

# **The effects of fumonisin B<sub>1</sub> in preeclampsia**

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## **AUTHOR'S DECLARATION**

This study titled “the effects of fumonisin B<sub>1</sub> in preeclampsia” represents the original work by the author and has not been submitted to any other University for a similar degree. Where other people’s work has been used, it has been duly acknowledged.

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Date: 13 December 2012

## **DEDICATION**

*To my wonderful parents Moraka and Sepodi Serumula*

## PRESENTATIONS AT CONFERENCES

**Conference:** College of Health Sciences Research Symposium

**Title of paper:** Determination of Fumonisin B<sub>1</sub> in maternal blood and placental tissue of hypertensive patients

**Date:** 28-29 September 2005

**Venue:** Nelson R. Mandela School of Medicine, Durban

**Conference:** South African Society for Biochemistry and Molecular Biochemistry

**Title of paper:** Effects of Fumonisin B<sub>1</sub> in Preeclampsia.

**Date:** 2-5 July 2006

**Venue:** University of Kwazulu Natal, Pietermaritzburg

**Conference:** 34<sup>th</sup> Meeting of the Physiology Society of Southern Africa.

**Title of paper:** Effects of Fumonisin B<sub>1</sub> in Preeclampsia.

**Date:** 26-29 September 2006

**Venue:** University of Kwazulu Natal, Durban

**Conference:** 35<sup>th</sup> Meeting of the Physiology Society of Southern Africa

**Title of paper:** The effects of fumonisin B<sub>1</sub> in pregnancies complicated by preeclampsia.

**Date:** 9-12 September 2007

**Venue:** Glenburn lodge, Gauteng

**Conference:** 47<sup>th</sup> Annual Microscopy Society of South Africa

**Title of paper:** Mycotoxins in preeclampsia: possible aetiological agents.

**Date:** 8-11 December 2009

**Venue:** University of Kwazulu Natal, Westville

**Conference:** College of Health Sciences Research Symposium

**Title of paper:** Fumonisin B<sub>1</sub> disrupts redox homeostasis in oesophageal epithelial cancer cells (SNO)

**Date:** 14-15 December 2011

**Venue:** University of Kwazulu Natal, Medical School

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## LIST OF ABBREVIATIONS

FB <sub>1</sub>	Fumonisin B <sub>1</sub>
Sa	Sphinganine
So	Sphingosine
DNA	Deoxyribonucleic acid
KEH	King Edward VIII Hospital
NCCEMD	National committee on confidential enquiries into maternal deaths
KZN	KwaZulu-Natal
NHBPEP	National High blood pressure education program
PIH	Pregnancy-induced hypertension
DBP	Diastolic blood pressure
SBP	Systolic blood pressure
HELLP	Haemolytic anaemia, elevated liver enzymes and low platelet count
LIF	Leukaemia inhibitory factor
IL	Interleukin
eNOS	Endothelial nitric oxide synthase
GST	Glutathione S-transferase
TNF	Tumour necrosis factor
TGF	Transforming growth factor
sFlt	Soluble fms-like tyrosine kinase
VEGF	Vascular endothelial growth factor
PGF	Placental growth factor
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
O <sub>2</sub> <sup>•</sup>	Superoxide radical
PROMECA	Programme on mycotoxins and experimental carcinogenesis
FP	Fumonisin P
TCA	Tricarballic acid
FA	Fumonisin A
FC	Fumonisin C
AFB <sub>1</sub>	Aflatoxin B <sub>1</sub>
USA	United States of America

HPLC	High performance liquid chromatography
RP-HPLC	Reverse phase-high performance liquid chromatography
GGT	$\gamma$ -Glutamyltransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphate
ALT	Alanine aminotransferase
OC	Oesophageal cancer
NTD	Neural tube defects
ELEM	Equine leukoencephalomalacia
MRC	Medical research council
PPE	Porcine pulmonary oedema
LDL	Low density lipoprotein
IUPAC	International union of pure and applied chemistry
SPT	Serine palmitoyltransferase
ATP	Adenosine triphosphate
PARP	Poly- (ADP ribose) polymerase
NAD	Nicotinamide adenine dinucleotide
ER	Endoplasmic reticulum
Ca <sup>2+</sup>	Calcium ion
Mg <sup>2+</sup>	Magnesium ion
FADD	Fas-associated death domain
Fas L	Fas ligand
CARD	Caspase recruitment domain
DED	Death effector domain
EDTA	Ethylene diamine tetra acetic acid
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
NaOH	Sodium hydroxide
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
K <sub>2</sub> EDTA	Dipotassium ethylene diamine tetra acetic acid
KH <sub>2</sub> PO <sub>4</sub>	Potassium dihydrogen phosphate
DTT	Dithiothreitol

Tris	Tris-hydroxymethylamino methane
PMSF	Phenyl methyl sulfonyl fluoride
TEMED	N,N,N',N'-Tetramethylethylenediamine
DAB	3,3-Diaminobenzidine tetrahydrochloride
PBS	Phosphate buffered saline
IHC	immunohistochemistry
PAP	Peroxidase antiperoxidase
HRP	Horse radish peroxidase
H &E	Haematoxylin and eosin
TTBS	Tris buffered saline with Tween-20
HIER	Heat induced epitope retrieval
RT	Room temperature
ADP	Adenosine diphosphate
NaH <sub>2</sub> PO <sub>4</sub>	Sodium dihydrogen orthophosphate
Na <sub>2</sub> HPO <sub>4</sub>	Disodium hydrogen orthophosphate
NaCl	Sodium chloride
PE	preeclampsia
IE	Imminent eclampsia
SD	Standard deviation
MDA	Malondialdehyde
PVDF	Polyvinylidenedifluoride
SAX	Strong anion exchange
OPA	O-phthaldialdehyde
NH <sub>4</sub> OH	Ammonium hydroxide
CHCL <sub>3</sub>	Chloroform
Na <sub>2</sub> SO <sub>4</sub>	Sodium sulphate
KOH	Potassium hydroxide
CH <sub>3</sub> OH	Methanol
TBARS	Thiobarbituric acid reactive substances
H <sub>3</sub> PO <sub>4</sub>	Phosphoric acid
TBA	Thiobarbituric acid
BHT	Butylated hydroxytoluene
HCl	Hydrochloric acid

SEM	Standard error of the mean
AF	Albumin spiked with fumonisin B <sub>1</sub>
A	Albumin
ROS	Reactive oxygen species
GC-MS	Gas chromatography-Mass spectrometry
IgG	Immunoglobulin G
S	Syncytiotrophoblastic cell layer
M	Mesenchymal cells
V	Blood vessels
R	erythrocytes
IVS	Intervillous space
N	Nuclear aggregates
SK	Syncytial knots
CT	cytotrophoblasts
E	Endothelial cells

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## ABSTRACT

Preeclampsia is the leading cause of foetal and maternal mortality and morbidity in developing countries. In South Africa, maize is a dietary staple for most black African populations and is susceptible to contamination by mycotoxins such as fumonisin B<sub>1</sub> (FB<sub>1</sub>). Fumonisin B<sub>1</sub> is a ubiquitous secondary metabolite of *Fusarium* fungi produced predominantly by *Fusarium verticillioides*. This mycotoxin shares structural similarities with the backbone of sphingoid bases (sphinganine and sphingosine) which are substrates for the biosynthesis of complex sphingolipids. The mechanism of FB<sub>1</sub> toxicity therefore is centred on the disruption of this process. The aim of the present study was to elucidate the possible causal link between FB<sub>1</sub> and preeclampsia. Following ethical approval, 20 normotensive and 20 preeclamptic patients were recruited into the study. Blood and placental tissue were collected and processed for further analysis. The presence of FB<sub>1</sub> was verified using standard immunohistochemical and electrophoretic techniques. The levels of FB<sub>1</sub> and sphingolipids were quantified using high performance liquid chromatography (HPLC). Western blotting was conducted to confirm the presence of FB<sub>1</sub> in the serum. Placental tissue apoptosis was evaluated using Hoechst staining and other markers. Lipid peroxidation was measured in serum and placental tissue of both groups. Fumonisin B<sub>1</sub> was immunolocalised within the endothelial cells and mesenchymal cells of placentas from both groups, while FB<sub>1</sub> was present in cytotrophoblastic cells of preeclamptic patients only. In addition, FB<sub>1</sub> concentrations were significantly higher in preeclamptic compared to normotensive serum samples. Sphinganine was significantly elevated in preeclamptic serum samples whilst there was no statistical difference in the sphingosine levels between the groups. Chromatin condensation was higher in the preeclamptic patients. Caspase 3 and Fas were present with greater intensity in preeclamptic samples. The levels of lipid peroxidation were significantly higher in both serum and placental tissue of preeclamptic patients. This study has

demonstrated not only the presence of FB<sub>1</sub> in the serum and placental tissues of pregnant women but also the potential effects of this mycotoxin in the humans.

# CHAPTER 1

## Introduction

Hypertensive disorders of pregnancy are the leading cause of maternal and foetal death worldwide (Duley, 2005). Preeclampsia is a multi-system disorder unique to human pregnancy and is characterised by hypertension and proteinuria in the second half of pregnancy (Walker, 2000). Approximately 7-10% of all pregnancies worldwide are affected by preeclampsia with incidences as high as 27% in under resourced countries (Walker, 2000, Moodley, 1996, Davey and MacGillivray, 1989, Noris *et al.*, 2005).

Although the aetiology of preeclampsia remains obscure, it is widely believed that preeclampsia is a trophoblastic disease. Microscopic analysis of placentas from preeclamptic women reveals shallow invasion of the spiral arteries by placental trophoblastic cells. This is due to an inability to differentiate into invasive trophoblasts leading to vasoconstricted spiral arteries and reduced placental perfusion (Robertson *et al.*, 1986, Khong *et al.*, 1986, Kaufmann *et al.*, 2003, Kharfi *et al.*, 2003). Cytotrophoblasts are of epithelial origin and in order for adequate invasion to occur, they have to differentiate by down-regulating cell adhesion molecules of epithelial origin (integrin $\alpha_6/\beta_4$ , E-cadherin) and up-regulate the adhesion molecules (VE-cadherin, platelet endothelial adhesion) that are important for endothelial cell function (Noris *et al.*, 2005, Robertson *et al.*, 1986, Khong *et al.*, 1986, Kharfi *et al.*, 2003, Voss *et al.*, 2002). Thus far the only effective cure for preeclampsia is the delivery of a placenta (Peters and Flack, 2003).

Oxidative stress is an imbalance between oxidants and antioxidants in the body. The levels of oxidative stress are generally high in pregnancy due to the increased energy demands from the developing foetus but this process seems to be exaggerated in preeclampsia indicating aberrant antioxidant response (Wang and Walsh, 1996, Serdar *et al.*, 2002). Due to the multi-system involvement in preeclampsia, it is less likely that a single agent will be responsible for all the defects observed in this syndrome and highly likely that a variety of factors will work together to influence the outcome of the disease.

The eastern coastline of South Africa is reported to have the highest incidence of preeclampsia and approximately 18% of all pregnancies in this area have some form of hypertension (Moodley, 1996). Environmental conditions in this geographical region are conducive for mould contamination of maize which is the staple diet for most rural populations. Contamination of maize by moulds results in the production of mycotoxins most of which remain stable under normal cooking conditions and therefore increases the risk of an individual's exposure to these mycotoxins (Moodley *et al.*, 2001, Chelule *et al.*, 2001). Fumonisin B<sub>1</sub> is a mycotoxin produced by the mould *Fusarium verticillioides* that has been implicated in numerous animal diseases such as porcine pulmonary oedema, equine leukoencephalomalacia and hepatocarcinoma in rats (Voss *et al.*, 2002, Harrison *et al.*, 1990, Ross *et al.*, 1991, Gelderblom *et al.*, 2001). In addition, it has been implicated as an aetiological agent in oesophageal cancer in humans in China and South Africa (Yoshizawa *et al.*, 1994, Chu and Li, 1994, Sydenham *et al.*, 1990). Fumonisin B<sub>1</sub> contains a hydrocarbon backbone similar to that of sphingoid bases (sphinganine (Sa) and sphingosine (So)), a terminal amino group and several tricarballic acid moieties. The mechanism of FB<sub>1</sub> toxicity is postulated to be through the inhibition of ceramide synthase, a key enzyme in *de novo* sphingolipid metabolism which requires the sphingoid bases as substrates (Merrill *et al.*,

1997, Riley *et al.*, 1996, Merrill *et al.*, 1996). The tricarballylic acid moieties bind to the fatty acid binding domain while the amino group binds on the sphingolipid-binding domain of the enzyme. Through this interaction the enzyme functions are obliterated by FB<sub>1</sub> and consequent to this is the depletion of complex sphingolipids (ceramides, sphingomyelin) and elevation in sphingoid bases. Furthermore, there is also increased DNA fragmentation, decreased cell viability, loss of regulation of differentiation and apoptotic morphology associated with FB<sub>1</sub> toxicity (Chu and Li, 1994, Sydenham *et al.*, 1990, Merrill *et al.*, 1996). The ratio of Sa/So has been used as a biomarker in animal and *in vitro* models but most studies have not produced significant results especially in human samples (van der Westhuizen *et al.*, 1999, Tran *et al.*, 2003, Cano-Sancho *et al.*, 2011). More recently, urinary FB<sub>1</sub> has been proposed as a reliable biomarker of exposure in rural populations (van der Westhuizen *et al.*, 2011).

## 1.1

## Objectives

The purpose of this study was to:

- Determine the presence of and quantify FB<sub>1</sub> in the blood and placental tissues of pregnant women
- Quantify the levels of Sa and So as possible biomarkers for FB<sub>1</sub> exposure in pregnancy
- Compare the levels of apoptosis between the pregnancies complicated by preeclampsia and the normotensive pregnancies
- Measure lipid peroxidation as indicated by the levels of malondialdehyde in serum and placental tissues of normotensive and preeclamptic patients



## CHAPTER 2

### *Literature review*

#### **2.1 Hypertensive disorders of pregnancy**

Hypertension is the leading cause of mortality and morbidity associated with pregnancy (Walker, 2000). The prevalence of these disorders varies between countries with the highest burden reported in developing countries. At King Edward VIII Hospital (KEH), Durban, South Africa, 18% of all admissions to the obstetric unit have some degree of hypertension (Moodley, 1996). The prevalence in Zimbabwe is 7.1% while 6-8% of all pregnancies in the United States are affected by some form of hypertension (Wacker *et al.*, 1998). The mortality rates from preeclampsia and eclampsia are also generally higher in developing countries compared to developed countries (Wacker *et al.*, 1998, Subramaniam, 2007). According to the South African National Committee on Confidential Enquiries into Maternal Deaths (NCCEMD), KwaZulu-Natal (KZN) Province has the highest maternal mortality rates in South Africa and of all complications of pregnancy hypertension is the leading cause of maternal death nationwide (NCCEMD, 2000). The high mortality rates reported in KZN may also be due to effective reporting systems in this province's health departments and may not be a true representative of the national situation. Interestingly, the NCCEMD also reported the highest mortality rates in black women compared to other races in South Africa. Various factors may contribute to these discrepancies including the inaccessibility of the antenatal care for rural black women, the lack of proper infrastructure and low socio-economic and nutritional status of these women (Okafor and Aniebue, 2004, Wacker *et al.*, 1998, Chelule *et al.*, 2001). Climatic conditions such as high temperatures and humidity have also been implicated in the incidence of hypertension (Wacker *et al.*, 1998, Subramaniam, 2007).

### **2.1.1 *Classification of hypertensive disorders of pregnancy***

The American College of Obstetricians and Gynaecologists and more recently the national high blood pressure education program working group on high blood pressure (NHBPEP) in Pregnancy have classified hypertension in pregnancy into four categories as follows 1) gestational hypertension, 2) chronic hypertension, 3) preeclampsia superimposed on chronic hypertension and 4) preeclampsia. The term pregnancy induced hypertension (PIH) has recently been removed from the classification as it is believed to be misleading (Leeman and Fontaine, 2008, Peters and Flack, 2003, Marik, 2009, Duley, 2005).

Gestational hypertension was previously known as PIH and is characterised by development of hypertension without proteinuria after the twentieth week of gestation in a previously normotensive individual with no history of hypertension. It is an interim diagnosis reserved for women who are eventually diagnosed with preeclampsia or chronic hypertension. Half of the women diagnosed with gestational hypertension between 24 and 35 weeks develop preeclampsia. Severe gestational hypertension is associated with poor perinatal outcomes compared to women with mild preeclampsia and management is similar to the one used in severe preeclampsia (Leeman and Fontaine, 2008, Peters and Flack, 2003, Marik, 2009, Duley, 2005).

Chronic hypertension is characterised by blood pressure measurement of 140/90 or higher, taken on two occasions before the 20<sup>th</sup> week of gestation and continuing beyond 12 weeks following delivery. Superimposed preeclampsia is the primary contributor to morbidity observed in chronic hypertension and usually results from sudden increase in blood pressure and new proteinuria. Treatment of mild to moderate chronic hypertension however seems to be unhelpful in preventing preeclampsia or of any benefit to the foetus. Excessive decrease in

blood pressure through the use of antihypertensive medications may also result in reduction in placental perfusion which will further impact on foetal development. However, treatment is warranted when the patient's BP persists above 150mmHg/110mmHg to prevent end-organ damage (Leeman *et al.*, 2008; Peters and Flack, 2004; Marik, 2009; Duley, 2003).

Preeclampsia superimposed on chronic hypertension is encountered in women with chronic hypertension who develop new signs or symptoms of preeclampsia in the second half of pregnancy. These signs include a raised blood pressure and new proteinuria (Sibai *et al.*, 2005, Leeman and Fontaine, 2008, Peters and Flack, 2003, Marik, 2009, Duley, 2005).

Preeclampsia is a multisystem disorder unique to human pregnancy with an unexplained aetiology. It is characterised by an abnormal response of the vasculature to placentation. The diagnosis is based on the high blood pressure of 140/90 mmHg taken on two occasions 6-hours apart and proteinuria of 300mg in a 24 hour-urine specimen after twenty weeks of gestation. Proteinuria is usually determined by means of Dipstix® (Table 2.1), which gives semi-quantitative measurements. False positives result if the urine is too concentrated or contaminated, while grossly diluted urine gives false negatives (Sibai *et al.*, 2005).

Table 2.1: Approximate protein concentrations in urine as determined by Dipstix® (Ames)  
Redrawn from (Moodley, 1996)

<b>Dipstix ® result</b>	<b>Approximate concentrations</b>
Trace	0.1g/l
1+	0.3 g/l
2+	1.0g/l
3+	3.0g/l
4+	>20.0g/l

Proteinuric hypertension is the most widely accepted definition of preeclampsia while oedema is now omitted from all definitions of preeclampsia. Preeclampsia is also known to affect multiple systems with varying severity depending on the system affected. The central nervous system complications include convulsions (eclamptic), cerebral and retinal oedema, and cortical blindness while necrosis of the renal cortex and tubules is the most common manifestation of renal involvement. Pulmonary oedema, jaundice, hepatic infarction and the haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome are also most commonly encountered in preeclampsia. Preeclamptic placentas are prone to infarction, uteroplacental bleeding and abruptio placentas (deSwiet, 1995, Sibai *et al.*, 2005).

While the aetiology remains elusive, abnormal placentation is the major cause of this disease. Abnormal placentation is characterised by the inability of cytotrophoblastic cells to invade maternal spiral arteries resulting in vasoconstricted vessels with reduced delivery of blood and nutrients to the placenta (Lyll *et al.*, 2001, Jauniaux *et al.*, 2006). The failure of invasive cytotrophoblasts to mimic the endothelial system by increasing expression of adhesion molecules of endothelial origin (VE-cadherin, vascular endothelial adhesion molecule-1) and down-regulation of adhesion molecules from their epithelial phenotype (E-cadherin, integrin  $\alpha_6$ ) is the major cause of this shallow invasion observed in preeclampsia (Kharfi *et al.*, 2003).

Routine nutritional supplementation in an attempt to prevent preeclampsia proved unsuccessful whilst calcium supplementation resulted in decreased risk of developing preeclampsia in high risk women. Low dose aspirin is also effective for use in women at increased risk of preeclampsia. Hydralazine has been used for treatment of preeclampsia for

many decades but due to undesirable side effects has been discontinued (Leeman and Fontaine, 2008, Peters and Flack, 2003, Marik, 2009, Duley, 2005, Walker, 2000).

## **2.2 The placental development and preeclampsia**

Mammalian placental tissue is an important organ required for maternal and foetal exchange of nutrients and gases (Hole, 1987, Tarrade *et al.*, 2001). With an inherent endocrine capability, this organ ensures an optimum supply of hormones required during gestation such as the human chorionic gonadotropin and human placental lactogen. In addition to this, the placental growth hormone which appears in the maternal blood from 12 to 20 weeks up to term regulates the maternal blood glucose and supply of nutrients to the placenta. Although its function is poorly understood, human chorionic somatomammotropin production is also gradually increased during pregnancy (Evain-Brion and Malassine, 2003, Tarrade *et al.*, 2001).

The prenatal period of development consists of a cleavage stage and begins with fertilisation in the uterine tube. Following fertilisation and formation of a zygote, two daughter cells are formed during mitosis and after a series of divisions form a hollow ball of cells called the blastocyst. These blastocysts eventually become the trophoblasts that release proteolytic enzymes (matrix metalloprotease-9) to digest a portion of the endometrium and sink slowly into the depression. The cells of the trophoblast begin to produce tiny, finger-like projections (microvilli) that grow into the endometrium to complete the cleavage period (Hole, 1987).

During a normal pregnancy, after implantation of the embryo the foetal trophoblastic tissue migrates in two phases down the spiral arteries (Figure 2.1), displacing the musculo-elastic structure of these arteries (Robertson *et al.*, 1986, Kaufmann *et al.*, 2003, Lyall *et al.*, 2001,

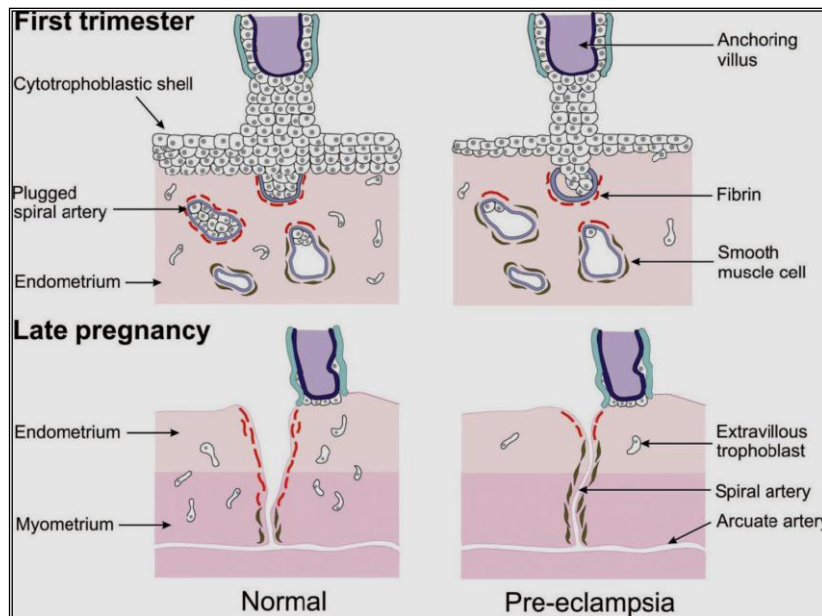
Jauniaux *et al.*, 2006). The first phase affects the decidual portions (8-10 weeks) and the second phase affects the myometrial portions (16-18 weeks) of the arteries resulting in dilatation. This physiological transformation is characterised by a gradual loss of the normal musculo-elastic structure of the arterial wall and replacement by amorphous fibrinoid material in which trophoblast cells are embedded. These physiological changes are necessary for a successful pregnancy and ensure a continuous supply of oxygen and nutrients from the maternal blood (Lyll *et al.*, 2001, Kaufmann *et al.*, 2003, Jauniaux *et al.*, 2006).

### **2.2.1 Pathogenesis and aetiology of preeclampsia**

In preeclampsia the second phase of trophoblastic invasion described above fails to occur and the myometrial portions of the spiral arteries remain muscular and undilated with a propensity towards vasoconstriction. This makes defective trophoblast invasion the main consistent histological feature in preeclampsia (Kaufmann *et al.*, 2003, Lyll *et al.*, 2001, Robertson *et al.*, 1975, Jauniaux *et al.*, 2006). Figure 2.1 represents the features that are observed in normal and preeclamptic trophoblast invasion. The cytotrophoblastic cells are the chief participants in this process and behave like pluripotent stem cells where they differentiate into 3 main types of cells important for placental development, the anchoring trophoblasts, the villous cytotrophoblasts and the extra villous (invasive) trophoblasts.

The villous trophoblasts are responsible for the endocrine properties of the placenta whilst the anchoring trophoblasts are important for attachment of the placenta to the uterine wall and thus express adhesion molecules important for this function. The invasive trophoblasts are responsible for the morphological changes observed in spiral arteries during pregnancy. A differentiation of cytotrophoblasts into invasive trophoblasts is achieved through loss of their epithelial phenotype and acquisition of an endothelial phenotype through increased

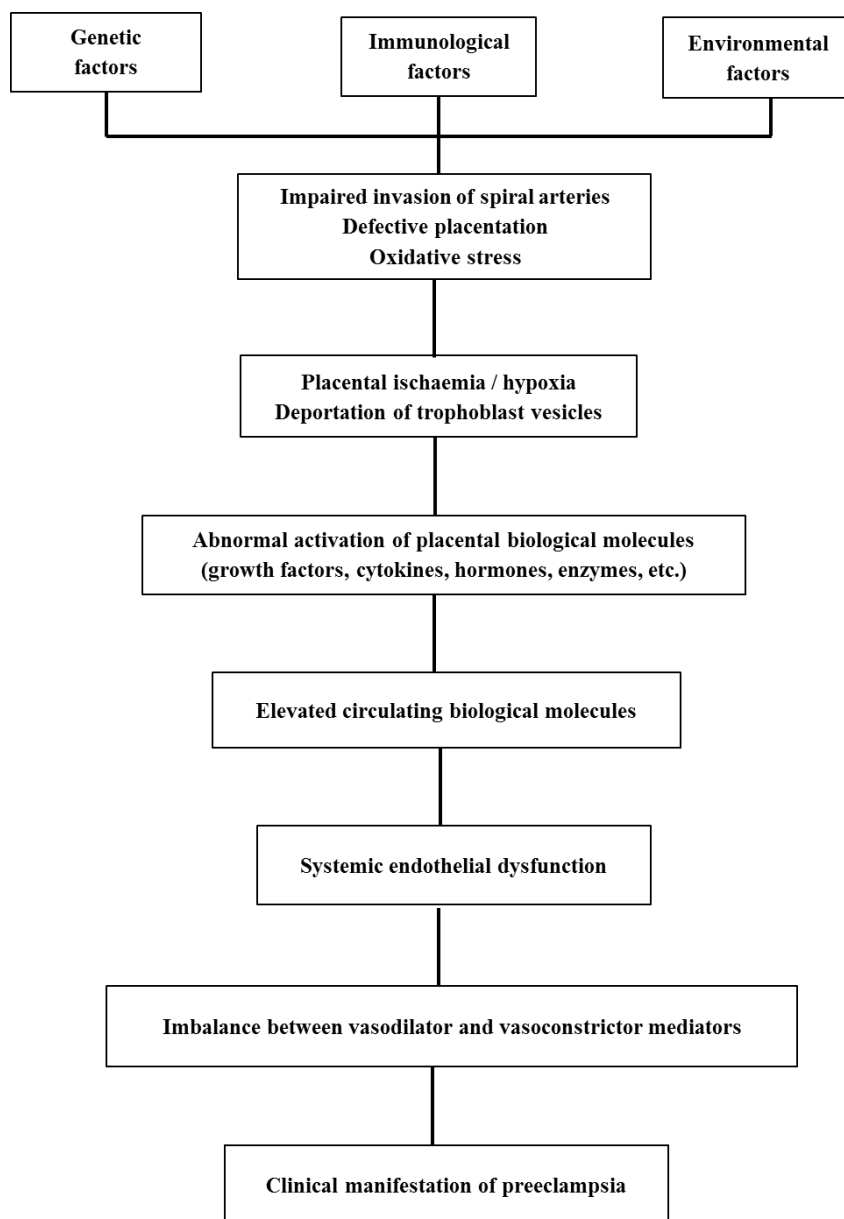
expression of vascular adhesion molecules (VE-cadherin, platelet endothelial adhesion molecule, vascular endothelial adhesion molecule 1 and  $\alpha 4$ -integrins) and down-regulation of epithelial adhesion molecules such as integrin  $\alpha_6/\beta_4$ ,  $\alpha_v/\beta_5$ , E-cadherin (Khong *et al.*, 1986, Zhou *et al.*, 1997). This hypothesis has however been challenged by studies that demonstrated that the adhesion molecules may not be entirely responsible for the aberrant invasion observed. Nonetheless, these changes are not evident in placentas from preeclamptic pregnancies (Zhou *et al.*, 1997, Kharfi *et al.*, 2003).



**Figure 2.1:** Trophoblast invasion in normotensive and preeclamptic placenta. Normotensive pregnancies show increased plugging of spiral arteries by invasive cytotrophoblasts, thus allowing adequate blood supply to the placenta. There is reduced plugging of spiral arteries in preeclamptic placentas which remain muscular and vasoconstricted resulting in reduced uteroplacental perfusion (Jauniaux *et al.*, 2006).

Despite the extensive research conducted in preeclampsia no single agent can be implicated in the syndrome. It is believed that the aetiology lies in the interaction between genetic, environmental and immunologic factors (Figure 2.2). Various genes have been surveyed for their possible involvement in preeclampsia including endothelial nitric oxide synthase

(eNOS), angiotensinogen, human leukocyte antigen-G, endothelin epoxide hydrolase and glutathione S-transferase (GST). However, none of the studies have successfully produced unequivocal evidence of the gene's aetiological role in preeclampsia (Moses *et al.*, 2000, Masse *et al.*, 2002, Guo *et al.*, 1999, Williams and Broughton Pipkin, 2011, Zhang *et al.*, 2008). The genetic studies have also been limited by the fact that preeclampsia is unique to pregnancy and resolves following delivery and therefore does not provide an opportunity to study after pregnancy.



**Figure 2.2:** Schematic representation of the proposed involvement of placental molecular mechanisms in pathogenesis of preeclampsia adapted from (Kharfi *et al.*, 2003).



The foeto-placental unit serves as an allograft because it contains paternal antigenic tissue that is foreign to the maternal host. A normal pregnancy represents a state of immune adaptation but the immune response in preeclamptic pregnancies is exaggerated (Dekker and van Geijn, 1996 , Southcombe *et al.*, 2011, Sibai *et al.*, 2005). Immune maladaptation and subsequent activation of decidual lymphoid cells lead to an increase in trophoblastic cell derived lipid peroxides in preeclampsia. These placental lipid peroxides may increase secretion of thromboxane and cause progressive vasoconstriction in the placental vasculature. During an immune response and/or prolonged period of ischaemia activated neutrophils undergo a 'respiratory burst' causing production of superoxide ( $O_2^{\bullet}$ ) and hydrogen peroxide ( $H_2O_2$ ). These free radicals destroy the integrity of endothelial cells, vascular basement membrane and sub-endothelial matrix which consequently results in endothelial dysfunction, a feature that is also characteristic of preeclampsia (Dekker and Sibai, 1998).

A plethora of growth factors/cytokines or their receptors studied during trophoblast invasion revealed their importance in placental development. Epidermal growth factor is a major regulator of the implantation process as it plays a role in the invasion, differentiation and proliferation of trophoblasts (Staun-Ram and Shalev, 2005). High levels of tumour necrosis factor (TNF)- $\alpha$  have been implicated in the induction of apoptosis in cytotrophoblasts and therefore is important in the maintenance of cellular homeostasis during placental development. It also enhances formation of endothelial cell molecules like endothelin whilst decreasing acetylcholine-mediated vasodilation. Prior to the development of preeclampsia, it is reported that serum levels of TNF- $\alpha$  and its soluble receptor are elevated (Sibai *et al.*, 2009, Hunt *et al.*, 1996). Interleukin (IL)-6 and leukaemia inhibitory factor are important in implantation (Robb *et al.*, 2002). Other cytokines important during trophoblast differentiation include epidermal growth factor, insulin-like growth factor-II and IL-1 $\beta$  which positively

regulate differentiation of trophoblasts along the invasive pathway. Transforming growth factor (TGF)- $\beta$  is a known inhibitor of migration and proliferation of extravillous trophoblasts and its serum concentrations are significantly higher in preeclamptic pregnancies than in normotensive pregnancies (Naicker *et al.*, 2002, Benyo *et al.*, 2001, Staun-Ram and Shalev, 2005).

Other risk factors such as nulliparity, limited sperm exposure and the extremes of age of first pregnancy are also associated with a high risk of developing preeclampsia. Subsequent pregnancies reduce the risk significantly except where there is a history of hypertension in previous pregnancies (Sibai *et al.*, 2005). In addition, pregnant women who attended antenatal care have a lower risk of developing preeclampsia compared to those with no antenatal care (Ansari *et al.*, 1995). Access to antenatal care in most developing countries is still a luxury and thus this may explain the high incidence of preeclampsia in developing countries.

Angiogenic imbalance may also play role in the pathogenesis of preeclampsia. There is up-regulation of anti-angiogenic factors such as soluble fms-like tyrosine kinase -1(sFlt-1) along with a reduction in the pro-angiogenic factors (vascular endothelial growth factor (VEGF) and placental growth factor (PGF) in preeclamptic placentas. sFlt-1 inhibits the pro-angiogenic factors, VEGF and PGF by interfering with their interaction with at the receptor level. The levels of sFlt-1 are reportedly high in preeclamptic pregnancies compared to their normotensive counterparts (Wolf *et al.*, 2005, Maynard *et al.*, 2003, Ferrara, 2001).

In addition to smoking, nutritional status and climatic conditions may also play a role in preeclampsia. Rural African communities are heavily reliant on maize as their staple and

numerous studies have demonstrated the contamination of maize by various species of moulds including *Fusaria* (Haschek *et al.*, 1992, Wilkes and Sutherland, 1998, Musser and Plattner, 1997). A consequence to this contamination is the exposure of the individuals to the mycotoxins produced by these moulds. Moodley *et al.* reported the highest levels of preeclampsia in the Eastern coastline of South Africa and postulated that this may be attributed to the dietary staple for this population being maize (Moodley *et al.*, 2001).

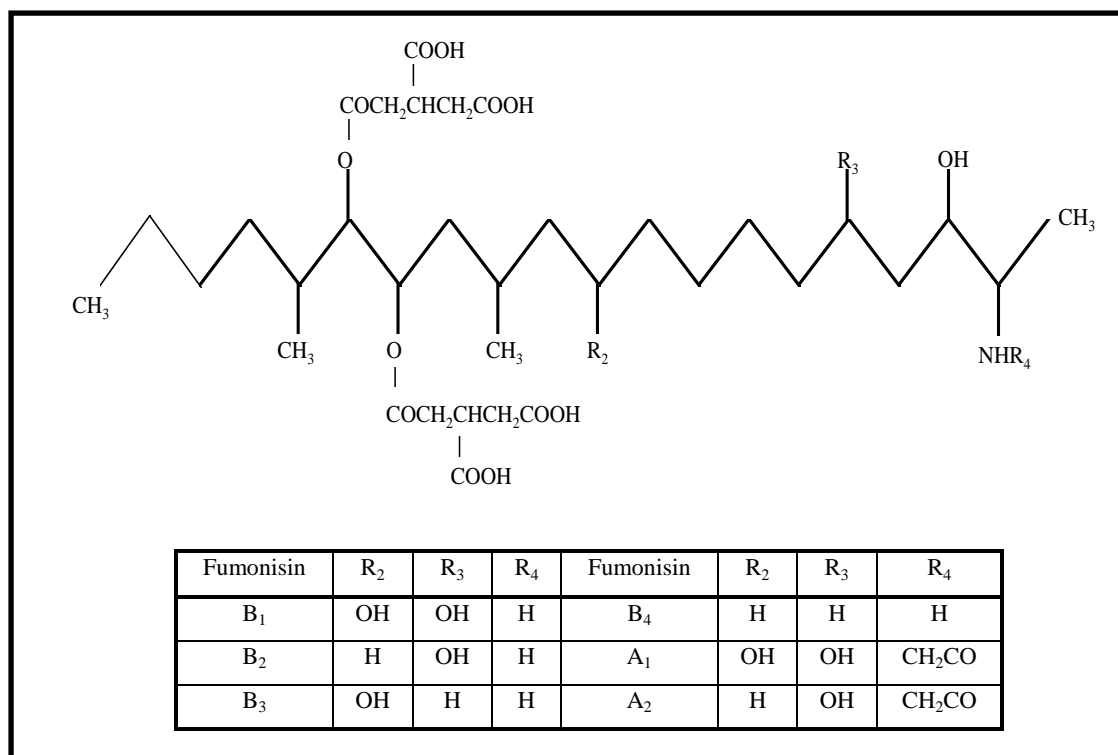
### **2.3 Fumonisin**

Fumonisin are a group of naturally occurring mycotoxins produced by moulds of the *Fusarium* species. These mycotoxins were originally isolated from *Fusarium moniliforme* (recently named *F. verticillioides*) MRC 826 by the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) group of South African researchers (Bezuidenhout *et al.*, 1988, Gelderblom *et al.*, 1988). *Fusarium proliferatum* and *F. nygamai* cultures were also shown to produce fumonisin (Thiel *et al.*, 1991). The fumonisin isolated from the maize cultures in Transkei was designated fumonisin B<sub>1</sub> (FB<sub>1</sub>) (Haschek *et al.*, 1992). Since then, over 28 types of fumonisins have been discovered and it is very likely that the list will grow even further as more fumonisins are discovered. The major fumonisin isolated from the *Fusaria* cultures include B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>4</sub>, A<sub>1</sub>, A<sub>2</sub>, C<sub>1</sub> and the P (FP<sub>1-3</sub>) (Wilkes and Sutherland, 1998, Musser and Plattner, 1997).

#### **2.3.1 Structure of fumonisins**

Fumonisin B<sub>1</sub> is the most toxicologically significant and most abundantly produced fumonisin. The backbone of the B series is 2-amino-12,16, dimethyl-3,5,10,14,15-pentahydroxyicosane joined in diester linkage with propane-1,2,3-tricarboxylic acid (tricarballic acid (TCA)) via hydroxyl groups at C-14 and C-15; and the terminal carboxyl

group of TCA. In addition to FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub> and FB<sub>4</sub> are also found in naturally contaminated foods and differ structurally in the number and position of hydroxyl groups on their hydrocarbon chain (Figure 2.3). The free amino group on the B series distinguishes them from the A series (FA<sub>1</sub> and FA<sub>2</sub>), which are the *N*-acetyl derivatives. Fumonisin C<sub>1</sub> also differs from FB<sub>1</sub> in that it lacks the amino-end terminal-methyl group. In the P series, 3-hydroxypyridinium replaces the amine at C-2 and can occur in amounts approximately one-third of the B-series (Bezuidenhout *et al.*, 1988, Gelderblom *et al.*, 1988) (Figure 2.4). The presence of a free amino and four free carboxyl groups makes the fumonisins strongly polar molecules.



**Figure 2.4:** The hydrocarbon backbone of fumonisins showing the position of TCA side chains, amino and hydroxyl groups. Differences in the position and number of hydroxyl groups and the acetylation of the amino group are depicted, redrawn from Norred, 1993.

The tricarballylic acid moieties and amide groups on the FB<sub>1</sub> backbone are important in its toxicity. This was established in a study that showed that nixtamalisation of maize during

production of tortillas resulted in cleavage of the TCA groups on FB<sub>1</sub> and yielded less toxic hydrolysed fumonisins with low biological activity (Palencia *et al.*, 2003). Furthermore, acetylation of the primary amino groups also resulted in reduction in toxicity (Gelderblom *et al.*, 1993). Norred demonstrated that both FB<sub>1</sub> and FB<sub>2</sub> were poorly absorbed from the gastrointestinal tract and rapidly cleared from the blood. Although very little accumulates in the tissues, the liver and the kidney have been shown as reservoirs for FB<sub>1</sub> in the body (Norred, 1993). Interestingly the kidney was shown to have significantly higher concentrations of FB<sub>1</sub> than the liver (Riley and Voss, 2006). Fumonisin B<sub>1</sub> is also thermostable at various temperatures (50°C, 75°C and 100°C) encountered during the cooking or processing of maize making it difficult to decrease its toxicity (Dupuy *et al.*, 1993, Alberts *et al.*, 1990). Fermentation of maize to ethanol also resulted in very little degradation of FB<sub>1</sub> whilst it was not recovered in distilled alcohol (Bothast *et al.*, 1992).

### **2.3.2 Occurrence of fumonisins in foods and feed**

One hundred countries have implemented legislation on the regulation of the amount of mycotoxins allowed in food for human consumption. Only 15% of these countries are African and there is huge variation between countries on the maximum level of mycotoxins allowed in food. The United States recommends the maximum amount of aflatoxin in food to be 20µg/kg with the exception of milk while the European Union is by far the strictest with only 4µg/kg total aflatoxin allowed. Rural African communities are at a much higher risk of exposure to mycotoxins due to their dependence on subsistence farming of maize (Wagacha and Muthomi, 2008, Wagacha *et al.*, 2012, Shephard *et al.*, 2007). The Eastern Cape province of South Africa has been the hub of mycotoxin research and is the area where high levels of fumonisins were discovered. There is also a high incidence of oesophageal cancer in this area (Shephard *et al.*, 2007). A study by Thiel *et al.* showed that maize in this region had

the highest FB<sub>1</sub> concentrations produced mainly by the *F. verticillioides* (Thiel *et al.*, 1991).Burger *et al.* also reported a huge variation in the amount of fumonisin between home-grown maize and commercial maize in the Transkei (Burger *et al.*, 2010).Maize samples from rural areas in KZN contained significantly higher concentrations of FB<sub>1</sub> compared to their urban counterparts (Chelule *et al.*, 2001).

Mycotoxin production can occur during harvest, storage and food processing (Wagacha and Muthomi, 2008). Environmental conditions such as humidity, temperature below 22<sup>0</sup>C and frequent rain during the first 2 weeks after silking contribute to production of mycotoxins by moulds infecting the maize (Chelule *et al.*, 2001). Therefore, depending on climatic conditions some areas around the world would have high concentrations of mycotoxins compared to others. In addition to climatic conditions, processing of maize-based products either commercially or domestically also affects the amount of mycotoxins produced. For example, nixtamalisation of maize to make tortillas results in decreased concentration of FB<sub>1</sub> while fermentation of maize to ethanol and high temperature (>100<sup>0</sup>C) have very little effect on the production of FB<sub>1</sub> by the moulds.

Analysis of food samples in Zimbabwe showed high concentrations of FB<sub>1</sub> and *F. verticillioides* concentrations were higher in maize, wheat, sorghum and rapoko (finger millet) compared to nuts (Gamanya and Sibanda,2001).In Ghana the predominant fungal contaminant is *Aspergillus spp.* (65%) which co-occurs with *Furarium spp.* (5.1%).Co-occurrence of fumonisins with other mycotoxins was also demonstrated in cereal and spice from Morocco (Zinedine *et al.*, 2006). In the Kenyan Kisii district, the highest concentration of FB<sub>1</sub> observed 1000ng/g (Kedera *et al.*, 1999).

The People's Republic of China (Shangqui, Cixian&Linxian Counties) reported co-occurrence of FB<sub>1</sub> (48%), trichothecenes (A and B) and aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) in the high risk areas for oesophageal cancer while only 25% of FB<sub>1</sub> was reported in the low risk area (Chu and Li, 1994, Yoshizawa *et al.*, 1994). Maize imported into Thailand from the USA contained less FB<sub>1</sub> than that imported from Australia. By far maize samples from the UK were the safest with regard to mycotoxin production (Patel *et al.*, 1997). Comparison of fumonisin levels in rice and corn from Argentina revealed that corn flour contained higher levels of FB<sub>1</sub> compared to rice (Lerda *et al.*, 2005). In India, FB<sub>1</sub> was found in rain-affected maize and sorghum samples and only a few in poultry feed samples (Shetty and Bhat, 1997A, Shetty and Bhat, 1997B). All these studies together demonstrate the ubiquitous nature of these mycotoxins and the potential risk they pose to human and animal health.

### **2.3.3 Toxic effects of fumonisins in animals**

Male BD IX rats fed with freeze- or oven-dried culture material of *F. moniliforme* MRC 826 developed hepatocellular and ductular liver carcinomas. Purified FB<sub>1</sub> caused marked reduction in weight gain, induction of  $\gamma$ -glutamyltransferase (GGT)-positive foci and toxic hepatitis. In addition, liver function enzymes such as alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were increased. Feeding various concentrations of FB<sub>1</sub> to rats and mice showed significant interspecies differences as mice showed mild hepatic injury while rats showed minimal mild nephrosis, demonstrating that mice were less sensitive to FB<sub>1</sub> than rats (Voss *et al.*, 1996). Other effects of fumonisin in rats include decreased food intake and body weight gain, decreased organ weights (absolute/relative), increased sphinganine (Sa) and sphingosine (So) ratio in urine, serum, liver and kidney (Voss *et al.*, 1996, Bondy *et al.*, 1997).

Equine leukoencephalomalacia (ELEM), a disease that is characterised by liquefactive necrosis of white matter in one or both cerebral hemispheres was associated with consumption of mouldy maize contaminated with *F. verticillioides* (Meireless *et al.*, 1994). Fumonisin B<sub>1</sub> was implicated as the causative agent of ELEM by Marasas *et al* in 1988; the study showed that dosing two horses with culture material of *F.moniliforme* MRC 826 caused brain oedema and hepatitis (Marasas *et al.*, 1988). Injection of extracted and purified FB<sub>1</sub> into a horse caused symptoms typical of ELEM such as apathy, nervousness, a wide-based staring stance, shuffling gait, incoordination and bumping into objects, trembling, ataxia, reluctance to move, paresis of the lower lip and tongue, inability to eat and drink with principal lesions being severe oedema of the brain and focal necrosis in the medulla oblongata (Wilkins *et al.*, 1994).

In pigs, consumption of feed contaminated with fumonisins caused symptoms of porcine pulmonary oedema (PPE) such as acute onset of dyspnoea, lethargy, weakness, increased respiratory rate, cyanosis and death, concurrent hydrothorax and pulmonary oedema. The risk of PPE increased as the concentration of fumonisins increased. Intravenous injection or feeding of pigs with FB<sub>1</sub> caused decreased feed consumption, increased total bilirubin, cholesterol and liver enzymes (GGT, ALP, ALT, AST, arginase), mild to severe respiratory distress and interstitial pulmonary oedema with increased respiration rate and marked cyanosis. Other hepatic changes include disorganisation and necrosis of hepatocytes, loss of sinusoidal microvilli, pancreatic acinar cell degradation and the presence of membranous material in hepatic sinusoids and pulmonary intravascular macrophages. Increased free Sa and Sa/So ratio in the liver, lung and kidney before histopathological or serum biochemistry changes were also observed (Riley *et al.*, 1993, Motelin *et al.*, 1994).



Poultry are less susceptible to toxic effects of fumonisins than other animals. However, diarrhoea, significant decreases in body weight and average daily gain (or feed) conversion were noted. In addition, increases in relative weights of liver, kidney, gizzard and proventriculus were observed in day-old broiler chicks fed diets containing FB<sub>1</sub> for 14 to 21 days. Biliary hyperplasia, multifactorial hepatic necrosis, muscle necrosis, intestinal goblet cell hyperplasia and rickets also occurred (Espada *et al.*, 1994, Wiebking *et al.*, 1993).

Baboons fed with culture material of *F. verticillioides* developed acute congestive heart failure and hepatic cirrhosis (Kriek *et al.*, 1981) while vervet monkeys developed hepatitis (Jaskiewicz *et al.*, 1987). A low fat diet with added fumonisins caused the development of an atherogenic profile, i.e. increased cholesterol, low density lipoprotein (LDL)-cholesterol, apoprotein B, liver enzymes, albumin, factor VII and fibrinogen which are possibly due to chronic liver toxicity (Fincham *et al.*, 1992).

In cattle, consumption of a fumonisin-contaminated diet for thirty days resulted in cattle showing signs of hepatobiliary changes (i.e. increased cholesterol, bilirubin and enzymes) but no observable change in feed intake, body weight or temperature. They were however languid, visibly distressed and ate their food sluggishly (Osweiler *et al.*, 1993, Smith and Thakur, 1996). Rabbits fed with fumonisin-contaminated diet developed central nervous system changes similar to ELEM and a dose of 1.75mg/kg/day diet resulted in liquefaction and haemorrhage in the brains of pregnant rabbits, hepatic and renal changes (predominantly apoptosis) were also observed (Bucci *et al.*, 1996). Hepatic and nephrotoxicities were also observed in lambs orally dosed with a fumonisin culture material (Edrington *et al.*, 1995).

#### **2.3.4 Effects of FB<sub>1</sub> in humans**

High incidence of neural tube defects (NTD) have been reported in Africa, China and Texas causing health authorities to question a possible relationship between consumption of maize products and a cluster of NTDs in these regions (Missmer *et al.*, 2006, Marasas *et al.*, 2004). In Texas, the increased risk of NTDs was related to the amount of tortillas consumed as well as the source (Missmer *et al.*, 2006). Women who consumed home-made tortillas had a higher risk of NTD compared to those who purchased their tortillas. Neural tube defects are often associated with a deficiency in folic acid and the mechanism by which FB<sub>1</sub> may induce NTDs is postulated to be via the disruption of sphingolipid metabolism (Collins *et al.*, 1998). Folate transporters are phosphatidylinositol anchored proteins and components of lipid rafts which are rich in cholesterol and sphingolipids. Therefore a disruption of sphingolipid metabolism by FB<sub>1</sub> results in decreased entry of the folate into the cell due to a decrease in receptors was demonstrated in various cell lines treated with FB<sub>1</sub> (Stevens and Tang, 1997, Marasas *et al.*, 2004).

Maize and maize based products are staple foods for most rural black populations of Southern Africa. The high risk of oesophageal cancer (OC) has been associated with consumption of mycotoxin-infected maize or maize based products (Rava *et al.*, 1996). Common contributing factors in the high incidence regions of OC include mineral poor soil with excessive alkalinity and low annual rainfall, resulting in stressed plants susceptible to heavy fungal contamination (e.g. *F. verticillioides*).

A strong link between OC and fumonisins was discovered by the PROMEC group studying low (Bizana and Lusikisiki) and high (Butterworth and Kentani) risk OC areas in the Transkei. During 1985 and 1986, cytological examinations were done on adults in the low,

intermediate and high OC rate areas from 12 households (Rheeder *et al.*, 1992). Mild and advanced cellular changes occurred more frequently in the high OC rate household occupants. In households whose members showed cytological abnormalities, both good and mouldy home-grown maize showed significantly higher prevalence of *F. verticillioides*. Furthermore, the healthy maize samples from the high OC incident areas had significantly higher levels of FB<sub>1</sub> and FB<sub>2</sub> compared with the low OC rate areas (Rheeder *et al.*, 1992). Yoshizawa *et al.* have also reported higher incidence of maize contamination with fumonisins in maize from the high OC risk area compared with the low risk areas in China (Yoshizawa *et al.*, 1994).

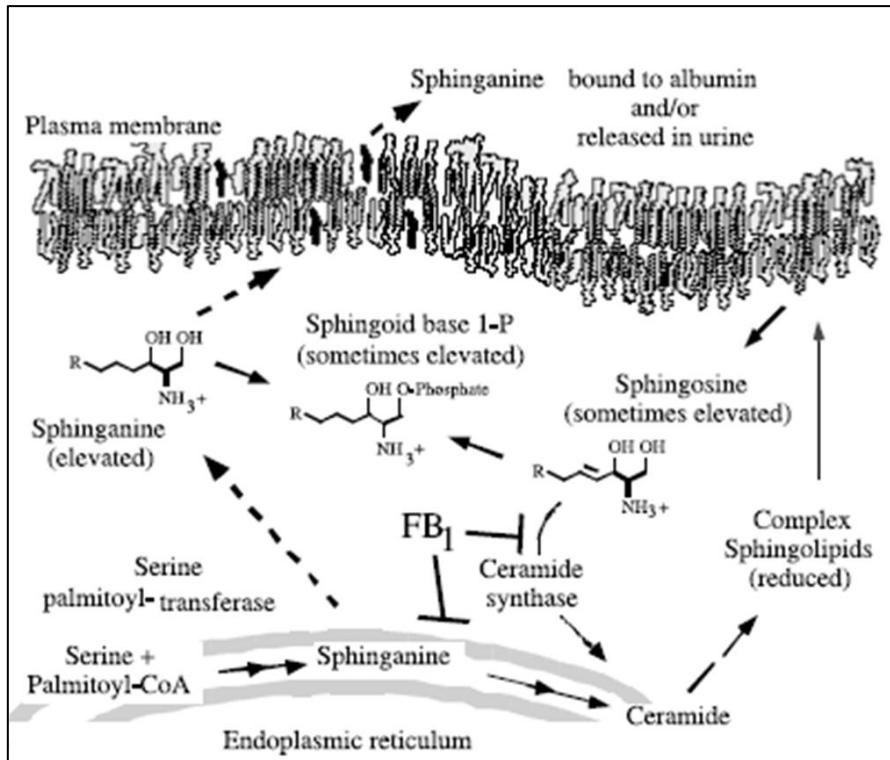
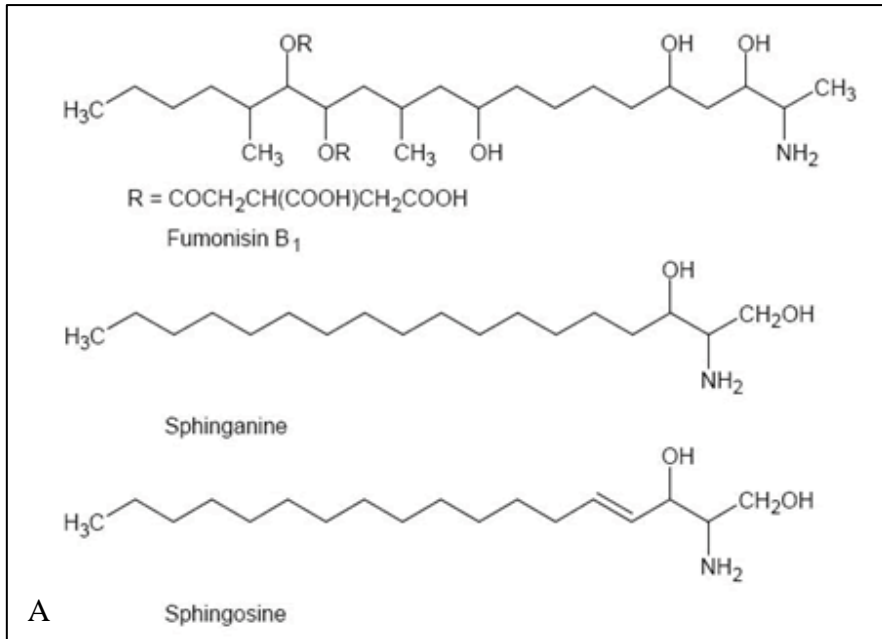
### **2.3.5 Mechanism of action of fumonisin B<sub>1</sub>**

Fumonisin B<sub>1</sub> is a structural analogue of sphingoid bases (Figure 2.4A), Sa and So and thus acts as a substrate for the enzyme ceramide synthase (sphingosine N-acyltransferase) important in the *de novo* sphingolipid biosynthesis (Figure 2.4B). As a result of this similarity, the sphingolipid-binding domain of the enzyme recognises the primary amino group on FB<sub>1</sub> while the tricarballic acid moieties are recognised by the fatty acyl binding domain of the enzyme resulting in inhibition (Riley *et al.*, 2001). Fumonisins lacking the tricarballic acid groups (partially hydrolysed) showed low biological activity and toxicity compared to unhydrolysed FB<sub>1</sub>. The acetylation or methylation of the amide groups on FB<sub>1</sub> was also unable to elicit any toxic effects in both *in vivo* and *in vitro* studies (Howard *et al.*, 2002, Norred *et al.*, 2001).

Although sphingolipids are available from the diet, the *de novo* pathway of sphingolipid synthesis plays a pivotal role in supplying the body with the complex lipids (Riley *et al.*, 2001). The *de novo* sphingolipid pathway begins with the condensation of palmitoyl-CoA

and L-serine to form 3-ketosphinganine (catalysed by serine palmitoyltransferase; SPT). Due to the inability to detect the 3-ketosphinganine derivative *in vivo*, the reduction of 3-Ketosphinganine (3-ketosphinganine reductase) to sphinganine is rapid. Acylation of the amino group on the sphingoid bases results in formation of dihydroceramides (catalysed by ceramide synthase). Additionally other O-acylated, phosphorylated and N-methylated derivatives also exist. Finally, the formation of ceramide that is catalysed by desaturase results in the insertion of a 4, 5-trans double bond into sphingoid base backbone.

Ceramide synthase is a target for mycotoxins such as FB<sub>1</sub>, *Alternaria* toxin, Australifungins and N-acylaminopentols. The increase in sphingoid bases observed following FB<sub>1</sub> exposure results when the capacity of sphingosine kinase to degrade sphingosine to its 1-phosphate derivative has been exceeded (Soriano *et al.*, 2005, Riley *et al.*, 2001). In the absence of an exogenous sphingoid base source, loss of this pathway by mutation on SPT or its metabolites affects growth and viability. Therefore through inhibition of this pathway FB<sub>1</sub> may indirectly affect the processes dependent on these molecules (van der Westhuizen *et al.*, 2001a, Desai *et al.*, 2002, Schroeder *et al.*, 1994, Merrill *et al.*, 1996).



**Figure 2.4:** The structural similarities between FB<sub>1</sub>sphinganine (Sa) and sphingosine(So) (A) and the consequent inhibition of ceramide synthase (X) in the de novo sphingolipid pathway (B) leading to accumulation of sphingoid bases and depletion of complex sphingolipids (Schroeder *et al.*, 1994, Wang *et al.*, 1999).

### **2.3.6 Sphingolipids**

Sphingolipids are ubiquitous cellular membrane components and mediators of cellular communication especially in the brain tissue and nerve cells. They contain a sphingosine backbone instead of glycerol, two alcohols and an amino group. These sphingolipids are precursors of important complex sphingolipids such as ceramide and sphingomyelin which is a component of the myelin sheath (Soriano *et al.*, 2005). The levels of sphingolipid metabolites are tightly regulated as they perform important biological functions in the cell. Sphingoid bases (Sa and So) are important in the regulation of cellular growth, differentiation, transformation, morphology, apoptosis and endothelial cell permeability while ceramide plays a role in cellular senescence, apoptosis and differentiation. The phosphorylated metabolite of sphingosine (sphingosine-1-phosphate) has proliferative and anti-apoptotic properties (Soriano *et al.*, 2005, Riley *et al.*, 2001). Sphingolipids also form part of the localised regions called membrane micro-domains (lipid rafts). These lipid rafts affect various cellular functions such as calcium homeostasis, endocytosis, and protein sorting and signalling. Of major importance is the presence of these lipid rafts in the folate transporter which has been implicated in development of NTDs (Rao and Acharya, 2008). There are currently more than 300 types of sphingolipids with sphingosine being the most commonly found in the backbone of mammalian sphingolipids (Menaldino *et al.*, 2003).

### **2.3.7 Sphinganine to sphingosine ratio as a useful biomarker for FB<sub>1</sub> exposure**

The associated increase in sphinganine and sometimes sphingosine has led to the recommendation for use of Sa/So ratio as an indicator of fumonisin exposure (van der Westhuizen *et al.*, 1999, Loiseau *et al.*, 2007, Kim *et al.*, 2001). Various studies have been conducted on animal, *in vitro* and human samples in order to demonstrate the importance of Sa/So ratio in FB<sub>1</sub> exposure (Kim *et al.*, 2001, van der Westhuizen *et al.*, 1999, Wang *et al.*,

1999, Loiseau *et al.*, 2007). Human plasma and urine from three rural African areas (Transkei, KZN & Bomet) were analysed for the concentrations of Sa and So. Although there was no statistical difference between the ratios in the three groups, plasma and urine samples from Transkei had the highest ratio compared to both KZN and Bomet (van der Westhuizen *et al.*, 1999). Wang *et al.* showed a dose-dependent increase in urinary Sa and So in rats and this was also evident in porcine epithelium (Wang *et al.*, 1999, Loiseau *et al.*, 2007). Loiseau *et al.* found that FB<sub>1</sub> affected sphingolipid metabolism in the porcine epithelium in a dose- and time-dependent manner (Loiseau *et al.*, 2007). A concentration of 100µM FB<sub>1</sub> elicited a significant increase in sphinganine levels after day 2 of treatment whereas the low concentration (20µM) took longer (6 days) to show a similar effect. The increase in sphingosine was however only observed after four days of treatment at both concentrations (20µM and 100µM) (Loiseau *et al.*, 2007). Sphingoid bases are known inducers of cell death and this is highly dependent on their intracellular concentrations.

## **2.4 Cell death**

Two morphologically distinct forms of cell death have been described, programmed cell death (apoptosis) and necrosis (Kerr *et al.*, 1972). Necrosis is defined as accidental death; it is a pathological process which occurs when cells are exposed to a serious physical or chemical insult. Apoptosis is defined as programmed cell death and is responsible for cellular homeostasis during development, normal tissue turnover, and atrophy induced by endocrine and other stimuli, negative selection in the immune system and a substantial proportion of T-cell killing. Morphological features for necrosis include loss of membrane integrity which begins with swelling of the cell and mitochondria, disintegration of other organelles and finally cell lysis. Biochemical features of necrosis include loss of regulation of ion homeostasis, random digestion of DNA and post-lytic DNA fragmentation. It is also

important to note that necrosis affects groups of cells, is not energy requiring, is evoked by non-physiological disturbance such as complement attack, lytic viruses etc. and also results in significant inflammatory response. The features that characterise apoptosis are membrane blebbing and aggregation of chromatin at the nuclear membrane. It begins with cytoplasmic shrinkage (decrease in cell volume) and nuclear condensation and ends with formation of apoptotic bodies (Graf *et al.*, 2007, Elmore, 2007). In contrast to necrosis, apoptosis affects individual cells, is induced by physiological stimuli and the apoptotic cell is phagocytosed by adjacent cells and macrophages. Thus the inflammatory response is evaded. In some diseases or injury states the two modes of cell death may co-exist and dependent on various factors the cells may die via necrosis or apoptosis. In the presence of high ATP, the cell undergoes apoptosis while reduced ATP levels result in necrotic death. Poly (ADP ribose) polymerase (PARP) I activity is another important determinant of the mode of cell death (Kiechle and Zhang, 2002).

#### **2.4.1 Apoptosis**

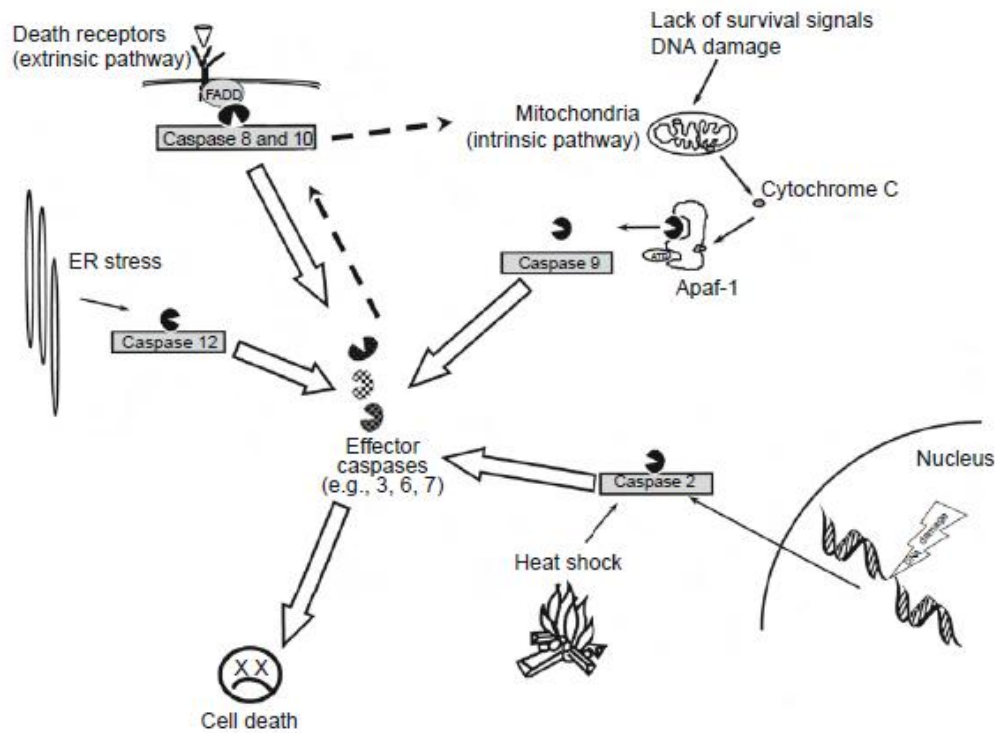
Apoptosis is an energy requiring process involving activation of proteins and enzymes. It is associated with pre-lytic DNA fragmentation, release of cytochrome C from mitochondria, activation of a caspase cascade and translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane (Krammer *et al.*, 2007, Fadok and Henson, 2003). Several stimuli are known to trigger apoptosis, such as death receptors (TNF- $\alpha$  and Fas Ligand), growth factor insufficiencies, toxins, oxidative stress and calcium influx through the plasma membrane. Apoptosis occurs via two mechanisms, the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (Figure 2.5). Both these pathways result in activation of a caspase cascade which eventually leads to cell death. The intrinsic pathway is mediated by mitochondrial membrane permeabilisation and the release of cytochrome C



(mitochondria) and calcium (endoplasmic reticulum (ER)). The extrinsic (death-receptor) pathway (TNF and Fas) is dependent on ligand (Fas L) binding to the death receptor (Fas/ CD 95) with resultant recruitment of the receptor-associated death domain resulting in formation of a signal complex which activates procaspase 8 and its downstream caspases. The CD95 (APO-1 or Fas) receptor ligand system is important in homeostasis of the peripheral lymphoid compartment and cytotoxic T-lymphocyte-mediated target cell killing (Rahman *et al.*, 2009, Neale and Mor, 2005).

Caspases are cysteinyl-aspartate specific proteinases which cleave the protein targets at the aspartate region. They are synthesised as inactive zymogens with an N-terminal prodomain followed by a 20kDa and 10kDa subunit. Cleavage of the prodomain at specific aspartate residues leads to the activation of caspases. Thus far 12 different members of caspases in mammalian cells have been identified. The caspases have been divided into three subgroups depending on their prodomain structure and substrate specificity. They are further differentiated according to their role in induction or execution of apoptosis as either initiator and/or execution caspases respectively. Initiator caspases (8, 9&10) possess a caspase recruitment domain (CARD) or death effector domain (DED). Activation of these caspases is responsible for the cleavage of cytoskeletal proteins such as actin, vimentin and fodrin which result in the characteristic blebbing of cell membranes. The characteristic “flip” of phosphatidylserine from the inner to the outer leaflet of the membrane is also thought to be due to the action of activated initiator caspases which directly or indirectly cleave translocases/flippases. During these early stages of apoptosis, the process is still thought to be reversible and is important in signalling for cell-cell recognition and induction of the coagulation cascade (Huppertz *et al.*, 2006, Elmore, 2007).

Activation of executioner/effector caspase (3, 6 &7) is the beginning of the irreversible component of the apoptotic process. These executioner caspases cleave a broad range of proteins which are critical for cellular survival such as nuclear envelope proteins (lamins A, B and C), proteins involved in DNA maintenance and repair (PARP), enzymes involved in the relaxation of the DNA-helix and separation of chromosomes during mitosis and intermediate filament proteins. Furthermore, executioner caspases also activate DNA fragmentation factor and other endonucleases which result in DNA fragmentation. Poly-(ADP-ribose) polymerase catalyses the ADP-ribosylation of nuclear proteins at spontaneous DNA strand breaks sites using NAD as a substrate. This facilitates the repair of damaged DNA. It also plays a role in cell death by depleting the cell of NAD and ATP. In addition, PARP also inhibits  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases which cleave DNA during apoptosis. Thus, as a consequence of caspase cleavage, there is reduced DNA repair and increased chromosome damage. Lamins are important in maintaining the structural stability of the nucleus and are found on the inner surface of the nuclear membrane. Their cleavage leads to nuclear collapse and fragmentation. The main caspase responsible for the cleavage of lamins is caspase 6 (Elmore, 2007, Huppertz *et al.*, 2006).



**Figure 2.5:** The figure depicts the two apoptotic pathways. The extrinsic pathway incorporates activation of death receptors and caspase 8 and the intrinsic (mitochondrial) pathway results in apoptosome formation and caspase 9 activation. Both pathways result in activation of initiator caspases specific for the pathway but converge at the level of activation of effector caspases that signify the beginning of the end (Rahman *et al.*, 2009).

## 2.5 The role of apoptosis in trophoblast development

As in any tissue development, apoptosis plays a major role in placental development. It is important in the control of trophoblast turnover as well as maintaining a balance between proliferating and differentiating cells. Apoptotic events within the placenta begin with apoptosis in the cytotrophoblasts linked to syncytial fusion, the release of syncytial knots then makes up the final (execution) stages of apoptosis in the syncytiotrophoblasts. In order to ensure continuous replenishment of the syncytiotrophoblast layer, the apoptotic cascade is limited to a subset of cells which express transcription factors that inhibit proliferation and promote syncytial fusion while others are allowed to proliferate (Huppertz *et al.*, 2006,

Kadyrov et al., 2006). Caspase 8 activity is evident in cytotrophoblast destined for syncytial fusion and hence apoptosis while the effector caspases are found in inactive forms in this layer. The presence of anti-apoptotic proteins within the syncytiotrophoblasts ensures that the cells do not progress immediately into the final stages of apoptosis, i.e. formation of syncytial knots. The apoptotic activity of both cytotrophoblasts and syncytiotrophoblast is controlled at the mitochondrial level by inhibitors of apoptosis such as Bcl-2 family. The fused syncytial layer contains a mixture of newly-fused cytotrophoblast and old syncytiotrophoblast nuclei. The shape of newly fused nuclei is large, ovoid and rich in euchromatin while the pre-apoptotic ones are smaller and denser. The activity of execution caspases increases in the syncytial cells (nuclei) destined for syncytial knot formation.

The apoptotic cascade in the extravillous cytotrophoblasts is poorly understood due to the inaccessibility of relevant tissue and cell types and as a result only end-stages of apoptosis have been documented. The Fas (CD95) and Fas ligand are present on the extravillous cytotrophoblasts. Similar to the differentiation of syncytiotrophoblasts, the extravillous trophoblasts also express different apoptotic markers at different stages. At the beginning of the invasive pathway they are resistant to apoptosis but later in the pathway the execution phases of apoptosis are activated. A study was done to evaluate the distribution of the different apoptotic markers within the placental tissue. The inactive form of caspase 8 (initiator caspase) was restricted to the apical part of the cytoplasm in the syncytiotrophoblast and only weaker staining within the villous cytotrophoblasts. Activated caspase 3 is distributed evenly in the cytoplasm of syncytiotrophoblasts (Mancini *et al.*, 1998, Huppertz *et al.*, 1999, Huppertz *et al.*, 2006, Heazell *et al.*, 2008, Huppertz and Kingdom, 2004). Villous trophoblast apoptosis is high in pregnancies complicated by preeclampsia and intrauterine growth restriction. The exaggerated shedding syncytium in these pregnancies may therefore

result in cells undergoing a necrotic form of cell death where the whole process of apoptosis has not been completed, a term called aponecrosis (Huppertz and Kingdom, 2004).

Apoptosis of invasive cytotrophoblasts from preeclamptic placentas is reportedly higher than in normotensive placentas (DiFederico *et al.*, 1999, Genbacev *et al.*, 1999, Kadyrov *et al.*, 2006, Huppertz *et al.*, 2005). This indicates the role this process may play in the defective invasion (Allaire *et al.*, 2000, Huppertz *et al.*, 2006, Zhou *et al.*, 1997). The Fas/FasL system is important in the immune protection of the developing foetus and hence is an integral part of the placental apoptotic machinery. Alterations in the Fas-FasL system in pregnancies complicated by preeclampsia have also been reported indicating the possible involvement of the Fas system in placental apoptosis (Neale *et al.*, 2003, Neale and Mor, 2005). The FasL was localised on the villous cytotrophoblasts mainly in the first trimester while the receptor was localised to the microvillous surface of the syncytiotrophoblasts (Huppertz *et al.*, 1998, Levy and Nelson, 2000, Neale *et al.*, 2003).

## **2.6 Fumonisin B<sub>1</sub> and apoptosis**

Fumonisin B<sub>1</sub> is known to induce apoptosis through various mechanisms. The disruption of sphingolipid biosynthesis is one of these mechanisms where the accumulation of sphingoid bases causes apoptosis. Fumonisin B<sub>1</sub> is also known to induce apoptosis through the death receptor pathway by stimulating production of TNF- $\alpha$  (He *et al.*, 2001, Seefelder *et al.*, 2003a).

In the presence of FB<sub>1</sub> there was increased expression of TNF- $\alpha$  along with increased apoptosis in the liver and kidney of mice treated with FB<sub>1</sub> while macrophages derived from animals treated with FB<sub>1</sub> also produced higher amounts of TNF- $\alpha$  (Voss *et al.*, 2002, Dugyala

*et al.*, 1998, Ciacci-Zanella and Jones, 1999). A rabbit kidney cell (RK13) treated with a range of FB<sub>1</sub> concentrations at varying duration showed no DNA fragmentation at all the time intervals studied but induced a significant concentration-dependent increase in micronucleus frequency (Rumora *et al.*, 2002). Fumonisin B<sub>1</sub> has also been shown to increase the activity of caspase 3 and also induce DNA damage in human fibroblasts (Galvano *et al.*, 2002, Seefelder *et al.*, 2003a).

## **2.7 Rationale for the study**

The aetiology of preeclampsia remains elusive while the incidence and mortality is on the increase especially in developing countries. The populations in developing countries are dependent on a diet constituted by dietary staples such as maize and maize-based products. It is unfortunate that these staples are frequently contaminated by moulds. The toxic metabolites produced by these moulds have been implicated in various human and animal diseases. In this context, the role of the diet in preeclampsia has not been fully investigated. Therefore this study aims to unravel the potential role of the mycotoxin FB<sub>1</sub> in preeclampsia.

## CHAPTER 3

### *Patient demographics*

#### **3.1 Collection of samples**

Ethical approval was obtained from the Nelson R. Mandela School of Medicine (Ref. no. H025/05) and informed consent to draw blood and placental tissue was obtained from all the patients participating in this study after a full explanation of the study (Appendix A1). All the samples were collected from the King Edward VIII Hospital Labour ward, Durban, South Africa.

A total of 40 black African patients were recruited in this study, 20 were preeclamptic (table 3.2) and 20 were normotensive (table 3.1) pregnant women as controls. Venous blood samples (5ml) were collected into a lithium heparin tube (5ml) and ethylene diamine tetra acetic acid (EDTA) tube (5ml). Placenta was collected immediately following delivery and blocks of tissue were cut. Both sections were washed in physiological saline (0.9%) and subsequently one block was immersed in formal saline (10%) and the other immediately frozen at -75<sup>0</sup>C.

Fixed placental tissue samples from both groups were processed for immunohistochemical analysis, routine haematoxylin and eosin staining and Hoechst. Serum samples were also analysed for the presence of fumonisin B<sub>1</sub> (western blotting and high performance liquid chromatography (HPLC)) and sphingolipids (HPLC). Lipid peroxidation was determined in both serum and placental tissues.

The mean age of the normotensive participants (Table 3.1) was  $29 \pm 7.8$  (range: 19-40 years) whilst the mean gestational age was  $37 \pm 4.9$  (range: 24-42 weeks). The mean systolic blood pressure was  $119 \pm 14.3$  (range: 95-148 mmHg) and diastolic blood pressure was  $72 \pm 9.3$  (range: 50-90 mmHg).

**Table 3.1: Demographics of normotensive patients**

	Age (years)	Gest Age (/40 Weeks)	Blood pressure (mmHg)	
			SBP	DBP
1	30	38	148	80
2	31	38	130	84
3	37	38	130	90
4	40	38	100	85
5	26	39	110	70
6	30	36	127	70
7	39	40	117	77
8	27	38	105	74
9	24	40	115	69
10	24	42	138	71
11	36	40	95	70
12	35	41	100	71
13	22	28	104	50
14	20	30	110	72
15	37	37	131	60
16	18	39	127	80
17	18	24	130	74
18	19	30	116	65
19	40	39	114	66
20	22	41	130	62
<b>Mean</b>	<b>29</b>	<b>37</b>	<b>119</b>	<b>72</b>
<b>SD</b>	<b>7.8</b>	<b>4.9</b>	<b>14.3</b>	<b>9.3</b>



The mean age of preeclamptic patients (table 3.2) was  $26 \pm 5.9$  (range: 19-40) while the mean gestational age was  $34 \pm 5.1$  (range: 24-40). The mean systolic blood pressure was  $156 \pm 9.1$  (range: 145-174) and diastolic blood pressure was  $95 \pm 9.3$  (range: 78-110). Proteinuria was a defining characteristic in these patients and ranged from 0.3g/l to 3.0g/l via dipstick test.

**Table 3.2: Demographics of hypertensive patients**

			Blood pressure (mmHg)			
	Age	Gestational Age (Weeks)	SBP	DBP	Proteinuria	Condition
1	27	35	155	100	++	PE
2	26	27	151	93	++	PE
3	22	28	170	100	++	IE
4	32	34	163	101	+	IE
5	40	27	150	90	++	PE
6	32	37	152	103	+++	PE
7	31	37	162	104	+	PE
8	27	36	162	80	+	PE
9	22	36	162	86	++	PE
10	34	38	145	83	+	PE
11	23	40	152	102	++	PE
12	21	39	168	110	+++	PE
13	31	28	160	101	+++	PE
14	20	39	150	110	+	PE
15	19	34	164	87	++	PE
16	21	40	151	95	+++	PE
17	22	24	148	90	+	PE
18	19	39	148	78	+	PE
19	30	29	174	99	++	PE
20	20	31	159	95	++	PE
<b>mean</b>	<b>26</b>	<b>34</b>	<b>157</b>	<b>95</b>		
<b>SD</b>	<b>6.0</b>	<b>5.1</b>	<b>8.2</b>	<b>9.3</b>		

**Key to the tables:**

SBP: Systolic blood pressure

DBP: Diastolic blood pressure

PE: preeclampsia

IE: Imminent eclampsia

SD: Standard deviation

Trace amounts of protein in the urine = 0.1g/l

+ = 0.3g/l

++ = 1.0g/l

+++ = 3.0g/l

++++ = > 20g/l

## CHAPTER 4

### *Qualitative and quantitative analysis of fumonisin B<sub>1</sub>, sphingolipids and lipid peroxides in the serum of preeclamptic and normotensive pregnant women.*

#### 4.1

#### INTRODUCTION

Serum represents an important route by which xenobiotics/digested foods are transported from their site of absorption to their target sites. For hydrophobic molecules, proteins present in the serum are responsible for performing this function. The most important serum protein involved in the transport of xenobiotics is albumin. Albumin is rich in lysine residues, which allows xenobiotics to bind to the positively charged side chain (Kawakami *et al.*, 2006). The carcinogenic mycotoxin, aflatoxin B<sub>1</sub>, forms adducts with lysine residues in albumin (Sabbioni *et al.*, 1987). Fumonisin B<sub>1</sub> is a polar mycotoxin that utilises the gastrointestinal tract as a route of exposure. Serum therefore represents an important mechanism for FB<sub>1</sub> delivery to target sites in the body.

The hydrophilicity of FB<sub>1</sub> means that it does not require a transport protein to reach its target sites. However, the structure of FB<sub>1</sub> would also facilitate binding of FB<sub>1</sub> to serum proteins through interactions with its carboxyl and amino groups. The carboxyl groups have been shown to interact with the positively charged groups on the protein whilst the terminal amino group interacts with negatively charged amino acids on the protein. Thus proteins rich in positively charged amino acids such as lysine would interact with FB<sub>1</sub> through the epsilon amino group found on the lysine residue.

The question therefore is ‘Does FB<sub>1</sub> bind to serum proteins?’ This question has been partially addressed in previous studies which looked at interaction of FB<sub>1</sub> with various

proteins from biological and non-biological samples. Seefelder *et al.* showed that FB<sub>1</sub> does interact with proteins and carbohydrates from food samples, whilst other studies showed the interaction in oesophageal and liver tissue samples (Seefelder *et al.*, 2003b, Myburg *et al.*, 2009, Cawood *et al.*, 1994).

It may now be considered an established fact that the mechanism of FB<sub>1</sub> toxicity is due to disruption of sphingolipid metabolism at the level of ceramide synthase. The inhibition of ceramide synthase results in an accumulation of sphinganine (Sa) and sphingosine (So). It is the alteration in the ratio of these key molecules in serum and also urine that is now considered a biomarker for FB<sub>1</sub> exposure.

Proteomics is an invaluable technique in environmental and occupational health studies. The environment plays a major role in many human processes and thus by using this method valuable information at the cellular and molecular levels can be obtained. Therefore this study qualitatively (western blotting) and quantitatively (high performance liquid chromatography (HPLC)) determined whether FB<sub>1</sub> and intermediates of the sphingolipid pathway, sphinganine and sphingosine, are present in serum samples of normotensive and preeclamptic pregnant women.

Fumonisin B<sub>1</sub> toxicity is often associated with increased oxidative stress through various mechanisms. Reactive oxygen species such as superoxide interact with polyunsaturated fatty acids to form lipid hydroperoxides. One of the by-products of this peroxidative process is malondialdehyde (MDA). Thus; the presence of lipid peroxides in the form of MDA in the serum from both groups was evaluated.

## 4.2

## MATERIALS AND METHODS

### 4.2.1 *Materials*

Bovine serum albumin (BSA) was purchased from Roche Diagnostics and diaminobenzidine (DAB) chromogen tablets were purchased from DAKO Corporation, Southern Cross Biotechnology. Monoclonal primary antibody (mouse anti-FB<sub>1</sub>) was purchased from Neogen (USA) and secondary antibody (rabbit anti-mouse IgG) was purchased from Capital Labs. All the sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) chemicals and reagents employed in the making of buffers were purchased from BioRad, South Africa. Thiobarbituric acid (TBA) and butylatedhydroxytoluene (BHT) were purchased from Capital laboratories (South Africa). High performance liquid chromatography (HPLC) grade consumables (methanol, acetonitrile, 2-mercaptoethanol, sodium dihydrogen phosphate, ammonium hydroxide, chloroform, silica, anhydrous sodium sulphate and potassium hydroxide) were also purchased from Merck Chemicals, South Africa whilst O-phthaldialdehyde and strong anion exchange cartridges were purchased from Capital Labs, South Africa. All other chemicals employed in the making of buffers and solvents were purchased from Merck, South Africa.

### 4.2.2 *Methods*

#### 4.2.2.1 **Sample preparation**

Venous blood, collected in vacutainers (clot activator) prior to delivery, and serum was obtained by centrifugation at 1491rpm for 30 minutes (Eppendorff 5408R centrifuge, Hamberg, Germany). The serum was aliquoted and then stored at -20<sup>0</sup>C until analysis.

#### **4.2.2.2 The Bicinchoninic Acid (BCA) Assay**

The BCA assay was used to determine crude protein concentrations for subsequent standardisation. A bovine serum albumin standard (1mg/ml) was prepared and serial dilutions (Appendix B1) were prepared with storage buffer, pH 7.5 (Appendix B2). Serum protein samples (25µl) from normotensive and preeclamptic patients were diluted (1:100) with storage buffer and then loaded into a 96-well plate along with the serial dilutions (25µl) in duplicate. A BCA working solution (200µl, Appendix B1.1) was then added into each well and incubated for 30minutes at 37<sup>0</sup>C. The plate was then spectrophotometrically read on a Biotek µQuant microplate reader at 562nm. A standard curve was then prepared using the averaged absorbance values obtained from the BSA standards and protein concentrations were extrapolated from the standard curve (Appendix B1.2). These protein concentrations were then standardised to 200µg/ml with storage buffer.

An aliquot of standardised sample (100µl) was mixed with 1xSDS reducing (sample) buffer(100µl) (Appendix B3.3). The protein samples were then heated to 100<sup>0</sup>C for 5 minutes to denature them.

#### **4.2.2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The 10% resolving gel was prepared (Appendix B3.1) and poured into the assembled glass plates (enough to leave space for the stacking gel). Water was poured over the separating gel in order to prevent oxygen from diffusing into the gel and preventing polymerisation. The gel was left to polymerise for approximately an hour. The water was then poured off and dried with fibre-free filter paper. Stacking gel (4%) (AppendixB3.2) was then overlaid on top of the resolving gel, the combs were inserted and the gel left to polymerise as before. The combs were then removed and gels were loaded on electrophoresis apparatus. Samples (30µl) and

5µl of molecular weight marker were then loaded into their respective wells and allowed to electrophorese at 150V for 1 hour in electrode (running) buffer, pH 8.3 (Appendix B5). The gels were then stained with a Coomassie blue stain (Appendix B4.1.1) for 30 minutes, destained with destaining solution 1 (Appendix 4.2) for 1 hour and finally left in destaining solution 2 (Appendix 4.3) overnight. The gels were analysed using a ChemiDoc XRS gel documentation system (BioRad, South Africa).

#### **4.2.2.4 Western blot analysis of serum for FB<sub>1</sub>**

Following electrophoresis, the separated proteins were electro-transferred to an equilibrated polyvinylidenedifluoride (PVDF) membrane for one hour in transfer buffer (Appendix B6). After transfer the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (Appendix B7), the membrane was immuno-probed with monoclonal anti-FB<sub>1</sub>(1:1000 in 1% BSA, Neogen, USA) for 1 hour at room temperature (RT). The PVDF membrane was then subjected to 5 washes (10 minutes each with gentle agitation) with TTBS. The membrane was then incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:10000; Bio-Rad) for 1 hour at RT. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Biorad, South Africa). Band intensity analysis was conducted on the resultant bands.

#### **4.2.2.5 High performance liquid chromatography -Quantitation of serum FB<sub>1</sub>**

The FB<sub>1</sub> in serum samples was extracted and purified using the method described by van der Westhuizen *et al.* (2001). Pre-conditioning of strong anion exchange (SAX) cartridges was

achieved by application of 5ml of methanol-water (3:1 v/v) to the column. The sorbent was successively washed with 5ml of methanol-water (3:1 v/v) and 5ml of methanol.

An aliquot (500 $\mu$ l) of serum was deproteinised by the addition of 2.5ml of methanol. The protein was precipitated by centrifugation at 5 000rpm for 10 minutes at RT (Eppendorff 5804R). The supernatant was removed and applied to a pre-conditioned strong anion exchange (SAX) cartridge. Fumonisin B<sub>1</sub> was then eluted from the sorbent with 10ml of 5% acetic acid in methanol using a Varian Vac Elute system (Capital laboratory supplies) at a flow rate of 1ml/min. The eluate was evaporated to dryness at 60°C and the residue was re-dissolved in 100 $\mu$ l of acetonitrile- water (1:1 v/v) and allowed to stand for 5 minutes prior to derivatisation. The FB<sub>1</sub> was derivatised using O-phthaldialdehyde (OPA) derivative (Appendix C1) prior to analysis by reverse phase HPLC (RP-HPLC). Derivatised samples were prepared by mixing 40 $\mu$ l of the extracted sample with 80 $\mu$ l of the OPA reagent. RP-HPLC analysis of the serum samples was carried out under isocratic conditions (flow rate 1ml/min) using methanol-0.1M NaH<sub>2</sub>PO<sub>4</sub> (29:71) buffer (pH3.3). Injection of 50 $\mu$ l of derivatised sample was performed in duplicate in order to confirm reproducibility. The HPLC system employed consisted of a spectra physics P2000 binary pump, an autosampler and an FL2000 fluorescence detector (excitation-335nm & emission 440nm ThermoSeparation production) with a Waters NovaPak C18 column ( Waters, Microsep, SA) and an HIRB-10C guard column (Hichrom Ltd, SMM Instruments) attached. The results were analysed using the Chromquest 4 software.

#### **4.2.2.6 Analysis of sphinganine and sphingosine from serum**

Serum (500 $\mu$ l) was deproteinised in methanol (2ml), vortexed and centrifuged for 10 minutes at 5 000rpm (Eppendorff 5408R). The supernatant (2ml) was transferred into a clean



centrifuge tube and 1.9ml of water, 1.2ml of 0.35M NH<sub>4</sub>OH and 4ml chloroform (CHCl<sub>3</sub>) were added and then centrifuged for 10 minutes at 5 000rpm. After centrifugation, the top layer was aspirated and discarded. A column was prepared by packing 0.5g of silica and 5g of anhydrous sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) and conditioned with CHCl<sub>3</sub> (4ml). The sample (3ml) was then loaded onto the column and washed with 1ml CHCl<sub>3</sub>, followed by elution with 10ml of CHCl<sub>3</sub>: CH<sub>3</sub>OH: NH<sub>4</sub>OH (20:20:0.8, v/v/v) and dried under a gentle stream of nitrogen at 38<sup>0</sup>C. The dried residue was re-dissolved in 1ml 0.125M potassium hydroxide (KOH)/ CH<sub>3</sub>OH: CHCl<sub>3</sub> (4:1) and kept at 38<sup>0</sup>C. After 1.5 hours the samples were cooled on ice and thereafter re-dissolved in 1ml of CHCl<sub>3</sub> and 5ml alkaline NH<sub>4</sub>OH. The mixture was centrifuged at 1200xg for 10 minutes. The top layer was discarded and bottom layer dried under a gentle stream of nitrogen and stored at 4<sup>0</sup>C.

High performance liquid chromatography was performed on the OPA derivatised samples. The derivatised sample (50µl) was injected into the HPLC system consisting of a spectra physics P2000 binary pump, an autosampler and an FL2000 fluorescence detector (excitation-335nm & emission 440nm;ThermoSeparation production) with a Waters NovaPak C18 column ( Waters, Microsep, SA) and an HIRB-10C guard column (Hichrom Ltd, SMM Instruments) attached. Samples were pumped in the isocratic mobile phase of methanol/0.05M potassium phosphate buffer (pH 7.4) (91:1) and the flow rate controlled at 2ml/min. The results were then analysed using the Chromquest 4 software.

#### **4.2.2.7 Thiobarbituric acid reactive substances (TBARS) - analysis of lipid peroxidation in serum**

Equal amounts (500µl) of serum and 2% H<sub>3</sub>PO<sub>4</sub> were added to a glass tube. The solution was then aliquoted into two glass tubes, one designated sample (400µl) and another, the

blank(400µl). To each tube, 7% H<sub>3</sub>PO<sub>4</sub> (200µl) was added followed by addition of TBA/BHT (400µl) to the sample tube and 3mM HCl (400µl) to the blank tube. The pH of all samples was adjusted to pH 1.5 using 1M HCL and then heated to 100<sup>0</sup>C for 15 minutes. Following cooling to room temperature, butanol (1.5ml) was added to each tube and vortexed for 40-60seconds.The butanol phase (top) was aliquoted into a centrifuge tube and centrifuged at 13200rpm for approximately 6 minutes. The lipid peroxides were then measured spectrophotometrically at dual wavelength (532nm, 600nm) and the following equation was used to calculate the concentration of MDA in the samples:

$$\frac{\text{average absorbance of sample}}{156\text{mM}}$$

#### **4.2.2.8 Statistical analysis**

The results were analysed using the Graphpad Prism 5 software. A student's t-test was use for analysis. Data is presented as mean± standard error of the mean (SEM). A p-value of <0.05 was considered statistically significant.

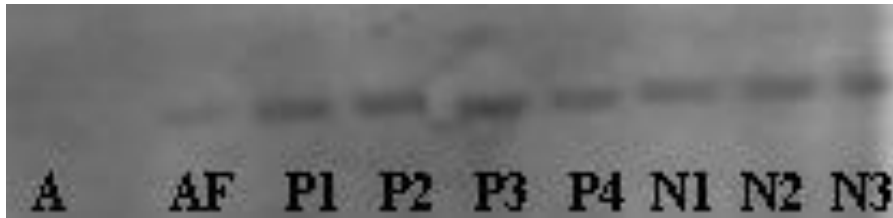
### **4.3**

## **RESULTS**

Serum samples were both quantitatively and qualitatively analysed for the presence of FB<sub>1</sub>. Western blotting was used to determine the presence of FB<sub>1</sub> in serum whilst HPLC was used to quantify the FB<sub>1</sub> in serum. An albumin standard was used as a method control; one albumin fraction was spiked with FB<sub>1</sub> whilst the other was unspiked and served as a negative control. In addition, the levels of MDA as an indicator of lipid peroxidation were also determined.

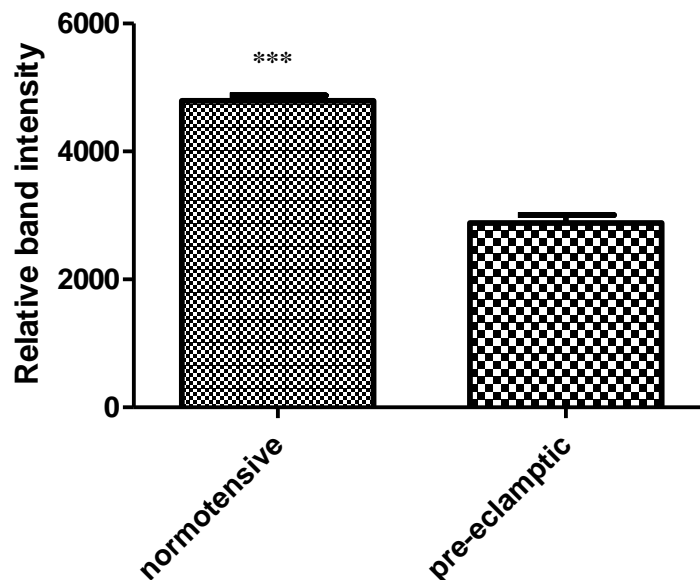
*Western blot analysis of serum*

Fumonisin B<sub>1</sub> was found in both normotensive and preeclamptic serum samples (Figure 4.1). The albumin negative control showed the absence of FB<sub>1</sub>, whilst the positive had a visible band.



**Figure 4.1:** A representation of western blot banding pattern for serum samples from normotensive patients (N1-N3) and preeclamptic patients (P1-P3). Pure albumin (A) and albumin spiked with FB<sub>1</sub> (AF) served as negative and positive controls respectively.

The densitometric analysis of the membrane (Figure 4.2) revealed significantly higher band intensity ( $p < 0.0001$ ) in normotensive samples compared to preeclamptic samples (Table 4.1).



**Figure 4.2:** Band intensity analysis of sera from preeclamptic and normotensive women. \* indicates  $p < 0.0001$ . Unpaired T-test with Welch correction, 95% confidence interval  $n=20$ .

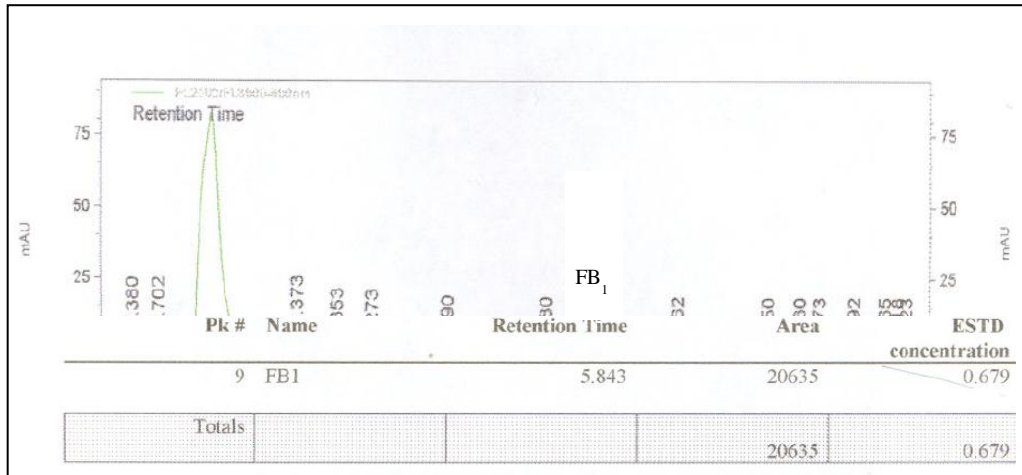
### Quantitation of FB<sub>1</sub> (HPLC)

Serum samples were also analysed for the presence of FB<sub>1</sub> and the levels of sphinganine and sphingosine using HPLC (Table 4.1).

**Table 4.1** Relevant patient demographics and FB<sub>1</sub>, sphinganine and sphingosine values (values presented as mean  $\pm$  SEM)

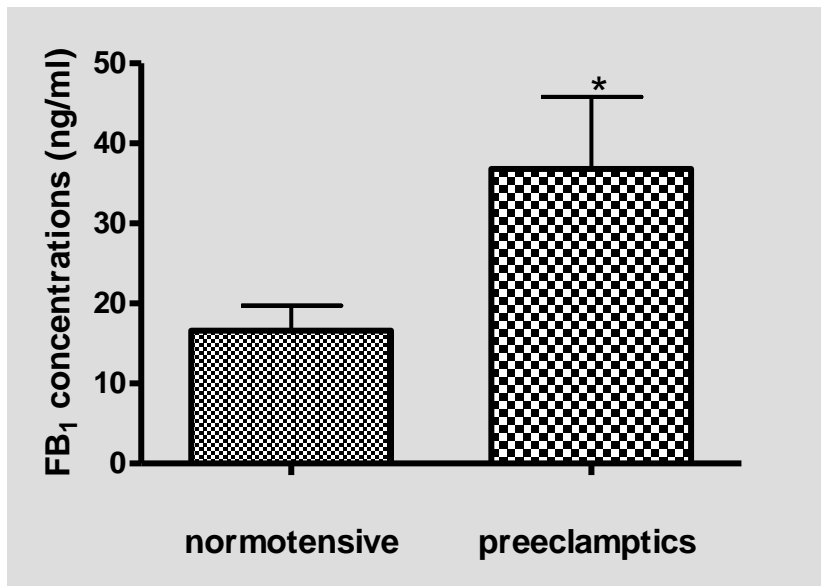
Variable	Normal (n=20)	Preeclampsia (n=20)	p-value
Age	28.8 $\pm$ 1.732	26 $\pm$ 1.335	
Gestational age	37 $\pm$ 4.9	34 $\pm$ 5.1	
Blood pressure			
Systolic	118.9 $\pm$ 3.201	157.3 $\pm$ 1.835	
Diastolic	72 $\pm$ 2.074	95.4 $\pm$ 2.075	
Proteinuria	trace	1.155 $\pm$ 0.223	
Mean concentration of FB <sub>1</sub>	16.59 $\pm$ 3.141	36.82 $\pm$ 9.007	0.03
Mean concentration of Sa	0.034 $\pm$ 0.010	0.088 $\pm$ 0.021	0.04
Mean concentration of So	0.017 $\pm$ 0.005	0.025 $\pm$ 0.006	0.3

The concentration of FB<sub>1</sub> in serum was analysed using HPLC and the concentration was calculated using the Chromquest 4 software. A typical chromatogram of FB<sub>1</sub> (Figure 4.3), demonstrating the retention time of FB<sub>1</sub> and the results are presented in Table 4.1. Retention time was used to calculate the concentration of FB<sub>1</sub> in the sample through the software mentioned above.



**Figure 4.3:** A representative HPLC chromatogram from patient’s serum showing FB<sub>1</sub> retention time (5.843) and an estimated concentration of 0.679µM.

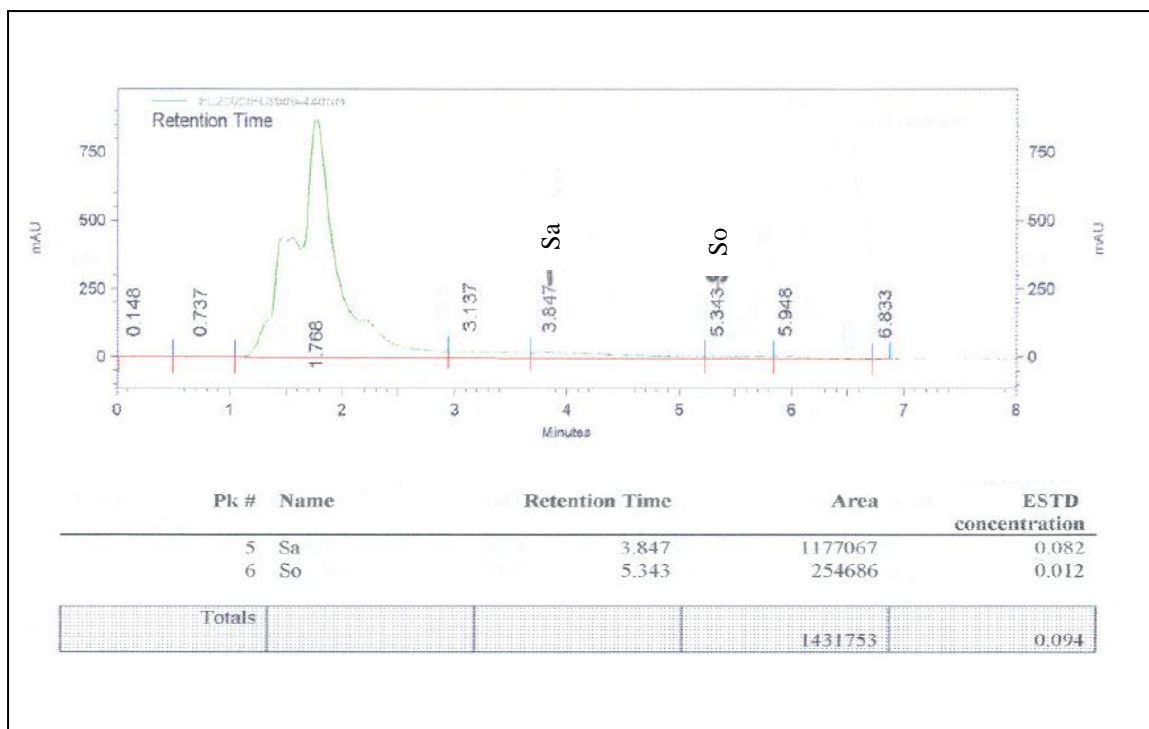
Preeclamptic sera had significantly higher ( $p = 0.03$ ) concentrations of FB<sub>1</sub> ( $36.82 \pm 9.007$ ; 0-114ng/ml) compared to the normotensives ( $16.59 \pm 3.141$ ; 0-25ng/ml) (Table 4.1 & Figure 4.4). Non-detectable levels of FB<sub>1</sub> were observed in one preeclamptic sample and two normotensive samples. The highest concentration detected in preeclamptic samples was 114ng/ml whilst 37ng/ml was the highest observed level in normotensive samples (Figure 4.4).



**Figure 4.4:** Graphical presentation of serum concentrations of FB<sub>1</sub> from normotensive (n=20) and preeclamptic (n=20) women. FB<sub>1</sub> was significantly higher in preeclamptics (p=0.03). Two-tailed 95% confidence interval\* indicates the results were significantly different from controls (Mann Whitney test).

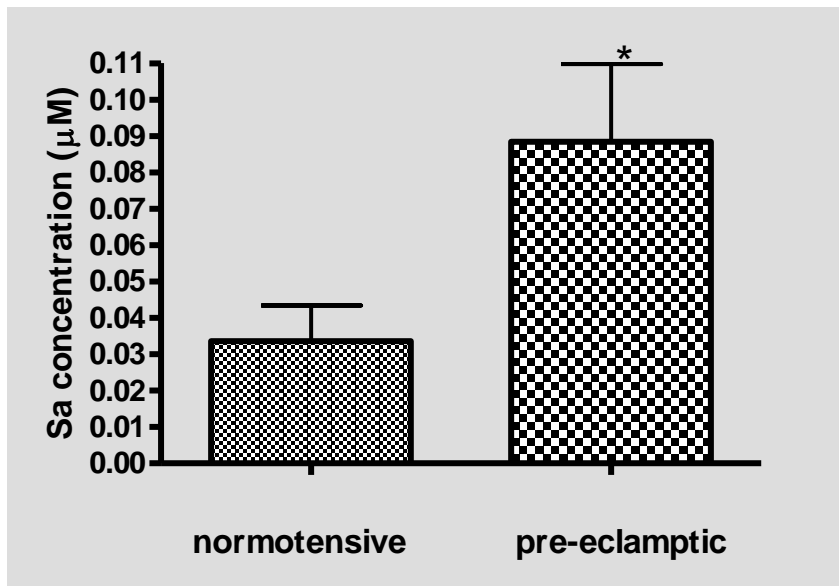
#### *Quantification of sphinganine and sphingosine (HPLC)*

Serum samples from both normotensive and preeclamptic women were analysed for the presence and concentration of sphinganine and sphingosine and a chromatograph was generated (Figure 4.5).

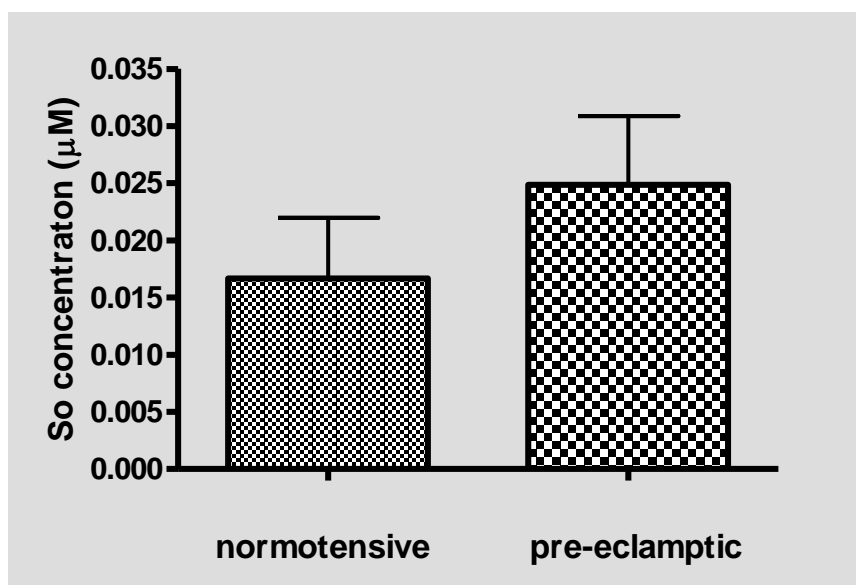


**Figure 4.5:** A representative chromatograph of sphinganine (Sa)/sphingosine (So) analysis from serum of pregnant women showing the retention times and peaks for Sa and So.

The mean concentration ( $0.088 \pm 0.021$ ; range 0- $0.192\mu\text{M}$ ) of sphinganine was significantly higher ( $p=0.04$ ) in preeclamptics compared to normotensives ( $0.034 \pm 0.010$ ; range 0- $0.086\mu\text{M}$ ) (Table 4.1 & Figure 4.6). There was no statistical difference in the sphingosine concentrations between the normotensives and preeclamptics ( $p=0.3$ ) (Table 4.1 & Figure 4.7). Preeclamptic samples ( $0.025 \pm 0.006$  range 0- $0.003\mu\text{M}$ ) were slightly higher than that found in normotensive samples ( $0.017 \pm 0.005$ ; 0.002- $0.028\mu\text{M}$ ). Two samples from each group had non-detectable levels of sphingosine whilst two samples in normotensives also had non-detectable levels of sphinganine.



**Figure 4.6:** Sphinganine concentrations from normotensive and preeclamptic sera. Significantly high concentrations of sphinganine were observed in preeclamptics compared to the normotensives. \* indicates statistically significant difference between the means ( $p=0.04$ ) using t-test with Welch correction, two-tailed, 95% confidence interval.

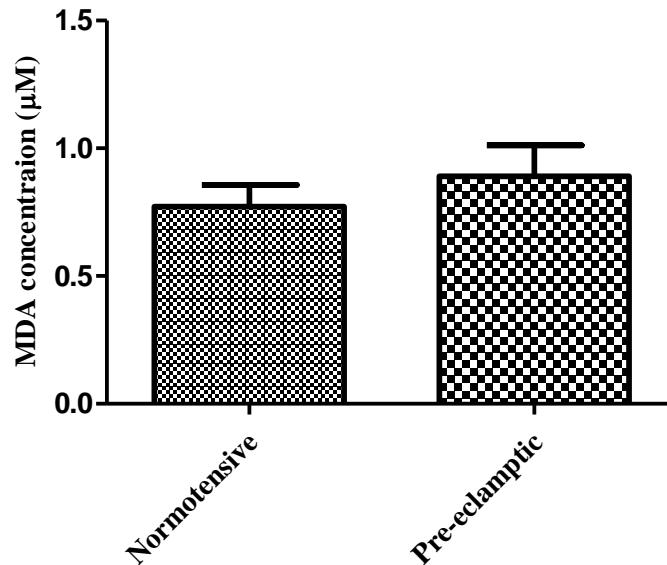


**Figure 4.7:** Graphical representations of sphingosine concentrations from normotensive and preeclamptic samples. There was no significant difference. T-test, Welch correction  $p>0.05$ , Two-tailed, 95% confidence interval

*Lipid peroxidation (MDA) in serum of pregnant women*



The levels of lipid peroxidation in serum were measured using the TBARS assay. There was a slight but non-significant increase in lipid peroxides as indicated by MDA in preeclamptic samples compared to normotensive controls (Figure 4.8).



**Figure 4.8:** Malondialdehyde (MDA) concentrations in normotensive and preeclamptic sera. The MDA concentration was non-significantly higher in preeclamptic sera compared to the normotensives.  $p > 0.05$ , unpaired t-test with Welch's correction.

#### 4.4

#### DISCUSSION

Although preeclampsia has been studied extensively, its aetiology still eludes researchers and thus it is imperative to investigate other possible aetiological agents. The diet of the African community is comprised primarily of maize and maize-based products and a study by Chelule *et al.* showed high levels of FB<sub>1</sub> in rural maize samples compared to their urban counterparts (Chelule *et al.*, 2001). This finding coupled with the fact that the Eastern coastline of South Africa has one of the highest incidence of preeclampsia in the country, may implicate FB<sub>1</sub> in the development of the disease (Moodley *et al.*, 2001).

To date, no study has shown the presence of FB<sub>1</sub>-protein conjugates in pregnant women. This current study demonstrated that FB<sub>1</sub> forms conjugates with albumin, a serum protein rich in lysine residues (Figure 4.1). The tricarballic acid (TCA) moieties in FB<sub>1</sub> will allow strong binding to the ε-amino groups of the lysine residues. Albumin is the major serum protein in plasma and its important functions are to regulate colloid osmotic pressure and transport (Baynes and Dominiczak, 2009).

This study showed that FB<sub>1</sub> is present in the blood of normotensive and preeclamptic women. It is important to note that the FB<sub>1</sub> detected using western blotting represents only bound/conjugated FB<sub>1</sub>, while HPLC quantifies both bound and free FB<sub>1</sub>. The levels of bound FB<sub>1</sub> (FB<sub>1</sub>-serum conjugates) were higher in normotensive sera compared to preeclamptics (Figure 4.2). This is in stark contrast to the quantitative analysis (HPLC) which showed the opposite effect (Figure 4.4). Preeclampsia is a condition of pregnancy marked by proteinuria which results in a loss of serum proteins such as albumin (Thangaratinam *et al.*, 2009). Thus, preeclamptics may be characterised as having reduced concentrations of albumin in their serum. Extensive loss of protein was observed in all hypertensive patients compared to normotensive women who have no (trace levels) protein loss (Table 4.1). Therefore due to proteinuria, very little albumin is available for binding to FB<sub>1</sub> in preeclamptic sera and much of the FB<sub>1</sub> remains free in the serum. In comparison, normal albumin levels prevail in normotensive sera leaving more available to bind FB<sub>1</sub> and hence the high relative band intensity observed in this group. This provides a plausible explanation for the reduced FB<sub>1</sub>-protein adducts observed in the preeclamptic sera.

In addition, preeclamptic sera contained high levels of lipid peroxides compared to normotensive counterparts indicating increased production of reactive oxygen species (ROS)

that may interact with cellular macromolecules such as protein, lipids and DNA. In the current study it is reasonable to hypothesise that the reduction in albumin-FB<sub>1</sub> adducts in preeclamptic sera may be due to the fact that in addition to the proteinuric loss, the available albumin is oxidised by ROS and is thus unavailable to bind to FB<sub>1</sub>. The levels of oxidised albumin have been measured in several diseases such as stroke, diabetes and renal failure as an indicator of oxidative stress. The use of MDA as an indicator of lipid peroxidation has come under scrutiny due to the non-specific interaction of TBA with other molecules such as sugars, urea, proteins and alkanals in addition to MDA. Therefore in addition to measuring MDA it is recommended that other products of lipid peroxidation be measured using highly specific methods such as HPLC and GC-MS. A measurement of oxidised albumin in the current study would have strengthened the arguments (Devasagayam *et al.*, 2003, Moon *et al.*, 2011). Another plausible explanation may be that the ROS interact with polyunsaturated fatty acids on the glomerular membrane, making it leaky and facilitating the loss of albumin via this route.

As mentioned, qualitative analysis of FB<sub>1</sub> by HPLC provides the total concentration of FB<sub>1</sub> present (bound and unbound). Thus, higher levels of FB<sub>1</sub> are seen in preeclamptic women compared to their normotensive counterparts (Figure 4.4). These results concur with a previous study by Moodley *et al.* who first reported on the presence of FB<sub>1</sub> in serum of black South African women from the same Hospital. Eclamptic and preeclamptic serum samples showed higher FB<sub>1</sub> concentrations compared to their normotensive counterparts (Moodley *et al.*, 2001). This is possible as the staple diet of all patients in the study is maize and as a result of this dietary pattern, the subjects would be chronically exposed to FB<sub>1</sub>. Fumonisin B<sub>1</sub> is a polar molecule and is rapidly excreted; therefore the high levels observed in the preeclamptic women may indicate an increase in absorption, reduced excretion or accumulation. Due to the

chronic exposure through maize it is reasonable to postulate that FB<sub>1</sub> would accumulate over time either through binding to serum or tissue proteins which would serve as reservoirs for the mycotoxin. This conjugation with proteins and reduced excretion may therefore prolong its toxic action(s) in these patients and possibly also *in utero*.

Fumonisin B<sub>1</sub> is known to interact with macromolecules through its TCA moieties and terminal amino group. The amino and thiol groups in proteins interact with the TCA and terminal amino group on the FB<sub>1</sub> backbone respectively (Seefelder *et al.*, 2003b). In addition, TCA moieties FB<sub>1</sub> are able to conjugate to other macromolecules such as carbohydrates (Seefelder *et al.*, 2003b, Hopmans *et al.*, 1997). Hopmans *et al.* showed the excretion of FB<sub>1</sub> and its metabolites (FB<sub>1</sub>-fructose adduct, hydrolysed FB<sub>1</sub>) in rats and found that 50% of FB<sub>1</sub> was excreted un-metabolised while the FB<sub>1</sub>-fructose adduct was absorbed to a higher degree than the hydrolysed form (Hopmans *et al.*, 1997). This therefore indicates the role that macromolecules play in prolonging the half-life of toxins. The binding of FB<sub>1</sub> to proteins such as albumin may therefore serve as a mechanism to enhance its absorption, prolong its toxicity by extending its retention in the body.

As a consequence of FB<sub>1</sub> toxicity, sphinganine is elevated in most tissues and cells to a greater degree than sphingosine (Desai *et al.*, 2002). The current study showed significantly higher levels of sphinganine and sphingosine in preeclamptic patients compared to their normotensive counterparts. This is not surprising considering the higher levels of FB<sub>1</sub> observed in these subjects would have a greater impact in inhibition of sphingolipid metabolism resulting in the observed increase in these sphingoid bases. These results concur with other human studies in that sphinganine concentrations were elevated in the presence of FB<sub>1</sub> (van der Westhuizen *et al.*, 1999, Castegnaro *et al.*, 1998). Van Der Westhuizen *et al.*

were the first to show the increase in Sa and associated Sa/So ratio in blood and urine of rural Eastern Cape communities (van der Westhuizen *et al.*, 1999). However, they concluded that the sphinganine/sphingosine ratio may not be sensitive enough to act as a biomarker for FB<sub>1</sub> exposure in humans. Various animals were also studied to demonstrate the importance of the sphinganine/sphingosine ratio as a biomarker for FB<sub>1</sub> exposure. Following a single gavage dose of FB<sub>1</sub>, the serum of vervet monkeys showed an elevation in the ratio compared to controls (van der Westhuizen *et al.*, 2001a). Low concentrations of FB<sub>1</sub> were shown to be effective in elevating the sphinganine/sphingosine ratio following repeated dosing (van der Westhuizen *et al.*, 2001b). Fumonisin B<sub>1</sub> was also shown to increase the ratio in male Wistar rats (Domijan *et al.*, 2007).

This is the first preliminary report on the sphingolipid concentrations in pregnant women and in agreement with van der Westhuizen *et al.* A comprehensive study of the sphinganine/sphingosine ratio in pregnant women will have to be conducted to support its use as a biomarker of FB<sub>1</sub> exposure. A recent report has provided evidence for the use of urinary FB<sub>1</sub> concentrations as a biomarker of exposure and therefore future studies in pregnant women should also look into using this method for biomarker purposes (van der Westhuizen *et al.*, 2001a, van der Westhuizen *et al.*, 2011). Elevated sphinganine has been associated with decreased cell viability, DNA fragmentation, loss of regulation of differentiation and apoptotic morphology. Thus the high levels of sphinganine observed in preeclamptic samples may contribute to the apoptosis reported in preeclampsia (Allaire *et al.*, 2000).

#### 4.5

#### CONCLUSION

The presence of FB<sub>1</sub> in sera of pregnant women indicates the chronic exposure of pregnant women to mycotoxins. The serum concentrations of FB<sub>1</sub> were higher in preeclamptic compared to normotensive patients. This study shows that FB<sub>1</sub> conjugates with albumin, thereby interfering with its normal physiological functions. It is tempting to say that due to the findings in the study FB<sub>1</sub> may be an aetiological agent in the development of preeclampsia. However it must be noted maize is not only contaminated by *Fusaria spp.* moulds but other species of moulds may co-occur and as a result affect the toxicity of FB<sub>1</sub>. Therefore a mycotoxin survey of the blood of rural communities which are heavily reliant on maize may demonstrate this possible interaction. Further studies also need to be conducted on the actual amino acid residues involved in the binding of FB<sub>1</sub> in serum.

## CHAPTER 5

### *Analysis of placental samples for the presence of fumonisin B<sub>1</sub>, apoptotic markers and lipid peroxides*

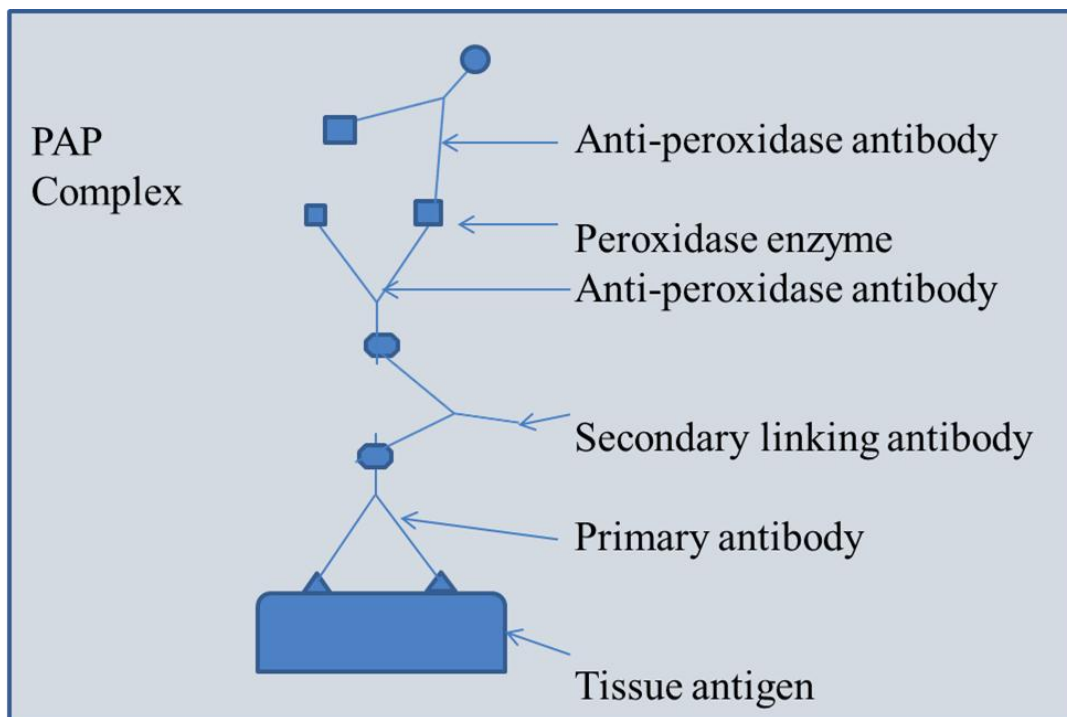
#### 5.1 INTRODUCTION

Since its discovery, microscopy has become an indispensable tool in the study of cellular structure and function. The use of the different types of microscopes like electron and light microscopy has provided the possibility of studying the ultra-structure of different organs and tissues and hence provide important information about their fine structure (Javois, 1999).

Light microscopy allows visualisation of tissue or cellular architecture using various dyes. Cell staining is a powerful method to demonstrate both the presence and subcellular location of a particular molecule of interest. Dyes are aromatic benzene ring compounds or derivatives that possess colour and the ability to bind to tissues. Chromophores confer colour to the dye and alter the light resonance properties of the compound resulting in unequal absorption when white light is passed through. The colour emitted by a specific object is dependent on the colour spectrum when white light falls on it. Primary stains highlight the element to be studied while secondary stains have a high affinity for background materials and are usually of the contrasting colour (Javois, 1999).

Although fixation is an integral part of tissue preservation, it also affects the interaction of tissues with various stains. Certain fixatives may either enhance or inhibit dye uptake through modifications of tissue proteins. Formalin for instance, forms crosslinks with proteins and masks cationic binding sites for anionic dyes suppressing acidic dye (eosin) uptake, while anionic binding sites are exposed enhancing the basophilic (haematoxylin) nature of nuclei.

Immunohistochemistry (IHC) is defined as the identification of a tissue constituent *in situ* by means of a specific antigen-antibody reaction where the antibody has been tagged with a visible label (Javois, 1999). It was used in this study to facilitate the detection of antigens at the light microscopy level. The peroxidase anti-peroxidase (PAP) method (Figure 5.1) was applied to immunolocalise FB<sub>1</sub> within placental tissues. This method, pioneered by Sternberger in 1979 involves the use of immunological sandwich amplification and the horse radish peroxidase enzyme (HRP) to evoke a signal that is rendered visible using 3, 3-diaminobenzidine tetrahydrochloride (DAB). The distinguishing feature of the procedure is the PAP immune complex. The anti-peroxidase antibodies are from the same animal species that produced the primary antibody. The secondary linking antibody is raised in a different animal species from the primary antibody. The HRP produced from the horse radish plant is a 40kDa protein with immunogenic capabilities.



**Figure 5.1:** Illustration of molecular interactions of the PAP technique depicting the various levels that facilitate amplification of the signal, adapted from (Javois, 1999).



The advantage of this procedure is that the PAP complex remains soluble and the enzymatic activity of the peroxidase is not affected by the attached antibodies. The background problems encountered with this method may be alleviated by the use of detergents and extensive washing steps.

Formalin fixation and embedding in paraffin wax are important in the preservation of tissue morphology. However, they have the potential to damage, destroy or mask antigenic sites and thus reduce the reactivity of antigens with antibodies during immunohistological preparations. Even though snap frozen tissue may be used as an alternative to paraffin embedded tissue, the former method still offers well-preserved tissue architecture and cellular morphology superior to frozen tissue (Javois, 1999, Shi *et al.*, 1991). Treatment of proteins or peptides with formaldehyde resulted in four major modifications including, formation of hydroxymethyl adducts, Schiff base adducts, 4-imidazolinone adducts and methylene bridges. In order to overcome these challenges, antigen retrieval techniques have been developed to unmask the epitopes. The earliest discovery was the use of protease digestion which was not effective in unmasking most epitopes and led to the discovery of heat induced epitope retrieval (HIER) by employing heating techniques such as microwave, pressure cooker and autoclave heating in various solutions (Shi *et al.*, 2001b, Shi *et al.*, 2001a, Fowler *et al.*, 2011, Pileri *et al.*, 1997). Due to its proven efficacy and affordability, the microwave oven method was employed in this study to unmask epitopes of interest.

### ***5.2.1 Materials***

All routine microscopy reagents were purchased from Merck chemicals (South Africa). The antibodies for Fumonisin B<sub>1</sub>, caspase 3 and Fas and secondary antibodies were purchased from Capital laboratories (South Africa). Bovine serum albumin (BSA) was purchased from Roche Diagnostics and diaminobenzidine (DAB) chromogen tablets were purchased from DAKO Corporation, Southern Cross Biotechnology. Mayer's haematoxylin and eosin stains were purchased from Merck Chemicals (South Africa). Hoechst 33258 stain was purchased from Capital Laboratories (South Africa). Thiobarbituric acid and butylated hydroxytoluene were purchased from Capital Laboratories (South Africa). All general laboratory consumables were purchased from Merck Chemicals (South Africa).

### ***5.2.2 Methods***

#### **5.2.2.1 Morphological analysis of placental samples: Tissue processing**

Placental tissues were fixed in formal saline as described in chapter 3. An adapted version of the automated tissue processor was used to manually process the tissue. Briefly, placental tissue samples were fixed in 10% formal saline for 12-24 hours at 24°C and then dehydrated through increasing changes of ethanol (10%-100%). The tissues were then cleared in 2 changes of xylene (1hr each) and transferred to melted paraffin wax for one hour and left in fresh wax overnight (60°C). The sections were embedded in fresh paraffin wax for 20 minutes (20°C) and allowed to solidify.

Prior to sectioning the tissue blocks, glass slides were coated with poly-L-lysine (Sigma). It is an adhesive subbing solution for immunohistochemistry and routine histological staining

preparations. The polycationic nature of poly-L-lysine allows interaction with anionic sites of tissue sections resulting in strong adhesive properties. Wax embedded tissue blocks were sectioned using a Leica RM 2025 microtome and mounted on poly-L-lysine coated slides. The sections were heat fixed (60°C) onto slides and stored at room temperature for further analysis.

#### ***Haematoxylin and Eosin (H & E) staining***

Heat fixed placental sections were deparaffinised in 2 changes of xylene (100%) (10 minutes each) followed by rehydration in decreasing grades of ethanol, absolute (2 changes, 5 minutes each); 95% and 70% ethanol (2 minutes each). The sections were then briefly washed in deionised water and stained with Mayer's haematoxylin (Appendix D1.1) for 5 minutes at room temperature. Thereafter, the sections were allowed to stand under a gentle stream of tap water for 10 minutes and rinsed in distilled water. The sections were then rinsed in 95% ethanol (10 dips) and counterstained with eosin-phloxine B solution (Appendix D1.2) for 30seconds to 1 minute. The sections were then dehydrated through increasing grades of ethanol and cleared in 2 changes of xylene (5 minutes each) and mounted with DPX. These sections were then viewed using a light microscope (Olympus IX51), images were captured using analysis LS software.

#### **5.2.2.2 Immunohistochemical analysis of placental tissue for fumonisin B<sub>1</sub>**

Tissue sections picked up on poly-L-lysine coated slides were analysed for the presence of FB<sub>1</sub> using IHC. All antibodies were diluted to a pre-determined appropriate dilution factor using 1% (w/v) BSA in Tris-buffered Saline (TBS) (0.05M, pH 7.2) solution as the diluent. The use of method controls involved omission of the primary antibody and its replacement with TBS on the tissue specimen. Antigen retrieval was conducted to unmask antigenic

determinants that would have been affected by fixation and subsequent processing (Javois, 1999).

### **5.2.2.3 Antigen retrieval**

Tissues sections were dewaxed and dehydrated as mentioned in 5.2.2.1 above. The slides were transferred to plastic Coplin jars filled with HIER buffer(10mM sodium citrate buffer, pH 6, Appendix D3) and placed in the microwave (with loose-fitting caps) and heated at maximum power (700-1000W). The solution was allowed to boil for 3-5 minutes while constantly replenishing evaporated solution. The cycle was repeated 2-3 times and then allowed to cool to room temperature (approximately 20 minutes). Immunohistochemistry was performed immediately following this procedure.

### **5.2.2.4 Immunohistochemistry**

A Pap pen was used to create reagent wells immediately following antigen retrieval. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol for 30 minutes at room temperature (Javois, 1999).

The blocking solution was discarded and drop-washed in TBS (0.05M, pH 7.2) at room temperature. Non-specific binding arising from the antibodies was blocked by incubation in 2% BSA for 15 minutes at room temperature, the excess BSA was blotted off with fibre-free paper. The slides were incubated in primary antibody (mouse monoclonal anti-FB<sub>1</sub>; 1:100) overnight (12-14hrs) at 4°C. Thereafter, the slides were drop-washed in TBS and TBS: Tween-20 (TTBS). The slides were incubated in secondary antibody (rabbit anti-mouse IgG conjugated to HRP; 1:100) and then washed as before. The excess TTBS was blotted off with fibre-free paper and stained with DAB (reconstituted according to manufacturer's (BioRad)

instructions) for 5-10 minutes in the dark constantly monitoring the colour change to avoid over-staining. The unbound DAB was drop-washed in deionised water and the slides were stained with Mayer's haematoxylin for 3-5 minutes. The slides were blued in hot water (60°C) and coverslipped using DPX. These slides were then analysed using the Olympus IX51 microscope with the analysis LS software. For immunolocalisation of caspase 3 and Fas the same procedure was followed with the primary antibodies corresponding to caspase 3 and Fas.

#### **5.2.2.5 Analysis of placental DNA integrity (Hoechst stain)**

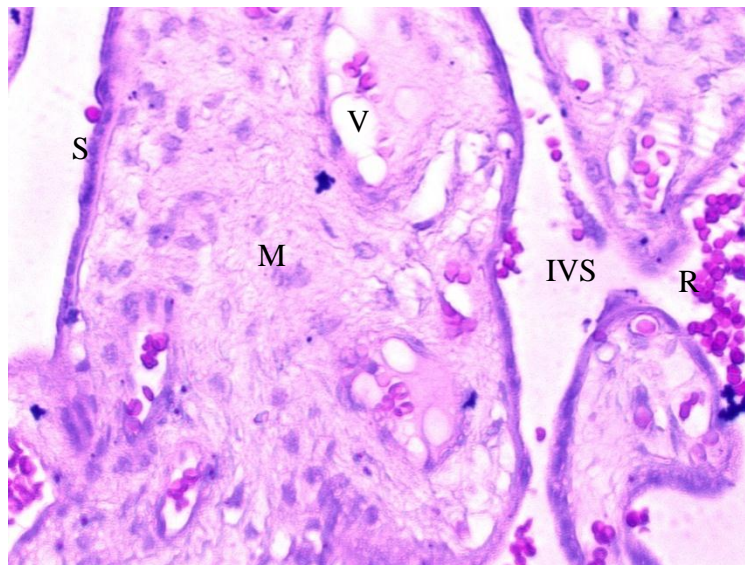
The formalin-fixed placental tissues were stained with Hoechst using the method of Farré *et al.* with minor modifications (Farré *et al.*, 2002). Briefly, 5µm placental tissue sections were dewaxed and permeabilised with 0.5% TritonX-100 in 0.5% sodium citrate for 8 minutes at RT. The cells were stained with Hoechst 33258 (Appendix D2) for 1 hour and then rinsed with deionised water. Sections were then mounted with DPX and viewed with a fluorescent microscope (Olympus IX51) at the absorption wavelength of 334nm and emission wavelength of 365 nm. Images were captured using the analysis LS software ensuring that fluorescence intensity remained constant.

#### **5.2.2.6 Analysis of lipid peroxidation in placental tissue**

Tissue (50mg) was homogenised in 500µl of 0.2% H<sub>3</sub>PO<sub>4</sub> and centrifuged at 1491rpm. Equal amounts (500µl) of supernatant and 2% H<sub>3</sub>PO<sub>4</sub> were added in a glass tube. Thereafter, the procedure described in section 4.2.2.7 was followed.

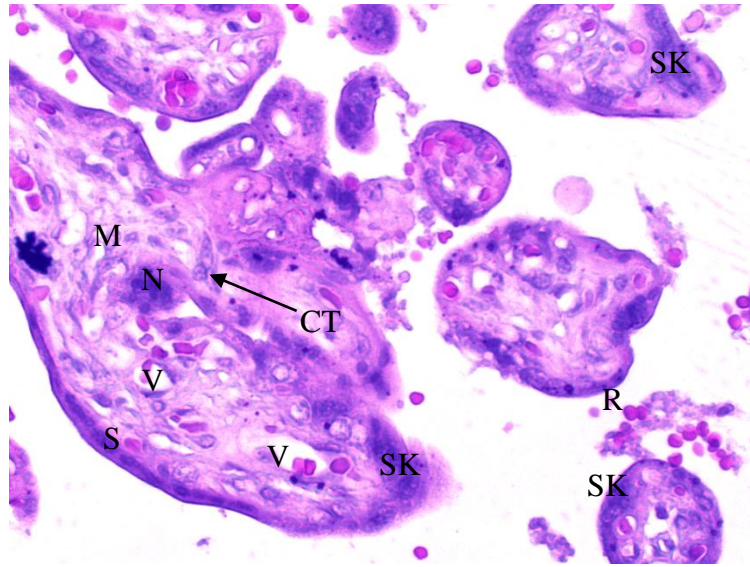
*Morphological analysis*

Placental tissue was analysed for morphological changes using H&E (Figure 5.2&5.3). All the cells that form part of a normal placenta were visualised including mesenchymal cell, syncytiotrophoblasts and endothelial cells. Both normotensive and preeclamptic placentas displayed a thinner and irregular syncytium due to the increase in number of capillaries in contact with the syncytial layer.



**Figure 5.2:** Normotensive placental villi showing numerous blood vessels (V) in close proximity to the syncytiotrophoblastic cell layer (S), the presence of mesenchymal cells (M) and erythrocytes (R) within the intervillous space (IVS) and in some capillaries [H&E] (600x).

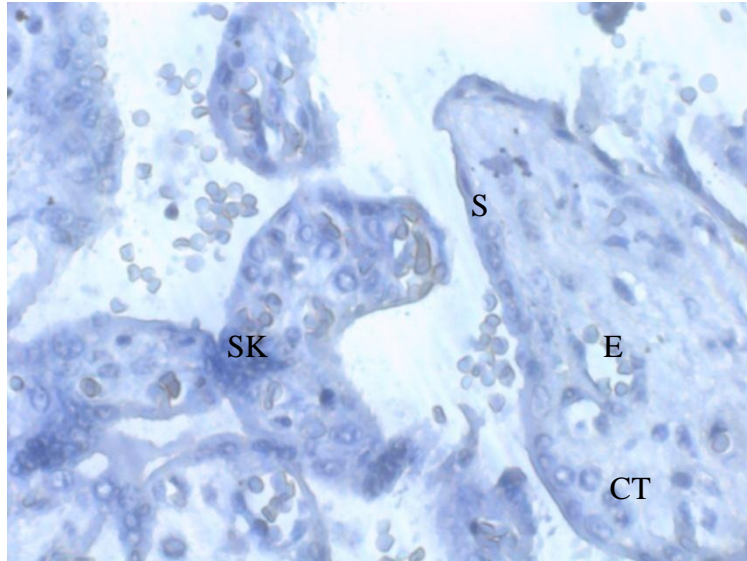
Another notable feature was the presence of numerous syncytial knots and nuclear aggregates predominantly in preeclamptic placentas compared to their normotensive counterparts (Figure 5.2, 5.3). There was a high number of syncytial knots and other placental aggregates in preeclamptic samples (>3 per frame) compared to that of normotensive placentas ( $\leq 3$ ).



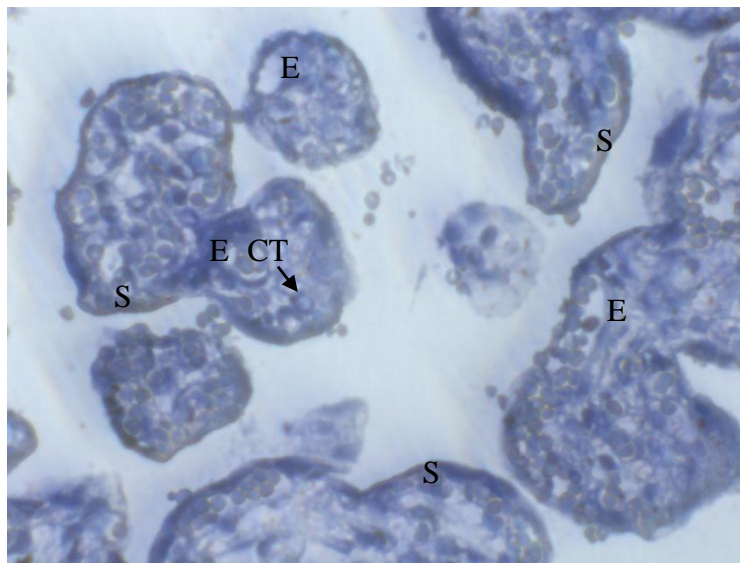
**Figure 5.3:** Preeclamptic placental villi showing a highly vascularised mesenchyme with numerous blood vessels (V), fusion of the syncytiotrophoblastic cell layer (S) with capillaries is also evident. Cytotrophoblasts (CT) visualised within the mesenchyme, numerous syncytial knots (SK) and nuclear aggregates (N) and erythrocytes (R) within the intervillous space (IVS) and in some capillaries [H&E] (600x).

#### *Immunolocalisation of FB<sub>1</sub> in placental tissue*

The presence of FB<sub>1</sub> within the placental tissue was analysed using IHC (Figure 5.4& 5.5). Fumonisin B<sub>1</sub> was immunolocalised predominantly within the syncytial cell layer of preeclamptic samples compared to normotensive samples and also in the endothelial cells. In addition, FB<sub>1</sub> was also present in the syncytial knots of both groups studied. However, an interesting feature was the presence of FB<sub>1</sub> in preeclamptic cytotrophoblasts only and not in their normotensive counterparts.



**Figure 5.4:** Mature normotensive placental villi probed for the presence of FB<sub>1</sub>. Fumonisin B<sub>1</sub> was immunolocalised within the syncytial layer (S) and endothelial cells (E). However there was no FB<sub>1</sub> within the cytotrophoblasts (CT) (400x).

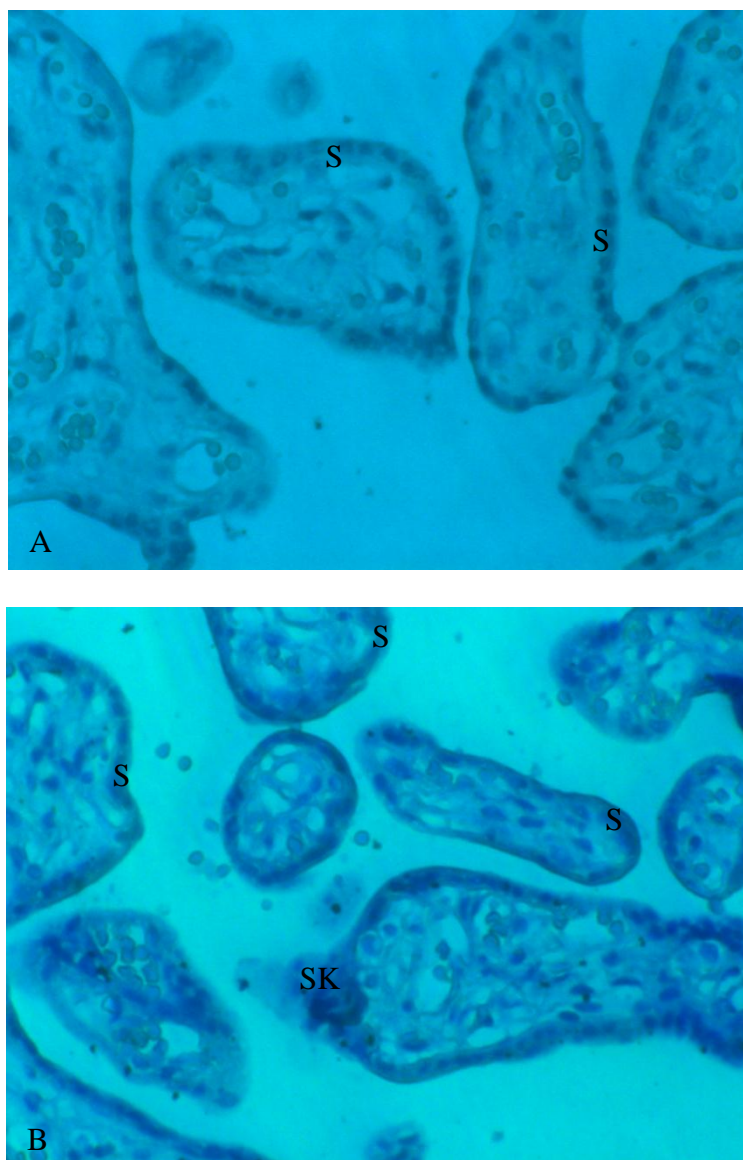


**Figure 5.5:** Placental villi from preeclamptic (B) samples showing positivity for FB<sub>1</sub> (Brown staining). Fumonisin B<sub>1</sub> was present within the syncytiotrophoblastic cells (S), endothelial cells (E) whose basement membrane has fused with the syncytial layer. Fumonisin B<sub>1</sub> was also present within the cytotrophoblastic cells (CT) of preeclamptic placenta and not the normotensive placenta (400x).

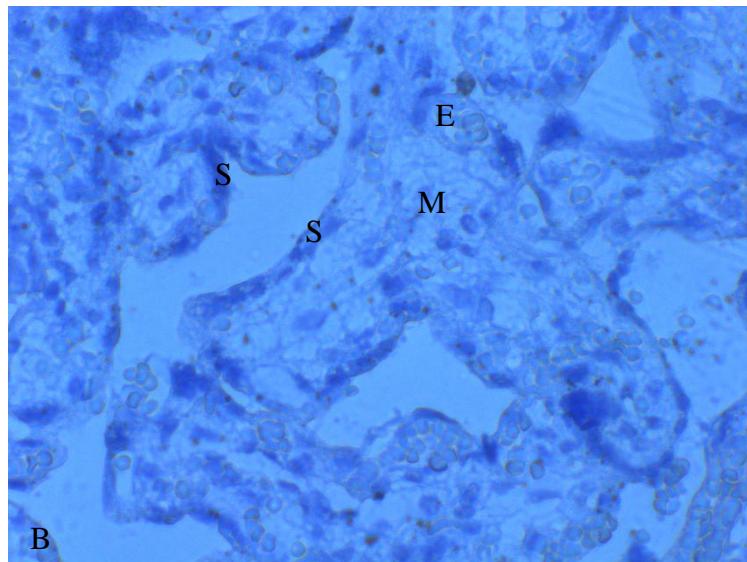
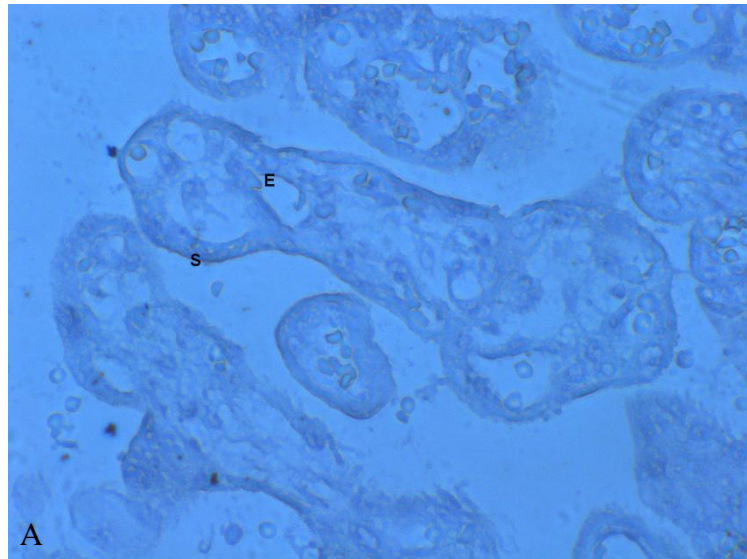


*Analysis of apoptotic markers in placental tissue*

Fas and caspase 3 were the markers localised in the placental tissue. Both Fas and caspase 3 were expressed at greater intensity in preeclamptic placentas (Figure 5.6 & 5.7). In addition to syncytiotrophoblast, syncytial knots also expressed Fas in preeclamptic tissue. Caspase 3 was expressed in mesenchymal cells, syncytiotrophoblasts and endothelial cells of preeclamptics while only the endothelial cells and the syncytiotrophoblasts expressed caspase 3 in normotensives (Figure 5.7).

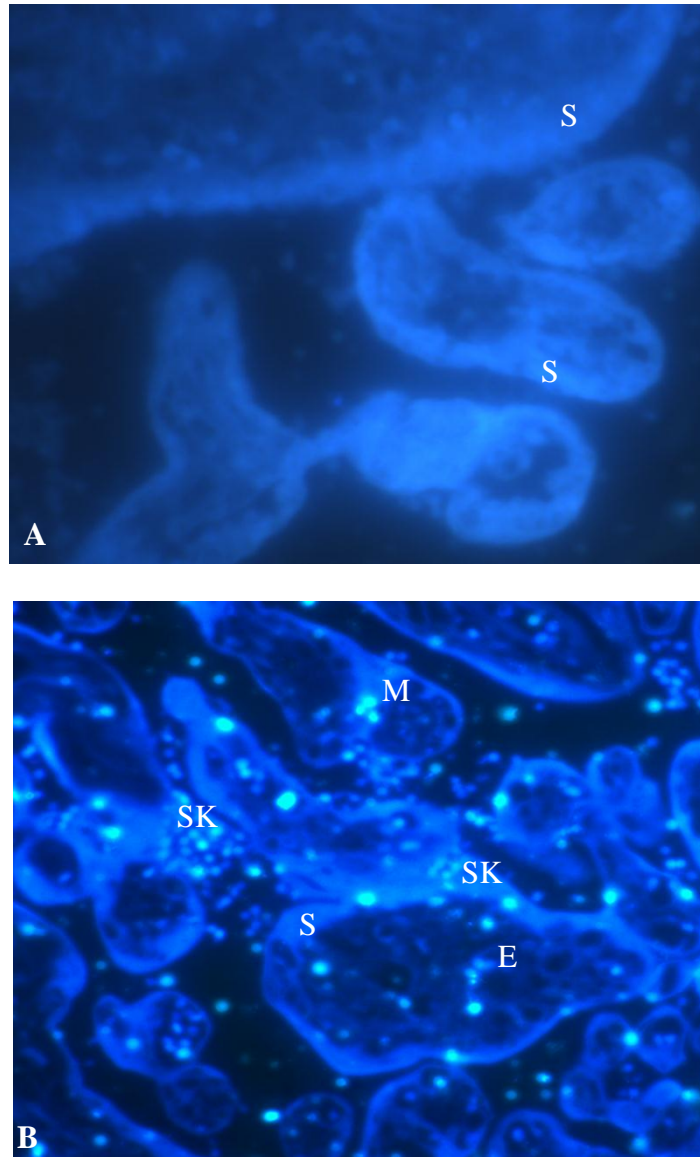


**Figure 5.6:** Normotensive (A) and preeclamptic (B) placental villi showing the presence of Fas along the apical membrane of the syncytiotrophoblasts (S) and syncytial knot (SK) (400x).



**Figure 5.7:** Mature normotensive (A) and preeclamptic (B) villi showing positive staining for caspase 3 (Brown staining). Caspase 3 was immunolocalised apically in the cytoplasm of syncytiotrophoblasts (S), endothelium (E) and the mesenchyme (M) (400x).

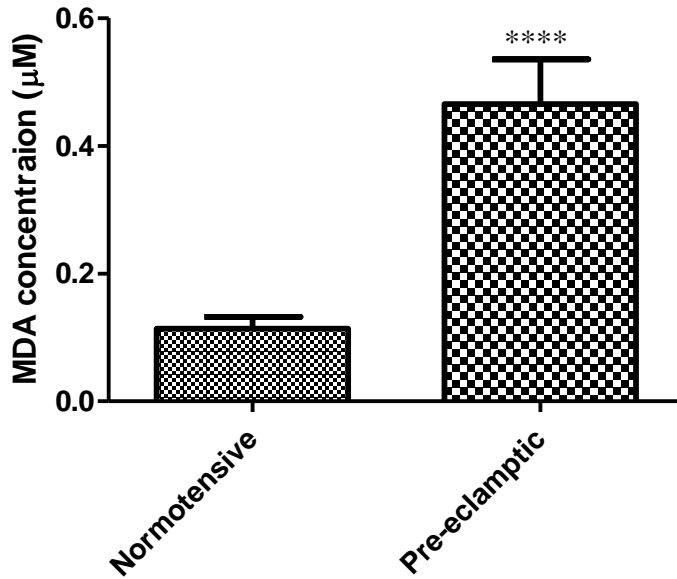
Fragmented DNA was higher in preeclamptic samples compared to normotensive counterparts. The syncytial layer, endothelial and mesenchymal cells showed higher levels of apoptosis in preeclamptic placentas.



**Figure 5.8:** Normotensive (A) and preeclamptic (B) villi showing uniform staining in the normotensive sample and an increased number of cells with condensed chromatin (arrow) predominantly within the syncytium (S) and also the endothelial cells (E) and syncytial knots (SK) of preeclamptic sample(B) (400x).

*Placental MDA levels as an indicator of lipid peroxidation*

Malondialdehyde (MDA) concentrations are used as indicators of lipid peroxidation in cells. The levels of MDA were significantly higher in preeclamptic placental samples compared to normotensive placentas (Figure 5.9). The mean MDA concentration in preeclamptic placentas ( $0.4653 \pm 0.07001$ ) was four-fold higher than normotensives ( $0.1139 \pm 0.01821$ )  $p < 0.0001$ .



**Figure 5.9:** Graphical representation of lipid peroxide levels in normotensive and preeclamptic placental tissue samples. The lipid peroxides (MDA) were significantly higher ( $p < 0.0001$ ) in preeclamptic placental tissues compared to the normotensive counterparts. Unpaired t-test with Welch’s correction.

#### 5.4 DISCUSSION

Morphological analysis of placental samples using H & E revealed fusion between the basement membrane of endothelial cells with that of the syncytium. This feature is common in the last weeks (third trimester) of gestation and is thought to reduce the diffusion barrier and facilitate exchange of substances between maternal and foetal circulation (Figures 5.2 & 5.3). The nuclei are usually absent or very sparsely distributed in these areas, a feature that was observed in this study (Huppertz, 2008). This study also demonstrated the presence of nuclear aggregates in preeclamptic placental samples compared to normotensive counterparts (Figures 5.2 & 5.3).

These nuclear aggregates have been previously characterised into three broad categories including, syncytial sprouts, syncytial bridges and syncytial knots. The sprouts are aggregates

of immature nuclei and are usually present in the first trimester of pregnancy where they act as precursors of newly formed villi. Fusion of the sprouts results in syncytial bridges which are thought to give support to the villous tree while aggregates of degenerating nuclei protruding from the villous surface are called syncytial knots (Jones and Fox, 1977). Boyd and Hamilton were the first to describe the presence of stromal trophoblastic buds which are found within the villi of most placentas possessing cytotrophoblasts on their periphery surrounding a central core of syncytium (Boyd and Hamilton, 1964).

Several studies have reported a significant increase in the frequency of syncytial knots in preeclamptic pregnancies compared to their normotensive counterparts. As previously described, these knots are aggregated nuclei of degenerating syncytial cells. Thus an increase in the number of syncytial knots may indicate a high level of syncytiotrophoblast apoptosis (Heazell *et al.*, 2007, Kadyrov *et al.*, 2006, Huppertz *et al.*, 1998, Mayhew *et al.*, 1999). Indeed, this study showed increased Fas and caspase 3 expression in preeclamptics compared to their normotensive counterparts thus confirming apoptosis in these cells. Austgulen *et al.* also showed an increased in apoptosis in the syncytial layer of preeclamptic placentas compared to the control groups (Austgulen *et al.*, 2004).

Fumonisin B<sub>1</sub> was also immunolocalised within the placental tissue using immunohistochemistry specifically within the syncytiotrophoblastic cell layer (predominant), endothelial cells, syncytial knots and mesenchymal cells of both groups. Of particular interest was the presence of FB<sub>1</sub> within the cytotrophoblasts of preeclamptic villi and not in normotensive villi. Fumonisin B<sub>1</sub> has been previously demonstrated to interact with proteins by binding to either the thiol or amino groups on the amino acid residues (Seefelder *et al.*, 2003b). One such amino acid is lysine, which contains alpha ( $\alpha$ ) and epsilon ( $\epsilon$ ) amino

groups. The alpha amino group is involved in peptide bond formation whilst the epsilon confers structural characteristics to lysine. The amino group on lysine would therefore interact with tricarballic acid moieties on FB<sub>1</sub>. The placenta is a highly vascularised tissue and thus would be very rich in extracellular matrix components that form the basement membrane. Various types of collagen have been identified in the placental basement membrane. This collagen is very rich in lysine and thus would also be able to interact with FB<sub>1</sub> through a similar mechanism described above. For this reason FB<sub>1</sub> was predominantly localised on the endothelial layer and syncytium possibly in their basement membranes.

Other lysine-rich proteins include albumin and histones. All of these proteins therefore are potential targets for FB<sub>1</sub>. This study has however not identified the amino acids or proteins involved in the interaction with FB<sub>1</sub> and therefore future investigations will aim to investigate the identity of the amino acids binding to FB<sub>1</sub> using mass spectrometry. This will confirm the types of interactions between FB<sub>1</sub> and macromolecules in biological samples. The intracellular localisation of FB<sub>1</sub> in tissue was also previously demonstrated both *in vivo* and *in vitro* thus indicating the ability of FB<sub>1</sub> to enter cells. The mechanism postulated is through its terminal amino group which would allow FB<sub>1</sub> to be recognised by protein transporters on the plasma membrane (Myburg *et al.*, 2009, Cawood *et al.*, 1994).

The syncytial layer forms a barrier between foetal and maternal blood and therefore if this layer is compromised in any way could result in maternally derived toxins gaining entry into the foetal blood due to the close proximity of foetal blood vessels to the syncytial layer. This study has demonstrated that FB<sub>1</sub> is able to gain entry into the syncytiotrophoblasts raising a question as to whether it is involved in the increased shedding of trophoblasts observed in preeclamptic samples. As a result of this compromised lifespan of syncytial cells, the

cytotrophoblastic cells needed to regenerate this layer are recruited to the area and need to differentiate into syncytial cells. Therefore the presence of FB<sub>1</sub> in the cytotrophoblasts of preeclamptic but not normotensive women warrants further investigation as it may affect the biological and functional ability of these cells. The importance of these cells in the invasion of spiral arteries has been previously demonstrated (Zhou *et al.*, 1997). The primary reason for the failure to invade spiral arteries is due to the inability of cytotrophoblasts to express several adhesion molecules such as integrin and cadherin important for this function (Zhou *et al.*, 1997, Khong *et al.*, 1986). The presence of FB<sub>1</sub> in these cells may induce production of reactive oxygen species through its interactions with molecules such as tumour necrosis factor (TNF- $\alpha$ ). Only a few studies have been conducted on the presence of FB<sub>1</sub> in the placental tissue and currently only one study has shown a similar pattern of binding in placental tissues as the one observed in this study (Coumi, 2000; unpublished data). The presence of FB<sub>1</sub> in these cells may also result in a disruption of sphingolipid molecules including sphinganine and sphingosine which are known inducers of apoptosis. This study demonstrated very high levels of apoptosis in preeclamptic tissues compared to normotensive tissues through analysis of key apoptotic markers (caspase 3, Hoechst, Fas). This finding is in agreement with various studies which also demonstrated a similar result (DiFederico *et al.*, 1999, Allaire *et al.*, 2000, Ishihara *et al.*, 2002, Genbacev *et al.*, 1999, Goswami *et al.*, 2006).

In addition, lipid peroxidation was significantly higher in preeclamptic compared to normotensive samples. This was also demonstrated in the serum samples (chapter 3) where lipid peroxidation was higher in preeclamptics compared to normotensives. Pregnancy is associated with high levels of oxidative stress due to the increased energy demand from the developing placenta and the resultant increase in mitochondrial activity which are the primary source of ROS. Oxidative stress is exaggerated in preeclamptic pregnancies and has been

attributed to the imbalance in antioxidants (Myatt and Cui, 2004, Serdar *et al.*, 2002). Although the levels of MDA were remarkably high in preeclamptic samples, it is important to mention that the TBA used in the measurement of MDA is not specific for MDA only but cross-reactivity with other molecules such as sugars, urea, alkanals and proteins has been demonstrated and therefore extreme caution should be exercised when making conclusions from the TBARS assay. Albumin has been shown to translocate in and out of the blood into various tissues and analysis of its oxidation status would therefore give a better indication of the overall oxidative status in the body (Terawaki *et al.*, 2004). In addition to oxidised albumin, MDA is known to interact with DNA and protein forming adducts and the use of HPLC and mass spectrometry would provide invaluable information on these adducts and provide a more sensitive indicator of lipid peroxidation and overall oxidative stress *in vivo*.

The higher levels of apoptosis observed in preeclamptic placentas may be due to various factors including the increased concentrations of sphingoid bases (sphinganine and sphingosine) and increased lipid peroxidation. The previous chapter (table 4.1 and Figures 4.6&4.7) in the current study demonstrated significantly high levels of Sa and So in preeclamptic sera compared to normotensive sera. Sphinganine is a known pro-apoptotic molecule and thus its presence at such high levels indicates the potential role it plays in the induction of apoptosis in trophoblasts. It is therefore reasonable to postulate that due to the increase in sphinganine, the trophoblasts are stimulated to undergo apoptosis whilst in normotensive samples the low levels of sphingoid bases may not be sufficient to induce apoptosis to such a high degree. The Hoechst stain demonstrated high levels of cells undergoing apoptosis in preeclamptic samples compared to normotensives. Fumonisin B<sub>1</sub> is a known inducer of apoptosis through various mechanisms (Ciacci-Zanella and Jones, 1999, Seefelder *et al.*, 2003a). The increase in sphinganine being one of them, in addition FB<sub>1</sub> has



been shown to use the death receptor pathway to induce apoptosis and through up-regulation of caspase 3 and all of these were demonstrated in this study (Seefelder *et al.*, 2003a).

## 5.5

## CONCLUSION

This study showed the presence of FB<sub>1</sub> in placental tissue and its potential risk to the foetus. There were also high levels of apoptosis and lipid peroxidation in preeclamptic placental samples which may indicate aberrant physiological processes such as a decreased antioxidant response. Fumonisin B<sub>1</sub> is postulated to induce apoptosis in the placental through the death receptor pathway (Fas), induction of caspase 3 activity, sphingolipid disruption and DNA fragmentation (Hoechst). The use of MDA as an indicator of apoptosis has been brought under scrutiny and therefore measurements of oxidised albumin, DNA and protein adducts may have strengthened the findings in this study. It is unlikely that FB<sub>1</sub> is solely responsible for the exaggerated apoptosis observed in preeclampsia. However, the evidence presented in this study indicates that it may have a role in the aetiology of preeclampsia.

## CHAPTER 6

### *Overall Conclusions*

This study demonstrated the presence of FB<sub>1</sub> and its associated metabolites in the blood and placenta of normotensive and preeclamptic women. The high levels of FB<sub>1</sub> observed in preeclamptic samples were attributed to the consumption of dietary staples and aberrant physiological processes including proteinuria and the conjugation to body proteins. Although it is tempting to conclude that FB<sub>1</sub> is the cause of all these observations, it is important to note that co-contamination of maize by fungi may result in multi-mycotoxin exposure thus indicating possible interactions that may have led to the observations in this study. To date no study has surveyed the different mycotoxins commonly known to colonise maize in humans and only one has probed for the presence of FB<sub>1</sub> in pregnant women. Other mycotoxins may be involved in the toxicity of FB<sub>1</sub> and therefore need to be determined in this population group to not only protect the mothers but also the developing foetus.

This study not only demonstrated the presence of FB<sub>1</sub> but also measured the sphingolipid changes associated with its toxicity. It is the first study to measure the levels of sphingoid bases, sphinganine and sphingosine in the blood of pregnant women. The increase in apoptosis and lipid peroxides in preeclamptic samples may be attributed to the observed elevation in sphinganine and sphingosine.

The samples used in the study were obtained from a single hospital in KZN which may not be a representative sample of the situation in the whole province. A comparison between pregnant women from rural and urban hospitals would have strengthened the hypothesis that rural communities were at a higher risk of FB<sub>1</sub> toxicity than their urban counterparts. A

dietary questionnaire should have been included to ascertain how often the women consumed maize and maize-based products.

Although this study showed the interaction of FB<sub>1</sub> with albumin, other proteins like collagen may also be involved especially in sequestering FB<sub>1</sub> in tissues like the placenta. Therefore the findings in this study highlighted the importance of food screening and also education around good agricultural practices. A recent intervention study in the Eastern Cape Province on the prevention of consumption of mouldy maize has provided some hope with regard to training of subsistence farmers on proper handling of maize and recognition of contaminated maize.

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## APPENDICES

### Appendix A: Information given to participants and informed consent document

#### 1. INFORMATION DOCUMENT

**Study title:** The effects of fumonisin B<sub>1</sub> in pre-eclampsia.

**Greeting:** Dear participant

#### **Introduction:**

I am Miss Metse Serumula, a researcher working with Professors A Chuturgoon and J Moodley. We are doing a research project studying the effects of a toxin called fumonisin B<sub>1</sub>, present in maize and maize meal, on placental DNA. Research is a process done in order to learn answer to question. Moulds are commonly known as fungi. These fungi sometimes produce poisonous substance called mycotoxins and fumonisin B<sub>1</sub> is a poisonous substance produced by fungi which are commonly found on maize (mealie) or mealie-based products. It has been shown in previous studies that people with high blood pressure in pregnancy had high levels of these toxins (fumonisin B<sub>1</sub>) in their blood. You have been informed that you have high blood pressure in pregnancy (pre-eclampsia). We would like to invite you to participate in our study.

#### **What does participation mean?**

This means that if you agree to participate, we will obtain a sample of your blood ( $\pm 15$ ml), a tablespoon full. The taking of blood samples will be done at the same time as the other routine bloods are taken so that no further discomfort is caused to you. In addition, once you have delivered, we would like to take four blocks of tissue, the **size of a pigeon's egg**

(2cmx2cm) from the afterbirth (placenta). Taking of blood samples and afterbirth tissue will not have any effect on you or your baby. Normally the “afterbirth” is destroyed.

**What is involved in the study?**

After collection of the bloods and afterbirth tissue samples, bloods will be separated into blood cells (lymphocytes) and blood fluid (plasma/serum) and stored in a freezer (-70<sup>0</sup>C). One block of afterbirth tissue (2cm x2cm, the size of a pigeon’s egg) will be stored in the freezer (-70<sup>0</sup>C) and 3 afterbirth tissues immersed in formalin will be stored in a cupboard (at 25 <sup>0</sup>C). The following methods will be used in order to help us understand the role of mycotoxins. The blood will be analysed/ tested using flow cytometry and high performance liquid chromatography while the tissue samples will be tested using immunohistochemistry, high performance liquid chromatography, Hoechst and JC-1 staining.

**Benefits of being in the study.** The study will not have any direct impact on you or your treatment but may help in providing further information given to pregnant women with high blood pressure or those women who are likely to develop high blood pressure in pregnancy.

**Participation is voluntary**, and if you do not decide to participate in this study, your treatment will not be different in any way. The doctors will manage you in the same way that they manage patients with your condition. You may discontinue your participation in the study and this will also not affect your treatment in any way.

**Confidentiality:** Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. We will be using the results of the study for publication; however no names of participants will be used. In addition note that I am doing

this research to obtain a higher degree and thus it is part of my studies to become a scientist/-experienced researcher. Personal information may be disclosed if required by law. Certain Organizations such as Research ethics committee may inspect and/or copy your research records for quality assurance and data analysis.

For further information please contact the following people all located in the Nelson R. Mandela School of Medicine:

1. **Miss Metse Serumula (Principal investigation), Room 547D, Division of Medical Biochemistry (031 260 4597). I will be willing to answer your queries in the language you are most comfortable with.**
2. **Professor Anil Chuturgoon (supervisor), Mycotoxin Research Unit-Doris Duke Medical Research Institute (031 260 4404)**
3. **Professor J. Moodley (co-supervisor), Room 108A, Obstetrics and Gynaecology( 031 260 4250),**
4. **Contact details of REC administrator and chair – for reporting of complaints / problems.**  
**Mrs Cheryl Borresen Room 211 MRC building (031 260 4495)**

## **SECTION 5 : INFORMED CONSENT :**

### **CONSENT DOCUMENT**

#### **Consent to Participate in Research**

You have been asked to participate in a research study.

You have been informed about the study by ...**Metse Serumula**.....

Where applicable: You have been informed about any available compensation or medical treatment if injury occurs as a result of study-related procedures;

You may contact **Metse Serumula**..... at 260 **4597**..... any time if you have questions about the research or if you are injured as a result of the research.

You may contact the Medical Research Office at the Nelson R Mandela School of Medicine at 031-260 4604 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop.

If you agree to participate, you will be given a signed copy of this document and the participant information sheet which is a written summary of the research.

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate.

\_\_\_\_\_  
Signature of Participant

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Witness  
(Where applicable)

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Translator  
(Where applicable)

\_\_\_\_\_  
Date

**Zulu translation of the informed consent document**

**IFOMU ELINIKEZA ULWAZI NGOCWANINGO NOKUYIFOMU**

**LESIVUMELWANO NGALOLUCWANINGO**

**Isihloko socwaningo:** Effects of fumonisin B<sub>1</sub> in pre-eclampsia

**Isibingelelo:** Volontiya elithandekayo

**Isingeniso:**

Ngingu Nkosazane Metse Serumula, umncwaningi osebenzisana no Profesa Chuturgoon kanye no Moodley. Senza ucwaningo ngemiphumela yobuthi obubizwa nge fumonisin B<sub>1</sub>, obutholakala embileni nase mpushini. Ucwaningo uhlelo olwenziwa ukuze kutholakale impendulo emubuzweni othile. Uboyana obutholakala entweni eyonakele noma ebolile kwaziwa njengesikhunta. Kwesinye isikhathi lesisikhunta siqhiqiza ubuthi obuthile okubizwa nge mycotoxins kanye ne fumonisin B<sub>1</sub> okuwubuthi obuqhiqizwa yisikhunta esitholakala embileni noma ekudleni okwenziwe ngombila. Kolunye ucwaningo oselwenziwa kubonakele ukuthi abantu besifazane abane BP (high blood pressure) ephezulu ngesikhathi bekhulelwe babenezinga eliphezulu lalobubuthi (fumonisin B<sub>1</sub>) egazini labo. Sewazisiwe ukuthi njengoba ukhulelwe une BP ephezulu. Sifisa ke ukukumema ukuba ungenele lolucwaningo lwethu.

### **Kusho ukuthini ukubayingxenye yalolucwaningo?**

Lokhu kusho ukuthi uma uvuma ukuba yingxenye yalolucwaningo, siyothatha kuwe isampula legazi elilinganiselwa ku ( $\pm 15$ ml), okungaba yigazi elingange sipunu sokudla esigcwele. Konke ukuthathwa kwegazi kuyokwenziwa ngesikhathi esifanayo noma esisodwa ukuze ungalokhu uzwa ubuhlungu. Ukwengeza kulokhu, ngemuva kokubeletha, sifisa ukuthatha izicubu ezine, ezilingana namaqanda ejuba (2cmx2cm) esiyoziithatha kumzanyana (placenta) ophuma ngemuva kokubeletha. Ukuthathwa kwegazi nezicubu kumzanyana angeke kukuphazamisa wena kanye nomntwana wakho. Ngokwenjwayelo “umzanyana” uyalahlwa.

### **Yini eyokwenzeka kulolucwaningo:**

Ngemuva kokuthatha amagazi nezicubu kumzanyana, amagazi ayohlukaniswa bese egcinwa endaweni eno ayisi. Isicubu esisisodwa esilingana neqanda lejuba siyogcinwa esiqandisini

esibanda ngendlela efanayo nokubekwe kuyo igazi bese kuthi izicubu ezintathu ziyocwiliswa oketshezini olubizwa nge formalin zigcinwe ekhabetheni. Lokhu kuyokwenziwa ukuze siqonde ukuthi ubuthi besikhunta budlayiphi indima. Igazi lakho kanye nezicubu ezithathwe kumzanyana wakho kuyocutshungulwa besekuhlolwa.

**Inzuzo yokuba kulolucwaningo.** Lolucwaningo alunakuku phazamisa noma luphazamise uhlelo lokunakekelwa noma ukwelashwa kwakho kepha lungasisiza ukuba sithole ulwazi kabanzi ngabantu besifazane ababa ne BP ephezulu ngesikhathi bekhulelwe noma kulabo besifazane okunokwenzeka babe ne BP ephezulu ngesikhathi bekhulelwe.

**Ukuba kulolucwaningo kuwubuvolontiya,** uma futhi uthatha isinqumo sokungabi yingxeny e yalolucwaningo ukunakekeleka kwakho akunakuphazamiseka. Odokotela bayokunakekela ngendlela efanayo nabanakekela ngayo iziguli ezisesimweni esifana nesakho. Ungaphuma futhi kulolucwaningo noma ingasiphi isikhathi lokhu akunakuphazamisa ukwelashwa kwakho.

**Imfihlo:** Kuyokwenziwa imizamo yokugcina imininingwane yakho iyimfihlo. Akunakuqinisekiswa imfihlo ephelile. Imiphumela yalolucwaningo iyosetshenziswa emibhalweni eyoshicilelwa, kepha angeke kusetshenziswe magama abantu ababeyingxeny e yalolucwaningo. Ngaphezu kwalokhu ngiyafisa ukuba wazi ukuthi ngenza lolucwaningo ukuze ngithole iziqu zemfundo ephezulu, kuyingxeny e yokufunda kwami ukuba ngibe ngumcwaningi osezingeni eliphezulu. Iminingwane yakho ingadalulwa uma lokhu kufunwa ngumthetho. Ezinye izinhlangano njengekomiti locwaningo lase Nyuvesi (Research ethics committee) zingabheka imibhalo yakho yalolucwaningo noma futhi zikopishe imininingwane yakho ukuze ziqinisekise ukuthi yonke into yenzeka ngendlela efanele.



Uma ufuna olunye ulwazi uyacelwa ukuba uxhumane nalababantu bonke abatholakala esikoleni sokufundela odokotela i Nelson R. Mandela School of Medicine:

5. **Unkosazane Metse Serumula (umcwaningi omkhulu), Room 547D, kuMnyango we Medical Biochemistry (031 260 4597). Ngiyozama ukuphendula imibuzo yakho ngolimi ofisa ukuba ngiyiphendule ngalo.**
6. **Profesa Anil Chuturgoon (ophethe lolucwaningo), Mycotoxin Research Unit-Doris Duke Medical Research Institute (031 260 4404)**
7. **Profesa J. Moodley (obambisene nophethe), Room 108A, Obstetrics and Gynaecology( 031 260 4250)**
8. **Imininingwane yophethe ikomiti locwaningo – uma ufisa ukubeka izikhalo zakho noma izinkinga zakho ngalolucwaningo. Mrs Cheryl Borresen Room 211 MRC building (031 260 4495)**

**INGXENYE YESIHLANU: IFOMU ELIKUNIKA ULWAZI NGALOLUCWANINGO**  
**NOKUYIFOMU LESIVUMELWANIO NGALOLUCWANINGO :**

**UMBHALO WESIVUMELWANO**

**Imvume yokuba yingxenywe kulolucwaningo**

Uceliwe ukuba ube yingxenywe yalolucwaningo.

Sewazisiwe ngalolucwaningo ngu .....

Lapho kufanele khona: wazisiwe ngesinxephezelo noma ukwelashwa uma kwenzeka ulimala ngenxa yalolucwaningo;

Ungaxhumana no **Metse Serumula..... ku 260 4597.....** noma ingasiphi isikhathi uma unemibuzo ngalolucwaningo noma uma ulimala ngenxa yalolucwaningo.

Uma unemibuzo ngamalungelo akho njengomuntu okulolucwaningo ungaxhumana ne Medical Research Office esikoleni sodokotela eNelson R. Mandela School of Medicine ku 031-260 4604.

Ukuba kulolucwaningo kuwukuthanda kwakho, angeke ujeziswe noma ulahlekelwe ngamalungelo akho uma unqaba ukuba kulolucwaningo noma uthatha isinqumo sokuphuma kulolucwaningo noma ingasiphi isikhathi.

Uma uvuma ukuba kulolucwaningo, uyonikezwa ikhophi yalelifomu esayiniwe kanye nefomu elinika ulwazi ngalolucwaningo okuyifomu elinolwazi olufingqiwe ngalolucwaningo.

Ngichazelwe ngomlomo ngalolucwaningo kanye nangalolulwazi olungenhla. Ngiyaqonda ukuthi kusho ukuthini ukuba kwami kulolucwaningo futhi ngiyazikhethela ngaphandle kokuphoqwa ukuthi ngibe kulolucwaningo.

\_\_\_\_\_  
Isiginesha yevolontiya                      Usuku

\_\_\_\_\_  
Isiginesha kafakazi                      Usuku  
(Uma kufanele)

\_\_\_\_\_  
Isiginesha katolika                      Usuku  
(Uma kufanele)

**Appendix B: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting reagents**

**1. Bovine serum albumin (BSA) serial dilutions**

**Table A1 BSA serial dilutions used in the bicinchoninic acid assay**

<b>Concentration</b>	<b>Volume of sample</b>	<b>Volume of storage buffer</b>
0mg/ml	0µl	1000µl
0.2mg/ml	200µl	800µl
0.4mg/ml	400µl	600µl
0.6mg/ml	600µl	400µl

0.8mg/ml	800µl	200µl
1.0mg/ml	1000µl	0µl

The volume of sample required to make a specific BSA concentration was calculated using the following formula

$$C_1V_1 = C_2V_2$$

$$V_1 = C_2V_2/C_1$$

$$= 0.2\text{mg/ml} \times 1\text{ml} / 1\text{mg/ml}$$

$$= 0.2\text{ml} = 200\mu\text{l of BSA Stock} + 800\mu\text{l of storage buffer to make up 1ml (1000}\mu\text{l)}$$

### 1.1 Bicinchoninic acid (BCA) working solution

4µl of  $\text{CuIISO}_4$  was combined with 198µl BCA per well.

### 1.2 Standard curve of BSA standards

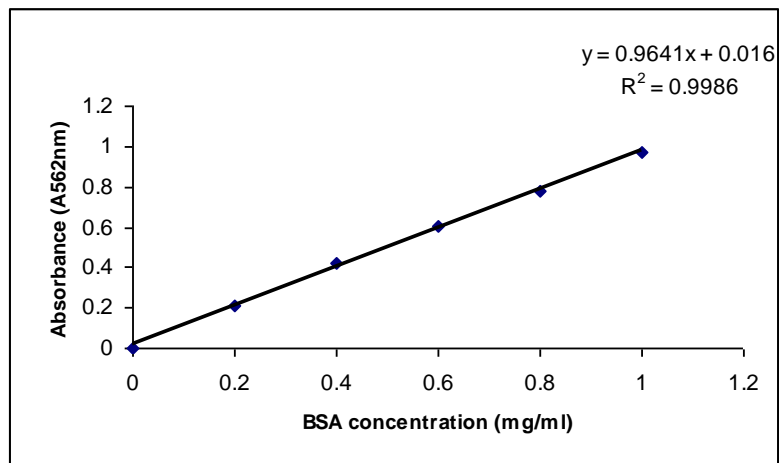


Figure A1. Standard curve of BSA serial dilutions for protein quantification.

The equation:  $y = 0.9641x + 0.016$  was then used to calculate protein concentrations in the sample

## 2. Storage buffer

### 0.5mM $\text{Na}_2\text{-EDTA}$

$$M_1V_1 = M_2V_2$$

$$100. \text{x} = 0.5\text{mM} \times 100\text{ml}$$

$$X = 0.5\text{ml}$$

### 0.1M KH<sub>2</sub>PO<sub>4</sub> (pH7.4)\*

$$N = C \times V$$

$$= 0.1 \times 0.1$$

$$= 0.01 \text{ moles}$$

$$\text{Mass} = n \times \text{Molar mass}$$

$$0.01 \times 136.04\text{g/mol}$$

$$1.3604\text{g}$$

Combine all ingredients and make it up to 100ml with deionised water (Adjust pH to 7.5) with HCL or NaOH)

### 0.1mM DTT

$$M_1 V_1 = M_2 V_2$$

$$1 \times x = 0.0001 \times 0.1$$

$$= 10\mu\text{l}$$

### 0.25 M Sucrose

$$N = C \times V$$

$$= 0.25\text{M} \times 0.11$$

$$= 0.025 \text{ moles}$$

$$\text{Mass} = n \times \text{molar mass}$$

$$= 0.025 \times 342.3\text{g/mol}$$

$$= 8.5575\text{g}$$

Combine all other ingredients except KH<sub>2</sub>PO<sub>4</sub> and use it to adjust volume to 100ml.

## 3. Resolving and stacking gel preparations

### 3.1 Table A2 Resolving gel preparation (0.375M Tris, pH 8.8)

	12%	10%
Deionised water	3.35ml	3.95 ml
0.5M Tris-HCL < pH 8.8	2.5ml	2.5 ml
10% (w/v) SDS stock (store at RT)	100μl	100μl
Acrylamide/Bis (30% stock) degas for 15 min at RT)	4.0ml	3.35ml
10% Ammonium persulfate (fresh daily)	50μl	100μl

Tetramethylethylenediamine(TEMED)	5µl	5µl
Total monomer*	10ml	10ml

### 3.2 Table A3 Stacking gel preparation 4% gel, 0.125M Tris, pH 6.8

Deionised water	6.1ml
0.5M Tris-HCL, pH 6.8	2.5ml
10% (w/v) SDS	100µl
Acrylamide/bis (30% stock)	1.33ml
10% ammonium persulfate (fresh daily)	50µl
TEMED	10µl
Total monomer*	10ml

\*Combine all reagents in the monomer solution except the TEMED and APS and degas under vacuum for 15minutes. Then add the APS and TEMED to initiate polymerization. Swirl gently to mix.

### 3.3 Table A4 Sample buffer (SDS reducing buffer) store at room temperature

Deionised water	3.8ml
0.5M Tris-HCL, pH 6.8	1.0ml
Glycerol	0.8ml
10% (w/v) SDS	1.6ml
2-mercaptoethanol	0.4ml
1%(w/v) bromophenol blue	0.4ml
TOTAL	8.0ml

## 4. Gel staining and destaining solution

### 4.1 Coomassie brilliant blue staining stock solution (1% coomassie brilliant blue R-250)

Coomassie brilliant blue (CB) R-250	1g
Deionised water (DH <sub>2</sub> O)	100ml

#### 4.1.1 Staining solution (0.125 CB-R250; 50% Methanol; 10% Acetic acid)

Coomassie brilliant blue R-250 (from stock)	62.5ml
Methanol	250ml
Acetic acid	50ml

DH<sub>2</sub>O to 500ml

**4.2 Destaining solution 1 (50% methanol, 10% acetic acid)**

Methanol 500ml

Acetic acid 100ml

DH<sub>2</sub>O to 1 litre

**4.3 Destaining solution 2 (7% Methanol, 5%acetic acid)**

Methanol 70ml

Acetic acid 50ml

DH<sub>2</sub>O to 1 litre

**5. 5X electrode (running) buffer, pH 8.3**

Tris base 9g 15g/l

Glycine 43.2g 72g/l

SDS 3g 5g/l

To 600ml with DH<sub>2</sub>O

Store at 4<sup>0</sup>C. Warm to room temperature if precipitation occurs.

Dilute 60ml of 5X stock with 240ml of DH<sub>2</sub>O for electrophoretic run.

**6. Transfer buffer**

192mM Glycine 14.4g

25mM Tris 3.03g

Methanol 100ml

DH<sub>2</sub>O to 1litre

**7. 0.05% Tris buffered Saline with Tween-20 (0.05%) (pH 7.5) [TTBS]**

20mM Tris	3g
150mM NaCl	8g
KCl	0.2g
0.05% Tween 20	5ml

Dissolve all ingredients in 1litre of DH<sub>2</sub>O then add 10% Tween 20 to make 0.05% TTBS

**Appendix C: High performance liquid chromatography (HPLC) reagents**

**1. O-phthaldialdehyde (OPA)**

Dissolve 20mg of OPA in 600µl of methanol

5ml of 0.1M di-sodium tetraborate (pH 10.5)

50µl of 2-mercaptoethanol and store in the dark at RT.

**Appendix D: Placental tissue processing and staining solutions**

**1: Haematoxylin and Eosin (H & E) staining solutions**

**1.1 Mayer's Haematoxylin solution**

Potassium or ammonium (alum)	50g
Haematoxylin (Mayer's)	1g
Sodium iodate	0.2g
Citric acid	1g

Distilled water 1Litre

Stir to dissolve the chemicals in the order listed above

Results: nuclei- blue, cytoplasm – pink to red

## 1.2 Eosin-Phloxine B solution

### Eosin Stock solution

Eosin Y	1g
Distilled water	100ml

Mix to dissolve and store at room temperature

### Phloxine B stock solution

Phloxine B	1g
Distilled water	100ml

Mix to dissolve

### Eosin-Phloxine B working solution (0.25%)

Eosin stock solution	100ml
Phloxine stock solution	10ml
Ethanol (95%)	780ml
Glacial acetic acid (concentrated)	4ml

Combine all ingredients and store at room temperature

## 2. Hoechst Staining solution

Dissolve 1mg of Hoechst 33258 in 1ml deionised water and then dilute to 20 $\mu$ g/ml with deionised water.

Use the following equation for calculation volume required

$$C_1V_1 = C_2V_2$$

$$V_1 = 0.02\text{mg/ml} \times 5\text{ml} / 1\text{mg/ml}$$

$$= 0.1\text{ml} = 100\mu\text{l}$$



Combine 0.1ml of Hoechst 33258 stock solution with 4.9ml of deionised water to make a final volume of 5ml and final concentration of 20µg/ml.

**3. Antigen retrieval buffer Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)**

Tri-sodium citrate (dihydrate) ----- 2.94 g

Distilled water ----- 1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4°C for longer storage.