo E2 has antioxidant properties as well, which are contingent on the dose. In support Andozia et al. (2010) showed that a low dose of E2 (early follicular phase) stimulated Nitric Oxide (NO) synthesis which afforded protection and increased viability in endothelial cells that were pre-incubated with hydrogen peroxide. Of particular interest, within the same study, Andozia et al. (2010) reported that even a low dose of EE failed to activate NO and thus did not protect the endothelial cells from oxidative stress. Overall it would seem there is

a relationship between oxidative stress (LPH) and estrogen, which warrants further investigation.

Clinical Implications

A recent meta-analysis confirmed oxidative stress (LPH and DNA/RNA damage) is elevated in persons with BD relative to controls (Brown, Andreazza, & Young, 2014b). This associated oxidative stress has been implicated in several disorders, for example mitochondrial dysfunction (Lin & Beal, 2006). Indeed oxidative stress can cause reduced expression of several mitochondrial electron transport chain subunits, increased mtDNA deletion and mutation, reduced pH and levels of high-energy phosphates have been reported to be decreased in the brains of BD patients relative to controls (Clay, Sullivan, & Konradi, 2011).

The preponderance of mitochondrial dysfunction found in individuals with BD is heavily supported in the literature (Ana C. Andreazza & Young, 2014) and confirmed within multi-dimensional studies (Cataldo et al., 2010; Fattal, Link, Quinn, Cohen, & Franco, 2007; Regenold et al., 2009). In addition mitochondrial dysfunction has been found to be significantly increased in individuals with autoimmune/inflammation disorders (Hernández-Aguilera et al., 2013). With respect to BD, the prevalence of autoimmune disorder comorbidity is increased which has led to the hypothesis that psychiatric disorders might be linked in part to autoimmune disorders (Hamdani, Doukhan, Kurtlucan, Tamouza, & Leboyer, 2013).

Autoimmune disorders occur when the body's immune system attacks healthy cells leading to disease (A.D.A.M. Medical Encyclopedia, 2013). This immune response directed to healthy tissue can cause prolonged inflammation (A.D.A.M. Medical Encyclopedia, 2013). Inflammation is a healthy response to infection, but chronic inflammation has been implicated in the pathogenesis of cancer. One documented source of inflammation is an increase in ROS (Rottner, Freyssinet, & Martinez, 2009). Indeed ROS have been reported to trigger inflammation through a pathway which involves a chain effect of promoting MAPKinase activation, followed by triggering NF-KB which leads to an inflammatory response with cytokines (Rottner et al., 2009). These inflammatory cytokines can actually in turn promote more ROS, creating a pathological feedback loop where tissue is consistently in a pro-inflammatory state (Rottner et al., 2009). This ultimately compromises the immune system which makes the body vulnerable to exogenous and endogenous toxic attacks (Rottner et al., 2009). With regards to our study we found that COC use induces oxidative stress through either a decrease in antioxidant defense system or an increase in reactive oxygen species. Currently studies are not consistent on whether a decrease in antioxidants can alone be responsible for an increase in oxidative stress. However, we hypothesize that COC can increase ROS (as discussed above) and has the potential to trigger the inflammation pathway placing the body into a pro-inflammatory state. This in turn might influence autoimmune disorders, which could lead to the entrainment or exacerbation of mood BD-related illness episodes (Figure 2). We see that mood can be linked to oxidative stress, with studies showing an increase corresponding to manic and depressive episodes

(Kapczinski et al., 2011; Kunz et al., 2008), which is congruent with our thesis results. Another biomarker for BD are inflammation markers, interestingly inflammation markers (IL-2, IL-4, IL-6) are heightened during mania and IL-6 during depression (Brietzke et al., 2009). We are beginning to see that different mood states correspond with different markers, this suggests that peripheral markers (oxidative stress and inflammation) could be promising biomarkers as hypothesized by Frey & Dias. (2014). Thus the next step is to determine and investigate the modulators of oxidative stress and pro-inflammatory inducing pathways. Within our study we found that YRMS scores correlated positively with 3-NT and LPH within the COC-using group. Perhaps one exogenous source for producing ROS, which creates oxidative stress and activates pro-inflammatory pathways, could be hormones.

The next step would be determining whether or not peripheral markers can be used to assess brain abnormalities. One recent study (Versace et al., 2014) addressed this exact question and found that the varying levels of lipid peroxidation were associated and could explain white matter abnormalities by 59% and 51% of fractional anisotropy and radial diffusivity differences, respectively.

Limitations and Future Directions

There are several limitations that need to be addressed in this study. First our inclusion criteria, while deliberately broad did not control for diet or comorbidity for diseases i.e. cardiovascular disease, all of which are known factors to affect oxidative stress. Secondly menstrual cycle day was not controlled for, making it difficult to be

precise in indentifying where in the cycle a participant was. This is important because women using combined oral contraceptives have a placebo week. Lastly we were limited by our small sample size.

Based on the findings from this study, longitudinal studies are required to further investigate this relationship between combined oral contraceptives and oxidative stress parameters. It is therefore advisable to apply the listed limitations to determine whether or not women with BD have increased oxidative stress markers relative to non-psychiatric women. In addition, further research is required to understand pharmacotherapy drug-drug dynamic interactions on oxidative stress. We found differences in mood stabilizer use and atypical use. The next direction would be to evaluate each individual mood stabilizer, atypical antipsychotic and combined use on oxidative stress markers in women with BD in both OCU and NCU conditions. All of these suggestions should be applied within large, double-blinded, placebo controlled studies.

Conclusion

To the best of our knowledge, no study has examined oxidative stress levels between women with BD OCU and NCU. After controlling for smoking, BMI and age it was found OCU women with BD have increased 3-Nitrotyrosine relative to NCU women with BD. The management of BD episodes in women can present additional challenges associated with the reproductive cycle. Recently these mood episodes have been found to be influenced by oxidative stress. Indeed within our study we found a relationship between the oxidative stress markers 3-NT and LPH, and YRMS scores to be significant

only within the COC users. Furthermore with respect to our additional analysis, LPH was significantly correlated to E2. Overall, we have found convincing evidence that the relationship between oxidative stress and mood episodes could be influenced by exogenous hormones.

Table 1
Demographic characteristics of participants.

Characteristic	BD		Control	
	OCU (n=11)	NCU (n=14)	OCU (n=5)	NCU (n=5)
Age (yrs +/- SD)	25.90 ± 5.90	23.07 ± 4.71	23.80 ± 4.27	27.20 ± 7.56
BMI (kg/m ²)	25.04 ± 4.30	24.59 ± 2.74	23.22 ± 2.62	24.59 ± 2.74
Smoking (% yes)	25%	11.1%	--	--
Mood Episode				
Within Past 6 Months				
Hypomanic (% yes)	63.6%	64.3%	--	--
Depression (% yes)	18.2%	14.3%	--	--
Medication(s) (%)				
None	9.1%	----	---	---
Lithium	---	14.3%	---	---
Divalproex	36.4%	14.3%	---	---
Quetiapine	---	7.1%	---	---
Lithium + Risperidone	18.2%	7.1%	---	---
Lithium + Divalproex	9.1%	7.1%	---	---
Lithium + Quetiapine	---	14.3%	---	---
Divalproex + Risperidone	---	14.3%	---	---
Divalproex + Olanzapine	9.1%	21.4%	---	---
Divalproex + Quetiapine	18.2%	---	---	---
YMRS	4 SD 8.40 [Range: 0 – 28]	1.50 SD 1.99 [Range: 0 – 5]	---	---
HAMD	5.36 SD 6.74 [Range: 0 – 18]	6.50 SD 9.04 [Range: 0 – 29]	---	---
MADRS	5.64 SD 6.70 [Range: 0 – 17]	4.72 SD 2.58 [Range: 0 – 19]	---	---

Note. YMRS – Young Manic Rating Scale; the maximum score is 60.

MADRS – Montgomery-Asberg Depression Scale; the maximum is 60.

HAMD – Hamilton Rating Scale Depression; the maximum score is 48.

Table 2

Means categorized by oral contraceptive use in women with bipolar disorder and healthy controls (n=35)

	Bipolar Disorder				Control			
	OCU (n=11)		NCU (n=14)		OCU (n=5)		NCU (n=5)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LPH (nmol/mL)	2.71	1.66	3.57	2.51	3.57	2.51	3.49	1.78
PCC (nmol/mg)	.11	.05	.08	.07	.08	.07	.09	.03
4-HNE (µg/mL)	.62	.36	.53	.28	.53	.28	.63	.32
3-NT (nmol/mL)	9.04	2.96	8.08	1.72	8.08	1.72	7.69	3.10

Note. LPH = Lipid Hydroperoxides

PCC = Protein Carbonyl Content

4-HNE = 4- Hydroxynonenal

3-NT = 3-Nitrotyrosine

OCU = Oral Contraceptive Use

NCU = Non Contraceptive Use

Table 3

Descriptive statistics for oxidative stress markers in Women with BD who took oral contraceptives and those who did not, smoking sample (n=17)

	Bipolar Disorder			
	OCU (n=8)		NCU (n=9)	
	Mean	SD	Mean	SD
LPH (nmol/mL)	2.58	1.40	3.97	1.92
PCC (nmol/mg)	.10	.04	.08	.03
HNE (µg/mL)	.60	.35	.67	.34
3-NT (nmol/mL)	8.95	1.63	6.74	2.22

Note. LPH = Lipid Hydroperoxides

PCC = Protein Carbonyl Content

4-HNE = 4- Hydroxynonenal

3-NT = 3-Nitrotyrosine

OCU = Oral Contraceptive Use

NCU = Non Contraceptive Use

Table 4

ANCOVA results of oxidative stress levels between women with Bipolar Disorder who took oral contraceptives (n=8) and those who did not (n=9), adjusted for smoking status, BMI and Age (N=17).

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
BMI	LPH (nmol/mL)	2.08	1	2.08	.62	.45
	3-NT (nmol/mL)	.03	1	.03	.01	.94
	PCC (nmol/mg)	.00	1	.00	.13	.72
	4-HNE (µg/mL)	.03	1	.03	.26	.62
Age	LPH (nmol/mL)	.33	1	.33	.10	.76
	3-NT (nmol/mL)	6.59	1	6.59	1.58	.23
	PCC (nmol/mg)	.00	1	.00	2.41	.15
	4-HNE (µg/mL)	.14	1	.14	1.04	.33
Smoke	LPH (nmol/mL)	.39	1	.39	.12	.74
	3-NT (nmol/mL)	.11	1	.11	.03	.87
	PCC (nmol/mg)	6.99E-007	1	6.99E-007	.00	.98
	4-HNE (µg/mL)	.05	1	.05	.38	.55
Group	LPH (nmol/mL)	7.58	1	7.58	2.25	.16
	3-NT (nmol/mL)	23.50	1	23.50	5.64	.04*
	PCC (nmol/mg)	.00	1	.00	1.23	.29
	4-HNE (µg/mL)	.02	1	.02	.18	.68
Error	LPH	40.43	12	3.37		
	3-NT	50.01	12	4.17		
	PCC	.01	12	.00		
	4-HNE	1.58	12	.13		

Note.

R Squared = .212 (Adjusted R Squared = -.051)

R Squared = .366 (Adjusted R Squared = .154)

R Squared = .278 (Adjusted R Squared = .037)

R Squared = .109 (Adjusted R Squared = -.188).

LPH = Lipid Hydroperoxides, 3-NT = 3-Nitrotyrosine, PCC = Protein Carbonyl Content, and 4-HNE = 4-Hydroxynonenal. Covariates met homogeneity of regression assumptions.

* p value 0.05

Table 5

Estimated means for oxidative stress markers for ANCOVA analysis

Dependent Variable	Group	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
LPH	OCU	2.58 ^a	.66	1.14	4.02
(nmol/mL)	NCU	3.97 ^a	.62	2.61	5.32
3-NT	OCU	9.07 ^a	.73	7.47	10.67
(nmol/mL)	NCU	6.63 ^a	.69	5.13	8.14
PCC	OCU	.10 ^a	.01	.07	.12
(nmol/mg)	NCU	.08 ^a	.01	.05	.10
4-HNE	OCU	.60 ^a	.13	.31	.88
(µg/mL)	NCU	.67 ^a	.12	.41	.94

Note.

a. Covariates appearing in the model are evaluated at the following values: BMI = 25.0765, Age = 25.2353, Smoke = 1.82.

Note. LPH = Lipid Hydroperoxides

PCC = Protein Carbonyl Content

4-HNE = 4- Hydroxynonal

3-NT = 3-Nitrotyrosine

OCU = Oral Contraceptive Use

NCU = Non Contraceptive Use

Table 6

Pairwise Comparisons for ANCOVA analysis, adjusted by Bonferroni

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig. ^b	95% Confidence Interval for Difference ^b	
						Lower Bound	Upper Bound
LPH	OCU	NCU	-1.38	.92	.16	-3.39	.63
(nmol/mL)	NCU	OCU	1.38	.92	.16	-.63	3.39
3-NT	OCU	NCU	2.44 [*]	1.03	.04 [*]	.20	4.67
(nmol/mL)	NCU	OCU	-2.44 [*]	1.03	.04 [*]	-4.67	-.20
PCC	OCU	NCU	.02	.02	.29	-.02	.05
(nmol/mg)	NCU	OCU	-.02	.02	.29	-.05	.02
4-HNE	OCU	NCU	-.08	.18	.68	-.47	.32
(µg/mL)	NCU	OCU	.08	.18	.68	-.32	.47

Based on estimated marginal means

*. The mean difference is significant at the $p < 0.05$

b. Adjustment for multiple comparisons: Bonferroni.

Table 7

ANCOVA results of 3-Nitrotyrosine between women with Bipolar Disorder who took oral contraceptives (n=7) and those who did not (n=8), and Mood Stabilizer use (yes), adjusted for smoking status, BMI and Age (N=15).

Dependent Variable: Nitrotyrosine

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Smoke	.071	1	.071	.016	.902
BMI	.093	1	.093	.021	.888
Age	4.384	1	4.384	.986	.344
Group	28.173	1	28.173	6.337	.031
Error	44.456	10	4.446		
Total	974.061	15			

R Squared = .429 (Adjusted R Squared = .200)

- p value < 0.05

Table 8

Estimate means for 3-Nitrotyrosine for ANCOVA

Dependent Variable: 3-Nitrotyrosine

Group	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
OCU	9.231 ^a	.808	7.432	11.031
NCU	6.416 ^a	.754	4.735	8.096

Table 9

Pairwise Comparisons between women with BD (OCU and NCU) and mood stabilizer use (yes), adjusted by Bonferroni

Dependent Variable: Nitrotyrosine

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig. ^b	95% Confidence Interval for Difference ^b	
					Lower Bound	Upper Bound
OCU	NCU	2.816*	1.119	.031	.324	5.308
NCU	OCU	-2.816*	1.119	.031	-5.308	-.324

Based on estimated marginal means

*. The mean difference is significant at the $p < 0.05$

b. Adjustment for multiple comparisons: Bonferroni.

Table 10

ANCOVA results of Protein Carbonyl Content between women with Bipolar Disorder who took oral contraceptives and those who did not, and type of Mood Stabilizer (Lithium or Divalproex), adjusted for smoking status, BMI and Age (N=9).

Dependent Variable: Protein Carbonyl Content

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Smoke	3.88E-007	1.00	3.88E-007	.00	.97
BMI	.00	1.00	.00	7.53	.02
Group	.00	2.00	.00	5.01	.03*
Error	.00	9.00	.00		

R Squared = .708 (Adjusted R Squared = .578). Covariates used: BMI and Smoking Status. MANCOVA met covariate assumptions for homogeneity of regression.

*. p value < 0.05

Table 11

Estimate means for Protein Carbonyl Content for ANCOVA

Dependent Variable: Protein Carbonyl Content

Group	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Lithium + NCU	.098 ^a	.007	.081	.115
Divalproex + OCU	.088 ^a	.006	.073	.102
Divalproex + NCU	.053 ^a	.012	.026	.081

a. Covariates appearing in the model are evaluated at the following values: Smoke = 1.79, BMI = 25.25.

Table 12

Pairwise Comparisons between women with BD (OCU and NCU) and mood stabilizer type (Lithium or Divalproex), adjusted by Bonferroni

Dependent Variable: Protein Carbonyl Content

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig. ^b	95% Confidence Interval for Difference ^b	
					Lower Bound	Upper Bound
Lithium + NCU	Divalproex + OCU	.010	.010	.952	-.018	.039
	Divalproex + NCU	.045*	.014	.034	.003	.086
Divalproex + OCU	Lithium + NCU	-.010	.010	.952	-.039	.018
	Divalproex + NCU	.034	.014	.105	-.006	.075
Divalproex + NCU	Lithium + NCU	-.045*	.014	.034	-.086	-.003
	Divalproex + OCU	-.034	.014	.105	-.075	.006

Based on estimated marginal means

*. The mean difference is significant at the $p < 0.05$

b. Adjustment for multiple comparisons: Bonferroni.

Table 13

ANCOVA results of 3-Nitrotyrosine between women with Bipolar Disorder who took oral contraceptives and those who did not, and Atypical Antipsychotic (Y/N), adjusted for smoking status, BMI and Age (N=10).

Dependent Variable: 3-Nitrotyrosine

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Smoke	2.199	1	2.199	.732	.412
Age	4.025	1	4.025	1.340	.274
BMI	.230	1	.230	.076	.788
Group	43.465	3	14.488	4.822	.025
Error	30.044	10	3.004		

R Squared = .619 (Adjusted R Squared = .390)

Table 14

Estimate means for 3-Nitrotyrosine for ANCOVA

Dependent Variable: 3-Nitrotyrosine

Group	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Atypical + OCU	9.33 ^a	.90	7.32	11.34
Atypical + NCU	8.13 ^a	.83	6.29	9.98
None + OCU	8.62 ^a	1.00	6.41	10.84
None + NCU	4.94 ^a	.88	2.97	6.91

a. Covariates appearing in the model are evaluated at the following values: Smoke = 1.82, Age = 25.23, BMI = 25.07.

Table 15

Pairwise Comparisons between women with BD (OCU and NCU) and atypical antipsychotic (Y/N), adjusted by Bonferroni

Dependent Variable: 3-Nitrotyrosine

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig. ^b	95% Confidence Interval for Difference ^b	
					Lower Bound	Upper Bound
Atypical + OCU	Atypical + NCU	1.195	1.167	1.000	-2.629	5.019
	None + OCU	.703	1.427	1.000	-3.975	5.380
	None + NCU	4.387*	1.290	.041*	.159	8.614
Atypical + NCU	Atypical + OCU	-1.195	1.167	1.000	-5.019	2.629
	None + OCU	-.492	1.398	1.000	-5.074	4.089
	None + NCU	3.192	1.239	.165	-.867	7.251
None + OCU	Atypical + OCU	-.703	1.427	1.000	-5.380	3.975
	Atypical + NCU	.492	1.398	1.000	-4.089	5.074
	None + NCU	3.684	1.293	.104	-.553	7.921
None + NCU	Atypical + OCU	-4.387*	1.290	.041*	-8.614	-.159
	Atypical + NCU	-3.192	1.239	.165	-7.251	.867
	None + OCU	-3.684	1.293	.104	-7.921	.553

Based on estimated marginal means

*. The mean difference is significant at the $p < 0.05$

b. Adjustment for multiple comparisons: Bonferroni.

Table 16

Correlations between YMRS scores and LPH, PCC, 4-HNE and 3-NT in women with BD taking oral contraceptives (N=11)

		LPH	PCC	4-HNE	3-NT	YMRS	
Spearman's rho	LPH	Correlation Coefficient	1.000	.573	.196	.382	.676*
		Sig. (2-tailed)	.	.066	.564	.247	.022
		N	11	11	11	11	11
	PCC	Correlation Coefficient	.573	1.000	.068	.127	.199
		Sig. (2-tailed)	.066	.	.842	.709	.558
		N	11	11	11	11	11
	4-HNE	Correlation Coefficient	.196	.068	1.000	.419	.371
		Sig. (2-tailed)	.564	.842	.	.199	.261
		N	11	11	11	11	11
	3-NT	Correlation Coefficient	.382	.127	.419	1.000	.711*
		Sig. (2-tailed)	.247	.709	.199	.	.014
		N	11	11	11	11	11
	YMRS	Correlation Coefficient	.676*	.199	.371	.711*	1.000
		Sig. (2-tailed)	.022	.558	.261	.014	.
		N	11	11	11	11	11

*. Correlations are significant at $p < 0.05$ level (two-tailed)

Note. LPH = Lipid Hydroperoxides
PCC = Protein Carbonyl Content
4-HNE = 4- Hydroxynonenal
3-NT = 3-Nitrotyrosine

Table 17

Means categorized by oral contraceptive use, mood stabilizer use (yes) in women with bipolar disorder and healthy controls.

	Bipolar Disorder				Control			
	OCU (n=10)		NCU (n=13)		OCU (n=5)		NCU (n=5)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LPH (nmol/mL)	2.74	1.74	3.53	1.85	3.57	2.51	3.05	1.56
PCC (nmol/mg)	.10	.05	.09	.03	.08	.07	.09	.05
4-HNE (µg/mL)	.65	.36	.59	.29	.53	.28	.47	.32
3-NT (nmol/mL)	9.19	3.07	7.61	3.21	8.08	1.72	9.56	2.43

Table 18

Means categorized by oral contraceptive use, mood stabilizer type (Lithium or Divalproex) in women with bipolar disorder.

Medication	Bipolar Disorder							
	Lithium				Divalproex			
	OCU (n=2)		NCU (n=5)		OCU (n=7)		NCU (n=7)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LPH (nmol/mL)	4.18	2.36	4.65	2.19	2.60	1.51	3.02	1.28
PCC (nmol/mg)	.16	.11	.10	.02	.09	.02	.09	.04
4-HNE (µg/mL)	.82	.51	.77	.27	.64	.36	.47	.28
3-NT (nmol/mL)	11.64	5.94	6.12	1.77	9.15	1.65	9.02	3.68

Table 19

Means categorized by oral contraceptive use, atypical antipsychotic use (yes/no) in women with bipolar disorder.

	Bipolar Disorder							
	Atypical Antipsychotic				No Atypical Antipsychotic			
	OCU (n=5)		NCU (n=9)		OCU (n=6)		NCU (n=5)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LPH (nmol/mL)	2.98	1.83	3.57	1.89	2.49	1.64	3.34	1.78
PCC (nmol/mg)	.11	.07	.09	.03	.11	.03	.08	.04
4-HNE (µg/mL)	.84	.37	.66	.34	.43	.23	.56	.30
3-NT (nmol/mL)	10.52	3.16	8.35	2.89	7.81	2.34	6.52	3.45

Table 20

Means categorized by oral contraceptive use, atypical antipsychotic type (Risperidone, Olanzapine, Quetiapine and no atypical antipsychotic) in women with bipolar disorder.

Med	Risperidone		Olanzapine		Quetiapine		No Atypical Use							
	OCU		NCU		OCU		NCU							
	(n=2)	(n=2)	(n=2)	(n=2)	(n=2)	(n=4)	(n=6)	(n=5)						
	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD
LPH	3.46	3.37	4.95	2.54	2.86	2.57	2.89	.91	2.90	.45	2.49	1.64	3.34	1.78
PCC	.17	.09	.08	.02	.08	.04	.08	.04	.10	.03	.11	.03	.08	.04
4-HNE	1.06	.18	.73	.31	.56	.34	.45	.06	.67	.43	.43	.23	.56	.30
3-NT	$\frac{12.7}{7}$	4.34	8.58	5.24	9.43	1.97	8.19	.34	7.64	1.08	7.81	2.34	6.52	3.45

Table 21

Means categorized by oral contraceptive use, medication combination (mood stabilizer + atypical) and mood stabilizer use (yes) in women with bipolar disorder.

Medication	Bipolar Disorder							
	Mood Stabilizer + Atypical				Mood Stabilizer			
	OCU		NCU		OCU		NCU	
	(n=5)	(n=8)	(n=4)	(n=4)	(n=4)	(n=4)	(n=4)	(n=4)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LPH (nmol/mL)	2.98	1.83	3.65	2.01	2.92	1.81	3.80	1.69
PCC (nmol/mg)	.11	.07	.09	.03	.09	.02	.09	.03
4-HNE (µg/mL)	.84	.37	.60	.31	.48	.28	.58	.34
3-NT (nmol/mL)	10.52	3.16	8.30	3.09	8.68	2.14	6.82	3.90

Table 22

Hormonal contraceptives used by participants

Hormonal Contraceptive	Bipolar Disorder (n=14)	Healthy Controls (n=5)
Yasmin®	3	
Ortho Tri-Cyclen®	3	
Minastrin®	1	
Loestrin®	1	
Cyclen®	1	
Marvelon®	1	
Depo Provera®	1	1
IUD	2	
NuvaRing®	1	
Brevicon®		1
Femcon®		1
Alesse®		1
Ortho Tri-Cyclen Lo®		1

Figure 1. Diagram of the Electron Transport Chain in the Mitochondria showing the process of Oxidative Phosphorylation; Image retrieved from biochemstyles.com (2014).

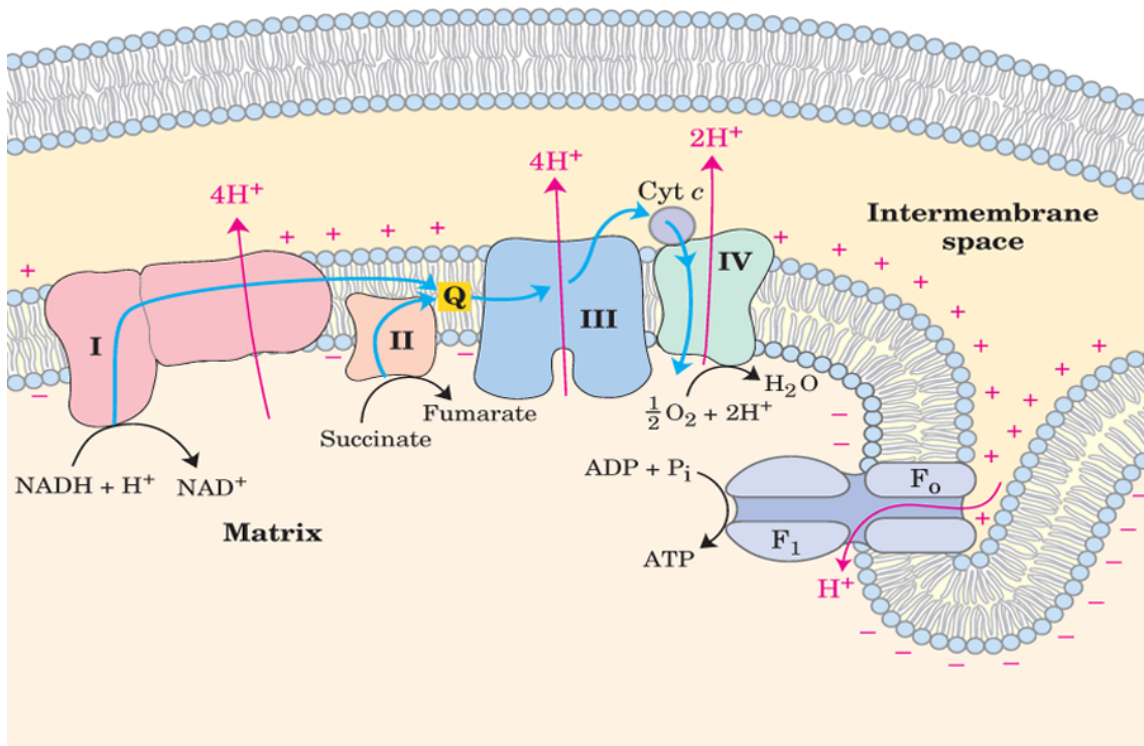


Figure 2. Diagram depicting how Combined Oral Contraceptive use induces ROS which leads to an increase in oxidative stress and activation of pro-inflammatory cytokines (Rottner et al., 2009). This associated increase in oxidative stress and pro-inflammatory cytokines has been hypothesized to influence Bipolar Disorder mood episodes.

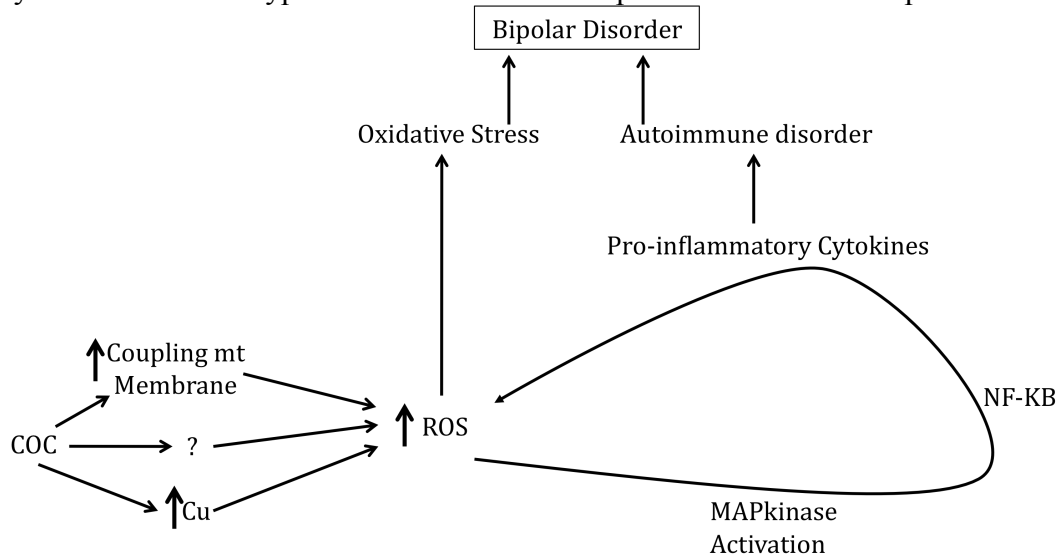


Figure 3. Normal Q-Q plot for 3-Nitrotyrosine

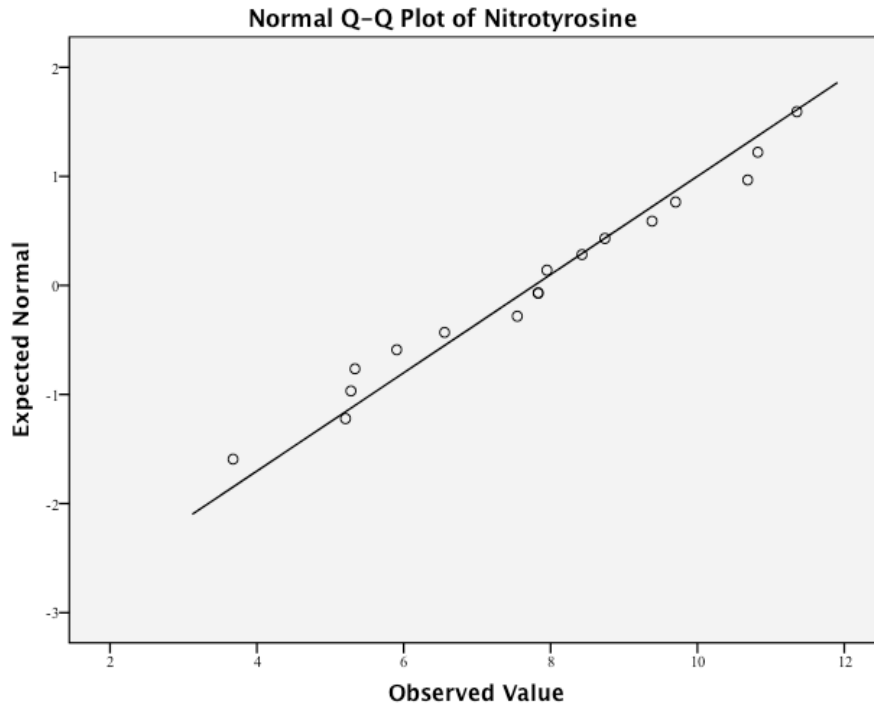


Figure 4. Normal Q-Q plot for Protein Carbonyl Content

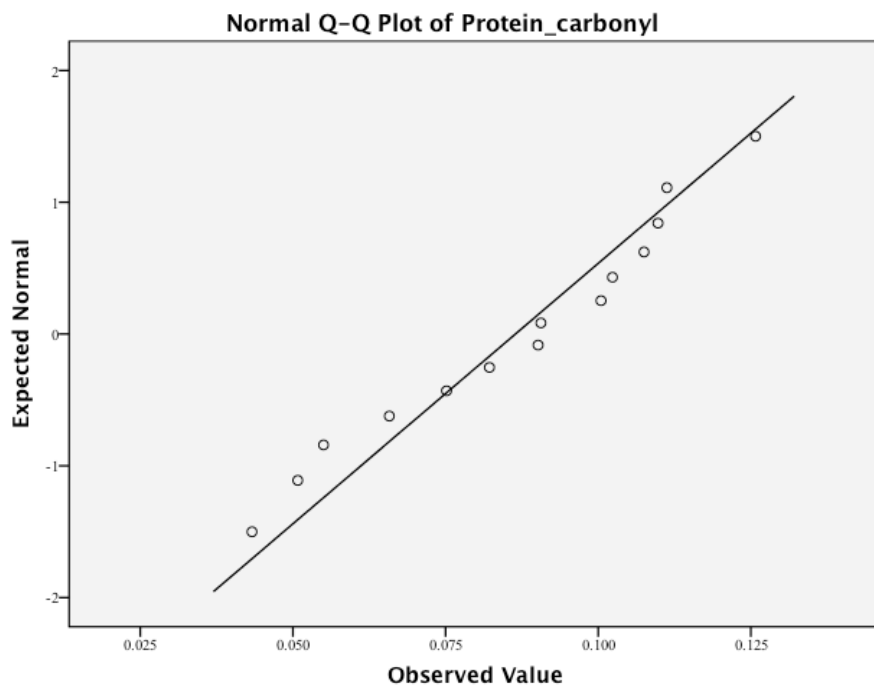
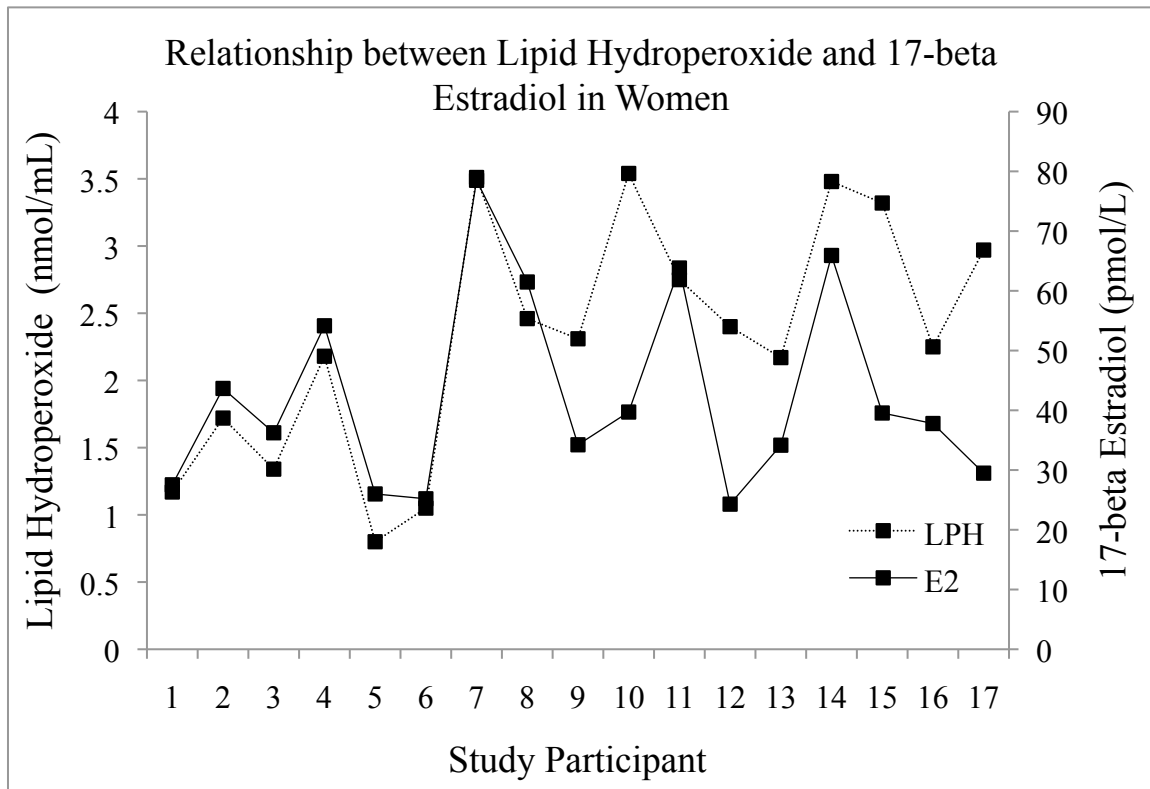


Figure 5. Line graph showing the relationship between Lipid Hydroperoxide and 17-beta Estradiol serum levels in women.



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