

# Effect of oral contraception on biotransformation, oxidative stress and oxidative damage

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**- Vince Lombardi**

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# ABSTRACT

The first hormonal oral contraceptive was released in 1960, giving women a more efficient way of controlling pregnancy and family planning. A combined oral contraceptive (COC) consists of an estrogen and a progestin compound. Initially, the concentrations of the hormones in COCs were very high and over the years there was a decline in the concentrations of the hormonal compounds in COCs, as well as the development of physiological similar hormonal compounds. However, the long term effects of COCs are not known.

The Biotransformation and Oxidative Stress Status Laboratory (North-West University) found that female patients using a specific COC formulation, namely ethinylestradiol/drospirenone (EE/DRSP) showed characteristically high levels of ROS and 2,3-DHBA, even though the antioxidant potential was high enough to be able to bind and eliminate the ROS – a discovery backed by several studies. High levels of ROS can cause oxidative stress and oxidative damage. A study was designed to determine the effect of the EE/DRSP formulation on female participants using the COC on biotransformation, oxidative stress and oxidative damage. For this study, 20 EE/DRSP users and 19 controls were recruited (NWU-0096-08-A1). Participants were required to take the standard biotransformation loading test. All samples were processed per standard procedures and the concentrations of 8-OHdG, 3-NT and lipid peroxidation were determined additionally.

The results confirmed the high levels of ROS, especially 2,3-DHBA in the EE/DRSP group. As a result of the increased ROS levels, the Phase II conjugation reactions, especially glutathionation and glucuronidation, which is the main Phase II reactions for the metabolism of EE, in the EE/DRSP group came under pressure and was systemically depleted. The depletion of the conjugation reactions led to a secondary increase in ROS. The conclusion could be made that the use of the EE/DRSP COC formulation had a negative effect on the biotransformation of its users and could have serious health risks if left untreated.

**Keywords:** biotransformation, oxidative stress, oxidative damage, estrogen, combined oral contraception, ethinylestradiol, drospirenone.

# LIST OF ABBREVIATIONS AND SYMBOLS

|                                       |                                               |
|---------------------------------------|-----------------------------------------------|
| $\alpha$                              | Alpha                                         |
| $\beta$                               | Beta                                          |
| $\mu$                                 | Micron                                        |
| $\mu\text{g}$                         | Micrograms                                    |
| $\mu\text{L}$                         | Microlitre                                    |
| $\mu\text{M}$                         | Micromolar                                    |
| %                                     | Percentage                                    |
| $^{\circ}\text{C}$                    | Degrees Celsius                               |
| 2-OH-E <sub>1</sub> (E <sub>2</sub> ) | 2-hydroxyestradiol                            |
| 3-NT                                  | 3-Nitrotyrosine                               |
| 4-OH-E <sub>1</sub> (E <sub>2</sub> ) | 4-hydroxyestradiol                            |
| 8-OHdG                                | 8-hydroxy-2'-deoxyguanosine                   |
| ADP                                   | Adenosine Diphosphate                         |
| ATP                                   | Adenosine Triphosphate                        |
| AUC                                   | Area Under Curve                              |
| BMI                                   | Body Mass Index                               |
| BOSS                                  | Biotransformation and Oxidative Stress Status |
| BSTFA                                 | (bis(trimethylsilyl)-trifluoroacetamid        |
| CAT                                   | Catalase                                      |
| CBG                                   | Corticosteroid Binding Globulin               |
| CE                                    | Catechol Estrogen                             |
| CID                                   | Collision Induced Dissociation                |

|                   |                                       |
|-------------------|---------------------------------------|
| CoA               | Coenzyme A                            |
| COC               | Combined Oral Contraception           |
| COMT              | Catechol- <i>O</i> -methyltransferase |
| CYP               | Cytochrome                            |
| DEPPD             | N,N-diethylparaphenylenediamine       |
| DHBA              | Dihydroxy Benzoic Acid                |
| DNA               | Deoxyribonucleic Acid                 |
| DRSP              | Drospirenone                          |
| E <sub>1</sub>    | 17β-estradiol                         |
| E <sub>2</sub>    | Estrone                               |
| EDTA              | Ethylenediaminetetraacetic acid       |
| EE                | Ethinylestradiol                      |
| ER                | Estrogen Receptor                     |
| ESI               | Electrospray Ionisation               |
| FDA               | Federal Drug Administration           |
| Fe <sup>2+</sup>  | Ferrous                               |
| Fe <sup>3+</sup>  | Ferric                                |
| FeSO <sub>4</sub> | Ferrous Sulphate                      |
| FRAP              | Ferric Reducing Antioxidant Potential |
| FSH               | Follicle Stimulating Hormone          |
| (g)               | Gas                                   |
| g                 | Gravitational Force                   |
| g-log             | Generalized log                       |
| GC                | Gas Chromatography                    |
| gluc              | Glucuronidation                       |
| glyc              | Glycination                           |
| GnRH              | Gonadotropin-releasing Hormone        |

|                               |                                     |
|-------------------------------|-------------------------------------|
| GPx                           | Glutathione Peroxidase              |
| GSH                           | Glutathione (reduced)               |
| GSSG                          | Glutathione (oxidized)              |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen Peroxide                   |
| HCl                           | Hydrochloric Acid                   |
| HDL                           | High Density Lipoproteins           |
| HIV                           | Human Immunodeficiency Virus        |
| HOCl                          | Hypochlorous Acid                   |
| HP                            | Hewlett-Packard                     |
| HPLC                          | High Pressure Liquid Chromatography |
| IU                            | Intrauterine                        |
| L                             | Litre                               |
| LC                            | Liquid Chromatography               |
| LDL                           | Low Density Lipoproteins            |
| LH                            | Luteinizing Hormone                 |
| MDA                           | Malondialdehyde                     |
| mg                            | Milligrams                          |
| mg/L                          | Milligram per Litre                 |
| mL                            | Millilitre                          |
| mL/min                        | Millilitre per Minute               |
| mm                            | millimetre                          |
| mmol/L                        | Millimole per Liter                 |
| MPA                           | Metaphosphoric Acid                 |
| MRM                           | Multiple Reaction Monitoring        |
| MS                            | Mass Spectrometer                   |
| MSQ                           | Medical Symptoms Questionnaire      |
| <i>m/z</i>                    | Mass to Charge Ration               |

|                                 |                                             |
|---------------------------------|---------------------------------------------|
| N <sub>2</sub>                  | Nitrogen                                    |
| NADP(H)                         | Nicotinamide Adenine Dinucleotide Phosphate |
| Na <sub>2</sub> SO <sub>4</sub> | Sodium Sulphate                             |
| nm                              | Nanometer                                   |
| NO <sub>2</sub> •               | Nitric Oxide                                |
| NWU                             | North-West University                       |
| O <sub>2</sub>                  | Oxygen                                      |
| O <sub>2</sub> -•               | Superoxide Anion                            |
| OC                              | Oral Contraception                          |
| OH                              | Hydroxyl Radical                            |
| PCA                             | Principle Component Analysis                |
| pH                              | Acidity                                     |
| PUFA                            | Polyunsaturated Fatty Acid                  |
| Q                               | Quinone                                     |
| R <sup>2</sup>                  | Regression Line                             |
| r.c.f.                          | Relative Centrifugal Force                  |
| RNS                             | Reactive Nitrogen Species                   |
| RO <sub>2</sub> •               | Peroxyl Radical                             |
| ROC                             | Receiver Operator Characteristics           |
| ROS                             | Reactive Oxygen Species                     |
| r.p.m                           | Rotations per Minute                        |
| RSD                             | Relative Standard Deviation                 |
| SHBG                            | Sex Hormone Binding Globulin                |
| SIM                             | Selected Ion Monitoring                     |
| SOD                             | Superoxide Dismutase                        |
| SPE                             | Solid Phase Extraction                      |
| SST                             | Serum Separating Tube                       |

|       |                                      |
|-------|--------------------------------------|
| sulph | Sulphonation                         |
| TAC   | Total Acyl-Carnitines                |
| TBA   | Thiobarbituric Acid                  |
| TBARS | Thiobarbituric Acid Reactive Species |
| TFA   | Trifluoroacetic Acid                 |
| TFC   | Total Free Carnitines                |
| TMCS  | Trimethylchlorosilane                |
| TPTZ  | tripyridylpyridine                   |
| UV    | Ultraviolet                          |
| VTE   | Venous Thromboembolism               |
| v/v   | Volume to Volume                     |

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# 1

# INTRODUCTION

Contraception as a way of preventing pregnancy has a long and interesting history going as far back as 15000 in France and the Ancient Egyptians. However, it was not until the Federal Drug Administration (FDA) approved the first hormonal contraceptive, Enovid<sup>®</sup>, in May 1960 that women all over the world were able to have an efficient way of controlling pregnancy and planning a family. The initial doses of hormones in the first generation contraception were very high, and over the years a lot have been done to reduce hormone doses and composition in order to reduce side-effects associated with oral contraception (OC) use. The prevalence of contraception use in women between the ages of 15 and 49 differs worldwide with a mean of 62.7% married women in 2011 using some form of contraception. Overall, more than 100 million women worldwide use a combined oral contraception (COC) which represents 8.8% of contraceptive method use (Christin-Maitre, 2013).

The long term effects that COCs have on biotransformation and metabolism is largely unknown (Rad *et al.*, 2011) and the effect hormonal contraceptives have on oxidative stress is controversial (De Groote *et al.*, 2009; Siddique *et al.*, 2005). It has been shown that estrogen have protective or pro-oxidant effects e.g. cardio-protective effects (Persky *et al.*, 2000). However, Rogan (2007) showed how the normal detoxification of estrogens can result in the formation of quinones which is able to bind to DNA, forming depurinating adducts which can result in the initiation of cancer (Rogan, 2007). To support the carcinogenic potential of COCs, the [International Agency for Research on Cancer \(2005\)](#) declared COCs to be carcinogenic with an increased risk for liver, cervical and breast cancer.

Previous studies showed that patients using exogenous hormones, including COCs, have a dramatic increase in free radicals, even though they possess the necessary antioxidant potential to clear these radicals. Finco *et al* (2012) proved that the conventional antioxidant treatments proved insufficient in reducing the levels of free radicals (Finco *et al.*, 2012). To the contrary studies on the combination of ethinylestradiol and drospirenone in contraception, as in the case of certain COCs provide for an overall well-tolerated contraceptive with

excellent cycle control and reliable contraceptive efficacy (Taneepanichskul & Phupong 2007).

When considering the controversial literature on the use of oral contraceptives, it is evident that these COC users have an increase in reactive oxygen species (ROS) levels. One of the key questions this study needs to address is whether biotransformation is affected by the use of COCs.

The study was designed to include female test subjects between the ages of 18 and 35. Recruited test subjects were divided in 2 groups: A “control” group (using no form of oral contraception), and an “EE/DRSP” group. Test subjects were asked to complete a biotransformation test (as standardized by the Biotransformation and Oxidative Stress Status or BOSS laboratory, North-West University Potchefstroom). The standard analysis for biotransformation and oxidative stress was performed, as well as additional methods for the analysis of oxidative damage markers of lipids, DNA and proteins.

This dissertation is divided into five chapters. **Chapter 1** (Introduction) gives a brief overview of the study, hypothesis, aim and objectives, and further chapter division. **Chapter 2** (Literature Review) gives a literature background of several key concepts in this study including a review on biotransformation, the menstrual cycle and associated hormones, and oral contraception with the focus on formulations containing drospirenone and ethinyl-estradiol. **Chapter 3** (Materials and Methods) describes the experimental approach in detail, as well as the materials and method for each of the analysis performed. **Chapter 4** (Results and Discussion) will give a systematic and logical overview of the results obtained in the analytical stage of this study as well as a detailed interpretation and discussion thereof. **Chapter 5** (Conclusion) will give a summary of the results and final conclusion as well as possibilities for other studies in this field. References were listed according to the Harvard referencing style.

# 2

# LITERATURE REVIEW

## 2.1 BIOTRANSFORMATION, OXIDATIVE STRESS AND ANTIOXIDANTS

### 2.1.1 BIOTRANSFORMATION/DETOXIFICATION

Biotransformation can be described as the chemical processes through which parent compounds (or toxicants) are enzymatically converted to their metabolites which then form conjugates. After conjugation, metabolites are usually more water soluble and polar and therefore ready to be excreted. The principle site for biotransformation reactions within the body is the liver, but other organs such as the lungs, stomach, intestine, skin and bladder also play an important role in biotransformation (Lu & Kacew, 2002). Biotransformation is considered to be a method of metabolic detoxification (Lu & Kacew, 2002).

The biotransformation or detoxification system is an integral part of drug metabolism, especially to protect tissues and cells from the damaging effects of these compounds and metabolites. Biotransformation is especially associated with drug metabolism. Drug biotransformation can affect the overall effectiveness of a drug in the body, and especially if bioactivation occurs through the metabolism of the parent compound (Brandon *et al.*, 2003). A collective name for all drugs, drug metabolites and environmental agents such as pesticides and pollutants and other chemical compounds foreign to the body, is xenobiotics (Online Medical Dictionary, 2012). Xenobiotics are usually external in origin. Endogenous “toxins” also exist and usually as a result of regular metabolism, but also due to bacterial metabolism (Liska, 1998).

Biotransformation takes place through two main phases, namely Phase I and II, but a third phase has also been described (Liska 1998). The Phase I reactions are the degradation reactions and three types of reactions occur: oxidation, reduction and hydrolysis (Lu & Kacew, 2002) as illustrated in Figure 2.1. Phase II reactions are the conjugation reactions: glucuronidation, sulphation, methylation, acetylation, amino acid conjugation and glutathione conjugation (Lu & Kacew, 2002). Phase III biotransformation involves antiporter activity and is an energy-dependent efflux pump located in the intestine. Its function is to move

intracellular xenobiotics out of the cell in order to decrease the intracellular concentration thereof. This mechanism allows for the reabsorption and re-exposure of xenobiotics not metabolized in Phase I, essentially increasing the efficacy of the detoxification process (Liska, 1998).

S

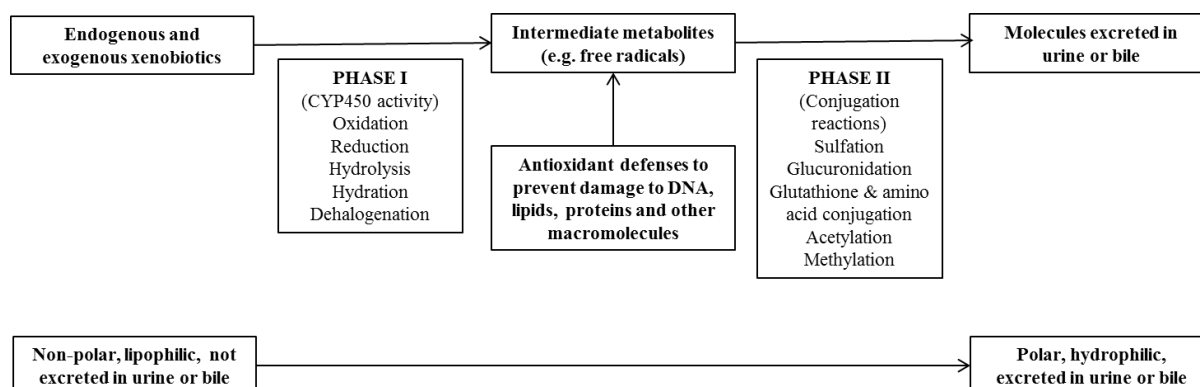


**Figure 2.1** A diagrammatical representation of the Phase I and II biotransformation in the liver during which non-polar, water-insoluble toxicants are converted to polar, water-soluble toxicants which can be excreted in the urine or bile.

Secondary tissue damage is the result of reactions with reactive species formed through Phase I reactions, the intermediate metabolites and Phase II conjugation reactions (Liska, 1998). The oxidative metabolism of drugs, environmental chemicals and endogenous substances such as steroids, are catalysed by the cytochrome enzyme system during phase I detoxification. This system consists of a superfamily of heme-containing mono-oxygenases. There are three families within this system namely CYP1, CYP2 and CYP3. These families are responsible for the metabolism of exogenous and endogenous compounds, with isoforms thereof responsible for the metabolism of estrogen (Tsuchiya *et al.*, 2005).

### 2.1.2 FREE RADICAL SPECIES

Free radicals can be defined as chemical species, or free low molecular weight molecules, containing at least one unpaired electron (Dictionary of Biology, 2004:277; Nordberg & Arnér, 2001). Some of the biologically significant free radicals (Table 2.1) include superoxide anions, hydroxyl radicals, peroxy radicals and nitric oxide (Aruoma, 1998; Finkel & Holbrook, 2000).

**Table 2.1** A summary of *in vivo* biologically important free radicals (Aruoma, 1998; Dictionary of Biology, 2004:675).

| Free radical                         | Description                                                                                                                                                                                                                                                                                   |
|--------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Superoxide anions ( $O_2^{\cdot-}$ ) | Produced as a result of incomplete reduction of oxygen during mitochondrial respiration, enzyme systems and auto-oxidation; superoxide dismutase (SOD) converts 2 $O_2^{\cdot-}$ to $H_2O_2$ and $O_2$ .                                                                                      |
| Hydroxyl radicals ( $OH^{\cdot}$ )   | Highly reactive radicals with very short half-life ( $10^{-9}$ s) that react with other molecules to form another radical; formed through the Fenton reaction especially in cases of altered homeostasis; attack proteins, DNA, polyunsaturated fatty acid and almost all other biomolecules. |
| Hydrogen peroxide ( $H_2O_2$ )       | Not a free radical, but a reactive oxygen species formed when $O_2^{\cdot-}$ is converted by superoxide dismutase and other oxidase enzymes; forms hydroxyl radicals in the presence of transition metals.                                                                                    |
| Hypochlorous acid (HOCl)             | Produced by the neutrophil-derived enzyme myeloperoxidase during inflammation when chloride ions are oxidized in the presence of $H_2O_2$ .                                                                                                                                                   |
| Nitric oxide ( $NO^{\cdot}$ )        | Free radical produced by damaged vascular endothelium; promotes vasodilation and oxidation of low-density lipoproteins.                                                                                                                                                                       |
| Peroxyl radicals ( $RO_2^{\cdot}$ )  | Intermediate species formed during lipid peroxidation chain reactions; increased production during oxidative stress as a result of smoking, xenobiotics, and inflammation.                                                                                                                    |

In everyday use, the terms “free radicals” and “reactive oxygen species” (“ROS”) are frequently referred to as synonyms of each other. ROS are generally defined as chemically reactive molecules derived from oxygen, with molecules such as the hydroxyl radical being extremely reactive and hydrogen peroxide and superoxide less reactive and include free radical and non-radical oxidants (Monaghan *et al.*, 2009; Nordberg & Arnér, 2001).

ROS are metabolic products of intracellular processes and are produced for example in the electron transport chain during energy production (Balaban *et al.*, 2005). Extracellular sources, such as UV-radiation and air pollutants, are also associated with an increase in ROS production. Even though ROS can react with biomolecules often causing oxidative damage, within physiological boundaries ROS can also play a biologically important role in cellular functions such as cell signalling, cell transformation, regulation of smooth muscle relaxation,

regulation of blood flow, and immunity (De Groote *et al.*, 2009; Devadas *et al.*, 2002; Dröge, 2002; Finkel, 2003).

Many diseases (e.g. neurodegenerative diseases, diabetes, atherosclerosis, ischemia-reperfusion injury, cancer, malaria, HIV infection and rheumatoid arthritis) are associated with increased ROS usually as a secondary response to the disease and can play a further deteriorating role in the pathological process thereof (Aruoma, 1998; Dröge, 2002; Finkel & Holbrook, 2000; Halliwell, 1989)

### 2.1.3 OXIDATIVE STRESS AND OXIDATIVE DAMAGE

Aruoma (1998) describes oxidative stress as “the imbalance between generation of ROS and the activity of the antioxidant defences”, also stating that “severe oxidative stress can cause cell damage and death”. Severe oxidative stress or an oxidative event can be the result of either increased ROS production, or a decrease in the antioxidant protection causing an altered homeostatic redox state (Aruoma, 1998).

Oxidative stress is the result of an imbalance in the production of ROS and the neutralizing action of both enzymatic and non-enzymatic antioxidants resulting in oxidation reactions with biomolecules and cellular components (Monaghan *et al.*, 2009). Oxidative stress can arise as a result of uncontrolled oxidation of biomolecules by ROS (Monaghan *et al.*, 2009). During the event of oxidative stress, an imbalance in the redox state of the cell is created resulting in oxidative damage to biomolecules such as DNA, lipids and proteins, as illustrated in Figure 2.2 (Aruoma, 1998; Matés *et al.*, 2008). Even though oxidative stress that exceed the intracellular antioxidant potential can lead to oxidative damage, low levels of oxidative stress can actually increase antioxidant potential (Niki *et al.* 2005).

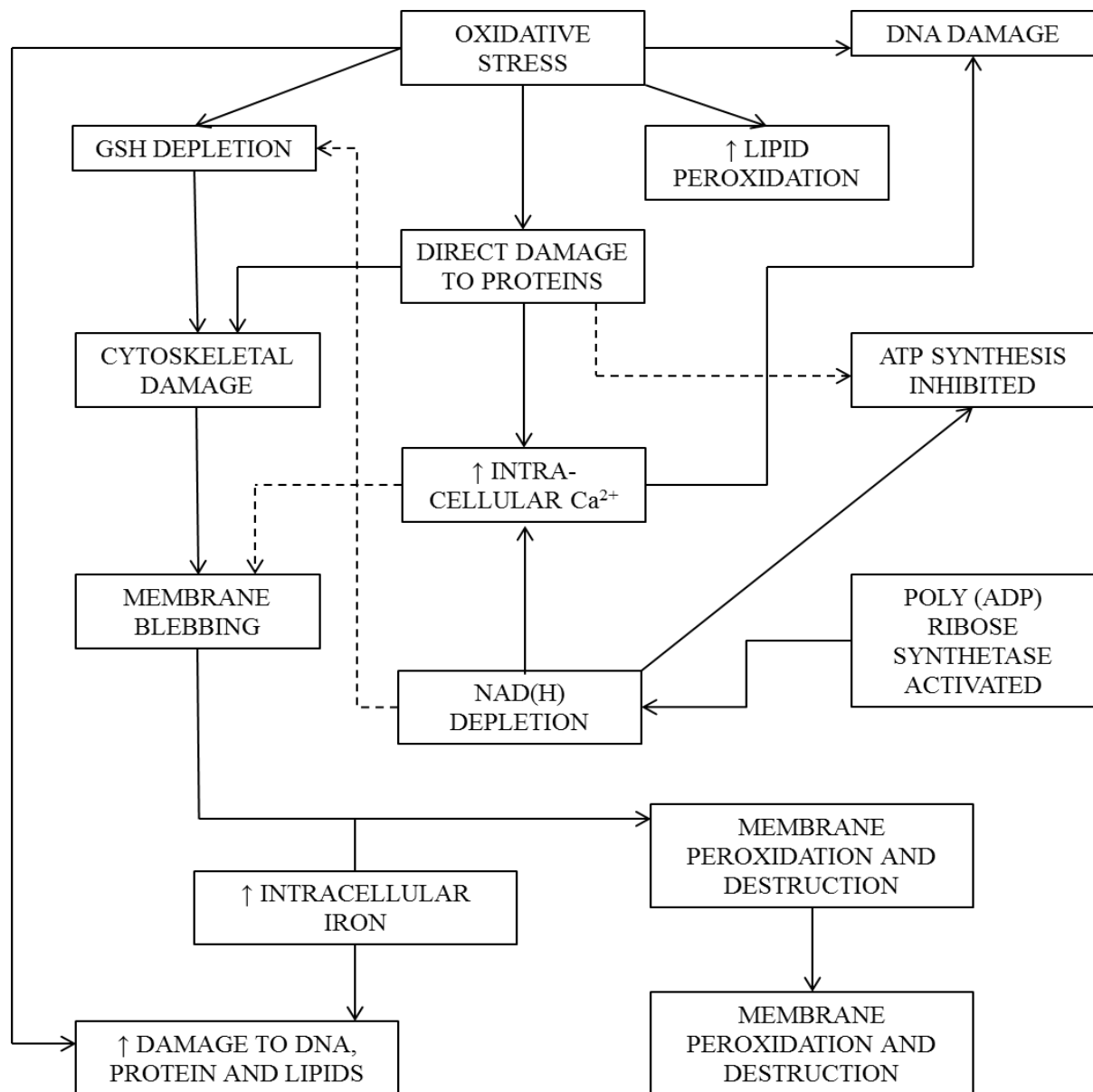


**Figure 2.2** A schematic summary of the mechanisms responsible for cellular damage during oxidative stress conditions (adapted from Aruoma (1998)).

### 2.1.3.1 DNA damage

Mitochondrial DNA seem to be more susceptible to the oxidative damage caused by ROS, since it is close to the production of ROS through the electron transport chain (ETC), and would therefore be more likely to suffer the consequences of increased production (Balaban *et al.*, 2005; Finkel & Holbrook, 2000).

One of the commonly used biomarkers for oxidative damage in DNA is 8-hydroxy-2'-deoxyguanosine (8-OHdG) and the increased excretion of the metabolite in urine is indicative

of oxidative DNA damage and DNA repair (Ogino & Wang, 2007; Rall *et al.*, 2000; Valavanidis *et al.*, 2009). 8-OHdG is the marker that reflect oxidative DNA damage the most accurate (Ogino & Wang, 2007). Therefore, there is a correlation between the increase in oxidative stress and excretion of 8-OHdG (Honda *et al.*, 2000; Valavanidis *et al.*, 2009). The 8-OHdG metabolite is produced when hydroxyl radicals and singlet oxygen species bind to the guanine bases on DNA to form a damaged based. Due to damage repair of DNA, the damaged bases are excised and then excreted in urine (Honda *et al.*, 2000; Valavanidis *et al.*, 2009).

#### **2.1.3.2 Protein damage**

Proteins can undergo reversible oxidation-reduction reactions at two sites, namely methionine and cysteine (Jones, 2008). Oxidation in cysteine occurs mainly through sulphur species and although this oxidation is reversible in most cases, oxidation through sulphinates and sulphonates are irreversible in the mammalian system (Jones, 2008).

Some amino acids (e.g. tryptophan, tyrosine, histidine and cysteine) are more prone to damage from free radicals. This leads to the secondary and tertiary damage to the protein structure of which these amino acids form a part of (Dröge, 2002; Monaghan *et al.*, 2009). Free radicals can cause conformational changes to occur in proteins – changing protein structures and active sites, resulting in loss of enzyme activity or altered functions of receptors and transport proteins (Aruoma, 1998; Butterfield *et al.*, 1998). Reversible disulphide bridges are also a product of protein oxidation. Even though these changes are reversible, it can lead to structural changes and impaired function of proteins (Dröge, 2002; Monaghan *et al.*, 2009).

When considering the oxidation damage of proteins, it is noticeable that the degree of damage are subjected to the amino acid content of the proteins, the structure of the protein and the location of the proteins with regards to the production site of ROS (Dröge, 2002; Monaghan *et al.*, 2009). There are several biomarkers that can indicate whether protein oxidation has occurred, including 3-nitrotyrosine (3-NT), chlorotyrosine, dityrosine, trityrosine and 3-bromotyrosine (Ogino & Wang, 2007).

#### **2.1.3.3 Lipid damage**

Polyunsaturated fatty acids (PUFAs) are a major component of cellular membranes, but are highly susceptible to oxidative damage which could result in the alteration in membrane

fluidity, of permeability to other molecules, and the intracellular metabolism (Bandyopadhyay *et al.*, 1999). PUFAs can be damaged by free radicals through free radical chain reactions. This process, known as lipid peroxidation, are the result of polyunsaturated fatty acids being exposed to  $O_2$  and other free radicals in the presence of trace metal ions (Jones, 2008). Figure 2.3 illustrates the process of lipid peroxidation and the various steps involved.

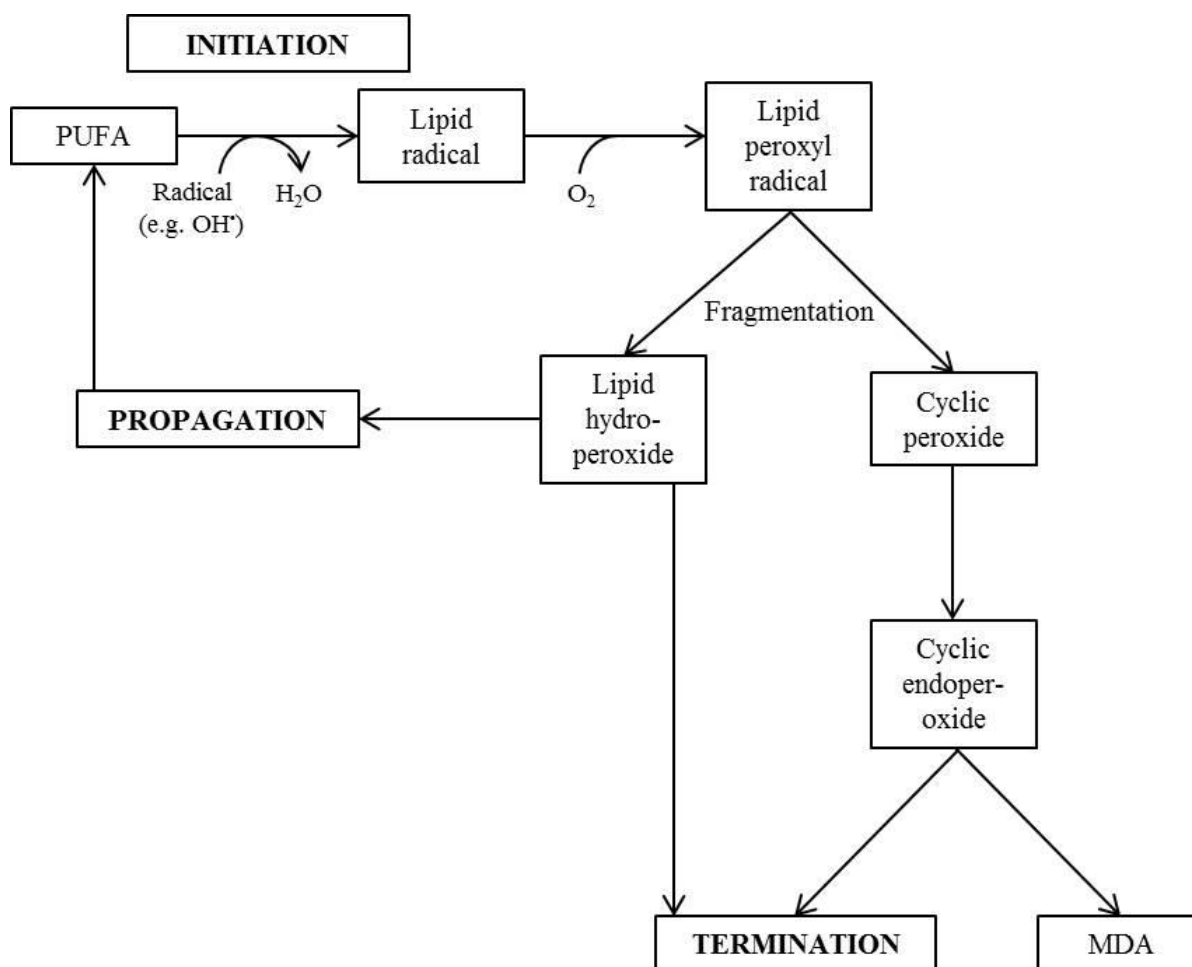


**Figure 2.3** A schematic summary of the process of lipid peroxidation and MDA production. Lipid peroxidation occurs in three stages, namely initiation, propagation, and termination. During initiation a chain activating free radical (e.g. hydroxyl, alkoxyl, or peroxy radicals) abstract a hydrogen atom from a methylene group on the PUFA, forming a lipid radical (lipid diene) which undergoes some conformational changes and react with molecular  $O_2$  to form a lipid peroxy radical. The peroxy radical can form hydroperoxides or cyclic peroxides. MDA is the end product of cyclic endoperoxides. Hydroperoxide can form part of the propagation step during which the hydroperoxide act as a free radical and can abstract a hydrogen atom from another PUFA, starting the process over. The termination step only

occurs when two lipid radicals bind to each other, or through the action of an antioxidant (Gutteridge (1995)).

The oxidation of PUFAs can lead to the formation of multiple products, of which malondialdehyde (MDA), a small molecule of a low molecular weight consisting of three carbons, is the most common biomarker of the peroxidation of lipids (Alía *et al.*, 2005; Grotto *et al.*, 2009; Ogino & Wang, 2007). MDA is able to react with other functional groups such as lipoproteins, proteins and DNA and plays a role in carcinogenesis (Grotto *et al.*, 2009).

#### 2.1.4 ANTIOXIDANT DEFENSES

##### 2.1.4.1 The role of antioxidants

Normal cellular function is only possible through the control of the intracellular redox balance. Oxidative stress due to metabolic processes such as mitochondrial energy generation and the actions of CYP450 in detoxification of toxins are counteracted by the activity of an antioxidant (Bandyopadhyay *et al.*, 1999).

Both Halliwell (1991) and Gutteridge (1999) describe an antioxidant to be a substance that have the ability to delay, inhibit or prevent the process of oxidation of a substrate, even when the concentration of the antioxidant is significantly lower than the substrate to be oxidized, with oxidizable substrates including protein, lipid, carbohydrate and DNA molecules (Gutteridge, 1999; Halliwell, 1991). Therefore, it can be said that antioxidants aid in buffering the redox environment and controlling the redox state in a biological system by either removing toxic levels of oxidants or by causing changes that can effect downstream signalling of oxidants (Bandyopadhyay *et al.*, 1999, Gutteridge, 1999).

The body has its own antioxidant system, giving it the ability to protect itself against oxidative damage due to increase in ROS. Antioxidants can act by scavenging free radicals, by preventing the formation of ROS or by repairing damage already done by ROS (Halliwell, 1991). The antioxidant system can be divided into two broad categories as illustrated in Figure 2.4, namely enzymatic antioxidants that can act as scavengers, e.g. catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), and non-enzymatic antioxidants including vitamin A (retinol), vitamin C (ascorbic acid) and vitamin E ( $\alpha$ -

tocopherol), glutathione (GSH) and uric acid (De Groote *et al.*, 2009). Cells have the ability to respond to a sudden increase in ROS or RNS by increasing glutathione production to increase intracellular scavenging activity to regulate the homeostatic balance. This response is known as the “oxidative stress response” and is essential in the maintenance of redox homeostasis (Dröge, 2002).

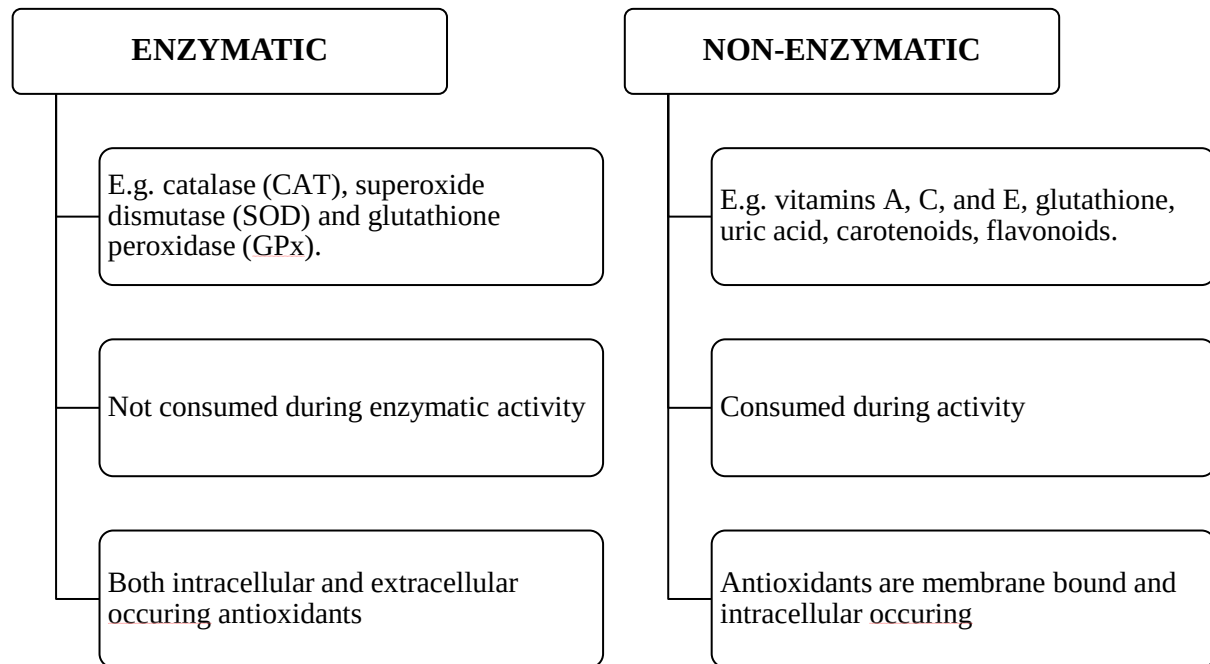


**Figure 2.4** A diagrammatic classification of antioxidants, their activity and location within the body (adapted from De Groote *et al* (2009), Gutteridge (1995), and Halliwell (1991)).

Antioxidants within cells are evolved to fit their oxidative metabolism environment in order to effectively protect against the effects of ROS and other oxidizing metabolites. These antioxidants include enzymes and act as enzymatic catalysts and are therefore not consumed during enzymatic actions. Membrane-bound antioxidants (e.g. vitamin E) act as scavengers to protect PUFAs against oxidative damage (Gutteridge 1999; Halliwell 1991). Extracellular occurring antioxidants such as catalase, superoxide dismutase (SOD), glutathione and glutathione peroxidase act as a buffer system protecting the body from extracellular free radicals (including metal ions).

### 2.1.4.2 Glutathionation

Glutathione ( $\gamma$ -glutamylcysteinylglycine) is a tripeptide consisting of three amino acids: glutamate, cysteine and glycine (see figure 2.5 for biosynthesis). Some very important biological functions of glutathione include the storage and transport of cysteine, biosynthesis leukotriene and prostaglandin, maintaining protein structure and function, storage and transport of metals through non-enzymatic binding, and regulation of enzyme activity through the reduction of disulphide bonds, but the primary function of glutathione is sustaining a homeostatic redox balance to protect the cell against ROS, RNS and xenobiotics. Glutathione can be present within the cell in both reduced (GSH) and oxidized (GSSG) form, with the ratio of GSH:GSSG in the cytosol and mitochondria 10:1 (Alía *et al.*, 2005). During oxidative stress conditions, the GSSG levels will rise resulting in a lower GSH:GSSG ratio, but the cell compensates by exporting GSSG out of the cell or by forming mixed disulphide bonds between GSSG and other thiol proteins or GSH to decrease the GSSG concentration.

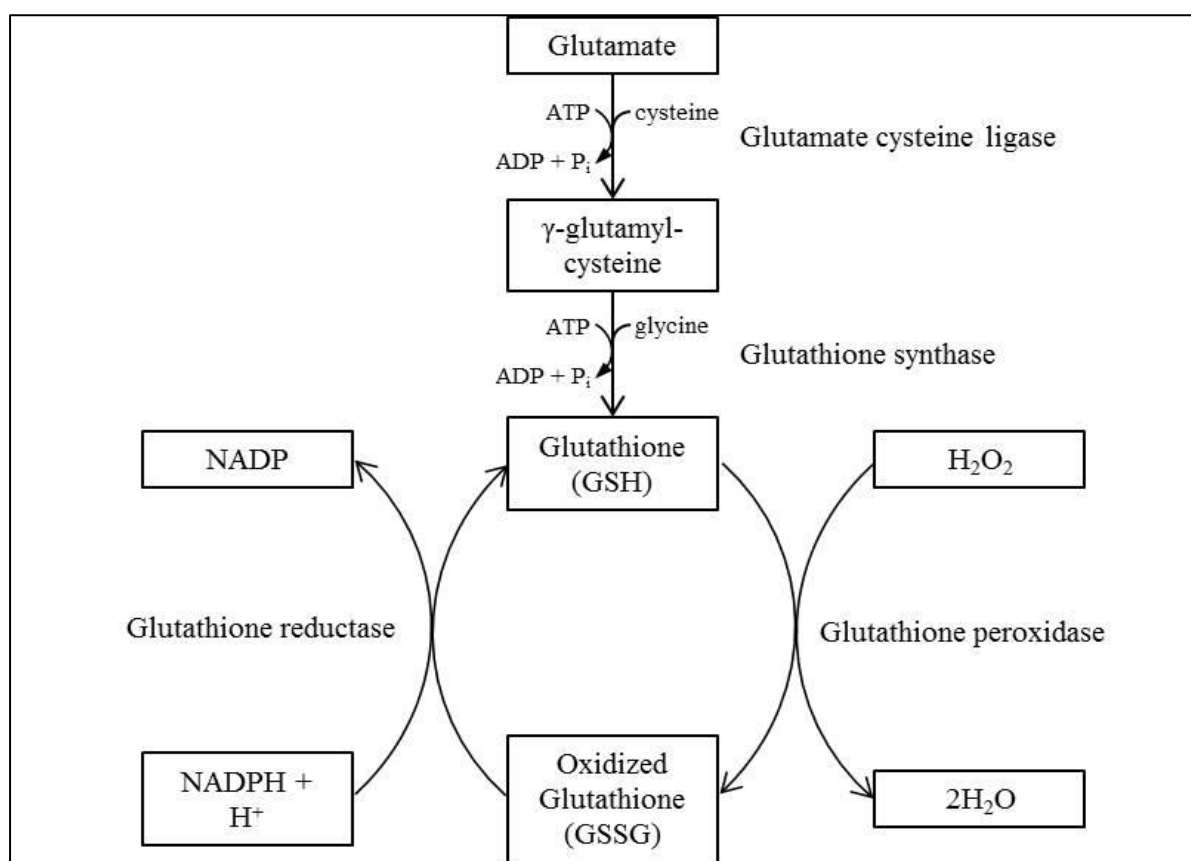


**Figure 2.5** The synthesis of glutathione (GSH) from glutamate, cysteine, and glycine, and the processes involved with maintaining an intracellular redox balance through the oxidation and reduction of glutathione (adapted from Salway, 2004:32-33).

When two GSH molecules are oxidized, they form a cross-link bond (disulphide bridge). Through the action of glutathione reductase in the presence of NADPH, GSSG are reduced to form two GSH molecules. Glutathione reductase is therefore responsible for keeping the GSH:GSSG ratio high. Glutathione transferase catalyses the conjugation of GSH to electrophilic molecules by activating the free sulfhydryl group of GSH in order to make it more susceptible for binding with these electrophilic molecules (Salway, 2004:32).

Since high levels of ROS do not necessarily indicate oxidative stress, just as high levels of antioxidants do not necessarily indicate a better *in vivo* redox state, it is important to measure more than one oxidative damage marker when determining oxidative stress status in patients (Monaghan et al. 2009). This will result in more complete information of the patient's biotransformation profile, oxidative status, antioxidant potential and oxidative damage on which an informed diagnosis can be made.

## **2.2 ORAL CONTRACEPTION, ESTROGEN AND XENOESTROGENS**

### **2.2.1 HORMONES OF THE MENSTRUAL CYCLE**

The basis of the female reproductive system is the menstrual cycle – the cyclic increase and decrease in female hormones. The normal length of a cycle is 28 days, but significant variation occurs from person to person as well as intra-personal, and a menstrual cycle of 20 to 45 days can be seen in some women. The menstrual cycle can be divided into three phases, namely the follicular phase characterized by follicle growth, ovulation during which the follicle releases the oocyte, and the luteal phase where the corpus luteum (remains of the follicle in the ovary) forms and will degenerate if fertilization did not occur (Guyton & Hall, 2006:1012).

During the menstrual cycle, six different hormones influence the course of the cycle, namely gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, progesterone and inhibin. The varying concentrations not only have an effect on each other, but also affect the uterus lining and the phases of the cycle. Figure 2.6 gives a schematic summary of four of the important hormones that determines the course of the menstrual cycle (Guyton & Hall, 2006: 1016; Widmaier, Raff & Strang, 2011:607).

It has been shown that high levels of estrogen during the luteal phase of the cycle are associated with increased lipid peroxidation products, namely malondialdehyde. Therefore, high MDA may play an important role in the initiation of menstruation (Akande & Akinyinka, 2005).

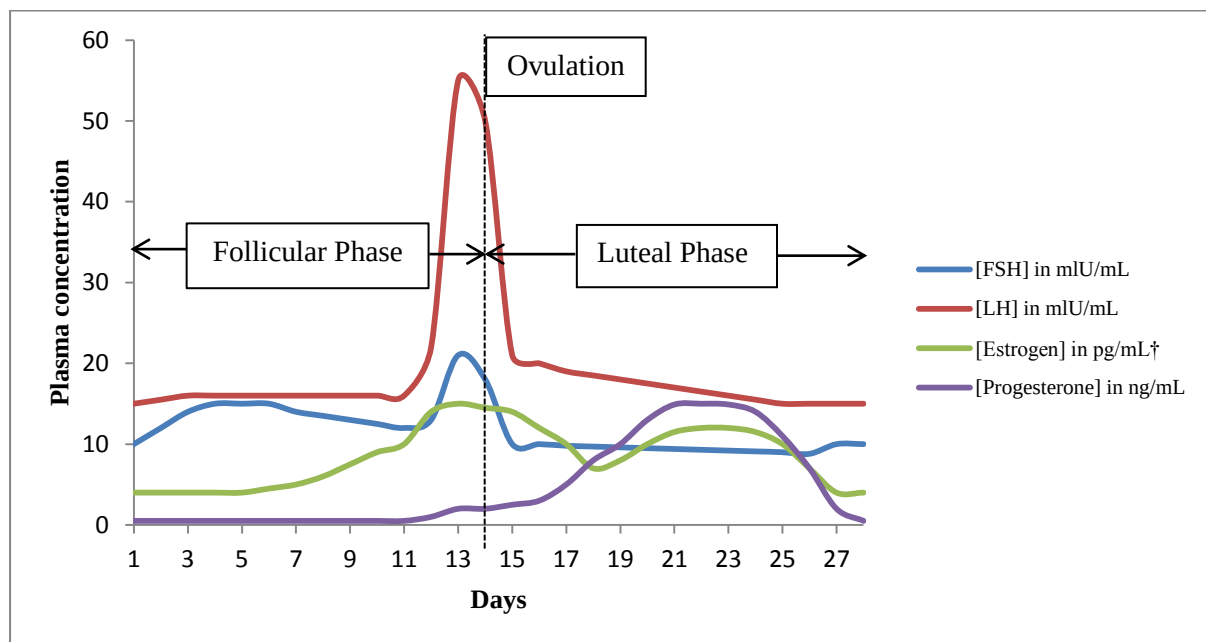


**Figure 2.6** This figure gives a summary of the fluctuations of four of the hormones that play an important role in the menstrual cycle. FSH will increase during the follicular phase, peaking just before ovulation and decreasing during the luteal phase. LH will stay constant during the first part of the cycle, increasing drastically 18 hours before ovulation and decreasing during the luteal phase. Estrogen concentration are low during the first week of the follicular phase, but increases during the second week, however before ovulation the concentration start dropping, and an increase can again be seen during the luteal phase. Progesterone secretion is very low during the follicular phase, but it shows a sharp increase after ovulation in the luteal phase (adapted from Widmaier, Raff & Strang, 2011:607). (For scale purposes, the estrogen concentrations given were [estrogen]<sup>-10</sup> pg/mL).

## 2.2.2 ESTROGEN METABOLISM

### 2.2.2.1 Synthesis of estrogen

Estrogen is one of the primary steroid hormones in the female body and is synthesized *de novo* from cholesterol or acetyl-CoA within the ovaries. Synthesis of all steroid hormones is

energy dependent and regulated by cytochrome P450 enzymes consuming NADPH (Salway, 2004:72). Estrogens are lipophilic and essential for growth, differentiation and function of tissues in humans. The term “estrogen” is usually used as a collective term to describe the *in vivo* female hormones estradiol, estriol and estrone. Estrogens have important functions in the female body, since it is important for the development of primary and secondary sexual characteristics, regulation of the menstrual cycle and pregnancy in females (Guyton & Hall, 2006:1017; [Report on Carcinogens, 2011:193](#)). Of the three forms in which estrogen is detected in plasma, 17 $\beta$ -estradiol (E<sub>1</sub>) is the primary estrogen and the most effective of the three: it is 12 times more effective than estrone (E<sub>2</sub>) and 80 times more effective than estriol (Guyton & Hall, 2006:1016).

#### **2.2.2.2 Metabolism of estrogen**

The liver plays an important role in estrogen degradation. It contains the enzymes responsible for conjugation of estrogens with glucuronides and sulphates. Approximately 20% of these conjugates are excreted in the bile, the rest mostly in urine. Furthermore, the function of the liver is essential in converting the potent E<sub>1</sub> and E<sub>2</sub> to less potent estriol in order to avoid prolonged activity of these two estrogens (Guyton & Hall, 2006:1016). The mechanisms responsible for estrogen metabolism enables the hormone to regulate several physiological processes in the body (Miller, 2010).

Estrogen undergo oxidative metabolism mainly in the liver through the action of isoforms of the CYP family which are expressed in the liver, and the level of CYP enzyme expression can influence the effects of estrogen on its target tissues (Tsuchiya *et al.* 2005). Approximately 80% of estradiol is converted to 2-hydroxyestradiol (2-OH) through the actions of CYP1A2 and CYP3A4 in the liver (and CYP1A1 in other tissues). 20% of estradiol is converted to 4-hydroxyestradiol (4-OH) by CYP1B1 and CYP3A5 (Tsuchiya *et al.*, 2005).

There are two major pathways for estrogen metabolism namely the formation of catechol estrogen (CE) and 16 $\alpha$ -hydroxylation.

#### **2.2.2.3 The CE metabolism pathway**

The first step in the CE pathway is the conversion of E<sub>1</sub> and E<sub>2</sub> by the cytochrome P450 enzymes to their hydroxyl products, also called their catechol estrogens, 2-OH-E<sub>1</sub>(E<sub>2</sub>) and 4-

OH-E<sub>1</sub>(E<sub>2</sub>). About 80% of E<sub>1</sub> and E<sub>2</sub> are converted to 2-OH-E<sub>1</sub>(E<sub>2</sub>) through CYP1A2 and CYP3A4 activity and 20% to 4-OH-E<sub>1</sub>(E<sub>2</sub>) through CYP1B and CYP3A5 activity in the liver.

The catechol estrogens can undergo inactivating enzyme reactions such as sulphation and glucuronidation, however, the most important conjugation pathway of catechol estrogens is *O*-methylation catalyzed by catechol-*O*-methyltransferase (COMT) to form methoxy estradiol products. If conjugation via COMT becomes insufficient or backed-up, competitive catalytic oxidation results in the formation of semiquinones through peroxidase or CYP450 enzyme reactions. Semiquinones are oxidized to quinones via peroxidase or CYP450 enzyme reactions. The different reactions that quinones can undergo can be seen in figure 2.7. These reactions include redox cycling to semiquinones, reduction to its catechol estrogen, conjugation with glutathione, and the formation of depurinating DNA adducts.

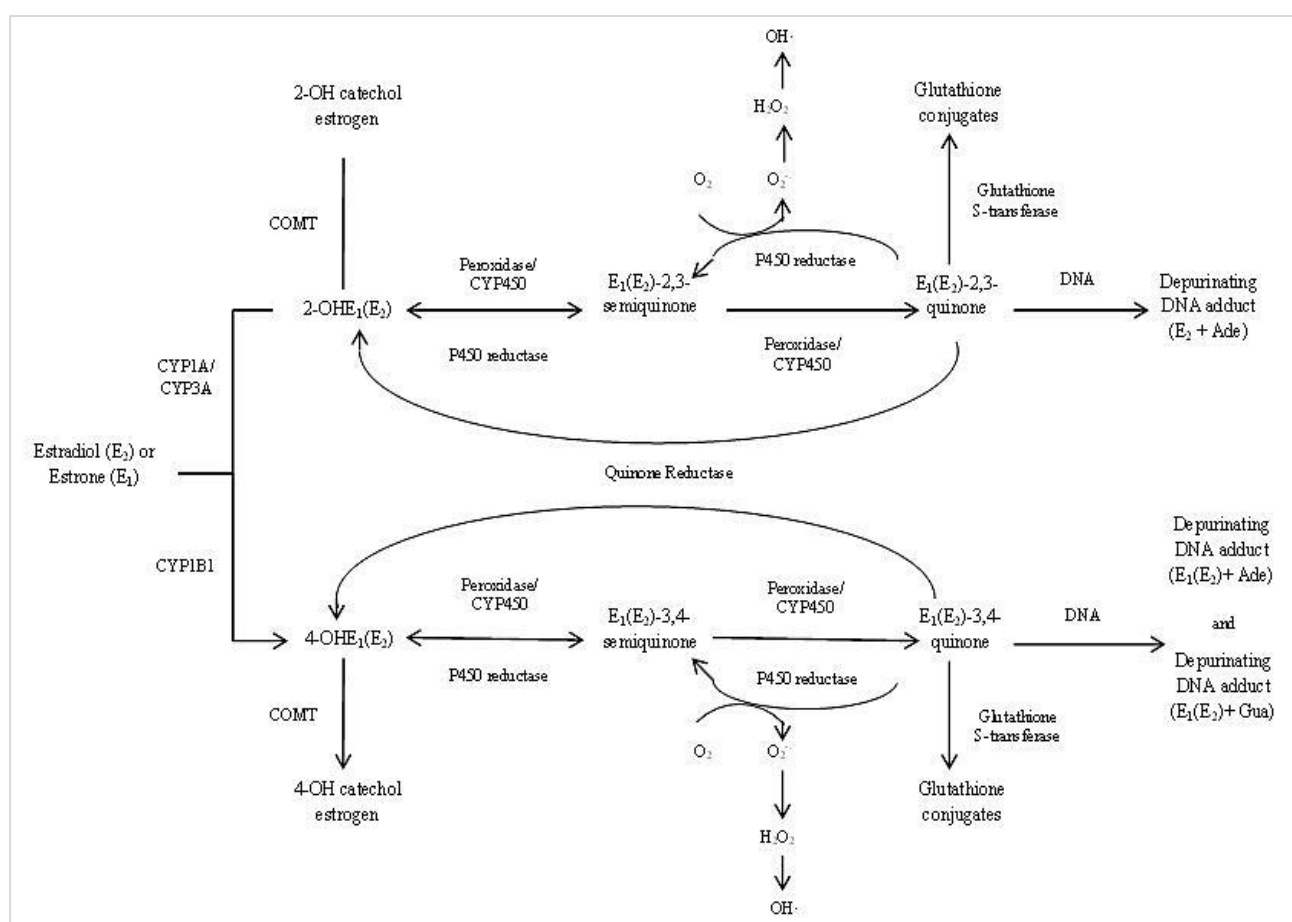


**Figure 2.7** The metabolism of estrone and estradiol through oxidative pathways (adapted from Rogan 2007).

2-OH-E<sub>1</sub>(E<sub>2</sub>) are a borderline carcinogen considered to have a low carcinogenic potential when compared to 4-OH-E<sub>1</sub>(E<sub>2</sub>) which has a high carcinogenic potential. The O-methylation of 2-OH-CE takes place at a higher rate than that of the 4-OH-CE and therefore, free radical production due to redox cycling takes place at a much lower rate (Tsuchiya *et al.*, 2005).

The estrogen 4-hydroxylase enzyme, CYP1B1, is mainly responsible for the initial hydroxylation of estrogens to 4-OH-CE. CYP1B1 expression is regulated by estradiol via the estrogen receptor (Tsuchiya *et al.* 2005). When high levels of CYP1B1 are present, more of the 4-OH catechol estrogen is formed. 4-OH-CE levels are associated with several types of malignant and benign cancers especially of the estrogen target tissues (mammary, ovary and uterus tissues). It was also found that the expression of CYP1B1 was higher in tumor tissues (Rogan, 2007). CYP1B1 activity decreases the estrogenic potential of estrone, but produces a metabolically active metabolite indicated in carcinogenesis (Tsuchiya *et al.*, 2005).

2-hydroxylation in the liver predominates 4-hydroxylation by 9:1 (Siddique *et al.*, 2005). Since O-methylation of 4-OH catechol estrogen is much slower than that of 2-OH catechol estrogen, it results in a higher rate of hydroxyl radical formation, therefore an increase in ROS. More depurinating adducts are formed when the quinone products bind to DNA resulting in a higher mutation rate and eventually carcinogenesis (Tsuchiya *et al.*, 2005). The quinone products of 2-OH-CE (E<sub>1</sub>(E<sub>2</sub>)-2,3-Q) have a poor competitive ability compared to E<sub>1</sub>(E<sub>2</sub>)-3,4-Q product of the 4-OH-CE resulting in a much lower carcinogenic potential (Rogan, 2007). It can be concluded that an imbalance in the homeostasis of estrogen metabolism can have detrimental effects on the body. Therefore it is very important to have the right balance in activating and deactivating pathways of estrogen metabolism to reduce the risk of carcinogenesis (Rogan, 2007).

#### **2.2.2.4 16 $\alpha$ -hydroxylation**

16 $\alpha$ -hydroxylation involves the hydroxylation of estrone at the 16 $\alpha$ -position through the action of the cytochrome enzymes CYP3A4/5 resulting in the formation of 16 $\alpha$ -hydroxyestrone (Huang *et al.*, 1998). 16 $\alpha$ -hydroxyestrone is implicated in breast cancer carcinogenesis and can also cause genotoxic DNA damage by binding covalently to DNA (Huang *et al.*, 1998). 16 $\alpha$ -hydroxyestrone has the ability to alkylate amino acid residues on proteins. It can also activate estrogen receptors, triggering growth-promoting genes and promoting breast cancer proliferation. The ratio of 2-OH estrone to 16 $\alpha$ -OH estrone should

be 2:1. A smaller ratio results in an increased long term risk for estrogen related cancers (Zhu & Conney, 1998).

### 2.2.3 ESTROGEN-RELATED CARCINOGENESIS

It is a well-known fact that increased concentrations of estrogen, both endogenous and exogenous, are associated with increased risk for estrogen related cancer such as endometrial and breast cancer, although the mechanisms of estrogen-related carcinogenesis are not completely understood (Bolton & Thatcher, 2008; Clemons & Goss, 2001; Hankinson *et al.*, 2004; Tsuchiya *et al.*, 2005; Yager, 2000). Important factors in the induction of estrogen related cancer, is the duration of prolonged exposure of cells to estrogen (through early menarche and late menopause or through estrogen replacement therapy) and the levels of estrogen cells are exposed (Bolton & Thatcher, 2008; Huang *et al.*, 1998). Studies in SENCAR mouse skin and ACI rat mammary gland suggested that 4-OH catechol estrogen or its metabolite E2-3,4-Q can induce mutations similar to the mutations associated with breast cancer, resulting in the hypothesis that E2-3,4-Q and 4-OH catechol estrogen are possible carcinogens via a genotoxic pathway and that faulty BER are the mechanism responsible for the mutagenesis in the presence of estrogen (Rogan, 2007).

Estrogen can be considered to be epigenetic carcinogens which function by stimulating abnormal cell proliferation and growth through estrogen mediated processes resulting in genetic damage, eventually causing carcinogenesis (Rogan, 2007; Tsuchiya *et al.* 2005). The formation of electrophilic or redox active quinones as metabolites of estrogen metabolism can cause alkylation or oxidative damage to cellular proteins and DNA (Bolton & Thatcher, 2008; Clemons & Goss, 2001) and metabolic activation of ethinylestradiol (EE) and the metabolism to reactive species are responsible for genotoxicity (Siddique *et al.*, 2005).

Studies found that unbalanced estrogen homeostasis may play an important role in the initiation of cancer (Rogan, 2007). It was proved that women with breast cancer had higher levels of estrogen and that the 4-OH catechol estrogen levels in these women were four times higher than the levels in women without cancer (Rogan, 2007). Considering that the oxidative pathway forming 4-OH catechol estrogen is a minor pathway in comparison to the pathway forming 2-OH estrogen, these results are troublesome. It is possible that 4-OH-CE induces genotoxicity (Clemons & Goss, 2001; Siddique *et al.*, 2005 Tsuchiya *et al.*, 2005). It was also found that CYP1B1 activity was higher in non-tumour tissues of breast cancer patients than in the same tissues of healthy women, and also COMT quinone reductase were

expressed in higher concentrations in healthy individuals, indicating the importance of catechol estrogen in breast cancer initiation (Rogan, 2007).

The 3,4-quinone product of 4-OH-estrone is responsible for the production of free radicals that could have DNA damaging effects. During its metabolism, EE produces reactive oxygen species which are converted to  $H_2O_2$  in the presence of superoxide dismutase.  $H_2O_2$  have the ability to cause DNA damage by inducing single strand and double strand breaks in DNA, chromosomal aberrations and sister chromatid exchange (Siddique *et al.*, 2005; Yager, 2000).

Regulation of estrogen pathways is important for the regulation of estrogen metabolism and regulation can occur both through genomic (nuclear) and non-genomic (extra-nuclear) pathways. DNA damage which can also occur through the binding or alkylation of estrogen receptors (ERs) by *o*-quinones can cause the selective targeting of estrogen sensitive genes and a higher risk of mutations during DNA replication (Bolton & Thatcher, 2008).

#### 2.2.4 COMBINED ORAL CONTRACEPTIVES (COCs)

The use of contraceptive steroids was first approved in 1960 and was considered to be a safe, effective and reversible fertility regulation method. Contraceptive steroids include oral pills, transdermal patches, vaginal rings, implants, injections and intra-uterine (IU) systems (Sitruk-Ware & Nath, 2011). The use of COCs is a popular form of cycle control and alleviating the symptoms associated with premenstrual stress syndrome including headaches, excessive menstrual bleeding, water retention, breast tenderness, anxiety, irritability, increased appetite, nausea and dysmenorrhoea (Guang-Sheng *et al.*, 2010; Rad *et al.*, 2011). The use of COCs creates an artificial hormonal environment to control the menstrual period (Guang-Sheng *et al.*, 2010). However, the long term effects of COCs, and thus continuous exposure to *ex vivo* hormones, on the metabolism are largely unknown (Rad *et al.*, 2011).

The use of contraceptive steroids brought on a lot of side effects including cardiovascular disease and venous thromboembolism (VTE). This led to the decrease of the EE and progestin dose, as well as the development of new molecules with safer physiological effects (Rad *et al.*, 2011; Sitruk-Ware & Nath, 2011). The metabolic effects of contraceptive steroids include altered lipid profiles, haemostatic variables and carbohydrate metabolism.

##### 2.2.4.1 Estrogen component of COCs

EE is an exogenous synthetic steroid hormone used in many contraceptives (Siddique *et al.*, 2005). Its metabolism is close to that of endogenous estrogen, namely metabolic oxidation to 2- and 4-catechol estrogen, which can be further broken down to quinones (Rogan, 2007). EE is absorbed rapidly and completely after ingestion. EE undergoes first-pass metabolism in the gut and forms significant amounts of sulphate conjugates in the jejunal mucosa through the action of sulphatase, with glucuronidation playing a less significant role in first-pass metabolism. It is transported in the blood via plasma (serum) proteins, in particular serum albumin. Although EE induces hepatic synthesis of SHBG and CBG, it does not bind to SHBG. The absolute bioavailability of EE is 20% to 65% with an average half-life of about 17 hours. It is metabolized completely (not excreted in unchanged form) and metabolites are excreted in the urine and bile. The metabolites excreted in the urine are glucuronide and sulphate conjugates and mono-oxidation products. The metabolites of EE in bile also include glucuronide conjugates, sulphate conjugates and oxidative products (Product A package insert, 2002; Zhang *et al.*, 2007).

*In vitro* studies showed that EE can induce reversible and irreversible inhibition of metabolizing enzymes (CYP450 isoforms). The acetylenic group on EE causes mechanism-based inactivation (irreversible) of CYP enzymes and NADPH-dependent inactivation have also been indicated. Oxidation of the acetylene group can result in an oxidative product involved in haem destruction or apoprotein modification (Zhang *et al.*, 2007).

#### **2.2.4.2 Progesterone-component of COCs**

The progestogen component in a COC should resemble natural progesterone as closely as possible, also with regards to pharmacological activity. Synthetic progestogens are mainly structurally related to either 19-nortestosterone or 17 $\alpha$ -hydroxyprogesterone (Krattenmacher, 2000; Sitruk-Ware & Nath, 2011). The side effects of these progestogens – ascribed to the absence of antihormone properties – include breast pain and tenderness, weight gain, increased blood pressure and mood swings (Krattenmacher, 2000; Parsey & Pong, 2000). Progestins, or synthetic progesterones, can be divided into four classes or generations based on their structural relationship to either testosterone or progesterone. First and second generation progestins resembled testosterone (19-nortestosterone derivatives) and was associated with negative side-effects due to androgenic properties such as acne, oily skin, hair growth and decreased HDL levels. Alternative progestins have been developed to resemble

progestogen more closely reducing the androgenic side-effects and increasing antimineralocorticoid and antiandrogenic action (Sitruk-Ware & Nath, 2011).

Drospirenone is a newly developed progestogen (6 $\beta$ ,7 $\beta$ ,15 $\beta$ ,16 $\beta$ -dimethylene-3-oxo-17 $\alpha$ -pregn-4-ene-21,17carbolactone) , unique in the sense that it is not structurally related to either of the above mentioned progesterones. Instead, drospirenone is a structural analogue to spironolactone, an aldosterone antagonist (Sitruk-Ware, 2005). Since drospirenone resembles progesterone more closely, it results in a biochemical and pharmacological profile similar to that of progesterone. Drospirenone has progestenic, antimineralocorticoid and antiandrogenic properties, resulting in a decrease in side effects observed in OCs containing other progestogens (Guang-Sheng *et al.*, 2010; Krattenmacher, 2000; Parsey & Pong, 2000; Sitruk-Ware, 2005). The anti-androgenic properties of drospirenone can be ascribed to the low binding affinity of drospirenone to the androgen receptor, in low concentrations inhibiting androgen associated gene transcription and androgen secretion (Guang-Sheng *et al.*, 2010; Muhn *et al.*, 1995; Teichmann, 2003). The contraceptive efficacy of drospirenone lies in its inhibition of ovulation. Drospirenone are 3 - 10 times more effective in inhibiting ovulation than progesterone (Guang-Sheng *et al.*, 2010; Muhn *et al.*, 1995).

**Table 2.2** Summary of types of progestins (Krattenmacher, 2000; Sitruk-Ware & Nath, 2011).

| Progesterone                     | Structurally similar progestogen                                                                   |
|----------------------------------|----------------------------------------------------------------------------------------------------|
| 19-nortestosterone               | Norethisterone<br>Norethisterone acetate<br>Levonorgestel<br>Gestodene<br>Desogestrel<br>Dienogest |
| 17 $\alpha$ -hydroxyprogesterone | Medroxyprogesterone acetate<br>Megestrol acetate<br>Chlormadinone acetate<br>Cyproterone acetate   |

Drospirenone is metabolised after ingestion to yield the acid of drospirenone and a sulphate metabolite; these metabolites are excreted in the faeces and urine (Krattenmacher, 2000).

The combination of drospirenone and EE proved to have no significant changes on blood pressure, a favourable effect on the lipid and triglyceride profiles and a trend towards weight loss. It was also found that drospirenone counteract the action of estrogen which leads to sodium retention, weight gain and high blood pressure, by inducing sodium loss and anti-mineralocorticoid activity (Krattenmacher, 2000; Parsey & Pong, 2000).

#### **2.2.4.3 Effects of COCs on metabolism**

Evidence showed that the use of combined oral contraceptives affected carbohydrate metabolism, lipid profiles, haemostatic variables, bone markers, and sex hormone-binding globulin (SHBG) (Rad *et al.*, 2011).

There is controversy on the pro- and antioxidant effects of the hormones, estrogen and progestin, used in combined oral contraceptives, with studies proving both protective and detrimental effects on oxidative stress. Estrogen exhibits antioxidant activity by inhibiting function and expression of NADP<sup>+</sup>/NADPH oxidase, by increasing the expression and level of activation of endothelial nitric oxide synthase, and stimulating the expression and activity of manganese and extracellular superoxide dismutase. However, progestin counteracts these antioxidant activities through activation of NADPH oxidase and inhibition of manganese and extracellular superoxide dismutase (De Groote *et al.*, 2009).

The use of OCs is associated with an increase in ROS. Reasons for this increase in ROS include an increase in lipid peroxides associated with increase copper concentrations and altered lipid soluble antioxidant defences, an increase in the amount of oxidized LDL, over-regulation of nitric oxide synthase, glutathione depletion as a result of estrogen and progestin metabolism, stimulation of the production of cellular energy which increases oxidant production, reduced concentrations of LH and FSH, and redox cycling between the *o*-quinones and semi-quinones, generating ROS such as superoxide, hydrogen peroxide and hydroxyl radicals (Finco *et al.*, 2011; Pincemail *et al.*, 2007; Siddique *et al.*, 2005).

De Groote *et al.* (2009) provided evidence for the link between oral contraceptives and oxidative stress by showing an increase in lipid peroxides and oxidized LDLs possibly due to the estrogen induced increase in serum copper (Finco *et al.* 2011). Furthermore, it was shown that contraceptives containing high estrogen concentrations of more than 50µg, can increase oxidative stress and ROS drastically from the start of contraceptive treatment (Finco *et al.*, 2011; Finco *et al.*, 2012).

Finco *et al.* (2012) proved that lower doses of estrogen can also induce oxidative stress conditions. Even though oxidative stress is present during two-thirds of the menstrual cycle, the use of oral contraceptives results in an increase of hormones well above the physiological level, creating a haematological peak, putting pressure on the antioxidant system. Ingested hormones in the form of oral contraception also disrupt the natural homeostasis of these hormones *in vivo*, and this could lead to the metabolism of estrogen via pathways known for the production of metabolites that increase the concentrations of ROS in the body and can also initiate carcinogenesis (Rogan, 2007).

It was found that the increased levels of ROS present in women using oral contraceptives, are not lowered by the conventional antioxidant treatments. ROS was also increased in women even though they possess the necessary *in vivo* antioxidant potential to reduce these drastically high levels of ROS. This suggested that the hormone-induced increase in ROS possess special characteristics.

Finco *et al.* (2011) proposed the use of the physiological modulator MF Templar<sup>®</sup> containing selenium yeast, pyridoxine hydrochloride,  $\alpha$ -lipoic acid, coenzyme Q10, 10%  $\beta$ -carotene, and decaffeinated green tea extract containing catechin. MF Templar<sup>®</sup> proved to decrease the oxidative stress induced by the use of contraceptives. The presence of catechin in the mixture proved to be of special importance as a strong antioxidant and the presence of lipoic acid and coenzyme Q10 support mitochondrial function and energy production. Administering catechins in its natural form (green tea) did not have any significant effect on the reduction in hormone-induced ROS, thereby highlighting the need for a multi-factor antioxidant. It is therefore useful to include water soluble as well as lipophilic compounds in the antioxidant mixture, utilizing the antioxidant capacity of different compounds instead of increasing the dosage of one of the antioxidants (Finco *et al.*, 2012).

#### 2.2.5 COCs CONTAINING ETHINYLESTRADIOL AND DROSPIRENONE

One well-known COC (Product A) is a combined oral contraceptive containing 30 $\mu$ g 17 $\alpha$ -ethinylestradiol and 3mg drospirenone. The contraceptive contains 21 active hormonal tablets containing both EE and drospirenone and 7 inert tablets. Another popular hormone product (Product B) is also a COC containing 20 $\mu$ g 17 $\alpha$ -ethinylestradiol and 3mg drospirenone with 24 active hormonal tablets and 4 inert tablets. Product A is considered to be well-tolerated, effective and safe OC, with good cycle control and low Pearl Index value (Guang-Sheng *et al.*, 2010; Parsey & Pong, 2000).

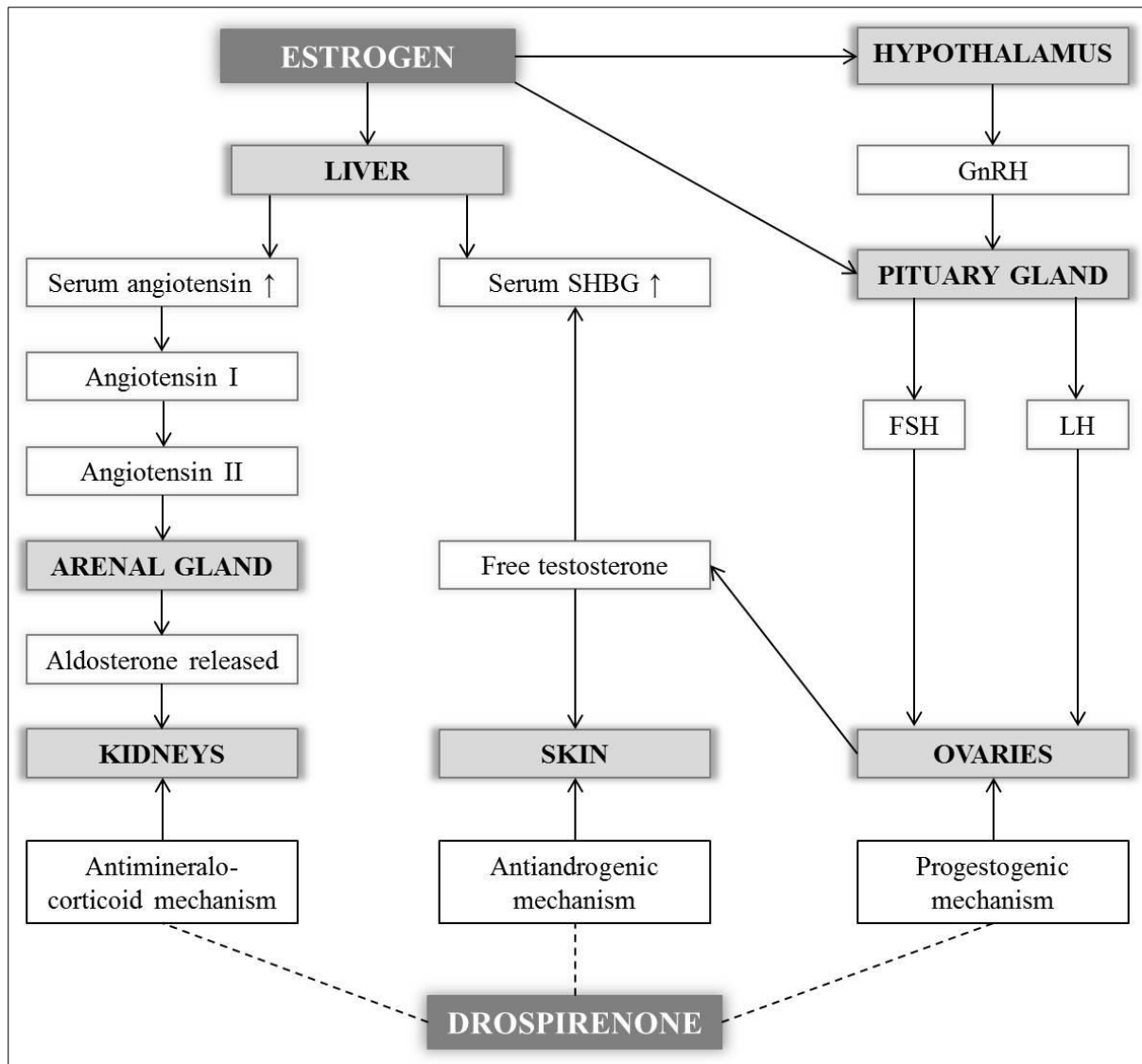


**Figure 2.8** This figure provides a summary of the effects of both estrogen and drospirenone on *in vivo* physiological markers. The three-way effect of drospirenone as antimineralocorticoid, antiandrogenic and progestogenic can be seen. Drospirenone was designed to counteract the effects of estrogen. FSH – Follicle stimulating hormone; LH – Luteinizing hormone; and SHBG – Sex hormone binding globulin (adapted from Krattenmacher (2000)).

The Pearl Index acts as an indicator of the effectiveness of pregnancy prevention of a contraceptive method (birth control). The combination of drospirenone and EE in this low-dose oral contraceptive provides health benefits reaching beyond contraceptive uses. It was found that Product A have favourable effects on menstrual symptoms such as water retention and negative as well as favourable impacts on the skin, weight and lipid profiles (Krattenmacher, 2000). Furthermore, the occurrence of intermenstrual bleeding, spotting and

breakthrough bleeding are greatly reduced over time with use of Product A (Guang-Sheng *et al.*, 2010). In a study it was found that due to the positive effects of Product A and the added medical benefits of using this product, the participants preferred using Product A over other forms of COCs containing alternative progestins (Guang-Sheng *et al.*, 2010). The effects that EE and drospirenone have on the liver and several glands and organs are shown in figure 2.8. Contradictory to the overall positive view of the combination of COCs containing EE and drospirenone, there have been recent reports of deaths associated with taking Product A and Product B.

Furthermore, previous biotransformation profiles done at the BOSS laboratory, showed a possible link between patients using specific oral contraceptives and increased levels of reactive species measured. These increased levels of reactive species can be seen in figure 2.9. This figure is an extract from a female patient's biotransformation profile. Dramatic increased levels of ROS can be seen: normal values would fall between 52.4 and 107.3 units, while the patient's profile showed ROS levels of 333.5 units. The same trend can be seen when considering the 2.3-DHBA concentration (a hydroxylradical marker) of the patient which is also elevated above normal concentrations. When the patients' antioxidant potential is taken into account (reference range 355 – 448  $\mu\text{M}$ ), it is evident that these women do possess the necessary antioxidant potential to reduce these increased levels of ROS, but without any effect.

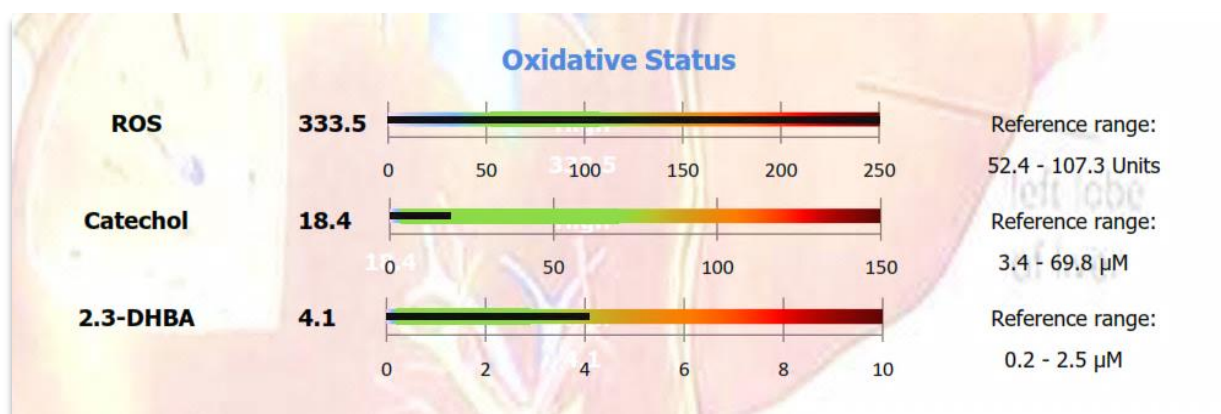


**Figure 2.9** An extract from the biotransformation profile of a female patient using a COC containing EE/drospirenone. The ROS concentrations are dangerously high indicating a high prevalence of ROS after the biotransformation test was performed (see Chapter 3). The

increase in 2,3-DHBA concentration is an indication of increased hydroxyl radicals as a result of aspirin-loading (see Chapter 3).

## 2.3 AIMS, OBJECTIVES AND EXPERIMENTAL APPROACH

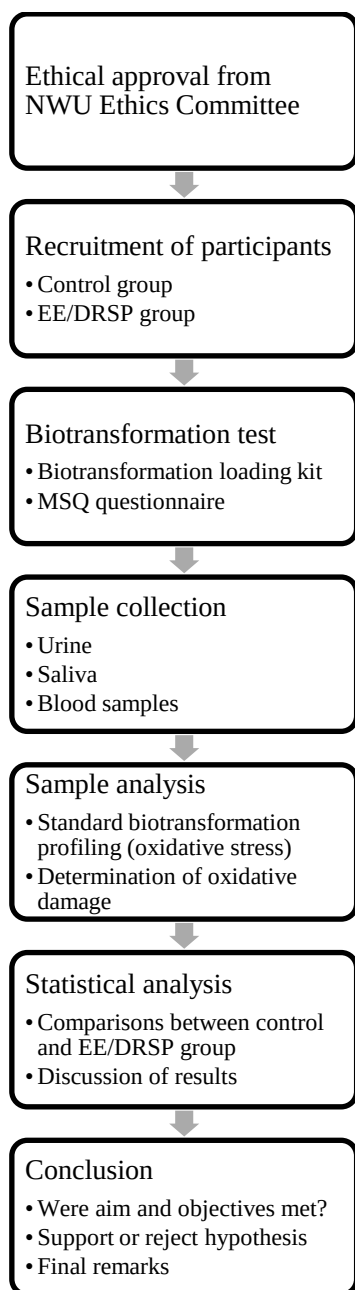


**Figure 2.10** A flow chart to show the outline of the experimental approach.

It was hypothesised that the use of two COCs with a similar formulation will alter the biotransformation, free radical levels and oxidative damage profiles in women using these contraceptives. The **aim** was therefore to investigate the effects of two specific combined

oral contraceptives, on the biotransformation, oxidative stress status and oxidative damage of its users. The following **objectives** were set in order to achieve the aim of this study and prove the hypothesis:

1. Conduct an age-matched controlled study with the COC users of the specific formulations as experimental and non-COC users as control groups.
2. Perform standardised biotransformation profiling and determine oxidative damage on samples of each participant collected during the luteal phase of the menstrual cycle.
3. Statistically process results to evaluate potential differences in biotransformation, free radical levels and oxidative damage between the control and experimental groups.

To investigate the effect of the use of the mentioned COCs (Product A and B) on the users' biotransformation, oxidative stress and oxidative damage, an experimental approach was determined. Because this study involved testing in humans, an application was submitted to the Ethics Committee of the North-West University, which was granted. Secondly, participants were recruited and placed into either the control group or the EE/DRSP group, depending on their use of oral contraceptives. Participants were asked to take the biotransformation test and complete a medical symptoms questionnaire. Urine, saliva and blood samples were collected and analysed for the quantification of acylcarnitines, organic acids such as 2,3-DHBA, phase II conjugates, caffeine clearance (Phase I), glutathione, antioxidant potential, and ROS as part of the standard biotransformation profile, and 8-OHdG, TBARS, and 3-NT were quantified to determine oxidative damage (Chapter 3). Statistical analysis was performed and data was interpreted in order to make comparisons between the EE/DRSP and control groups (Chapter 4). A conclusion was drawn, as described in Chapter 5, which either supported or discarded the hypothesis, and the aims and objectives were evaluated to confirm whether the study reached its goals.

# 3

# MATERIALS AND METHODS

## 3.1 INTRODUCTION

The **primary study** took place in two stages: the first a sample collection stage and the second an analytical stage.

The **sample collection** stage was conducted in the form of an age-matched controlled study with Product A and Product B users as the experimental and non-COC users as the control groups. With the help of a medical doctor, participants were clinically selected. A total of 20 patients using the specified COCs as an oral contraceptive were selected, and paired for comparison with a control group of 19 participants within the same age range.

Ethical approval for the study was received from the NWU Ethics Committee (NWU-0096-08-A1). All participants signed a consent form. Before sample collection, participants were subjected to a biotransformation loading test consisting of caffeine, paracetamol and aspirin. Blood, urine and saliva samples were collected. Participants were also asked to complete a Medical Symptoms Questionnaire (MSQ) at sample collection to monitor their perceived changes in clinical symptoms.

The **analytical** stage consisted of biotransformation profiling, determination of *in vivo* antioxidant potential and oxidative stress status, and quantification of oxidative damage markers. All tests were standardized. For biotransformation profiling, the following tests were performed: carnitine analysis, organic acid extraction for determination of catechol and 2,3-DHBA, determination of Phase I metabolism, and determination of Phase II metabolism. For determination of *in vivo* antioxidant potential and oxidative stress status, the following analysis was done: quantification of total glutathione, reactive oxygen species and FRAP assay. Lastly, for the determination of oxidative damage, the following markers will be considered: malondialdehyde, nitrotyrosine and 8-hydroxy-2-deoxyguanosine.

### 3.1.1 SAMPLE COLLECTION: BIOTRANSFORMATION LOADING KIT

Biotransformation loading was developed to test the detoxification ability of the liver. Since the liver is the primary organ responsible for the detoxification of substances such as caffeine, aspirin and paracetamol, a standardised loading test consisting of these three substances was developed in order to measure the different aspects of a patient's biotransformation in urine, blood and saliva samples. These aspects included Phase I and II biotransformation pathways, oxidative stress status and antioxidant capacity. Patients/participants were clearly instructed on how to prepare for the kit, as well as times when tablets should be taken and samples be collected. The kit contained 150mg caffeine tablet, two 300mg aspirin tablets and two 500mg paracetamol tablets, as well as a 1L urine container, small urine container, and two salivettes.

**Table 3.1** Sample collection for the biotransformation loading kit.

| Day                                                                                                            | Time          | Instructions                                                                                                                          |
|----------------------------------------------------------------------------------------------------------------|---------------|---------------------------------------------------------------------------------------------------------------------------------------|
| 1                                                                                                              | Early morning | A baseline urine sample was collected in the small urine container, sample was frozen.                                                |
|                                                                                                                | 08:00         | Caffeine tablet was taken before breakfast                                                                                            |
|                                                                                                                | 10:00         | First saliva sample was collected in a salivette, sample was frozen.                                                                  |
|                                                                                                                | 16:00         | Second saliva sample was collected in a salivette, sample was frozen.                                                                 |
|                                                                                                                | 21:00         | Eating and drinking (except water) were discontinued. Bladder was emptied and the two aspirin and two paracetamol tablets were taken. |
| Collect all overnight urine in the large urine container until 07:00 of Day 2, freeze sample after collection. |               |                                                                                                                                       |
| 2                                                                                                              |               | Frozen samples took to pathology laboratory. Pathologist collected three blood samples: two with EDTA and one without.                |

Sample collection took place according to Table 3.1. The urine samples were used to determine Phase II metabolism, saliva samples were used to determine caffeine clearance, and blood samples were collected to determine oxidative stress status and antioxidant capacity. During the test participants were advised to avoid certain beverages (alcohol, coffee, tea, cocoa, chocolate, cola soft drinks, rooibos tea and health tea), medication (containing caffeine, salicylates/aspirin and paracetamol), stone fruits (e.g. sultanas, currants, raisins), spices (e.g. curry, dill, oregano, paprika), and brassica vegetables (broccoli, cabbage, brusselsprouts, cauliflower and tomato).

## 3.2 ANALYTICAL METHODS

### 3.2.1 QUANTITATIVE ANALYSIS OF ACYLCARNITINES IN URINE

#### 3.2.1.1 Principle of method

Carnitine is a trimethylated amino acid that plays important roles in the body, including the modulation of acyl-CoA:CoA ratio, the storage of energy (acetylcarnitine), biotransformation of accumulated acyl groups and xenobiotic carboxylic acids, and co-factor in fatty acid transport to mitochondria. Carnitine can therefore be a good indicator of oxidative stress: if the levels of free carnitine decrease (due to depletion) and those of acyl-carnitine increase, it can be indicative of an oxidative state.

Tandem mass spectrometry (MS-MS) and electro spray ionization (ESI) were used in combination with controlled collision induced dissociation to identify and quantify concentrations of carnitine and acyl-carnitine present in the samples. Samples were butylated in the presence of butanolic hydrochloric acid (HCl). MS1 was set up in scanning mode in the mass range of 200 to 600. MS2 was set up to scan for precursor ions with a fragment of mass-to-charge ratio of 85 ( $m/z$ ), which is a common mass found after CID of butylated acylcarnitines. Therefore, all species of butylated carnitine and acylcarnitine were detected.

#### 3.2.1.2 Method

##### (i) *Extraction*

Two sets of eppendorf tubes (Merck) were marked for each sample. 100 $\mu$ L urine of each sample was transferred to the first set of eppendorf tubes with a micropipette (Eppendorf) and centrifuged (Labotec) for 20 minutes at 1300 rpm. 410 $\mu$ L of acylcarnitine isotope solution (VU Medical centre, Utrecht) were added as internal standard to each of the second set of eppendorf tubes. After centrifugation, 10 $\mu$ L of the urine samples were transferred to corresponding marked eppendorf tubes containing isotope solution. An evaporator (Stargate Scientific) was used to evaporate fluid in the tubes under nitrogen (Afrox) at 65°C for approximately 15 minutes until completely dry. 200 $\mu$ L butanolic HCl (Sigma) were added to the dried samples and incubated for 15 minutes at 65°C. Butylated samples were again evaporated under nitrogen at 65°C for approximately 20 minutes until completely dry. 100 $\mu$ L acetonitrile:water (50:50) (v/v) (Burdick & Jackson, Millipore) containing 0.1%

formic acid (Merck), were added to the dried samples to reconstitute samples in the mobile phase (acetonitrile:water 50:50 v/v). Samples were vortexed briefly at high speed (VELP Scientifica) to ensure butylated samples dissolve in mobile phase. 100µL of the sample were then transferred to a 96-well plate (Merck) covered in aluminium foil.

*(ii) MS/MS analysis*

The plate was placed in the Agilent LC 1200, where 10µL sample was injected. The flow rate of the mobile phase was maintained at 0.2 mL/min throughout the run. MS-MS analysis was performed on an Agilent 1640 Triple Quadrupole with positive ion electron spray. The first mass spectrometer (MS1) was set to scanning mode (mass range 200 – 600 (m/z)), while the second mass spectrometer (MS2) was used as a mass filter for the product ion of 85 (m/z). Therefore, all butylated products with a product ion of 85 were detected. The signal intensity of the carnitine species were relatively quantified to that of their corresponding deuterated internal standards for quantification purposes. For carnitines that do not have a stable isotope, signal intensity was quantified relative to another stable isotope in the mixture.

### 3.2.2 QUANTIFICATION OF PHASE II CONJUGATES IN URINE

#### 3.2.2.1 Principle of method

Phase II conjugates such as glucuronide conjugation, sulphate conjugation and glutathione conjugation can be measured by using paracetamol as a probe drug. Glucuronide conjugation and sulphate conjugation are the major metabolites of paracetamol metabolism, while a lower amount of a highly reactive metabolite is conjugated with glutathione. Aspirin (acetyl salicylic acid) can be used to determine glycine conjugation, since salicylic acid (glycine conjugate) is one of its major metabolites. Concentrations of Phase II metabolites were determined by means of linear regression from the calibration curves.

#### 3.2.2.2 Method

*(i) Extraction*

A medispot tube (Plastpro Scientific) was marked for every sample. 200µL of each participant's urine were transferred to the marked tubes. 100µL of a 25mmol/L acetamidophenol (Sigma) in water (Millipore) internal standard solution was added to each

tube and the tubes were mixed well. Before freezing, holes were made in the caps of the tubes as preparation for freeze drying. The samples were frozen at -80°C in a -80°C freezer (Forma Scientific). Samples were lyophilized overnight in a freeze dryer (United Scientific). After samples were completely dry, 400µL of the mobile phase was added to each sample to reconstitute the samples. Samples were vortexed (VELP Scientifica) to ensure that the dry sample mix completely with the mobile phase, and centrifuged quickly. 200µL of the every sample were transferred to HPLC vials (Agilent), and put into the HPLC autosampler (Agilent).

(ii) *HPLC analysis*

A Phenomenex Luna 5µ C18(2) 100A, 250×4.60mm or Phenomenex Luna 5µ C18(2) 100A, 150 × 2.00mm HPLC column was used at a temperature 35°C. Acquisition was set to monitor chromatography at 254nm. Flow rate of the mobile phase was set at either 1mL/min or 0.2mL/min, depending on the diameter of the column used. The mobile phase was run against a gradient consisting of mobile phase A (5% acetonitrile (Honeywell) in water with 0.05% trifluoroacetic acid (TFA) (Sigma)) and mobile phase B (acetonitrile). The composition of the mobile phase used during Phase II analysis can be explained through table 3.2.

**Table 3.2** Mobile phase gradient over time.

| <b>Sequential times</b> | <b>Mobile phase A</b> | <b>Mobile phase B</b> |
|-------------------------|-----------------------|-----------------------|
| First 3 minutes         | 100%                  | 0%                    |
| 4 – 11 minutes          | 75%                   | 25%                   |
| 12 – 13 minutes         | 15%                   | 85%                   |
| 14 – 20 minutes         | 0%                    | 100%                  |
| 21 – 23 minutes         | 0%                    | 100%                  |

After 28 minutes, data acquisition was stopped and the mobile phase changed back to 100% HPLC mobile phase A and 0% HPLC mobile phase B over 5 minutes. HP Chemstation software was used to integrate the conjugates and internal standard (acetamidophenol). The response ratios were determined and the percentages of the different conjugates excreted were determined.

### 3.2.3 QUANTITATIVE ANALYSIS OF PHASE I BIOTRANSFORMATION (CAFFEINE CLEARANCE) IN SALIVA

#### 3.2.3.1 Principle of method

Caffeine can be used to measure CYP1A2 activity as an indication of Phase I metabolism. During metabolism, caffeine undergoes N-demethylation through CYP1A2 catalyses to form paraxanthine, theobromine, and theophylline.

#### 3.2.3.2 Method

##### (i) *Solid Phase Extraction*

Since saliva sample collection takes place during two separate times, there were two samples for each participant. Two eppendorf tubes (A and B) (Merck) and two medispo tubes (A and B) (Plastpro Scientific) were marked for each participant, which would contain the two samples to be analysed for each participant. 200µL saliva sample and 200µL of a 4.6 mg/L 2-acetamidophenol (Sigma) in water internal standard were added to each eppendorf tube and then vortex to ensure the mixture is well mixed. Next, HF Bond Elut C<sub>18</sub> SPE columns (100mg/ml) (Varian) were placed on the solid phase manifold equipped with vacuum pump (Phenomenex) and conditioned for solid phase extraction of the sample: First, columns were slowly (<1mL/min) washed with ~2mL methanol (Honeywell), secondly the columns were activated with ~2mL laboratory grade water (Millipore). Before the resin in the column dried due to the vacuum, valves were closed and 400µL sample mixture was added to the columns. The valves were opened and the samples eluted slowly allowing compounds to bind to the resin. Columns were again washed with laboratory grade water and vacuumed until completely dry. The medispo tubes were positioned under the solid phase extraction columns and analytes were eluted with ~2mL methanol-sodium acetate (Sigma) buffer (pH 3.5) until columns were completely dry. The eluates were dried under nitrogen (Afrox) until approximately 50% of methanol had evaporated (half of the eluate), after which samples were frozen at -80°C until completely frozen (Forma Scientific). Samples were lyophilized in a freeze dryer (United Scientific) and the dried sample were reconstituted in 200µL 5% acetonitrile (Honeywell) in water with 1% formic acid (Merck). Samples were transferred to inserts (Agilent) in HPLC vials (Agilent) and put in an HPLC auto-sampler (Agilent).

(ii) *HPLC analysis*

A Phenomenex Luna 5 $\mu$  C18 column was used for chromatography. The HPLC conditions were as follow: Column temperature was set at 35°C, acquisition monitored chromatography at 254nm, mobile phase flow was set at 0.2mL/min running a gradient of mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile). The HPLC UV/VIS detector was auto-zeroed before sample injection. The composition of the mobile phase used during Phase I analysis can be explained by the following table.

**Table 3.3** Mobile phase gradient over time.

| Sequential times           | Mobile phase A | Mobile phase B |
|----------------------------|----------------|----------------|
| First 5 minutes            | 100%           | 0%             |
| 6 – 15 minutes             | 75%            | 25%            |
| 16 – 17 minutes            | 15%            | 85%            |
| 18 – 33 minutes (constant) | 15%            | 85%            |
| 33 <sup>rd</sup> minute    | 100%           | 0%             |

After 33 minutes, data acquisition was stopped and the mobile phase changed back to 100% A and 0% B. Agilent Chemstation software was used for data integration and caffeine concentration was determined by measuring response against the response of a calibration curve.

### 3.2.4 QUANTITATIVE GC/MS METHOD FOR THE ANALYSIS OF CATECHOL AND 2,3-DHBA IN URINE

#### 3.2.4.1 Principle of method

During conditions of oxidative stress, highly reactive hydroxyl radicals can form as a result. Aspirin can react spontaneously with these hydroxyl radicals. Therefore aspirin can be used as a probe substance to measure two hydroxyl radicals, namely catechol and 2,3-dihydroxybenzoic acid. Catechol and 2,3-DHBA are organic acids which can be analysed quantitatively (after derivatisation) using gas-chromatography mass-spectrometry (GC-MS) in selected ion monitoring (SIM) mode.

### 3.2.4.2 Method

A work list was created for every batch of samples analysed. The creatinine value was converted from  $\mu\text{mol/L}$  to  $\text{mg}\%$  by multiplying the value with a factor of 11.312. The volume of urine needed is calculated according to the following table:

**Table 3.4** Volume of urine needed when taking the  $\text{mg}\%$  creatinine into account.

| <b>Creatinine in <math>\text{mg}\%</math></b> | <b>Volume urine needed</b> |
|-----------------------------------------------|----------------------------|
| Creatinine > 100 $\text{mg}\%$                | 0.5mL                      |
| Creatinine < 100 $\text{mg}\%$                | 1mL                        |
| Creatinine < 5 $\text{mg}\%$                  | 2mL                        |
| Creatinine < 2 $\text{mg}\%$                  | 3mL                        |

The amount of internal standard used was calculated as follow:

$$\text{Internal Standard } (\mu\text{L}) = \text{creatinine } (\text{mg}\%) \times \text{volume urine (mL)} \times 5 \quad (\text{Formula 3.1})$$

#### (i) *Organic acid extraction*

Three kimax tubes (Lasec) for each sample were marked: 2 large kimax tubes and 1 small kimax tube. The calculated volume of urine of each sample was transferred to large kimax tubes. The amount of internal standard added was calculated by multiplying five times the  $\text{mg}\%$  creatinine with the volume of urine (see table 3.4). Internal standard, 3-phenylbutyric acid solution (Fluka) was added to the samples. 230 $\mu\text{L}$  of 5M hydrochloric acid (Merck) was added to each sample to reduce the pH of the samples to 1. 6mL of HPLC grade distilled ethylacetate (Sigma) was then added to each of the samples, after which the samples were shaken on a rotor (Cole-Parmer Instrument Company) for 30 minutes and centrifuged (LasecSA) at 2800r.p.m. (910r.c.f) for 3 minutes. The top organic phases of all the samples were aspirated with Pasteur pipettes (Merck) into the second set of large kimax tubes. Care was taken not to extract any of the lower aqueous phase with the organic phase. 3mL of HPLC grade distilled diethylether (Sigma) was added to the aqueous phase. The samples were again shaken on a roto-torque for 10 minutes and centrifuged for 3 minutes at 2800r.p.m. The organic phases were again aspirated and added to the first organic phases. The organic phases were dried by adding two spatulas sodium sulphate ( $\text{Na}_2\text{SO}_4$ ) (Merck) to every tube. Samples were vortexed well in order to ensure maximum contact of  $\text{Na}_2\text{SO}_4$  with

possible water molecules extracted – since Na<sub>2</sub>SO<sub>4</sub> is hygroscopic, it will bind to any residue water molecules to ensure the organic phase is completely water free. The samples were centrifuged for 5 minutes at 2800r.p.m. The organic phase was aspirated from the Na<sub>2</sub>SO<sub>4</sub> into the smaller kimax tubes. The samples were dried under nitrogen (Afrox) at 38°C for approximately 1 hour on a heating block with nitrogen evaporator (Stargate Scientifica).

(ii) *Derivatisation*

The derivatisation reagents were (bis(trimethylsilyl)-trifluoroacetamid) (BSTFA) (Supelco), trimethylchlorosilane (TMCS) (Sigma), and pyridine (Merck). Derivatisation reagents were added to the dried extract with a Hamilton syringe (250µL and 100µL) (Separations). The volumes of the reagents were calculated as follow:

**Table 3.5** Volume of derivatisation reagents added when taking the mg% creatinine into account.

| Reagent  | Creatinine calculations |
|----------|-------------------------|
| BSTFA    | mg% creatinine x 3      |
| TMCS     | mg% creatinine x 0.6    |
| Pyridine | mg% creatinine x 0.6    |

Reagents were added in the above mentioned order. The syringes were thoroughly cleaned with hexane. After adding the derivatisation reagents, the samples were incubated on a sand bath hotplate (VELP Scientifica) at 85°C for 45 minutes. Tubes were allowed to reach room temperature before samples were transferred to GC-MS vials (Agilent) with a Pasteur pipette.

(iii) *GC-MS analysis*

The DETOXSIM method was used to analyse samples on the GC-MS on a capillary column (JW Scientific). 1µL sample was injected in split mode with a split ratio of 5:1 at a front inlet temperature of 280°C at a pressure of 10.523psi. The GC temperature program started at 100°C for 2 minutes, followed by an increase in temperature at 4°C per minute to 120°C, followed by an increase of 6°C per minute to 215°C, followed by 20°C per minute increase to 300°C and kept for 2 minutes. Post run was at 295°C for 1 minute. Helium was used as a carrier gas and flow was kept constant at 1mL/min. The MS was operated in SIM (selected ion monitoring) and the compounds were identified according to the following table:

**Table 3.6** The retention times and ions of the different compounds detected by the MS.

| Compounds         | Retention time (min) | Ion monitored |
|-------------------|----------------------|---------------|
| Catechol          | 7.00 – 10.00         | 254.10        |
| Internal Standard | 10.00 – 12.00        | 221.10        |
| Salicylic Acid    | 12.00 – 16.00        | 267.10        |
| 2,3-DHBA          | 16.00 – 17.80        | 355.20        |
| 2,5-DHBA          | 17.80 – 21.00        | 355.20        |
| Salicyluric Acid  | 21.00 – 29.00        | 324.00        |

HP Chemstation software was used to integrate the peaks of catechol, 2,3-DHBA and the internal standard. The response ratio of the compounds to the internal standard was determined.

### 3.2.5 REACTIVE OXYGEN SPECIES (ROS) ASSAY

#### 3.2.5.1 Principle of method

The basic principle of this method is the colorimetric reaction of ROS with transition metals to form alkoxyl and peroxy radicals, which in turn oxidize N,N-diethylparaphenylenediamine to its cation. The reaction can be monitored spectrophotometrically at 546nm for 10 minutes.

#### 3.2.5.2 Method

##### (i) *Sample handling*

Blood samples were collected in SST vacutainers and stored in a -80°C freezer (Forma Scientific). On day of analysis, blood samples were thawed, vortexed and centrifuged at 10000g for 10 minutes in order to separate serum from the rest of the components of blood, e.g. red blood cells. Serum was aspirated into eppendorf tubes (Merck).

##### (ii) *Extraction and quantification*

A multichannel pipette (Thermo Scientific) was used to add 140µL ROS buffer to the wells in a 96 well plate (Sterilin). Next, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Sigma) aliquot concentrations

0mg/mL, 0.06mg/mL, 0.12mg/mL, 0.18mg/mL, 0.24mg/mL, and 0.3mg/mL were prepared and 2.5µL of each was added to the ROS buffer in the wells. Samples were vortexed and 2.5µL of each sample was added in triplicate to the ROS buffer. The plate was allowed to incubate at 25°C for 10 minutes. During this time, a mixture containing N,N-diethylparaphenylenediamine (DEPPD) (Sigma) ( $R_1$ ) and ferrous sulphate ( $FeSO_4$ ) (Labchem) ( $R_2$ ) were prepared in the ratio  $R_1:R_2 = 1:25$ . The mixture was mixed well and incubated in a water bath at 25°C for 5 minutes. After incubation, the  $R_1R_2$  mixture was added to the wells with a multichannel pipette. The plate reader (BioTek) program (GEN 5.1.1) was started exactly 50 seconds after the first addition of the  $R_1R_2$ . The plate was read at 546nm for 10 minutes each minute.

A regression line was determined from the standards that were added to the plate in duplicate, and  $R^2 > 0.97$  was accepted. The samples were added to the plate in triplicate. The average, standard deviation, and relative standard deviation (RSD) of each sample was determined. A RSD of <5% showed an acceptable result.

### 3.2.6 FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

#### 3.2.6.1 Principle of method

The FRAP assay are used to determine the antioxidant potential of biological fluids (e.g. blood). It is based on the colorimetric reaction when  $Fe^{iii}$ -TPTZ (ferric-tripyridylpyridine) is reduced to  $Fe^{ii}$ -TPTZ at low pH. The change can be measured spectrophotometrically at 593nm.

#### 3.2.6.2 Method

##### (i) *Sample handling*

Blood samples were collected in SST vacutainers and stored in a -80°C (Forma Scientific). On day of analysis, blood samples were thawed, vortexed and centrifuged at 10000g for 10 minutes in order to separate serum from the rest of the components of blood, e.g. red blood cells. Serum was aspirated into eppendorf tubes (Merck).

##### (ii) *Extraction and quantification*

A 10mM stock solution Ferrous sulphate heptahydrate ( $\text{FeSO}_4$ ) (Labchem) was diluted with laboratory grade water (Millipore) to a concentration of 0.1mM in an eppendorf tube (Merck).  $\text{FeSO}_4$  (0.1mM) was used to create a concentration gradient in a 96 well plate (Sterilin) for the standard curve at concentrations 0.010, 0.008, 0.006, 0.004, 0.002, and 0 mM. After 15 $\mu\text{L}$  concentration gradient stock was added to the 96 well plate, 85 $\mu\text{L}$  laboratory grade water was added with a multichannel pipette (Thermo Scientific) to the wells to contain the samples. Samples were vortexed and 15 $\mu\text{L}$  of each sample was added to the plate in triplicate, bringing the total volume in wells containing the samples also to 100 $\mu\text{L}$ . The plate was incubated at 25°C for 10 minutes on the plate reader (BioTek). During incubation of the plate, the sodium acetate trihydrate (FRAP) (BDH Analar Limited Poole England) reagent was prepared by taking into account the amount of wells used and mixing FRAP acetate buffer, 2,4,6-Tris-2-(pyridyl)-1,3,5-triazine (TPTZ) (Sigma) and Ferric chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) (Sigma), in the ratio FRAP acetate buffer:TPTZ: $\text{FeCl}_3$  = 10:1:1. The FRAP reagent was incubated in 25°C for 5 minutes. After the plate incubated for 10 minutes, 250 $\mu\text{L}$  of the FRAP reagent was added to the wells with a multichannel pipette. The plate reader program (GEN 5.1.1) was started at exactly 1 minute 20 seconds after the FRAP reagent was added to the first wells.

Concentration of FRAP was measured against a regression line which was determined from the standards that were added to the plate in duplicate, and  $R^2 > 0.97$  was accepted. The samples were added to the plate in triplicate. The average, standard deviation, and relative standard deviation (RSD) of each sample was determined. A RSD of <5% showed an acceptable result.

### 3.2.7 TOTAL GLUTATHIONE (GSH/GSSG) ASSAY

#### 3.2.7.1 Principle of method

Glutathione is an important *in vivo* antioxidant that plays an important role in neutralizing reactive species. It also plays a role in clearance of toxins by acting as a substrate for conjugation and reduction reactions in the body. Glutathione can exist in two forms in the body: a reduced (GSH) or oxidized (GSSG) state. Glutathione becomes oxidized (GSSG) when reacting with ROS and reduced through the action of glutathione reductase in the presence of NADPH which acts as an electron donor. Glutathione is also used as a phase II

liver biotransformation substrate Glutathione can therefore become depleted with a high toxin load, resulting in too little glutathione for antioxidant function. This could have harmful effects on the body.

In this assay, Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid or DTNB) reacts with GSH resulting in the formation of a spectrophotometric detectable product at 412nm. GSSG is also reduced to the GSH, therefore total glutathione levels in whole blood is measured in this assay. The rate of colour change is proportional to total glutathione concentrations.

### **3.2.7.2 Method**

#### *(i) Preparation of reagents*

Metaphosphoric Acid (MPA) (Sigma) was prepared by weighing 0.7247g MPA for every 5mL of sample. The MPA was dissolved in 5mL laboratory grade water (Millipore) for every 0.7247g MPA, vortexed and refrigerated prior to sample analysis.

NADPH was weighed very accurately prior to start of sample analysis by weighing 0.005g of NADPH to dissolve in 3mL GSH (Oxis International). GSH buffer was placed in a 25°C water bath.

#### *(ii) Extraction and quantification*

Blood samples were collected in EDTA vacutainers and stored at -80°C.

Two sets eppendorf tubes (Merck) were labelled for every whole blood sample.

The whole blood samples were thawed, inverted and vortexed prior to analysis. 50µL of every whole blood sample were pipetted (Eppendorf) into the accordingly marked eppendorf tube. MPA was kept on ice. 350µL of the ice cold MPA were added to a sample in an eppendorf tube and the sample vortexed for 20 seconds. This was repeated for every sample. Samples were kept on ice. Eppendorf tubes containing the MPA-sample mix were centrifuged at 10000g and 4°C for 5 minutes. 750µL GSH buffer were added to the second set of Eppendorf tubes. 12.5µL of the centrifuged MPA-sample mix was added to the second set of eppendorf tubes. Samples were kept on ice. 50 µL of the prepared standards (0.00, 0.10, 0.25, 0.50, 1.50 and 3.00uM GSH) were added in duplicate to the 96 well plate. Samples were vortexed for 12 seconds and 50µL of each sample was added in triplicate to the 96-well plate. 50µL chromogen was added to each well with a multi-channel pipette

(Thermoscientific). 50 $\mu$ L of the enzyme were added to each well with a multi-channel pipette. The 96-well plate was subsequently incubated at 25°C for 5 minutes (part of the plate reader program) (BioTek Synergy). NADPH buffer was prepared by dissolving the weighed NADPH in GSH buffer. The amount of GSH buffer used was calculated by multiplying the number of wells (both standard and sample wells) by 50 $\mu$ L. After incubation of the 96-well plate, 50 $\mu$ L NADPH buffer were added to each well with a multi-channel pipette. NADPH buffer were added to the wells within 30 seconds of addition to the first row. The 96-well plate were read at 412nm for 3 minutes

Concentration of total glutathione was measured against a regression line which was determined from the standards that were added to the plate in duplicate, and an  $R^2$  of more than 0.97 was accepted. The samples were added to the plate in triplicate. The average, standard deviation, and relative standard deviation (RSD) of each sample was determined. A RSD of <5% showed an acceptable result.

### 3.2.8 TBARS ASSAY IN URINE.

#### 3.2.8.1 Principle of method

MDA can be determined in biological samples such as urine and blood through the optimized TBARS assay, as described by Bothma (2012) (Buege & Aust 1978). Thiobarbituric acid (TBA) reacts with the MDA in a sample at low pH to form adducts (pink chromogen) which can be quantified through a spectrophotometric method measuring absorbance at 532nm (Gutteridge, 1995, Bothma, 2012).

#### 3.2.8.2 Method

##### (i) *Preparation of reagents*

The reagent mix (15% TCA reagent (m/v), 0.375% TBA reagent (m/v) and 0.25N HCl) for the TBARS assay was prepared in laboratory grade water (Millipore) and sonicated until dissolved completely.

A 10mM MDA standard stock solution was prepared by adding 16.64 $\mu$ L tetramethoxypropane to 10mL milli-Q water. A 1mM MDA dilution was prepared by adding

100µL of the stock solution to 900µL milli-Q water. This was used to prepare a calibration range with MDA concentrations 0, 0.98, 1.95, 3.91, 7.81, 15.63, and 31.15µM.

*(ii) Extraction and quantification*

Before use, urine samples were centrifuged at 15400g for 4 minutes. 800µL of the sample or MDA standards were added to a microcentrifuge tube (Eppendorf). 800µL of the reagent mix was then added to each standard and sample. The tubes were incubated at 90°C for 1 hour. After incubation, the tubes were again centrifuged at 15400g for 4 minutes. For the standards 150µL of each were added to a 96-well plate in duplicate for the calibration range and 150µL of each sample were added to the 96-well plate in triplicate. The absorbance was measured at 532nm (MDA absorbance) and 600nm (background) with a spectrophotometer plate reader (BioTek Synergy). The MDA standards were used to construct a calibration curve and a R<sup>2</sup>-value of 0.99 or higher were accepted as an adequate value for the linear calibration curve.

### 3.2.9 LC-MS/MS QUANTIFICATION OF NITROTYROSINE IN URINE

#### 3.2.9.1 Principle of method

Bothma (2012) described an analytical method for the quantification of 3-NT in urine samples using LC-MS/MS. 3-NT in a urine sample was quantified with a calibration curve in the presence of an internal standard (phenylalanine isotope) and samples were butylated in the presence of R-2 butanolic/HCl as derivatisation agent. LC-MS/MS were done in positive electrospray ionisation (+ESI) and in multiple reaction monitoring (MRM) to determine the transition of  $283.13m/z \rightarrow 181 m/z$ .

#### 3.2.9.2 Method

*(i) Preparation of reagents*

The stock solution (175mg/L) for the standard range of the calibration curve was prepared by adding 12.28mg 3-Nitrotyrosine (Sigma) to a mixture containing 61.4mL methanol and 8.6mL water. This stock solution was used to prepare, through serial dilution, a sample with concentration 0.1 mg/L in methanol (Sigma Aldrich). 1mL of this sample was added to a microcentrifuge tube (Sigma Aldrich) and dried under a stream of N<sub>2</sub>(g) at 65°C. The sample

was derivatised by adding 200µL R-(-2)-buthanol solution (prepared by adding 250µL acetylchoride, 50µL at a time, to each container of R-(-2)-buthanol on ice) and incubating the sample at 65°C for 15 minutes. The sample was again evaporated to dryness under a stream of N<sub>2</sub>(g) at 65°C. The dried derivatised sample was dissolved in 1mL of the mobile phase. The sample was used in a serial dilution (in mobile phase) to 3-NT concentrations of 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.025, 0.05, 0.075, and 0.1mg/L. 100µL of each concentration was transferred to pulled point inserts (Agilent) in HPLC caps with vials (Agilent).

(ii) *Extraction*

For the extraction of 3-NT from urine samples, 1 mL urine was aliquoted into microcentrifuge tubes and centrifuged at 15700g for 2 minutes at room temperature. 100µL of the supernatant of each sample was transferred to a clean eppendorf tube (with corresponding number). 100µL methanol was added to each sample and the mixture was vortexed briefly before being dried under N<sub>2</sub>(g) at 65°C for approximately 40 minutes until dry. The samples were derivatised by adding 100µL of the R-(-2)-buthanol solution, the samples were incubated at 65°C for 15 minutes. Samples were again dried under N<sub>2</sub>(g) at 65°C for 15-20 minutes and the dried sample was dissolved in mobile phase. 100µL of each sample was transferred to pulled point inserts in HPLC caps with vials.

(iii) *LC-MS/MS analysis*

Extracted samples were analysed on an LC 1290 infinity (Agilent). The injection volume was 5µL and sample separation took place on a Zorbax SB-18, 1.8 µM, 2.1 x 50mm C18 column (Agilent) using a flow rate of 200µL/min. The analyte was eluted by using a mobile phase gradient (see table 3.7) – mobile phase A was acetonitrile containing 0.1% formic acid and mobile phase B was water containing 0.1% formic acid. Pre and post run (4 minutes) equilibration of the system was performed with a ratio of mobile phase A: mobile phase B (10:90 v/v). The following table gives the change in gradient over time:

**Table 3.7** The retention times and ions of the different compounds detected by the MS.

| Sequential times | Mobile Phase A | Mobile Phase B |
|------------------|----------------|----------------|
| Start            | 10%            | 90%            |
| 1– 15 minutes    | 10 to 30%      | 90 to 70%      |
| 15 – 17 minutes  | 30 to 90%      | 70 to 10%      |

|                            |             |           |
|----------------------------|-------------|-----------|
| 17 – 18 minutes (constant) | 90%         | 10%       |
| 18 – 19 minutes            | 90 to 100%  | 10 to 0%  |
| 19 – 21 minutes            | 100%        | 0%        |
| 22 – 24 minutes            | 100% to 10% | 0% to 90% |

A 6460 series tandem mass spectrometer (Agilent) equipped with a Jetstream electrospray source was set to multiple reaction monitoring (MRM) and used to detect 3-NT in extracted samples in positive ESI mode at a retention time of ~10.44 minutes. The transition which was monitored for derivatised 3-NT in the MRM mode, was 283.13 to 181 *m/z*.

### 3.2.10 LC-MS/MS QUANTIFICATION OF 8-OHDG IN URINE

#### 3.2.10.1 Principle of method

As mentioned previously, 8-OHdG is the result of oxidative damage to DNA when OH<sup>-</sup> bind to guanine bases, triggering DNA repair and the excision of damaged 8-OHdG bases. Since a correlation between increased ROS and 8-OHdG metabolites excreted in urine exist (Honda et al. 2000), 8-OHdG in urine can be quantified as an indication of ROS-induced DNA damage.

LC-MS/MS was the platform used to quantify 8-OHdG in participants, measuring the metabolite abundance against 2-acetamidophenol as an internal standard. Liquid chromatography (LC) was performed for chromatographic separation of compounds as well as mobile phase delivery. Mass spectrometry was used as detector and performed as by conducting a precursor and product ion scan for both 8-OHdG and 2acetamidophenol with positive ionisation. Data quantification was performed with MassHunter software (Steenkamp, 2010, Weimann *et al.*, 2001).

#### 3.2.10.2 Method

##### (i) Extraction

Urine samples were defrosted overnight at ~5°C. 3mL of each sample was aliquoted and 200 µL of an ammonium formate buffer (pH 3.75) (Sigma Aldrich) and 50 µL of the internal standard (acetamidophenol) (Sigma) in water (Millipore) were added to each aliquot. Varian

STRATA-X C18 solid phase columns (200mg/6mL) (Separations) were put on a Visiprep™ vacuum manifold (Supelco) and conditioned with 6 mL methanol (Merck) and 6 mL water, allowing solutions to flow through without positive pressure. The aliquoted samples (3mL urine, 200µL ammonium formate, and 50µL internal standard) were loaded on the SPE columns. The columns were again washed with 2 mL water. The 8-OHdG metabolites were eluted from the SPE columns with 6mL of 40% methanol into medispo tubes (Plastpro Scientific). The samples were dried under a stream of nitrogen (Afrox) for approximately 40 min at 37°C on a heating block with evaporator needles (Stargate Scientific). The samples were then frozen for approximately two hours and then freeze-dried (United Scientific) overnight. Dried samples were reconstituted in 100µL of a 1mM ammonium formate buffer (pH 3.75).

(ii) *LC-MS/MS analysis*

Chromatography was performed on a Zorbax SB-Aq (2.1mm x 150mm, 3.5 µM) (Agilent) with a Zorbax SB-Aq guard column (2.1mm x 12.5mm, 5µm) (Agilent). Chromatographic separation was achieved through a mobile phase gradient (see table 3.8), mobile phase A was a 1mM ammonium formate buffer (pH 3.75) and mobile phase B was 100% acetonitrile (Agilent) with a flow rate of 200µL/min and an injection volume of 20µL.

**Table 3.8** The retention times and ions of the different compounds detected by the MS.

| Sequential times | Mobile Phase A | Mobile Phase B |
|------------------|----------------|----------------|
| Start (0)        | 95%            | 5%             |
| 2 minutes        | 95%            | 5%             |
| 10 minutes       | 74%            | 26%            |
| 12 minutes       | 50%            | 50%            |
| 13 minutes       | 50%            | 50%            |
| 15 minutes       | 0%             | 100%           |
| 21 minutes       | 0%             | 100%           |
| 26 minutes       | 95%            | 5%             |
| 41 minutes       | 95%            | 5%             |

MRM was performed with ESI in positive ion mode, which monitored the transition  $284.1\ m/z \rightarrow 168.0\ m/z$ . During 0 – 5 minutes and 16 – 41 minutes, the eluting compounds were directed to waste.

# 4

## RESULTS AND DISCUSSION

### 4.1 STATISTICS

Statistical analysis was performed with MetaboAnalyst Version 3.0. First, the raw data was divided in the different participant groups. The zero values or missing values were replaced with half the detection limit or lowest value of the variable. All negative values were also treated as missing values and replaced with half the detection limit.

First, univariate analysis was performed on the data set in order to explore each variable in the set separately. The Shapiro-Wilk test showed that not all variables in the data set were distributed normally. Non-normal distributed variables were log transformed before Student's t-test and fold-change were determined. Multivariate analysis was also performed to highlight the relationships between variables. For the multivariate analysis, all variables were transformed using generalized log (g-log) in order to limit the impact of higher value variables.

The overall statistics showed that only two variables had statistically significant differences ( $p\text{-value} \leq 0.001$ ) between the EE/DRSP and the control group. These two variables were ROS ( $p\text{-value} = 0.000008$ ) and Phase II sulphonation ( $p\text{-value} 0.005$ ). A principal component analysis (PCA) score plots was drawn of all the variables in the data set, but no significant separation between the two groups could be detected, as evident in figure 4.1(a). The analysis was repeated when the variables with low variance between the two groups and an effect size of less than 0.3 were taken out of account, as can be seen in figure 4.1(b). No separation was evident in this plot as well.

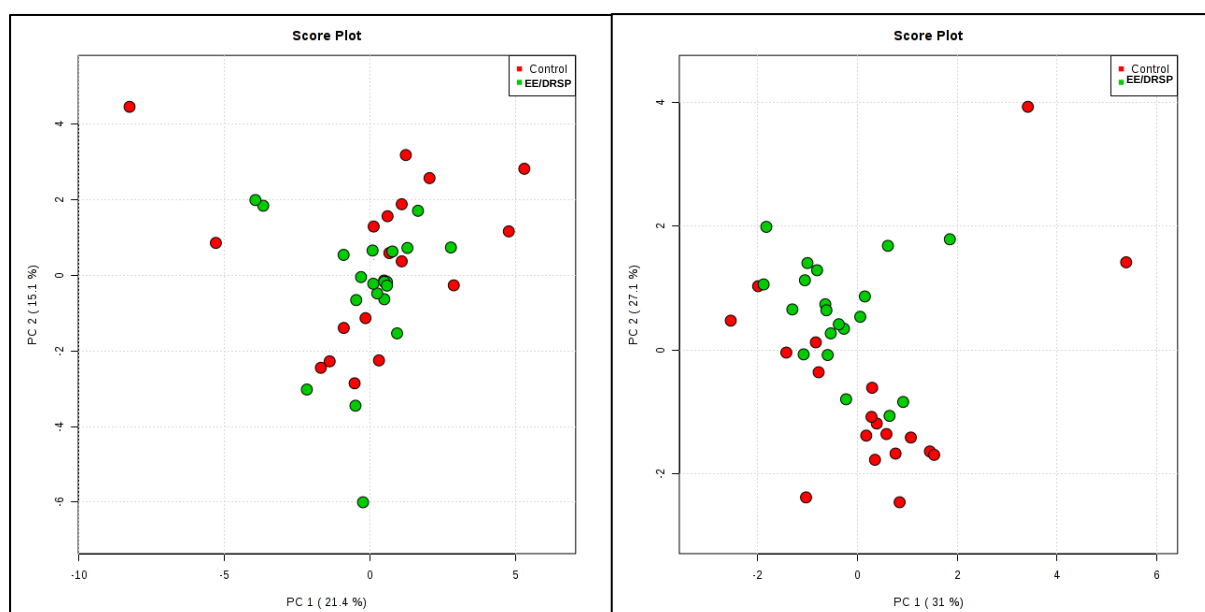


**Figure 4.1** Results of the PCA score plots of (a) all variables and (b) variables with an effect size larger than 0.3.

The next step was to draw receiver operator characteristics curves (ROC curves) of all markers on univariate and bivariate levels to show relationships between different variables. Variables with good discrimination potential – considered as an area under curve (AUC) of larger than 0.7 – are listed in table 4.1. Interesting patterns emerged such as the relationships between ROS and variables such as BMI, height, weight and age. Even though these trends are interesting and show that these physiological factors do have an effect on ROS production, the results fall outside the scope of this study. However, the ROC curves show that statistical trends do exist within the dataset, even though these trends might not be statistically significant. These trends will be discussed further with the discussion of each subsection in the rest of the chapter.

**Table 4.1** ROC curve results of all the univariate and bivariate variables having an AUC of more than 0.7

| Name       | AUC     | T-tests   | Fold Change |
|------------|---------|-----------|-------------|
| Height/ROS | 0.89211 | 9.4066E-7 | 0.76648     |
| ROS        | 0.88947 | 1.0747E-6 | -0.7632     |
| Age/ROS    | 0.88421 | 7.5873E-6 | 0.68327     |
| BMI/ROS    | 0.87632 | 1.8934E-5 | 0.65964     |
| ROS/GSHt   | 0.86579 | 1.2405E-5 | -0.85253    |

|                      |         |           |          |
|----------------------|---------|-----------|----------|
| Weight/ROS           | 0.86316 | 3.1912E-5 | 0.66621  |
| ROS/FRAP             | 0.85263 | 4.6586E-5 | -0.86747 |
| Sulph/ROS            | 0.84474 | 0.0025352 | 1.0942   |
| U to C ratio/ROS     | 0.83421 | 2.9271E-4 | 0.82353  |
| 2,5-DHBA/ROS         | 0.82368 | 0.02557   | 0.76375  |
| Gluc/ROS             | 0.80263 | 0.030495  | 0.81139  |
| ROS/3-Nitrotyrosine  | 0.8     | 0.0028317 | -0.9796  |
| Mercap/ROS           | 0.79737 | 0.027677  | 0.85793  |
| SAU/ROS              | 0.77368 | 0.030086  | 0.74698  |
| ROS/Ratio TAC to TFC | 0.76579 | 0.0092475 | -0.72917 |
| Catechol/ROS         | 0.76053 | 0.0054447 | 1.0413   |
| Sulph                | 0.75789 | 0.26448   | 0.33096  |
| 2,3-DHBA/ROS         | 0.75526 | 0.0085871 | 0.67744  |
| ROS/TBARS            | 0.74737 | 0.018046  | -1.1192  |
| Uric acid/ROS        | 0.71316 | 0.010789  | 0.81951  |

## 4.2 PHASE I: CAFFEINE CLEARANCE

Caffeine clearance was measured as an indicator of CYP1A2 activity which gives an indication of Phase I metabolism. Two saliva samples were collected for each participant over a period of 6 hours. Since caffeine is metabolised by CYP1A2 to its metabolites (paraxanthine, theobromine and theophylline), the rate of caffeine metabolism can give an indication of the activity of Phase I metabolism. A normal caffeine clearance profile will lie between 0.1 and 1.5 ml/min/kg. The following table gives the caffeine clearance of the EE/DRSP and Control groups in ml/min/kg.

**Table 4.2** Results of Phase I: Caffeine clearance.

| Phase I: Caffeine Clearance |                          |             |                          |
|-----------------------------|--------------------------|-------------|--------------------------|
| EE/DRSP                     |                          | Control     |                          |
| Participant                 | Clearance<br>(ml/min/kg) | Participant | Clearance<br>(ml/min/kg) |
| 1                           | 0,3                      | 6           | 0,7                      |
| 2                           | 0,5                      | 9           | 0,4                      |
| 5                           | 0,1                      | 10          | 0,0                      |

|                |            |                |            |
|----------------|------------|----------------|------------|
| 7              | 0,5        | 11             | 0,2        |
| 8              | 0,2        | 12             | 0,7        |
| 15             | 0,4        | 13             | 0,7        |
| 16             | 0,8        | 14             | 0,5        |
| 20             | 0,2        | 17             | 0,2        |
| 21             | 0,2        | 18             | 0,8        |
| 29             | 0,5        | 19             | 1,3        |
| 30             | 0,5        | 22             | 0,1        |
| 34             | 0,1        | 24             | 0,3        |
| 35             | 0,4        | 25             | 0,2        |
| 40             | 0,6        | 26             | 0,3        |
| 42             | 0,1        | 27             | 2,0        |
| 43             | 0,5        | 28             | 0,1        |
| 44             | 1,0        | 32             | 0,6        |
| 45             | 0,7        | 38             | 0,1        |
| 46             | 0,5        | 41             | 0,8        |
| 47             | 0,3        |                |            |
| <b>Average</b> | <b>0,4</b> | <b>Average</b> | <b>0,5</b> |
| <b>Std Dev</b> | <b>0,2</b> | <b>Std Dev</b> | <b>0,5</b> |

From table 4.2 it can be seen that only participant 27 (control) had a caffeine clearance level higher than the normal range, and only participant 10 (control) had a lower than normal caffeine clearance. All other participants fell within the normal range. When considering the averages of both the EE/DRSP and the control groups, there is little difference between these two groups. The control group had a slightly higher average than the EE/DRSP group, but does not have any statistical significance and cannot be seen as a biological trend. There is also high variation between participants within a group as is evident from the standard deviation, with the control group showing a higher deviation between subjects.

Ethinylestradiol (EE) is a known inhibitor of the cytochrome P450 enzymes. In a study by Balogh et al. (1995) it was found that COC formulations containing EE combined with either levonorgestrel or gestodene had an inhibition effect on CYP1A2 resulting in a 55% decrease in caffeine clearance. This supports the slightly higher caffeine clearance profile of the control group. However, due to intrapersonal and interpersonal variation in metabolism – evident from the high standard deviation – it is difficult to determine whether the interaction

of EE and caffeine clearance are biologically significant or just due to variation in metabolism (Zhang *et al.*, 2007).

### 4.3 PHASE II: CONJUGATION REACTIONS

Paracetamol and aspirin (acetyl salicylic acid) were used as probe substances to determine the percentage recovery of their metabolites in urine samples of participants. Paracetamol metabolism mainly results in glucuronide and sulphation conjugates as well as glutathione conjugates in small amounts. Aspirin metabolism result in the formation of salicyluric acid which is a glycine conjugate.

The results of the conjugation reactions involving paracetamol and aspirin as probe drugs are given in table 4.3 and 4.4.

In table 4.3 the percentage recovery of two of the conjugation reactions, namely glucuronidation and sulphonation, with paracetamol as probe substance are given.

#### 4.3.1 GLUCURONIDE CONJUGATION

When considering the results for Glucuronidation-conjugates, it can be seen that on average the control group showed higher recovery of glucuronide (34.4%) than the EE/DRSP group (29.0%). This could indicate that the female participants not using any form of hormonal contraceptive had a higher level of Phase II glucuronidation metabolism. The normal range of recovery for glucuronide is between 9.2 and 34.4%. Therefore, both groups fall well within the normal range with the control group high on the reference scale.

It can also be seen that the deviation between participants within a group are quite high. Participants 17 and 18 of the control group had very low glucuronide recovery, while participants 9, 22, 24, 25, 27, 28, 38, and 41 had a recovery of glucuronide of more than 34.4%. Participant 1 of the EE/DRSP group had a low glucuronide recovery, while participants 8, 15, 16, 42, 43, and 47 had a glucuronide conjugate recovery of more than 34.4%.

**Table 4.3** Results of Phase II Glucuronidation and Sulphonation reactions in the EE/DRSP and Control groups.

| Phase II : Glucuronidation |             |                |             | Phase II: Sulphonation |             |                |             |
|----------------------------|-------------|----------------|-------------|------------------------|-------------|----------------|-------------|
| EE/DRSP                    |             | Control        |             | EE/DRSP                |             | Control        |             |
| Participant                | Value       | Participant    | Value       | Participant            | Value       | Participant    | Value       |
| 1                          | 8,3         | 6              | 21,8        | 1                      | 5,0         | 6              | 17,1        |
| 2                          | 26,2        | 9              | 36,9        | 2                      | 21,4        | 9              | 15,5        |
| 5                          | 13,7        | 10             | 30,1        | 5                      | 13,3        | 10             | 19,5        |
| 7                          | 19,5        | 11             | 34,3        | 7                      | 14,7        | 11             | 21,3        |
| 8                          | 39,8        | 12             | 31,5        | 8                      | 11,5        | 12             | 28,3        |
| 15                         | 41,2        | 13             | 22,1        | 15                     | 14,8        | 13             | 26,7        |
| 16                         | 44,9        | 14             | 37,0        | 16                     | 17,5        | 14             | 25,6        |
| 20                         | 25,2        | 17             | 2,7         | 20                     | 23,5        | 17             | 1,7         |
| 21                         | 18,4        | 18             | 2,4         | 21                     | 9,7         | 18             | 2,1         |
| 29                         | 29,0        | 19             | 23,1        | 29                     | 10,3        | 19             | 22,9        |
| 30                         | 31,5        | 22             | 43,3        | 30                     | 18,9        | 22             | 15,0        |
| 34                         | 34,4        | 24             | 40,4        | 34                     | 13,8        | 24             | 16,1        |
| 35                         | 33,0        | 25             | 39,4        | 35                     | 19,3        | 25             | 17,3        |
| 40                         | 32,3        | 26             | 33,0        | 40                     | 20,6        | 26             | 31,8        |
| 42                         | 47,1        | 27             | 37,0        | 42                     | 9,4         | 27             | 20,5        |
| 43                         | 43,7        | 28             | 49,0        | 43                     | 15,8        | 28             | 20,5        |
| 44                         | 20,4        | 32             | 34,2        | 44                     | 16,6        | 32             | 28,4        |
| 45                         | 12,5        | 38             | 83,1        | 45                     | 7,5         | 38             | 28,4        |
| 46                         | 23,0        | 41             | 52,6        | 46                     | 9,3         | 41             | 32,4        |
| 47                         | 35,6        |                |             | 47                     | 17,5        |                |             |
| <b>Average</b>             | <b>29</b>   | <b>Average</b> | <b>34,4</b> | <b>Average</b>         | <b>14,5</b> | <b>Average</b> | <b>20,6</b> |
| <b>Std Dev</b>             | <b>11,3</b> | <b>Std Dev</b> | <b>17,1</b> | <b>Std Dev</b>         | <b>5,0</b>  | <b>Std Dev</b> | <b>8,3</b>  |

According to the ROC curves (AUC = 0.80263) (results in table 4.1) there is a biological trend when considering the relationship between glucuronidation and ROS production between the control and EE/DRSP groups. The control group have a better glucuronidation to ROS relationship than the EE/DRSP group.

EE is excreted as water soluble products of metabolism mainly through glucuronide and sulphate conjugate (Product A package insert, 2012). Glucuronide conjugation mainly performs an excretory role in estrogen metabolism through a group of enzymes named UDP-glucuronosyltransferase (UGT) ((Raftogianis *et al.*, 2000). Even though both groups seemingly have the necessary antioxidant potential to counteract the high levels of ROS, it is

evident that the free radicals generated in the EE/DRSP group pose a specific problem to the antioxidant capacity of these individuals (Finco *et al.*, 2011; Finco *et al.*, 2012).

#### 4.3.2 SULPHATE CONJUGATION

Post-extraction recovery of sulphate conjugates during Phase II metabolism of paracetamol showed that, on average, the control group had a higher percentage recovery (20.6%) of the sulphate conjugates than the EE/DRSP group (14.5%). This could indicate that the female participants in the control group had a higher level of Phase II Sulphonation metabolism when using a probe substance. However, when looking at the normal reference range of sulphate-conjugate recovery (a normal range is considered to be 6.4 – 20.9%), both groups fall well within the normal range. The deviation between subjects were also lower, but interpersonal variation between the participants within the two groups were still high, especially considering the low recovery of participants 17 and 18 of the control group and the high recovery of participants 11, 12, 13, 14, 19, 26, 32, 38, and 41. Only one participant (participant 1) had a lower than reference range recovery, and also only one participant (participant 20) had a higher than reference range recovery. The interpersonal differences within the EE/DRSP group are less.

Statistical analysis (see section 1) showed that the normalized differences between the control and EE/DRSP groups were statistically significant (see boxplots in figure 4.2). Therefore, the results definitely showed that sulphonation metabolism is higher in the control group than in the EE/DRSP group, which indicates that EE/DRSP users have a lower Phase II sulphonation metabolism or a depletion of systemic sulphate levels in the presence of a probe substance.

When looking at the results of the ROC curves, the AUC of sulphonation to ROS also showed a biological trend (AUC = 0.84474). This is indicative, and supportive of the statistical difference, of the fact that the sulphonation reaction as part of Phase II metabolism of the control group are better able to control the high ROS-induced state of the loading test.

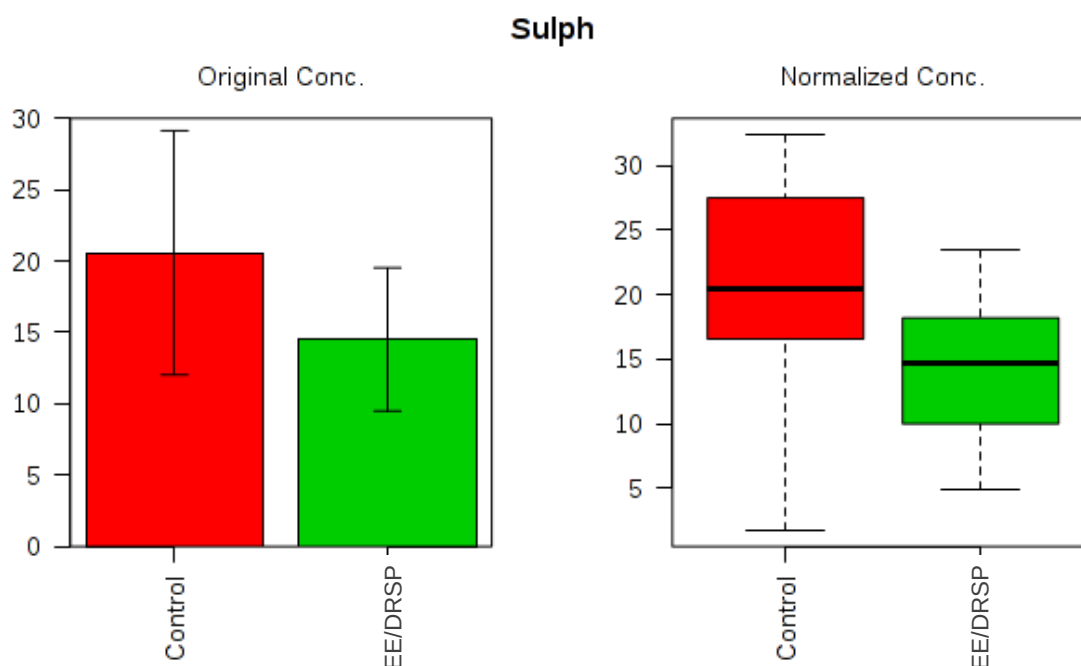


**Figure 4.2** Box-plots indicating the difference between the control and EE/DRSP group with regards to sulphonation. In the figure (a) shows the original concentration of analysed samples and (b) the normalized concentrations indicating a statistical significance between the two groups. The red box shows the results for the control group, while the green box shows for the EE/DRSP group. P-value = 0.0052109 (p-value < 0.001).

When considering the abovementioned results of sulphate conjugation, it is clear that the EE/DRSP group have a systemic depletion of sulphates. This could be ascribed to the chronic increased ROS levels in these participants, as can be seen in section 5.1 of this chapter. Chronic high levels of ROS would result in a higher level of activity of the Phase II metabolism on a daily basis. When the metabolism of these participants were challenged with the loading test which further induced high levels of ROS, the activity of sulphate conjugation simply could not control the levels of ROS. Because the control group was not subjected to chronic high levels of ROS, this depletion was not evident.

### 4.3.3 GLUTATHIONATION

During Phase II metabolism of paracetamol, a small amount of toxic metabolites bind to glutathione via the cytochrome P450 pathway to form mercapturic acid conjugates, which could be measured in participant urine samples.

When looking at table 4.4, it is evident that even though the control group had a slightly higher average percentage recovery of mercapturic acid metabolite, the two groups are very

similar, both falling within the reference range determined by the BOSS laboratory of 1.6 – 4.0% recovery. Participants 6, 11, 13, 14, 17, 18, 19, 24, and 28 of the control group had a low recovery, while participant 38 of the control group had a percentage recovery higher than the reference range. In the EE/DRSP group participants 1, 5, 7, 8, 15, 21, 29, 30, 35, 40, 44, 45, 46, and 47 had a low percentage recovery of the metabolite, while participant 20 had a high percentage recovery. The results of the ROC curves (table 4.1) showed that the relationship of glutathione conjugation of paracetamol metabolites to ROS had an AUC of 0.79737 between the two groups.

**Table 4.4** Results of Phase II Glutathione and Glycine reactions in the EE/DRSP and Control groups.

| Phase II: Glutathionation |            |                |            | Phase II: Glycination |             |                |             |
|---------------------------|------------|----------------|------------|-----------------------|-------------|----------------|-------------|
| EE/DRSP                   |            | Control        |            | EE/DRSP               |             | Control        |             |
| Participant               | Value      | Participant    | Value      | Participant           | Value       | Participant    | Value       |
| 1                         | 0,8        | 6              | 1,3        | 1                     | 2,6         | 6              | 6,1         |
| 2                         | 3,7        | 9              | 2,0        | 2                     | 9,2         | 9              | 12,5        |
| 5                         | 0,7        | 10             | 1,8        | 5                     | 4,6         | 10             | 12,9        |
| 7                         | 1,5        | 11             | 1,3        | 7                     | 11,6        | 11             | 13,3        |
| 8                         | 0,7        | 12             | 2,6        | 8                     | 7,8         | 12             | 11,2        |
| 15                        | 1,3        | 13             | 1,5        | 15                    | 14,7        | 13             | 6,9         |
| 16                        | 2,1        | 14             | 1,5        | 16                    | 15,2        | 14             | 12,3        |
| 20                        | 4,5        | 17             | 0,2        | 20                    | 16,9        | 17             | 1,5         |
| 21                        | 0,9        | 18             | 0,1        | 21                    | 7,5         | 18             | 1,7         |
| 29                        | 1,1        | 19             | 1,1        | 29                    | 10,4        | 19             | 10,8        |
| 30                        | 1,3        | 22             | 2,3        | 30                    | 12,2        | 22             | 13,9        |
| 34                        | 2,2        | 24             | 1,2        | 34                    | 14,9        | 24             | 9,1         |
| 35                        | 1,4        | 25             | 2,3        | 35                    | 13,7        | 25             | 13,4        |
| 40                        | 1,4        | 26             | 2,1        | 40                    | 11,5        | 26             | 17,7        |
| 42                        | 1,6        | 27             | 2,7        | 42                    | 14,8        | 27             | 10,6        |
| 43                        | 2,4        | 28             | 1,4        | 43                    | 14,3        | 28             | 9,1         |
| 44                        | 0,8        | 32             | 2,1        | 44                    | 12,3        | 32             | 17,7        |
| 45                        | 0,5        | 38             | 4,5        | 45                    | 4,9         | 38             | 26,3        |
| 46                        | 1,4        | 41             | 3,0        | 46                    | 7,6         | 41             | 17,7        |
| 47                        | 1,4        |                |            | 47                    | 12,4        |                |             |
| <b>Average</b>            | <b>1,6</b> | <b>Average</b> | <b>1,8</b> | <b>Average</b>        | <b>11,0</b> | <b>Average</b> | <b>11,8</b> |
| <b>Std Dev</b>            | <b>1,0</b> | <b>Std Dev</b> | <b>1,0</b> | <b>Std Dev</b>        | <b>4,0</b>  | <b>Std Dev</b> | <b>5,7</b>  |

Glutathione conjugation was lower in the EE/DRSP group as a result of the systemic depletion of glutathione which were caused by the continuous binding to hormone metabolites. Therefore, this chronic depletion of this physiological important antioxidant could lead to a secondary increase in ROS, since there is a lower availability of free glutathione to bind and neutralize ROS. Because a high recovery of the mercapturic acid conjugate is associated with an overload of the glutathione antioxidant system – an overload which could lead to possible liver damage by the highly reactive metabolite of paracetamol. Glutathione conjugation of catecholestrogens has been indicated as a major pathway for binding these reactive species that are products of estrogen metabolism (Raftogianis *et al.*, 2000). Due to systemic depletion of glutathione as a result of binding to hormone metabolites, glutathione conjugation was lower on average for the EE/DRSP group than for the control group. The control group showed higher glutathione conjugation as a result of more readily available glutathione which could decrease the induced increase in ROS. Finco *et al.* (2011) showed that the oxidative stress that arose with the use of an EE/DRSP contraceptive formulation was somehow resistant to treatment with a physiological modulator with antioxidant potential.

#### 4.3.4 GLYCINATION

During Phase II metabolism, aspirin forms conjugates with glycine. These glycine conjugates are then measured in the urine samples of participants. The reference range of glycine conjugate recovery in urine is considered by the BOSS laboratory to be 9.5 to 26.6%. Considering the results in table 4.4, it is evident that both the control and EE/DRSP groups fall well within this criteria, with very little variation on average between the two groups. Within the control group, participants 6, 13, 17, 18, 24, and 28 had a low percentage recovery, while no participants had a higher recovery than indicated by the reference range. Looking at the EE/DRSP group, participants 1, 2, 5, 8, 21, 45, and 46 had a low percentage recovery, and no participants had a higher than average recovery. The ROC analysis did not indicate any biological trends between glycation and ROS or any other variable within the dataset. No reference could be found that glycation are a significant metabolic pathway in the metabolism of endogenous and xenoestrogens, and considering the main conjugation pathways for conjugation of EE are sulphonation and glucuronidation, the results that found

no biological trends relating to the increase in ROS to the increase in glycine conjugates, are supported.

#### **4.4 PHASE I:PHASE II RATIOS**

The ratios of Phase I to Phase II metabolism are indicative of the effectiveness of the Phase I and II metabolism as a whole. It is essential that all the reactive metabolites that form during Phase I metabolism are bound and rendered harmless during Phase II, otherwise this could potentially lead to accumulation of reactive intermediates, oxidative stress and possibly oxidative damage. The effectiveness of Phase I metabolism was determined through a caffeine loading test and caffeine clearance was measured over time. Phase II conjugation metabolism was monitored with paracetamol and aspirin as probe substances to determine the percentage recovery in urine of conjugates that form during the metabolism of these two substances. The ratios are given in table 4.5.

The reference range for Phase I: Sulphation is considered by the BOSS laboratory to be 1.6 – 7.0. Both the Control and EE/DRSP group fall well within this reference range. The EE/DRSP group had showed on average a lower ratio of Phase I: Sulphation metabolism than the control group. Within the control group participants 10, 11, 22, 26, and 28 had a low ratio, while participants 17, 18, and 27 had a ratio higher than the reference range. The standard deviation between participants within the EE/DRSP group is much lower than the deviation between the participants of the control group.

The reference range for Phase I: Glycination is 1.1 – 5.5, as determined by the BOSS laboratory. The results in table 4.5 show that the EE/DRSP group had an average ratio that fell within the reference range while the control group had an average higher than the reference range. The high ratio of the control group could be the result of the very high values of participants 6, 13, 18, 19, and 27, resulting in a high standard deviation in this group. High ratios could also be seen in the EE/DRSP group with participant 1 and 45. The variation between subjects was much smaller than in the control group.

The reference range considered to be the normal range by the BOSS laboratory for Phase I: Glucuronidation is 1.1 – 4.2. Looking at the results in Table 4.5, it can be seen that both groups fall within the normal range, but the control group had an average ratio of 3.8 while the EE/DRSP group had a ratio of 1.8. Again it is evident that the ratio of the control group is affected by the high ratio of participant 18, which also affected the standard deviation.

No statistical trends or statistical significant results were reported for these ratios. However, it seems as if the deviation between participants seemed to be lower in the EE/DRSP group than the control for all three ratios measured, possible indicating that the EE/DRSP hormonal contraceptive have a uniform effect on females using this form of contraceptive, resulting in little variation between subjects when it comes to Phase I:Phase II metabolism ratios.

**Table 4.5** Ratios of Phase I metabolism to Phase II reactions (Sulphation, Glycination and Glucuronidation).

| Phase I Sulphation |       |             |       | Phase I Glycination |       |             |       | Phase I Glucuronidation |       |             |       |
|--------------------|-------|-------------|-------|---------------------|-------|-------------|-------|-------------------------|-------|-------------|-------|
| EE/DRSP            |       | Control     |       | EE/DRSP             |       | Control     |       | EE/DRSP                 |       | Control     |       |
| Participant        | Value | Participant | Value | Participant         | Value | Participant | Value | Participant             | Value | Participant | Value |
| 1                  | 6,0   | 6           | 4,1   | 1                   | 11,4  | 6           | 11,5  | 1                       | 3,6   | 6           | 3,2   |
| 2                  | 2,5   | 9           | 2,4   | 2                   | 5,8   | 9           | 2,9   | 2                       | 2,0   | 9           | 1,0   |
| 5                  | 0,4   | 10          | 0,1   | 5                   | 1,1   | 10          | 0,1   | 5                       | 0,4   | 10          | 0,0   |
| 7                  | 3,3   | 11          | 0,9   | 7                   | 4,2   | 11          | 1,5   | 7                       | 2,5   | 11          | 0,6   |
| 8                  | 1,7   | 12          | 2,4   | 8                   | 2,5   | 12          | 6,0   | 8                       | 0,5   | 12          | 2,1   |
| 15                 | 2,7   | 13          | 2,6   | 15                  | 2,7   | 13          | 10,1  | 15                      | 1,0   | 13          | 3,2   |
| 16                 | 4,7   | 14          | 1,8   | 16                  | 5,4   | 14          | 3,8   | 16                      | 1,8   | 14          | 1,3   |
| 20                 | 0,9   | 17          | 9,4   | 20                  | 1,2   | 17          | 11,1  | 20                      | 0,8   | 17          | 6,0   |
| 21                 | 2,1   | 18          | 37,8  | 21                  | 2,8   | 18          | 46,2  | 21                      | 1,1   | 18          | 32,7  |
| 29                 | 4,5   | 19          | 5,7   | 29                  | 4,5   | 19          | 12,1  | 29                      | 1,6   | 19          | 5,7   |
| 30                 | 2,4   | 22          | 0,4   | 30                  | 3,8   | 22          | 0,5   | 30                      | 1,5   | 22          | 0,2   |
| 34                 | 1,0   | 24          | 1,7   | 34                  | 0,9   | 24          | 3,1   | 34                      | 0,4   | 24          | 0,7   |
| 35                 | 2,1   | 25          | 1,3   | 35                  | 2,9   | 25          | 1,7   | 35                      | 1,2   | 25          | 0,6   |
| 40                 | 2,9   | 26          | 0,8   | 40                  | 5,2   | 26          | 1,5   | 40                      | 1,8   | 26          | 0,8   |
| 42                 | 0,6   | 27          | 9,5   | 42                  | 0,4   | 27          | 18,4  | 42                      | 0,1   | 27          | 5,3   |
| 43                 | 3,3   | 28          | 0,5   | 43                  | 3,7   | 28          | 1,1   | 43                      | 1,2   | 28          | 0,2   |
| 44                 | 6,0   | 32          | 2,0   | 44                  | 8,1   | 32          | 3,2   | 44                      | 4,9   | 32          | 1,7   |
| 45                 | 9,8   | 38          | 0,3   | 45                  | 15,0  | 38          | 0,3   | 45                      | 5,9   | 38          | 0,1   |
| 46                 | 4,9   | 41          | 2,4   | 46                  | 5,9   | 41          | 4,4   | 46                      | 2,0   | 41          | 1,5   |
| 47                 | 1,6   |             |       | 47                  | 2,3   |             |       | 47                      | 0,8   |             |       |
| Average            | 3,2   | Average     | 4,5   | Average             | 4,5   | Average     | 7,3   | Average                 | 1,8   | Average     | 3,5   |
| Std Dev            | 2,3   | Std Dev     | 8,3   | Std Dev             | 3,6   | Std Dev     | 10,4  | Std Dev                 | 1,5   | Std Dev     | 7,1   |

It is notable that for each of the measured ratios, the EE/DRSP group had lower averages than the control group. This is because the percentage recoveries for the respective conjugates measured are consistently lower in the EE/DRSP than the control group, resulting in a smaller ratio. The assumption could be made that the Phase II metabolism of the control group are better able to deal with the metabolites generated through Phase I metabolism.

#### 4.5 OXIDATIVE STRESS AND ANTIOXIDANT POTENTIAL

Oxidative stress are determined by the amount of ROS, catechol, 2,3-DHBA, and 2,5-DHBA present in the blood or urine of a participant.

**Table 4.6** Results of spectrophotometric determination of ROS and the organic acid extraction of catechols.

| ROS         |       |             |       | Catechol    |       |             |       |
|-------------|-------|-------------|-------|-------------|-------|-------------|-------|
| EE/DRSP     |       | Control     |       | EE/DRSP     |       | Control     |       |
| Participant | Value | Participant | Value | Participant | Value | Participant | Value |
| 1           | 142,1 | 6           | 104,8 | 1           | 2,5   | 6           | 7,5   |
| 2           | 170,2 | 9           | 79,6  | 2           | 6,4   | 9           | 11,7  |
| 5           | 181,2 | 10          | 76,1  | 5           | 8,4   | 10          | 15,7  |
| 7           | 133,0 | 11          | 78,1  | 7           | 1,3   | 11          | 4,4   |
| 8           | 138,5 | 12          | 106,5 | 8           | 25,6  | 12          | 2,9   |
| 15          | 259,5 | 13          | 126,6 | 15          | 3,9   | 13          | 17,2  |
| 16          | 145,2 | 14          | 74,4  | 16          | 12,9  | 14          | 9,7   |
| 20          | 82,6  | 17          | 172,3 | 20          | 4,1   | 17          | 6,6   |
| 21          | 126,3 | 18          | 58,6  | 21          | 6,2   | 18          | 5,9   |
| 29          | 132,4 | 19          | 95,9  | 29          | 21,3  | 19          | 12,2  |
| 30          | 107,8 | 22          | 149,7 | 30          | 23,8  | 22          | 7,9   |
| 34          | 131,1 | 24          | 79,6  | 34          | 3,9   | 24          | 15,3  |
| 35          | 145,3 | 25          | 74,4  | 35          | 7,2   | 25          | 7,0   |
| 40          | 132,7 | 26          | 61,6  | 40          | 5,1   | 26          | 7,7   |
| 42          | 158,1 | 27          | 71,2  | 42          | 1,9   | 27          | 17,3  |
| 43          | 168,2 | 28          | 58,8  | 43          | 3,9   | 28          | 4,1   |
| 44          | 164,6 | 32          | 82,3  | 44          | 13,8  | 32          | 7,0   |
| 45          | 171,5 | 38          | 87,5  | 45          | 9,0   | 38          | 5,1   |
| 46          | 210,9 | 41          | 139,6 | 46          | 9,0   | 41          | 4,1   |

|         |       |         |      |         |     |         |     |
|---------|-------|---------|------|---------|-----|---------|-----|
| 47      | 213,3 |         |      | 47      | 4,6 |         |     |
| Average | 155,7 | Average | 93,6 | Average | 8,7 | Average | 8,9 |
| Std Dev | 39,7  | Std Dev | 31,3 | Std Dev | 7,2 | Std Dev | 4,5 |

#### 4.5.1 REACTIVE OXYGEN SPECIES

ROS was spectrophotometrically determined by the conversion of DEPPD to its cation in the presence of alkoxyl and peroxy radicals in the blood sample. When looking at table 4.6 it is evident that on average the EE/DRSP had a much higher level of ROS than the control group. The reference range of ROS in blood samples is 52.4 – 107.3 units. Considering this, it is evident that the ROS levels of the control group fall within these criteria (93.6 units), while the ROS levels of the EE/DRSP group (155.7 units) are much higher than the reference range criteria. Within the EE/DRSP group only participant 20 had a ROS level that fell within the reference range, the rest of the participants had measured levels of more than the reference range. The highest measured ROS levels was for participant 15 (259.5 units), an amount more than twice that of the highest value within the reference range. In the control group participants 13, 17, 22, and 41 had higher than reference range values, while the rest of the participants in the control group fell well within the reference range.

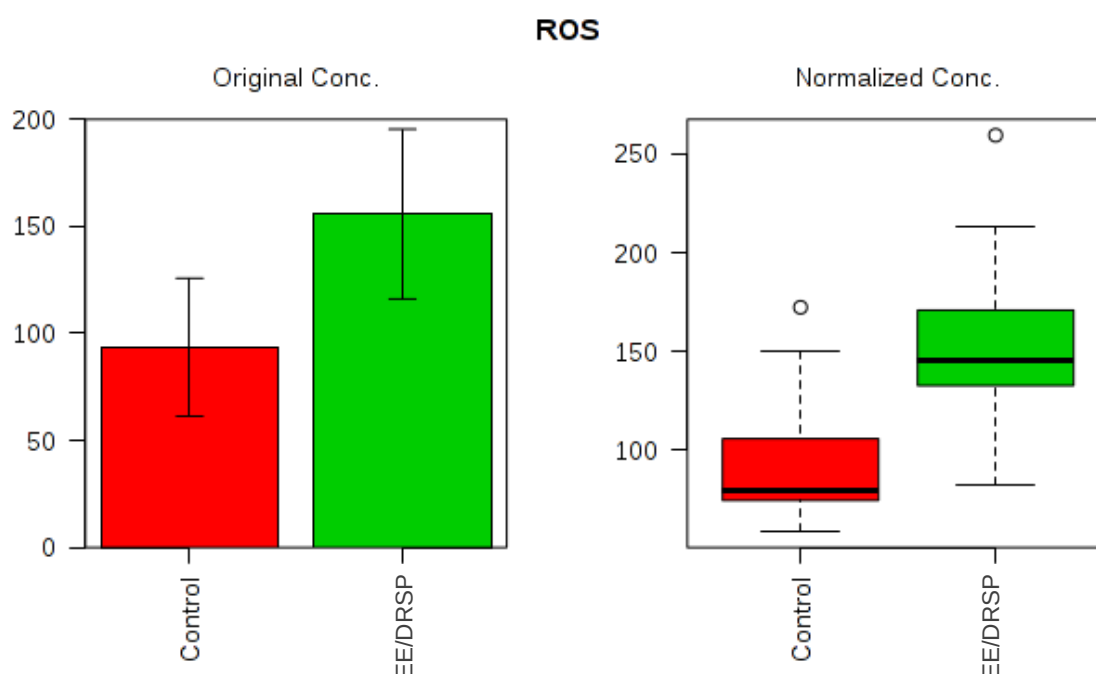


**Figure 4.3** Box-plots indicating the difference between the control and EE/DRSP group with regards to ROS production. In the figure (a) shows the original measured ROS units of analysed samples and (b) the normalized measured ROS units indicating a statistical

significance between the two groups. The red box shows the results for the control group, while the green box shows for the EE/DRSP group. P-value = 0.000008 (p-value < 0.001)

Statistical analysis (see section 1 of this chapter) showed that the normalised values for ROS between the two groups were statistically significant (see the boxplots in figure 4.3, p-value = 0.000008). The results therefore indicate that there is a definite difference in the amount of ROS produced by the participants within the two groups with the EE/DRSP group having much higher levels of ROS, which are possibly produced during the metabolism of the EE/DRSP hormonal contraceptive formulation.

When looking at the ROC analysis (see figure 4.5), the AUC showed a biological trend in the amount of ROS produced (AUC = 0.88974) in the two separate groups with a definite indication that the ROS in the EE/DRSP group are much higher than in the control group. This biological trend further supports the statistical difference between the two groups.

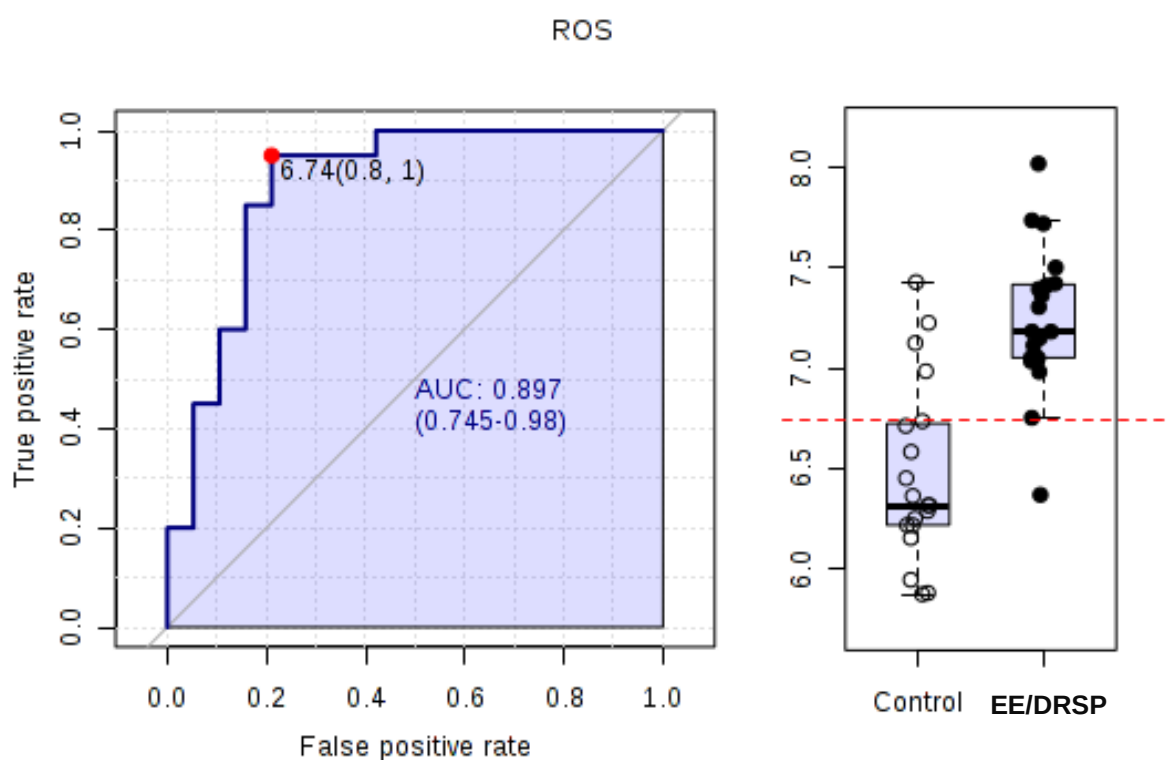


**Figure 4.4** ROC curve indicating an AUC of 0.8897, which indicates a definite biological trend between the levels of ROS in participants in the control group and participants in the EE/DRSP group. The box plots to the right indicate a good separation between the results of the two groups.

Oxidative stress, as evident from high ROS levels, is caused by COCs such as the EE/DRSP formulation, even though the mechanisms behind this drastic increase is not known and controversial (Finco *et al.*, 2012; Pincemail *et al.*, 2007). Evidence exist that increased ROS are the result of redox cycling between the *o*-quinones and semi-quinones, metabolites in the metabolism of estrogen (see figure 2.7), generating ROS such as superoxide, hydrogen peroxide and hydroxyl radicals responsible for DNA damage in the process (Siddique *et al.*, 2005). When considering the increase in both 2,3-DHBA and 2,5-DHBA (see section 5.2) hydroxyl radicals seem to form predominantly, indicating that the EE/DRSP group are more susceptible to oxidative stress. CYP450 reductase converts quinones back to semiquinones during estrogen metabolism and in the presence of oxygen, hydrogen peroxide and hydroxyl radicals are formed (Rogan, 2007).

#### 4.5.2 2,3-DHBA AND 2,5-DHBA

Aspirin, as a probe substance in this instance, can bind to hydroxyl radicals during high (induced) oxidative conditions. Therefore a loading test with aspirin is used to determine the presence of hydroxyl radicals such as catechol (see results in table 4.6) and 2,3-DHBA and 2,5-DHBA (results in table 4.7).

When looking at the results for catechols in table 4.6, we can see there is a little difference on average between the two groups. The reference range concentration for catechol formation is considered to be 3.4 – 69.8µM by the BOSS laboratory. Both groups fall within this reference range. No participants from either group had a catechol concentration higher than that of the reference range. However, participants 1, 7, and 42 of the EE/DRSP group had lower concentrations than the reference range and participant 12 of the control group had a lower concentration than the reference range.

The ROC analysis (table 4.1) showed a biological trend (AUC = 0.76053) between the ratio of catechol to ROS production for the two groups. This indicates that catechol production are relevant to the amount of ROS produced, with the EE/DRSP group showing a higher catechol to ROS ratio than the control group.

As mentioned earlier, 2,3-DHBA and 2,5-DHBA are organic compounds which form as a result of hydroxylation of aspirin (2,3 –DHBA from spontaneous chemical interaction and 2,5 –DHBA as a result of enzymatic hydroxylation). From table 4.7 it can be seen that, on average, the EE/DRSP group had a higher concentration of 2,3-DHBA than the control

group. However, the concentration reference range for the production of 2,3-DHBA is considered by the BOSS laboratory to be 0.2 – 2.5µM, resulting in both groups falling outside the concentration criteria for the reference range. This may be attributed to the fact that the 2,3 –DHBA range was determined initially for both males and females, while the range for females alone may be slightly different because of hormonal induced free radical formation.

**Table 4.7** Results of the organic extraction of 2,3-DHBA and 2,5-DHBA.

| 2,3-DHBA       |            |                |            | 2,5-DHBA       |             |                |             |
|----------------|------------|----------------|------------|----------------|-------------|----------------|-------------|
| EE/DRSP        |            | Control        |            | EE/DRSP        |             | Control        |             |
| Participant    | Value      | Participant    | Value      | Participant    | Value       | Participant    | Value       |
| 1              | 3,5        | 6              | 5,7        | 1              | 24,1        | 6              | 141,4       |
| 2              | 4,8        | 9              | 1,2        | 2              | 47,0        | 9              | 43,8        |
| 5              | 3,9        | 10             | 2,9        | 5              | 48,5        | 10             | 51,9        |
| 7              | 0,7        | 11             | 2,2        | 7              | 13,1        | 11             | 40,7        |
| 8              | 3,3        | 12             | 2,4        | 8              | 40,6        | 12             | 53,9        |
| 15             | 2,9        | 13             | 4,1        | 15             | 80,4        | 13             | 42,0        |
| 16             | 2,5        | 14             | 2,9        | 16             | 31,8        | 14             | 48,0        |
| 20             | 1,7        | 17             | 5,7        | 20             | 24,9        | 17             | 62,8        |
| 21             | 3,4        | 18             | 0,7        | 21             | 43,0        | 18             | 4,5         |
| 29             | 2,9        | 19             | 2,9        | 29             | 57,1        | 19             | 37,7        |
| 30             | 3,7        | 22             | 2,9        | 30             | 56,7        | 22             | 59,0        |
| 34             | 3,0        | 24             | 4,1        | 34             | 53,9        | 24             | 56,6        |
| 35             | 3,3        | 25             | 1,9        | 35             | 46,4        | 25             | 45,8        |
| 40             | 3,3        | 26             | 2,7        | 40             | 45,7        | 26             | 43,4        |
| 42             | 2,0        | 27             | 3,1        | 42             | 63,1        | 27             | 49,7        |
| 43             | 2,3        | 28             | 2,4        | 43             | 46,7        | 28             | 44,0        |
| 44             | 4,0        | 32             | 1,3        | 44             | 34,7        | 32             | 35,1        |
| 45             | 0,5        | 38             | 2,7        | 45             | 5,5         | 38             | 45,8        |
| 46             | 4,7        | 41             | 1,9        | 46             | 644,5*      | 41             | 44,5        |
| 47             | 4,4        |                |            | 47             | 62,9        |                |             |
| <b>Average</b> | <b>3,0</b> | <b>Average</b> | <b>2,8</b> | <b>Average</b> | <b>41.1</b> | <b>Average</b> | <b>50,0</b> |
| <b>Std Dev</b> | <b>1,2</b> | <b>Std Dev</b> | <b>1,3</b> | <b>Std Dev</b> | <b>20.2</b> | <b>Std Dev</b> | <b>24,5</b> |

\* This value is an outlier and the average and standard deviation were calculated without taking this value into account.

The results in table 4.1 for the ROC analysis indicated a biological trend in the concentration of 2,3-DHBA to ROS production (AUC = 0.75526). These results support the

abovementioned indication that aspirin bind to hydroxyl radicals resulting in the urinary excretion of organic compounds such as 2,3-DHBA under high ROS conditions.

The same statistical trend was evident for 2,5-DHBA. On average, the EE/DRSP group had lower concentrations of 2,5-DHBA in their urine than the control group. The ROC curves indicated a biological trend in the amount of 2,5-DHBA excreted to the amount of ROS production (AUC = 0.82368). This further underlines the apparent increased binding of aspirin to hydroxyl radicals during its metabolism in high ROS conditions. However, 2,3-DHBA are considered to be a better indicator of free radical reactions than 2,5-DHBA.

When the results of the hydroxylation of aspirin is considered, it can be seen that when salicylic acid undergoes Phase II metabolism, the formed metabolites bind to hydroxyl radicals to form primarily 2,3-DHBA and to a lesser extent 2,5-DHBA. According to Dupont et al. (1999) phase II metabolism sees that most of the salicylic acid metabolites conjugate with either glycine or glucuronide, but about 60% of metabolites bind to hydroxyl radicals forming either 2,3-DHBA or 2,5-DHBA (Dupont et al. 1999). 2,5-DHBA can also form through the CYP450 pathways, therefore 2,3-DHBA are a good marker for oxidative stress *in vivo* (Coudray and Favier, 2000). Since glycation is one of the conjugation pathways of salicylic acid, and considering the results in section 3.4 of this chapter and the biological trends found between both 2,3-DHBA and 2,5-DHBA and the increase in ROS within the EE/DRSP group, the conclusion can be made that due to the induced oxidative stress conditions, more hydroxyl radicals were present to bind to the salicylic acid metabolites, indicating oxidative stress (Coudray and Favier, 2000).

#### 4.5.3 ANTIOXIDANT POTENTIAL

The potential of a participant biological fluid (in this case blood) to neutralize and eliminate ROS and other oxidants is determined in this study by two factors, namely the antioxidant potential of the body and the total glutathione present in the body.

The FRAP assay determines the antioxidant potential in a participant's blood, by reducing  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  at a low pH. The results in table 4.8 showed that on average, both the EE/DRSP group had a lower concentration of antioxidants in the blood than the control group. The reference range for FRAP was determined by the BOSS laboratory to be 355.4 – 448.0  $\mu\text{M}$ . In comparison with the reference range, it is evident that both groups fall within the reference range, but the EE/DRSP group are at the lowest end of the range. Within the EE/DRSP

group it can be seen that one participant (47) had an extremely low FRAP value, and participants 8, 20, 29, 30, and 40 had an antioxidant potential less than the reference range. Participants 1, 43, and 45 of the EE/DRSP group had an antioxidant potential higher than that of the reference range. Within the control group participants 12, 17, 18, 19, 22, 24, 26, and 27 had antioxidant potential values less than the reference range, while participants 6, 28, and 38 had reference values higher than that of the reference range. Although not statistically significant, it seems that the EE/DRSP group displayed a trend towards lower FRAP values in general.

**Table 4.8** Results for antioxidant potential and total glutathione present in blood samples of participants.

| FRAP        |       |             |       | GSHt        |        |             |        |
|-------------|-------|-------------|-------|-------------|--------|-------------|--------|
| EE/DRSP     |       | Control     |       | EE/DRSP     |        | Control     |        |
| Participant | Value | Participant | Value | Participant | Value  | Participant | Value  |
| 1           | 517,1 | 6           | 517,1 | 1           | 944,0  | 6           | 979,3  |
| 2           | 447,1 | 9           | 447,1 | 2           | 865,3  | 9           | 839,4  |
| 5           | 388,9 | 10          | 388,9 | 5           | 745,0  | 10          | 858,4  |
| 7           | 410,3 | 11          | 410,3 | 7           | 940,2  | 11          | 1187,7 |
| 8           | 268,6 | 12          | 268,6 | 8           | 878,4  | 12          | 847,3  |
| 15          | 367,9 | 13          | 367,9 | 15          | 965,7  | 13          | 632,7  |
| 16          | 373,1 | 14          | 373,1 | 16          | 966,2  | 14          | 943,5  |
| 20          | 275,1 | 17          | 275,1 | 20          | 1128,9 | 17          | 1086,6 |
| 21          | 411,7 | 18          | 411,7 | 21          | 742,6  | 18          | 1233,0 |
| 29          | 328,0 | 19          | 328,0 | 29          | 732,4  | 19          | 956,5  |
| 30          | 257,5 | 22          | 257,5 | 30          | 833,1  | 22          | 752,5  |
| 34          | 308,0 | 24          | 308,0 | 34          | 1002,0 | 24          | 755,8  |
| 35          | 393,1 | 25          | 393,1 | 35          | 650,7  | 25          | 1155,9 |
| 40          | 244,2 | 26          | 244,2 | 40          | 1119,2 | 26          | 1190,6 |
| 42          | 309,1 | 27          | 309,1 | 42          | 882,8  | 27          | 940,7  |
| 43          | 477,6 | 28          | 477,6 | 43          | 874,1  | 28          | 878,2  |
| 44          | 432,7 | 32          | 432,7 | 44          | 788,9  | 32          | 859,1  |
| 45          | 537,3 | 38          | 537,3 | 45          | 907,2  | 38          | 752,7  |
| 46          | 362,1 | 41          | 362,1 | 46          | 824,4  | 41          | 1011,1 |
| 47          | 4,4   |             |       | 47          | 772,9  |             |        |
| Average     | 355,7 | Average     | 374,2 | Average     | 878,2  | Average     | 940,0  |

|         |       |         |      |         |       |         |       |
|---------|-------|---------|------|---------|-------|---------|-------|
| Std Dev | 117,4 | Std Dev | 83,4 | Std Dev | 124,4 | Std Dev | 165,3 |
|---------|-------|---------|------|---------|-------|---------|-------|

The statistical results in table 4.1 showed that there was a trend between the ratio of ROS to FRAP between the two groups (AUC = 0.85263). Due to the chronic high ROS conditions found in the EE/DRSP group, this group are subjected to systemic depletion of antioxidants and therefore antioxidant potential. Therefore, the average antioxidant potential in the EE/DRSP group is lower than in the control group.

This is consistent with the results found by Fince *et al.* (2011) and Finco *et al.* (2012). Both these studies focused on the oxidative stress that are caused by the use of COCs and the fact that the users thereof seemingly possess the necessary antioxidant potential to bind and clear ROS. Also, these studies found that the administration of commonly used antioxidants such as catechins found in green tea did not have any significant effect on the reduction of ROS in these cases. A very specific physiological modulator containing catechins, polyphenols, Q10, and lipoic acid was administered and the ROS was lowered successfully.

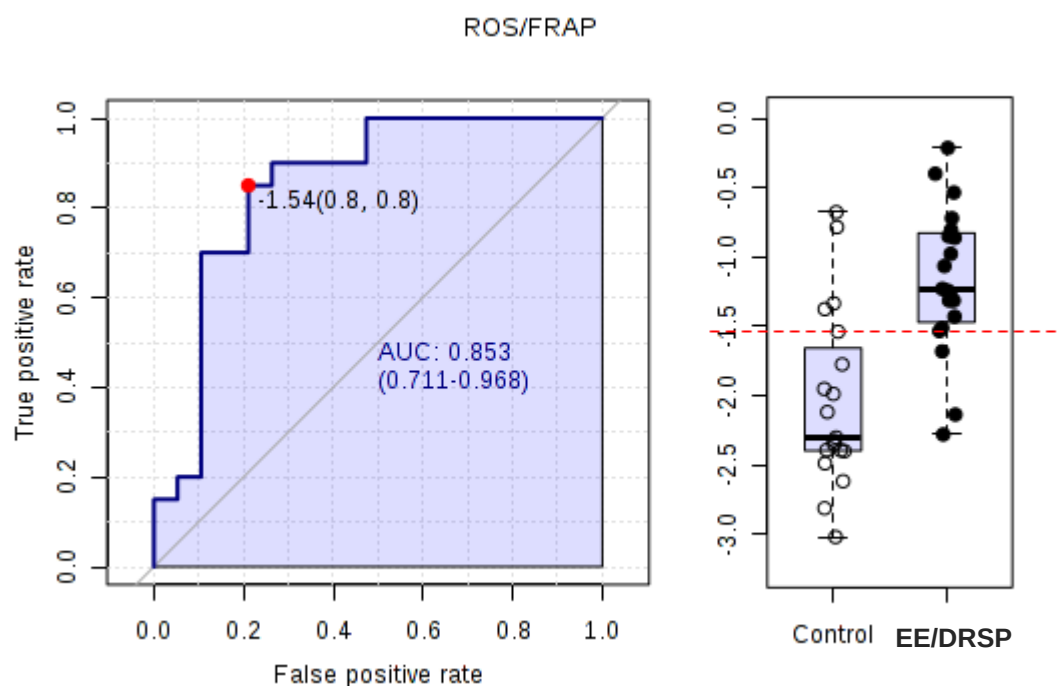


**Figure 4.5** ROC curve indicating an AUC of 0.85263, which indicates a definite biological trend between the levels of ROS to FRAP in participants within the control group and participants in the EE/DRSP group. The box plots to the right indicate a good separation between the results of the two groups.

#### 4.5.4 TOTAL GLUTATHIONE

Total glutathione was measured by converting oxidised glutathione to reduced glutathione to spectrophotometrically determine the amount of total glutathione in blood samples. The reference range for total glutathione (GSHt) according to the BOSS laboratory is 875 – 1026  $\mu\text{M}$  in blood samples. When looking at table 4.8 it can be seen that, on average, the EE/DRSP group had a lower total glutathione concentration than the control group. When comparing this to the reference range, it can be seen that even though both groups fall within the accepted reference range, the EE/DRSP group fall at the lower end of the range. Participants 2, 21, 29, 30, 35, 43, and 46 had GSHt concentrations lower than the reference range, while participant 20 and 34 had GSHt concentrations higher than the reference range. Within the control group, participants 9, 10, 12, 22, 24, 32, and 38 had GSHt concentrations lower than the reference range, while participants 11, 17, 18, 25, 26, and 41 had GSHt higher than the reference range. The statistical results in table 4.1 showed that there was a trend between ROS to GSHt between the two groups ( $\text{AUC} = 0.86579$ ). The biological trend (see figure 4.6) indicated that the control group had lower concentrations of ROS to GSHt, whereas this was not the case for the EE/DRSP group. Chronic conjugation of hormone metabolites in the EE/DRSP group and chronic high ROS levels resulted in the systemic depletion of glutathione which in turn result in decreased glutathione available to neutralize induced high levels of ROS. Therefore, the control group, who had high concentrations of total glutathione readily available, could bind and neutralize the induced levels of ROS.

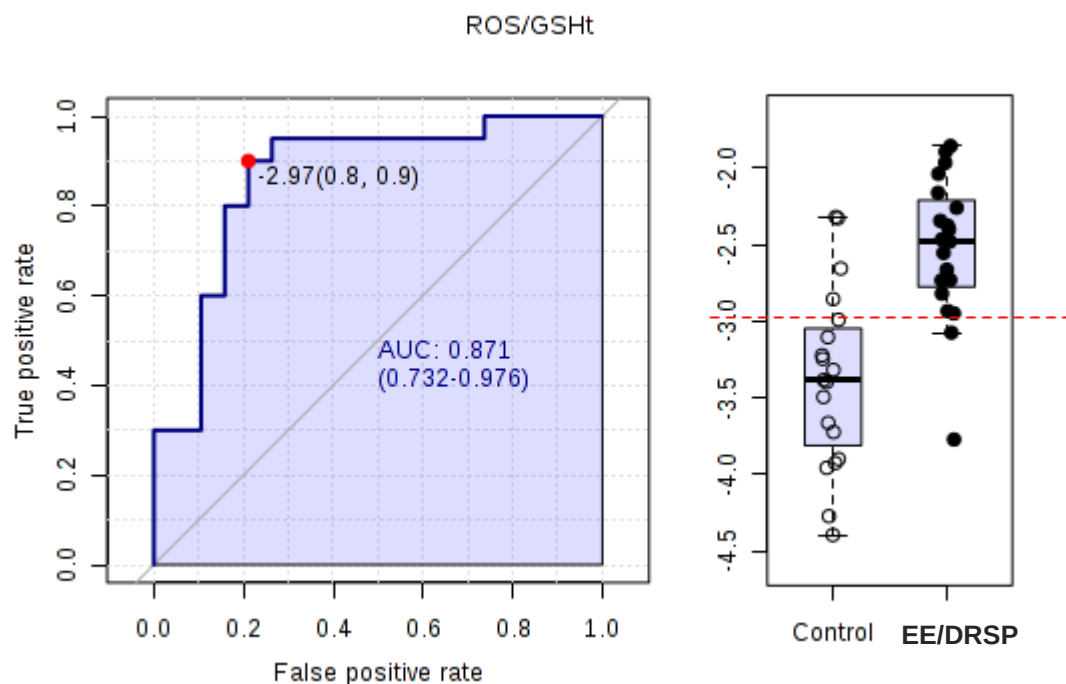


**Figure 4.6** ROC curve indicating an AUC of 0.86579, which indicates a definite biological trend between the levels of ROS to GSHt concentrations in participants within the control group and participants in the EE/DRSP group. The box plots to the right indicate a good separation between the results of the two groups.

Glutathione is also responsible for binding quinone-products, as well as catecholestrogens, formed during the oxidative metabolism of estrogen (Lakhani *et al.*, 2003; Raftogianis *et al.*, 2000). Glutathione binds to these reactive products in the presence of the enzyme Glutathione S-Transferase (GST) (see chapter 2). Quinones are formed as products of oxidative estrogen metabolism from 2-OH or 4-OH estrogen. This conversion, especially the reactions involving the 4-OH estrogen, to quinones have the ability to inhibit the reactivity of GST (Chang *et al.*, 1998).

#### 4.6 CARNITINE PROFILES

Carnitine plays an imported role in the binding and excretion of accumulated fatty acids and xenobiotic carboxylic compounds during conditions of high oxidative stress. In table 4.9 the results for the carnitine analysis are given. Butylated urinary carnitines were quantified in urine samples with tandem mass spectrometry, using a precursor ion of 85 *m/z*.

On average, the EE/DRSP group had slightly lower concentrations of free carnitine (TFC) than the control group, but both groups fell well within the BOSS laboratory determined reference range of 0.6 – 5.1 mmol/mol creatinine.

Looking at the results for total acyl-carnitines (TAC), it can be seen that the EE/DRSP group had an average concentration slightly higher than the control group, again both groups fell well within the BOSS laboratory determined reference range of 0.8 – 9.8 mmol/mol creatinine.

The average ratio of TAC to TFC showed that the EE/DRSP group had a slightly bigger ratio than the control group. The reference range for the TAC:TFC ratio is considered to be <1.9. Both groups therefore fell within the reference range criteria. Within the EE/DRSP group, participants 15, 20, 21, 40, 43, and 47 had TAC:TFC ratios higher than the reference range, and in the control group, participants 6, 13, 32, and 41 had higher ratios than the reference range. When levels of free carnitine decline (e.g. depletion due to binding with xenobiotic carboxylic acids) and the levels of TAC incline, it could indicate that more acylgroups bind to free carnitine as a result of higher circulating triacylglycerols associated with hormone therapy in women. When considering these results, it can be seen that participants in both groups did not show oxidative stress as a result of carnitine activity. The statistical analysis (table 4.1) showed that there was a biological trend (AUC = 0.76579) in the ratio of ROS:TAC to TFC. This is indicative that the TAC:TFC ratio increases when there is an increase in ROS production.

**Table 4.9** Results for total free carnitine, total acyl-carnitines and ratio of acylcarnitines to free carnitine.

| Total-Free C    |       |                 |       | Total Acyl-C    |       |                 |       | Ratio TAC to TFC |       |                 |       |
|-----------------|-------|-----------------|-------|-----------------|-------|-----------------|-------|------------------|-------|-----------------|-------|
| EE/DRSP         |       | Control         |       | EE/DRSP         |       | Control         |       | EE/DRSP          |       | Control         |       |
| Partici<br>pant | Value | Partici<br>pant | Value | Partici<br>pant | Value | Partici<br>pant | Value | Partici<br>pant  | Value | Partici<br>pant | Value |
| 1               | 2,9   | 6               | 3,3   | 1               | 3,0   | 6               | 12,3  | 1                | 1,0   | 6               | 3,8   |
| 2               | 6,3   | 9               | 1,6   | 2               | 6,9   | 9               | 1,8   | 2                | 1,1   | 9               | 1,2   |
| 5               | 12,8  | 10              | 14,7  | 5               | 8,6   | 10              | 13,3  | 5                | 0,7   | 10              | 0,9   |
| 7               | 0,9   | 11              | 3,4   | 7               | 0,9   | 11              | 5,1   | 7                | 0,9   | 11              | 1,5   |
| 8               | 0,7   | 12              | 0,8   | 8               | 1,2   | 12              | 1,1   | 8                | 1,7   | 12              | 1,3   |
| 15              | 1,4   | 13              | 1,3   | 15              | 4,3   | 13              | 2,7   | 15               | 3,1   | 13              | 2,0   |
| 16              | 8,5   | 14              | 1,7   | 16              | 10,2  | 14              | 3,3   | 16               | 1,2   | 14              | 1,9   |

|                |            |                |            |                |            |                |            |                |            |                |            |
|----------------|------------|----------------|------------|----------------|------------|----------------|------------|----------------|------------|----------------|------------|
| 20             | 1,6        | 17             | 2,4        | 20             | 6,6        | 17             | 3,1        | 20             | 4,1        | 17             | 1,3        |
| 21             | 1,4        | 18             | 8,2        | 21             | 4,7        | 18             | 7,2        | 21             | 3,4        | 18             | 0,9        |
| 29             | 3,7        | 19             | 5,7        | 29             | 4,0        | 19             | 8,2        | 29             | 1,1        | 19             | 1,4        |
| 30             | 2,3        | 22             | 7,9        | 30             | 4,1        | 22             | 6,6        | 30             | 1,8        | 22             | 0,8        |
| 34             | 9,4        | 24             | 2,4        | 34             | 6,1        | 24             | 3,2        | 34             | 0,6        | 24             | 1,3        |
| 35             | 3,5        | 25             | 1,6        | 35             | 5,4        | 25             | 3,0        | 35             | 1,6        | 25             | 1,9        |
| 40             | 3,8        | 26             | 5,4        | 40             | 11,1       | 26             | 4,3        | 40             | 2,9        | 26             | 0,8        |
| 42             | 6,6        | 27             | 2,1        | 42             | 6,0        | 27             | 1,9        | 42             | 0,9        | 27             | 0,9        |
| 43             | 2,0        | 28             | 5,1        | 43             | 4,8        | 28             | 7,3        | 43             | 2,5        | 28             | 1,4        |
| 44             | 1,0        | 32             | 0,5        | 44             | 0,8        | 32             | 1,5        | 44             | 0,8        | 32             | 3,0        |
| 45             | 2,4        | 38             | 6,3        | 45             | 4,4        | 38             | 7,2        | 45             | 1,8        | 38             | 1,1        |
| 46             | 3,5        | 41             | 0,6        | 46             | 3,3        | 41             | 2,2        | 46             | 0,9        | 41             | 3,5        |
| 47             | 2,2        |                |            | 47             | 5,5        |                |            | 47             | 2,5        |                |            |
| <b>Ave</b>     | <b>3,8</b> | <b>Ave</b>     | <b>4,0</b> | <b>Ave</b>     | <b>5,1</b> | <b>Ave</b>     | <b>5,0</b> | <b>Ave</b>     | <b>1,7</b> | <b>Ave</b>     | <b>1,6</b> |
| <b>Std Dev</b> | <b>3,3</b> | <b>Std Dev</b> | <b>3,4</b> | <b>Std Dev</b> | <b>2,8</b> | <b>Std Dev</b> | <b>3,4</b> | <b>Std Dev</b> | <b>1,0</b> | <b>Std Dev</b> | <b>0,9</b> |

These results are supported by the fact that women using COCs showed increased levels of triglycerides over the course of three years (Guazzelli *et al.*, 2005). Other studies found a similar increase in triglyceride levels women using the EE/DRSP formulation (Klipping & Marr, 2005; Taneepanichskul & Phupong, 2007). It is well-proven that the use of COCs increases the lipid profile of users and an increased lipid profile can also result in increased risks for thromboembolism and other cardiovascular diseases (De Groote *et al.*, 2009; Matés *et al.*, 2008).

#### 4.7 OXIDATIVE DAMAGE: DNA, PROTEIN AND LIPID DAMAGE

The tests for oxidative damage in 8-OHdG, 3-Nitrotyrosine and MDA (TBARS) were additional test performed to assess whether there is an early indication of oxidative damage in the EE/DRSP group, especially considering the very high levels of ROS production within this group. The results are given in table 4.10.

##### 4.7.1 MALONDIALDEHYDE (TBARS ASSAY)

The TBARS test were used to determine the presence of malodialdehyde (MDA) in urine samples. The concentration of MDA was determined spectrophotometrically. When looking

at the results, it can be seen that, on average, there was no difference between the concentrations of MDA between the EE/DRSP group and control group. However, ROC analysis showed that there is a biological trend between the production of ROS and the concentration of MDA produced (AUC = 0.74737). Therefore, in a case of increased ROS production (as evident in the EE/DRSP group) this could be responsible for an increased ratio of ROS to MDA.

It was found that the concentrations of MDA (TBARS levels) were significantly higher in hypertensive males having high ROS values when compared to normotensive males having normal ROS values (Bothma 2012; Gutteridge 1995). In this case it can be seen that the EE/DRSP group, having high levels of ROS, showed an increase in the concentrations of MDA. Because of the estrogen-associated increase in fatty acids and also the high levels of ROS in the EE/DRSP group, the assumption can be made that the increased concentrations of MDA in this group are the result of lipid peroxidation brought on by higher amounts of lipids that are readily oxidized by the high levels of ROS present. Since long term use of COCs can increase lipids in the blood of users (De Groote *et al.*, 2009; Guazzelli *et al.*, 2005; Matés *et al.*, 2008; Pincemail *et al.*, 2007), it can be assumed that the increase in lipid peroxidation will occur chronically as a result of the chronic high ROS state. The presence of MDA can also be associated with an increased cancer risk, since these metabolites are mutagenic (Matés *et al.*, 2008).

**Table 4.10** Results for the determination of oxidative damage of the DNA, protein and lipid markers.

| TBARS       |       |             |       | 3-Nitrotyrosine |       |             |        | 8-OHdG      |       |             |       |
|-------------|-------|-------------|-------|-----------------|-------|-------------|--------|-------------|-------|-------------|-------|
| EE/DRSP     |       | Control     |       | EE/DRSP         |       | Control     |        | EE/DRSP     |       | Control     |       |
| Participant | Value | Participant | Value | Participant     | Value | Participant | Value  | Participant | Value | Participant | Value |
| 1           | 7,9   | 6           | 6,7   | 1               | 26,4  | 6           | 30,5   | 1           | 0,3   | 6           | 21,9  |
| 2           | 7,2   | 9           | 2,1   | 2               | 72,2  | 9           | 31,1   | 2           | 3,5   | 9           | 6,8   |
| 5           | 1,8   | 10          | 2,1   | 5               | 35,9  | 10          | 26,2   | 5           | 5,0   | 10          | 4,6   |
| 7           | 0,6   | 11          | 3,1   | 7               | 40,2  | 11          | 56,5   | 7           | 1,3   | 11          | 6,1   |
| 8           | 0,1   | 12          | 2,4   | 8               | 31,9  | 12          | 236,2* | 8           | 26,2  | 12          | 7,2   |
| 15          | 2,5   | 13          | 1,3   | 15              | 46,0  | 13          | 42,2   | 15          | 4,9   | 13          | 5,3   |

|                |            |                |            |                |             |                |             |                |             |                |            |
|----------------|------------|----------------|------------|----------------|-------------|----------------|-------------|----------------|-------------|----------------|------------|
| 16             | 2,8        | 14             | 0,1        | 16             | 37,6        | 14             | 24,0        | 16             | 11,8        | 14             | 0,6        |
| 20             | 2,6        | 17             | 3,0        | 20             | 24,4        | 17             | 37,5        | 20             | 12,3        | 17             | 10,9       |
| 21             | 1,5        | 18             | 4,1        | 21             | 31,3        | 18             | 52,3        | 21             | 13,3        | 18             | 23,0       |
| 29             | 0,8        | 19             | 1,8        | 29             | 40,0        | 19             | 17,8        | 29             | 7,2         | 19             | 10,2       |
| 30             | 2,0        | 22             | 4,0        | 30             | 96,7        | 22             | 27,4        | 30             | 17,8        | 22             | 10,8       |
| 34             | 6,9        | 24             | 2,5        | 34             | 64,8        | 24             | 34,7        | 34             | 11,3        | 24             | 12,3       |
| 35             | 2,5        | 25             | 2,1        | 35             | 43,6        | 25             | 39,8        | 35             | 17,7        | 25             | 10,7       |
| 40             | 1,4        | 26             | 3,4        | 40             | 36,2        | 26             | 215,8*      | 40             | 12,0        | 26             | 3,2        |
| 42             | 2,6        | 27             | 2,9        | 42             | 42,3        | 27             | 45,8        | 42             | 9,1         | 27             | 15,1       |
| 43             | 0,3        | 28             | 1,3        | 43             | 29,9        | 28             | 36,5        | 43             | 10,8        | 28             | 4,2        |
| 44             | 2,3        | 32             | 2,0        | 44             | 29,5        | 32             | 38,8        | 44             | 14,4        | 32             | 10,9       |
| 45             | 4,4        | 38             | 2,1        | 45             | 42,1        | 38             | 33,0        | 45             | 11,9        | 38             | 5,5        |
| 46             | 0,3        | 41             | 2,4        | 46             | 9,7         | 41             | 58,2        | 46             | 5,4         | 41             | 12,5       |
| 47             | 2,1        |                |            | 47             | 36,7        |                |             | 47             | 15,6        |                |            |
| <b>Average</b> | <b>2,6</b> | <b>Average</b> | <b>2,6</b> | <b>Average</b> | <b>40,9</b> | <b>Average</b> | <b>37,2</b> | <b>Average</b> | <b>10,6</b> | <b>Average</b> | <b>9,6</b> |
| <b>Std Dev</b> | <b>2,3</b> | <b>Std Dev</b> | <b>1,3</b> | <b>Std Dev</b> | <b>18,7</b> | <b>Std Dev</b> | <b>10,9</b> | <b>Std Dev</b> | <b>6,3</b>  | <b>Std Dev</b> | <b>5,7</b> |

\* These values were very high in relationship to the other values found; therefore the average and standard deviation were calculated without taking these values into account.

#### 4.7.2 3-NITROTYROSINE

3-Nitrotyrosine (3-NT) is a biomarker for oxidative damage to proteins. Butylated 3-NT were extracted from urine samples and the concentration of 3-NT was determined based on a calibration curve. Looking at the results, it can be seen that the EE/DRSP group had, on average, higher concentrations of 3-NT compared to the control group. ROC curve analysis showed that the ROS to 3-NT ratio showed a biological trend (AUC = 0.8). In light of the high ROS levels in the EE/DRSP group it can therefore be assumed that protein oxidation did occur as a result of reactive nitrogen species present which would bind to ROS. These products are responsible for protein oxidation. Therefore, higher levels of 3-NT present would cause higher binding to the chronic high levels of ROS possibly leading to higher protein oxidation in the EE/DRSP group. Even though Bothma (2012) did not find a significant link between oxidative stress and 3-NT, the link between increased protein oxidation and oxidative stress conditions is well known (Berlett & Stadtman, 1997).

#### 4.7.3 8-HYDROXY 2'-DEOXYGUANOSINE

Increased 8-OHdG are the result of oxidation of guanine bases in DNA which are then removed through excision repair. 8-OHdG was extracted through solid phase extraction and quantified on an LC-MS/MS. The results showed that the EE/DRSP group had a slightly higher average concentration of 8-OHdG than the control group. When considering this result in the light of the high ROS levels it can be assumed that there is a slightly higher excision of 8-OHdG in participants using the EE/DRSP hormonal formulation. However, this was not enough to indicate a statistical trend between increased ROS production and an increased excretion of 8-OHdG.

It was found by Steenkamp (2010) that a higher level of ROS was not necessarily associated with a higher level of 8-OHdG. Therefore, it is possible that 8-OHdG is either not a good marker for oxidative DNA damage, or that oxidative DNA damage would only occur over a long term chronic oxidative stress state (e.g. in cancer patients).

These three assays for the determination of further oxidative damage as a result of high ROS levels, showed no immediate damaging effect on DNA. However, the biological trends between lipid peroxidation and protein oxidation in relationship to chronic high levels of ROS, are cause for concern. In this study 3-NT and MDA can be seen as early biomarkers for intracellular oxidative damage.

#### 4.8 SUMMARY

In this chapter, Phase I and Phase II metabolism as a result of the biotransformation loading test was described, as well as other factors such as antioxidant potential, the formation of reactive species, the role of carnitines, and the possible oxidative damage to cellular components.

Two results showed statistical significance: the very high increase in ROS and the low Phase II sulphate conjugation in the EE/DRSP group when compared to a control group not using COCs. Other biological trends in the EE/DRSP group also emerged, such as the apparent inhibition of glutathione S-transferase or systemic depletion of glutathione, lower overall Phase II conjugation, and increased triglycerides suggesting that although the antioxidant potential in this group tested within the normal range, this somehow did not result in effective neutralization of free radical levels, possibly due to the depletion of antioxidants as a result of chronic high levels of ROS. Early signs of intracellular oxidative damage could also be seen

in the increased protein and lipid peroxidation. When taking all of these results into account and considering the fact that ROS caused by the use of COCs such as the EE/DRSP formulation have unique characteristics, it is evident that a unique treatment would be necessary to reduce ROS to physiological normal levels. It is quite possible that the increase in hormones administered through oral contraception, increases the concentrations of these hormones in the blood to such an extent that the concentrations are much higher than under physiological conditions, leading finally to aberrant free radical metabolism.

# 5

# CONCLUSION

## 5.1 INTRODUCTION

Hormonal contraceptives, including OCs, have gained popularity since it was first approved and brought on the market in the 1960's. Since then, changes to both the progestin and estrogen components in COCs have been improved and changed to be more representative of their *in vivo* hormonal counterparts. However, several authors have highlighted the lack of understanding the long term effects COCs have on the biotransformation and metabolism of users. Increased levels of ROS that lead to oxidative stress – when the *in vivo* ROS levels are increased above the physiological threshold – is an occurrence in females using the COCs. Through routine screening of patients doing the biotransformation loading test at the BOSS Laboratory, North-West University, Potchefstroom Campus, it was found that females using specifically the EE/DRSP formulation as a COC, had ROS levels higher than that found in many other pathological conditions. Furthermore, it is concerning that these patients do have the inherent antioxidant potential to lower these high levels of ROS, but for some reason the body's antioxidant system cannot decrease ROS levels. It was also found by the BOSS Laboratory through treatment of these patients that the conventional antioxidant treatments prescribed did not prove to be successful in decreasing ROS levels. This was underlined by literature references that proved that a specific antioxidant formulation was necessary in order to successfully reduce ROS levels in patients using the EE/DRSP COC. Therefore, it can be seen that the effect this formulation has on the biotransformation profile of its users are unique and the reasons are not fully understood.

## 5.2 SUMMARY OF STUDY DESIGN AND METHODS USED

In all, 47 female participants between the ages of 18 and 35 were recruited to take part in the study. Of those, 20 participants were using the EE/DRSP COC and 19 participants were placed in the control group that did not use any form of hormone treatment. These participants were asked to complete the biotransformation loading test on the 14<sup>th</sup> day of their

menstrual cycle. Samples were collected by the BOSS Laboratory and the standard biotransformation analyses were performed (Phase I detoxification, Phase II detoxification, carnitine profiles, determination of 2,3-DHBA and 2,5-DHBA, and the determination of ROS, FRAP, and total glutathione), including three additional tests for determining oxidative damage (determining 8-OHdG, 3-NT and TBARS). Results were then statistically processed, with the two results showing statistical significance being the ROS and Phase II: sulphate conjugation reactions ( $p < 0.001$ ). Multivariate analysis was performed and the ROC curves drawn to determine the relationships between some of the biotransformation metabolites in order to see whether a biological trend could be seen in the data. The most significant trends were those that showed the relationship of a metabolite with the increase in ROS within the EE/DRSP group.

### 5.3 SUMMARY OF RESULTS

As mentioned, two results showed strong statistical significance ( $p < 0.001$ ), the first being the very high levels of ROS in the EE/DRSP group that was not evident in the control group, while the second was the apparent inhibition of the sulphate conjugation reaction in the EE/DRSP group.

Multivariate analysis indicated the biological trends or biological relationships ( $AUC \geq 0.7$ ) between ROS and several of the biotransformation reactions monitored during the loading test. The relationship between ROS and glucuronidation, one of the major pathways for EE metabolite conjugation, showed that this conjugation pathway was under pressure as a result of the systemic depletion of Phase II enzymes in the EE/DRSP group, which could be seen in the lower recovery of glucuronide conjugates in this group. Glutathionation, as one of the main conjugation pathways in the binding of quinones, showed chronic depletion as a result of the consistent metabolism of ethinylestradiol due to chronic administration of the EE/DRSP formulation. Tying in with the results of glutathione was the result of the FRAP test which showed that the EE/DRSP did have the necessary antioxidant potential *in vivo* to reduce the levels of ROS, but the biological relationship of antioxidant potential to ROS indicated that the body's antioxidant system prove ineffective in reducing the high levels of ROS. Furthermore, in the results of both 2,3-DHBA and 2,5-DHBA showed that as a result of the increased ROS levels, more hydroxyl radicals were present to bind to salicylic uric acid to form 2,3-DHBA and 2,5-DHBA in the EE/DRSP group. The carnitine profiles showed an

increased presence of lipids as a result of oxidative stress in the presence of ROS. Also, the amount of free carnitines present was less in the EE/DRSP group, increasing the ratio of TAC:TFC. The additional analyses for the determination of possible oxidative damage to intracellular components showed that protein oxidation and lipid peroxidation already started to take place in EE/DRSP group, while oxidative damage to DNA could not be proven.

#### 5.4 AIMS AND OBJECTIVES

It was hypothesised that the use of the COCs containing the EE/DRSP combination will alter the biotransformation, free radical levels and oxidative damage profiles in women using these contraceptives. The **aim** was to investigate the effects of the combined oral contraceptives, with an EE/DRSP formulation on the biotransformation, oxidative stress status and oxidative damage of its users. The **objectives** were to conduct an age-matched controlled study with EE/DRSP users as experimental and non-COC users as control groups, to perform standard biotransformation profiling and determine oxidative damage on samples of each participant collected during the luteal phase of the menstrual cycle, and statistically process results to evaluate potential differences in biotransformation, free radical levels and oxidative damage between the control and experimental groups.

The aim and all the objectives were met through the course of this study. From the results it was obvious that the hypothesis could not be rejected. A very broad hypothesis was set at the onset of this study because this area of research is fairly new and very little was known of the potential effects this COC formulation could have on biotransformation, free radical levels and the consequent oxidative damage in its users. It was confirmed that the EE/DRSP group had statistical significant higher levels of ROS than the control group, as well as a biological trend showing higher catechols to ROS, higher 2,3-DHBA to ROS and higher 2,5-DHBA to ROS ratios in the EE/DRSP group. From the results it was evident that the measured biotransformation parameters (Phase I and II metabolism, carnitine metabolism antioxidant potential and the additional damage markers) were also affected by the dramatically increased ROS production in the EE/DRSP group. Sulphonation tested overall lower in the EE/DRSP group, while biological trends could be observed that indicated that, with the exception of glycination, Phase II conjugation reactions were less effective in clearing the possible causes of the high levels of ROS. Furthermore, the ratio of TAC:TFC was higher with the increase in ROS in the EE/DRSP group. It was also clearly evident from the results that the EE/DRSP

group did have the necessary antioxidant potential to reduce the high levels of ROS but were somehow ineffective in this task.

## **5.5 CONCLUSION**

When taking all the results in consideration, an overall picture starts to emerge. In females using the EE/DRSP formulation, a very high increase in the levels of free radicals can undoubtedly be seen. One of the main free radicals produced, seemed to be hydroxyl radicals evident from the results of 2,3-DHBA. Even though the reasons for this radical increase in ROS are highly disputed in literature, from the metabolism of estrogen it can be seen that free radicals can either form as a result of CYP450 reductase activity, or as a secondary result because of the systemic depletion of conjugation metabolites and antioxidants. This systemic depletion could be seen in the lower recovery of glucuronide and sulphate – glucuronidation and sulphation are two of the essential pathways in the metabolism of EE. Furthermore, glutathionation – a direct antioxidant pathway in the metabolism of EE – also showed systemic depletion, putting the antioxidant capacity of the liver under pressure. Therefore a secondary increase in free radicals could be seen. Because of the estrogen and oxidative stress related increase in lipids, TAC increased while TFC decreased, while oxidative damage to readily available lipids already started to take place as can be seen in the increased MDA.

The results of this study had proven that users of the EE/DRSP group had a systemic reduction of glucuronide, glutathione, and sulphates as a result of the chronic hormone metabolism. The reduction of these conjugation metabolites could result in a secondary increase of ROS. The increase in ROS found in the EE/DRSP group caused oxidative cellular damage to lipids and proteins. Therefore, the use of an EE/DRSP formulation causes a strain on the biotransformation of its users, which could result in serious health risks if left untreated.

## **5.6 FUTURE RECOMMENDATIONS**

There is an estrogen concentrations difference between Product A and B. Even though the concentrations over a month are fairly similar, the difference can lead to variable results within a single group. As a recommendation, I would advise that in future Product A and B be split into two experimental groups instead of combining them in one experimental group.

For this study, test samples were taken on day 14 of the menstrual cycle, based on the fact that the ovulation occurs mainly on day 14. However, because of the hormonal variation in the control group and the fact that ovulation does not necessarily occur on day 14, it would be interesting if the study was performed over time in order to modulate all the markers over time against hormonal levels. This could possibly lead to a better supported hypothesis, especially if the differences between the two groups are better highlighted.

# BIBLIOGRAPHY

Akande, A.A. & Akinyinka, A.O. 2005. Serum malondialdehyde levels during the menstrual cycle. *African Journal of Biotechnology*, 4(11):1297-1299. Date of Access: 13 Oct. 2014.

Alía, M., Mateos, R., Ramos, S., Lecumberri, E., Bravo, L., & Goya, L. 2005. Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *Journal of Biochemical and Molecular Toxicology*, 19(2):19-28. Date of Access: 16 Feb. 2012.

Aruoma, O.I. 1998. Free Radicals, Oxidative Stress, and Antioxidants in Human Health and Disease. *Journal of the American Oil Chemists' Society*, 75(2):199-212. Date of Access: 15 Mar. 2012.

Balaban, R.S., Nemoto, S. & Finkel, T. 2005. Mitochondria, Oxidants, and Aging. *Cell*, 120(4):483-495. Date of Access: 17 Jul. 2012.

Balogh, A., Klinger, G., Henschel, L., Börner, A., Vollanth, R. & Kuhn, W. 1995. Influence of ethinylestradiol-containing combination oral contraceptives with gestoden or levonorgestrel on caffeine elimination. *European Journal of Clinical Pharmacology*, 48:161-166. Date of Access: 13 Oct. 2014.

Bandyopadhyay, U., Das, D. & Banerjee, R.K. 1999. Reactive oxygen species: Oxidative damage and pathogenesis. *Current Science*, 77(5):658-666. Date of Access: 11 Jul. 2012.

Berlett, B.S. & Stadtman, E.R., 1997. Protein Oxidation in Aging, Disease, and Oxidative Stress. *The Journal of Biological Chemistry*, 272(33):20313-20316. Date of Access: 11 Jul. 2013.

Bolton, J.L. & Thatcher, G.R.J., 2008. Potential mechanisms of estrogen quinone carcinogenesis. *Chemical research in toxicology*, 21(1), pp.93–101. Date of Access: 21 Feb. 2012.

- Bothma, K., 2012. The involvement of lipid and protein oxidation in hypertension: the SABPA study. Potchefstroom: NWU. (Dissertation - MSc).
- Brandon, E.F.A., Raap, C.D., Meijerman, I., Beijnen, J.H., Schellens, J.H.M. 2003. An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicology and Applied Pharmacology*, 189:233–246. Date of Access: 11 Jul. 2012.
- Buege, J.A. & Aust, S.D. 1978. Microsomal lipid peroxidation. *Methods in Enzymology*, 6879:302–310. Date of Access: 11 Jul. 2012.
- Butterfield, D.A., Koppal, T., Howard, B., Yatin, S., Allen, K., Aksenov, M., Aksenov, M., & Carney, J. 1998. Structural and Functional Changes in Proteins Induced by Free Radical-mediated Oxidative Stress and Protective Action of the Antioxidants N-tert-Butyl-. *Annals of the New York Academy of Sciences*, 854:448–462. Date of Access: 13 Oct. 2014.
- Chang, M., Zhang, F., Shen, L., Pauss, N., Alam, I., Van Breemen, R.B., Blond, S. & Bolton, J.D. 1998. Inhibition of Glutathione S-Transferase Activity by the Quinoid Metabolites of Equine Estrogens. *Chemical research in toxicology*, 11(97):758–765. Date of Access: 29 Dec. 2014.
- Christin-Maitre, S. 2013. History of oral contraceptive drugs and their use worldwide. *Best Practice & Research Clinical Endocrinology & Metabolism*, 27(1):3-12. Date of Access: 13 Oct. 2014.
- Clemons, M. & Goss, P. 2001. Estrogen and the Risk of Breast Cancer. *The New England Journal of Medicine*, 344(4), pp.276–286. Date of Access: 11 Apr. 2015.
- Coudray, C. & Favier, A. 2000. Determination of salicylate hydroxylation as an in vivo oxidative stress marker. *Free Radical Biology and Medicine*, 29(11):1064-1070. Date of Access 13 Oct. 2014.
- De Groote, D., Perrier d'Hauterive, S., Pintiaux, A., Balteau, B., Gerday, C., Claesen, J. & Foidart, J., 2009. Effects of oral contraception with ethinylestradiol and drospirenone on oxidative stress in women 18-35 years old. *Contraception*, 80, pp.187–93. Date of Access: 21 Feb. 2012.

Devadas, S., Zaritskaya, L., Rhee, S.G., Oberley, L. & Williams, M.S. 2002. Discrete Generation of Superoxide and Hydrogen Peroxide by T Cell Receptor Stimulation: Selective Regulation of Mitogen-Activated Protein Kinase Activation and Fas Ligand Expression. *The Journal of Experimental Medicine*, 195(1):59–70. Date of Access: 25 Jul. 2012.

Dröge, W. 2002. Free radicals in the physiological control of cell function. *Physiological Reviews*, 82(1):47–95. Date of Access: 17 Jul. 2012.

Dictionary of Biology. 2004. London, UK: Pengiun Books Ltd.

Dupont, I., Berthou, F., Bodenez, P., Bardou, L., Guirriec, C., Stephan, N., Dreano, Y., & Lucas, D. 1999. Involvement of cytochromes P-450 2E1 and 3A4 in the 5-hydroxylation of salicylate in humans. *Drug Metabolism and Disposition*, 27(3), pp.322–326. Date of Access: 20 Dec. 2014.

Finco, A., Belcaro, G. & Cesarone, M.R. 2011. Assessment of the activity of an oral contraceptive on the levels of oxidative stress and changes in oxidative stress after co-treatment with two different types of physiological modulators with antioxidant action. *Contraception*, 84(4):418–422. Date of Access: 15 May 2012.

Finco, A., Belcaro, G. & Cesarone, M.R. 2012. Evaluation of oxidative stress after treatment with low estrogen contraceptive either alone or associated with specific antioxidant therapy. *Contraception*, 85(5):503–508. Date of Access: 15 May 2012.

Finkel, T. 2003. Oxidant signals and oxidative stress. *Current Opinion*, 15:247–254. Date of Access: 11 Jul. 2012.

Finkel, T. & Holbrook, N.J. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature*, 408:239–247. Date of Access: 16 Jul. 2012.

Grotto, D., Santa Maria, L., Valentini, J., Paniz, C., Schmitt, G., Garcia, S.C., Pomblum, V.J., Rocha, J. B.T. & Farina, M. 2009. Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. *Quimica Nova*, 32(1):169–174. 24 Sep. 2015.

- Guang-Sheng, F., Mei-Lu, B., LiNnan, C., Xiao-Ming, C., Zi-Rong, H., Zi-Yan, H., Xiao-Ping, J., Jian, L., Shu-Ying, W., Cheng-Liang, X. & Zheng-Ai, X., Tian-Fu, Y. 2010. Efficacy and Safety of the Combined Oral Contraceptive Ethinylestradiol / Drospirenone (Yasmin®) in Healthy Chinese Women. *Clinical Drug Investigations*, 30(6):387–396. Date of Access: 13 Oct. 2014.
- Guazzelli, C.A.F., Lindsey, P.C., de Araújo, F.F., Barbieri, M., Petta, C.A. & Aldrighi, J.M. 2005. Evaluation of lipid profile in adolescents during long-term use of combined oral hormonal contraceptives. *Contraception*, 71(2):118–121. Date of Acces: 07 Mar. 2012.
- Gutteridge, J.M.C. 1995. Lipid Peroxidation and Antioxidants as Biomarkers of Tissue Damage. *Clinical Chemistry*, 41(12):1819–1828. Date of Access: 13 Oct. 2014.
- Gutteridge, J.M.C. 1999. Hydroxyl radical formation from the auto-reduction of ferric-citrate complex. *Free Radical Biology and Medicine*, 11(4):401-406. Date of Access: 11 Jul. 2011.
- Guyton, A.C. & Hall, J.E. 2006. Textbook of Medical Physiology. 11<sup>th</sup> ed. Philadelphia, PA: Elsevier Saunders.
- Halliwell, B. & Gutteridge, J.M.C. 1989. Free Radicals in Biology and Medicine. 2<sup>nd</sup> ed. Oxford: Clarendon Press.  
[https://books.google.co.in/books?id=\\_QdrAAAAMAAJ&source=gbs\\_book\\_other\\_versions](https://books.google.co.in/books?id=_QdrAAAAMAAJ&source=gbs_book_other_versions)  
 Date of Access: 13 Oct. 2014.
- Halliwell, B. 1991. Reactive oxygen species in living systems: Source, biochemistry and, role in human disease. *The American Journal of Medicine*, 91(3):S14-S22. Date of Access: 07 Mar. 2012.
- Hankinson, S.E., Colditz, G.A. & Willett, W.C. 2004. Towards an integrated model for breast cancer etiology: The lifelong interplay of genes, lifestyle, and hormones. *Breast Cancer Research*, 6:213–218. Date of Access: 15 May 2012.
- Honda, M., Yamada, Y., Tomonaga, M., Ichinose, H. & Kamihira, S. 2000. Correlation of urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage,

and clinical features of hematological disorders: a pilot study. *Leukemia Research*, 24:461–468. Date of Access: 13 Oct. 2014.

Huang, Z., Guengerich, F.P. & Kaminsky, L.S. 1998. 16 $\alpha$ -Hydroxylation of estrone by human cytochrome P4503A4/5. *Carcinogenesis*, 19(5):867–872. Date of Access: 05 Jun. 2012.

Jones, D.P. 2008. Radical-free biology of oxidative stress Radical-free biology of oxidative stress. *American Journal of Physiology and Cell Physiology*, 295:C849–C868. Date of Access: 15 May 2012.

Klipping, C. & Marr, J. 2005. Effects of two combined oral contraceptives containing ethinyl estradiol 20  $\mu$ g combined with either drospirenone or desogestrel on lipids, hemostatic parameters and carbohydrate metabolism. *Contraception*, 71(6):409–416. Date of Access: 07 Mar. 2012.

Krattenmacher, R. 2000. Drospirenone: pharmacology and pharmacokinetics of a unique progestogen. *Contraception*, 62(1):29–38. Date of Access: 20 Feb. 2012.

Lakhani, N.J., Sarkar, M.A., Venitz, J. & Figg, W.D. 2003. 2-Methoxyestradiol, a Promising Anticancer Agent. *Pharmacotherapy*, 23(2):165–172. Date of Access: 20 Feb. 2012.

Liska, D.J. 1998. The Detoxification Enzyme Systems. *Alternative Medicine Review*, 3(3):187–198. Date of Access: 11 Jul. 2011.

Lu, F.C. & Kacew, S. 2002. Lu's basic toxicology: fundamentals, target organs and risk assessment. 4<sup>th</sup> ed. New York, NJ: Taylor & Francis.

Matés, J.M., Segura, J., Alonso, F.J. & Márquez, J. 2008. Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis. *Archives of toxicology*, 82(5):73–99. Date of Access: 21 Feb. 2012.

Miller, V.M. 2010. Estrogen Metabolomics: A Physiologist's Perspective. *Hypertension*, 56(5):816–818. Date of Access: 27 Feb. 2012.

Monaghan, P., Metcalfe, N.B. & Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology Letters*, 12:75–92. Date of Access: 15 May 2012.

Muhn, P., Krattenmacher, R., Beier, S., Elger, W. & Schillinger, E. 1995. Drospirenone: A Novel Progestogen with Antimineralocorticoid and Antiandrogenic Activity. *Contraception*, 51:99–110. Date of Access: 13 Oct. 2014.

Niki, E., Yoshida, Y., Saito, Y. & Noguchi, N. 2005. Lipid peroxidation: Mechanisms, inhibition, and biological effects. *Biochemical and Biophysical Research Communications*, 338(1):668-676. Date of Access: 25 Sep. 2015.

Nordberg, J. & Arnér, E.S.J. 2001. Reactive Oxygen Species, Antioxidants, and the Mammalian Thioredoxin System. *Free Radical Biology & Medicine*, 31(11):1287–1312. Date of Access: 11 Jul. 2012.

Ogino, K. & Wang, D.H. 2007. Biomarkers of Oxidative Stress/Nitrosative Stress: An Approach to Disease Prevention. *Acta Medica Okayama*, 61(4):181–189. Date of Access: 24 Sep 2015.

Online Medical Dictionary. 2012. Xenobiotics. <http://www.online-medical-dictionary.org/definitions-x/xenobiotics.html> Date of access: 12 Jun. 2012.

Parsey, K.S. & Pong, A. 2000. An Open-Label , Multicenter Study to Evaluate Yasmin, a Low-Dose Combination Oral Contraceptive Containing Drospirenone, a New Progestogen. *Contraception*, 61:105-111. Date of Access: 13 Oct. 2014.

Persky, A.M., Green, P.S., Stublely, L., Howell, C.O., Zaulyanove, L., Brazeau, G.A. & Simpkins, J.W. 2000. Protective Effect of Estrogens Against Oxidative Damage to Heart and Skeletal Muscle In Vivo and In Vitro. *Proceedings of the Society of Experimental Biology and Medicine*, 223:59–66. Date of Access: 21 Feb. 2012.

Pincemail, J., Vanbelle, S., Gaspard, U., Collette, G., Haleng, J., Cheramy-Bien, J.P., Charlier, C., Chapelle, J.P., Giet, D., Albert, A., Limet, R. & Defraigne, J.O. 2007. Effect of different contraceptive methods on the oxidative stress status in women aged 40 – 48 years

from the ELAN study in the province of Liège, Belgium. *Human Reproduction*, 22(8):2335–2343. Date of Access: 25 Oct. 2012.

Rad, M., Kluft, C., De Kam, M.L., Meijer, P., Cohen, A.F., Grubb, G.S., Constantine, G.D. & Burggraaf, J. 2011. Metabolic profile of a continuous versus a cyclic low-dose combined oral contraceptive after one year of use. *The European Journal of Contraception & Reproductive Health Care*, 16(2):85–94. Date of Access: 05 Mar. 2012.

Raftogianis, R., Creveling, C., Weinshilboum, R. & Weisz, J. 2000. Chapter 6: Estrogen Metabolism by Conjugation. *Journal of the National Cancer Institute Monographs*, 27:113–124. Date of Access: 19 Nov. 2014.

Rall, L.C. et al., 2000. Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) as a marker of oxidative stress in rheumatoid arthritis and aging: Effect of progressive resistance training. *The Journal of Nutritional Biochemistry*, 11, pp.581–584.

Rogan, E. et al., 2007. Xenoestrogens, Biotransformation, and Differential Risks for Breast Cancer. (In Hyman, M. ed. Boulder, CO: Innovision. p. 112-121).

Salway, J.G. 2004. *Metabolism at a Glance*. 3<sup>rd</sup> ed. Oxford, UK: Blackwell Publishing Ltd.

Siddique, Y.H., Beg, T. & Afzal, M. 2005. Genotoxic potential of ethinylestradiol in cultured mammalian cells. *Chemico-Biological Interactions*, 151(2):133–141. Date of Access: 21 Feb. 2012.

Sitruk-Ware, R. & Nath, A. 2011. Metabolic effects of contraceptive steroids. *Reviews in endocrine & metabolic disorders*, 12(2):63–75. Date of Access: 05 Mar. 2012.

South African Electronic Package Inserts. 2002. Product A.  
<http://home.intekom.com/pharm/schering/yasmin.html> Date of Access: 27 Feb. 2012.

Steenkamp, L. 2010. Urinary 1,4-dihydroxynonene mercapturic acid (DHN-MA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) as markers of oxidative damage : The SABPA study. Potchefstroom: NWU. (Dissertation - MSc).

Taneepanichskul, S. & Phupong, V. 2007. Influence of a new oral contraceptive with drospirenone on lipid metabolism. *Gynecological Endocrinology*, 23:347–350. Date of Access: 07 Mar. 2012.

Teichmann, A. 2003. Pharmacology of estradiol valerate/dienogest. *Climacteric*, 6(2):17-23. Date of Access: 19 Nov 2014.

Tsuchiya, Y., Nakajima, M. & Yokoi, T. 2005. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer letters*, 227(2):115–24. Date of Access: 20 Feb. 2012.

U.S. Department of Health and Human Services. 2011. Report on Carcinogens. 12<sup>th</sup> ed. Washington D.C.

Valavanidis, A., Vlachogianni, T. & Fiotakis, C. 2009. 8-hydroxy-2'-deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *Journal of environmental science and health. Part C, Environmental carcinogenesis & ecotoxicology reviews*, 27(2):120–39. Date of Access: 13 Oct. 2014.

Weimann, A., Belling, D. & Poulsen, H.E. 2001. Measurement of 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine in DNA and human urine by high performance liquid chromatography-electrospray tandem mass spectrometry. *Free Radical Biology and Medicine*, 30(7):757–764. Date of Access: 13 Oct. 2014.

Widmaier, E.P., Raff, H. & Strang, K.T. 2011. Vander's Human Physiology: The mechanisms of body function. 12<sup>th</sup> ed. New York, NJ: McGraw Hill.

Yager, J.D. 2000. Chapter 3: Endogenous Estrogens as Carcinogens Through Metabolic Activation. *Journal Of The National Cancer Institute Monographs*, 27:67–73. Date of Access: 15 May 2012.

Zhang, H., Cui, D., Wang, B., Yong-Hae, H., Zheng, Y., Michael, S. & Rodrigues, A.D. 2007. Pharmacokinetic Drug Interactions Involving 17alpha-Ethinylestradiol: A New Look at an Old Drug. *Clinical Pharmacokinetics*, 46(2):133–157. Date of Access: 07 Mar. 2012.

Zhu, B.T. & Conney, A.H. 1998. Functional roles of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis*, 19(1):1-27. Date of Access: 11 Nov. 2014.