NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
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Investigation of the correlation between oxidative stress and hypertension: The SABPA Study.

By

Cynthia Antoinette Botha, B.Sc. (Hons.)

Dissertation submitted for the degree Magister Scientiae (M.Sc.) in Biochemistry at the Potchefstroom Campus of the North-West University

Supervisor: Dr. R. Louw
School for Physical and Chemical Sciences, North-West University (Potchefstroom Campus), South Africa.

Co-Supervisor: Prof. F.H. van der Westhuizen
School for Physical and Chemical Sciences, North-West University (Potchefstroom Campus), South Africa.

Co-Supervisor: Dr. R. Schutte
School for Physiology, Nutrition and Consumer Sciences, North-West University (Potchefstroom Campus), South Africa.

December 2008
Potchefstroom

Table of contents:

Acknowledgements:

What good is the feeling of success without someone to enjoy it with?

First and foremost I would like to share my greatest appreciation for the Lord, my Savior, without Whom nothing would be possible. Thank You for the gift of knowledge and the search for truth and for blessing me with a bright and inquisitive mind.

The realization of this dissertation would not have been possible without the help of the following people:

Roan, thank you for being my mentor the past two years. For your willingness to help and your (never-ending..?) patience with me! I really look up to you and I am greatly thankful for all you've done for me.

To Buks, my greatest love! Thank you for always believing in me and for standing by me even when things were tough! We really make the best team in the world! I love you with everything I have.

Mom, thank you for working hard to give me the opportunity to study and for taking care of me and motivating me.

Thank you Nadia, for your help with the dissertation and for always listening and being there for me!

My sincere gratitude goes out to the following people for their contributions: Prof Francois for help with the molecular work in the study and the help with the reviewing of all the chapters, as well as all the (much needed) advice. Dr. Leone Malan for her help and for giving me the opportunity to take part in the SABPA study. To Rudolph, for his help and suggestions with the dissertation. To Mrs. Hettie Sieberhagen for the editing. To my family and all my friends for their love and support!

Abstract:

Recently, an alarmingly high prevalence of hypertension is seen in urbanised black South African communities, compared to their Caucasian counterparts. Therefore a study was organised to assess these lifestyle changes and incidence of hypertension with regards to the individual's reaction to elevated stress levels. This study was dubbed the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study.

The present study (as part of the SABPA study) was initiated to investigate whether this high blood pressure had any influence on a person's oxidative stress profile. Therefore, several tests were carried out on samples from the 200 SABPA participants. The tests consisted of oxidative stress assays, including ROS levels, FRAP values and the GSH concentration in blood. A newly developed PCR and RFLP approach was also followed to screen the samples for a specific single nucleotide polymorphism (SNP) in the tyrosine hydroxylase (TH) gene. This enzyme takes part in catecholamine biosynthesis, which is a pathway activated in times of stress. Along with the assays performed, the daytime ambulatory blood pressure values (08h00 to 18h00) were also obtained.

After analyses were performed, several statistical methods were carried out on the data and results were graphically represented. Preliminary results showed gender differences with regards to oxidative stress parameters and thus all subsequent data was divided for the two genders. Results from the male group in this study support the hypothesised connection between oxidative stress and hypertension, as the ROS levels were higher in hypertensive males than in normotensive males. However, the hypothesised connection between the TH C-824T base change and high blood pressure could not be proven. It was concluded that there is indeed a positive correlation between oxidative stress and hypertension, as hypothesised.

Opsomming:

Onlangs is 'n kommerwekkende hoë voorkoms van hipertensie waargeneem by verstedelike swart Suid-Afrikaanse gemeenskappe, in vergelyking met hul blanke eweknieë. 'n Studie is dus georganiseer om hierdie veranderinge in leefstyl en voorkoms van hipertensie te ondersoek in terme van die individu se reaksie tot verhoogde stresvlakke. Hierdie studie is die Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) studie genoem.

Die huidige studie (soos deel van die SABPA studie) is geïnisieer om te ondersoek of hierdie hoë bloeddruk enige invloed het op 'n persoon se oksidatiewe stresprofiel. Verskillende toetse is uitgevoer met die monsters van die 200 SABPA deelnemers. Hierdie toetse het bestaan uit oksidatiewe stres metings, insluitende ROS-vlakke, FRAP-waardes en die GSH-konsentrasie in bloed. 'n Nuwe PCR- en RFLP-benadering is ook gevolg om 'n spesifieke enkelnukleotied polimorfisme (SNP) in die tirosienhidroksilase (TH) geen aan te toon. Hierdie ensiem neem deel aan katesjolamienbiosintese, 'n metaboliese weg wat geaktiveer word in tye van stres. Saam met die toetse wat uitgevoer is, is die dag ambulatoriese bloeddruk data (08h00 tot 18h00) ook verkry.

Nadat analises uitgevoer is, was verskeie statistiese metodes op die data toegepas. Voorlopige resultate het geslagsverskille aangetoon in terme van oksidatiewe stres parameters en alle daaropvolgende data is dus tussen die twee geslagte verdeel. Resultate van die manlike groep in hierdie studie ondersteun die voorgestelde hipotese van die konneksie tussen oksidatiewe stres en hipertensie, omdat die ROS-vlakke hoër was in hipertensiewe mans as in normotensiewe mans. Die hipotese van die konneksie tussen die C-824T baseverandering en hoë bloeddruk kon nie ondersteun word nie. Die afleiding dat daar 'n positiewe korrelasie is tussen oksidatiewe stres en hipertensie ondersteun wel die gestelde hipotese.

List of Abbreviations:

%	:	percent
$[A\text{-}NH_2\bullet]^+$	:	coloured radical cation of the chromogenic substrate (DEPPD)
® or ™	:	trademark
°C	:	degrees Celsius
µL	:	microlitre
µM	:	micromole
$\bullet O_2^-$	:	superoxide
$\bullet OH^-$	:	hydroxyl radical
$^1O_2$	:	singlet oxygen
A	:	adenine
AIDS	:	acquired immunodeficiency syndrome
$A\text{-}NH_2$	:	chromogenic substrate (DEPPD)
ANOVA	:	analysis of variance
ATP	:	adenosine triphosphate
BMI	:	body mass index
Bp	:	base pairs
BP	:	blood pressure
BSO	:	buthionine sulfoximine
C	:	cytosine
$C_2H_3O_2Na$	:	anhydrous sodium acetate
CAD	:	coronary artery disease
cGMP	:	cyclic guanosine monophosphate
CH_3COOH	:	acetic acid
CO_2	:	carbon dioxide
CytP450	:	cytochrome P_{450}
DBP	:	diastolic blood pressure
DEPPD	:	N,N-diethyl-*para*-phenylenediamine
DNA	:	deoxyribonucleic acid

DOPA	:	dihydroxy phenylalanine
dROM	:	derivatives of reactive oxygen metabolites
dsDNA	:	double-stranded deoxyribonucleic acid
DTNB	:	5,5'-dithiobis-2-nitrobenzoic acid
e^-	:	electron
e.g.	:	for example
EC	:	enzyme commission
ECG	:	electrocardiogram
EDTA	:	ethylenediaminetetraacetic acid
eNOS	:	endothelial nitric oxide synthase
EP	:	epinephrine
et al.	:	and others
EtBr	:	ethidium bromide
FAD	:	flavin adenine dinucleotide
$FeCl_3.6H_2O$	:	ferric chloride
$FeSO_4.7H_2O$	:	ferrous sulphate
FRAP	:	ferric reducing antioxidant power
FSH	:	follicle stimulating hormone
g	:	gravitational force
g	:	gram
G	:	guanine
G Protein	:	guanine nucleotide-binding protein
GR	:	glutathione reductase
GSH	:	reduced glutathione
GSSG	:	oxidised glutathione
H^+	:	hydrogen ion
H_2O	:	water
H_2O_2	:	hydrogen peroxide
HCl	:	hydrochloric acid
HIV	:	human immunodeficiency virus
HSD	:	honestly significant difference
HT	:	hypertensive
i.e.	:	that is

iNOS	:	inducible nitric oxide synthase
NOHLA	:	N^{ω}-hydroxy-L-arginine
JHB	:	Johannesburg
K^+	:	potassium
kDa	:	kilodalton
Kg/m^2	:	kilogram per metre squared
KPO_4	:	potassium phosphate
LC-MS	:	liquid chromatography-mass spectrometry
LH	:	luteinizing hormone
Ltd.	:	private company limited by shares
M	:	metre
M2VP	:	1-methyl-2-vinyl-pyridium trifluoromethanesulfonate
MA.	:	Massachusetts
MDA	:	malondialdehyde
mg	:	milligram
mg/L	:	milligram per litre
min	:	minutes
mL	:	millilitre
mm Hg	:	millimetre of mercury
MPA	:	metaphosphoric acid
MSE	:	mean squared error
$Na.PO_4$	:	sodium phosphate
$NaAc.3H_2O$	:	sodium acetate (hydrous)
$NADP^+$	:	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	:	nicotinamide adenine dinucleotide phosphate (reduced)
NE	:	norepinephrine
NF-κB	:	nuclear factor kappa beta
nm	:	nanometre
nmol	:	nanomoles
nNOS	:	neuronal nitric oxide synthase
NO	:	nitric oxide

NOS	:	nitric oxide synthase
NT	:	normotensive
NWU	:	North-West University
O_2	:	molecular oxygen
O_3	:	ozone
$OONO^-$	:	peroxynitrite
OR.	:	Oregon
OXPHOS	:	oxidative phosphorylation
PCR	:	polymerase chain reaction
Phox	:	phagocytic oxidase
pmoles	:	picomoles
Pty.	:	proprietary limited company
Q_{10}	:	coenzyme quinone ten
RAAS	:	renin angiotensin aldosterone system
Rac	:	ras-related C3 botulinum toxin substrate
RE	:	restriction endonuclease
RFLP	:	restriction fragment length polymorphism
RNS	:	reactive nitrogen species
R-O•	:	alkoxyl radical
R-OO•	:	peroxyl radical
R-OOH	:	generic hydroperoxide
ROS	:	reactive oxygen species
s	:	seconds
S.A.	:	South Africa
SABPA	:	sympathetic activity and ambulatory blood pressure in Africans
SBP	:	systolic blood pressure
SD	:	standard deviation
SHR	:	spontaneously hypertensive rats
SNP	:	single nucleotide polymorphism
SNS	:	sympathetic nervous system
SOD	:	superoxide dismutase
T	:	thymine
TAE	:	tris-acetate-EDTA buffer

Taq	:	*Thermus aquaticus*
TH	:	tyrosine hydroxylase
T_m	:	melting temperature
TPTZ	:	2,4,6-tris(2-pyridyl)-s-triazine
U	:	units
U.K.	:	United Kingdom
U.S.A.	:	United States of America
UV	:	ultraviolet
UVB	:	medium wave ultraviolet
V/cm	:	volt per centimetre
Vit C	:	ascorbic acid (Vitamin C)
Vit E	:	α-tocopherol (Vitamin E)
WT	:	wild type
w/v	:	weight per volume

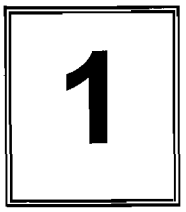
List of Equations:

List of Figures:

List of Tables:

Chapter 1:

Introduction



Recent relocation of black people in South Africa from rural areas to westernised urban areas associates with changes in diet from a traditional diet with high carbohydrate content, to a more western diet with high fatty content. This change goes together with activation of the sympathetic nervous system as a result of stress, which leads to higher catecholamine metabolism and elevated levels of these molecules in the body contributing to oxidative stress. Accompanying the change in diet and physical activity (leading to obesity), is a change in blood pressure, which is influenced by catecholamines and reactive metabolites of oxygen and nitrogen. These black people have higher blood pressures than their Caucasian counterparts (Brewster *et al.*, 2004).

The aim of this study (as part of the SABPA study, where the effect of urbanisation on catecholamine metabolism and hypertension was investigated), was to investigate an association between the oxidative stress profiles of these newly urbanised black South Africans and the incidence of hypertension in this group of people. Such a study has never been done in South Africa, although it has been widely accepted that African people have, on average, higher blood pressure values than Caucasian people.

Along with the blood pressure data on the SABPA participants, several other parameters were measured, and although these parameters were not used in this study, it is nonetheless important data for constructing a reference range of values in the black community in South Africa, which has never been done before.

With the valuable data obtained from this study, future insights into diagnostics and treatment of hypertension in this very unique South African population can be made. This dissertation consists of the following chapters: Some literature background is given in Chapter 2, while in Chapter 3 the materials and methods

used in this study are discussed. In Chapter 4 details are given on the standardisation of a newly developed polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method for detecting the C-824T base change in the human tyrosine hydroxylase gene. Chapter 5 consists of graphical representation of the data and a discussion of the findings. In Chapter 6 the results are discussed and final conclusions are given. Some recommendations are proposed for future studies and the shortcomings of the present study are outlined.

Chapter 2:

Literature review

2

2.1. Oxidative stress.

2.1.1. Introduction:

The unique molecular structure of oxygen enables it to perform its important life-sustaining duties most elegantly. Molecular oxygen (O_2) has two electrons with parallel spins in its outermost orbital. Because of this feature, oxygen can accept four electrons in a tetravalent reduction reaction to form water during aerobic respiration or oxidative phosphorylation (OXPHOS) in the mitochondrion. A by-product of this reaction is adenosine-5'-triphosphate (ATP), the high-energy molecule so vital to life. Ironically, oxygen can also undergo a series of univalent reduction reactions to form potentially very dangerous free radicals. These molecules damage virtually all cellular components from DNA molecules to proteins and lipids and also form important mediators of signal transduction pathways in the cell. Oxygen is thus vital for life, but also takes part in oxidation reactions which produce molecules that do not sustain life. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidising agent. Thus, all organisms living in an aerobic environment are perpetually exposed to free radicals (Singal *et al.*, 1998). An experiment by Lavoisier in 1785, where guinea pigs exposed to high concentrations of oxygen developed congestion of the heart and lungs and died before the oxygen was fully utilised, also demonstrated the harmful effects of oxygen.

Four steps univalently reduce oxygen and cause free radicals to form. These radicals are listed below and the processes of reduction are given in Figure 2.1.

- Superoxide anion ($\bullet O_2^-$)
- Hydrogen peroxide (H_2O_2)
- Hydroxyl radical ($\bullet OH^-$) by means of Fenton chemistry.
- Singlet oxygen ($^1O_2$)

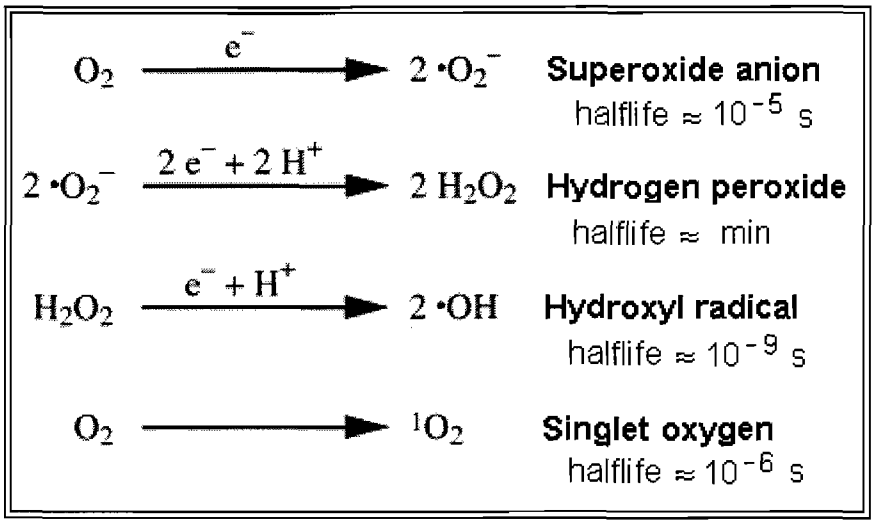


Figure 2.1. Redox reactions and half-lives of some oxygen-derived metabolites. (Adapted from Winkler *et al.*, 1999)

Although the mammalian body is not defenceless against these harmful molecules, in certain pathophysiological instances, the natural enzymatic and non-enzymatic antioxidant systems in the body (that maintain a reducing state in cells) can be overwhelmed by the high concentration of free radicals. In these situations, a build-up of free radicals can result in a systemic oxidising state. This phenomenon is known as oxidative stress and since its discovery, it has drawn much attention from various different scientific disciplines. Oxidative stress has since been implicated in a host of different pathologies from cancer to vascular heart disease and aging (Sies, 1997).

2.1.2. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS):

Free radicals are highly reactive molecules, each with an unpaired electron in its outermost orbital. They are toxic for cells and tissues in the body and cause a myriad of deleterious effects. A fundamental fact about free radicals is that the unpaired electrons in their outer shells do not affect the charge on the resultant molecule. Free radicals can be negatively charged, positively charged or electrically neutral. This is because charge is concerned with the number of negatively charged electrons in relation to the positively charged protons, whereas free radicals are related only to the spatial arrangement of the outer electron (Cheeseman and Slater, 1993).

The free radicals derived from molecular oxygen constitute the largest concentration of free radicals in the cell and are therefore the most important class of such substances in living systems, although not the only class. Reactive Nitrogen Species (RNS) are also produced. ROS, and other free radicals can be produced endogenously, but can also be acquired from exogenous sources (Dreher and Junod, 1996).

2.1.2.1. Endogenous sources of oxidative species:

The most significant amount of ROS formed endogenously can be attributed to the leakage of activated oxygen from mitochondria during normal oxidative respiration. Mitochondria produce about 2-3 nmol of superoxide/min per mg of protein (Valko *et al.*, 2006). Although the mitochondrial electron transport chain is a very efficient system, the very nature of the alternating one electron oxidation-reduction reactions it catalyses, predisposes each electron carrier to side reactions with molecular oxygen. Superoxide is considered the primary ROS and subsequent reactions with other molecules generate secondary ROS, such as hydrogen peroxide and the hydroxyl radical.

Xanthine oxidase is a large enzyme that is widely distributed among species and within various types of tissues. It is a member of the molybdenum iron-sulfur flavin hydroxylases. It catalyzes the hydroxylation of hypoxanthine to xanthine (a purine) and from xanthine to uric acid, reactions which also produce superoxide and hydrogen peroxide (Hille and Nishino, 1995).

Each molecule of this enzyme is composed of a 20 kDa domain containing two iron sulfur centers, a central 40 kDa flavin adenine dinucleotide (FAD) domain, and an 85 kDa molybdopterin-binding domain with the four redox centers aligned in an almost linear fashion (Enroth *et al.*, 2000).

NADPH (nicotinamide adenine dinucleotide phosphate) oxidase is a membrane bound enzyme complex, found in the plasma membrane of phagocytes and other cells, made up of six subunits:

- A rho guanosine triphosphate (GTPase) (rac 2 or rac 1)
- Five phagocytic oxidase (phox) units:
    - p91phox
    - p22phox
    - p40phox
    - p47phox
    - p67phox

The structure of NADPH oxidase is quite complex, consisting of two membrane-bound elements (p91phox and p22phox), three cytosolic components (p67phox, p47phox and p40phox), and a low-molecular-weight G protein (either rac 2 or rac 1). Activation of NADPH oxidase is associated with, and probably caused by, the migration of the cytosolic components to the cell membrane so that the complete oxidase can be assembled (Babior, 2004).

This enzyme complex generates superoxide by transferring electrons from NADPH in the cell, across the membrane, to molecular oxygen.

Equation 2.1. The enzymatic reaction catalysed by NADPH oxidase.

$$NADPH + 2O_2 \leftrightarrow NADP^+ + 2{\bullet}O_2^- + H^+$$

Nitric oxide (NO) is a radical with several functions in the cell. A suitable level of NO has beneficial abilities, such as protection of the liver against ischemic damage. On the other hand, chronic overproduction of NO can contribute to various carcinomas and inflammatory conditions (Tylor *et al.*, 1997). The major function of NO is vasodilation by means of relaxation of the smooth muscle cells (Kajiya *et al.*, 2007). NO induces relaxation by activating guanylate cyclase, which results in increased intracellular cGMP. This inhibits calcium entry into the cell. K^+ channels are activated and hyperpolarization and relaxation are induced. NO also stimulates a cGMP-dependant protein kinase that activates the enzyme, myosin

light chain phosphatase. Myosin light chains are then dephosphorylated, which leads to muscle relaxation, regulation of blood pressure and vasomotor tone, platelet aggregation and adhesion, neurotransmission, and killing of bacteria, viruses, and tumor cells. It has also been suggested to inhibit various neutrophil functions, such as adhesion and migration, and activation of neutrophil NADPH oxidase (van der Vliet and Cross, 2000).

The enzyme responsible for production of NO from arginine and oxygen is nitric oxide synthase (NOS), a dimeric calmodulin dependant Cytochrome P_{450} hemoprotein. There are three known isoforms of this enzyme. Neuronal NOS (nNOS) is found in nervous tissue and produces NO with a function of cell communication. Inducible NOS (iNOS) is found in cells in the immune system and is expressed after stimulation by cytokines, lipopolysaccharides and other immunologically relevant agents. It produces NO that takes part (as a free radical) in the fight against pathogens that invade the body. Endothelial NOS (eNOS) produces NO in blood vessels and is involved in vascular function, in particular, vasodilation (Knowles and Moncada, 1994). Two moles of O_2 and 1.5 moles of NADPH are consumed with every one mole of NO produced. This enzyme catalyses the conversion of L-arginine to NO and citrulline in two consecutive reactions. A by-product of the first reaction is N^{ω}-hydroxy-L-arginine (NOHLA), which is used in the second reaction as substrate. The enzyme contains two bound flavin cofactors and a bound heme.

Equation 2.2. Formula of the first reaction in the enzymatic function of NOS.

$$\text{L-Arg} + \text{NADPH} + H^+ + O_2 \rightarrow \text{NOHLA} + NADP^+ + H_2O$$

Equation 2.3. Formula of the second reaction in the enzymatic function of NOS.

$$\text{NOHLA} + \tfrac{1}{2}\,\text{NADPH} + \tfrac{1}{2}\,H^+ + O_2 \rightarrow \text{L-citrulline} + \tfrac{1}{2}\,NADP^+ + NO + H_2O$$

Cytochrome P_{450} (CytP450), part of a group of highly diverse detoxification enzymes, has a very complicated biological function. Numerous CytP450 isomers exist which catalyse a whole host of reactions and with hundreds and thousands of organic and xenobiotic substrates - this is one of the most biologically versatile group of enzymes. Reduced CytP450 isozymes seem to be a major intracellular source of ROS, as demonstrated by Siraki *et al.* in 2002. These scientists

confirmed that endogenous ROS formation is inhibited by CytP450 inhibitors. The CytP450 catalytic mechanism involves reductive activation of molecular oxygen by electrons supplied by CytP450 reductase and NADPH (Coon *et al.*, 1998). The product of oxygen reduction is mostly water, but uncoupling of the CytP450 catalytic cycle results in autoxidation of the oxycytochrome P450 complex to form $\bullet O_2^-$, and protonation and decay of the P450 peroxy complex to form H_2O_2 (Kuthan and Ullrich, 1982).

The peroxisome is an organelle (discovered by Christian de Duve in 1967) found in virtually all eukaryotic cells where it participates in detoxification pathways, doing away with various toxins in the cell, such as toxic peroxides. It also participates in the metabolism of fatty acids (β-oxidation). Its structure is made up of a lipid bilayer plasma membrane with a crystalline core. It contains certain oxidative enzymes, such as catalase, D-amino acid oxidase and uric acid oxidase. The enzymes in peroxisomes utilise molecular oxygen to remove hydrogen atoms from specific organic substrates, producing hydrogen peroxide (H_2O_2). This reactive molecule is quickly used by catalase to oxidise other substrates, and thus the potentially dangerous hydrogen peroxide is removed from the cellular environment (Schluter *et al.*, 2007).

The harmful effects of free radicals are used to the advantage of the body in the immune system by the phagocytes and inflammatory cells. These cells generate various types of oxidising species as a central part of their mechanism of killing pathogens, such as bacterial cells. Activated phagocytes are capable of producing ROS as well as RNS, e.g. superoxide anion, nitric oxide and their reactive product, peroxinitrite (Nathan and Shiloh, 2000). This sudden release of oxidising agents after activation by immunogenic substances, is called the 'Oxidative Burst'. An advantageous characteristic of these molecules that are aids in the function of the immune cells, is that these free radicals act in a non-specific way, damaging anything they come into contact with. This feature may not seem helpful, but it does enable the immune cells to attack any type of pathogen in a non-specific way. This prevents the escape of a pathogen by mutation of a single molecular target (McCord, 2000).

Not all endogenous sources of ROS and RNS are enzymatic. Some organic compounds can also produce reactive species. Of these compounds, the ubiquinones (e.g. coenzyme Q_{10}) are the most important (They are so called for their ubiquitous nature in the body). These molecules, among others, are present as electron acceptors in the electron transport chain of mitochondria. They can undergo a redox cycle with their conjugate semiquinones and hydroquinones, catalysing the production of superoxide and, subsequently, hydrogen peroxide (Lass *et al.,* 1997).

The generation of free radicals is coupled with the concentration and involvement of redox active metals, such as iron and copper, found in many classes of proteins. Although strict regulation mechanisms ensure that there is no free intracellular iron, an excess of superoxide causes the release of free iron from iron-containing molecules. The released iron can then participate in Fenton chemistry, thereby producing the highly reactive hydroxyl radical from hydrogen peroxide (Valko *et al.*, 2006). This radical has a half-life of less than one nanosecond, suggesting that it reacts close to its formation site.

Emotional stress in humans is associated with an increase in biomarkers for oxidative stress. Emotional stress increases catecholamine metabolism (by activation of the sympathetic nervous system), which in turn increases oxidative stress by increasing the production of free radicals. Accordingly, cognitive tension as well as sleep deprivation is linked to a lower antioxidant capacity. It has been seen that children with autism also present with higher markers of oxidative stress (isoprostane, a marker of lipid damage) as a result of psychological stress (Ming *et al.*, 2005). In contrast to this, it has also been found that meditation practitioners have higher antioxidant enzyme levels and lower oxidised lipid levels (Schneider *et al.*, 1998).

Several studies have suggested the role of psychological stress in the formation of ROS and thus the appearance of an oxidative stress situation in the body. In a study done by Yamaguchi *et al.* in 2002, it was shown that an oxidative metabolite of billirubin was elevated during times of psychological stress. In other studies, emotional stress enhanced oxidative stress by an increase of plasma superoxide

levels (Cernak *et al.*, 2000). Following emotional stimuli, levels of catecholamines increase in cerebral circulation, which induces an increase in systolic blood pressure and therefore it is possible that psychological stress can induce mild cerebrum ischemia reperfusion injuries and thus oxidative stress (Yamaguchi *et al.*, 2002).

2.1.2.2. Exogenous sources of oxidative species:

It is widely known that oxidants in tobacco smoke exist in sufficient amounts to play a major role in injuring the respiratory tract. These include various aldehydes, epoxides, peroxides and other free radicals that may be sufficiently long lived in order to survive till they cause damage to the alveoli and surrounding tissues (Carnevali *et al.*, 2003).

Radiotherapy may cause tissue injury by means of free radicals. Ionizing radiation consists of electromagnetic radiation (photons), including X-rays and gamma rays, and particulate radiation, such as electrons, protons, and neutrons. Radiation damages cells by direct ionisation of DNA and other cellular targets and by indirect effect through ROS (Borek, 2004).

Chaung and colleagues (2007) investigated the effect of urban air pollution on oxidative stress. In this study it was concluded that urban air pollution is associated with inflammation, oxidative stress, blood coagulation and autonomic dysfunction simultaneously in healthy young humans, with sulfate and ozone (O_3) as two major traffic-related pollutants contributing to such effects.

Although $O_3$is not a free radical, it is a very powerful oxidising agent. It contains two unpaired electrons and degrades under physiological conditions to •OH, suggesting that free radicals are formed when ozone reacts with biological substrates (Ueno *et al.,* 1998).

Chronic exposure to sunlight, particularly medium wave ultraviolet (UVB) radiation, causes among other harmful effects, an increase in the production of ROS and other free radicals. These molecules can overwhelm the elaborate antioxidant

system in the cutaneous tissue leading to oxidative damage and ultimately to skin cancer, immune suppression and premature aging (Katiyar *et al.*, 2001).

There are many sources of free radicals and reactive species in the body, and some sources outside the body. It is a widely known fact that these molecules wreak havoc with biological molecules and cause all sorts of damage. Fortunately, the body is not defenceless against these radicals and their deleterious effects. There are various defence mechanisms present in the body. Of these, antioxidants are most important and will be discussed in the following section.

2.1.3. Antioxidants:

An antioxidant is a substance that prevents or slows the oxidation of other molecules. Antioxidants scavenge free radicals by donating electrons to the free radical molecule, thus neutralising it and stopping the chain reaction of radical formation. The antioxidant molecule, however, is oxidised in the process and forms the radical derivative of the antioxidant molecule. Thus, antioxidants inhibit the oxidation of other important biological molecules by being oxidised themselves. Antioxidants are normally reducing agents, such as thiols or polyphenols (Halliwell, 2000). Figure 2.2 illustrates the types of antioxidants and their functions.

In living cells there are two major classes of antioxidants, namely enzymatic and non-enzymatic and these classes are either water soluble (hydrophilic) or lipid soluble (hydrophobic). Hydrophilic antioxidant molecules neutralise free radicals in the cytoplasm and extracellular fluid and hydrophobic antioxidants work in the lipid bilayer of the plasma membrane, while other antioxidant molecules are metal chelators that remove free transition metal ions, so they cannot participate in reactions that produce free radicals like the hydroxyl radical. The functionality of one antioxidant will therefore depend largely on the workings of the other antioxidants in the system (Sies, 1997).

The best-studied cellular antioxidants are the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase. Superoxide generated by various processes is first converted to hydrogen peroxide by superoxide dismutases. The

hydrogen peroxide is then removed by catalases and peroxidases. These enzymes function together and their contribution to the defence against oxidative stress can be hard to separate (Ho *et al.*, 1998).

SOD is an enzyme, found in almost all eukaryotic cells and in extracellular fluid, that catalyses the conversion of superoxide into oxygen and hydrogen peroxide. There are three types of SOD's in the body and they all contain metal ion cofactors. SOD found in the cytosol is the copper/zinc isoform, while manganese SOD is found in the mitochondria. The third form is present in the extracellular fluids and contains copper and zinc cofactors (Zelko *et al.*, 2002).

Catalases convert hydrogen peroxide to water and oxygen in the peroxisomes, with either iron or manganese as cofactor (Chelikani *et al.*, 2004).

Glutathione peroxidase is a selenium-containing enzyme that catalyses the conversion of hydrogen peroxide to water together with the conversion of reduced glutathione (GSH) to its oxidised form (GSSG) (Mezes *et al.*, 2003).

Equation 2.4. Enzymatic reaction of glutathione peroxidase.

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

Gluthathione reductase then reduces the GSSG (with NADPH as an electron acceptor) to from GSH, completing the cycle.

Equation 2.5. Enzymatic reaction of glutathione reductase.

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

Non-enzymatic antioxidants include ascorbic acid, or Vitamin C, a water-soluble antioxidant that reduces radicals and can also participate in recycling oxidised Vitamin E (α-tocopherol) molecules. It cannot be synthesised by humans and is thus obtained from the diet. Vitamin E is a major lipid soluble antioxidant and protects against lipid peroxidation in the membranes. Glutathione is a tripeptide (glutamyl-cysteinyl-glycine) with a free sulphydryl group that is a prime target for oxidation by free radicals (Wu *et al.*, 2004). GSSG can then be recycled by

glutathione reductase to revert back to GSH. In a healthy organism, more than 90 % of the total glutathione is in the reduced form. A decrease in the ratio of GSH to GSSG is indicative of oxidative stress. Adding to the above-mentioned three molecules, there are various other molecules that act as antioxidants. Examples include uric acid, bilirubin, flavonoids, α-lipoic acid and carotenoids (Willcox *et al.*, 2004).

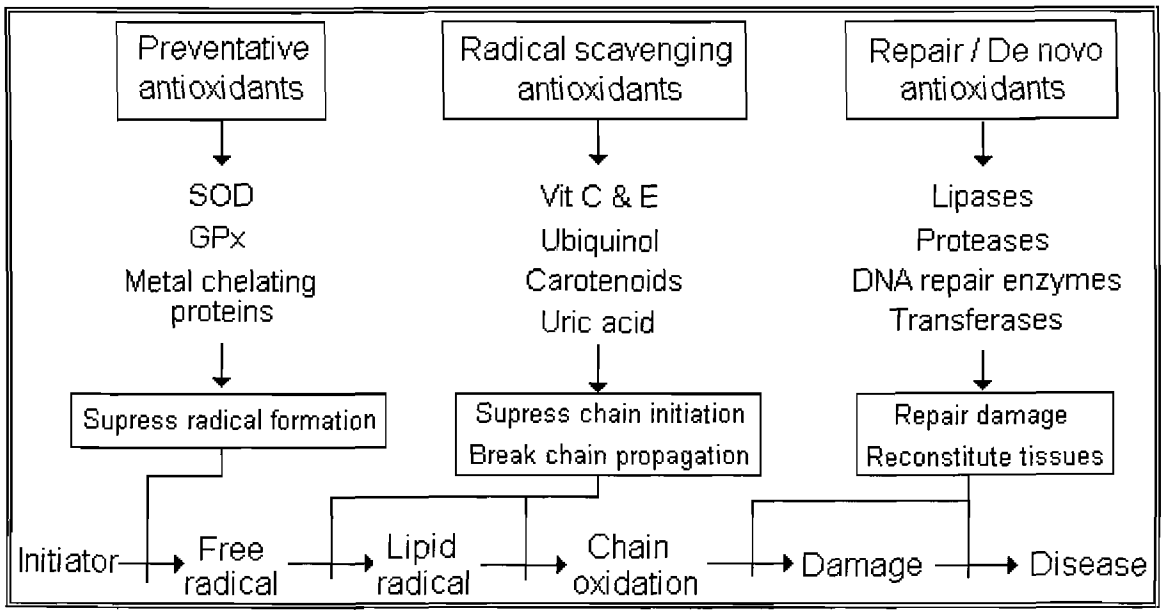


Figure 2.2: Antioxidant groups and actions against free radical formation.
(Adapted from Willcox *et al.*, 2004)

The therapeutic and preventative properties of antioxidants have been extensively researched, although many results and conclusions remain largely controversial (Bjelakovic *et al.*, 2007). Several similar studies, of which some are described next, have been reported where hypertension were involved.

In a study by Midaoui and de Champlain (2002), it was reported that the anti-hypertensive action and the prevention of insulin resistance by lipoic acid appeared to be due to its antioxidative properties. These properties are seen in Figure 2.3. In the study it was proposed that this was because it prevented the rise in oxidative stress, as reflected by normalisation of the superoxide anion production in the aorta and the prevention in the fall in activity of glutathione peroxidase in glucose fed rats.

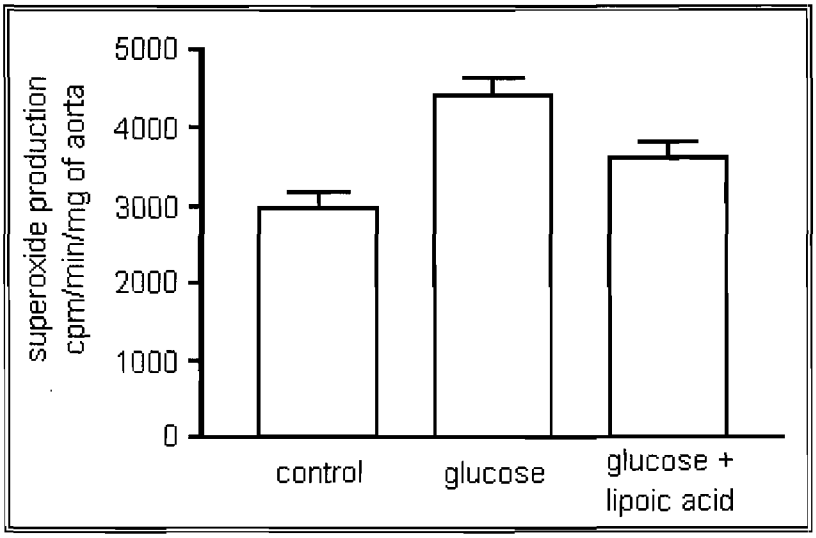


Figure 2.3. Results of Midaoui and de Champlain showing the antioxidative properties of lipoic acid (LA). (Adapted from Midaoui and de Champlain, 2002).

Levels of ROS scavengers have been reported to be depressed in hypertensive patients (Sagar *et al.*, 1992). However, Vitamin C recovers endothelial function by restoring the NO-mediated vasodilation of the endothelium in hypertensive patients (Taddei *et al.*,1998).

In a study done by Vaziri *et al.* (2000) rats were subjected to oxidative stress by glutathione depletion. This was achieved by means of the GSH synthase inhibitor buthionine sulfoximine (BSO) in drinking water for two weeks, after which a threefold decrease in tissue GSH content, as well as a marked elevation in blood pressure and a significant reduction in NO bioavailability was observed (Figure 2.4). In a group of rats given BSO and also Vitamin E-fortified chow and Vitamin C-supplemented drinking water, the hypertension was ameliorated and urinary metabolites of NO were improved, but no changes were seen in the control group. These findings suggest a therapeutic role of antioxidants against oxidative stress-induced hypertension.

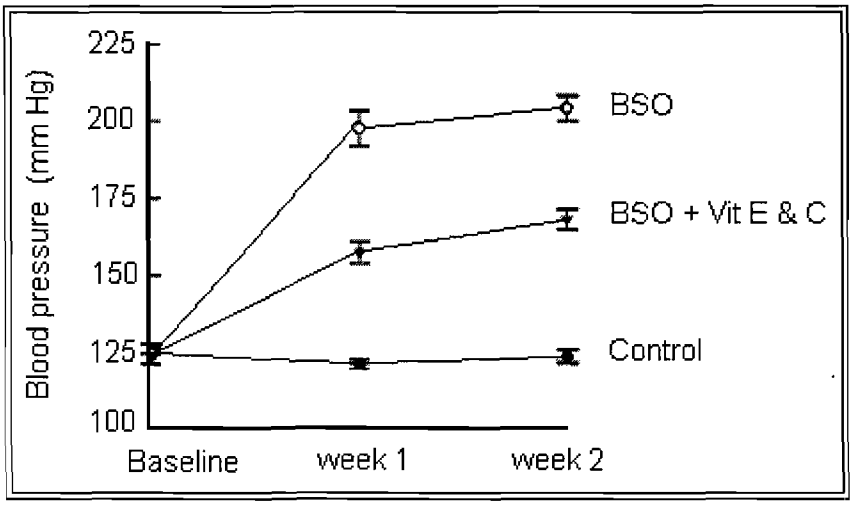


Figure 2.4. Results from Vaziri *et al.* showing increase in systolic blood pressure (mm Hg) in rats exposed to oxidative stress. (Adapted from Vaziri *et al.*, 2000)

2.1.4. Oxidative stress in pathology:

ROS production and excess free radicals in the body (oxidative stress) are thought to contribute to a variety of different diseases including neurological diseases (neurodegeneration like Alzheimer's and Parkinson's diseases) and other diseases related to aging, diabetes and cardiovascular diseases (hypertension, atherosclerosis). A short overview of the various cardiovascular diseases in which oxidative stress is involved or contributes to, will be given here.

In a study done by Ramakrishna and Jailkhani (2007), it was seen that higher values of protein carbonyl groups and malondialdehyde (MDA) as lipid peroxides were observed in diabetic patients, accompanied by a slight reduction in NO synthesis. An increase in lipid and protein oxidation was also seen together with a decrease in antioxidant levels. Hyperglycaemia stimulates the overproduction of free radicals and consequently increases protein and lipid peroxidation. Increased production of ROS has been linked to glucose oxidation, glycation of proteins and subsequent oxidation of these glycated proteins which contribute to the complications associated with diabetes (Maritim *et al.*, 2003).

Contributing factors in essential hypertension include increased sympathetic nervous system activity (Reaven *et al.*, 1996), perhaps as a result of heightened exposure to psychosocial and emotional stress (Levenstein *et al.*, 2001), overproduction of sodium retaining hormones and vasoconstrictors (Alarcon,

2006), high sodium intake (Koepke and DiBona, 1985), inadequate dietary intake of potassium and inappropriate renin secretion with resultant overproduction of Angiotensin II (activator of NAD(P)H oxidase) (Touyz, 2000). Reduction of NO production and resultant overproduction of reactive species, mainly superoxide, may promote endothelial dysfunction (Higashi *et al.*, 2002). At physiological conditions, both ROS and NO exert beneficial effects and can function as second messengers (Abraham *et al.*, 1998). Thus, a balance between ambient levels of superoxide and the release of NO has a critical role in the maintenance of normal endothelial function. Endothelial dysfunction plays an important role in the development of cardiovascular diseases. An important pathogenetic factor for the development of endothelial dysfunction is lack of NO, which is a potent endothelium-derived vasodilating substance (Von Haehling *et al.*, 2003).

Atherosclerosis is currently thought to be the consequence of a subtle imbalance between pro- and antioxidants (Cook, 2006). NO plays a crucial role throughout the coronary artery disease (CAD) spectrum, from its biosynthesis to the outcome after acute events. Defective eNOS-driven NO synthesis contributes to the development of major cardiovascular risk factors (insulin resistance, arterial hypertension and dyslipidaemia). eNOS is expressed in skeletal muscle, where it is involved in metabolic processes, and in the vascular endothelium, where it regulates arterial pressure (Stamler *et al.*, 1994). In a study done by Duplain *et al.* (2001) the link between eNOS and the control of the metabolic action of insulin was investigated. It was found that eNOS deficient mice were hypertensive and had a 40 % lower insulin-stimulated glucose uptake than the control group. These results suggest that eNOS is crucial for the control of arterial pressure, but also for the control of glucose homeostasis.

Recently, five polymorphisms in the p22phox promoter region has been discovered that results in over expression of this subunit of NADPH oxidase in the vascular wall of the spontaneously hypertensive rat (SHR). These findings suggest that the presence of polymorphisms in the promoter region of the p22phox gene might contribute to up regulation of p22phox in the vessel wall of SHR. Increased expression of this gene is also attenuated by SOD's in hypertensive rats, suggesting a role for superoxide in the regulation of p22phox expression. There

has also been evidence of strong binding sites for NF-κB in the strong positive regulatory region of the rat p22phox promoter (Zalba *et al.*, 2001).

In a study done by Landmesser *et al.* (2002), the role of NAD(P)H oxidase in vascular oxidative stress and hypertension caused by Angiotensin II was investigated. It was found that mice deficient in the p47phox subunit of the NAD(P)H oxidase enzyme did not show marked increases in blood pressure after infusion with Angiotensin II (a known vasoconstrictor and thus inducer of high blood pressure). Infusion of Angiotensin II in wild type mice increased systolic blood pressure and increased vascular superoxide production two- to three-fold. These results suggest a crucial role of NAD(P)H in vascular oxidative stress and blood pressure response to Angiotensin II infusion *in vivo*.

According to Touyz (2004), evidence at multiple levels suggests a role for oxidative stress in the pathogenesis of hypertension. In human hypertension, markers of systemic oxidative stress are increased, while treatment with a SOD mimetic or other antioxidants, improves vascular and renal function, and reduces blood pressure. Mouse models deficient in ROS-producing enzymes have generally lower blood pressures when compared to wild-type equals. Furthermore, infusion of Angiotensin II fails to induce hypertension in these mice (Landmesser *et al.*, 2002). In another study, an experimental model with compromised antioxidant capacity developed hypertension (Tanito *et al.*, 2004).

Various studies support the association between hypertension and increased oxidative stress; however, there is still a debate whether oxidative stress is a cause or consequence of hypertension. Studies on animals have generally supported the hypothesis that increased blood pressure is associated with increased oxidative stress, but studies on humans have been conflicting. Oxidative stress (perhaps through decreased bioavailability of NO) promotes vascular smooth muscle cell proliferation, leading to thickening of the vasculature. Oxidative stress also directly damages the endothelium and impairs its function. It is worth mentioning that treatment with drugs that lower blood pressure is associated with a drop in oxidative stress, which does seem to suggest that oxidative stress is not a cause, but rather a consequence of hypertension (Grossman, 2008).

Superoxide and NO can combine in a very rapid and favourable reaction to form peroxynitrite ($OONO^-$), enhancing *per se* proatherogenic mechanisms (leukocyte adherence, impaired vasorelaxation, platelet aggregation) (Cook, 2006), and can also oxidise arachidonic acid to form F_2-isoprostanes that exert potent vasoconstrictor effects (Pryor and Squadrito, 1995). Many clinicians have argued that essential hypertension must be related to the renin-angiotensin system. Studies have shown that Angiotensin II can stimulate oxidative stress, which could activate several vasopressor mechanisms which could potentiate the vasoconstrictor effect of Angiotensin II (Rajagopalan *et al.*, 1996). Angiotensin has been shown to stimulate production of superoxide which quenches NO. Additionally Angiotensin II may also stimulate endothelin production. Therefore, the decreased NO, increased isoprostane, and increased endothelin represent potent vasoconstrictor effects that can enhance the vasopressor action of Angiotensin II and may explain how hypertension is maintained in pathophysiological conditions (Romero and Reckelhoff, 1999).

2.2. Hypertension.

2.2.1. Introduction:

Hypertension or high blood pressure is a medical condition in which the blood pressure is chronically elevated. There are several types of hypertension with a myriad of different causes, and although several studies have shown possible causes, a single clear cause has never been elucidated. It is therefore often referred to as "essential hypertension".

According to, among others, Mari Hudson (2006), by the year 2010 more South Africans will die from heart-related conditions than from AIDS. More than six million South Africans suffer from high blood pressure, a risk factor for heart disease, and this figure is on the increase.

There are several different classification systems globally that provide guidelines for blood pressure measurement and normal ranges and cut-off points for hypertension. Therefore, choosing which system to go by can be a challenge for

clinicians and researchers alike. However, the ranges for ambulatory blood pressure measurement as given by O'Brien *et al.* (2005) on behalf of the European Society of Hypertension will be used in this study. In their paper, the following table was given for reference values:

Table 2.1. Reference values for blood pressures according to O'Brien *et al.* (2005).

	Blood pressure value (mmHg)		
	Optimal	Normal	Abnormal
Awake	<130/80	<135/85	>140/90
Asleep	<115/65	<120/70	>125/75

Types of hypertension include essential (primary) hypertension and secondary hypertension. Primary hypertension designates that no specific cause of the high blood pressure can be found. In these cases, which include up to 90 % of diagnosed hypertensive patients, there are several contributing risk factors:

Lifestyle: People who experience chronic elevated psychological stress are prone to hypertension (possibly through activation of the sympathetic nervous system and production of catecholamines that raise blood pressure). High alcohol intake and smoking also increase the risk for hypertension and other cardiovascular diseases (MacMahon, 1987).

Physical inactivity: Although too much exercise can also cause hypertension, an active lifestyle is crucial for keeping a healthy blood pressure range. Obesity is also associated with high blood pressure, where a body mass index (BMI) greater than 25 kg/m^2 is considered to be a risk factor (Bell *et al.*, 2002).

Diet: Diets with high sodium levels can cause salt sensitivity. When there is a high concentration of sodium in the bloodstream, cells will release water to balance the osmotic differences. As a result, the pressure in the arteries rises. Salt sensitivity is a phenomenon seen more commonly in African people. In a study done by Campese *et al.* (1991) on 17 black and nine white patients with essential hypertension, it was observed that a higher percentage of the black patients were

salt sensitive. In contrast, all of the white patients were salt resistant after treatment with a low sodium diet for nine days, followed by a high sodium diet for 14 days.

Age: Over time, collagen fibres accumulate in the arterial wall and the artery stiffens. This can lead to a raised blood pressure (McEniery *et al.,* 2007).

Oxidative stress: Several studies have indicated the role of oxidative stress (directly or indirectly) in the pathogenesis of hypertension as discussed in Section 2.1.4.

2.2.2. The catecholamines:

The catecholamines, as illustrated in Figure 2.5, are chemical compounds produced from the amino acid, tyrosine. They contain amines and catechol groups and act as hormones and neurotransmitters in the body. Tyrosine can be produced from phenylalanine by the enzyme phenylalanine hydroxylase, and is ingested in the diet. The catecholamines include 3,4-dihydroxy-l-phenylalanine (l-DOPA), dopamine, norepinephrine (NE) and epinephrine (EP).

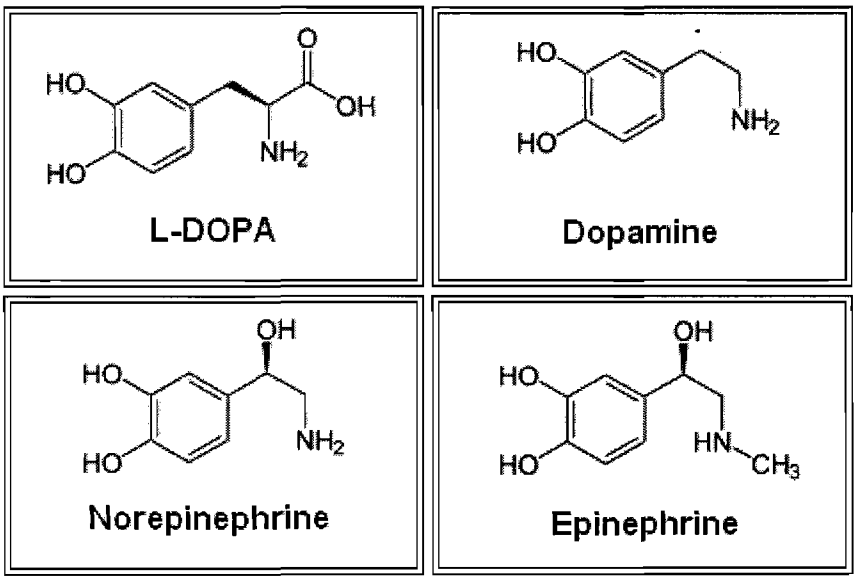


Figure 2.5. The catecholamines.

The catecholamine biosynthesis pathway starts with conversion of tyrosine to 3,4-dihydroxy-l-phenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH),

using O_2 as substrate and tetrahydrobiopterin as cofactor (Shiman *et al.*, 1971) and releasing water and dihydrobiopterin. L-DOPA is then converted by the enzyme DOPA-decarboxylase into dopamine, releasing CO_2 in the process. Dopamine-β-hydroxylase enzyme catalyses the subsequent conversion of dopamine into NE by hydroxylation of the dopamine molecule. This enzyme uses O_2 and dopamine as substrates and ascorbic acid as cofactor and releases water and dehydro-ascorbic acid during the reaction. Phenylethanolamine-*N*-methyltransferase is the last enzyme in the pathway. It uses S-adenosyl-methionine to convert NE into EP and releases homocysteine in the process (Nagatsu, 1991).

Cells that produce catecholamines include the chromaffin cells in the adrenal medulla and post ganglionic cells of the sympathetic nervous system and organs with sympathetic innervations, such as the heart, blood vessels and the brain (Pyatskowit and Prohaska, 2007). Catecholamines also circulate in the blood. The production of catecholamines is up-regulated by input from the sympathetic nervous system (SNS). After activation by various stimulants and stressors, the SNS releases acetylcholine which binds to receptors on postganglionic neurons. These neurons are then stimulated to produce and release higher concentrations of catecholamines, in particular, NE and EP, which can then bind to various adrenergic receptors on peripheral tissues to elicit the effects of the so-called fight-or-flight (sympatho-adrenal) response (Jansen *et al.*, 1995). These effects include pupil dilation, increased heart rate and a sudden rise in blood pressure. The SNS is thought to function without conscious input. For example, moments before waking, sympathetic outflow spontaneously increases, which can account for the sudden rise in blood pressure in the morning after waking. It is possible that chronic stimulation of the SNS, for example by the stress associated with urbanised living, can cause hypertension through constant high concentrations of catecholamines in circulation and the radical products of their break-down (Goldstein, 1983).

2.2.3. Tyrosine hydroxylase (TH):

TH (also known as tyrosine 3-monooxygenase) catalyses the rate-limiting step in the catecholamine biosynthesis pathway. Because of this fact, it has been extensively researched. TH is a homotetrameric mixed-function oxidase that uses molecular O_2 and tyrosine as its substrates and tetrahydrobiopterin as its cofactor. It catalyses the addition of a hydroxyl group to the meta position of tyrosine, thus forming L-DOPA. Stimulation of the adrenergic nerves, for instance in times of stress or emergencies, increases TH activity, thus speeding up the formation of catecholamines to function in response to these stressors (Plut *et al.*, 2002).

Several polymorphisms have been identified in the TH gene, and the implications in biochemical and physiological manifestations have been characterised. In a study done by Rao *et al.* (2007) on the genetics of this enzyme, it was seen that human catecholamine secretory traits are heritable, showing pleitropy with autonomic activity (and thus sympathetic activity, as the SNS is part of the autonomic nervous system) and with blood pressure. They discovered a single nucleotide polymorphism (SNP) in the promoter region of the TH gene that causes overexpression of the enzyme, resulting in higher concentrations of circulating catecholamines and a lowered baroreceptor function (which is the function of various stretch-sensitive baroreceptors in the carotid and aortic sinuses that regulate blood pressure by negative feedback). With this altered baroreflex, the changes in blood pressure associated with stressors are exaggerated and result in hypertension (Rao *et al.*, 2007).

Rao and colleagues (2007) found that the most common base change in the human TH gene is in the promoter region at position -824 (C→T). This single nucleotide polymorphism (SNP) and its associated haplotype had shown the strongest pleiotropy, increasing both norepinephrine secretion and blood pressure during stress. This heritable mutation is thought to be a (genetic) causative factor of essential hypertension.

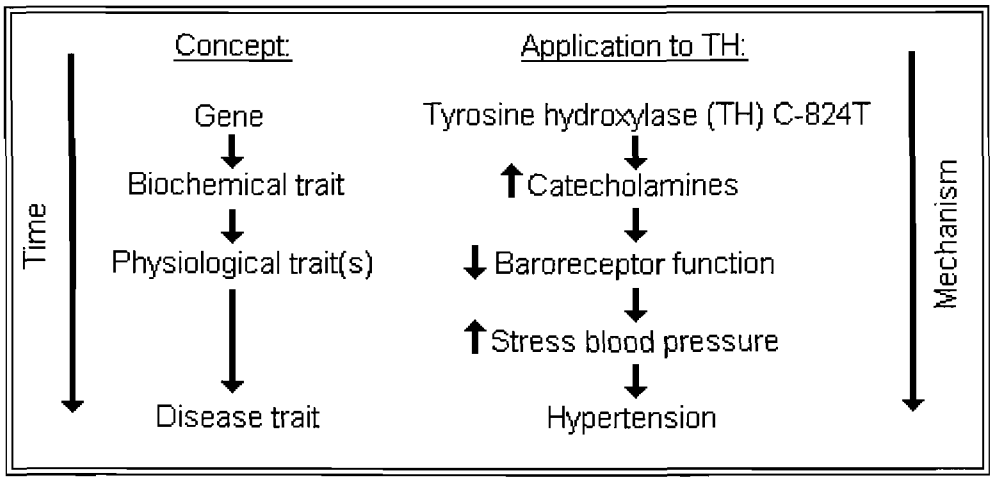


Figure 2.6. Flow diagram of events that lead to hypertension with the C-824T TH polymorphism. (Adapted from Rao *et al.*, 2007)

These results are also consistent with findings from Goldstein (1983), where the results indicated that decreased baroreflex-cardiac sensitivity, increased sympathetic outflow, and pressor hyper-responsiveness tend to occur together in some patients with essential hypertension.

2.2.4. Hypertension and South Africans of African origin:

Walker (1972) predicted that increasing urbanisation and a rise in socio-economic status in developing populations would increase their proneness to obesity, hypertension, diabetes and stroke. As we shall see, this prediction has since become reality.

As discussed before, hypertension is a multifactorial disease and less than a third of patients with high blood pressure are adequately treated (Moore and Williams, 2002; Whelton *et al.,* 2004). Hypertension occurs more frequently and is generally more severe in black persons than in Caucasian persons, leading to excess cardiovascular morbidity and mortality (Brewster *et al.*, 2004). Almost three quarters of the worldwide population with hypertension are in developing countries, with this occurrence fuelled by urbanisation (Kaplan and Opie, 2006).

With urbanisation, black South Africans undergo a nutritional transition from traditional, rural, carbohydrate-rich food with a low glycaemic index to a diet high in fat and poor quality carbohydrates, often found in so-called "fast foods". This

causes obesity, one of the risk factors for cardiovascular diseases, and hypertension. Apart from the change in diet, urbanisation is also associated with higher levels of psychosocial stress that accompany a modern lifestyle (Malan *et al.*, 2006). One can see this in the fact that unwesternised societies like the San (bushmen) in Southern Africa have blood pressures that do not increase with age, but for most others, the stress of modern life is difficult to avoid (Kaplan and Opie, 2006).

Hypertension in sub Saharan Africa is a widespread problem of immense economic importance because of its high prevalence in urban areas, its frequent underdiagnosis and the severity of its complications. The African Union has called hypertension one of the continent's greatest health challenges after AIDS (Opie and Seedat, 2005). In a study done by Poulter *et al.* (1985) in a Ghanaian rural community, it was observed that blood pressures rose steadily with age, probably due to westernisation. Thus, the migration of people from rural areas to urban settings of Nairobi was associated with an increase in blood pressure.

Hypertensive African subjects show a greater sensitivity to the pressure effects of norepinephrine. This sensitivity is enhanced by a high sodium diet (Dimsdale *et al.*, 1987). High sodium intake has been known to activate many pressor mechanisms, including an increase in intracellular calcium, a rise in plasma catecholamines and a worsening of insulin resistance (Schutte *et al.*, 2003). In the Hypertension Detection and Follow-up Programme in the U.S.A., it was seen that the subjects of lower socio-economic group had a higher prevalence of hypertension and also a lower level of education, suggesting a certain level of ignorance in developing communities (Tyroler, 1989).

2.3. The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study.

This project is the first study done on black South Africans to evaluate their coping styles and catecholamine metabolic markers contributing to higher sympathetic output and poorer psychosocial wellbeing. Lifestyle diseases such as diabetes and hypertension are generally associated with urbanisation, especially in Africans.

The South African Demographic and Health Survey in 2006 indicated that hypertension and type two diabetes in the black African population group are a major source of concern, and the scientific measurement of stress as precipitating factor for hypertension is rarely reported in South Africa (Steyn *et al.*, 2006).

The purpose of this study was to investigate biological markers associated with higher SNS activity in urbanised teachers with a specific coping style. There is clearly a need for examining lifestyle changes and its influence on health, contributing to a decreased quality of life of black South Africans, as well as a need for a research project in order to identify the psycho-physiological interaction of coping styles with sympathetic activity markers (cardiovascular, inflammatory and stress hormone) indices in black as well as in white urbanised Africans. Understanding the contributing role of associated factors to an enhanced sympathetic vascular activity, the lack of knowledge about the reactions of individuals to changes in the environment could be addressed.

This project will investigate the interaction of sympathetic activity and a specific coping style in urbanised subjects. Understanding the contributing factors of an enhanced sympathetic activity might lead to focused prevention and intervention strategies for lifestyle diseases such as hypertension.

This study was assembled to include inputs from a multidisciplinary team of experts from both the health, natural and social sciences at the North-West University, Potchefstroom Campus. The main hypothesis in the SABPA study was that increased sympathetic nervous system activity (as reflected by the renin-angiotensin II-aldosterone system (RAAS), stress profile, catecholamine metabolites, obesity, inflammatory markers and certain coping styles) promote vascular dysfunction, hypertension and metabolic syndrome prevalence in urbanised black South Africans.

To assess this hypothesis the relationship between lifestyle changes and increased sympathetic nervous system activity as well as vascular dysfunction in black urbanised South African teachers was investigated. Specific attention was given to the link between coping and renin-angiotensin-aldosterone system, stress

profile, catecholamine metabolites, obesity and inflammatory markers, cardiovascular and metabolic syndrome indicators. Results from this study could lead to recommendations to be used by health professionals for early preventative methods in the development of hypertension.

The SABPA study was approved by the ethics committee of the North-West University and was given the number: NWU-00036-07-S6.

2.4. Problem statement and hypothesis.

Recently an urbanisation trend has manifested in black communities in South Africa. This changing lifestyle brings about a change in diet and an increase in psychosocial and emotional stress. As described in Sections 2.1 and 2.2, this can lead to increased oxidative stress. There is a need to investigate the effect that this trend has on the oxidative stress profile of these communities. It is also important to investigate how it relates to hypertension prevalence, which is a big problem in South Africa, especially in the North-West province (Opie and Seedat, 2005).

Based on this knowledge, the following hypotheses were formulated in this study: Firstly, an increase in ROS values and thus oxidative stress may be seen in participants with hypertension. An increase in blood pressure may therefore be associated with an increase in oxidative stress markers. Secondly, according to the literature the C-824T mutation in the TH gene is associated with a rise in blood pressure, therefore participants with the mutation may have higher blood pressures.

2.5. Strategy and approach.

To investigate the role of oxidative stress in hypertension, as part of the SABPA study mentioned in Section 2.3, a battery of tests was assembled to measure the oxidative stress profile of the 200 black South African teachers who participated in the SABPA study. The strategy for this study is summarized in Figure 2.7 and will be described in detail in forthcoming chapters. The tests used to evaluate oxidative stress included the ROS assay, the FRAP assay and the GSH/GSSG ratio on blood samples (Chapter 3). In addition to the oxidative stress profile, a SNP analysis for the C-824T mutation in the TH promoter was also done for each participant (Chapter 4). Blood pressure analyses were performed by investigators at the School for Physiology, Nutrition and Consumer Sciences at the Potchefstroom campus of the North-West University. Although in the SABPA study a 24-hour blood pressure measurement was taken with the Cardiotens apparatus, only the day measurements were used (i.e. from 08h00 to 18h00 in half-hour increments). The pooled results from blood pressure values, oxidative stress markers and mutation analysis will be valuable in investigating the putative correlation that may exist between oxidative stress and hypertension.

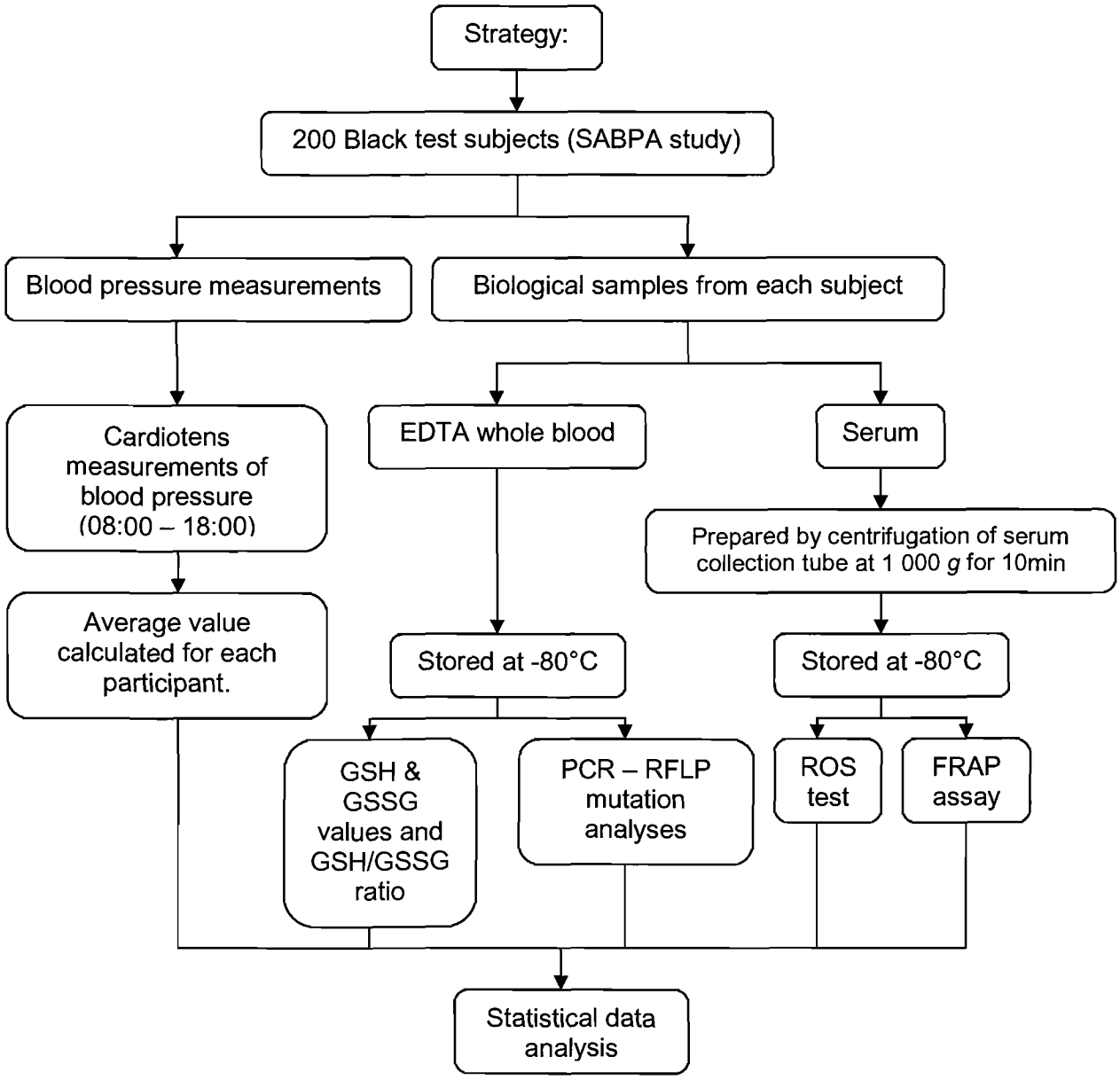


Figure 2.7. Visual representation of the strategy used in this study.

Chapter 3:

Materials and Methods

3

3.1. The SABPA study: Participants and methodological approach.

The SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) study commenced in February 2008 on apparently healthy, black urbanised South African school teachers (99 men and 101 women, aged 25–60 years). In this project sympathetic activity responses at baseline and when exposed to two laboratory stressors (i.e. the colour-word conflict chart and the cold pressor test) were measured. However, for the present study, only baseline/resting values were measured.

Recruitment was done in November 2007 and all potential subjects were given a standard information sheet at a screening visit prior to the start of the study. After selection, the details of the study were given in the participant's home language and they were given the opportunity to ask questions. All subjects willing to proceed were requested to sign the study informed consent form. The study was approved by the Research Ethics Committee of the North-West University.

Data collection for the participants involved two consecutive days (15 minutes on day one and 2 hours on day two). To avoid the occurrence of the white-coat effect, a 24 hour blood pressure and ECG measurement were taken with the Cardiotens apparatus (CEO120 Meditech). The apparatus was fitted on the first day at 07:30 am and the educators continued with their daily activities. At 16:30 pm on the first day, participants were transported from their schools to an overnight Metabolic Unit Research Facility of the North-West University (research unit for human studies and equipped with 10 well furnished bedrooms, a kitchen, two bathrooms and a living room). After a standard dinner, all the participants completed a set of questionnaires including a general socio-demographic health questionnaire.

At 06:00 am on the second day, the Cardiotens apparatus was removed and a fasting midstream urine sarnple obtained. Thereafter anthropometric measurements were taken. Measurements were taken in triplicate according to standardised methods. These included measurement of waist- and hip circumference with a Holtain non-stretchable flexible 7 mm metal tape. Subjects were weighed using a Precision Health Scale, and their heights were determined using the Invicta Stadiometer (IP 1465, UK). A finger prick for fasting blood glucose and HIV/AIDS (Firstly, the First Response Kit test and secondly, when positive, the Pareekshak test) were done. Uncitrated saliva cortisol and catecholamine metabolites samples (Sarstedt Salivette®, Dickerson and Kemeny, 2004) were obtained. Resting blood samples were obtained from the antecubital vein with a winged infusion set for preparation of serum, EDTA and citrated plasma.

3.2. Sample collection and storage.

All biological samples in this study were obtained from the 200 black test subjects of the SABPA study from February to July, 2008. All the samples were fasting, resting samples and consisted of resting samples of EDTA whole blood (drawn in purple top EDTA tubes), resting blood samples drawn in yellow top serum collection tubes and a fasting, midstream urine sample (first urine of the day). Whole blood samples were stored in appropriately numbered 1.5 mL Eppendorf® centrifuge tubes. 50 µL and 100 µL aliquots were stored separately for the GSH and GSSG assays, respectively. 10 µL of scavenger (see Section 3.4.3) was added to the GSSG sample and vortexed for ten seconds before freezing. Serum was prepared by centrifuging the yellow top serum collection tube for 10 minutes at 1000 x *g* at room temperature. The serum was removed and stored in appropriately numbered 1.5 mL Eppendorf® centrifuge tubes. All blood samples were stored at -80 °C until the analysis could be performed. Urine samples were kept on ice for transportation to the laboratory and were then stored at -20 °C.

3.3. PCR and RFLP mutation analysis for the detection of the C-824T SNP in the human tyrosine hydroxylase gene.

3.3.1. Introduction:

A protocol for PCR amplification of a region of the promoter of the **TH** locus, as well as restriction fragment length polymorphism (RFLP) was developed to screen the 200 test subjects from the SABPA study for the TH C-824**T** SNP only. The restriction endonuclease (RE) best suited to show the C-824T SNP, is *Tru*1I (*Mse*I). This RE cuts at the following sequence: 5'..T↓TAA..3'. It can be seen from Appendix A, that the only occurrence in the amplicon (after PCR) of this particular sequence is at the mutation site. This RE will therefore only digest the fragment if the SNP is present. The resulting digested fragments will also be of different sizes, so that results can easily be interpreted on a 2 % agarose gel.

3.3.2. Primer design:

An RFLP-approach (restriction fragment length polymorphism) was followed to test the participants for the C-824T SNP. Firstly, primers were designed to amplify an estimated 1000 bp region containing the mutation area. The primers were designed using Primer 3, version 0.4.0 (Rozen and Skaletsky, 2000) keeping the following critical parameters in mind: Firstly, the length of the primers should be 17 – 28 base pairs long with a GC content of between 50 and 60 %. The T_m values of the primers should be similar and between 55 °C and 65 °C. Lastly, there should be minimum self-complimentarity (hairpin formation) within the primers and no complimentarity between the forward and reverse primers to prevent primer-dimer formation. Using these parameters, the following primers were designed (Table 3.1):

Table 3.1. Sequence of PCR primers utilised for RFLP analysis.

Primer	Primer sequence (5' – 3')	T_m (°C)	Size (bp)
TH Forward	ccaggggctattgttgaaga	60.4	20
TH Reverse	caggccgtgtgtcttgtaga	62.5	20

T_m indicates the average calculated melting temperature in °C according to OLIGONUCLEOTIDE PROPERTIES CALCULATOR (2003), Inqaba Biotechnical Industries (supplier).

3.3.3. The optimised PCR and RFLP reactions:

The PCR reaction was optimised by altering the annealing temperature as well as the volume of EDTA blood used per reaction (Section 4.2). After the PCR reaction was optimised, the RFLP method was optimised by altering the reaction buffer, the buffer capacity as well as the incubation times for the restriction analysis (Section 4.3).

The final PCR and RFLP conditions used for the detection of the C-824T SNP in the TH gene can be summarised as follows: The PCR mixture consisted of 25 µL KAPA Blood Direct ReadyMix (KAPA Biosystems; Boston, MA., U.S.A.), 23.5 µL PCR grade water (Bioline; Taunton, MA., U.S.A.), 1 µL TH Forward primer (20 pmoles; Inqaba Biotechnical Industries (Pty) Ltd; Pretoria, Gauteng, S.A.), 1 µL TH Reverse primer (20 pmoles; Inqaba Biotechnical Industries) and 0.5 µL EDTA treated blood. Table 3.2 summarizes the PCR conditions for the amplification of the TH fragment in the Thermo Hybaid®[1] Multiblock System 0.2 G thermocycler.

Table 3.2. PCR conditions for the amplification of a fragment from the TH gene.

PCR step	# of cycles	Action	Temperature (°C)	Duration (min)
1	1	Denaturation	95	10:00
2	30	Denaturation	94	00:30
		Annealing	61	00:30
		Extension	72	00:30
3	1	Extension	72	05:00
4	1	Cooling	4	Hold

The RFLP analysis consisted of 2 µL Buffer R (supplied with the restriction endonuclease), 7.8 µL PCR grade water, 0.2 µL *Tru*1 RE (10 Units/µL; Fermentas

[1] Thermo Hybaid® is a registered trademark of the Hybaid Limited, Ashford, Middlesex, United Kingdom.

Life Sciences; Burlington, Ontario, Canada) and 10 µL PCR product containing the amplicon. No clean-up step was used to purify the amplicon prior to the restriction analysis. The PCR mixture was transferred directly from the PCR tube to the new restriction analysis tube with a micropipette. The reaction mixture was incubated for 4 hours at 65 °C in the Thermo Hybaid® Multiblock System 0.2G thermocycler.

Restriction analysis of the amplicons was observed by separation of these products with agarose gel electrophoresis. A 2 % (w/v) agarose gel was prepared with TAE buffer (consisting of 40 mM Tris base, 40 mM acetic acid, 1 mM EDTA). A loading dye containing bromophenol blue (0.05 % w/v) and glycerol (30 %) was used to assist with the loading of the products onto the agarose gel. The voltage applied varied according to the size of the gel, with an average of 10 $V.cm^{-1}$. The fragments were visualized using a UV fluorescence transilluminator at 254 nm with the use of ethidium bromide (EtBr, 0.5 µg/mL gel). The results were photographed with a ChemiGenius^{Q} Bio imaging system[2] from Syngene, using GeneSnap version 6.05 (2004) as bio imaging software.

3.4. The total Glutathione BIOXYTECH® GSH/GSSG-412™ assay.

3.4.1. Introduction:

In this study both GSH and GSSG were measured in whole blood. To have an accurate view of the state of oxidation vs. antioxidant power in the body, and thus a measure of the oxidative stress status, a ratio of GSH/GSSG was used. In times of oxidative stress the ratio would theoretically be lower, as a result of accumulation of oxidised glutathione (GSSG).

3.4.2. Principle of the method:

This method uses Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid or DTNB), which reacts with GSH to form a spectrophotometrically detectable product at 412 nm. In the same way, GSSG can be measured by first reducing GSSG to GSH, followed by reaction with Ellman's reagent. This method is based on the article from Tietze (1969), where the change in colour development during the reaction is

[2] ChemiGenius^{Q} Gel documentation systems from Syngene, a division of Synoptics Ltd., Cambridge, England.

proportional to the concentration of GSH or GSSG, depending on the sample (The reactions of this method are given in Figure 3.1).

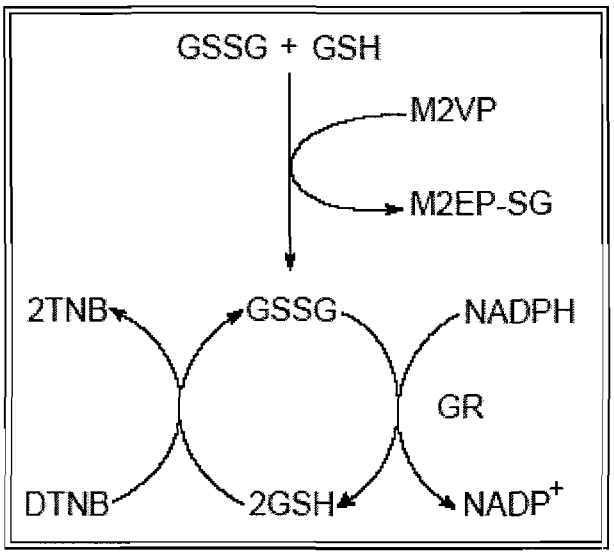


Figure 3.1. Schematic representation of reaction used for GSH/GSSG analysis. In this figure the principle of the method is given as a series of reactions where GSSG is the total oxidised glutathione, GSH is the total reduced glutathione, M2VP is the scavenger included in the kit, DTNB is Ellman's reagent (described above) and GR is the enzyme glutathione reductase.

These assays were done using the BIOXYTECH®[3] GSH/GSSG-412™ kit from *Oxis*Research™, a division of OXIS Health Products Inc. (Portland, OR., U.S.A.) The method described in the standard protocol included with the kit was followed, however some modifications were made. The method was modified for use in a 96 well plate format. Although some volumes have been adapted, the principles and final concentrations in the assay remained the same.

The following reagents are included in the kit: Assay buffer ($Na.PO_4$ with EDTA), GSSG buffer ($Na.PO_4$ with EDTA), the enzyme glutathione reductase (GR) in $Na.PO_4$ with EDTA, NADPH (β-Nicotinamide adenine dinucleotide phosphate) with Tris base and mannitol, the GSH scavenger 1-Methyl-2-vinyl-pyridium trifluoromethanesulfonate[4] (M2VP) in HCl and chromogen (5.5'-Dithiobis-2-nitrobenzoic acid) (DTNB) in $Na.PO_4$ with EDTA and ethanol. Standards were GSSG in KPO_4 buffer with EDTA. Each GSSG molecule is equivalent to two GSH molecules (Table 3.3).

[3] BIOXYTECH® is a registered trademark of OXIS International, Inc.
[4] US Patent 5,543,298

Table 3.3. Concentrations of the standards included in the GSH/GSSG kit.

	GSSG, µM	GSH, µM
1	0.000	0.00
2	0.050	0.10
3	0.125	0.25
4	0.250	0.50
5	0.750	1.50
6	1.500	3.00

Reagents and materials used, but not provided in the kit included metaphosphoric acid (MPA) (Sigma-Aldrich (Pty) Ltd., Aston Manor 1630, South Africa) with a final concentration in the assay of 5 % (w/v). The plate reader used was a Bio-Tek® FL600 microplate fluorescence reader with filter set at 410 nm.

3.4.3. Procedure:

Note:

The NADPH (12.5 mg in 7.5 mL Assay buffer) and MPA (2.8986 g in 20 mL Milli-Q®[5] water) were prepared fresh before every assay.

Sample preparation: (As from standard protocol.)

GSSG sample:

Whole blood (100 µL) was added to 10 µL M2VP (scavenger) in a centrifuge tube. After vortex mixing for 10 seconds, the samples were frozen at -80 °C until use. The samples were thawed, mixed and incubated at room temperature for 2-10 min. Cold 5 % (w/v) MPA (290 µL) was added to each tube, followed by immediate vortex mixing for 15-20 seconds and centrifugation at 1 000 x *g* for 10 minutes at 4 °C. After centrifugation, 50 µL of the MPA extract was added to 700 µL GSSG buffer and the diluted extract kept on ice until use.

GSH sample:

Whole blood (50 µL) was carefully added to the bottom of a centrifuge tube and frozen at -80 °C until use. The samples were thawed and 350 µL of cold 5 % (w/v)

[5] Milli-Q® is a registered trademark of Millipore Corporation, Billerica, MA, U.S.A. It refers to water treated with a Milli-Q® Ultrapure Water Purification System as per manufacturer's instructions.

MPA was added to each tube, followed by immediate vortex mixing for 15-20 seconds and centrifugation at 1 000 x *g* for 10 minutes at 4 °C. After centrifugation, 12.5 µL of the MPA extract was added to 750 µL assay buffer and the diluted extract kept on ice until use.

Assay: (Same for GSH and GSSG samples)

All samples were done in triplicate to ensure accurate results. 50 µL of diluted extract was added to 50 µL glutathione reductase (1 U) and 50 µL (75 µM) DTNB (chromogen). The mixture was incubated for 5 minutes at room temperature. The reaction was initiated by addition of 50 µL (0.25 mM) NADPH to a final volume of 200 µL. The change of absorbance was immediately measured at 412 nm for 3 minutes at room temperature. The concentrations of the analyte in the sample are given in µM. The formulae to calculate the concentrations of GSH_t, GSH and GSSG are given in Equations 3.1 – 3.3. After calculation of the concentration of the GSH and GSSG, the ratio was then calculated and the formula is given in Equation 3.4.

Equation 3.1. Formula for calculating the total glutathione concentration. The dilution factor for GSH is 488 X 2 = 976, as the standard series is given in GSSG concentration.

$$GSH_t = \frac{Net\ rate - Intercept}{Slope} \bullet Dilution\ Factor$$

Equation 3.2. Formula for calculating the total oxidised glutathione concentration. The dilution factor for GSSG is x 60.

$$GSSG = \frac{Net\ rate - Intercept}{Slope} \bullet Dilution\ factor$$

Equation 3.3. Formula for calculating the total reduced glutathione concentration.

$$GSH = GSH_t - GSSG$$

Equation 3.4. Formula for calculating the ratio of GSH/GSSG.

$$Ratio = \frac{GSH_t - 2GSSG}{GSSG}$$

3.5. The Ferric Reducing Antioxidant Power (FRAP) assay.

3.5.1. Introduction:

The FRAP assay is used to measure the reducing ability of serum, in other words, the antioxidant capacity of the serum. This value gives an accurate view of the antioxidant status of an individual. This method is based on the method published by Benzie and Strain, 1996.

3.5.2. Principle of the method:

Ferric to ferrous ion reduction at low pH causes a coloured ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range of complex mixtures of antioxidants (such as the antioxidant mixture in serum).

3.5.3. Reagents, buffers and solutions:

3.5.3.1. Reagents:

Sodium acetate ($NaAc.3H_2O$) was purchased from BDH AnalaR® (Merck; Darmstadt, Germany). Glacial acetic acid (CH_3COOH) was purchased from Saarchem[6] (Merck). Hydrochloric acid (HCl) was purchased from Bio-Zone Chemicals. Ferrous sulphate ($FeSO_4.7H_2O$) was purchased from Labchem. 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and Ferric chloride ($FeCl_3.6H_2O$) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). All reagents used were of highest purity.

3.5.3.2. Buffers and solutions:

For the reaction buffer, 1.55 g sodium acetate was dissolved in 8 mL acetic acid and made up to a volume of 500 mL with Milli-Q® water for a final concentration of 300 mM. 312.4 mg TPTZ was dissolved in 100 mL (40 mM) HCl for a final concentration of 10 mM. 4.56 mg ferric chloride ($FeCl_3.6H_2O$) was dissolved in 10 mL acetate buffer (300 mM) for a final concentration of 1.687 mM. Ferrous sulphate standard (10 mM; 2.78 mg/mL) aliquots were diluted 100x in Milli-Q® water for a working solution of 0.1 mM for the standard series. The freshly

[6] Saarchem (PTY) Ltd. is a company of Merck, JHB, South Africa.

prepared FRAP reagent used in the assay was made up as follows: 25 mL acetate buffer with 2.5 mL TPTZ and 2.5 mL ferric chloride.

3.5.4. Procedure:

A standard series was prepared in duplicate in a 96 well plate as follows:

Table 3.4. FRAP standard series constructed from the 0.1 mM diluted stock of $FeSO_4$.

Standard:	1	2	3	4	5	6
µL $FeSO_4$ (0.1 mM)	0	20	40	60	80	100
µL H_2O	100	80	60	40	20	0
Final Volume (µL)	**100**	**100**	**100**	**100**	**100**	**100**

To each well, 15 µL sample (serum) was added to 85 µL Milli-Q® water for a final volume of 100 µL. Of the FRAP reagent, 250 µL (prepared freshly for each assay) was added to each well with a 12 channel pipette. After a three minute incubation period the absorbance was measured at 593 nm and the concentrations extrapolated from the standard series. The formula for calculating the concentration of antioxidants (in µM) is given by Equation 3.5.

Equation 3.5. Formula for calculating the concentration of antioxidants in serum.

$$Concentration = \frac{Value\ from\ plate\ reader}{(15 \times 100)}$$

3.6. The Reactive Oxygen Species (ROS) assay.

3.6.1. Introduction:

The dROM test has widely been used to test for reactive oxygen species (ROS) in serum (Cesarone *et al.*, 1999). An improved version of the dROM test as proposed by Hayashi *et al.* in 2007 was modified by Botha in 2007 (unpublished results) and named the ROS assay. This assay allows high throughput and automated analysis of numerous serum samples with high reproducibility, consistent accuracy and much smaller amounts of sera and reagents than with the conventional dROM test.

3.6.2. Principle of the method:

This method is based on the fact that in an acidic medium, ROS will react with transition metals, such as iron, to form alkoxyl and peroxyl radicals. These radicals can then oxidize an additive, such as DEPPD, to its corresponding radical cation and the concentration of the cation can be measured at 505 nm or 546 nm. Equation 3.6 shows the reactions of the system.

Equation 3.6. The reactions/principle of the ROS assay system.

$$1.\ R\text{-}OOH + Fe^{2+} \rightarrow R\text{-}O\bullet + Fe^{3+} + OH^{-} \text{ or}$$

$$R\text{-}OOH + Fe^{3+} \rightarrow R\text{-}OO\bullet + H^{+} \text{ or}$$

$$R\text{-}O\bullet + [Fe{=}O]^{2+} + H^{+}.$$

$$2.\ R\text{-}O\bullet \text{ or } R\text{-}OO\bullet + A\text{-}NH_2 \rightarrow R\text{-}O^{-} \text{ or } R\text{-}OO^{-} + [A\text{-}NH_2\bullet]^{+}$$

R-OOH, R-O•, R-OO• and A-NH_2 are generic hydroperoxides, the alkoxyl radical of a generic hydroperoxide, the peroxyl radical of a generic hydroperoxide and DEPPD (chromogenic substrate), respectively. $[A\text{-}NH_2\bullet]^{+}$ is the coloured radical cation of the chromogenic substrate.

3.6.3. Reagents, buffers and solutions:

3.6.3.1. Reagents:

N,N-diethyl-*para*-phenylenediamine (DEPPD), sodium acetate (anhydrous) ($C_2H_3O_2Na$) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich. Ferrous sulphate ($FeSO_4.7H_2O$) was purchased from Labchem (Edenvale, S.A.). All reagents were of highest purity.

3.6.3.2. Buffers and solutions:

For the reaction buffer, sodium acetate was dissolved in Milli-Q® water for a concentration of 0.1 M. The pH was adjusted to 4.8 with acetic acid. N,N-diethyl-*para*-phenylenediamine (DEPPD) was dissolved in 0.1 M sodium acetate buffer for a final concentration of 100 mM (R_1). Ferrous sulphate was dissolved in 0.1 M sodium acetate buffer for a final concentration of 4.37 µM (R_2). Hydrogen peroxide was diluted with Milli-Q® water for constructing a standard series at various concentrations (0, 60, 120, 180, 240 and 300 mg/L).

3.6.4. Procedure:

In a well of a 96 well plate, 140 µL sodium acetate buffer (0.1 M; pH 4.8) was added. To that, 2.5 µL of sample (serum) or standard (hydrogen peroxide) was added. Then 100 µL of a mixed solution, prepared from R_1 and R_2 at a ratio of 1:25 was added with a 12 channel pipette. The plate was then incubated for at room temperature for 1 minute. The absorbance was then measured with a Bio-Tek® [7] FL600 microplate fluorescence reader (Filter: 560/15 nm) at 546 nm for a fixed time from 1 to 10 minutes at 1 minute intervals. ROS serum levels are extrapolated from the H_2O_2 standards. The formula for calculating ROS units is given in Equation 3.7. (1 Unit = 1.0 mg H_2O_2 / L).

Equation 3.7. Formula for calculating ROS units.

$$1\,Unit = 1.0mg\ H_2O_2 / L$$

3.7. Statistical analysis and presentation of the data.

STATISTICA version 8.0, StatSoft, Inc. (2007) was used to perform statistical analyses on the data and to schematically represent the data. Firstly, the oxidative stress data of the males and females was compared to determine if gender differences in oxidative stress existed. Since the study population consisted of 99 males and 101 females, one can consider the sample size as large enough for the Central Limit Theorem to hold, i.e. that the arithmetic mean of a large enough sample is normally distributed even though the data set is not normally distributed (Steyn *et al.,* 1994). Box plots were drawn and a Student's T-test was used to detect statistical differences between the males and females. A p-value < 0.05 was considered statistically significant throughout this study.

Since oxidative stress/antioxidant capacity differed by gender (Section 5.2), the subjects could not be pooled and in all subsequent data analyses, were performed separately in males and females. Each gender was divided into normotensive and hypertensive according to their daytime ambulatory blood pressure (08h00 – 18h00). Subjects were regarded as hypertensive if their blood pressure was ≥140 and/or ≥90 mmHg. The mean values for a given parameter were calculated for

[7] BIO-TEK® is a registered trademark of Bio-Tek instruments, Inc. Winooski, VT, U.S.A.

the normotensive and hypertensive groups and the Student's T-test was performed to determine differences.

Test subjects were additionally divided into three groups according to their genotype for the C-824T TH SNP, independent of hypertensive status: normal (wild type), heterozygous and homozygous for the SNP. A one-way analysis of variance (ANOVA) was performed on the data to detect differences between the mean values of the three groups for each of the blood pressure and oxidative stress parameters. If a statistically significant difference was detected with the ANOVA, a Post hoc analysis (Tukey HSD for unequal groups) was performed to determine between which of the three groups the statistically significant difference exists. Pearson correlations were performed between blood pressure and markers of oxidative stress. To interpret the strength of the relationship, Cohen (1977) gives the following guidelines: r = 0.1 (small effects); r = 0.3 (medium effects that can be considered visible to a researcher) and r = 0.5 (a large effect that can be considered practically significant to a researcher).

Chapter 4:

Optimisation of the RFLP analysis to detect the C-824T SNP in the human tyrosine hydroxylase gene

4

4.1. Introduction.

Tyrosine hydroxylase (TH) (EC number 1.14.16.2) is the enzyme responsible for catalyzing the rate-limiting step in the catecholamine biosynthesis pathway. Several mutations have been characterised in this gene. Rao *et al.* (2007) identified the C-824T single nucleotide polymorphism (SNP) as the most common base change in this gene. This SNP was present in 60.3 % of the 25 sub-Saharan African participants investigated by Rao *et al.* (2007). The mutation was connected to altered catecholamine secretion and was also the most significant predictor of diastolic blood pressure. Haplotypes of the four most common promoter SNP's (C-824T, G-801C, A-581G and G-494A) were formed and it was found that haplotype 2 [TGGG] was the most common in black participants (34.6 %) (Rao *et al.*, 2007).

Since the C-824T mutation in the TH gene was positively associated with increased diastolic blood pressure in sub-Saharan Africans (Shih and O'Connor, 2008), it was decided to screen the SABPA participants for this SNP. A novel restriction fragment length polymorphism (RFLP) analysis was developed which involves polymerase chain reaction (PCR) amplification of the region of the gene containing the SNP, followed by enzyme digestion and gel electrophoresis.

The sequence of the TH gene was obtained from GenBank (Appendix A). The gene is 13329 bp long and consists of 13 exons and 12 introns. The PCR reaction was designed to amplify the region containing the C-824T mutation. A fragment length of ±1000 bp was chosen because it and the resulting fragments can easily be separated and visualised on standard gel electrophoresis applications with commonly used DNA size markers. After PCR amplification, an RFLP approach

was followed to show the presence of the SNP. A restriction endonuclease (RE) was chosen that would cut the amplicon when the base change is present. If the amplicon did not contain the specific base change (C-824T), the RE would not be able to cut the amplicon at all. Since the amplicon sequence of the wild type would be 5' TCAA 3' and the sequence of the mutation 5' TTAA 3', *Tru*1I (or *Mse*1) was chosen for the RFLP analysis. This enzyme cuts at 5' T↓TAA 3' and would therefore cut amplicons containing the mutation (C-824T), but not the wild type that does not contain the mutation.

As shown in Appendix A, the position of the primers was chosen to amplify a region containing the mutation, but not with the base change in the middle of the amplicon. The rationale behind this was that if the amplicon contained the base change, the amplicon would be cut by the RE, resulting in two fragments of different lengths. If the fragments were identical in length, it would not be possible to separate them on a 2 % agarose gel. The primers were therefore chosen to amplify a 1013 bp fragment (containing the SNP). If the mutation is present in the amplicon, *Tru*1I would cut the amplicon in two fragments, 668 bp and 345 bp long. These fragments can easily be separated on a 2 % agarose gel. People that do not have the SNP in either alleles of the gene (wild type) will only show the 1013 bp fragment (uncut), whereas people who have the SNP in one of their TH gene (heterozygous for SNP) will produce the 1013 bp uncut fragment as well as the 668 bp and 345 bp fragments. In the case of people who have the SNP in both alleles, digestion with the RE will result in the 668 bp and 345 bp fragments and no 1013 bp fragment. In this way, the genotypes for the C-824T SNP on the TH gene of participants can be identified.

4.2. Optimisation of PCR to amplify a region of the human TH gene containing the C-824T SNP.

As no PCR method for amplification of this particular gene target has been described in the literature, a new PCR method was developed. In order to standardise the PCR, numerous parameters were altered, adjusted and optimised before the method would be used to screen the SABPA test subjects for the C-824T SNP in the TH gene.

4.2.1. Optimisation of the annealing temperature:

For the PCR reaction to work optimally, the primers have to anneal at a temperature where it will anneal well but also specifically to its target. The melting temperature (T_m-value) gives an indication where 50 % of the primer is annealed. A temperature gradient was done on the thermal cycler to determine the optimum annealing temperature needed for optimal amplicon formation. Ten different temperatures were chosen, ranging from 52.6 °C to 61.9 °C, flanking the calculated T_m values of the primers. If the annealing temperature is too low, non-specific binding of the primers can occur, resulting in the formation of unwanted fragments. If the annealing temperature is too high, it may result in lower product yield.

The PCR mixtures contained 25 µL KAPA Blood Direct ReadyMix, 23.5 µL PCR grade water, 1 µL TH Forward primer, 1 µL TH Reverse primer and 1 µL EDTA treated blood. Table 4.1 summarises the PCR conditions for the amplification of the TH fragment in the Thermo Hybaid®[8] Multiblock System 0.2 G thermocycler.

Table 4.1. PCR conditions for the amplification of a fragment from the TH gene

PCR step	# of cycles	Action	Temperature (°C)	Duration (min)
1	1	Denaturation	95	10:00
2	40	Denaturation	94	00:30
		Annealing	61	00:30
		Extension	72	00:30
3	1	Extension	72	05:00
4	1	Cooling	4	Hold

The PCR products were separated using agarose gel electrophoresis. A 2 % (w/v) agarose gel was prepared in TAE buffer (consisting of 40 mM Tris base, 40 mM acetic acid, 1 mM EDTA) containing ethidium bromide (0.5 µg/ml gel). The DNA fragments were visualised using a UV fluorescence transilluminator at 254 nm which visualises the intercalated ethidium bromide into the double stranded DNA

[8] Thermo Hybaid® is a registered trademark of the Hybaid Limited, Ashford, Middlesex, United Kingdom.

fragments. Figure 4.1 shows the results from the annealing temperature optimisation.

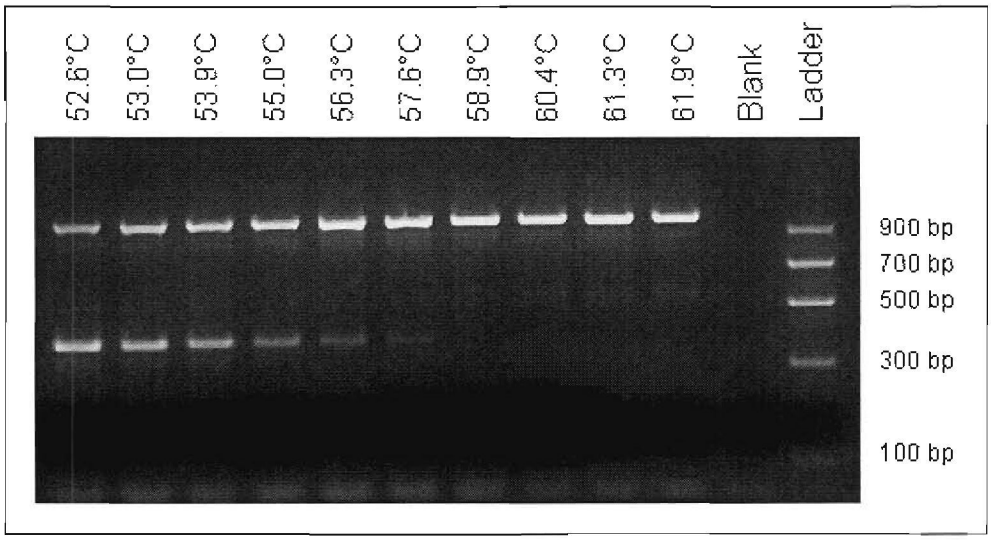


Figure 4.1. Optimisation of the annealing temperature for TH PCR. This figure is a photographic representation of a 2 % agarose gel with EtBr staining, where 20 µl of ten PCR mixtures were loaded, as well as a blank sample where no blood was added to the reaction. The DNA size markers that were used in this gel and the following experiments were self-created using PCR as follows: primers were designed to be used with pIRESneo vector as a PCR template. Using different combinations of the primers for PCR, amplicons with different lengths were obtained and used as the DNA size markers with lengths as indicated.

It can be observed from the results in Figure 4.1 that that lower annealing temperatures (i.e. from 52.6 °C to 57.6 °C) produced the desired fragment of 1013 bp, but also produced a non-specific fragment of approximately 350 bp. These lower temperatures were thus not considered to be ideal for this reaction. At temperatures above 58.9 °C (i.e. 58.9 °C to 61.9 °C), the 350 bp fragment seemed to disappear completely and only the desired fragment was detected. The intensity of the desired amplicon on the gel was highest at 60.4 °C and at 61.3 °C. However, at 61.9 °C, the intensity of the desired amplicon was slightly less compared to the two lower temperatures. Therefore, the optimum temperature for annealing was chosen to be 61.0 °C.

4.2.2. Optimisation of the volume of EDTA whole blood to be used:

Because EDTA whole blood, containing white blood cells with DNA as template, was used directly in the PCR reaction without prior isolation of DNA, the exact concentration of DNA template in the reaction mixture was unknown. In a PCR

reaction, the optimal amount of DNA template is needed for the specific concentration of primers to bind optimally and to ensure successful amplification. Too much template would use all the primers available in the first couple of cycles, resulting in the early depletion of the primers and very low amplicon yield. Too little DNA would result in low-level amplification and would thus lead to very low amplicon yield. Therefore the amount (µL) of EDTA whole blood to be used in the 50 µL reaction mixtures was varied from 1 % to 20 % (0.5, 2.5, 5.0, 7.5 and 10 µL respectively) and analysed in duplicate (reaction mixtures 1 – 10). The reaction mixtures also contained 1 µL forward primer, 1 µL reverse primer and 25 µL Blood Direct ReadyMix. PCR grade water was used to fill the reaction volume to the final 50 µL. The reactions were analysed with agarose gel electrophoresis and shown in Figure 4.2.

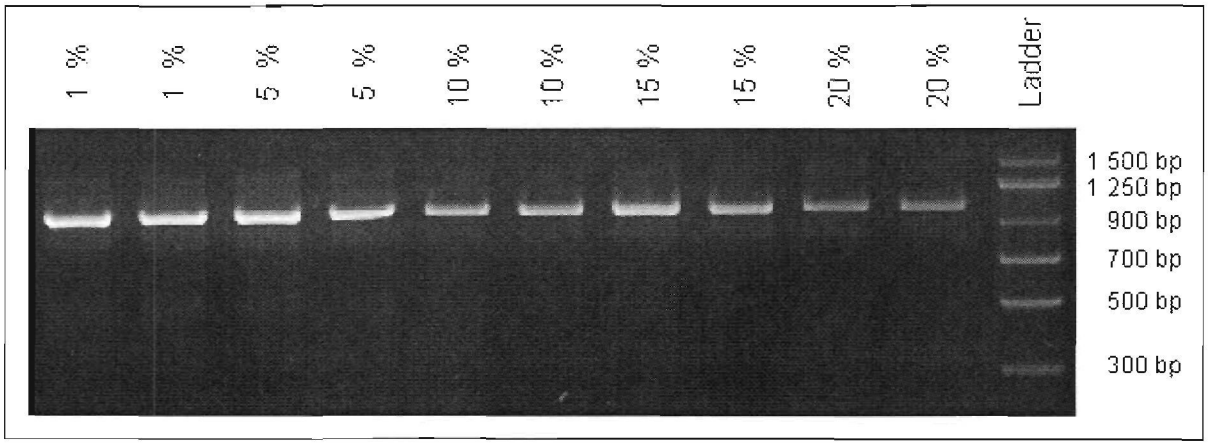


Figure 4.2. Optimisation of the blood content in reaction mixtures for TH PCR. This figure is a photographic representation of a 2 % agarose gel with EtBr staining, where 20 µl of ten PCR mixtures were loaded. An in-house pIRESneo Vector DNA marker was used as a size marker.

From the results shown in Figure 4.2 it is clear that a higher concentration of EDTA blood per PCR results in lower amplicon formation. The 1 % blood mixtures produced the strongest visual amplicon. Since higher blood concentrations results in lower product yield, a 1 % EDTA blood content was chosen for all subsequent PCR reactions.

4.2.3. Reproducibility of PCR analysis using the final reaction mixture:

Amplification of DNA directly from a biological sample (in this case fresh EDTA treated whole blood, not dried on Guthrie cards) is a relatively new concept.

Because the Taq polymerase enzyme is easily inhibited by proteins (Al-Soud and Rådström, 2001), one would usually isolate DNA from the sample before amplifying the DNA. In order to be certain that the PCR will not be inhibited by adding EDTA blood directly to the PCR, the reproducibility of the PCR was assessed. One of the SABPA samples (test person number 009) was therefore chosen to test the reproducibility of the PCR conditions. Six identical reaction mixtures were prepared, all containing blood from sample 009. The final conditions, as given in Section 3.3.3 were used and the results are shown in Figure 4.3.

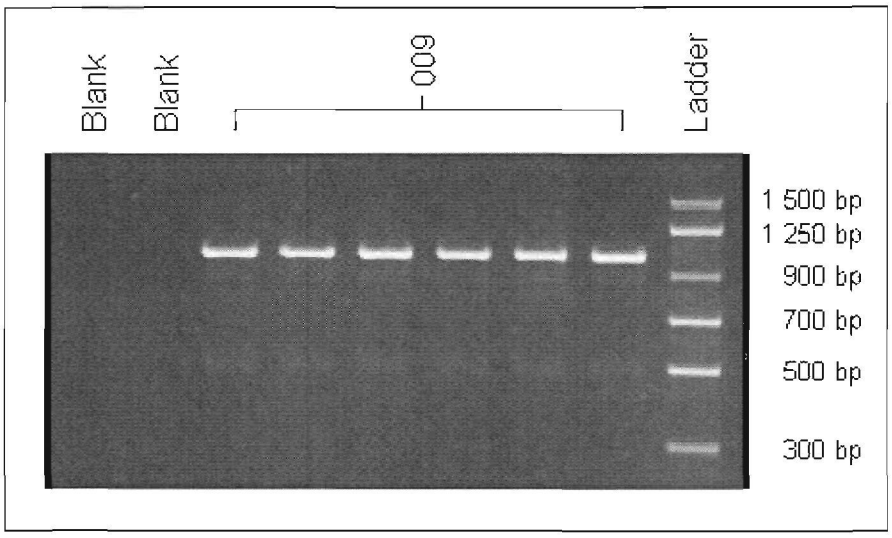


Figure 4.3. Reproducibility of TH PCR. This figure is a photographic representation of a 2 % agarose gel with EtBr staining, where 20 µl of six PCR mixtures were loaded, as well as two blank mixtures containing no template. An in-house pIRESneo Vector DNA marker was used as a DNA size marker.

From Figure 4.3, it can be observed that all the reactions produced a fragment of equal intensity. In none of the reactions was any reduced intensity observed, which could have been interpreted as partial inhibition of DNA polymerase by protein interference. Thus the reproducibility of the PCR was extremely good. The two negative controls showed no amplicon formation at all, confirming that the amplicons seen on the agarose gel was due to the presence of the DNA in the EDTA blood and not due to background contamination. The optimised PCR assay therefore delivered reproducible results and could thus be used to amplify the DNA containing the C-824T mutation in the SABPA samples.

4.3. Optimisation of the RFLP analysis for the detection of the C-824T mutation in the human tyrosine hydroxylase gene.

The RFLP analysis of this specific SNP has not been described in the literature, thus a new RFLP method had to be optimised to screen the SABPA participants for the mutation. After optimisation of the PCR reaction, the RFLP enzyme reaction was optimised by measuring the effect of several different parameters on the enzyme digestion. The RE chosen for this digestion was *Tru*1I, purchased from Fermentas Life Sciences (Burlington, Ontario, Canada).

4.3.1. Optimisation of the incubation time of enzyme digestion:

According to the manufacturer, the optimal temperature for *Tru*1I digestion of dsDNA is 65 °C. The first parameter to optimise was the incubation time at 65 °C. A blood sample from one of the SABPA participants was chosen for the optimisation of the restriction analysis. After the DNA was amplified with the optimised PCR (Section 3.3.3), the PCR mixture was used directly for the restriction analysis (no clean-up step was used to purify the amplified DNA). Four reaction mixtures were prepared in 0.2 mL PCR tubes, each containing 10 µL PCR product, 0.2 µL *Tru*1I (2 U), 2 µL of buffer R (10X) and 7.8 µL PCR grade water. The reaction mixtures were incubated in the Hybaid thermocycler at 65 °C for 1, 3, 5 and 18 hours, respectively. After the given incubation time, the samples were taken from the thermocycler and cooled to 4°C to stop the enzyme reaction. The samples were analysed on a 2 % (w/v) agarose gel. The results of the time-study are shown in Figure 4.4.

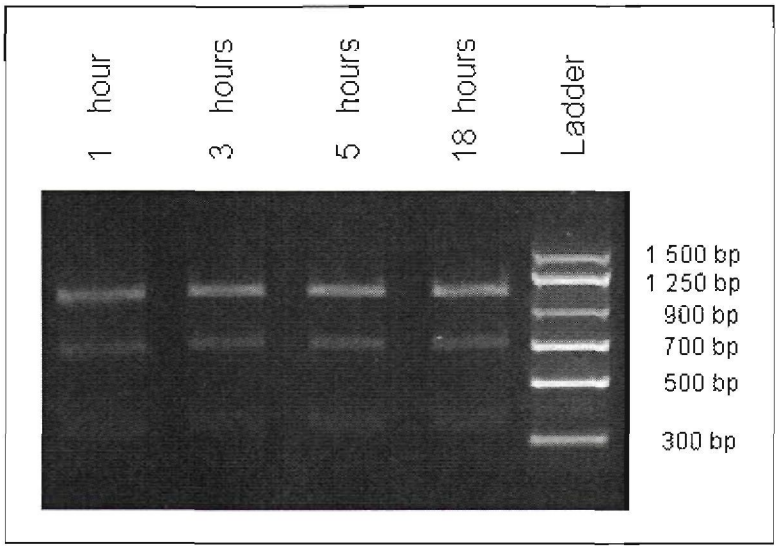


Figure 4.4. Optimisation of the length of enzyme digestion for RFLP. This figure is a photographic representation of a 2 % agarose gel with EtBr staining, where 20 µl of 4 RFLP products were loaded. An in-house pIRESneo Vector DNA marker was used as a size marker.

The ideal sample to use for this experiment should have been a homozygous sample for the SNP, but after visualisation of the results, it was clear that that the specific participant was a heterozygote, as three fragments could be seen on the visual representation. Therefore one cannot decide what the optimum incubation time was based on the disappearance of the top (1013 bp) band, because its presence could either be interpreted as ineffective digestion (if it contains the SNP) or undigested due to absence of the SNP. However, another way to assess the efficiency of the enzyme reaction, is by looking at the formation of the 668 bp band. This band should become more intense with longer periods of digestion. After inspection of the results (Figure 4.4), it seems that digestion for only one hour resulted in slightly lower band intensity and was interpreted to be not as well digested as the amplicons of the longer incubation reactions. From these results, it was concluded that the optimal incubation time for the digestion was between three and five hours. It was then decided to set the digestion time at four hours for the subsequent experiments. Taking into account the result from this time study, preliminary conditions and reaction mixture composition was used to screen several SABPA samples to identify a possible homozygous sample. This homozygote sample was then used for all subsequent RFLP optimisation experiments since the 1013 bp fragment will theoretically completely disappear

during RE digestion if the conditions are optimal, resulting in a 668 bp and a 345 bp fragment.

4.3.2. Variation of buffers in RFLP analysis:

Along with the buffer specifically made to work with *Tru*1I (buffer R), a universal buffer (buffer Tango; 10X) was also supplied with the RE. To test which of these two buffers were best suited to use in this RFLP reaction, three (20 µL) mixtures were prepared, one containing 2 µL buffer R (10X), a second with 2 µL buffer Tango and a third with 4 µL buffer Tango. Along with the various buffers in the reaction mixtures, 7.8 µL of PCR grade water was used in reaction mixture 1 and 2 and 5.8 µL in reaction mixture 3. 0.2 µL of RE (*Tru*1I) was used in the reaction mixtures along with 10 µL of crude PCR product for a total of 20 µL. A blank reaction mixture was also prepared, identical to reaction 1, but replacing the PCR mixture with 10 µL of PCR grade water. The four reaction mixtures were incubated at 65 °C for 4 hours. The samples were analysed on a 2 % (w/v) agarose gel together with an uncut PCR product. A pIRESneo Vector DNA size markers was used to assist with size calculation (Figure 4.5).

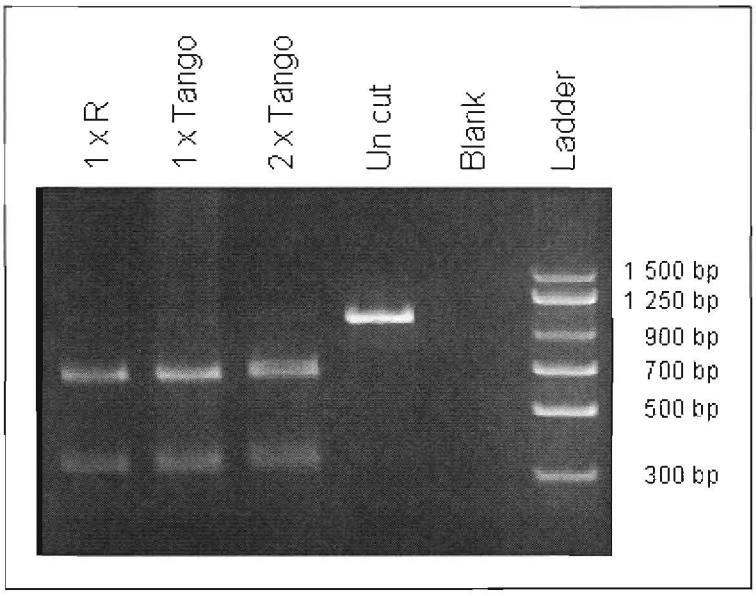


Figure 4.5. Optimisation of the buffer to be used for RFLP. This figure is a photographic representation of a 2 % agarose gel with EtBr staining, where 20 µl of three RFLP products were loaded. An un cut sample was also loaded, as well as a blank mixture containing no PCR product. An in-house pIRESneo Vector DNA marker was used as a size marker.

From the results (Figure 4.5) it is clear that the most suitable buffer was buffer R. This buffer allowed near complete digestion to take place. Complete digestion was not seen, but as this was the first in a series of parameters to test, it could have been because of one or more of the other parameters that were less than optimum for the reaction. Digestion with buffer Tango produced good results, but some so-called star activity was seen which can occur under reaction conditions that differ significantly from those optimum for the enzyme. Star activity is a loss of specificity of the RE, resulting from sub-optimal buffer conditions. Digestion was less complete with buffer Tango. Buffer R was chosen to use in all subsequent reactions.

4.3.3. Variation of the amount of enzyme to use in RFLP reaction:

For the digestion reaction to work optimally, the amount of enzyme added should not be too little, or too much. Too little enzyme will leave residual template that can give false negative results, whereas too much enzyme may result in star activity. In this study a series of reactions were prepared with differing amounts of enzyme (i.e. 1, 2, 5 and 10 units per reaction). Reaction mixtures 1 – 4 contained 0.1, 0.2, 0.5 and 1.0 µL of enzyme and they were made up (20 µL) with 7.9, 7.8, 7.5 and 7.0 µL of PCR grade water, respectively. Furthermore the mixtures also contained 10 µL of PCR product and 2 µL of buffer R, each. A blank was also prepared containing reaction mixture without any PCR product. The samples were incubated at 65 °C for four hours in the Hybaid thermocycler. The samples were separated on a 2 % (w/v) agarose gel together with an uncut PCR product (Figure 4.6).

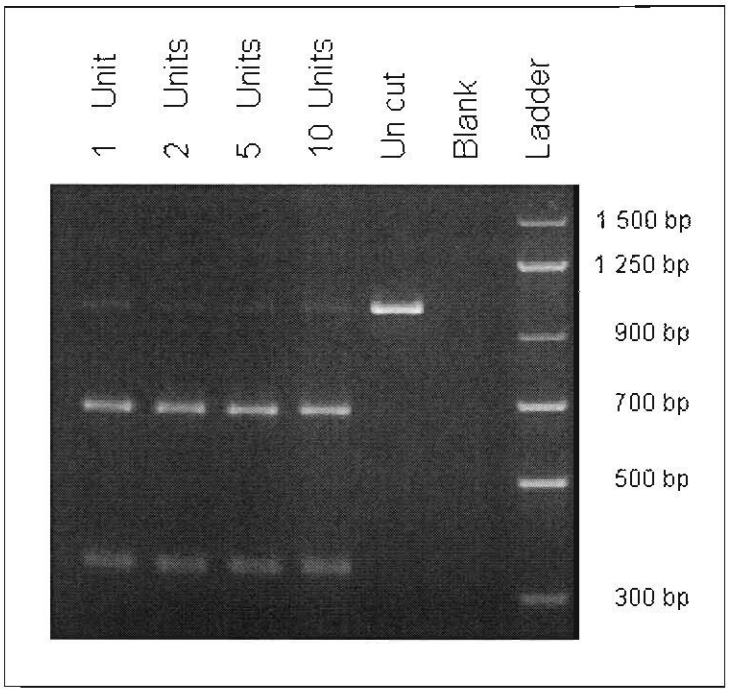


Figure 4.6. Optimisation of the amount of enzyme to be used for RFLP. This figure is a photographic representation of a 2 % agarose gel with EtBr staining, where 20 µl of 4 RFLP products were loaded. An uncut sample was also loaded, as well as a blank mixture containing no PCR product. An in-house pIRESneo Vector DNA marker was used as a size marker.

From Figure 4.6 it can be seen that 1 unit of enzyme was not enough for complete digestion. Thus, 2 units (0.2. µL) of enzyme per reaction was regarded a suitable for the digestion.

4.3.4. Variation of the percentage of PCR mixture in restriction analysis:

After PCR amplification, the amplicon obtained was cut in an RFLP digestion reaction. The newly developed PCR reaction used in this study consisted of only 30 cycles to limit non-specific amplification and thus delivered less amplicons per µL than conventional PCR reactions done from pure DNA extractions. In such cases, addition of 5 µL DNA is sufficient for RFLP digestion. However, after digestion, the resulting cut fragments would be even less substantial. In this case, it was necessary to include no less than 10 µL of DNA product into the RFLP reaction mixture to ensure adequate intensity of resulting fragments on an agarose gel.

Restriction endonucleases digest optimally in the right buffering conditions. Because the restriction reaction contained 50 % PCR mixture (without clean-up of

the PCR products), it is possible that the buffer in the PCR mixture could therefore influence the buffer conditions of the RFLP reaction and result in sub-optimum enzyme activity. In order to investigate this possibility, we constructed a series of reaction mixtures containing differing amounts (%) of PCR mixtures.

Reaction mixture 1 consisted of 10 µL of PCR product, 0.2 µL (2 U) of *Tru*1I, 2 µL of buffer R and 7.8 µL PCR grade water for a total volume of 20 µL. Reaction mixture 2 consisted of 10 µL of PCR product, 0.2 µL (2 U) of *Tru*1I, 3 µL of buffer R and 16.8 µL PCR grade water for a total volume of 30 µL. The final mixture contained 10 µL of PCR product, 0.2 µL (2 U) of *Tru*1I, 4 µL of buffer R and 25.8 µL PCR grade water for a total volume of 40 µL. The three reaction mixtures thus ranged from 50 % PCR content in reaction mixture one (a ratio of 1:1) to 25 % PCR content (a ratio of 1:3) in reaction three, with reaction two containing 33 % PCR product (a ratio of 1:2).

The reaction mixtures were incubated at 65 °C for 4 hours. After the incubation step, the samples were evaporated in a SpeedVac[9] SVC100 system at room temperature to equal volumes of approximately 10 µL, visually inspected and confirmed by means of micro pipette. PCR grade water was added to each mixture to a final volume of 20 µL. Although the initial volume of reaction mixture differed between the samples, each contained 10 µL of PCR mixture. The final volume (after evaporation) was adjusted to 20 µL, resulting in the mixtures all containing the same amount of DNA in the same volume, therefore the results from the agarose gel (Figure 4.7) can be visually compared to one another without compensating for a dilution factor.

[9] SpeedVac is from Savant instruments, Inc., Farmingdale, NY, U.S.A.

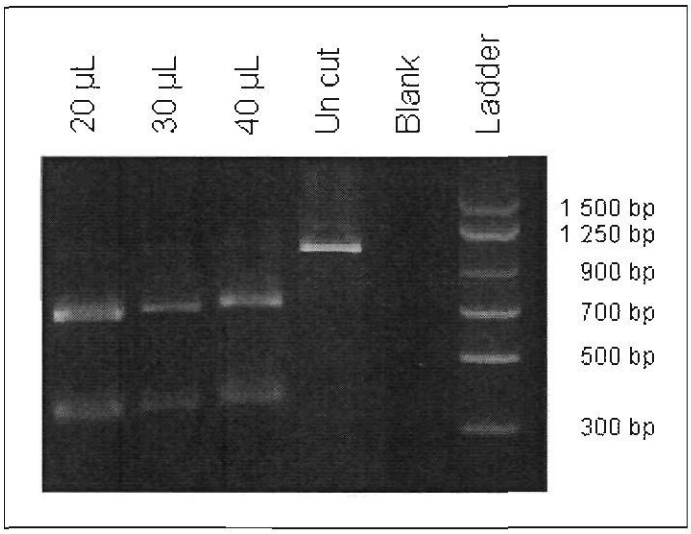


Figure 4.7. Variation of the PCR buffer content in the RFLP reaction. This figure is a photographic representation of a 2 % agarose gel with EtBr staining, where 20 µl of three RFLP products were loaded. An un cut sample was also loaded, as well as a blank mixture containing no PCR product. An in-house pIRESneo Vector DNA marker was used as a size marker.

In these three reactions, the buffer content was varied to compensate for changes in reaction composition, resulting from added PCR buffer. Thus, the different concentrations influenced the movement of DNA fragments. From the results it can be seen that the 20 µL reaction (containing 50 % PCR mixture) showed slightly more clear fragments than the 40 µL reaction (containing 25 % PCR mixture). Since the PCR buffer content was least influential in the reaction mixture containing 50 % PCR product, the 20 µL reaction composition was chosen for subsequent reactions.

4.4. The optimised mutation analysis for the detection of the C-824T SNP in the human tyrosine hydroxylase gene

During the optimisation experiments of the PCR and RE reactions, a very faint band was persistently present at 1013 bp when DNA obtained from a homozygote for the SNP was used (see Figure 4.7 as example). Thus the digestion of the 1013 bp fragment was incomplete or some contaminant wild type fragment was present. Since the PCR blank reaction (containing all reagents but no DNA) resulted in a very faint product at 1013 bp, the remaining fragment in the homozygote RE analysis was thought to be due to contamination.

The original PCR consisted of 40 cycles, which was a relatively high number of cycles and may be prone to amplification of a contaminant template and thus the faint product forming at 1013 bp (presumably wild type human TH). The number of cycles were thus reduced to 30 to avoid the formation of this PCR product. In these reactions, of which Figure 4.8 is an example, no faint fragment was ever observed at 1013 bp again in any of the PCR blanks. The homozygotes did not show any fragment at 1013 bp after RE digestion, confirming that the restriction conditions were optimal and resulted in complete digestion of the 1013 bp fragment when the DNA contained the C-824T mutation.

The optimised conditions are summarised in Section 3.3.3. Figure 4.8 shows a typical result for the optimised mutation analysis for the detection of the C-824T mutation in the test subjects.

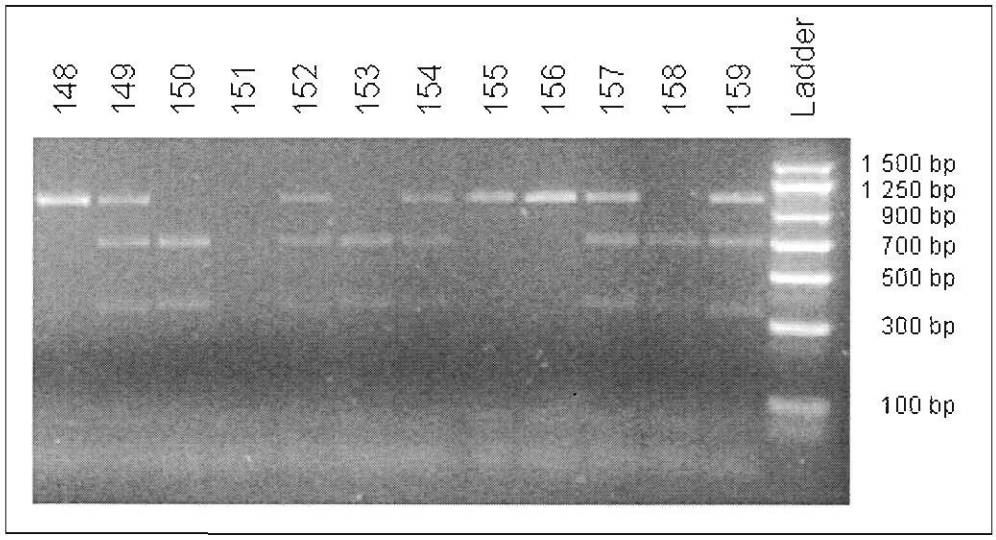


Figure 4.8. Typical result for the optimised mutation analysis for detection of the C-824T SNP in test subjects. This visualisation shows results from test subjects 148 – 159. One fragment at ± 1 000 bp indicates that the mutation is not present, while three fragments indicate a heterozygous sample and two fragments a homozygous sample. An in-house pIRESneo Vector DNA size markers was used as a size marker.

Chapter 5:

Results and Discussion

5.1. Introduction.

The association between hypertension and oxidative stress was investigated in this study. Daytime blood pressure values (08h00 to 18h00) from a 24 hour reading by a Cardiotens apparatus were collected for each of the SABPA participants and an average value was calculated. Several assays were carried out on resting blood samples obtained from each participant, including systemic oxidative stress measurement (ROS and the GSH/GSSG ratio) and antioxidant capacity (FRAP, GSH and GSSG). Concentrations obtained from ROS, FRAP and GSH/GSSG spectrophotometric assays were calculated automatically from standard graphs. All standard graphs used in this study were linear with r^2 values of above 0.99. The participants were also screened for a SNP in the TH promoter region (C-824T), since elevated blood pressure was positively connected to this SNP by Rao *et al.* (2007).

5.2. Oxidative stress in males and females.

Several studies reported that males and females have different levels of oxidative stress (Collins *et al.*, 1998; Nielsen *et al.*, 1997 and Ide *et al.,* 2002). However, Sartori-Valinotti *et al.* (2007) reported that neither human nor animal studies are consistent in terms of whether oxidative stress levels are higher in males or females. Because of this discrepancy on the role of gender in oxidative stress, it was decided to analyse male and female oxidative stress data separately in this study. The male and female data was compared to establish if a statistical significant difference exists between the oxidative stress parameters in the two gender groups. The data is graphically represented, showing the mean value with 95 % confidence intervals and also the spread (minimum and maximum values) for each parameter (Figure 5.1 – 5.6).

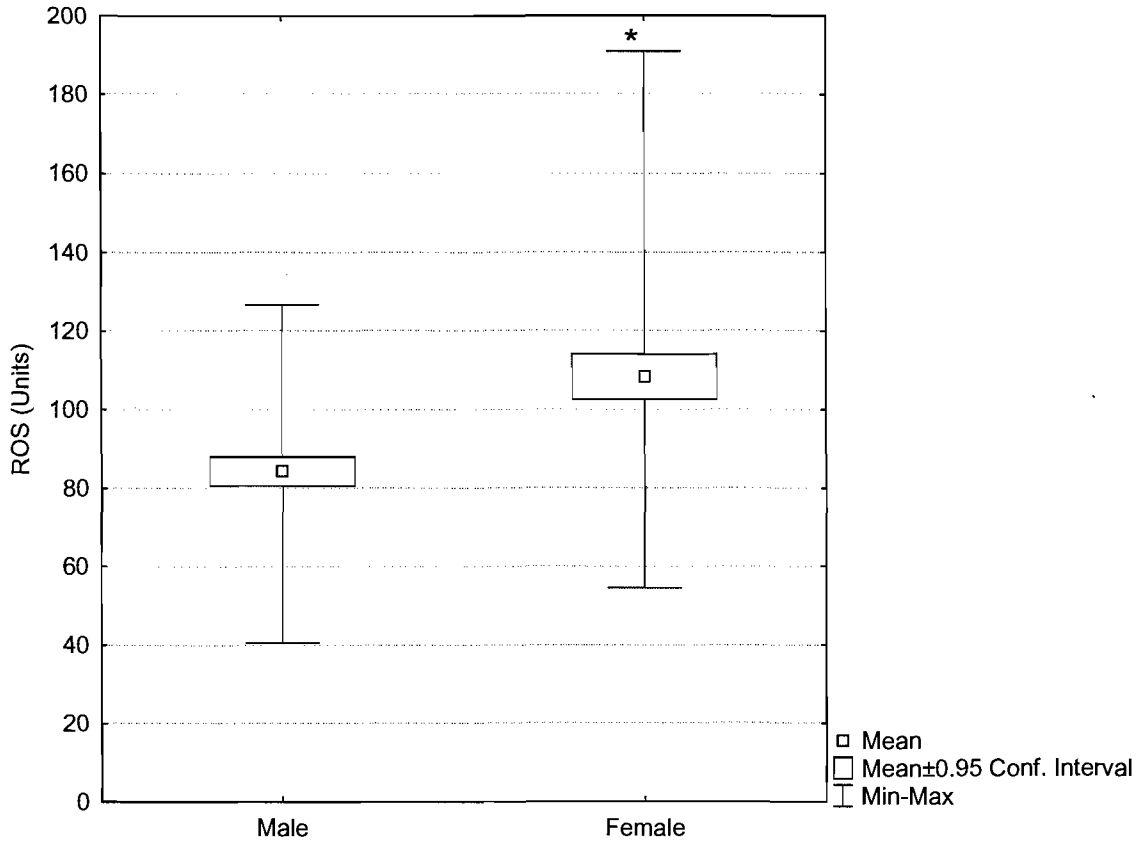


Figure 5.1. Comparison of ROS values between males and females. As the legend indicates, the plot shows the mean ± 95 % confidence interval values, as well as minimum and maximum values. *:$p < 0.01$. One unit is equivalent to 1.0 mg H_2O_2/L.

Figure 5.1 shows the comparison between ROS values of African males and females. From the figure, it can be observed that the mean ROS value of males was 84 units compared to the mean ROS value of females, which was 108 units. The ROS values in the male group ranged between 40.7 and 126.7 units. However, the ROS values in the female group ranged between 54.4 and 190.8 units. The Student's T test shows that the mean ROS value of the females is significantly higher than that of the males ($p<0.01$). The mean ROS value of the males was 84.4 units, compared to the mean value of 108.3 observed in the females, which is 28.3 % higher. ROS levels was thus influenced by gender and it was concluded that the male and female data should be separated in this study.

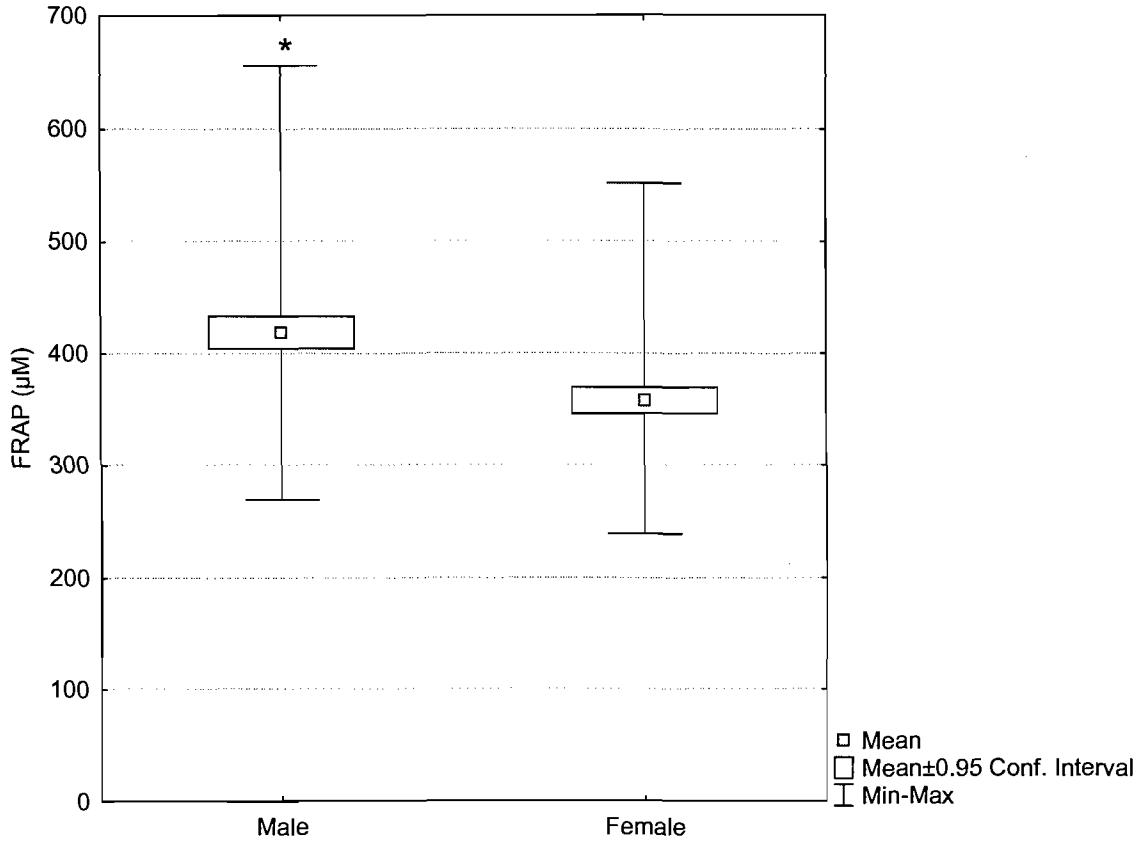


Figure 5.2. Comparison of FRAP values between males and females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values. *: $p < 0.01$.

Figure 5.2 illustrates the comparison between FRAP values of males and females. From the figure it is seen that the mean value of the male group (419 µM) was significantly higher compared to the mean value of the female group, which was 358 µM ($p < 0.01$), 17 % higher. The FRAP values in males ranged from 269.4 µM to 655.4 µM and were also higher than in the female group, which ranged from 239.2 µM to 551.9 µM. This means that the antioxidant capacity (as measured by the FRAP assay) is higher in men than in women. According to the results from Figures 5.1 and 5.2, ROS values were higher and FRAP values were lower in females, suggesting higher oxidative stress levels (including lower antioxidant capacity) in females.

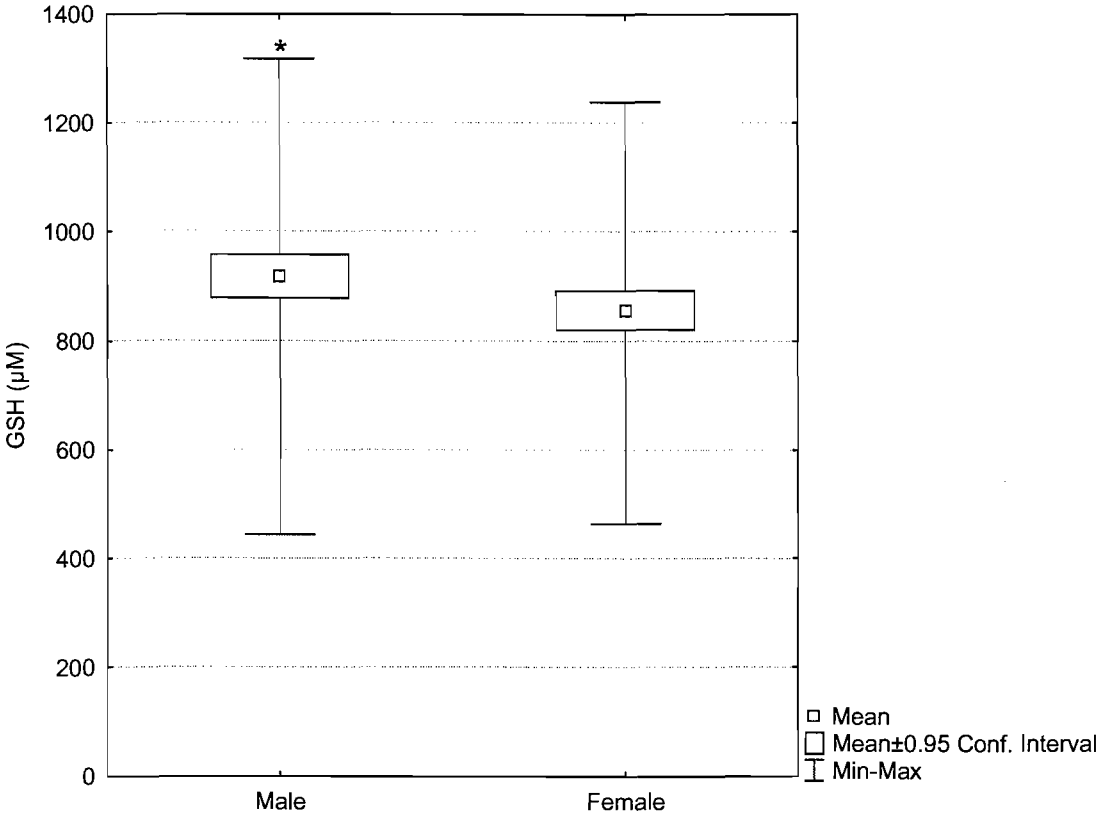


Figure 5.3. Comparison of GSH values between males and females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values. *: p = 0.02.

Another indication of the antioxidant capacity of a person is the reduced glutathione (GSH) concentration in blood. Figure 5.3 shows the difference in GSH values observed between men and women. Again, it is seen that the mean value in the male group (918 µM) is higher than the female group (857 µM), which is a difference of 7.1 %. It is also seen that the spread of values from the male group (i.e. 445.1 µM to 1316.9 µM) lies over a wider range than female values (i.e. 464.9 µM to 1239 µM). The difference between the male and female groups is statistically significant (p = 0.02). This result is in accordance with the difference in FRAP values observed, as both of these assays give an indication of the antioxidant capacity.

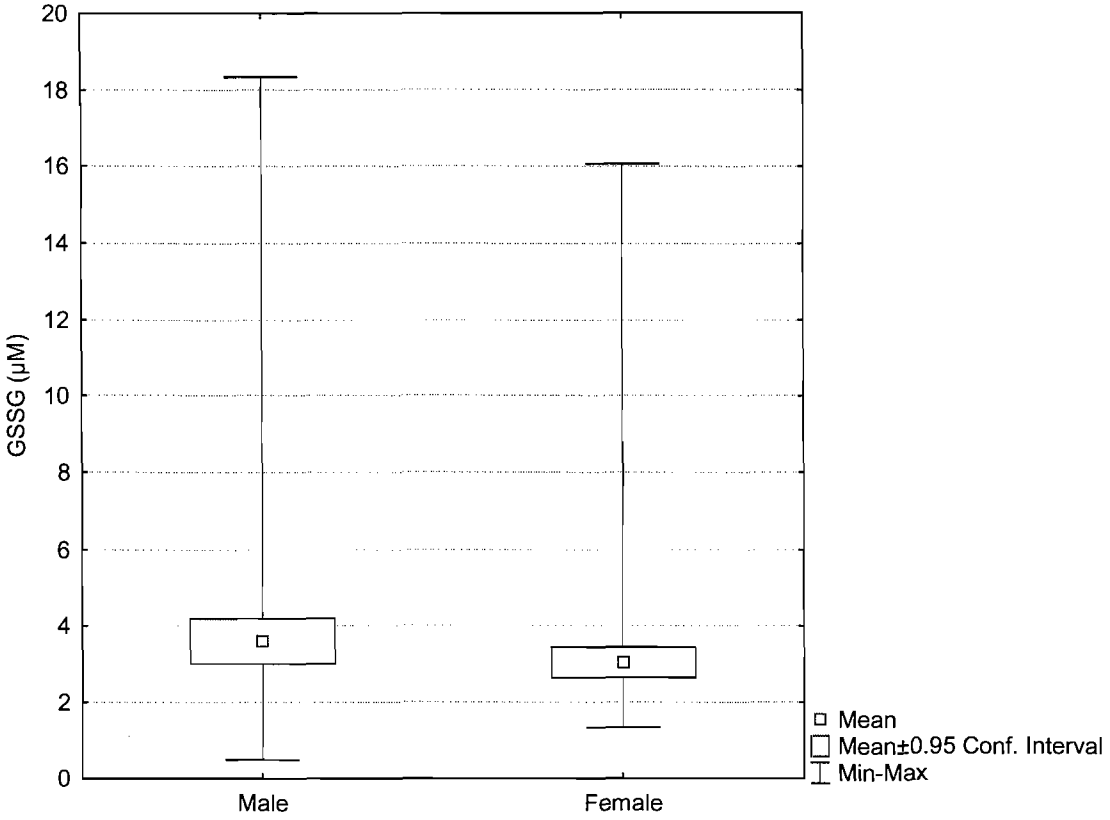


Figure 5.4. Comparison of GSSG values between males and females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

Along with the GSH values, oxidised glutathione (GSSG) was also measured. Although it was done with the same kit as for the GSH samples, the variance between triplicate measurements of the same GSSG sample was large. The mean value for the male group was 3.6 µM and the mean value for females was 3.0 µM, which is a 20 % difference, but not statistically meaningful ($p = 0.12$). It is also seen that the spread of values for the male group ranges from 0.5 µM to 18.3 µM, whereas the range of values for the female group is form 1.3 µM to 16.1 µM. The range of values for females is thus slightly narrower than that for the males, although differences between males and females were not significant.

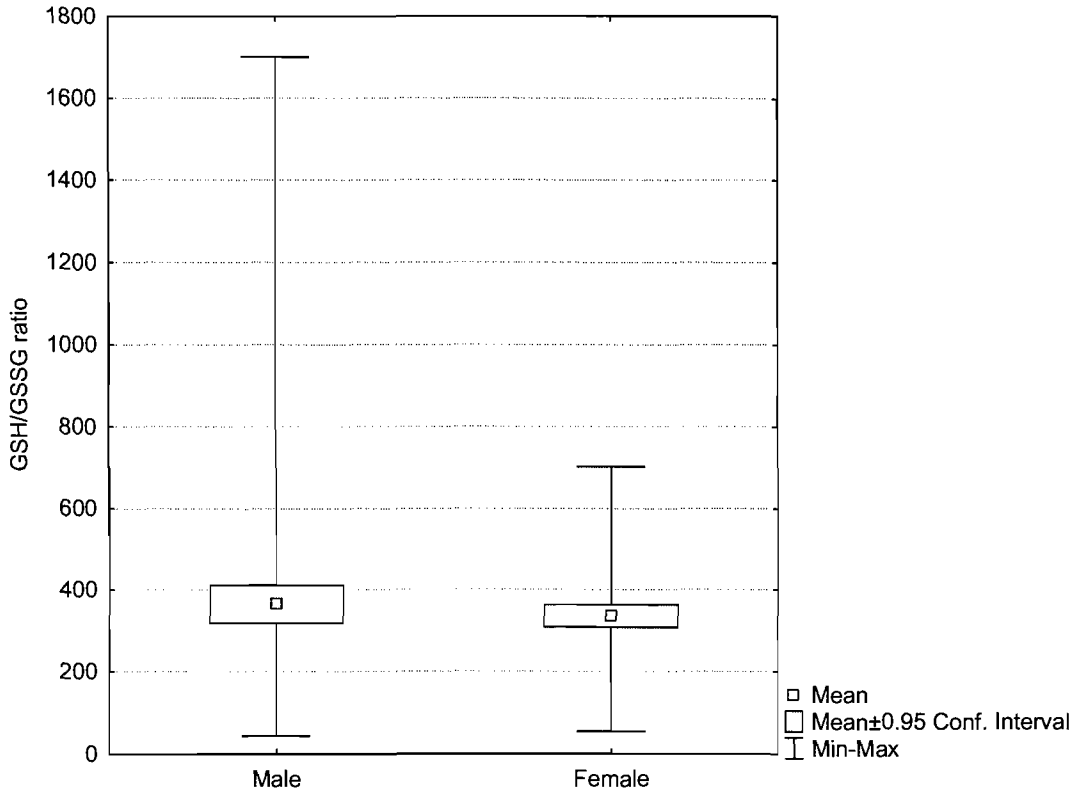


Figure 5.5. Comparison of GSH/GSSG ratio values between males and females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

The ratio of GSH to GSSG values were calculated for each participant to serve as another indication of systemic oxidative stress. As the GSSG values showed great variation, a ratio of GSH to GSSG would also deliver results with great variation. Figure 5.5 shows the comparison of the GSH/GSSG ratio between men and women. Again, no significant difference was observed between the males and the females. The mean value for the male group is 365, compared to the mean value of the female group, which is 336. This is 8.3 % higher, but not statistically meaningful ($p = 0.28$). The range of values for the male group (i.e. 43.2 to 1700.2) was also much wider than that of the female group (i.e. 54.4 to 701.6).

In studying the putative association between the five parameters of oxidative stress/antioxidant capacity between men and women, several observations were made. The ROS values, the parameter that demonstrates oxidative stress, were lower in males than in females. In the case of the parameters that reflect antioxidant capacity, the GSH and FRAP values, males had higher values than females. Therefore it is deduced that males have a higher antioxidant capacity

than women, and also lower oxidative stress levels. This trend is seen in the literature where men and women presented with differences in oxidative stress levels (Collins *et al.*, 1998). It was therefore concluded from the results described in Section 5.2 that all data for males and females should be handled separately in subsequent sections.

5.3. Hypertension and oxidative stress.

Oxidative stress/antioxidant capacity was significantly different between males and females (Section 5.2). The males showed lower ROS values and higher FRAP and GSH levels compared to the females. However, the GSSG levels and GSH/GSSG ratio did not differ between the male and the female groups. Since most of the oxidative stress/antioxidant capacity markers in this study was influenced by gender, the males and females were compared separately in the case of blood pressure markers. If oxidative stress was not influenced by gender, the data could be combined and the combined data set compared to blood pressure.

Males and females were divided into normotensive (NT) or hypertensive (HT) according to the European Standard (O'Brien *et al.,* 2005). According to this standard, a person is considered to be hypertensive is the systolic blood pressure exceeds 140mm Hg and/or if the diastolic blood pressure exceeds 90 mm Hg. In the males, the NT group contained 37 (36.6 %) participants and the HT group 64 (63.4 %). The NT female group contained 60 (63.8 %) participants and the HT group 34 (36.2 %).

5.3.1. Hypertension and oxidative stress in males.

The normotensive and hypertensive groups were compared according to their oxidative stress/antioxidant capacity parameters. Figures 5.6 – 5.10 show the results obtained.

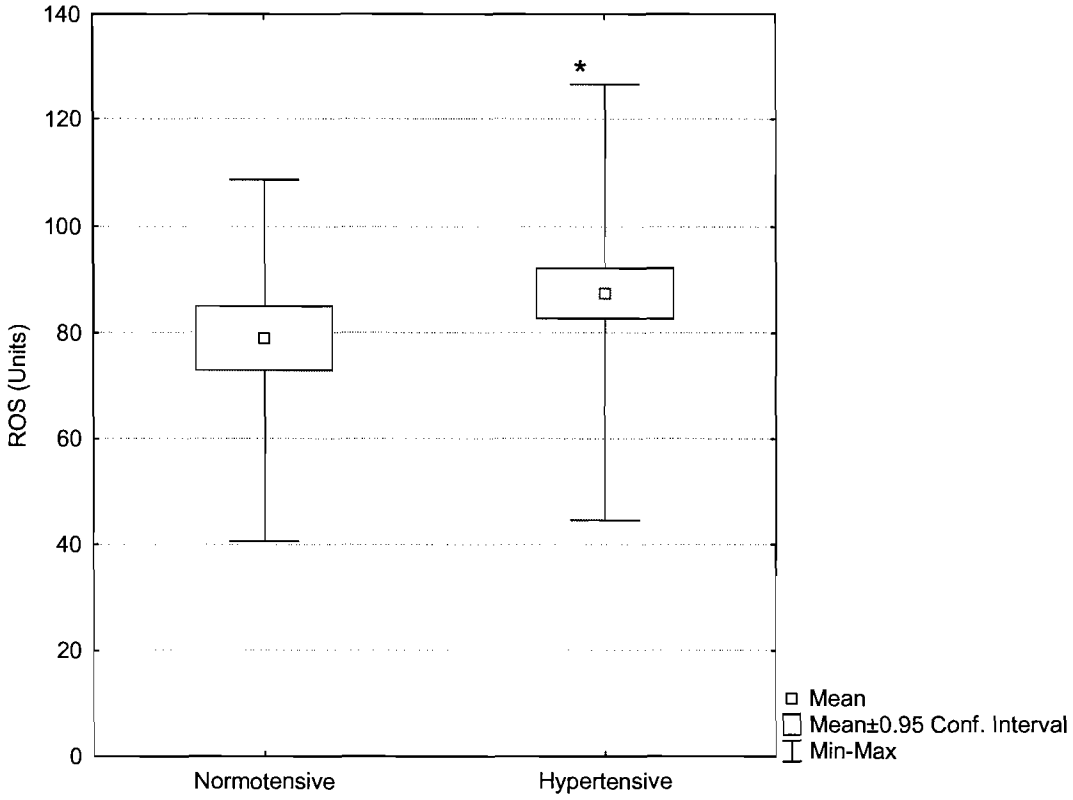


Figure 5.6. Comparison of ROS units between NT and HT males. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values. *: p = 0.03. 1 Unit is equal to 1.0 mg H_2O_2/L.

Figure 5.6 is a representation of the comparison of ROS values between normotensive (NT) males and hypertensive (HT) males. In this figure it is seen that the mean ROS value for HT males (88 units) was 11 % higher than the mean value for NT males (79 units). The spread of values for the HT group (44.7 units to 126.7 units) is also wider than that of the NT group (40.7 units to 108.07 units). From the Student's T test a statistical difference was seen between the NT and HT group with regards to the ROS levels (p = 0.03).

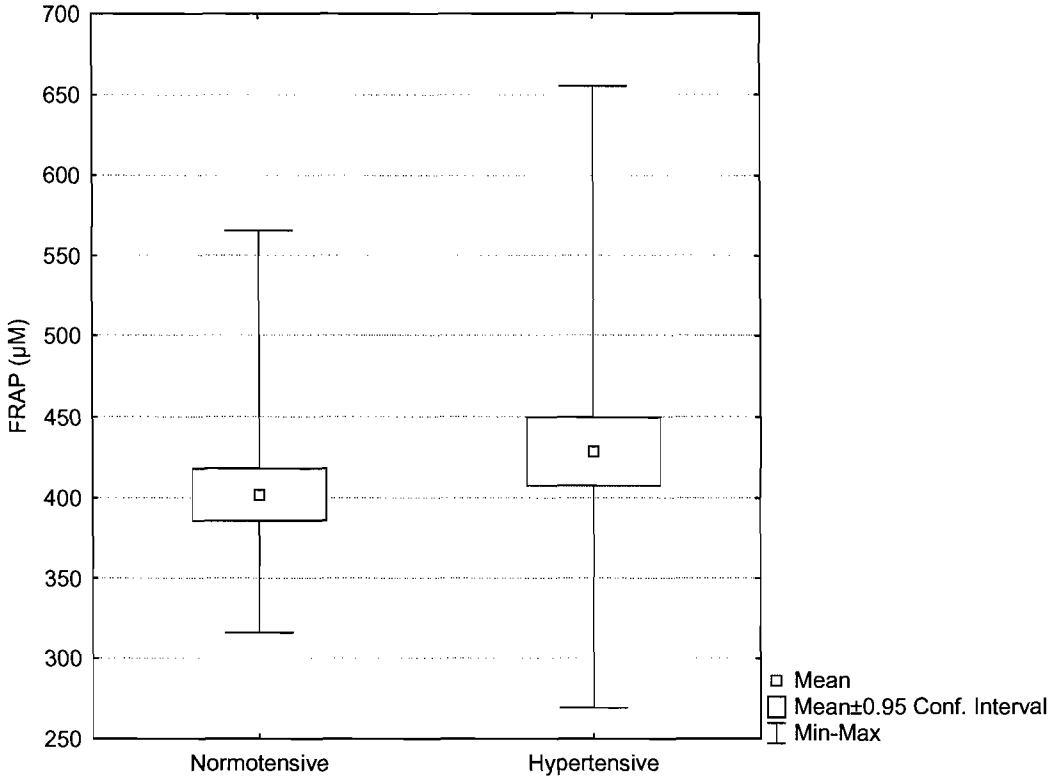


Figure 5.7. Comparison of FRAP values between NT and HT males. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

In Figure 5.7 FRAP values were compared between NT and HT males. From the figure it is seen that the mean value for the NT group was 402 µM, compared to the mean value for the HT group, which was 428 µM, which is 6.5 % higher. The minimum and maximum values for the HT group (269.4 µM to 655.4µM) fall over a much wider range than the NT group (315.8 µM to 565.6 µM), indicating a greater variance. Although the mean of the HT group are clearly higher, the difference between NT and HT males was only of borderline significance ($p = 0.08$).

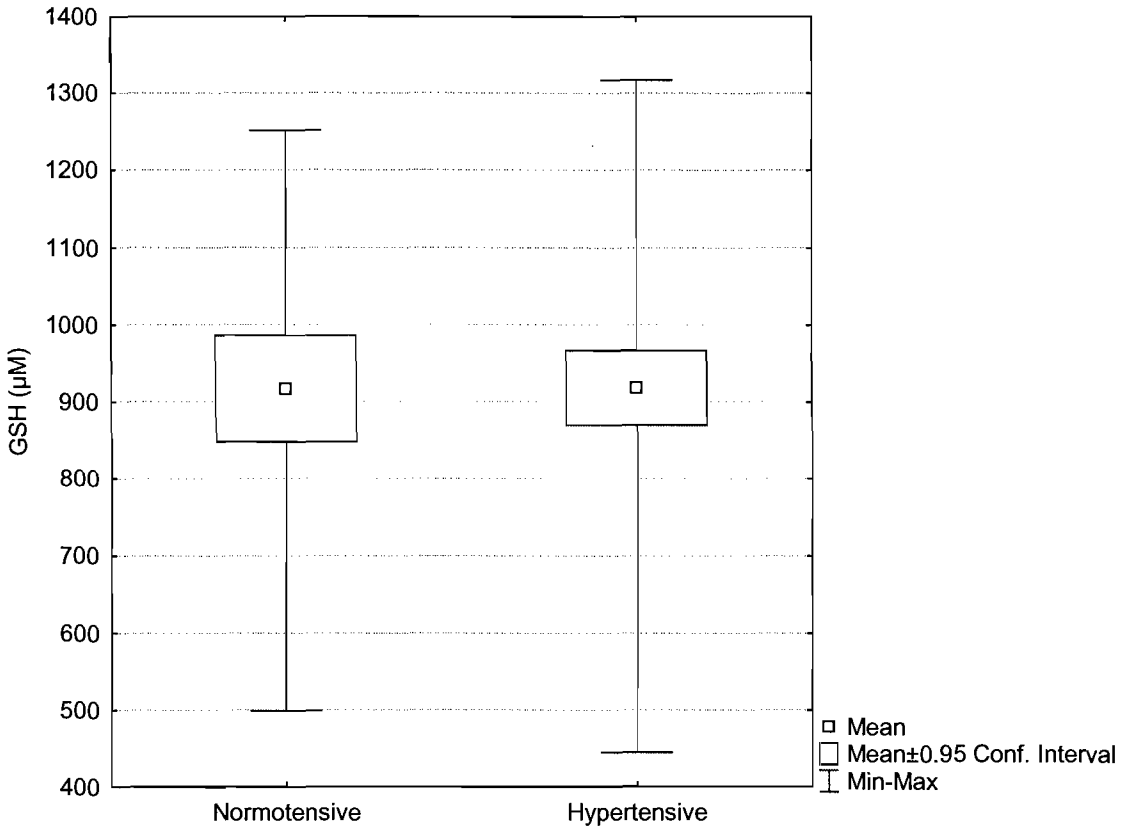


Figure 5.8. Comparison of GSH values between NT and HT males. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

In Figure 5.8 the comparison of GSH values between NT males and HT males is shown. From the figure the mean values for the two groups are 919 µM in the NT group and 918 µM in the HT group, showing that the HT GSH levels are only 0.1 % lower than the NT GSH levels. It is also seen that the spread of values in the HT group (445.1 µM to 1316.9 µM) is wider than in the NT group (499.2 µM to 1215.8 µM). The Student's T test showed no significant difference between these two groups ($p = 0.98$).

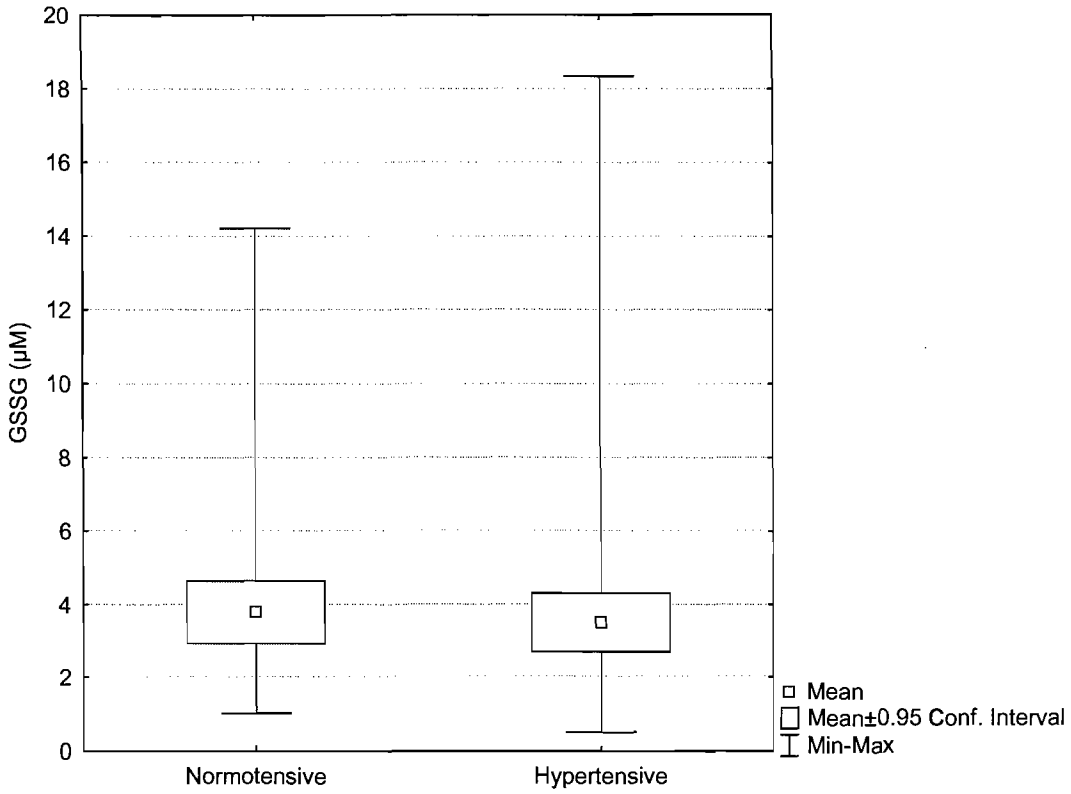


Figure 5.9. Comparison of GSSG values between NT and HT males. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

The GSSG values of NT and HT males were compared in Figure 5.9. From the figure one can see that the mean values are 3.8 µM in the NT group and 3.5 µM in the HT group. There is also a much wider spread in the HT group (0.5 µM to 18.3 µM), compared to the NT group (1.0 µM to 14.2 µM), but again no statistically significant difference could be observed between these two groups, according to the Student's T test ($p = 0.65$).

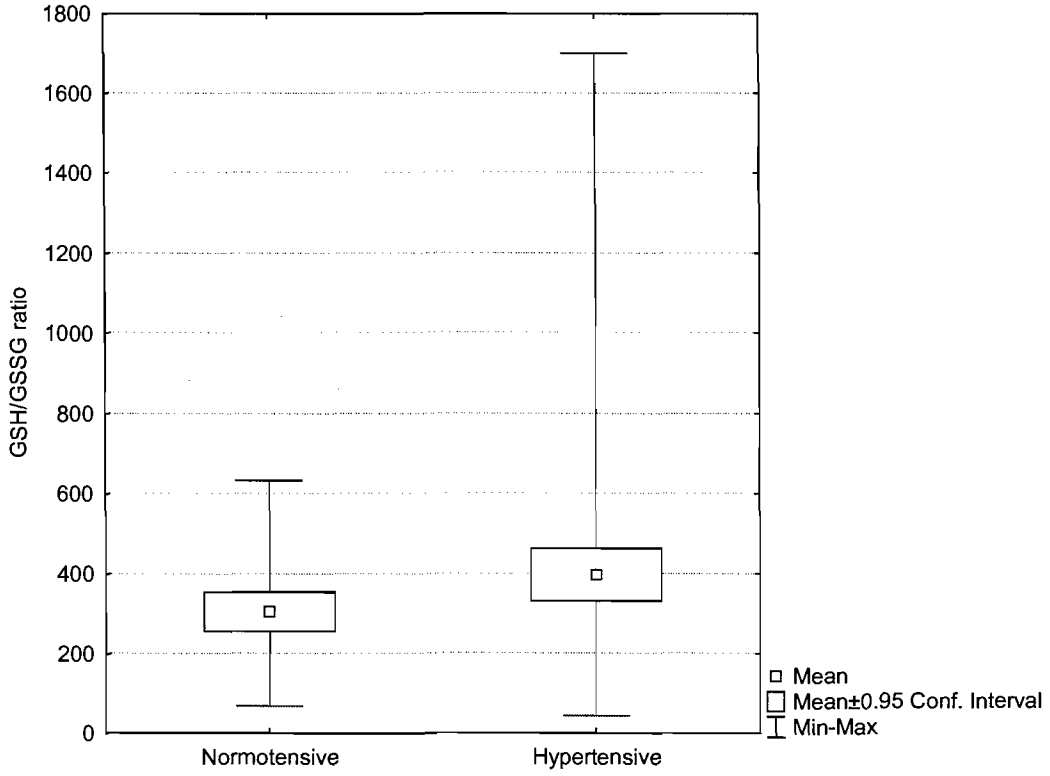


Figure 5.10. Comparison of GSH/GSSG ratio values between NT and HT males. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

Figure 5.10 shows the comparison of the GSH/GSSG ratio between NT and HT males and it can be seen that the mean value in HT males (397) is 30 % higher than the mean value in NT males (305). It is also observed that the spread in the HT group (i.e. 43.2 to 1700.2) is much wider than the spread in the NT group (i.e. 105.7 to 701.6) From the Student's T test, borderline significance was seen with a p-value of 0.06.

In Section 5.3.1 the oxidative stress/antioxidant capacity parameters were compared in NT and HT males. Firstly, the ROS values in the HT male group differed significantly from the ROS values in the NT group. This result is in accordance with the hypothesis stated in Section 2.4. Therefore, oxidative stress does increase with a rise in blood pressure in the black male participants. The antioxidant capacity (as measured by the FRAP assay) showed conflicting results. It is theoretically expected that the antioxidant capacity lowers in individuals with high oxidative stress as a result of exhaustion of the antioxidant concentration by the free radicals in the body, or inadequate intake of antioxidants. However,

although the ROS levels did increase with an increase in blood pressure, the FRAP values also *increased* in the HT male group. A possible explanation can be because of adaptive processes that strengthens the antioxidant system (Bobillier-Chaumont *et al.,* 2003). Furthermore, no significant difference was observed between NT and HT groups with regards to GSH content. Therefore, it is concluded that the GSH levels were not influenced by blood pressure at all. Similarly, GSSG levels did not differ between the two groups. Also, the expected lower GSH/GSSG ratio could not be observed in HT males, where a higher oxidative stress status was seen. This can be because of the unreliability of GSSG values (an inadequacy of the method). The unchanged GSH content of NT and HT males is contrary to the literature where a lower GSH content has been reported in individuals with high blood pressure (Kennedy *et al.,* 2005).

5.3.2. Hypertension and oxidative stress in females.

In the following five figures the oxidative stress/antioxidant capacity markers were compared between NT and HT females. Results are given in Figures 5.11 to 5.15.

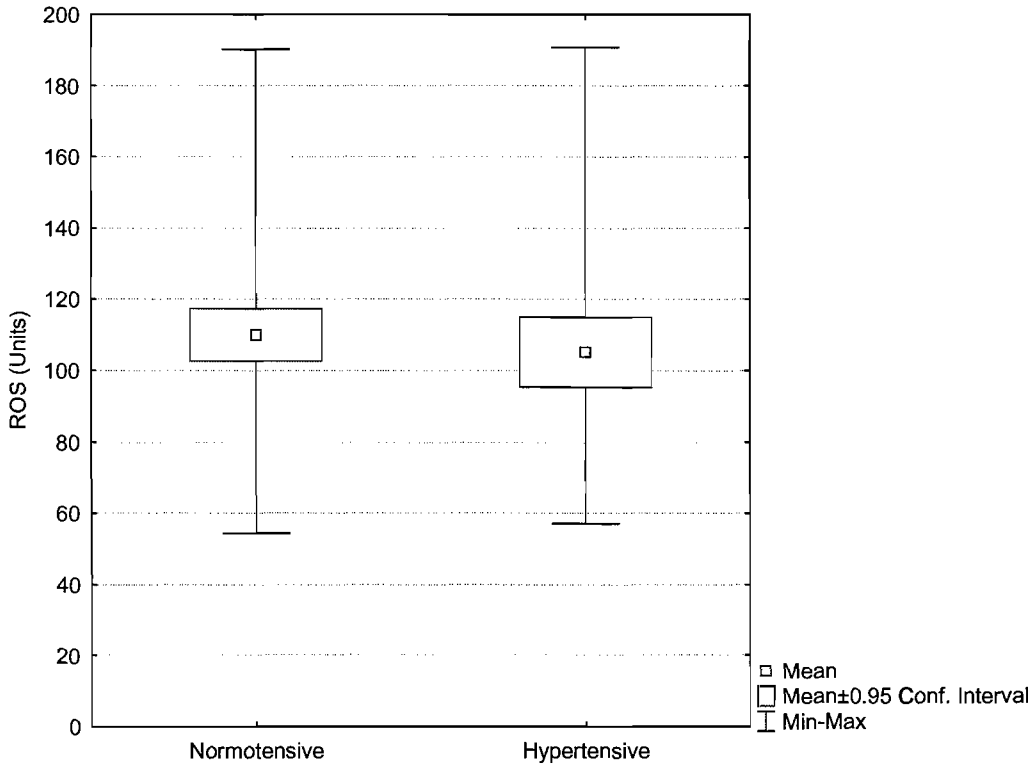


Figure 5.11. Comparison of ROS units between NT and HT females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

Figure 5.11 shows the comparison of ROS values between NT and HT females. As seen from the figure, the mean values and spreads of the two groups were similar. The mean value of the NT group was 105 units and the mean value for the HT group was 110 units, which is 4.5 % lower. The spread of the NT group was 54.4 units to 190.1 units and for the HT group, 57.1 units to 190.8 units. From the Student's T test, it is seen that the ROS values did not differ between NT and HT females ($p = 0.43$).

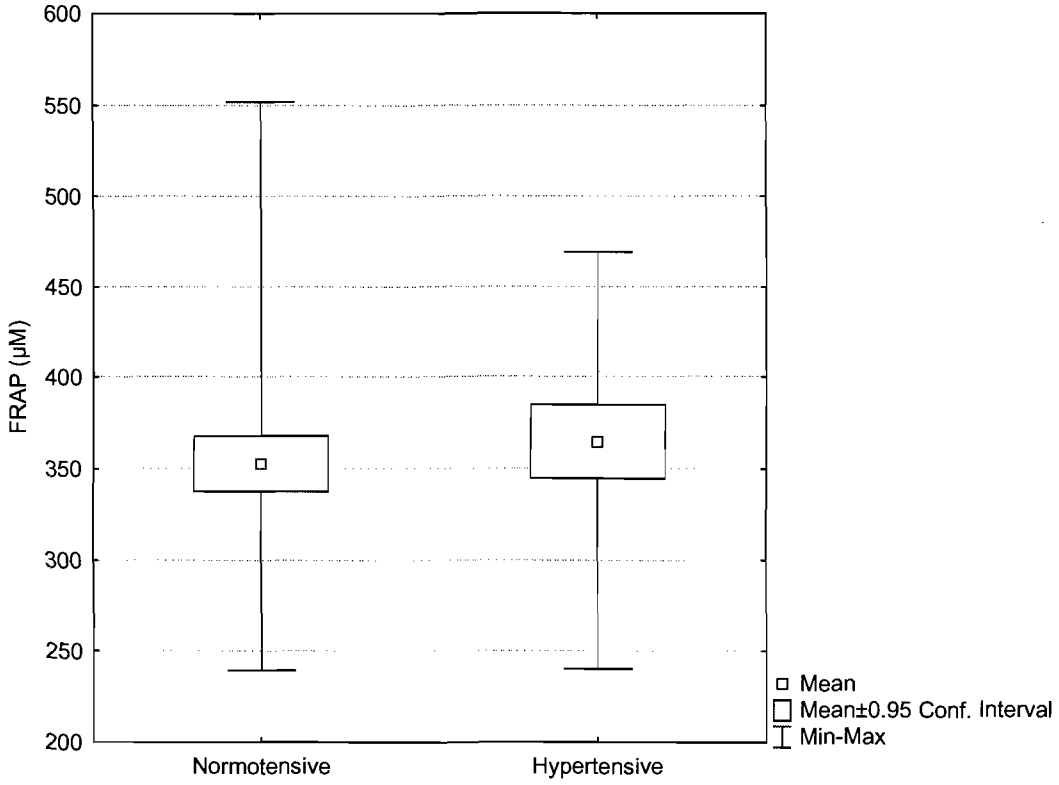


Figure 5.12. Comparison of FRAP values between NT and HT females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

Figure 5.12 presents the FRAP values compared between NT and HT females and it is seen form this figure that the mean value for the HT group (365 µM) was slightly higher than that of the NT group (353 µM), which is 3.4 % higher. The spread in the NT group (i.e. 239.2 µM to 551.9 µM) was wider than the spread of values in the HT group (240.0 µM to 469.3 µM). The Student's T test showed no statistical difference between the two groups ($p = 0.34$). The antioxidant capacity was almost the same for both groups.

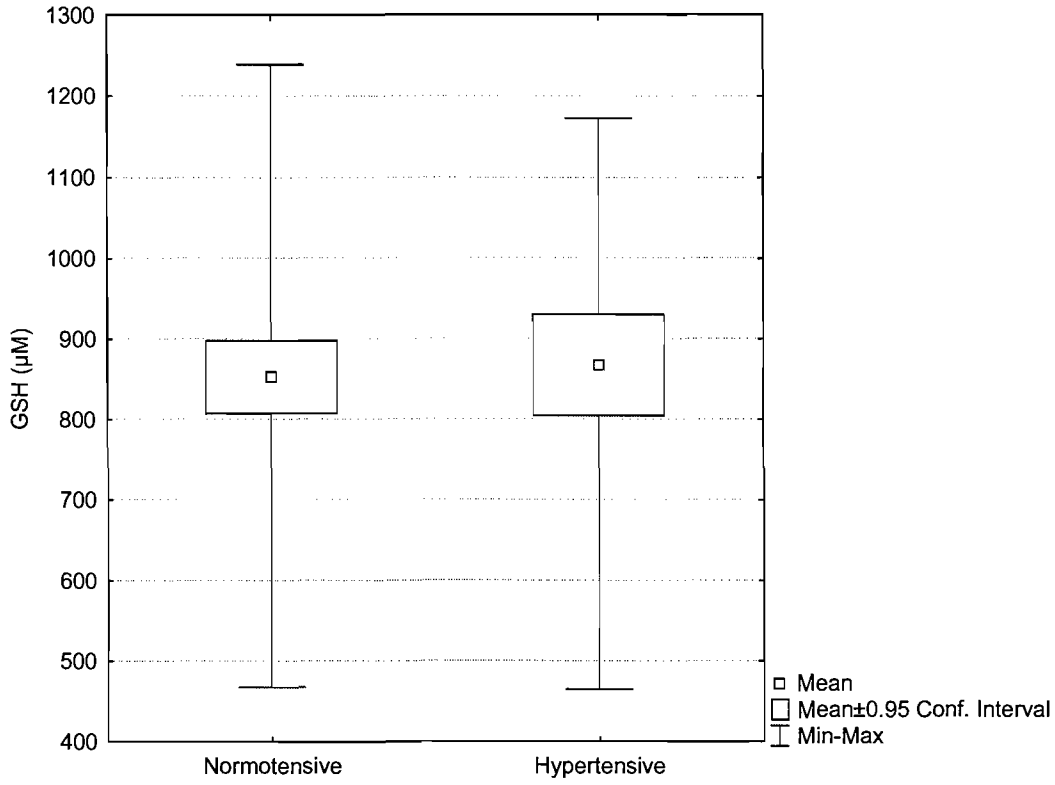


Figure 5.13. Comparison of GSH values between NT and HT females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

Figure 5.13 shows the comparison in GSH levels between NT and HT females. The two groups had similar mean values (868 µM for the NT group and 853 µM for the HT group). The spread for the NT group (467.9 µM to 1239.0 µM) was also wider than the HT spread of values (464.9 µM to 1173.0 µM). The Student's T test showed that there is no difference between the two groups ($p = 0.70$).

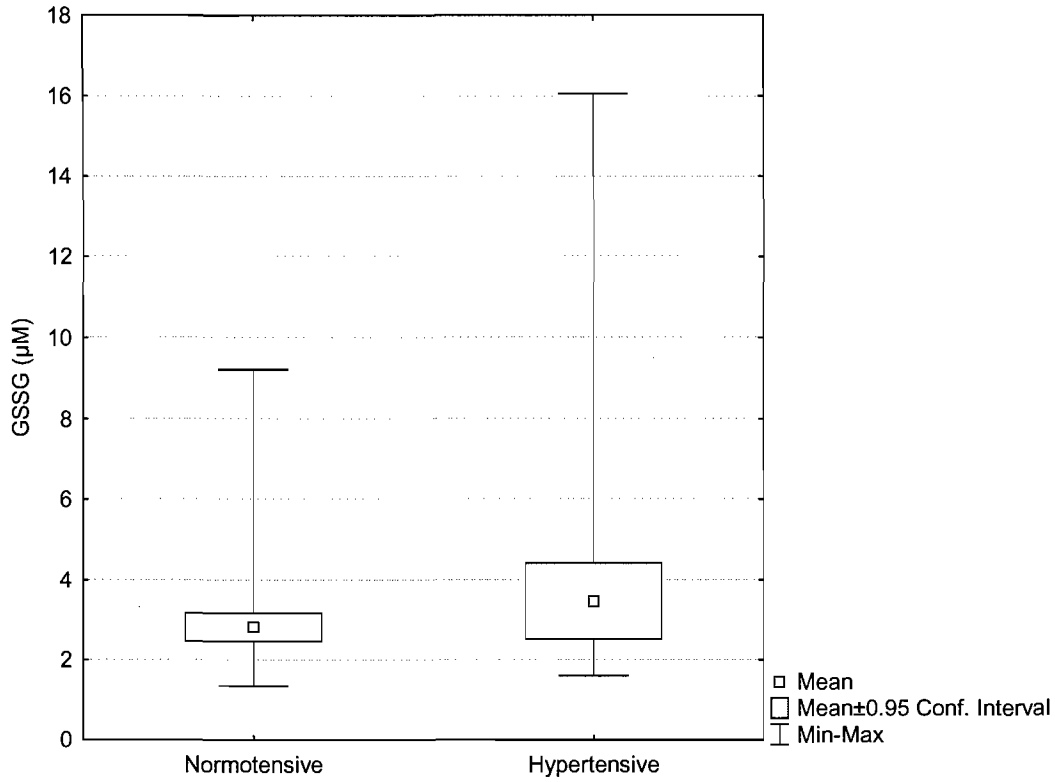


Figure 5.14. Comparison of GSSG values between NT and HT females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

Figure 5.14 shows the comparison in GSSG content between NT and HT female groups. The mean value for the HT group was 3.5 µM and for the NT group, 2.9 µM. The mean value for the HT group is thus 20.7 % higher. The spread of values in the NT group ranged from 1.3 µM to 9.2 µM, while the range in the HT group was 1.6 µM to 16.1 µM. Again, according to the Student's T test, the GSSG values for the NT and HT did not deliver any significant difference ($p = 0.13$).

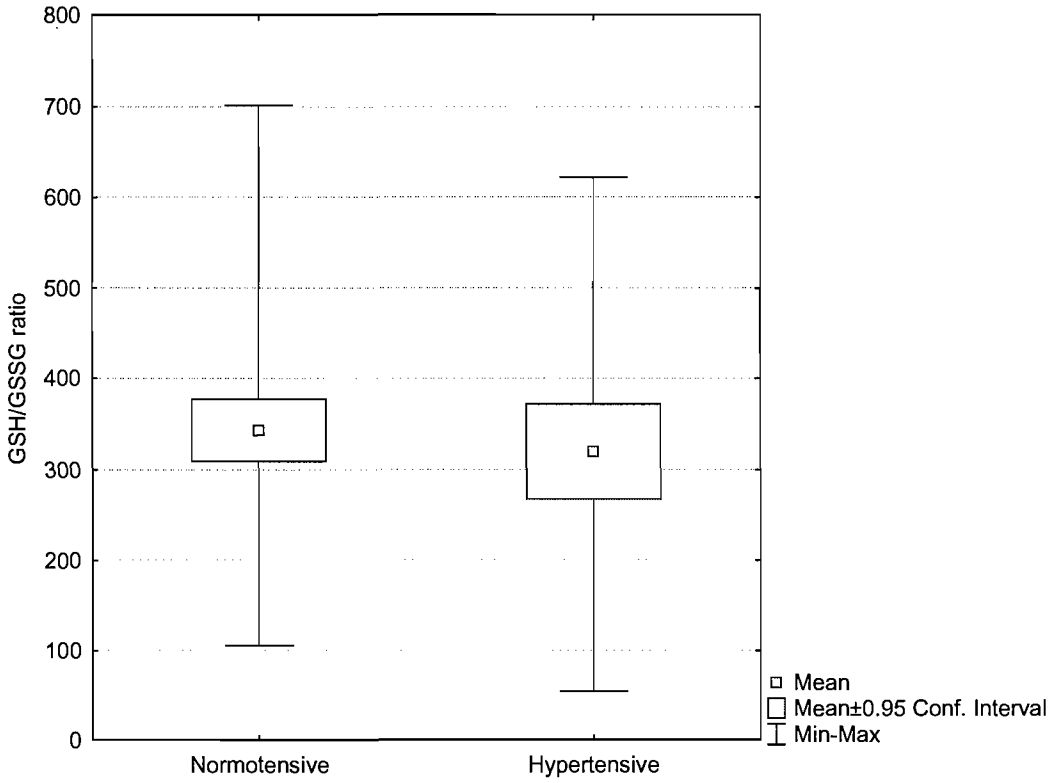


Figure 5.15. Comparison of GSH/GSSG ratio values between NT and HT females. As the legend indicates, the plot shows mean ± 95 % confidence interval values, as well as minimum and maximum values.

Figure 5.15 represents the comparison in the GSH/GSSG ratio between NT and HT females. The range of values in the NT female group was 105.7 to 701.6 and in the HT group, 54.4 to 622.3. The maximum and minimum values for the HT group, as well as the mean value (320) was lower than that of the NT group (344). Although, from the Student's T test, no difference existed for the GSH/GSSG ratio between NT and HT females ($p = 0.43$).

In Section 5.3.2 the oxidative stress/antioxidant capacity parameters, when compared between NT and HT females, did not deliver useful results. Firstly, the mean ROS value of the HT group was slightly lower than that of the NT group, which is contrary to the literature (Chaves *et al.,* 2007), although there was no statistical difference between NT and HT groups. FRAP values, as well as GSH values were also slightly higher in the HT group than the NT group. This would suggest a higher antioxidant capacity, but there was no statistical difference. This can possibly be because of the fact that females have a more complex hormonal

balance than males. Other possible explanations for this phenomenon is given in Chapter 6.

5.4. Association between the tyrosine hydroxylase C-824T SNP and blood pressure.

After the participants were screened for the TH C-824T SNP, they were divided into three groups: the wild types (WT) without the C-824T SNP, heterozygotes with the SNP on only one allele and homozygotes with the SNP present on both alleles. Since oxidative stress/antioxidant capacity differed between the two gender groups (Section 5.2), the males and females were again considered as two separate groups. In Chapter 2, the possible effect of the C-824T SNP on blood pressure was discussed (Rao *et al.*, 2007). However, this possible association was investigated in sub-Saharan Africans and not yet in black South Africans. Therefore, the role of the C-824T SNP on blood pressure in the SABPA participants remained unclear.

The three different genetic groups (WT, heterozygote and homozygote) were compared to blood pressure values of the participants. This was done for male and female groups. Firstly, Figures 5.16 and 5.19 show the number of participants in each of the genetic groups for the males and females, respectively. This was done to show the distribution of the participants between the three groups. The blood pressure parameters of the males compared to the different genotype groups are illustrated in Figures 5.17 and 5.18. Results from the female blood pressure against the three genotype groups are shown is Figures 5.20 and 5.21.

5.4.1. The TH C-824T SNP and blood pressure in males.

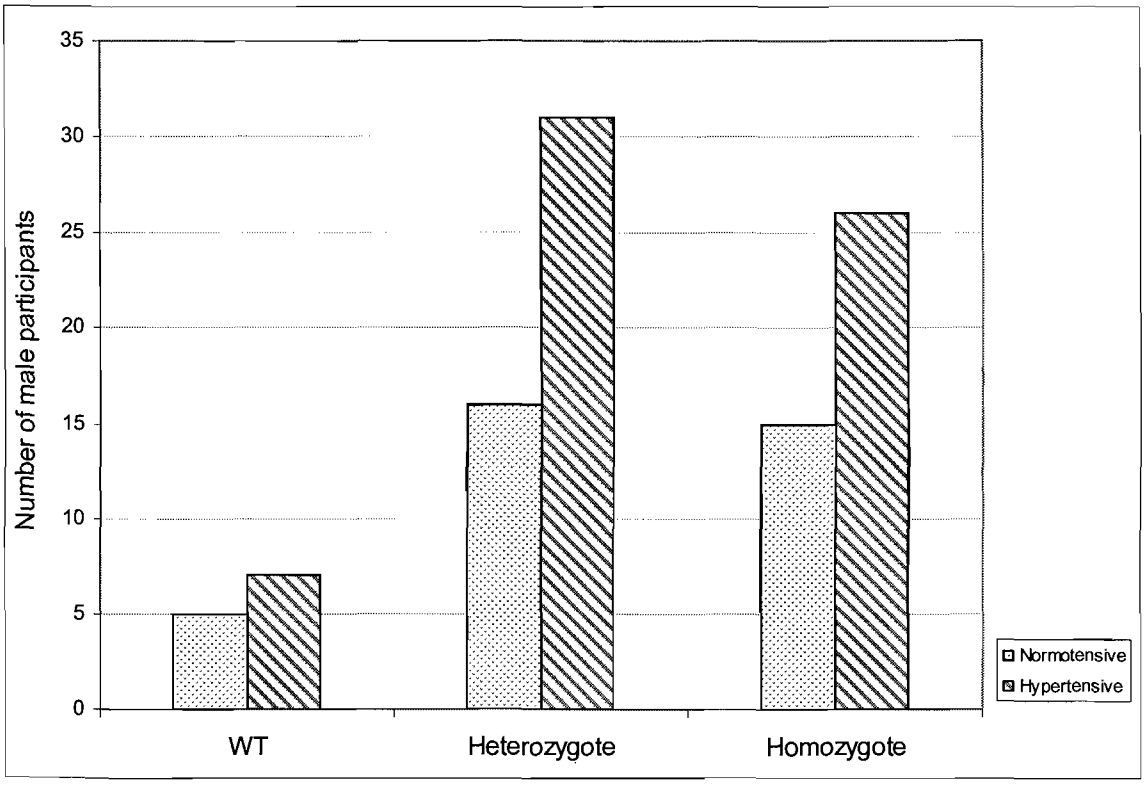


Figure 5.16. The number (n) of participants in the three genotypes. Figure shows Normotensive (NT) and hypertensive (HT) male participants in the three genotypes for the C-824T TH SNP. WT is the wild types.

From Figure 5.16 it is seen that a very large percentage of male participants had the SNP (88 %), with the groups being either heterozygotes (47 %) or homozygotes with the SNP (41 %). This finding is in accordance with the study of Rao *et al.* (2007), where they also found a higher percentage of black participants having the SNP (60.3 %), compared to Caucasian participants (16.7 %), although male and female groups were combined in that study. When these numbers are further broken down into hypertensive and normotensive groups, it is seen that overall there was a higher percentage of hypertensive male participants in each group; wild type (58.3 % vs. 41.7 %), heterozygotes (66 % vs. 34 %) and homozygotes (63.4 % vs. 36.6 %).

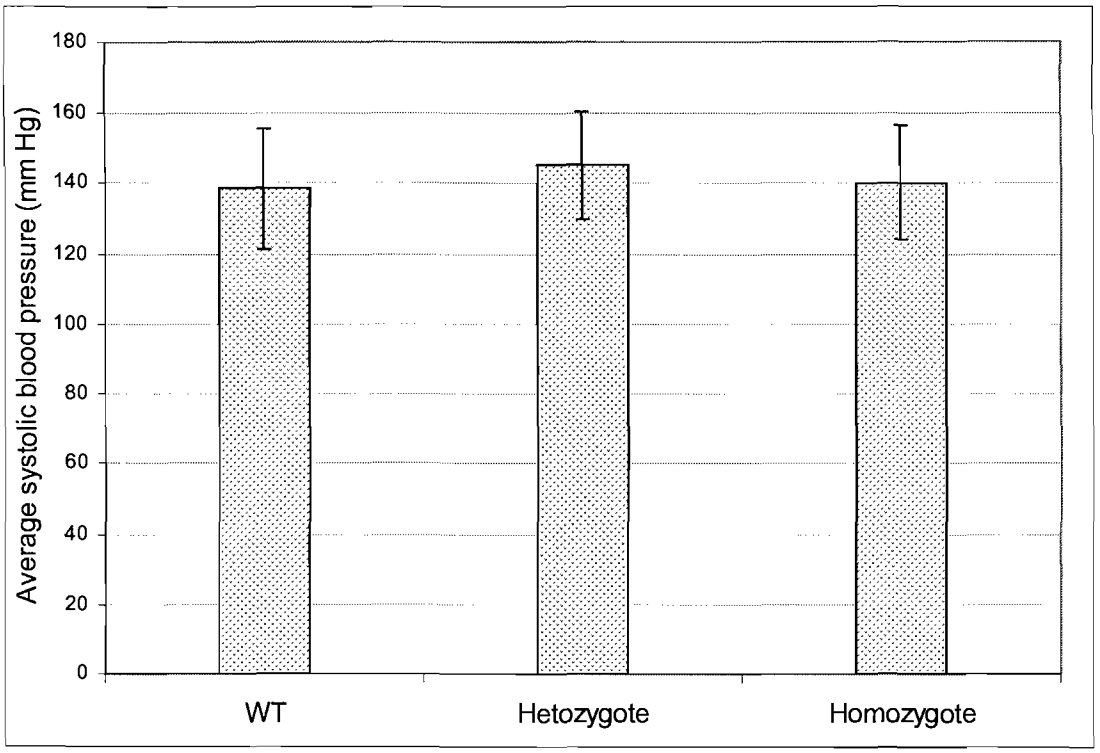


Figure 5.17. Average systolic blood pressure of the three genotypes. Wild type (WT), heterozygous and homozygous males are shown. Bars represent mean value ± 1 standard deviation (SD) ($p = 0.24$).

In Figure 5.17 the average systolic blood pressure values for the male participants are compared to the three genetic groups obtained after RFLP analysis. After performing a One-way ANOVA on the results to calculate differences in mean blood pressure, no statistically significant difference between the three groups with regards to systolic blood pressure was found ($p = 0.24$).

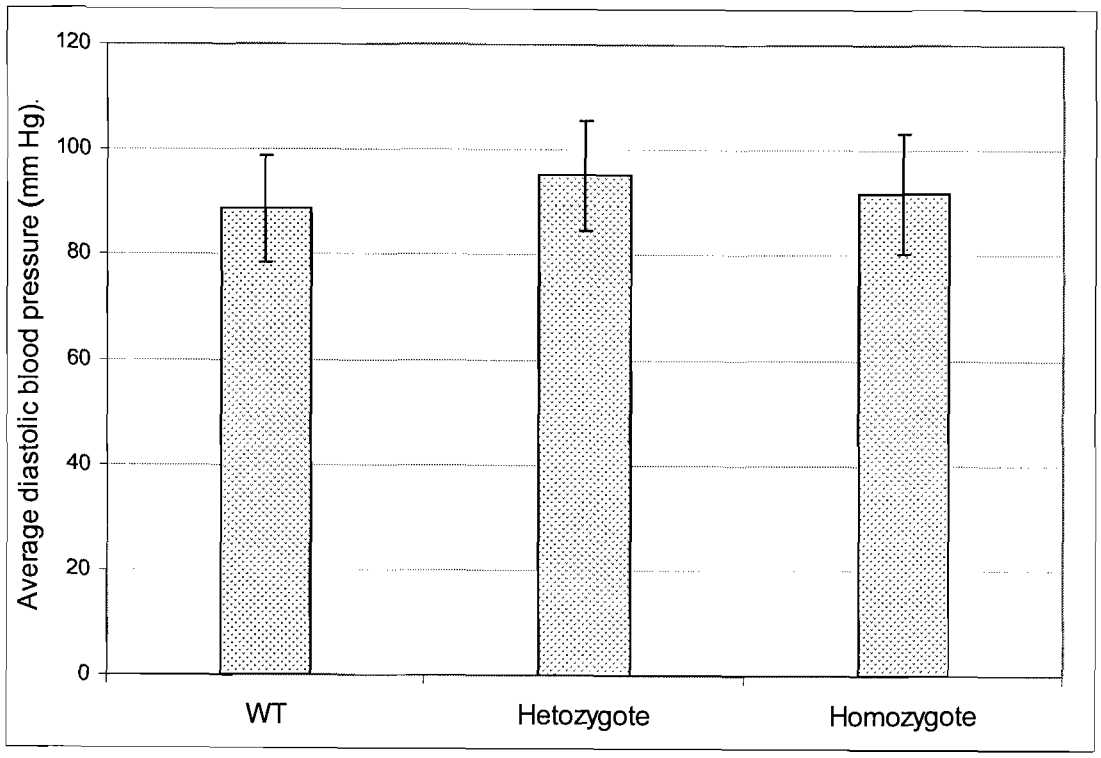


Figure 5.18. Average diastolic blood pressure of the three genotypes. Wild type (WT), heterozygous and homozygous males are shown. Bars represent mean value ± 1 standard deviation (SD) ($p = 0.13$).

Figure 5.18 shows the average diastolic blood pressure compared between the three male genetic groups, wild type, heterozygotes and homozygotes with the SNP. It is seen that the heterozygotes present with a slightly higher diastolic blood pressure than the wild types, but this increase is not seen in the homozygotes, as can be expected. After one-way ANOVA analysis, a non-significant p-value (0.13) was obtained.

5.4.2. The TH C-824T SNP and blood pressure in females.

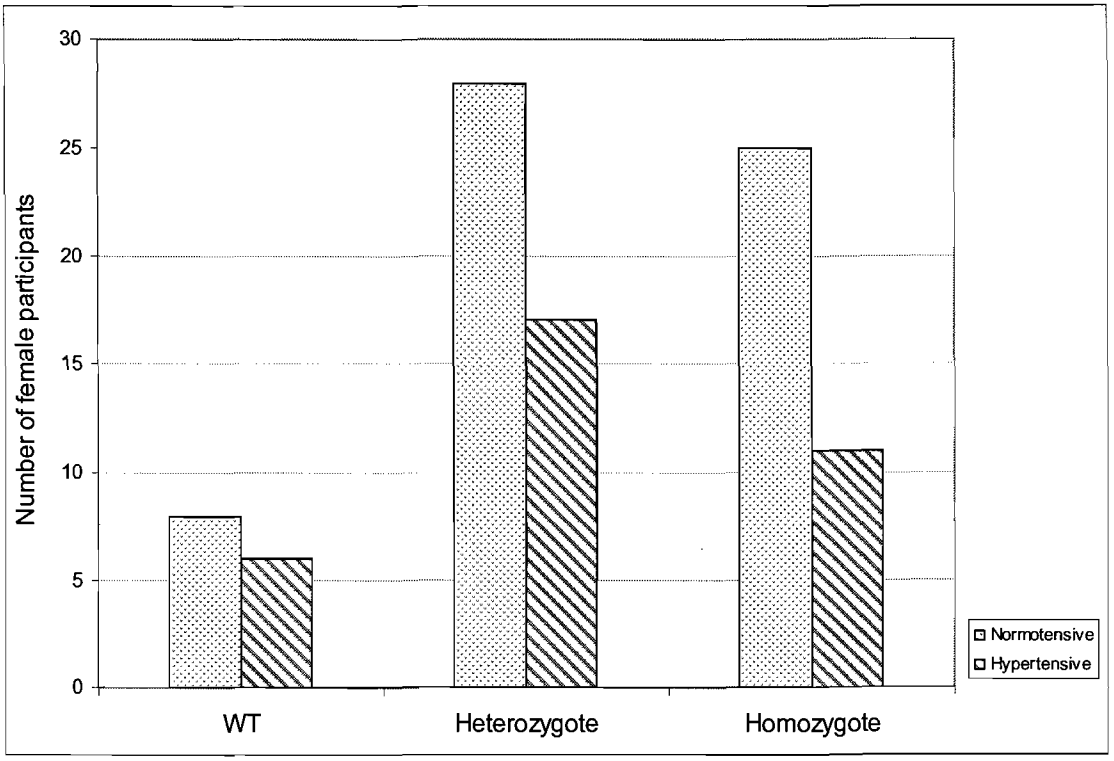


Figure 5.19. The number (n) of participants in the three genotypes. Figure shows Normotensive (NT) and hypertensive (HT) female participants in the three genotypes for the C-824T TH SNP. WT is the wild types.

From Figure 5.19 it is seen that a very large percentage of female participants had the SNP (86.3 %), but most of them were normotensive, compared to the high percentage of hypertensive males with the SNP. The three genetic groups were wild types (14.7 %) heterozygotes (47.4 %) or homozygotes with the SNP (37.9 %). This finding is in accordance with the study of Rao *et al.* (2007), where they found a higher percentage of black participants having the SNP (60.3 %), compared to Caucasian participants (16.7 %), although male and female groups were pooled in that study. If these percentages are further broken down into hypertensive and normotensive groups, it is seen that overall there were a higher percentage of normotensive female participants in each group; wild type (57.1 % vs. 42.9 %), heterozygotes (62.2 % vs. 37.8 %) and homozygotes (69.4 % vs. 30.6 %).

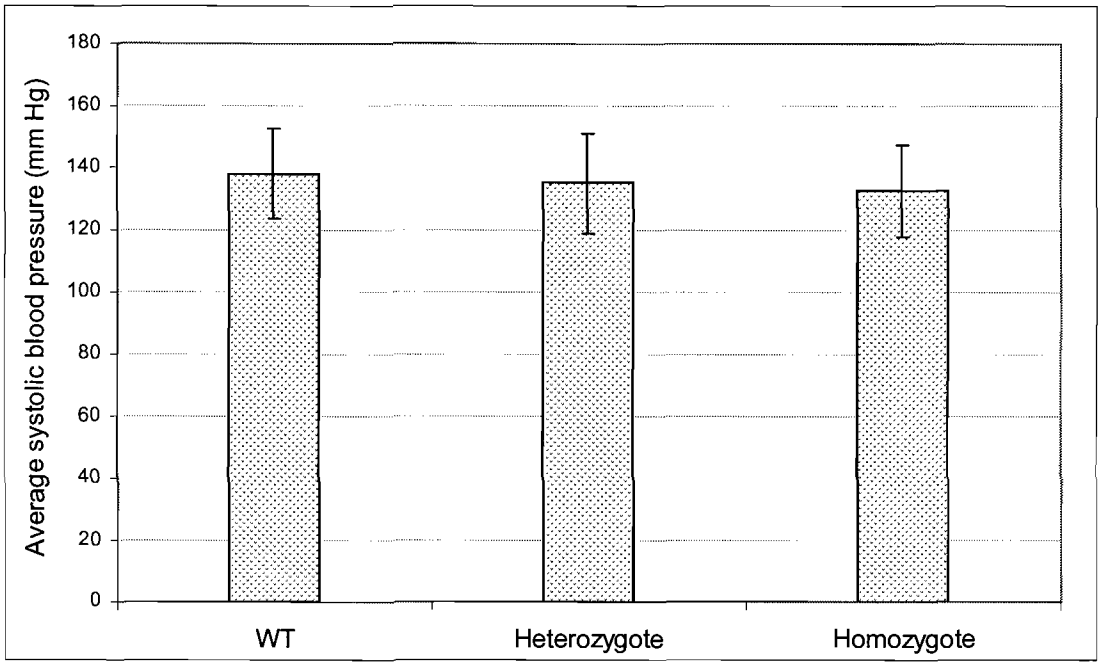


Figure 5.20. Average systolic blood pressure of the three genotypes. Wild type (WT), heterozygous and homozygous females are shown. Bars represent mean value ± 1 standard deviation (SD) ($p = 0.52$).

Blood pressure values from female participants were compared to the three genetic groups after RFLP analysis. Systolic (Figure 5.20) and diastolic (Figure 5.21) blood pressure values were compared separately and again, no statistical difference was seen.

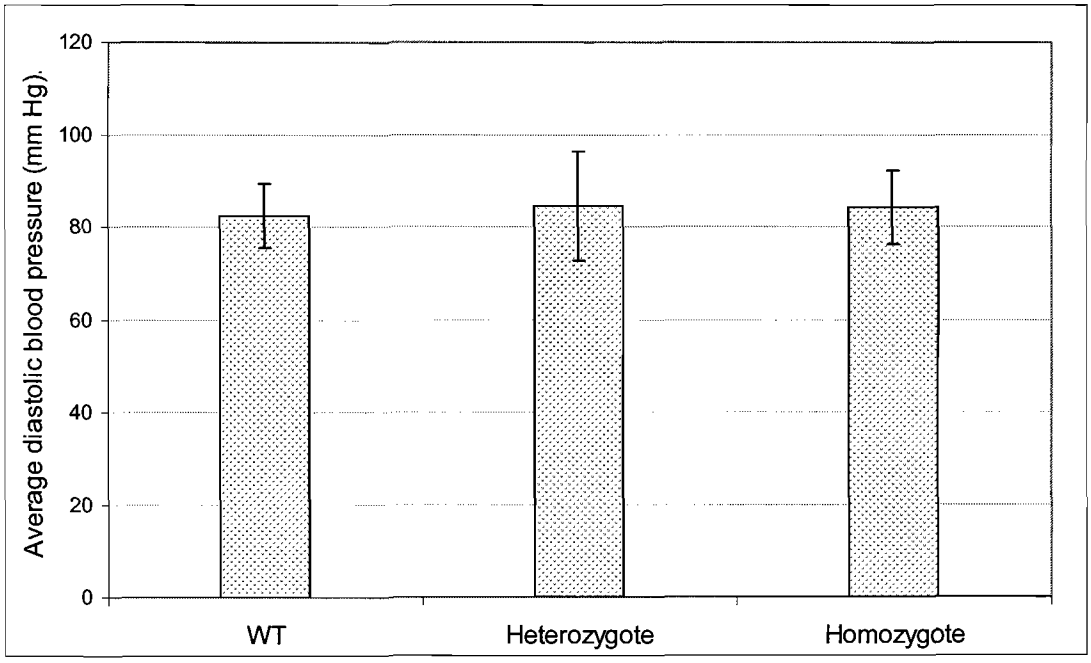


Figure 5.21. Average diastolic blood pressure of three genotypes. Wild type (WT), heterozygous and homozygous females are shown. Bars represent mean value ± 1 standard deviation (SD) ($p = 0.81$).

Considering the above-mentioned results, it was concluded that the SNP has no influence on the blood pressure in both the male and female groups participating in this study. This is contradictory to the results from Rao *et al.* (2007), who reported a correlation between this SNP and blood pressure. It is evident that in the black South African population on which this study was conducted, the SNP has no effect on blood pressure. These findings do not support the second hypothesis.

5.5. The TH C-824T SNP and oxidative stress.

Results from the RFLP analysis on the 200 African participants were also compared to oxidative stress parameters. The p-values, after One-way ANOVA analyses, are given in Table 5.1.

Table 5.1. One-way ANOVA analyses for oxidative stress/antioxidant capacity parameters and The TH C-824T SNP genotypes.

	Males	**Females**
ROS	0.05	0.61
FRAP	0.71	0.22
GSH	< 0.01	0.85
GSSG	0.44	0.24
GSH/GSSG	0.69	0.86

This table shows p-values obtained from comparison of the parameters in One-way ANOVA tests. Results are considered statistically significant with p-values ≤ 0.05. Each p-value denotes the difference between a said oxidative stress/antioxidant capacity parameter and the three genotypes groups seen as a whole, therefore, only one p-value is given per parameter.

As can be observed from Table 5.1, the only parameters which gave statistically significant results were in males with ROS and GSH values ($p<0.05$). All the other parameters gave higher p-values, indicating that these parameters were not influenced by the SNP. For the parameters that were influenced by the SNP (ROS and GSH), a Post hoc analysis (Tukey HSD for unequal groups) was performed to determine if statistically significant difference exists between the genotypes. The results are shown in Figures 5.22 and 5.23.

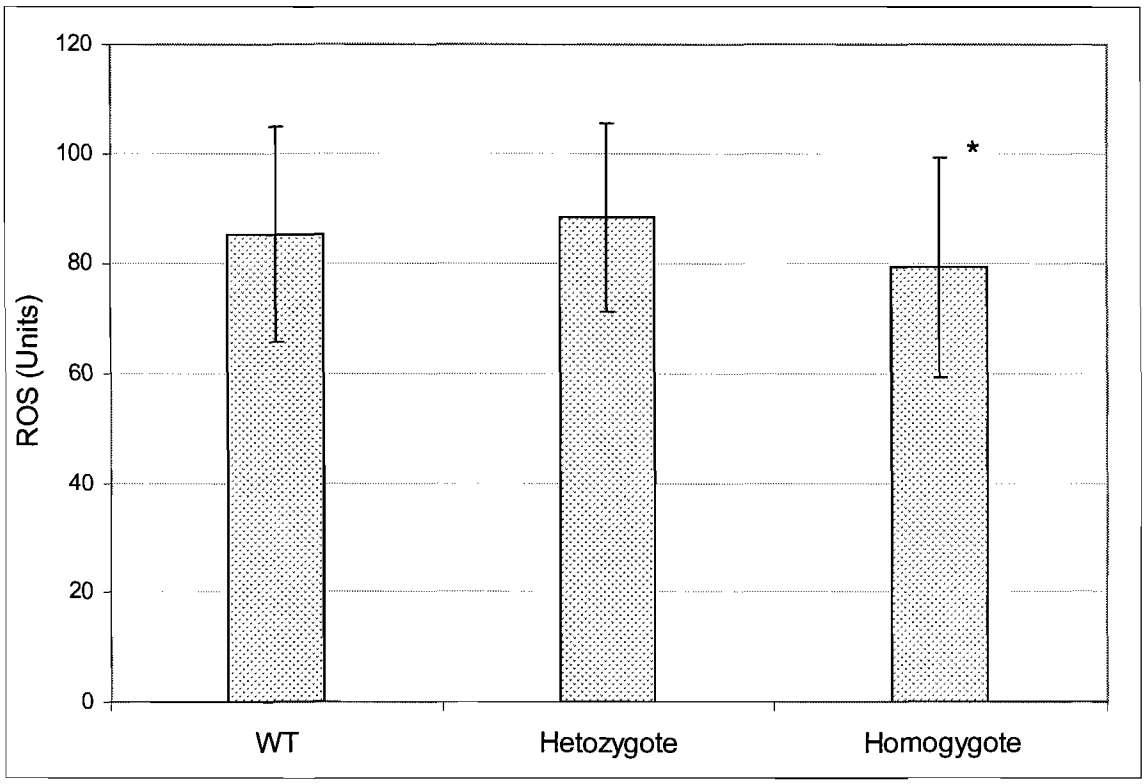


Figure 5.22. ROS values of wild type (WT), heterozygous and homozygous males. This graph shows one way ANOVA results between the three genotype groups (p = 0.05). Bars represent mean value ± 1 SD. *Significant p-value (0.05) obtained from difference between heterozygotes and homozygotes.

Table 5.2. Post hoc analysis showing p-values between the three genetic groups and ROS values for males.

	WT	Heterozygote	Homozygote
WT	-	0.91	0.65
Heterozygote	0.91	-	0.05
Homozygote	0.65	0.05	-

This table shows the results from performing Tukey's unequal HSD test. This test shows where a significant p-value lies (between which of the various groups). WT is wild type.

In Figure 5.22 the ROS units are compared between the three genotypes groups obtained after RFLP analysis in males: wild type (WT), heterozygous and homozygous. From Table 5.1, a p-value of 0.05 was observed and after analysis with Tukey HSD for unequal groups. The resulting p-values suggested a difference between the heterozygote and homozygote groups only. Therefore, no difference is observed between WT and heterozygous (p = 0.91) and also between WT and homozygous (p = 0.65). This means that ROS values are lower in participants with the mutation in both alleles than that of the heterozygotes, but there is no statistical difference between the wild types and the heterozygotes, or

the wild types and the homozygotes. These results are contrary to the hypothesis stated in Section 2.4. These findings seem to disprove the hypothesised indirect association between the SNP and resulting oxidative stress. This can be because of the relatively small group this study was done on.

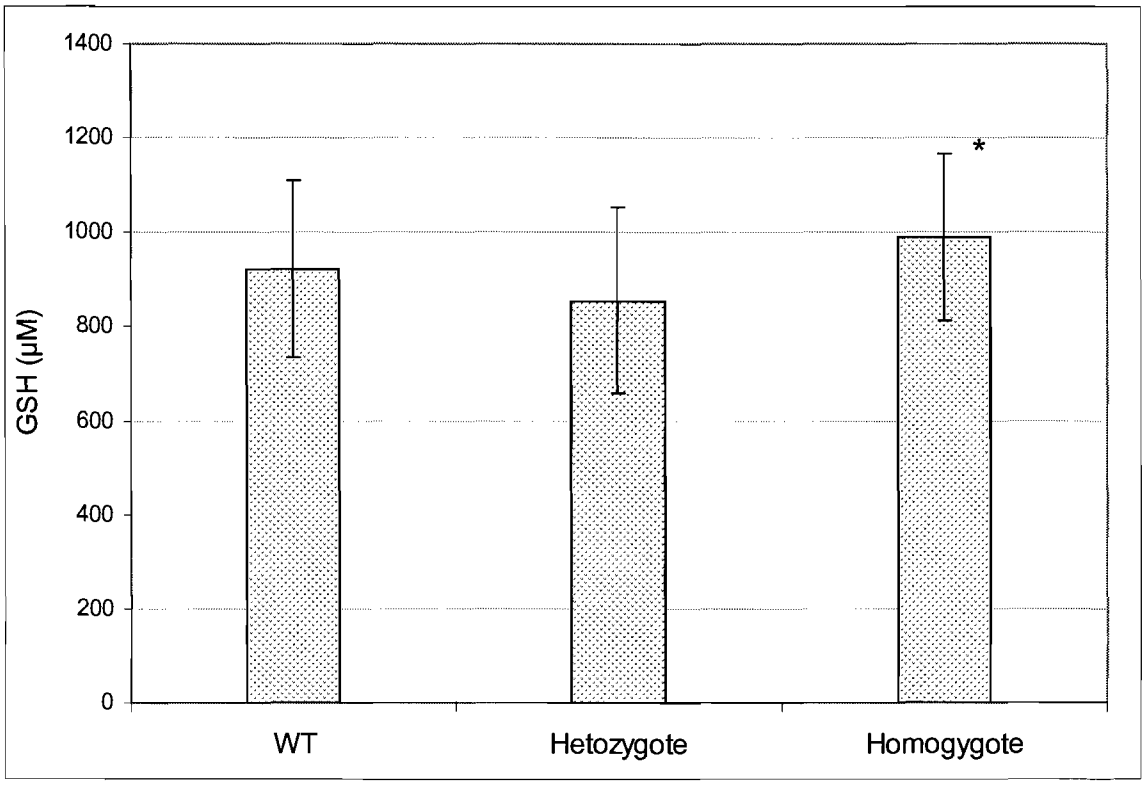


Figure 5.23. GSH values of wild type (WT), heterozygous and homozygous males. This graph shows one way ANOVA results between the three genetic groups ($p < 0.01$). Bars represent mean value ± 1 SD. *: Significant p-value (< 0.01) obtained from difference between heterozygotes and homozygotes.

Table 5.3. Post hoc analysis showing p-values between the three genetic groups and GSH values for males.

	WT	Heterozygote	Homozygote
WT	-	0.67	0.66
Heterozygote	0.67	-	0.01
Homozygote	0.66	0.01	-

This table shows the results from performing Tukey's unequal HSD test. This test shows where a significant p-value lies (between which of the various groups). WT is wild type.

In Figure 5.23 the GSH units are compared between the three genetic groups obtained after RFLP analysis in males: wild type (WT), heterozygous and homozygous. From Table 5.2 a p value of < 0.01 was observed and after analysis with Tukey HSD for unequal groups, the resulting p-values showed a difference

between the heterozygote and homozygote groups only. Therefore, no difference is seen between WT and heterozygous ($p = 0.67$) and also between WT and homozygous ($p = 0.66$). This indicates that GSH concentration is higher in individuals with the mutation on both alleles. Again, this is contrary to the stated hypothesis, as it is clear that the antioxidant capacity (only in terms of GSH content) is higher in homozygotes. Perhaps it is due to the body's ability to adapt and change to the consequences of the presence of the SNP. Thus presenting with a higher antioxidant capacity.

5.6. Correlation between blood pressure and oxidative stress.

ROS is highly associated with increased blood pressure in men (Section 5.3). Although no apparent association of the other parameters was shown with blood pressure, Pearson correlations were used to show (by means of r-values) the strength of association between the different parameters. Where there was a medium to strong correlation between parameters, scatter plots were constructed, showing the regression line, as well as 95 % confidence interval.

Table 5.4 and 5.5 show the results from Pearson correlation analyses done on the various parameters from the male and female groups. This means that each parameter was compared with every other parameter to show the strength of correlation between the two parameters. The correlation coefficient, r-value was calculated for each parameter to see whether the correlation was biologically significant. The table shows two values for each parameter. Firstly, it shows an r-value which ranges from -1 to 1. A negative r-value indicates a negative correlation. This means that if parameter A increases, parameter B will decrease and *vice versa*. A positive r-value indicates a positive correlation which means that if parameter A increases, so will parameter B. The second value given in the table is the p-value. This value indicates the statistical significance of the observed r-value.

Table 5.4. Pearson correlations for all measured parameters in males:

	Daytime SBP	Daytime DBP	ROS	FRAP	GSH	GSSG	GSH/ GSSG
Daytime SBP	-	0.87 p < 0.01	0.35 p < 0.01	0.22 p = 0.03	-0.13 p = 0.20	-0.11 p = 0.30	0.28 p < 0.01
Daytime DBP	0.87 p < 0.01	-	0.28 p < 0.01	0.20 p = 0.04	-0.04 p = 0.70	-0.10 p = 0.31	0.28 p < 0.01
ROS	0.35 p < 0.01	0.28 p < 0.01	-	-0.13 p = 0.22	-0.19 p = 0.06	-0.01 p = 0.92	0.06 p = 0.57
FRAP	0.22 p = 0.03	0.20 p = 0.04	-0.13 p = 0.22	-	0.05 p = 0.61	< 0.01 p = 0.98	0.12 p = 0.24
GSH	-0.13 p = 0.20	-0.04 p = 0.70	-0.19 p = 0.06	0.05 p = 0.61	-	0.03 p = 0.75	0.25 p = 0.01
GSSG	-0.11 p = 0.30	-0.10 p = 0.31	-0.01 p = 0.92	< 0.01 p = 0.98	0.03 p = 0.75	-	-0.59 p < 0.01
GSH/ GSSG	0.28 p < 0.01	0.28 p < 0.01	0.06 p = 0.57	0.12 p = 0.24	0.25 p = 0.01	-0.59 p < 0.01	-

SBP indicates average systolic blood pressure, DBP indicates average diastolic blood pressure.

Table 5.4 shows the correlation studies done on parameters from the male group. From the table it is clearly observed that the parameters that showed medium to strong correlations were ROS values compared with daytime systolic blood pressure ($r = 0.35$) and daytime diastolic blood pressure ($r = 0.28$) in males. Both of these correlation coefficients are statistically significant with p-values of < 0.01. Another observable trend in the table is the strong correlation between systolic blood pressure and diastolic blood pressure ($r = 0.87$), although it is generally seen that the two measurements of blood pressure rise and fall *in sync*. This correlation is thus expected and not mentioned further. Another strong correlation seen from the table is the correlations between GSSG and the GSH/GSSG ratio ($r = -0.59$); and GSH and the GSH/GSSG ratio ($r = 0.25$). Again, these correlations are not of practical importance, as the ratio is calculated from GSH and GSSG values. Thus, only ROS values compared to blood pressure gave significant results with medium strength correlation coefficients.

Table 5.5. Pearson correlations for all measured parameters in females:

	Daytime SBP	Daytime DBP	ROS	FRAP	GSH	GSSG	GSH/ GSSG
Daytime SBP	-	0.69 p < 0.01	-0.06 p = 0.54	0.20 p = 0.06	-0.04 p = 0.73	0.11 p = 0.30	-0.08 p = 0.45
Daytime DBP	0.69 p < 0.01	-	-0.12 p = 0.24	0.16 p = 0.14	0.02 p = 0.84	0.10 p = 0.34	-0.04 p = 0.72
ROS	-0.06 p = 0.54	-0.12 p = 0.24	-	0.24 p = 0.02	-0.13 p = 0.21	-0.16 p = 0.13	-0.05 p = 0.64
FRAP	0.20 p = 0.06	0.16 p = 0.14	0.24 p = 0.02	-	-0.15 p = 0.16	< 0.01 p = 0.99	-0.14 p = 0.18
GSH	-0.04 p = 0.73	0.02 p = 0.84	-0.13 p = 0.21	-0.15 p = 0.16	-	-0.01 p = 0.91	0.53 p < 0.01
GSSG	0.11 p = 0.30	0.10 p = 0.34	-0.16 p = 0.13	< 0.01 p = 0.99	-0.01 p = 0.91	-	-0.65 p < 0.01
GSH/GSSG	-0.08 p=.450	-0.04 p = 0.72	-0.05 p = 0.64	-0.14 p = 0.18	0.53 p < 0.01	-0.65 p < 0.01	-

SBP indicates average systolic blood pressure, DBP indicates average diastolic blood pressure.

From Table 5.5 the only parameters with medium to strong correlation coefficients in the female group, were the correlations between systolic and diastolic blood pressure and between GSH, GSSG and the ratio of GSH/GSSG. These correlation coefficients are not practically significant in this study and are discussed above. No other comparison between parameters delivered any useful results. It can thus be assumed that there are no strong correlations between any other parameter in the female group. Possible reasons for this lack of strong correlations in the female group, are given in Chapter 6.

After perusal of r-values in the male and female groups, the strongest effect sizes that were also significant in this study were selected and presented graphically. These were only done with data from the male group and not from the female group, because no strong correlation was seen in the table for females. These visual representations are given in Figures 5.24 and 5.25.

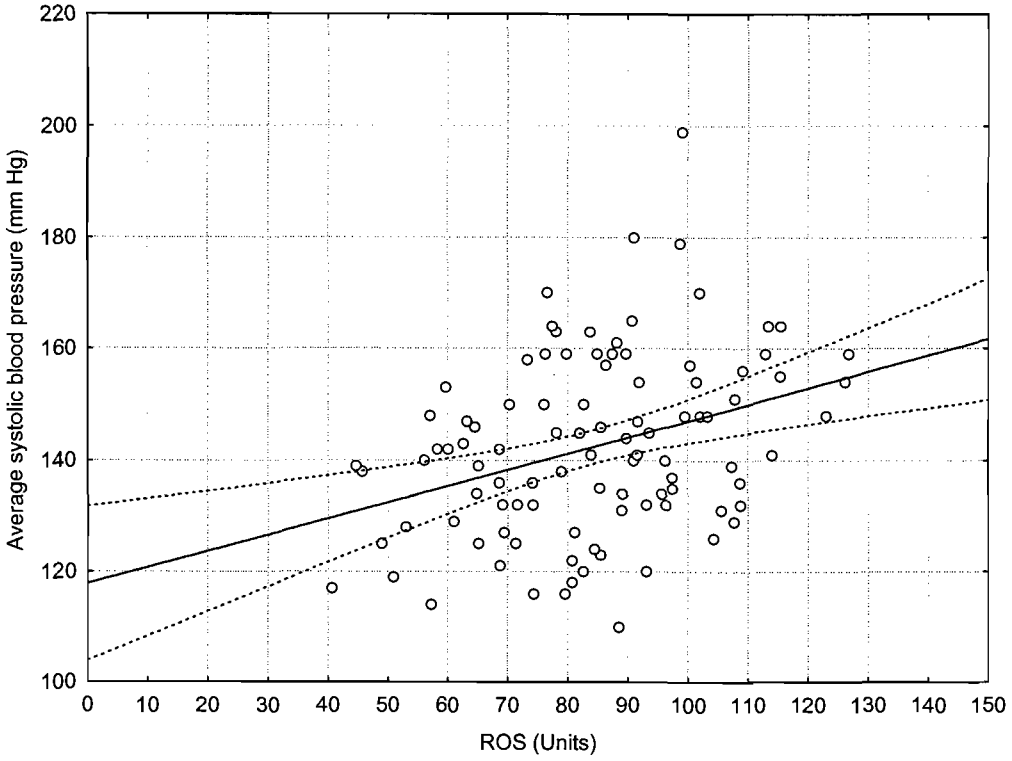


Figure 5.24. Correlation between average systolic blood pressure and ROS in males. The solid line represents the regression line, while the dotted line represents 95% confidence interval (r = 0.35).

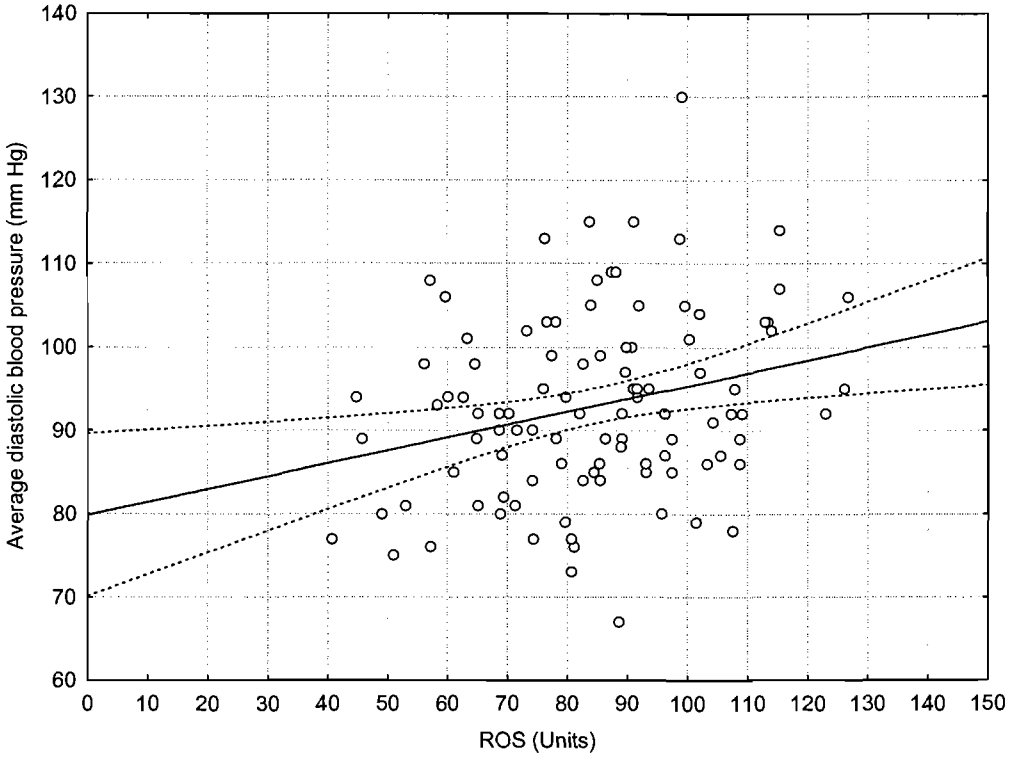


Figure 5.25. Correlation between average diastolic blood pressure and ROS in males. The solid line represents the regression line, while the dotted line represents 95% confidence interval (r = 0.28).

Figure 5.24 and 5.25 represented the correlations between ROS values and systolic and diastolic blood pressures in males. The figures show the blood pressures on the y-axis and the ROS values on the x-axis. In both figures a positive correlation is seen with an r-value of 0.35 between systolic blood pressure and ROS and an r-value of 0.28 between diastolic blood pressure and ROS. It is therefore concluded that ROS values do, in fact, rise with a rise in blood pressure in males, but not in females.

Some of the results presented in this chapter seem to support the hypothesis formulated for this study, while other parameters delivered rather conflicting results. The possible explanations of the conflicting results are given in Chapter 6, along with final conclusions and recommendations for future studies.

Chapter 6:

Conclusions and Recommendations

6.1. Introduction.

The aim of this study was to investigate the possible association between oxidative stress and hypertension in black South African men and women. It was hypothesised that with a rise in oxidative stress, an increase in blood pressure (and subsequently, hypertension) will be seen. Also, if a specific single nucleotide polymorphism (SNP) is present in the promoter region of the tyrosine hydroxylase gene (the enzyme responsible for the rate-limiting step in catecholamine biosynthesis), i.e. C-824T, hypertension is likely to be more prevalent. The study was done on 200 black South African urbanised educators from the North-West province.

To test the two hypotheses stated in Section 2.4, ambulatory blood pressure measurements were taken and several tests and assays were carried out on the biological samples obtained from the participants. These included oxidative stress/antioxidant capacity markers and a newly developed polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method to test for the presence of the SNP.

After sample collection and analyses, statistical methods were carried out using Statistica version 8 (StatSoft, Inc., Tulsa, OK). Several graphs were used to represent and compare the results as shown in Chapter 5. What is immediately evident from the data is that an alarmingly large number of participants (63.4 % of the males and 36.2 % of the females) were considered to be hypertensive. These findings are in accordance with the literature reviewed in Section 2.2.4. It is thus clear that the SABPA participants in this study are a good source for investigating factors associated with hypertension.

6.2. Discussion.

Although somewhat contradictory, it is evident from the literature that there is a possible gender difference in oxidative stress parameters (Section 5.2). This prompted us to separate the oxidative stress data of this study into male and female groups to see whether a gender difference indeed exists. With regards to the oxidative stress parameters measured, a difference was seen. Therefore it was decided to separate all subsequent data into male and female groups.

Oxidative stress parameters were measured and compared between male and female groups and several differences were observed. Firstly, the reactive oxygen species (ROS) levels in the male group were significantly lower than the ROS levels in the female group. Note that the groups still consisted of normotensive (NT) and hypertensive (HT) individuals at this stage, and that the only comparison was between the male group as a whole and the female group as a whole. In the FRAP results (a measure of the antioxidant capacity), another meaningful difference was evident. In this case the males had higher mean values than the females, suggesting a higher antioxidant capacity in males than in females. The GSH level is another indication of the antioxidant capacity of an individual, as the reduced glutathione molecule is one of the most important radical scavengers in the body. From the results of the GSH levels in the two groups, it was seen that again, males had a higher mean value than females. These results would suggest a higher antioxidant capacity and lower oxidative stress levels in males than in females of similar age and health status. Lastly, in neither the GSSG results, nor the ratio of GSH/GSSG significant differences between the two groups were observed. This can be because of the method used to measure the GSSG levels. This was done with the same kit as for the GSH levels and as the concentrations of GSH are orders of magnitude higher than the GSSG levels, it was thought that the GSSG levels were near the detection-limit of the method. Poor results can also arise as a result of the instability of the GSSG samples *in vitro*.

To explain the gender difference seen in the groups, some speculations can be made. As the ROS values differed, one can assume that the men had indeed lower ROS values. On the other hand, one can also say that ROS values were

higher in females to begin with. The same argument can be made for the antioxidant capacity markers, where one can argue that in the female group with the higher ROS values (and thus oxidative stress), the antioxidant capacity is depleted. Thus it appears that there is some unknown cause or source of oxidative stress in females that is not present in males. A possible explanation for this unusual occurrence, is that women have a much more complex hormonal system than men. For example, different stages in the menstrual cycle are regulated by different hormones and varying concentrations of these hormones in the body. Because of this and the fact that not all women are in the same stage at the same time in their menstrual cycles, it is safe to assume that data from any biological sample from a group of women, would differ significantly at any time. Hormones like estradiol (an oestrogen), follicle stimulating hormone (FSH), luteinizing hormone (LH) and progesterone influence and regulate not only the menstrual cycle, but have various other effects on different systems of the body, like the immune system (Da Silva, 1999).

Because of the observed gender differences in some of the oxidative stress parameters (Section 5.2), the association between blood pressure and oxidative stress was investigated separately in African men and women. Oxidative stress parameters were separated into NT and HT male groups. The main finding of this study was that ROS values (and thus oxidative stress) are elevated in HT males, compared to NT males. The exact biological reason for this is still unclear as it was not the aim of this study to elucidate the mechanism of action behind this phenomenon, but only to investigate if there was an association between oxidative stress and hypertension. However, these results do support the hypothesis proposed that blood pressure will increase with increased oxidative stress, as determined by ROS levels, although only in males. Therefore it is postulated that a higher blood pressure somehow affects the oxidative stress status of males. On the other hand, it is not known whether the higher oxidative stress levels are a cause or consequence of hypertension. FRAP values and GSH levels did not differ significantly between HT and NT males. This would suggest that antioxidant capacity is not influenced by a higher blood pressure, even if there is a higher oxidative stress status. One would assume that with a higher oxidative stress status, the body would try to compensate this change by up-regulating the

antioxidant systems and perhaps this is exactly what can be inferred from the results. The fact that the antioxidant capacity in HT males is not significantly lower than that of the NT males would suggest just this: The body is up-regulating its antioxidant defence, although not enough to cause the mean value of HT males to be significantly higher than the mean value of NT males. Again, no difference is seen in the GSSG values or the GSH/GSSG ratio.

In the female group a rather opposite effect is seen with regards to meaningful differences between the various parameters of oxidative stress. In this group no statistical difference was observed throughout the oxidative stress parameters. However, it was also observed that normotensive females presented with higher ROS values than normotensive males, which is a surprising result as mentioned before. Therefore, it is possible that the already high values in normotensive females, as a result of some other cause of oxidative stress that is present only in females, masks any additional increase that may be due to hypertension. A smaller percentage of females were hypertensive (36 %), compared to males (63 %). It is interesting to note that although oxidative stress parameters are higher in females, the prevalence of hypertension is lower. Therefore, some other factor that increases oxidative stress may be at work in females that does not influence the prevalence of high blood pressure. Also, the FRAP values were positively associated with ROS values in women, which is contrary to what is expected theoretically. It is thus apparent that with influences in various biological systems, general measurements like those in oxidative stress profiling, may also be influenced by these hormones, possibly explaining the conflicting FRAP results. As discussed above, this force would seem to be the complex hormonal balance existing in females. On the other hand one can speculate that a higher blood pressure in females does not influence any oxidative stress parameter in females. But there would really be no useful explanation for this phenomenon. One other possible explanation for the conflicting FRAP results in women, is that it is known that the FRAP assay measure the antioxidant capacity of serum as a whole. This means that each and every molecule in serum that is capable of reducing iron ions *in vitro* is measured by this test, however only a small percentage of these molecules are antioxidants that specifically scavenge ROS. Therefore, only a

small percentage of the measured value will fluctuate in accordance with the measured ROS values.

The most common single nucleotide polymorphism (SNP) in the promoter region of the tyrosine hydroxylase gene (C-824T), and its associated haplotype (TGGG) is reported to be associated with a rise in blood pressure and a causative factor of essential hypertension (Rao *et al.*, 2007). However, results from RFLP analysis in this study did not support this association in the black participants. According to these results, the SNP does not measurably influence blood pressure, and also has no significant effect on oxidative stress parameters. These findings would serve to disprove the second part of the hypothesis stated in Section 2.4. The present study was the first where this particular SNP analysis was performed on specifically black urbanised South Africans. These results indicate that this particular SNP does not seem to influence blood pressure in black South Africans on any level. The only statistically significant differences obtained were in the male group, where ROS levels were lower and GSH levels were higher in homozygotes compared to heterozygotes for the SNP. These are conflicting results, because in their report, Rao *et al.* (2007) stated that this SNP causes a greater response in blood pressure to stressors and thus hypertension. With regards to this C-824T SNP, no observable change in any of the oxidative stress parameters, or the blood pressure data could be seen in either the male group or the female group.

Furthermore, methodological shortcomings of this study included the unreliability of data obtained from the measurement of oxidised glutathione (GSSG). The method used for this measurement was based on the same method used for reduced glutathione (GSH) measurement and was done with the same kit. However, the GSSG values were orders of magnitude less than GSH values and were thus at the detection limits of the method. The GSSG in the blood samples is also less stable than the GSH. For this reason, neither the GSSG values, nor the GSH/GSSG ratio values produced accurate results.

The final outcome of this study and final conclusion to be made, is that blood pressure can be associated with higher oxidative stress, as reflected by increased ROS levels, in black urbanised South African males and these results would serve

to accept the first part of the hypothesis stated in Section 2.4. Although not measured, one could speculate that these higher ROS levels can possibly contribute to the degeneration process in the vessel walls seen in hypertension and the associated cardiovascular damage. This study, however did not investigate exactly what the cause of the oxidative stress increase was, or if the increase in blood pressure was a cause or consequence of the oxidative stress.

6.3. Recommendations.

In the present study only daytime ambulatory blood pressure values were used, together with oxidative stress parameters and the SNP analysis. The data was not corrected for possible confounders such as age, HIV status, body mass index (BMI), smoking, alcohol use and the intake of medications and supplement. In future studies investigating the association between oxidative stress and hypertension, one important aspect to consider would be to define the exclusion criteria and inclusion criteria more clearly, to avoid high occurrence of variability in the dataset. Examples of exclusion criteria would be smoking and recreational drug usage, medication and supplement intake, hormonal replacement or supplementation therapy, hormone contraceptive usage, genetic metabolic defects, pregnancy and lactation, unhealthy participants (i.e. no HIV, TB, infectious or other diseases) and cardiovascular diseases. Examples of inclusion criteria would include participants of equal social status and similar ages and lifestyles, also similar diets and alcohol usage. Suggestions for future studies would include standardisation of a reproducible and reliable LC-MS method for measuring GSSG in biological samples, as the ratio of GSH/GSSG gives a much more accurate measurement of systemic oxidative stress.

The possible connection between blood pressure and oxidative stress has not been determined in Africans. This study therefore investigated the association between hypertension and oxidative stress, to determine whether there was such an association. In this regard the study achieved its aims and provided data to show that this association is indeed a significant topic to investigate. It provided a base for future studies to investigate this association and its mechanisms, confounders, consequences and possible interventions.

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Appendix A :

DNA sequence of the promoter region of the human TH gene showing positions and sequences of primers and recognition sequence of the restriction enzyme, Tru1I.

```
  5'…tgaggtcttg gaggcaaatc cctccaacgc ccttctgagc aggcacccag acctactgtg
 9061 ggcaggaccc acaggaggtg gaggcctttg gggaacaccg tggaggggca tagcatctcc
 9121 gagagaggac agggtctgca ctgggtgctg agagacagca ggggccgagc ggtaggcttc
 9181 cctgccccca gggatgttcc aggggagcgc aagggagggg cattaatatc gtggcaagaa
 9241 agggcaggca ttgcagagtg agcagcgacg gaactgggtt ttgtgggatg cataggagtt
 9301 cacccggata agaggtgggt gaggaatgac actgcaaacc ggggatcacg gagccccaaa
 9361 tccttctggg ccaggaagtg ggaagggttg gggggtcttc cctttgcttt gactgagcac
 9421 tcagcctgcc tgcagagggc agcgaggagc cacggagggg tgtgggacag ggatgccatg
 9481 gctgaagcag ttttaggaaa ggtcccaggg gctattgttg aagagagaac ggggagcggg
 9541 gagtcccaca gctgacagga gcagagtggg ccctgagaga tgccagctct gggtgccaca
 9601 gtgaccagcc ggggtaggcc ttcgagaagt cagggagcgt ctagggcttc tggctcctgc
 9661 tgggcccagg gtgtcatctt gggctgccaa caccagaaag cccagcagat acaggaagcc
 9721 ccaagccctg tcggaaacgg ttcttctcca ggagggacag cggtggcagc gttcagccgc
 9781 aggccatgca ctctggggcc acgtccttcc ctctgtacag tccagcattg tcaaggcggg
 9841 ctctggccat ctctgctgac cccagaggga tggggaggcc tccccttcca ccagaagggc
 9901 cagaagccac cctgggcagg ggcatcactc tccctgggtg gggcagcggc ggggagcagg
 9961 aggtgccagt gggcgtgggc tggatgcggg tgcctgcggg gcggacatgg aacttggggg
10021 aggctctagg ctggggttgt cctcaaggga gttctcaggt caccccaggg tcaccctcaa
10081 cccggggcct ggtggggtag aggagaaact gcaaaggtct ctccaagggg aaggcatcag
10141 ggccctcagc actgagggac gtgcgtgctc ttcaaagaag gggccacagg accccgaggg
                                      t (C-824T)
                                          Tru1I (T/TAA)

10201 aagccaggag ctagcagtgg gccatagagg ggctgagtgg ggtgggtgga agccgtccct


10261 ggccctggtc gccctggcaa ccctggtggg gactgtgatg caggaggtgg cagccatttg
10321 gaaacgcgtg gcgtctcctt agagatgtct tcttcagcct cccagggtcc tccacactgg
10381 acaggtgggc cctcctggga cattctggac cccacagggc gagcttggga agccgctgca
10441 agggccacac ctgcagggcc cgggggctgt gggcagatgg cactcctagg aaccacgtct
10501 acaagacaca cggcctggaa tcttctggag aagcaaacaa attgcctcct gacatctgag
10561 gctggaggct ggattccccg tcttggggct ttctgggtcg gtctgccacg aggttctggt
10621 gttcattaaa agtgtgcccc tgggctgcca gaaagccccc ccctgtgtgc tctcttgagg
10681 gctgtggggc caaggggacc ctggctgtct cagccccccg cagagcacga gcccctggtc
10741 cccgcaagcc cgcgggctga ggatgattca gacagggctg gggagtgaag gcaattagat
10801 tccacggacg agccctttct cctgcgcctc cctccttcct cacccacccc cgcctccatc…3'
```

Sequence numbers refer to the human *TH* gene, accession number AF536811 (GenBank) on the U.S.A. National Library of Medicine database (http://www.ncbi.nlm.nih.gov/pubmed/). The bold sequences indicate the annealing positions of the forward and reverse primers, respectively. The sequence highlighted in yellow indicates the incomplete recognition site for the restriction endonuclease, *Tru*1I. The SNP at position 824, where a T replaces a C, generates the complete recognition site, TTAA.