ALTERED LIPID METABOLISM AS A POSSIBLE MECHANISM IN FUMONISIN-INDUCED HEPATOCARCINOGENESIS IN RATS AND INVESTIGATIONS INTO RISK ASSESSMENT IN HUMANS

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SUMMARY

Exposure to food contaminates such as mycotoxins have been associated with a variety of animal and human diseases worldwide. In South Africa, maize is the most important commercial grain crop not just economically but also as a local food commodity both commercially and in subsistence rural farming communities. In order to control and manage mycotoxin contamination in food, evidence-based risk assessment is needed that includes mechanistic and human exposure studies. From this perspective the current study was conducted and aimed in further unravelling fumonisin B_1 (FB_1) mycotoxin induced hepatocarcinogenesis via the disruption of the lipid metabolism. The study also critically evaluates aspects of human risk assessment due to its relevance and importance to food safety known to impact on food security. This entails mycotoxin distribution during maize dry milling and the assessment of mycotoxin exposure in the South African population and vulnerable rural communities at risk.

Fumonisin B_1 affects the integrity of biological membranes by altering key lipid and fatty acid parameter in plasma, microsomal, mitochondrial and nuclear subcellular membrane fractions in rat liver. Changes in the major lipid constituents entailing an increase in cholesterol (CHOL) and phosphatidylethanolamine (PE) whilst sphingomyelin (SM) and phosphatidylcholine (PC) tended to decrease. Isolated plasma membrane lipid rafts, from rat primary hepatocytes exposed to FB_1 augments the intricate effects exerted on the lipid metabolism regarding CHOL, SM and PE. The disruption of lipid and fatty acid constituents, such as arachidonic acid and ceramide, are likely to be key determinants affecting growth regulatory signaling pathways relevant to the critical balance between cell proliferation and apoptosis during cancer promotion. These changes provide further evidence that FB_1 induce cancer promotion by differential inhibition and/or stimulation process whereby a few resistant “initiated” hepatocytes proliferate in an environment where the growth of normal cells is inhibited. A specific lipogenic phenotype is effected by FB_1 which is closely associated with cancer development and considered to occur via an epigenetic-type of mechanism. These effects are not adequately addressed in defining risk assessment parameters.

To further refine risk assessment in the socio-demographic heterogeneous population of South Africa, the development and evaluation of a sensitive and interactive model the Mycotoxin Risk Assessment Model (MYCORAM) proofed to be more sensitive compared to the classical probable daily intake (PDI). The development of the MYCORAM was based on mycotoxin distribution during dry milling of maize in milling fractions intended for human consumption which was superimposed on the maize intake profiles of the South African population. Although dry milling, including a degerming step, is an effective way to reduce mycotoxins, risk and exposure assessment are influenced by maize dietary intakes, gender and ethnicity. This became evident when considering FB dietary exposure in rural maize subsistence farming communities in the Eastern Cape Province, South Africa confirmed the vulnerability of this subpopulation to risk of fumonisin exposure. Specific maximum tolerated maximum levels (MTL) to safeguard these communities fall outside the international regulatory processes and need to be urgently addressed. With the complex nature of cancer development in mind, integration of basic science and nutritional epidemiology will be important to contribute to our understanding of the adverse effects of FB and to define relevant risk assessment parameters.

OPSOMMING

Die blootstelling aan voedsel-kontaminante soos mikotoksienes word wêreldwyd met ‘n verskeidenheid van dierlike en menslike siektes geassosiseer. In Suid-Afrika word mielies as ‘n belangrike graanoes beskou, nie net vir die ekonomie nie maar ook as ‘n plaaslike voedselproduk beide kommersieel en vir bestaansboere in landelike gemeenskappe. Ten einde mikotoksien-kontaminasie van voedsel te kan beheer en bestuur, vereis bewys-gebaseerde risiko-evaluering wat insluit meganistiese en menslike blootstelling studies. Vanuit hierdie perspektief is die huidige studie uitgevoer en gemik op die verdere ontleding van die fumonisin B_1 (FB_1) mikotoksien geïnduseerde lewer-karsinogenese deur die ontwrigting van die lipiedmetabolisme. Die studie ondersoek terselfdetyd aspekte van menslike risiko-evaluering ingevolge die relevansie en belangrikheid hiervan in voedselveiligheid wat ook ‘n impak op voedselsekerheid sal maak. Dit sluit in die verspreiding van mikotoksiene gedurende die droëmaalproses van mielies en mikotoksien blootstelling in Suid-Afrika asook onder kwesbare landelike gemeenskappe.

Fumonisin B_1 beïnvloed die integriteit van biologiese membrane deur die modulasie van die belangrike lipied en vetsuur samestelling van plasma, mikrosomale, mitochondriale en kern subsellulêre membraan-fraksies in rot lewer. Veranderinge in die belangrike lipiedbestanddele, insluitende ‘n verhoging in cholesterol (CHOL) en phosphatidylethanolamine (PE), terwyl sphingomyelin (SM) en phosphatidylcholine (PC) geneig was om te verlaag. Geïsoleerde plasma membraan lipied vlotte (lipid rafts), vanaf primêre rot hepatosiete blootgestel aan FB_1, versterk die ingewikkelde gevolge wat uitgeoefen word op die lipiedmetabolisme insluitende die voorgestelde veranderings in CHOL, SM en PE vlakke. Die versteuring van lipiede en vetsure soos aragidoonsuur (arachidonic acid) en ceramied kan beskou word as belangrike determinante wat inmeng in groei-regulerende seinbane verwant aan die kritiese balans tussen selgroei en seldood. Die versteurings verskaf verdere bewyse dat FB_1 kanker bevorder deur ‘n seleksie proses wat onderskeidelike die onderdrukking en\of die stimulasie van ‘n paar weerstandige of geneties veranderde hepatosiete laat vermeerder in ‘n omgewing waar die groei van normale selle geïnhibeer word. Die spesifieke lipogeniese fenotipe wat FB_1 versoorsaak hou ten nouste verband met

kankerontwikkeling en die voorkoms van epigenetiese-soort meganismes word voorgestel. Hierdie oorsake word tans nie voldoende aangespreek tydens die bepaling van risiko-evaluerings limiete nie.

Om risiko-bepaling verder te verbeter in die sosio-demografies heterogene populasie van Suid-Afrika, was die ontwikkeling en evalueering van ‘n sensitiewe en interaktiewe model, die “Mycotoxin Risk Assessment Model” (MYCORAM) meer doeltreffend vergeleke met die gewone waarskynlike daaglikse inname. Die ontwikkeling van die MYCORAM was gebaseer op die mikotoksien verspreiding tydens die droëmaalproses van mielies in fraksies wat vir menslike verbruik bedoel was tesame met mielie dieetinnames van die Suid-Afrikaanse populasie. Alhoewel, die droëmaalproses van mielies, insluitende die verwydering van die kiem doeltreffende maniere is om mikotoksienes te verminder, word risiko- en blootstellings evaluering beinvloed deur mielie dieetinnames, geslag en etniese-verbandskap. Hierdie was veral opmerklik gedurende blootstelling aan FB in die dieet van landelike mielie bestaansboer gemeenskappe in die Oos-Kaap van Suid-Afrika en bevestig hoe kwesbaar hierdie populasie is. Spesifieke maksimum toelaatbare vlakke om hierdie gemeenskappe te beskerm val buite die huidige internasionale regulatoriese prosesse en benodig dringende aandag. Met die ingewikkelde aard van kankerontwikkeling in gedagte, sal die integrasie van basiese wetenskappe en voedingsepidemiologie, ‘n belangrik bydrae lewer tot die kennis van die negatiewe eienskappe van FB om toepaslike risiko-evaluerings limiete te kan bepaal.

Dedicated to my family:

Albert, Johanna and Hendrik Burger

Immeasurable grace from my Heavenly Father

In gratitude towards my Saviour and Lord, Christ Jesus

"Therefore, behold, I will allure her and bring her into the wilderness, *and* I will speak tenderly *and* to her heart. There I will give her, her vineyards *and* make the Valley of Achor (*Valley of trouble*) to be for her a door of hope *and* expectation. And she shall sing there *and* respond as in the days of her youth *and* as at the time when she came up out of the land of Egypt. And it shall be in that day, says the Lord, that you will call Me Ishi (*my Husband*), *and* you shall no more call Me Baali (*my Master*)".

"And I will betroth you to Me forever; yes, I will betroth you to Me in righteousness *and* justice, in steadfast love, *and* in mercy. I will even betroth you to Me in stability *and* in faithfulness, *and* you shall know the Lord."

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ABBREVIATIONS

%	Percentage
α	Alpha
ω	Omega
β	Beta
γ	Gamma
˚C	Degrees Celsius
µ	Micro
µg	Microgram
AA	Arachidonic acid
ACP	Acyl-carrier protein
ADI	Acceptable daily intake
AF	Aflatoxin
AIN	American Institute of Nutrition
Akt	Protein kinase B
ALA	Alpha- (α) linoleic acid
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
bw	Body weight
C	Carbon
$CaCl_2$	Calcium chloride
CANSA	Cancer Association of South Africa
Cat	Catalogue
CDase	Ceramidase
CDP	Cytidine 5'-diphosphocholine citocoline
CHOL	Cholesterol

CI	Confidence Interval
CK	Ceramide kinase
CL	Cardiolipin
CO_2	Carbon dioxide
CoA	Coenzyme A
COM	Commercial
COX	Cyclooxygenase
CPT	Cholinephosphotransferase
CS	Ceramide synthase
DAFNE	Data Food Networking
DAG	Diacylglycerol
ddH_2O	Double distilled water
DG	Degermed
DGLA	Dihomo-gamma (γ)-linolenic acid
DHA	Docosahexaenoic acid
DHET/s	Dihydroxyeicosatrienoic acid
DIG	Detergent-insoluble glycolipid-enriched
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DPA	Docosapentaenoic acid
DRMs	Detergent-resistant membranes
EC	Eastern Cape Province
ECRA	Ethics Committee for Research on Animals
EDTA	Ethylenediaminetetraacetic acid
EET/s	Epoxyeicosatrienoic acid
EFSA	European Food Safety Authority

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ELEM	Equine leukoencephalomalacia
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
F.	*Fusarium*
FA/s	Fatty acid/s
FAME	Fatty acid methyl esters
FAO	The Food and Agriculture Organisation of the United Nations
FB	Fumonisin
FBS	Foetal bovine serum
FB_T	Total Fumonisin ($FB_T = FB_1 + FB_2$)
FDA	United States Food and Drug Administration
FFQ	Food frequency questionnaire
FR	Folate receptor
FS	Free State Province
g	Gram
g	Acceleration of gravity
GCS	Glucosylceramide synthase
GEM	Glycolsphingolipid-enriched membranes
GEMS	Global Environment Monitoring System
GLA	Gamma- (γ) linolenic acid
GM	Geometrical mean
GM_1	Monosialotetrahexosylganglioside
GP	Gauteng Province

GPI	Glycosyl phosphatidylinositol
h	Hour
HDL	High density lipoproteins
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HETE	Hydroxyeicosatetraenoic acid
HG	Home-grown
HG_COM	Home-grown and commercial
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HIV/AIDS	Human immunodeficiency virus infection / acquired immunodeficiency syndrome
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HPLC	High performance liquid chromatography
HPV	Human papilloma virus
HRP-CTB	Horseradish peroxidase cholera toxin subunit B
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Kilogram
KOH	Potassium hydroxide
KZN	KwaZulu-Natal Province
L	Litre
LA	Linoleic acid
LCAT	Lecithin-cholesterol acyltransferase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
l_d	Liquid-disordered
LDL	Low-density lipoprotein
l_o	Liquid-ordered

LOAEL/LOEL	Lowest observed adverse (effect) levels
LOD	Limit of detection
LOX	Lipooxygenase
LP	Limpopo Province
LR/s	Lipid raft/s
LT	Leukotrienes
M	Molar
m	Meter
MAPK	Mitogen-activated protein kinase
MEM	Mitochondrial associated membrane
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mL	Millilitres
mM	Millimolar
mm	Millimetre
MP	Mpumalanga Province
MRC	Medical Research Council
MTL/s	Maximum tolerable level/s
MUFA/s	Monounsaturated fatty acid/s
MYCORAM	Mycotoxin risk assessment model
n	Sample size
N	Normal
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Northern Cape Province

ND	Not detected
ng	nanogram
NICUS	Nutrition Information Centre, University of Stellenbosch
No.	Number
NOAEL/NOEL	No observed adverse (effect) levels
NTC	National Technikon Certificate
NTD/s	Neural tubes defect/s
NW	North West Province
OA	Oleic acid
OC	Oesophageal cancer
OH	Hydroxy
OTA	Ochratoxin A
P	Pellet
PA	Phosphatidic acid
PC	Phosphatidylcholine
PDI	Probable daily intake
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine *N*-methyltransferase
PG	Prostaglandins
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol-4-monophosphate
PIP_2	Phosphatidylinositol-4,5-biphosphate
PL/s	Phospholipid/s
PLA_2	Phospholipase A_2
PMTDI	Provisional Maximum Tolerable Intake

PNS	Post nuclear supernatant
PROMEC Unit	Programme on Mycotoxins and Experimental Carcinogenesis
PS	Phosphatidylserine
PSS2	Phosphatidylserine synthase 2
PTP	Mitochondrial permeability spores
PUFA/s	Polyunsaturated fatty acid/s
QFFQ	Quantitative Food Frequency Questionnaire
RAPP tool	Ratio And Portion size Photo tool
rpm	Rounds per minute
S	Supernatant
Sa	Sphinganine
SAGL	Southern African Grain Laboratory
SCAPs	Sterol cleavage-activating proteins
SCC	Squarmous cell carcinoma
SCD	Stearoyl-CoA desaturase
SD	Standard deviation
SEM	Semolina
SFA/s	Saturated fatty acid/s
SK	Sphingosine kinase
SL/s	Sphingolipid/s
SM	Sphingomyelin
SMase	Sphingomyelinase
SMS	Sphingomyelin synthase
So	Sphingosine
spp.	Species
SPT	Serine palmitoytransferase

SR-B1	Scavenger receptor class B type 1
SREBPs	Sterol regulatory element-binding proteins
STD	Standard deviation
TDI	Tolerable daily intake
TGF	Transforming growth factor
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TRMC	Triton X-100 resistance membrane complexes
USD	American dollar
UV	Ultraviolet
v	Volume
VLDL	Very low-density lipoproteins
w	Weight
WC	Western Cape Province
WE	Williams E
WHO	World Health Organisation
yrs.	Years
ZEA	Zearalenone

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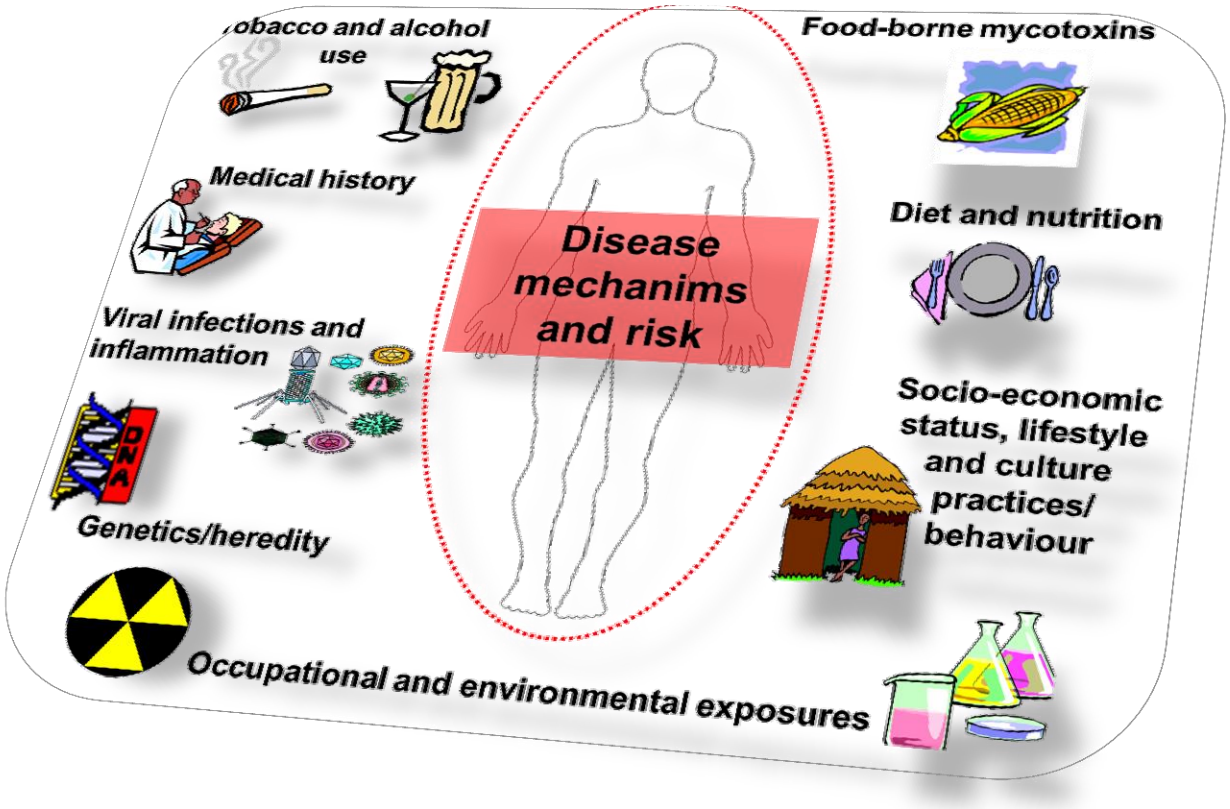

CHAPTER 1

GENERAL INTRODUCTION

Cancer is considered to be one of the leading causes of death worldwide, with lifestyle and environment factors contributing between 90 to 95% of all cancers (Hahn and Weinberg, 2002; Anand *et al*., 2008). In 1971, "War on Cancer" was declared by President Nixon of the United States of America. Forty years later and despite substantial advances in diagnoses, treatment and management, both cancer incidences and prevalence are still increasing worldwide, especially in developing countries (Coebergh *et al*., 2010). Substantial challenges in cancer research remain the causes, mechanisms involved, low survival rates and ultimately prevention (Lakdawalla *et al*., 2010). It is expected that by 2020, 15 million new cases of cancer will be diagnosed and 12 million would have succumbed to cancer in a world population of about 7.5 billion (Bray and Møller, 2006). The concept that cancer is mostly a disease of genetic origin has contributed very little to our understanding of the aetiology of cancer or how to prevent it (Anand *et al*., 2008). Perhaps a shift towards exploring environmental factors that may affect cell phenotype or directly initiate carcinogenesis via the "environment-gene" interactions will provide more insight. Environmental factors include exogenous (toxins, industrial chemicals, allergens, radiation, pollutants, bacteria, etc.) and endogenous (lifestyle and behaviour including diet, physical activity, socio-economic status, etc.) parameters (McCormack and Boffetta, 2011).

The disruption of the lipid metabolism is known to contribute to a "lipogenic" phenotype of tumour cells as specific genes affecting lipid homeostasis are overexpressed in cancer cells. These changes are associated with different cell growth patterns via altering cell proliferative indices through disrupting cellular redox balances and by inducing oxidative stress resistance (Milgraum *et al*., 1997; Hirch *et al*., 2010; Santos and Schulze, 2012). Together these lipogenic effects are now being recognised as part of the "hallmarks" and "gene signature" of cancer (Milgraum *et al*., 1997; Piyathilake *et al*., 2000; Menendez and Lupu, 2007; Hirch *et al*., 2010). Therefore, changes in lipid metabolism provide a unique opportunity to investigate non-genetic mechanisms associated with cancer development. In this regard, biological membranes are lipid metabolism-faculties that are vital to the homeostasis of every living cell. These intricate structures are not just boundaries to separate cellular content from an external microenvironment; membranes also partake in numerous biological processes (Szachowicz-Petelska *et al*., 2010). Structural

organisation and interactions between lipid constituents, including proteins, make membranes a central point for studying most diseases including cancer, aging, inflammation, neurodegenerative and cardiovascular (Escribá *et al*., 2003). Cell membranes consist of more than a thousand lipid moieties of which the main components are glycerolphospholipids (phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidylserine, phosphatidylinositol), sterols (cholesterol) and sphingolipids (sphingomyelin) (Van Meer *et al*., 2008). Recently, the lateral assembly of bilayer cell membranes was challenged by the existence of special "membrane-organising principle" or microdomains (Lingwood and Simons, 2010). The presence of microdomains has been regarded as highly controversial, however with the recent development of sophisticated quantitative imaging technology their existence has become more evident (Neumann *et al*., 2010). Microdomains such as lipid rafts, known to contain cholesterol, sphingolipids, saturated glycerolphospholipids and specific proteins, are associated with signal transduction, intracellular trafficking pathogen invasion/uptake, secretion and endocytosis (Pike, 2003; Frank and Lisanti, 2006; Shaw, 2006). The current knowledge regarding cellular membranes, and altered lipid metabolism in unraveling multifactorial diseases such as cancer, highlight the structural and functional complexity of lipids and their basic components the fatty acids.

Substantial evidence links food as a source of natural occurring carcinogens and specific dietary patterns with the incidence of cancer (Kaput, 2004).The effect of these food contaminants on the deregulation of the lipid metabolism including cell membranes, has not been studied well. The discovery of the carcinogenic mycotoxin, fumonisin B_1 (FB_1) as a liver cancer promoter, involved the disruption of the lipid metabolism and the subsequent modification of membrane integrity and function as underlying mechanisms (Gelderblom *et al*., 2001; 2008; Riley *et al*, 2001). These effects included (i) disruption of the membrane by altering cholesterol, phospholipids and sphingomyelin metabolism; (ii) inhibition of ceramide synthase disrupting sphingolipid metabolism; (iii) impairment of delta-6-desaturase enzyme with the modulation of membrane fatty acids and a shift towards prostanoid synthesis of the E2 series (Gelderblom *et al*., 2001).

Food contaminated by mycotoxins is considered to be a global public health priority and pose a threat to humans and animals as well as world economies regarding industry and international (Miller, 1998; Bryden, 2007) (Giacometti *et al*., 2012). In this regard, mycotoxins have the capacity to cause numerous adverse health effects with aflatoxin (AF), ochratoxin A (OTA), deoxynivalenol (DON, zearalenone (ZEA) and fumonisin B (FB) considered as the most important (Bennet and Klich, 2003; Marasas *et al*., 2008; Maresca and Fantini, 2010). These mycotoxins are produced by food-borne fungi and include *Aspergillus spp*. (AF and OTA), *Penicillium spp.* (OTA) and *Fusarium spp*. (DON, ZEA and FB) (Miller, 2002). Associated human diseases and disorders include hepatitis, liver cancer, stunting and immune suppression (AF), nephropathy (OTA), gastro-intestinal disorders, anorexia, nausea, emesis, headache, chills, giddiness and convulsions (DON), precocious pubertal changes in children, early menarche and possibly infertility (ZEA) and an increased risk of oesophageal and liver cancer, neural tube defects and stunting (FB) (Hult *et al*., 1982; Marquardt. and Frohlich, 1992; IARC, 1993a, 1993b, 2002a; 2002b; Li *et al*., 2001; Marasas *et al*., 2001, 2004; Maresca *et al*., 2002; Turner e*t al*., 2002; Kimanya *et al*., 2010; Amuzie and Pestka, 2010).

Outbreaks of the related mycotoxicoses are clustered, with mostly animals and vulnerable human populations from the developing world that are at risk. Vulnerable populations with a daily staple diet such as rural subsistence-farming populations with poor agricultural practices are susceptible to a chronic exposure to highly contaminated foods (Marasas *et al*., 2008). Chronic exposure to mycotoxins, even at low levels in an unvaried diet, may incur adverse health outcomes or possibly exacerbate other existing disease conditions (Bryden, 2007). Furthermore, the co-occurrence of mycotoxins, their possible synergistic and/or additive effect is currently poorly understood (Eaton and Klaassen, 2001; Scudamore and Patel, 2009; Waśkiewicz *et al*., 2012). South Africa, as part of the developing world, is both a maize consuming and producing country where food contaminants and safety are an unheeded conundrum (Figure 1.1). Food safety and security are linked (Hanning *et al*., 2012), however adequate definition of these concepts within a South African contexts are ill-defined and/or lacking (Labadarios *et al*., 2011). Relevant mycotoxins in a South African context include the FB-mycotoxins, DON and ZEA that co-occur in commercial maize and maize-based food and feed products (Shephard *et al*., 2005;

Shephard *et al*., 2007, SAGL, 2012). Of these mycotoxins, FB are the major contaminants especially in poor rural communities where maize is used as the major dietary staple and exposure is often comprised by food insecurity.

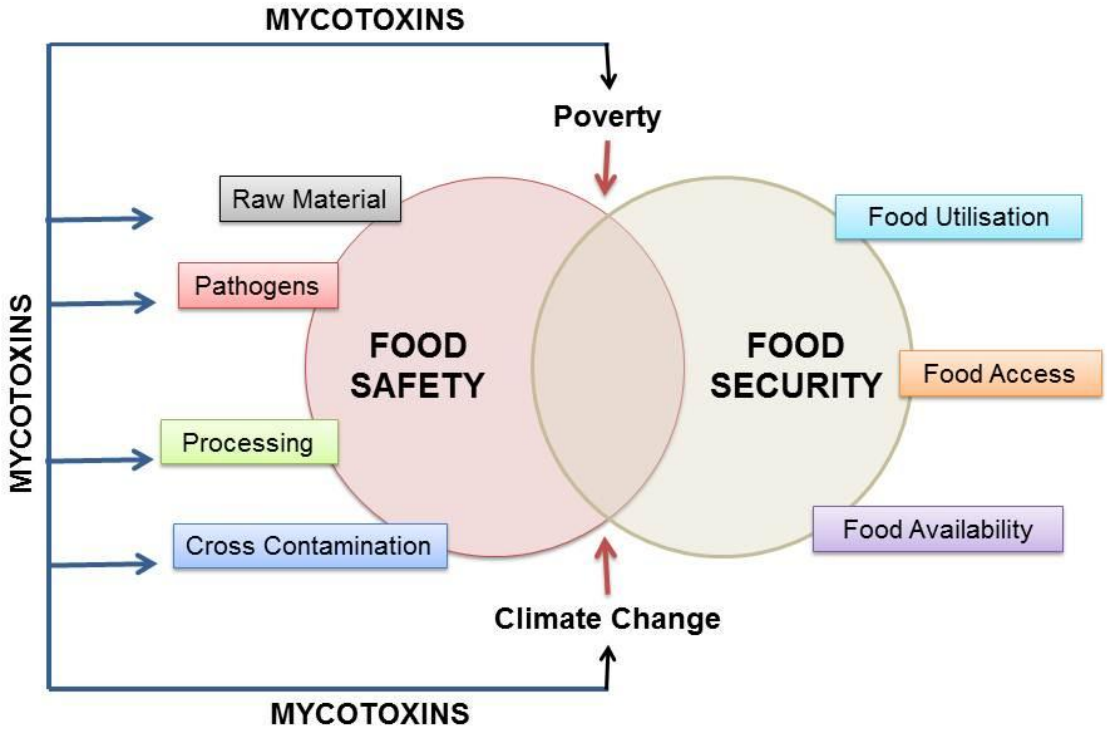

Figure 1.1: Multiphase impact of mycotoxins on aspects related to food safety that interlinks with food security
(Adapted from Hanning *et al*., 2012)

To assess the complexities of the impact of mycotoxins in South Africa, a multidisciplinary approach is warranted. The coalescing of mechanistic and human epidemiology investigations has the advantage of not only expanding basic scientific knowledge but also confronts public health aspects. The purpose of this dissertation is to contribute to the risk analysis framework of mycotoxins by integrating research on mycotoxin mechanisms and human epidemiological studies related to diet/nutrition, exposure/hazard and risk assessment.

The dissertation comprises eight chapters with Chapter 2 providing a comprehensive overview of the different themes that will be addressed. The effects of the carcinogenic FB_1 mycotoxin on important membrane lipid and fatty acid parameters

in isolated rat hepatic subcellular membrane fractions will be discussed in Chapter 3. Chapter 4 expands on this theme and the effect of FB_1 on nanosized microdomains or lipid rafts known to exist within cell membranes are highlighted. The joint aim of these two chapters is to further unravel the mechanisms by which FB_1 disrupts lipid metabolism and membrane integrity related to cancer promotion in the liver. FB_1 is known to promote neoplastic transformed cells by creating a growth differential whereby initiated (or resistant) cells proliferate, while normal cells are shunted towards apoptosis. From a food safety perspective, Chapter 5 describes the fate of mycotoxins in experimental and commercial dry milled maize fractions intended for human consumption. The development of a novel and interactive mycotoxin risk assessment model (MYCORAM) based on the maize consumption profiles of the South Africa population are presented in Chapter 6. In Chapter 7 the potential risk of exposure to FB is further expanded to include rural subsistence maize farming communities. The General Discussion and Conclusions section (Chapter 8) provides a critical evaluation and integration of the main findings and imparts relevant conclusions and recommendations.

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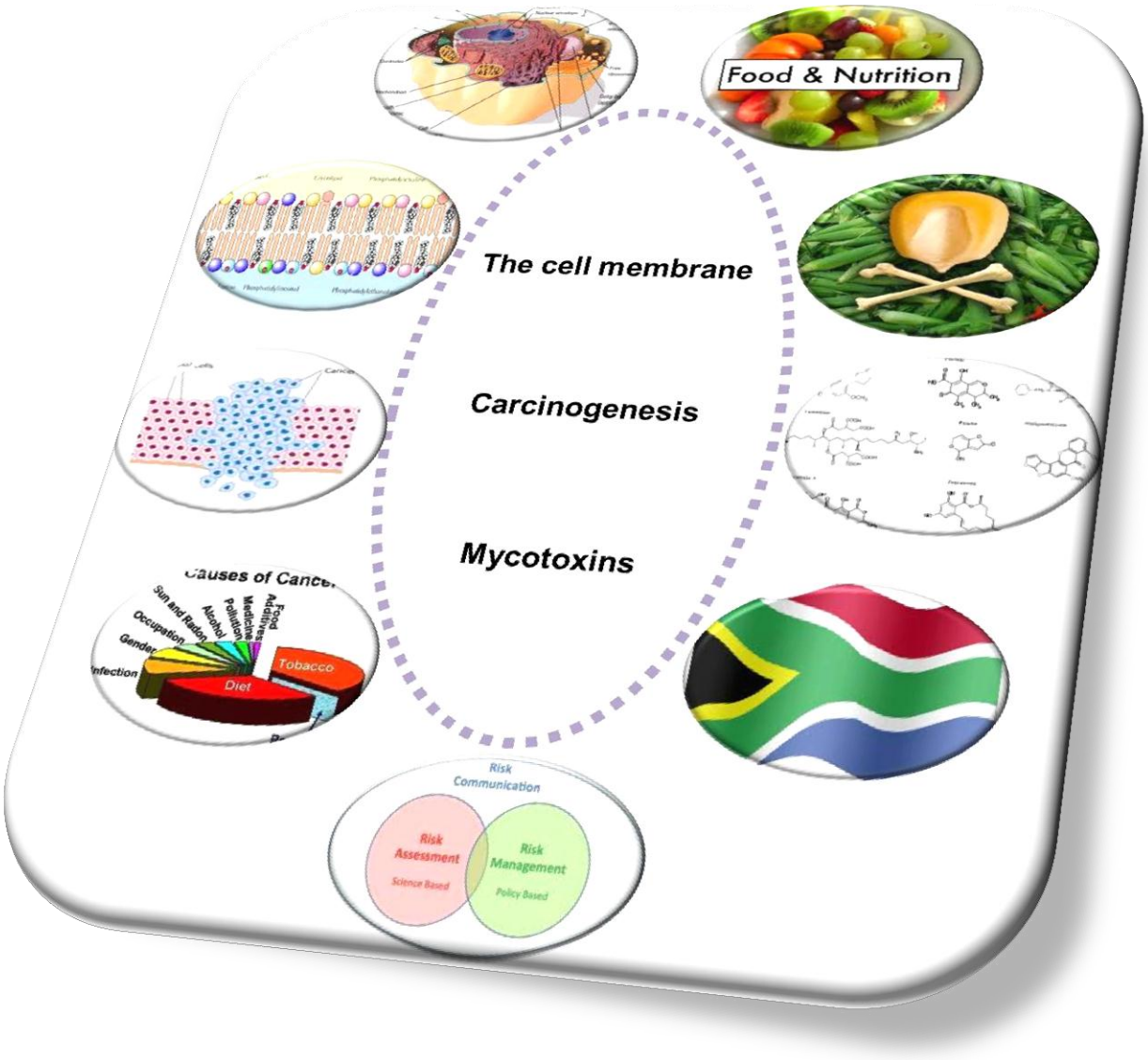

CHAPTER 2

LITERATURE REVIEW

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2.1. LIPIDS AND THE LIVER CELL

2.1.1. Introduction

The structural and functional diversity of biomolecules such as lipids are crucial to sustain homeostasis and ultimately life. Eukaryotic cells use a mere 5% of their genes to produce thousands of different types of lipids (Sud *et al*., 2007). The disruption of this intricate lipid metabolism has been associated with numerous diseases such as cardiovascular diseases, diabetes, obesity, inflammation, autoimmune diseases, neurological diseases, aging and cancer (Escribá *et al*., 2003; Hagen *et al*., 2010). Unraveling the role of these complex biomolecules during both normal and pathological conditions inevitably highlights the important role of biological cell membranes that forms a reservoir for lipid metabolism.

2.1.2. The liver cell

To fully appreciate the lipid metabolism and its role in cell membranes, the liver cell forms an ideal point of departure. The liver organ with its diversity of essential functions plays an important role in the lipid- and detoxification metabolism. The liver consists of multiple phenotypically distinct cells including polarized epithelial or parenchymal cells called hepatocytes and sinusoidal lining nonparenchymal cells containing fenestrated endothelial cells, hepatic stellate cells (Ito cells) and macrophages such as Kupffer, natural killer and natural killer-T cells (Figure 2.1) (Berry *et al*., 1991; Eckl and Bresgen, 2003). Cholangiocytes or biliary epithelial cells give rise to the bile ducts.

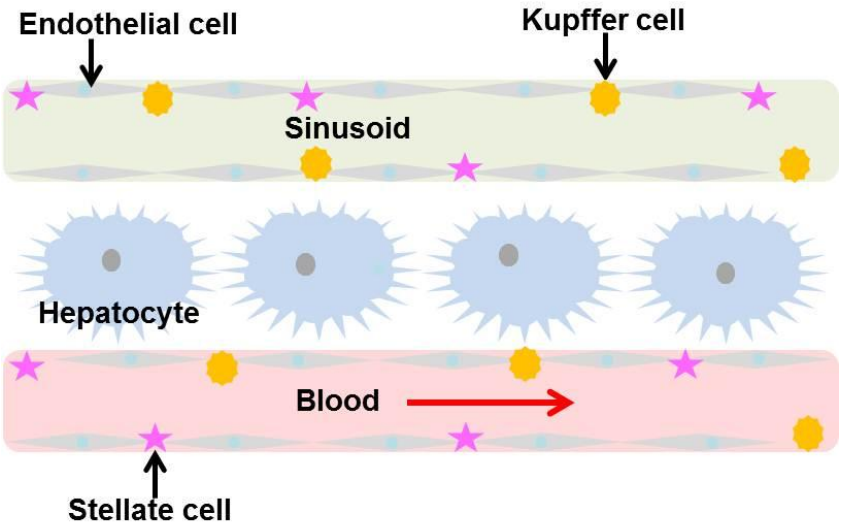

Figure 2 1: Cellular components of the liver
(Adapted from Roberts *et al*., 2007)

Hepatocytes are regarded as central to the metabolic activity of the liver and compromises 60% of the total cell number and 80% of the total liver mass (Fawcett, 1994, Williams and Latropoulos, 2002). Although hepatocytes in the adult liver are quiescent where only 1 out of 20 000 cells will undergo mitosis, it retains its replicative potential (Fausto, 2000). During cell loss it regulate its own growth by adapting this proliferate capacity in a synchronized manner in what is called compensatory regeneration or compensatory hyperplasia (Michalopoulos and DeFrances, 1997). The primary function of hepatocytes include regulate intermediary metabolism, detoxifying endo- and xenobiotics, production of circulation proteins and generate bile acid-dependent flow (Malhi *et al*., 2010). To fulfill its function, hepatocytes are abundant in rough endoplasmic reticulum (ER), mitochondria, several intricate Golgi-complexes, peroxisomes, smooth ER and lysosomes (Table 2.1). Some hepatocytes are also known to contain two nuclei.

Table 2.1: Comparative volumes of the major intracellular components in a single hepatocyte

Intracellular compartment	Percentage of the total cell volume
Cytosol	54
Mitochondria	22
Rough endoplasmic reticulum (ER)	9
Smooth ER including Golgi-complexes	6
Nucleus	6
Peroxisomes	1
Lysosomes	1
Endosomes	1

Adapted from: Alberts *et al*., 2002.

The hepatocyte is the focal point in response to toxin exposure. During the ingestion of xenobiotics, they are absorbed from the gastrointestinal tract and transported to the liver via the portal vain circulation. The livers ability to biotransform xenobiotics and noxious endotoxins includes hydroxylation, oxidation and conjugation reactions utilising complex detoxifying enzyme systems (phase I and II enzymes). The liver is therefore a prime target for chemical compounds that where either not successfully eliminated or converted to highly reactive intermediate compounds. To study the xenobiotic-mechanism associated with toxicity and/or carcinogenesis, the liver organ forms an ideal *in vitro* model. Therefore, the use of animal liver models to investigate the metabolic fate of toxins has been very effective not only because of the controlled laboratory set-up but the results can be applied and extrapolate to predict effects in humans (Williams and Latropoulos, 2002). Also, cultured primary hepatocytes from rodent models provide the closets scenario to the *in vivo* environment due to the preservation of its capacity to metabolise chemicals (Strom and Michalopoulos, 1982; Strom *et al*., 1983).

2.2. THE CELL MEMBRANE AND ITS MAJOR CONSTITUENTS

2.2.1. Introduction

Biological cell membranes are heterogeneous asymmetrical bilayers with structural, functional and conductive properties. These properties are accomplished by a diversity of highly dynamic and functional lipid constituents that interact with integral (transmembrane) or peripheral (amphitropic, extrinsic) membrane proteins. This enables cell membranes to maintain intracellular homeostasis in a demanding environment where continuous communication between the intra and extracellular environment is crucial to the cells' survival. Essential cellular processes impacted on by cell membranes include bioenergetics, T-cell receptor activation, pathogen internalisation, apoptosis, blood coagulation, microdomain formation and signal transduction (Ladha, 2000; Williams, 1998; Ohvo-Rekilä *et al*., 2002; Vereb *et al*., 2003; Ivanova *et al*., 2004; Engelmann and Wiedmann, 2010; Lajoie *et al*., 2009; Vance and Steenbergen, 2005). The structural breakdown of cell membranes yields a fluid mosaic model where a gel-, liquid-ordered- (l_o) and liquid-disordered (l_d) phase coexists, allowing the mobility and flexibility of lipid constituents to undergo structural changes (Singer and Nicolson, 1972; Gibbons, 2003; Chauhan, 2003; Vereb *et al*., 2003, Zhang *et al*., 2007; Escribá *et al*., 2007, Albanese and Dainiak, 2003). The basic lipid constituents of membranes are the glycerolipids, sphingolipids (SLs) and sterols with unique compositions for each different intracellular membrane. The chemical nature of membrane lipids are distinguished by their (i) head-group or backbone structure, (ii) hydrocarbon chain length, (iii) degree of unsaturation, (iv) chirality, (v) ionization, (vi) chelating properties and vii) the concentration or amount of lipids present (Ohvo-Rekilä *et al*., 2002; Hains, 2001; Maxfield and Tabas, 2005; Olsson *et al*., 1991; Cullis and Hope., 1985; Catalá, 2009). The above mentioned biophysical parameters will affect membrane fluidity, permeability, curvature, tension, membrane-associated proteins and ultimately numerous biological processes. At the exterior and interior surfaces of membrane bilayers, the phospholipids' ionic and polar head group*s* exist in a liquid-phase causing strong hydrophilic interactions. The non-polar saturated fatty acids (SFAs) chains are found within the interior of the bilayer forming a hydrophobic matrix that accommodates both, cholesterol (CHOL) and proteins (Hąc-Wydro and Wydro, 2007; Sankaram and Thompson, 1990; Simon

and Ikonen, 1997; Kang *et al*., 1995; Ingraham *et al*., 1981). The important membrane constituents are summarised in Figure 2.2.

2.2.2. Phospholipids

2.2.2.1. Phospholipid structure and function

Phospholipids (PLs) or phosphate-containing lipids are the structural backbone of membranes and highly concentrated within all cellular membranes. The glycerolphospholipids are the most predominant membrane PL and consists of two acyl (diacyl) groups linked to a glycerol backbone (Gurr *et al*., 1990; Kol *et al*., 2002, Dowhan and Bogdanov, 2002). The complex interaction of the various PLs are due to their polarity, amphipathic properties and the presence of both hydrophobic head and hydrophilic acyl chain domains (Figure 2.3) (Dowhan and Bogdanov, 2002). The specific head group of the diacylglycerol (choline, serine, inositol or ethanolamine) is responsible for the classification of the phosphatidic acid into several lipids including: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) (Figure 2.6) and other minor PLs, phosphatidic acid (PA), cardiolipin (CL), phosphatidylglycerol, phosphatidylinositol-4-monophosphate (PIP), phosphatidylinositol-4,5-biphosphate (PIP_2) (Dowhan and Bogdanov, 2002)

Phosphatidylcholine, the most abundant PL is localized to the outer surface of the membrane bilayer (Li *et al*., 2006; Zhang *et al*., 2007; Ohvo-Rekilä *et al*., 2002; Quinn, 2002; Daleke, 2003; Lange and Steck, 2008) whereas the rest (PE, PS, PI, PA, CL, phosphatidylglycerol, PIP and PIP_2) are found in the cytoplasmic or inner membrane surface (Quinn, 2002; Ivanova *et al*., 2004; Lange and Steck, 2008; Cullis *et al*., 1996). Phospholipids are of structural importance to cell membrane not only to ensure its function and integrity but are also precursor of signaling molecules and some lipids can also act as second messengers (for example, PA and PIP_2) (Exton, 1994; Huwiler *et al*., 2000, Raucher *et al*., 2000; Noga and Vance, 2003; Monaco *et al*., 2006). Table 2.2 summarises the percentages of important PLs and SLs within various cellular membranes.

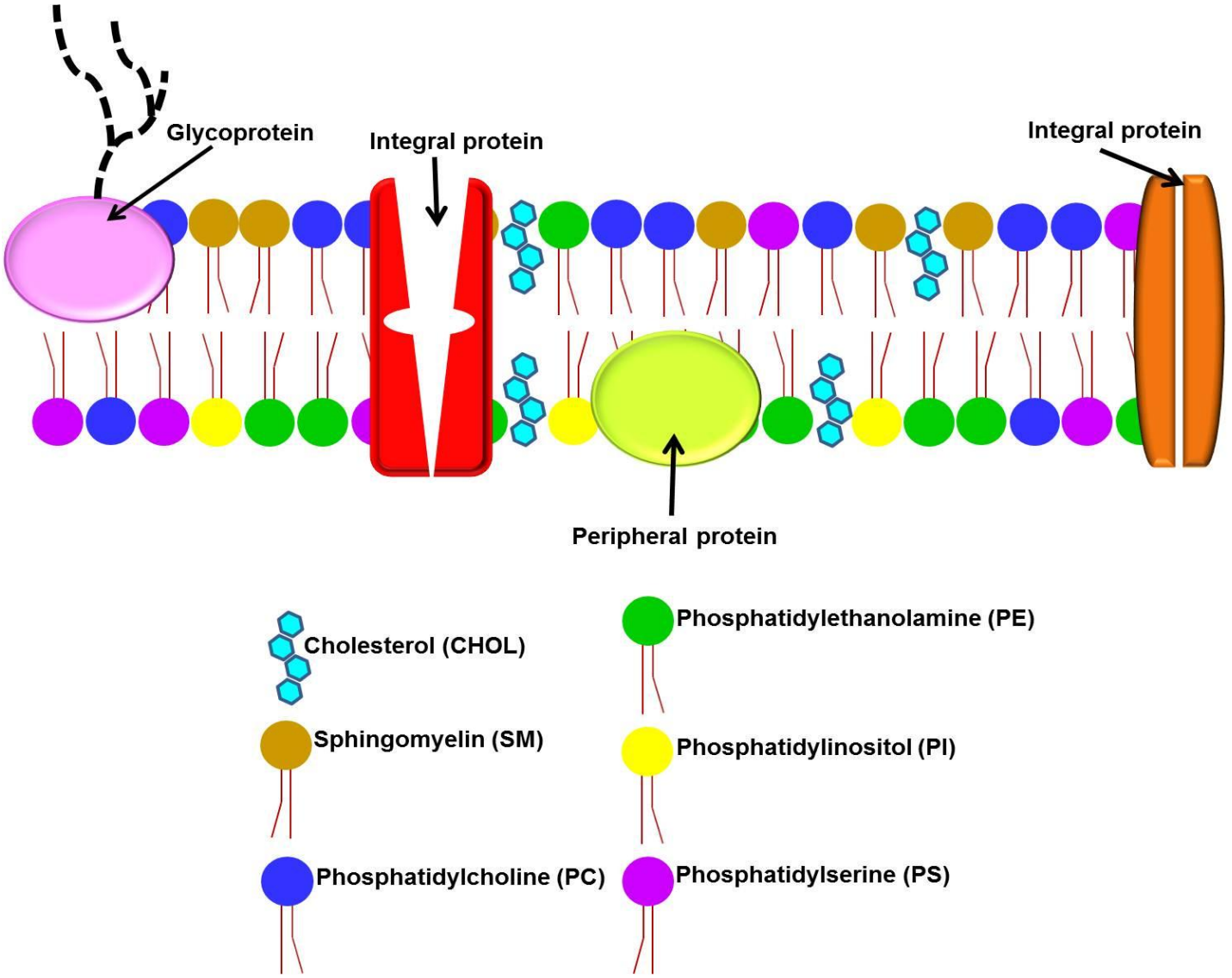

Figure 2.2: Structure of a cell membrane and its major lipid constituents

Table 2.2: Percentage (%) of phospholipids and sphingolipids in various cell membranes

Membrane fraction	%						
	PC	PE	PS	PI	PA	CL	SM
Plasma membrane	39	23	9	8	1	1	16
Mitochondria	40	35	1	5	-	18	1
Microsome	58	22	2	10	1	1	1
Nuclei	55	13	3	10	2	4	3
Lysosomes	40	14	2	5	1	1	20
Golgi-complexes	50	20	6	12	<1	1	8

Abbreviations: PC: phosphatidylcholine, PE: phosphatidylethanolamine, S: phosphatidylserine, PI: phosphatidylinositol (PI), PA: phosphatidic acid, CL: cardiolipin and SM: sphingomyelin. Adapted from Yeagle, 2004 and Escribá *et al*., 2008.

2.2.2.2. Synthesis of the four major phospholipids (PC, PE, PI and PS)

The PL metabolism is a rapid and tightly regulated process involving the interaction of extracellular growth factors and plasma membrane-bound corresponding receptors. The *de novo* synthesis sites for PLs include the ER, Golgi-apparatus, peroxisomes and the mitochondria (Fagone and Jackowski, 2009; Bell *et al*., 1981; Daleke, 2003). Phosphatidylcholine is synthesised in most mammalian cells either via the Kennedy pathway (cytidine 5'-diphosphocholine, citicoline or CDP-choline pathway) in the ER or by diacylglycerol cholinephosphotransferase (CPT) situated in the Golgi-apparatus both utilising diacylglycerol as precursor (Voelker *et al* 1996; Witz, 1997; Li and Vance, 2008; Fagone and Jackowski, 2009). In liver cells however, PC is formed by the methylation of PE by phosphatidylethanolamine *N*-methyltransferase (PEMT) located in the mitochondrial associated membranes (MEM) (Vance *et al.,* 1997).

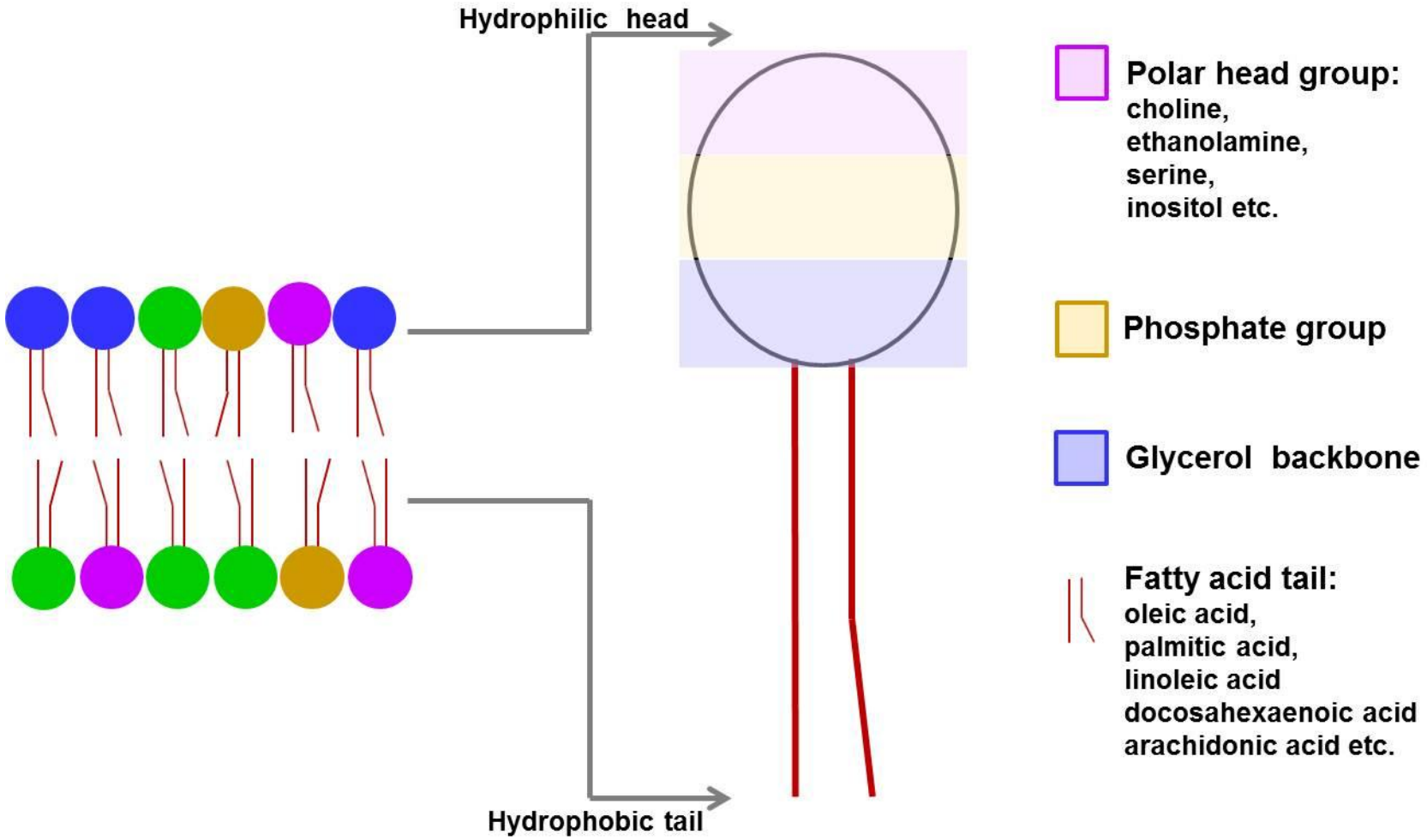

Figure 2.3: General structure of a phospholipid

Phosphatidylethanolamine can be synthesised by four pathways which include the Kennedy pathway involving the ER, the PS decarboxylation pathway in the inner mitochondrial membrane, acylation of lyso-PE localized to MEM and a base-exchange reaction between PS and ethanolamine catalyzed by PS synthase 2 (PSS2) also in the ER (Kennedy, and Weiss, 1956; Borkenhagen *et al*., 1961; Sundler *et al*., 1974; Zborowski *et al*., 1983; Vance and Tasseva., 2013). The production of PI located in the ER consists of a *de novo* or CDP-diacylglycerol (DAG/myo-inositol 3-phophatidyltransferase) and a salvage (or exchange) biosynthesis pathway (Takenawa *et al*., 1980; Justin *et al.,* 2002; Nuwahidy *et al*., 2006). Phosphatidylserine biosynthesis is made possible by a simple base-exchanges reaction were the head group of PC or PE is exchanged for serine in the presences of PSS1 and 2 in the ER.

2.2.3. Sphingolipids

2.2.3.1. Sphingolipid structure and function

Sphingolipids (SLs) are structurally heterogeneous molecules due to the large number of combinations that can occur in both the backbones and head groups (Figure 2.4) (Merrill *et al*., 2005). Sphingolipids consist of three structural components which include i) a straight long-chained amino alcohol (18 to 20 carbon atoms) or sphingoid base such as sphingosine (So), sphinganine (Sa, or dihydrosphingosine) or 4-hydroxysphinganine (phytosphingosine); ii) a long-chained SFA acid group linked to the sphingoid base amino group (C2 position) via an amide bond; iii) a hydrophilic head group, attached to the sphingoid base at the C1 position (OH-group) is responsible for the classification of SLs into complex phosphosphingolipids or glycosphingolipids (Figure 2.6) (Holthuis *et al*., 2001; Futerman and Riezman, 2005). The sphingoid base plus long-chained saturated fatty acid group yields ceramide and with the incorporation of a phosphorylcholine headgroup, sphingomyelin (SM) is formed. The phosphosphingolipids include SM whereas glycosphingolipids can be divided in to neutral or acidic or broadly into glucosphingolipids or galactosphingolipids, example of these include cerebrosides, sulfatides, globosides and gangliosides (Merrill and Sandhoff, 2002). Membrane SLs localized to the external surface of plasma membrane

bilayer, the lumen of intracellular vesicles and organelles (endosomes, Golgi membranes etc.). Sphingomyelin is restricted to eukaryotic cells and the brain and peripheral nerve tissue is especially abundant in SM. Overall, SLs including SM, are not just important structural components of cell membranes and lipoproteins, they also play an important role in the conduction of electrical nerve signals, cell growth, signaling, cell differentiation, migration and transformation of malignant cells, proliferation, membrane trafficking, autophagy, apoptosis, protein secretion and endothelial cell permeability (Merrill *et al*., 1996, Merrill *et al*., 1997; Ramstedt and Slotte, 2002; Soriano *et al*., 2005). Sphingolipids effect on membranes include lipid phase, curvature and thickness as well as the preferential association with sterols to form specialised microdomains known as lipid rafts (LRs) (Lingwood and Simons, 2010; Lippincott-Schwartz and Phair, 2010). The role of SM in LRs is currently unclear. A study conducted by Li *et al*., (2007) showed that SM deficiency by sphingomyelin synthase knockout had no influence on CHOL levels in LRs whereas Asano *et al.* (2012) confirmed this by illustrating the preservation of LRs and caveolae specific proteins in SM-deficient cells. Investigation into the regulation of SM is complicated due to numerous SM synthesis subcellular sites present within cells, such as the *cis-, medial*-Golgi-apparatus, plasma membranes and chromatin (Li *et al*., 2007). Whereas the, two isoforms of the enzyme sphingomyelin synthase (SMS) present in mammalian cells, SMS1 (active in the Golgi-apparatus) and SMS2 (plasma membrane associated) utilised different subcellular cites for *de novo* SM synthesis with SMS2 also responsible for the remodeling of SM (Huitema *et al*., 2004; Tafesse *et al*., 2006; Li *et al*., 2007).

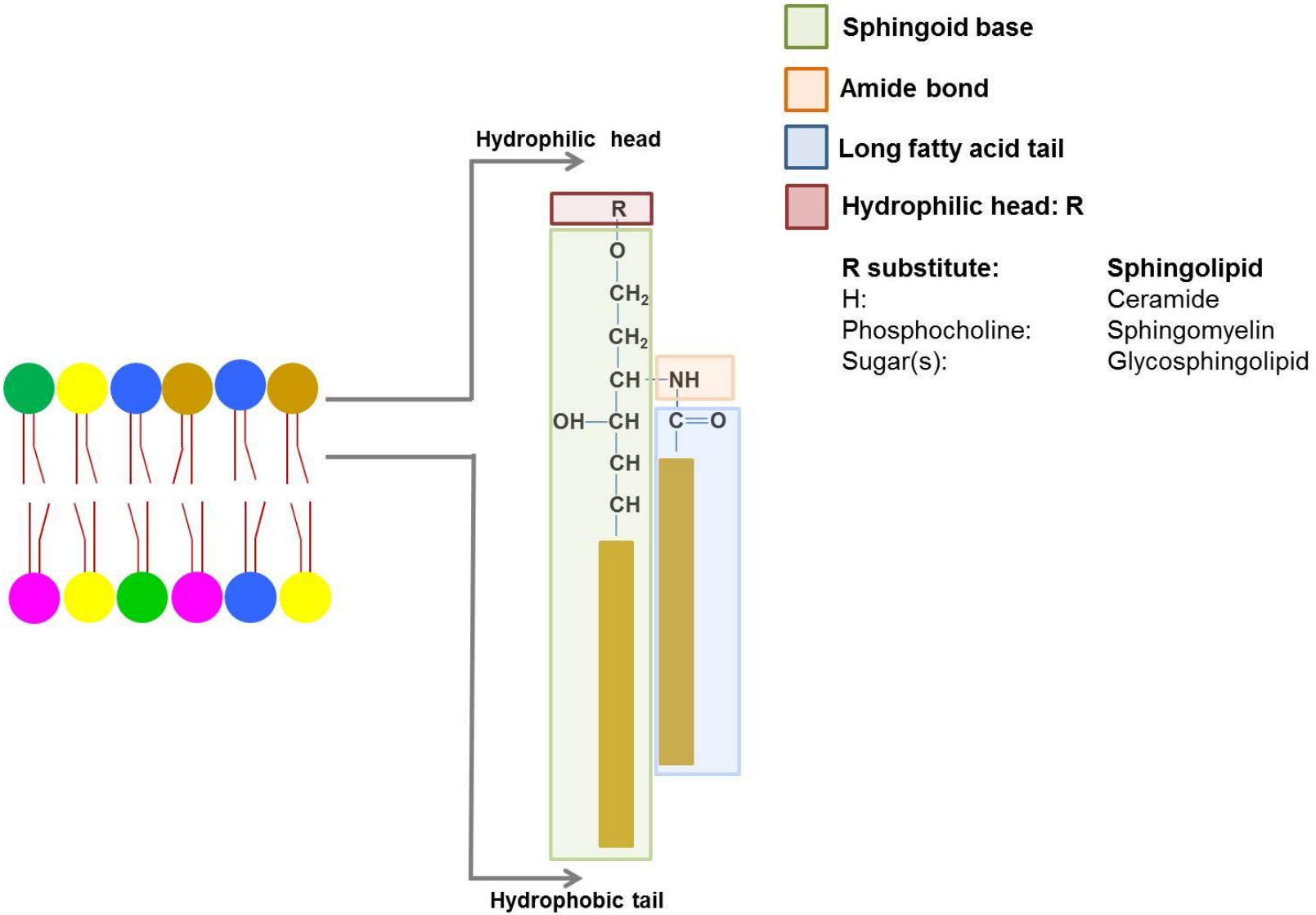

Figure 2.4: General structure of a sphingolipid

2.2.3.2. Sphingolipid synthesis

The syntheses of SLs involve various subcellular compartments such as the ER and Golgi-apparatus. *De novo* SM synthesis include both PC and ceramide as precursors directed by the enzyme SMS. The conversion of ceramide to SM is reversible utilising sphingomyelinase (SMase) (Zhang and Kolesnik, 1994; Zxa *et al*., 1998; Cremesti *et al*., 2002). Endogenous ceramide is formed by the condensation of serine and palmitoyl-CoA by the piridoxal-dependent enzyme, serine palmitoyltranferase (SPT) in the cytosolic side of the ER. The resultant 3'-keto-sphinganine is reduced to Sa and in return Sa can either be phosphorylated to Sa 1-phosphate or N-acylated (addition of a fatty acid CoA), the latter reaction activated by ceramide synthase (CS) to form dihydroceramide. Dihydroceramide is converted to ceramide by dihydroceramide desaturase. Ceramide (Figure 2.5) can be converted to i) glucosylceramide for the formation of complex glycosphingolipids or gangliosides via glucosylceramide synthase (GCS); ii) the production of galactosylceramide using galactosyltransferase; iii) ceramide 1-phosphate activated by ceramide kinase (CK) and iv) So, utilising ceramidase (CDase). Sphingosine can be transformed to So 1-phosphate through the enzyme sphingosine kinase (SK) whereas Sa can be converted to Sa 1-phosphate (Ryland *et a*l., 2011).

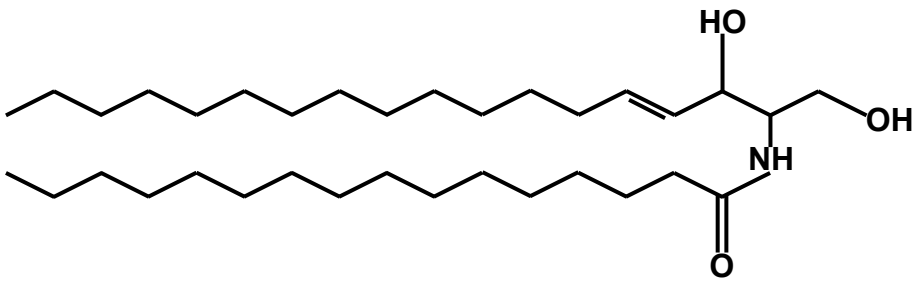

Figure 2.5: Chemical structure of ceramide

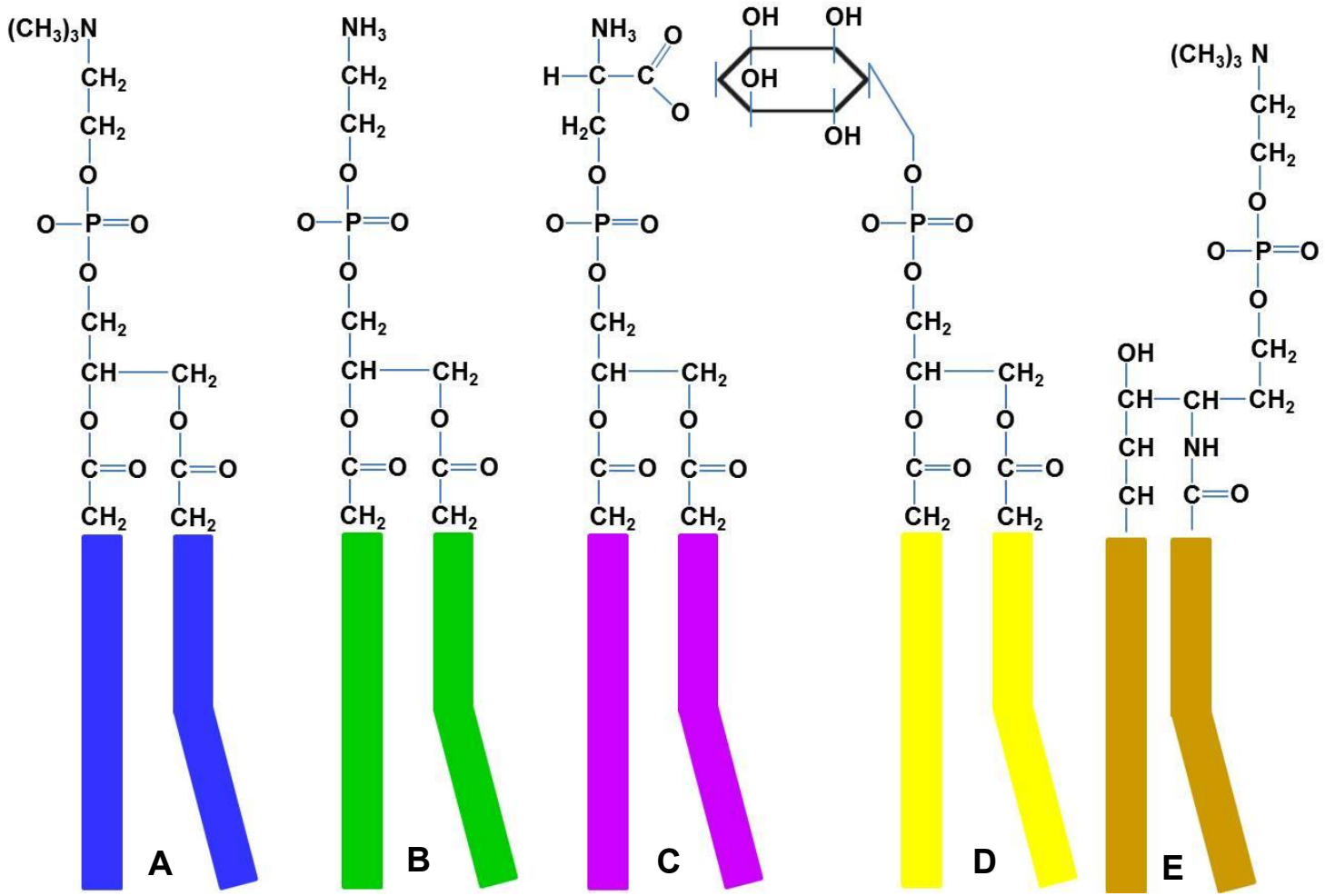

Figure 2.6: Structures of the most common phospholipids and sphingolipid
A: Phosphatidylcholine (PC); **B**: Phosphatidylethanolamine (PE); **C:** Phosphatidylserine (PS); **D:** Phosphatidylinositol (PI); **E:** Sphingomyelin (SM)

2.2.4. Fatty acids

2.2.4.1. Fatty acid structure and function

At the core of each PL molecule is its fatty acid (FA) constituents that are joined to the PL polar head groups by phospholipid esters. Generally FAs contains even numbers of carbon atoms and can be grouped into three classes: i) the saturated fatty acids (SFAs) with carbon atom length of 12 to 22 with no double bonds between the carbon atoms; ii) monounsaturated fatty acids (MUFAs) containing 16 to 22 carbon atoms with one carbon-carbon double bond in the *cis* configuration and iii) the polyunsaturated fatty acids (PUFAs) with two or more double bonds separated by methylene groups. Table 2.3 provides more detailed information on the important FAs. Polyunsaturated FAs are long-chained FAs grouped as ω-3, ω-6 or ω-9 depending on the location of the last double bond in relation to the terminal methyl (ω) end of the molecule. The difference in quantity, type, chain length and degree of unsaturation, stability of FAs are crucial factors and are maintained by the cells to ensure functional efficiency. These include i) a source of energy; ii) precursors for bioactive molecules; iii) maintaining cellular structure as building blocks of PLs; iv) cell signaling; v) gene expression; vi) regulation of nuclear receptors and transcription factors; vii) cell metabolism and homeostasis (Göttlicher *et al.*, 1992; Alexander, 1998; Kolonel *et al*., 1999; Jump, 2004). Of interest is the highly flexibility and disorder of especially the PUFA acyl chains compared to those of SFAs and MUFAs (Feller *et al*., 2002; Shaikh *et al*., 2003). These characteristics of PUFA acyl chains also differ between ω-3 and ω-6 FAs and will impact on the membrane structure and function (Shaikh and Eddidin, 2006).

Table 2.3: Name and symbol of the important fatty acids

Common name (name derivation and/or acronym) *History and/or information*	Scientific name	Symbol	Common name (name derivation and/or acronym) *History and/or information*	Scientific name	Symbol
Saturated fatty acids					
Lauric acid (Latin: *laurus*, "laurel plant") *Lauric acid was discovered in 1842 by T. Marrsson.*	*n*-Dodecanoic acid	C12:0	*Arachidic acid* *(Latin: Arachis, legume genus, **AA**)* *Arachidic acid occurs in large quantities in groundnut (Arachis hypogea) oil where it was also discovered by A. Gössmann in 1854.*	*n*-Eicosanoic acid	C20:0
Myristic acid (Latin: *Myristica*, nutmeg genus) *Myristic acid was discovered by L. Playfair in 1841 in* Myristica fragrans or seeds of the *family Myristicaceae.*	*n*-Tetradecanoic acid	C14:0	Behenic acid *Behenic acid is a major constituent of Ben oil (or behen oil), this oil is extracted from the seeds of the Ben-oil tree (Moringa oleifera). This tree was named after the Persian month Bahman, when the roots of this tree are normally harvested.*	Docosanoic acid	C22:0
Palmitic acid (Latin: *palma*, "palm tree") *Palmitic acid was discovered by E. Frémy in 1840, in saponified palm oil.*	*n*-Hexadecanoic acid	C16:0	Lignoceric acid (Latin*: lignum*, "wood" and *cera*, "wax") **Other name includes**: Tetracosanoic Acid *Tetracosanoic acid was isolated by J. Cason and R.J.J. Anderson in 1983 from the hydrolysis products of bovine wax.*	*n*-Tetracosanoic acid	C24:0
Stearic acid (Greek: *stear*, "hard fat") *Stearic acid was first described by M.E.* ***Chevreul*** *in 1823.*	*n*-Octadecanoic acid	C18:0			

Common name (name derivation and/or acronym) ***History and/or information***	**Scientific name**	**Symbol**	**Common name (name derivation and/or acronym)** ***History and/or information***	**Scientific name**	**Symbol**
Unsaturated fatty acids					
Palmitoleic acid *Palmitoleic acid was first described by P.G. Hofstädter in 1854 in whale sperm oil and named physetoleic acid. The molecular composition ($C_{16}H_{30}O_2$) was proposed by H. Bull in 1906 whereas the actual name was given by Lewkowitsch. Only in 1925 was the structure finalised by E.F. Armstrong and co-workers.*	*cis*-9-Hexadecenoic acid	C16:1ω-7	Linoleic acid (Greek: *linon*, "flax", **LA**) *The essentiality of the linoleic and linolenic acids was discovered by G.O. Burr and M.L. Burr in 1930. In 1940 D.E. Dolby and co-workers identified linoleic acid as a possible precursor for arachidonic acid.*	*cis*,*cis*-9,12-Octadecadienoic acid	C18:2ω-6
Vaccenic acid (Italian: *Vacca* "cow"), also known as asclepic acid *Vaccenic acid was discovered in 1928 by S.H. Bertram in animal fats and butter.*	*trans*-1-Octadenoic acid	C18:1ω-7	Alpha (α)-linolenic acid (**ALA**) *In 1923, essential fatty acids were discovered and called vitamin F. In 1930 G.O. Burr, M.M. Burr and E. Miller classified EFAs as fats and not vitamins. In 1950, R. Holman and C. Widmer, showed that PUFAs consist of two separate families of fatty acids: linoleic acid is the precursor for arachidonic acid (ω-6 family) while α-linolenic acid is the precursor for docosahexaenoic acid (ω-3 family).*	*cis*,*cis*,*cis*-9,12,15-Octadecatrienoic acid	C18:3ω-3

Common name (name derivation and/or acronym) ***History and/or information***	**Scientific name**	**Symbol**	**Common name (name derivation and/or acronym)** ***History and/or information***	**Scientific name**	**Symbol**
Unsaturated fatty acids					
Oleic acid (Latin*: oleum*, "oil", **OA**) *Oleic acid was discovered in 1846 by M. E. Chevreul and in 1898 F.G. Edmed formalized the structure of oleic acid.*	*cis*-9-Octadecenoic acid	C18:1ω-9	*Eicosadienoic acid* (**EDA**) ***Other names include:*** (11Z,14Z)-Eicosadecadienoic acid; 11,14-all-cis-eicosadienoic acid; all-cis-11,14-eicosadienoic acid; cis,cis-delta11,14-eicosadienoic acid; homo-gamma-linoleic acid *Eicosadienoic acid is a rare but natural occurring ω-6 PUFA and is exclusive to animal tissues (Huang et al., 2011.)*	*cis,cis*-11,14-Eicosadienoic acid	C20:2ω-6
Gamma(γ)-linolenic acid (**GLA**) *Gamma-linoleic acid was first described by A. Heiduschka and co-workers in evening primrose and its structure was elucidated in 1927 by A. Eibner and co-workers.*	*cis,cis,cis*-6,9,12-Octadecatrienoic acid	C18:3ω-6	Eicosatrienoic acid (**ETE**) *Eicosatrienoic Acid is also a rare ω-3 PUFA but is known to be one of the most active essential fatty acids (Holman, 1986).*	*cis,cis,cis*-11,14,17-Eicosatrienoic acid	C20:3ω-3
Gondoic acid **Other names include**: *cis*-Gondoic acid *cis*-11-Eicosenoic acid 11-Eicosenoic acid 11Z-Eicosenoic acid *cis*-11-Icosenoic acid (11Z)-Icos-11-enoic acid	*cis*-11-Eicosanoic acid	C20:1ω-9	Dihomo-gamma (γ)-linolenic acid (**DGLA**) *Dihomo-γ-linolenic acid was isolated in 1964 by D. van Dorp.*	*cis,cis*-8,11,14-Eicosadienoic acid	C20:3ω-6

Common name (name derivation and/or acronym) ***History and/or information***	**Scientific name**	**Symbol**	**Common name (name derivation and/or acronym)** ***History and/or information***	**Scientific name**	**Symbol**
Unsaturated fatty acids					
Arachidonic acid (Latin: arachidic, *of the groundnut*, Arachis, *groundnut genus,* **AA**) *Arachidonic acid was first isolated in by G.Y. Shinowara and co-workers in 1940 from PLs from beef suprarenal glands and its structure was elucidated three years later (in 1943) by C.L. Arens and co-workers.*	*cis,cis,cis,cis*-5,8,11,14-Eicosatetraenoic acid	C20:4ω-6	Erucic acid (New Latin*: cabbage genus*, Latin: *rocket, cabbage)* **Other name includes***:* 13-docosenoic acid *Erucic acid was first descried in 1849 by S. Darby in rapeseed oil and followed by the first isolation by F. Websky in 1953.*	*cis*-13-Docosenoic acid	C22:1ω-9
Eicosapentaenoic acid (Greek: *eikosa*, "twenty" and *pentaene*, "five double bonds", **EPA**) **Other name includes:** Icosapentaenoic acid Timnodonic acid *Eicosapentaenoic acid named timnodonic acid by Y. Toyama and co-workers who isolated this fatty acid 1935 from sardine oil.*	*cis,cis,cis,cis,cis*-5,8,11,14,17-Eicosapentaenoic acid	C20:5ω-3	Docosadienoic acid *In 1950, C. Widmer and R. T. Holman described the conversion of linolenic acid to the highly unsaturated fatty acids of 20 and 22 carbon atoms (Mohrhauer and Holman, 1963)*	*cis,cis*-13-16-Docosadenoic acid	C22:2ω-6

Common name (name derivation and/or acronym) ***History and/or information***	**Scientific name**	**Symbol**	**Common name (name derivation and/or acronym)** ***History and/or information***	**Scientific name**	**Symbol**
Unsaturated fatty acids					
Adrenic acid *In 1963, T.C. Lochang and C.C. Sweeley while developing a method to locate double bonds in polyenoic fatty acids from lipids isolated from canine adrenal glands, they identified a novel fatty acid (7,10,13,16-docosatetraenoic acid) and called it adrenic acid because of its apparent abundance in the adrenal gland.*	*cis,cis,cis,cis*-7,10,13,16-Docosatetraeonic acid	C22:4ω-6	Docosapentaenoic acid (**DPA**) **Other name includes:** Clupanodonic acid *In 1960, E. Klenk and H. Mohrhauer fed synthetic C^{l4}-1abeled compounds to rats and described the intermediate steps in the synthesis of docosahexaenoic acid from linolenic acid via eicosapentaenoic acid and docosapentaenoic acid.*	*cis,cis,cis,cis,cis*-7,10,13,16,19-Docosapentaenoic acid	C22:5ω-3
Omega (ω)-6 Docosapentaenoic acid **Other name includes:** Osbond acid *In 1961, J.C. Kirschman and co-workers observed an age-related increased in docospentaenoic acid (C22:5ω6) in rat testes and concluded that lipids may have an essential role in the maturation of the testis.*	*cis,cis,cis,cis,cis*-4,7,10,13,16-Docosapentaenoic acid	C22:5ω-6	Docosahexaenoic acid (Greek: *docosa*, "twenty-two" and *hexa*, "six", **DHA**) **Other name includes:** Cervonic acid *Docosahexaenoic acid was isolated from fish oil in 1942 by S. Matsuda and the first total synthesis was done by H.J.J. Pabon and co-workers in 1965.*	*cis,cis,cis,cis,cis,cis*-4,7,10,13,16,19-Decosahexaenoic acid	C22:6ω-3

Common name (name derivation and/or acronym) *History and/or information*	Scientific name	Symbol
Unsaturated fatty acids		
Nervonic acid *Nervonic acid, was described in 1972 by M. Tsujimoto in Elasmobranch fish (original name: selacholeic acid) whereas E. Klenk (1927) isolated and elucidated the structure of this fatty acid in cerebrosides from brain tissue.*	*cis*-15-Tetracoseanoic acid	C24:1ω-9

Adapted from Mohrhauer and Holman, 1963; Du Pont, 1990; Huang *et al*., 2011.

2.2.4.2. Fatty acid synthesis

The synthesis of FAs are relatively low in normal adult cells expect in the brain, liver, adipose and lung tissue (Jayakumar *et al*., 1995; Semenkovich *et al*., 1995). Dietary sources of fat also form an important way of cells to meet their metabolic requirements of these biomolecules. Synthesis starts in the cell cytosol and include two major steps: i) ATP-dependent carboxylation of acetyl-CoA by the enzyme acetyl-CoA carboxylase to form malonyl-CoA and ii) cyclic process where a acetyl primer and seven malonyl extender molecules undergo Claisen condensation, followed by three steps of ketoreduction-dehydration-enoyl reduction by different FAs synthase enzymes and finally hydrolysis to form palmitic acid (C16:0) (Wakil, 1989; Rangan and Smith., 2002) (Figure 2.7). Longer chained FAs such stearic acid (C18:0) up to C26 are synthesised from C16:0 by elongase and desaturase enzymes within the ER and to a lesser extend in the mitochondria.

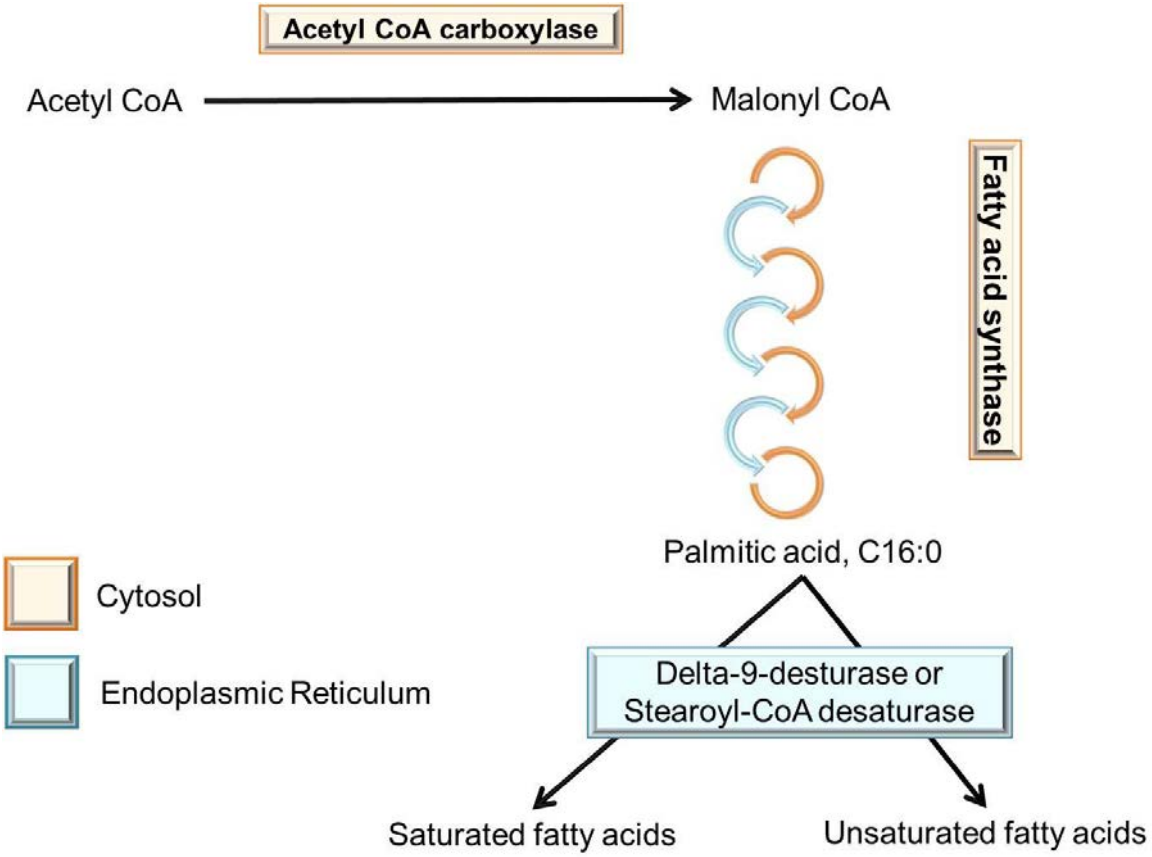

Figure 2.7: Fatty acid biosynthesis

Mammalian cells are able to synthesised *de novo* PLs, CHOL, SFAs and MUFAs however they are not capable of introducing a double bond beyond the delta-9 position in FA chains. Only the ω9 PUFAs are synthesised endogenously by eukaryotic cells via the enzyme delta-9-destaurase. Therefore the essential PUFAs, linoleic acid (LA, C18:2ω-6) and α-linoleic acid (ALA, C18:2ω-3), precursors for all the C20 and C22-carbon polyenioc products needs to be ingested via the diet (Ziboh and Chapkin, 1984; Benatti *et al*., 2004; Catalá, 2009). Following ingestion, these non-esterified essential FAs are transported by FA transporters and converted to FA acyl-CoA thioesters. Before undergoing elongation, saturation and desaturation the FA acyl-CoA substrates are utilised for the production of ATP via β-oxidation. Various lipid-metabolising enzymes is responsible for transforming C18:2ω-6 and C18:2ω-3 into their long chained unsaturated derivatives such as arachidonic acid (AA, C20:4ω-6), eicosapentaenoic acid (EPA, C20:5ω-3) and docosahexaenoic (DHA, C22:6ω-3). Of particular importance is the rate-limiting delta-6-desaturase

enzyme that converts C18:2ω-6 and C18:2ω-3 into γ-linolenic acid (GLA, 18:3ω-3) and octadecatetraenoic acid (stearidonic acid, C18:4ω-3), respectively. This enzyme together with other desaturase and elongate enzymes have a higher affinity for C18:2ω-3 (ω-3 pathway) than C18:2ω-6 (ω-6 pathway) followed by oleic acid (a non-essential FA, OA or C18:1ω9) (Kassem *et al*., 2012). This competition that exists between C18:2ω-6, C18:2ω-3 and C18:1ω9 affect a regulatory mechanism whereby low concentrations of C18:2ω-3 can inhibit the C18:2ω-6 metabolism and higher levels of C18:2ω-6 is required to suppress the C18:2ω-3 pathway. C18:1ω9 would require even higher levels to suppress the C18:2ω-6 pathway. It is because of this affinity or competition that dietary intake of FAs, the type, amount and ratio (ω-3 to ω-6) will influence the FA composition of PLs, cell membranes, plasma and tissue. The regulation of PUFAs and especially long chain PUFAs remains an important field of research. For instance during high dietary C18:2ω-3 intake, both C20:5ω-3 and docosapentaenoic acid (DPA, C225ω-3) levels increased but the production of C22:6ω-3 is limited (Sanders and Roshanai, 1983; Emken *et al*., 1992; Burdge and Wootton, 2002; Burdge *et al*., 2002). Possible reasons for this include the different metabolic destinies of C18:2ω-3 and C22:6ω-3 and the competition between ω-3 and ω-6 as substrates for the respective desaturase enzymes (Sprecher, 2002). In addition it has also been shown that tetracosapentaenoic acid (C24:5ω-3) and C18:2ω-3 also compete for the same desaturase enzyme placing a further metabolic strain on the conversion of C18:2ω-3 to C22:6ω-3 (Portolesi *et al*., 2007). Tetracosahexaenoic acid (C24:6ω-3) and tetracosapentaenoic acid (C24:5ω-6) are formed via two elongation and one desaturase (by delta-6-desaturase) steps and moved from the ER to the peroxisomes by the so-called Sprecher's shunt (Sprecher and Chen, 1999). In the peroxisomes these two PUFAs undergo beta-oxidation to produce C22:6ω-3 and docosapentaenoic acid (osbond acid, C22:5ω-6) (Sprecher and Chen, 1999).

2.2.4.3. Eicosanoids

Arachidonic acid, dihomo-γ-linolenic acid (C20:3ω6), C20:5ω-3 and C22:6ω-3 and are substrate for the production of potent transient hormone-like lipids called eicosanoids (eicosa = 20) such as prostaglandins (PG), prostacyclins, thromboxanes

hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT) (Ziboh, 1973; Marcelo and Voorhees, 1983; Leaf and Weber, 1998; Shaikh and Edidin, 2006). Eicosanoids are formed during the release of specific PUFAs substrates from membrane PLs via the phospholipase enzymes. Once released these PUFA precursors are used for the synthesis of eicosanoids via the cyclooxygenase (COX); lipooxygenase (LOX), epoxygenase and cytochrome P450 dependent enzyme families. The function of eicosanoids include cell growth and differentiation, platelet aggregation, inflammation, hemorrhage, vasoconstriction and vasodilatation, blood pressure and immune functions (Zhou and Nilson, 2001; Larsson *et al*., 2004, Berquin *et al*., 2008). Generally, ω-3 derived eicosanoids are known to have anti-inflammatory effects whereas ω-6 derived eicosanoids are pro-inflammatory (Calder *et al*., 2002). Omega-3 PUFAs can also be metabolised to form docosanoids (resolvins and protectins) that also have anti-inflammatory and immune-modulatory functions (Serhan *et al*., 2002, Serhan *et al*., 2004). Apart from the opposing biological effects of ω-3- and ω-6 derived eicosanoids, ω-3 PUFAs at higher concentration and thus availability, will compete for the COX and LOX enzymes at the expense of ω-6 PUFA eicosanoid precursors such as C20:4ω-6 (Needleman *et al*., 1976; Wada *et al*., 2007). Arachidonic acid is metabolised to produce the "2"-series prostaglandins (PGE_2, PGI_2 PGD_2 and PGE_2), thromboxane A_2 and B_2, 5-HETE, 5-hydroperoxyeicosatetraenoic acid, the "4"-series leukotrienes (LTA_4, LTB_4 and LTE_4), and lipoxin A_4. Additional epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acid (DHETs) can also be formed from the C20:4ω-6-epoxide hydrolase dependent metabolism. Another ω-6 PUFA, C20:3ω6 are metabolised to eicosanoids of the "1"–series such as PGE_1, PGD_1 and 15-HETE (Iversen et al., 1992; Dooper *et al*., 2002). The ω-3 precursor C20:5ω-3, function as a substrate for the formation of the "3- series prostaglandins (PGE_3), the "5"-series leukotrienes (LTB_5), "3"-series thromboxanes and docosanoids (Larsson *et al*., 2004; Zamaria, 2004; Rizzo *et al*., 2010). Whereas C22:6ω-3 can be metabolised for the production of various resolvins and docosanoids molecules (Serhan *et al*., 2004). Figure 2.8 summarises the ω-3, ω-6 PUFA and eicosanoid biosynthesis.

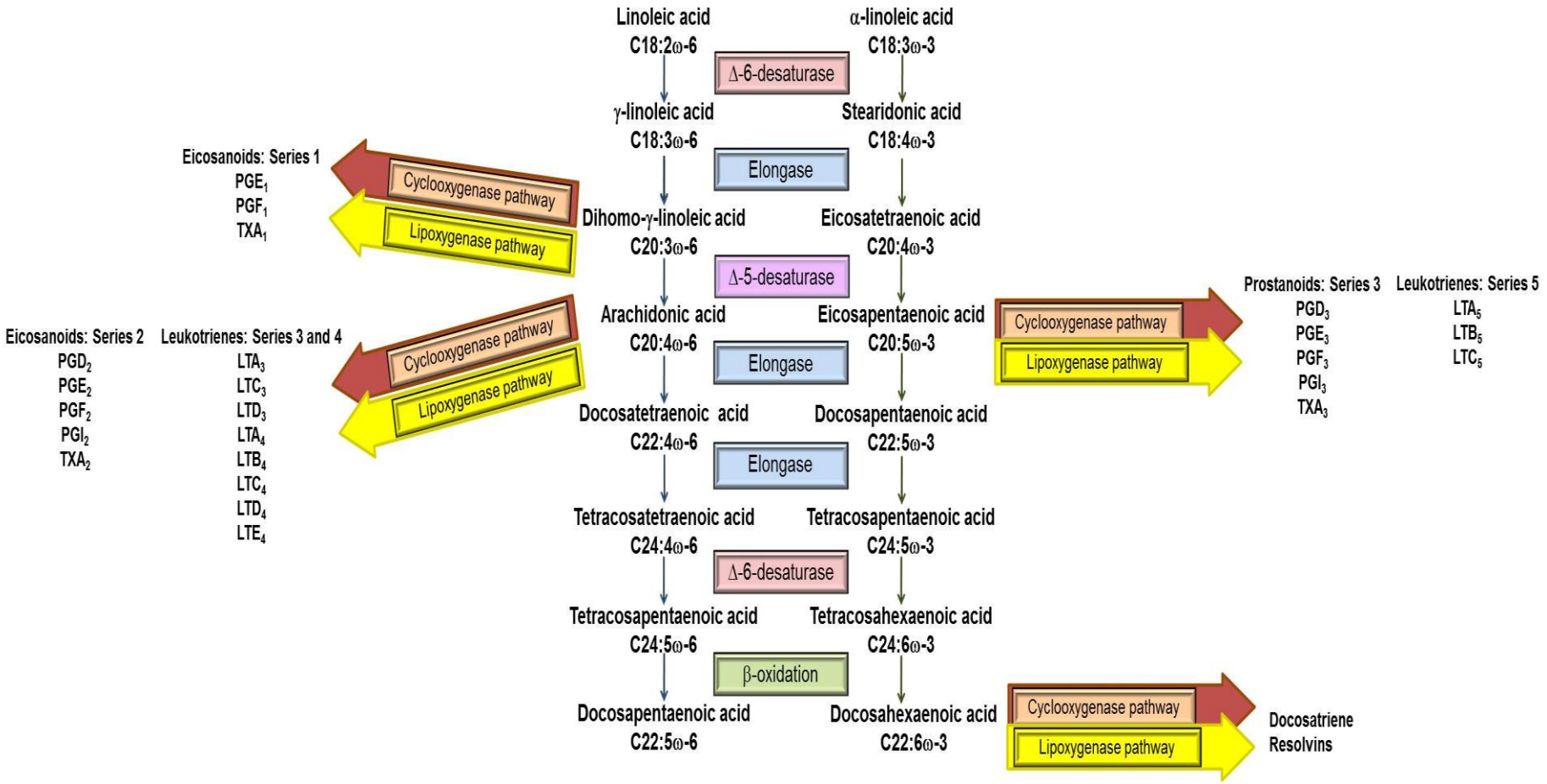

Figure 2.8: Synthesis of ω-3, ω-6 polyunsaturated fatty acids and eicosanoids

2.2.5. Cholesterol

2.2.5.1. Cholesterol structure and function

Cholesterol is a sterol isoprenoid composed of a semi-rigid tetracyclic ring with a hydroxyl group and an 8-carbon chain attached to the carbon-17 position (Figure 2.9). Cholesterol forms a major constituent of all eukaryotic cell membranes including the membranes of cytoplasmic vesicles such endosomes, lysosomes and distal Golgi-apparatus (Lange *et al*., 1999; Maio *et al*., 2002; Ohvo-Rekila *et al*., 2002; Mouritsen and Zukermann, 2004; Maxfield and Menon, 2006; Brusselman *et al*., 2007; Buhaescu and Izzedine, 2007; Montero *et al*., 2008). The distribution of CHOL in bilayer membrane is asymmetrically with more (60 to 70%) of the sterols situated in the inner membrane leaflet (Shroeder *et al*., 1991; Mondal *et al*., 2009). The interaction between CHOL and PLs in membranes is complex and currently there are four conceptual models which include i) the Condensed Complex model (Radhakrishnan *et al*., 2005); ii) the Superlattice model (Chong, 1994); iii) the Umbrella model (Huang and Feigenson, 1999) and iv) the Collective ordering process (Martinez-Seara *et al.*, 2010).

The chemical structure of CHOL with its small head group and rigid ring formation prevents sterol-sterol interaction and exposure of the hydrophobic membrane to water (Martinez-Seara *et al.*, 2010). In return PC, often present between two CHOL molecules can shield CHOL from water with its large polar head. The stereochemical complexes that form between CHOL and PLs in membrane bilayers play an important role in membrane fluidity; permeability and function by influencing the PL's lateral FA acid chain-order and membrane condensation (Bloom *et al*., 1991; Leventis and Silvius, 2001; Gibbons, 2003; Lange and Steck, 2008). An important functional relationship also exists between CHOL and SM due to their close proximity due to CHOL preference to saturated PL molecules with large polar heads such as PC and SM (Wattenberg *et al*., 1983; Van Bitterswijk *et al*., 1987; Lund-Katz *et al*., 1988; Leventis and Silvius, 2001; Martinez-Seara *et al.*, 2010). Cholesterol has a poor affinity for PUFAs acyl chains and the incorporation of PUFAs into cell membrane leads to the removal of CHOL. This non-homogenous distribution of CHOL and its association with SM possibly enhances the formation of defined microdomains called LRs (Brown and Rose, 1992; Rietveld and Simons, 1998;

Simons and Ikonene, 1997; Niu and Litman, 2002; Gibbons, 2003; Garmy *et al*., 2005). Apart from being an important structural molecule, CHOL is also a functional substrate for the formation of bile acids in the liver, steroid hormones in steroidogenic tissue such as the adrenal glands, ovaries, testes and vitamin D in the skin. Oxidation of CHOL, the oxysterols are also important cell signaling molecules that associates with nuclear receptors to affect gene expression (Maxfield and Menon, 2006; Schroepfer, 2000).

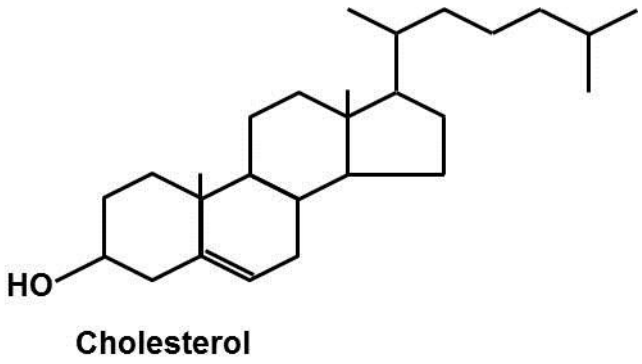

Figure 2.9: Chemical structure of cholesterol

2.2.5.2. Cholesterol synthesis

All cells can synthesise CHOL however the removal of CHOL is restricted to the liver and steroidogenic tissue. Regulation of the CHOL metabolism and homeostasis consist of a feedback mechanism operational at a transcriptional and posttranscriptional level (Goldstein and Brown, 1990; Okazaki *et al*., 2006). The transcriptional level includes the regulation by Insig- (ER membrane-bound proteins derived from INSIG: insulin-induced genes) SCAP-SREBP complexes within the ER (Horton *et al*., 2002; Maxfield and Tabas, 2005; Du *et al*., 2006). Sterol regulatory element-binding proteins cleavage activating proteins (SCAP) enable the translocations of membrane-bound sterol regulatory element-binding proteins (SREBP) to migrate from the ER to the Golgi-apparatus in response to CHOL depletion within the cell. At the Golgi-apparatus, SREBP are proteolytically cleaved and enters the nucleus where it activates the transcription of specific genes encoding CHOL biosynthetic enzymes and the lipoprotein receptors involved in the uptake and synthesis of CHOL (Sakai *et al*., 1996; Brown and Goldstein, 1997; DeBose-Boyd *et al*., 1999; Heemers *et al*., 2001; Nohturfft *et al*., 2000; Horton *et al.,* 2002). In the

liver, SREBP enhances the expression of low-density lipoproteins receptors which in return bind to low density lipoproteins (LDL) molecules resulting in the internalization of circulating CHOL. Cells treated with CHOL or oxysterols such as 25-hydroxycholesterol, the formed SCAP-SREBP complexes remains within the ER and transport from the ER to the Golgi-apparatus is abolished by the binding of SCAP to Insigs. The resultant block in SREBP activation and effect on gene transcription reduce the biosynthesis of CHOL (Adams *et al*., 2004).

CHOL can be synthesized *de novo* in the liver via the mevalonate pathway or made available by endocytosis of lipoproteic carriers that transports dietary-absorbed CHOL. Esterified CHOL, cholesterylesters, are hydrolyzed within the core of the lipoproteins' endosomes and lysosomes and release within the cell (Goldstein *et al*., 2006; Maxfield and Menon, 2006). These lipoprotein carriers consist of apolipoproteins and PLs aggregating into spherical shaped particles enclosing neutral lipids such as CHOL, cholesteryl esters and triglycerides (Tosi and Tugnoli, 2005). Lipoproteins are grouped into four main classes according to their densities (i) chylomicrons; (ii) very low-density lipoproteins (VLDL); (iii) low-density lipoproteins (LDL) and (iv) high-density lipoproteins (HDL). Free cellular CHOL in excess can be esterified and stored in the cytoplasm in lipid droplets, these droplets can be hydrolysed by neutral esterases that will release CHOL and other FAs (Yeaman, 2004; Maxfield and Menon, 2006). Human studies have shown that individuals that synthesize CHOL has a lower dietary absorption in effect of the feedback control exerted by cellular CHOL has on the mevalonate pathway (Miettinen *et al*., 2000; Ness *et al*., 2000). The reverse was also true for individuals with a higher dietary absorption resulting in a reduced *de novo* synthesis of CHOL (Miettinen *et al*., 2000).

2.2.5.3. Cholesterol esters

The form in which CHOL is transported throughout the blood is also the way it accumulates in the cell, by the addition of a long-chain FA or esterification of CHOL to form cholesterylesters (Glomset and Norum, 1973; Glomset *et al*., 1995). These insoluble CHOL esters are constituents of the hydrophobic core of all plasma lipoprotein particles (Schwartz *et al*., 2004) and are produced by lecithin-cholesterol acyltransferase (LCAT) from fee CHOL on the surface of the plasma HDL (Glomset and Norum, 1973; Glomset *et al*., 1995). The uptake of CHOL esters from HDL are

regulated by SM and ceramide in the lipoprotein or from the cell membrane via a scavenger receptor class B type 1 (SR-B1) (Subbaiah *et al*., 2005)

2.2.6. Specialised membrane structures or microdomains: Lipid rafts

The existence of specialised membrane nanometric microdomains with unique lipid composition that compartmentalise cellular functions is a highly investigated and contested research topic. A central consensus about the structural constituents of microdomains has however remained and microdomains are regarded as a platform or LRs containing CHOL, SM and glycolphospholipids (PC, PE and PS) and specific proteins with lipid-modifications (Figure 2.10) (Brown and Rose, 1992; Simons and Ikonene, 1997; Fridriksson *et al*., 1999; Simons and Toomre, 2000; London, 2002; Pike *et al*., 2002; Bonnin *et al*., 2003, Pike *et al*, 2005; Patra, 2008; Wassall and Stillwell, 2009, Tekpli *et al*., 2010). During the earlier studies the saturation state of CHOL and SM (acyl chain) and its resistance to non-ionic detergents at 4°C was utilised as a method to isolate LRs and became a widely used method (Brown and Rose, 1992; Schroeder *et al*., 1994a; Parton and Simon, 1995; Brown, 1998). This method has been associated with the disruption of the natural LR constituents and the artificial formation of microdomains, often referred to as detergent-resistant membranes (DRMs), Triton X-100 resistant membrane complexes (TRMC), detergent-insoluble glycolipid-enriched (DIG) fraction or glycolsphingolipid-enriched membranes (GEM) (Zegers and Hoekstra, 1998; Hooper, 1999; Simons and Toomre, 2000; Brown, 2002).

Different detergent isolation methods also lead to vast differences between the qualitative and quantitative LR-associated lipid content and proteins (Locke *et al*., 2005). In this regard variability exists among extracted protein LR-constituents due to their resistance or solubility to a specific detergent used (Pike *et al*., 2005, Delaunay *et al*., 2008). Recent advances in both microscopic and spectroscopic techniques such as single-particle tracking, fluorescence correlation spectroscopy, fluorescence resonance energy transfer; stimulated emission depletion and fluorescence photoactivation localization microscopy has produced correlating evidence of “subcompertamentalisation” in cell membranes (Lingwood and Simons, 2010, Neumann *et al*., 2010). Lipid rafts is thought to exist asymmetrically in both the

extracellular and cytoplasmic monolayer of the membrane bilayer (Harder *et al*., 1998, Subczynski and Kusumi, 2003; Devaux and Morris, 2004; Fadeel and Xue, 2009). This segregation between outer an inner membrane LRs is made possible by the coexistence of different lipid phases (l_d – l_o phases) (Devaux and Morris, 2004). However, it is expected that inner and outer membrane LR will differ in constituents with the outer LRs thought to be more unstable compared to inner membrane LRs (Devaux and Morris, 2004; Yao *et al*., 2009). In instances where the outer and inner leaflet LR co-localised is due to involvement of transmembrane proteins (Pyenta *et al*., 2001; Kunzelman-Marche *et al*., 2002). The existence of these membrane organizing principles are seemingly not exclusive to plasma membranes and although controversial have been shown to exits in membranes of the Golgi-complexes, ER, nuclei and mitochondria (Eberle *et al*., 2002; Foster *et al*., 2003; Hayashi *et al*., 2003, Zheng *et al*., 2009; Bartoccini *et al*., 2011; Poston *et al*., 2011; Sorice *et al*., 2012).

The immiscibility of membrane lipids supports this segregation into ordered functional platforms that will associate with specific proteins (Simon and Toomre, 2000; Pike, 2003). The formation of LRs starts with the synthesis of CHOL in the ER and is assembled in the Golgi-apparatus where it is moved towards the cell surface (Simons and Ikonen, 1997). From the cell surface LR can be removed by endosomes back to the Golgi-apparatus or recycled (Brown and London, 1998). Two models have been proposed to elucidate the existence of LR within cell membranes. Model one describes the joining of SLs via the interaction between their head groups, the amide and hydroxyl/carboxyl groups with CHOL spaced between the SL head groups via the interaction of hydrogen bonds and van der Waals links between the SL amide group and the CHOL 3-OH-groups (Simons and Ikonen, 1997; Filippov *et al*., 2006). The second model ascribes the formation of these specialised platforms on the interaction of saturated acyl chains which in returns favours the incorporation of CHOL (London and Brown, 2000). Both peripheral and integral proteins that are associated with SFAs (palmitoyl or myristoyl moieties) or attached via a glycosyl phosphatidylinositol (GPI) respectively are abundant within LRs (Calder and Yaqoob, 2007). A subclass of LR the caveolea are flask-shaped invaginations of the bilayer cell membrane and are known for the distinct expression of the protein caveolin-1 (Pike *et al*., 2002; Li *et al*., 2006). Lipid rafts are essential

for numerous cellular functions including sorting of proteins (Keller and Simon, 1998), cell signaling (Stauffer and Meyer, 1997, Brown and London, 1998), production of reactive oxygen species and apoptosis due to LR clustering or aggregation by death receptor ligands and apoptotic factors (Zhang *et al*., 2006; Aliche-Djoudi *et al*., 2011) and transport (membrane trafficking and pathogen entry) (Kiyokawa *et al*., 2005; Garmy et al., 2005; Pike, 2003; Edidin, 2003 and Taïeb *et al*., 2004; Simon and Ikonene, 1997; Patra, 2008, Harder, 2004). Although many unresolved and controversial aspects of LRs still exist, their characteristics (size, constituents and form) are seemingly governed by metabolic demands rather than pure thermodynamics (Devaux and Morris, 2004).

2.2.7. Membrane proteins

An important function of lipid constituents within membranes is their interaction with integral or peripheral membrane proteins. The diverse biophysical properties of membrane lipid will also preferentially interact with different proteins constituting a converse relationship between membrane lipids and proteins (Tillman and Cascio, 2003). In this regard the changes in physical properties, membrane fluidity and thickness, membrane charge distribution and lateral pressure will affect the structure and function of membrane proteins (Tillman and Cascio, 2003). In model membranes proteins showed a distinct partiality to a particular phase (liquid or solid) within the membrane bilayer (Shogomori *et al*., 2005, Brown, 2006). Although the recruitment of proteins to biological membranes remains unclear, both the membrane lipids and proteins in effect have a part to play (Van Meer *et al*., 2008). Protein-lipid interactions are complex and divers in type and effect and apart from play important role in stabilising membrane proteins, cell signaling, enzyme activity and transport, they may exert chaperon-like function during the insertion, folding or assembling of proteins (Palsdottir and Hunte, 2004). Important membrane lipid such as PE can also assist with the folding of membrane proteins by acting as molecular chaperones (Bogdanov and Dowhan, 1998). Membrane proteins induce functional microdomains where a specific protein will interact and re-organise specific membrane lipid species to increase the bilayer thickness (Epand *et al*., 2001; Tillman and Cascio, 2003). The interaction between membrane lipids and proteins are

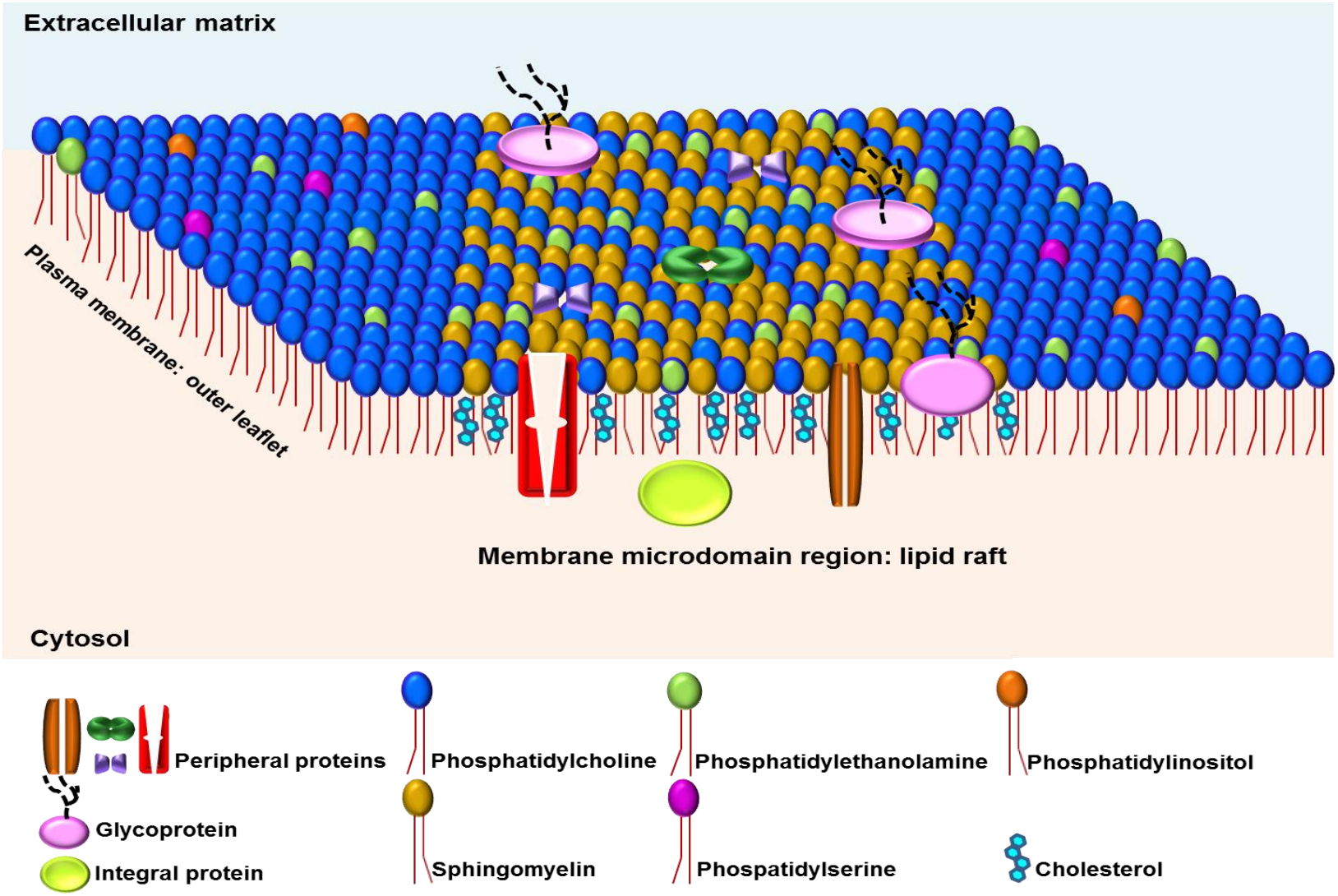

Figure 2.10: Outer membrane leaflets containing a lipid raft

complex with both parties able to exert functional and structural changes on the other.

2.2.8. Membrane fluidity

The fluid-nature of membranes governed by its lipid composition is crucial for the biological functions of both the membrane itself and membrane proteins. Membrane constituents like PLs and CHOL have been regarded as membrane fluidity modifying parameters. The specific PL content (80% of most PLs are PC, SM and PE), the size and hydration status of its head group, the fatty acyl chain length and FA composition responsible for the PLs degree of unsaturation have been implicated (Crowe *et al*., 1987; Treen *et al*., 1992; Escriba *et al*., 2008; M'Baye *et al*., 2008; Van Meer *et al*., 2008). For instance the head group of PC, choline is large with a cylindrical molecule geometry compared to PE with a small conical molecular geometry that allows for tither packaging of membrane constituents and thus a relative decrease in membrane fluidity (Van Meer *et al*., 2008). The ratio between MUFAs and SFA chains of PLs is known to directly affect fluidity. Phospholipids with saturated and monounsaturated acyl chains contribute to a more ordered membrane environment (decrease in membrane fluidity) compared to PUFA acyl chains, the latter having a high conformational flexibility (Feller *et al*., 2002; Shaikh *et al*., 2003, Feller and Gawrisch, 2005). Furthermore this high conformational flexibility depended on the acyl chain length and number of double bonds inevitably varies among PUFAs. Therefore, it would be expected that ω-3 and ω-6 acyl chains will affect membrane fluidity differently. In this regard C20:4ω-6-containing PL compared to C22:5ω-6-containing LPs, are more disordered and flexible (Rajamoorthi *et al*., 2005). For this purpose the inclusion of high dietary ω-3 PUFAs, especially C22:5ω-6 with its established health benefits also created the expectations of increasing membrane fluidity.

However, some studies failed to show an increase in membrane fluidity with increase fish oils consumption (Stillwell and Wassall, 2003). Possible explanation of this included the intricacy of changes in membranes already opulent in unsaturated FAs and C22:5ω-6 may affect fluidity only laterally. The lateral modulation of membrane

fluidity is seemingly accomplished by long-chain FAs (20 or more carbons) with their double bonds that affect disorder in the membrane bilayer's head groups (Stubbs and Smit, 1984; Stillwell and Wassall, 2003). Also Yang *et al*., (2011) indicated that PUFAs with 4 or more double bonds (C20:4ω-6, C20:5ω-3 and C22:5ω-6) increased membrane fluidity. The different functions of subcellular organelle also transpire into different membrane fluidity states, for example the plasma membrane is more rigid relative to that of the ER (Van Meer *et al*., 2008). Measuring membrane fluidity, ratios such as the PL/CHOL, PC/PE or PC/SM (the unsaturation index) has been regularly applied (Shinitzky and Inbar, 1976; Cooper, 1977; Owen *et al*., 1982; Abel *et al*., 2001). Recently, Fajardo *et al*. (2011) developed and compared an inclusive PL fluidity (PC/ (PE+SM) index to other ratios (PC/PE and PC/SM) and validated this index utilising a meta-analysis of studies done on mammalian membranes. Membrane fluidity is directly impacted on by temperature- and osmotic stress, diet, chemicals and genetic alterations (Los and Murata, 2004; Baum *et al.*, 2012).

2.3. CARCINOGENESIS, LIPID METABOLISM AND THE CELL MEMBRANE

2.3.1. Introduction

Cancer is a deleterious disease where a higher rate of cell proliferation exists at the expense of cell death in a compromised environment that is able to sustain an altered genotype. In 1924, Otto Warburg described for the first time the demand for cancer cells to increase their metabolism to sustain their rapid rate of proliferation (Warburg *et al.*, 1924). The so-called "Warburg effect" described the increased absorption and glycolysis of glucose in tumour cells compared to normal cells (Warburg, 1956). The shift from glucose catabolism by oxidative phosphorylation as observed in normal cells to an alternative glycolysis pathway in proliferation cancer cells, is necessary to provide energy and crucial building block such as carbohydrates, proteins, lipids and nuclei acids (Van der Heiden *et al*., 2009, 2010). The complex process of carcinogenesis (Figure 2.11) can be summarised in three steps: initiation (normal cell transformed); promotion (clonal expansion of initiated cells to preneoplastic cell) and progression (preneoplastic cells develop to neoplastic cells). During the process of carcinogenesis activating mutations in oncogenes are

progressively acquired while inactivating recessive mutations in tumour suppressor genes are taking place (Hanahan, and Weinberg, 2000).

2.3.2. Carcinogens

The conventional classification of carcinogens are: genotoxic or non-genotoxic (also known as tumour promoters, non-DNA reactive carcinogens or epigenetic carcinogens) (Weisburger, 2000; Bolt and Degen, 2004). Genotoxic carcinogens binds covalently to DNA causing the formation of DNA adducts, DNA damage, gene point mutations, deletions, insertions, recombination, rearrangement, amplification and chromosomal aberrations (Minamoto *et al*., 1999; Sugimura, 2000). The non-genotoxic carcinogens exhort their effect on numerous biochemical and cellular targets and are associated with chronic cell injury, chronic inflammation, generation of reactive oxygen/nitrogen species; lipid peroxidation, epigenetic effects, alterations in cellular signaling, inhibition of apoptosis, post-translational modifications, immunosuppression, endocrine modification and inhibition of gap-junction communication (Weisburger and Williiams., 1983; Purchase, 1994; Cohen, 1995; Cunningham, 1996; Klaunig *et al*., 1998; Minamoto *et al*., 1999; Lima and Van der Laan, 2000; Williams, 2001; Ellinger-Ziegelbauer *et al*., 2005). Genotoxic carcinogens play an important role during cancer initiation whereas non-genotoxins aid the clonal expansion of initiated cells, formation of preneoplastic foci and tumour growth (Schulte-Hermann *et al*., 1993; Stinchcombe *et al*., 1995; Kolaja *et al*., 1996; Mally and Chipman, 2002; Schrenk *et al*., 2004).

Non-genotoxins are characteristics by: being single organ- or tissue-specific, species- and gender-specific, induce response at high levels during chronic exposure and display a threshold dose effect where below no effects are observed (Ashby and Tennant, 1991; Barlow and Schlatter, 2010). The ability of non-genotoxins to inhibit apoptosis and mitotic suppression as well as to enhance replicative DNA synthesis and tumourigenesis (proliferation) remains both a challenge and point of much scientific interest (Cowles *et al*., 2007; Ellinger-Ziegelbauer *et al*., 2009). A correlation between regenerative cell proliferation and the induction of tumourigenesis have been established (Swenberg, 1993). Williams

(2008) applied different terminology to genotoxins and non-genotoxins using the same definitions, that of DNA-reactive and epigenetic carcinogen, respectively.

2.3.3. Initiation

The initiation of cancer entails the transportation, distribution and activation of a carcinogen to cause direct DNA damage (formation of DNA adducts) or induce epigenetic changes. These, together with germline or inherited genetic mutations can result in the activation of oncogenes (mutated proto-oncogenes) and the down regulation of tumour-suppressor genes. For a normal cell to become malignant four to five sequential genetic lesions need to accumulate in their key signaling pathways (Armitage and Doll, 1954; Laura and Pedraza-Fariña, 2009).

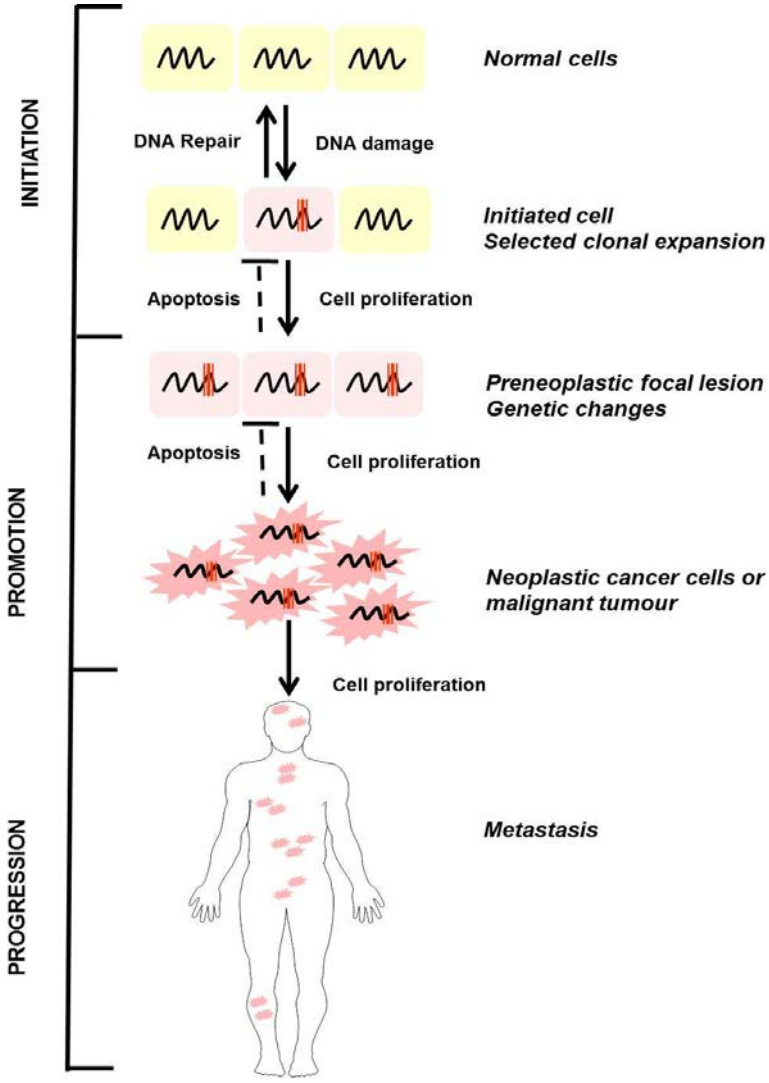

Figure 2.11: Carcinogenesis, initiation, promotion and progression

2.3.4. Promotion

Following cell replication of an initiated cell, its altered genotype now becomes a transformed phenotype within a favourable environment. The clonal expansion of initiated cells leads to tumour formation. Tumour promoters will have different effects on normal cells compared to initiated cell, by creating a growth differential where initiated cells escapes normal growth inhibitory signals and normal cells are removed (Solt and Faber, 1976; Andersen *et al*., 1995). The mechanism involved may include the cytotoxic induction of regenerative growth, hormone-stimulated growth and nuclear receptor antagonism involved in signaling (Pitot, 1990; Bock and Köhle, 2005). In this regard the role of COX-2 overexpression and consequential production of prostanoids and lipid peroxidation needs to be highlighted. These alteration reflecting the an increased AA-metabolism has been regarded as one of the critical events during human cancer promotion and a feature that is associated with no less than two third of all human neoplasms (Marks *et al*., 2007; Rizzo, 2011).

On the other hand, the inhibition of the relevant enzymes has shown great potential as chemopreventative strategies. Cyclooxygenase-2, activate during inflammatory and mitogenic stimuli, is able to act as a cancer promoter due to its ability to convert procarcinogens to active carcinogens and thereby increasing their oncogenic effects (Rizzo, 2011). The disruption of apoptosis by COX-2 a rate limiting enzyme in the production of PGs, include the reduction of the availability of intracellular levels of free AA (a known pro-apoptotic signal molecule) that is use a substrate for the production of various PGs (Prescott and Fitzpatrick, 2000). The alterations in important growth signaling pathways associated with overexpression of COX-2 includes the down-regulation of the transforming growth factor-beta (TGF-β) type II receptor and activation of the epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR) (Tsujii *et al*., 1995, Coffey *et al*., 1997; Dannenberg *et al*., 1999; Pai *et al*., 2002, 2003; Buchanan *et al*., 2003; Greenhough *et al*., 2009). Collectively these events lead to enhanced proliferation, survival, angiogenesis and migration as observed during tumour development and metastases. The resultant production of PGE_2 by COX-2 is responsible for the transactivation of the EGFR. In regards to the expression of EGFR, a surface

receptor with intrinsic tyrosine kinase activity has also been associated with numerous human cancers (Herbst and Shin, 2002). However, a positive feedback loop also exists between the EGFR and COX-2 signaling pathways (Choe *et al*., 2005). Epidermal growth factor (EGF) ligands bind to the EGFR causing an induction of COX-2 expression involving the ras/raf/mitogen-activated protein kinase (MAPK) cascades depending on the type of cell or stimuli. This interaction between COX-2/PGE_2 and EGFR is well documented and the inhibition of any one of these two pathways or both is warranted during chemotherapy.

2.3.5. Progression

During this step tumour cells are gradually transformed into invasive cells that have the ability to propagate and colonise (metastasise) elsewhere in the body. The elegant and comprehensive “hallmarks of cancer” developed by Hanahan and Weinberg (2011) outlines the biological characteristics of carcinogenesis and contributes substantial to our understanding of the complexity of cancer. These concepts are summarised in Figure 12.

2.3.6 Epigenetic control of cancer

Carcinogenesis is currently view as the consequence of both genetic and epigenetic aberrations (Kanwal and Gupta, 2010). DNA methylation, chromatin modifications (covalent changes in the core histones), physical alterations to nucleosome (or nucleosome positioning) and the noncoding micro-RNA regulation of posttranscriptional genes are the central process associated with normal epigenetic regulation (Ducass and Brown, 2006; Kanwal and Gupta, 2010). The reprogramming of normal epigenetic control during carcinogenesis is currently not fully understood. Seemingly, alterations in DNA methylation and histone modifications can operate independently to silence regulatory genes (such as tumour suppressor genes, p16 and p53) (Hitchler and Domann, 2009; Sharma *et al*., 2010). This, together with altered expression of chromatin-regulating enzymes possible by the loss of cofactors, induces an epigenetic switch and cancer cells with a new epigenotype compared to normal cells. This aberrant epigenomics or “epigenetic switch” play a role during all the stages of carcinogenesis (cancer initiation, promotion, invasion,

metastases) including chemotherapy resistance (Gupta and Massague, 2006; Gronbaek *et al*., 2007; Chiang and Massague, 2008). The cancer phenotype or "hallmarks of cancer" developed by Hanahan and Weinberg (2011) (Figure 2.12) complements the loss of epigenetic control. Therefore, cancer epigenetics can be associated with key metabolic changes (Hitchler and Domann, 2012). For instance histone acetylation (epigenetic alteration where acetyl-CoA acts as a cofactor during protein acetylation) and increased FA production (where a concomitant increase in fatty acid synthase expression will utilise acetyl-CoA as a precursor for FA production) are linked by sharing a cytosolic acetyl-CoA pool that is produced during the increased glycolytic activity of the Warburg effect in tumour cells (Wellen *et al.*, 2009, Hitchler and Domann, 2012; Zhou *et al*., 2012).

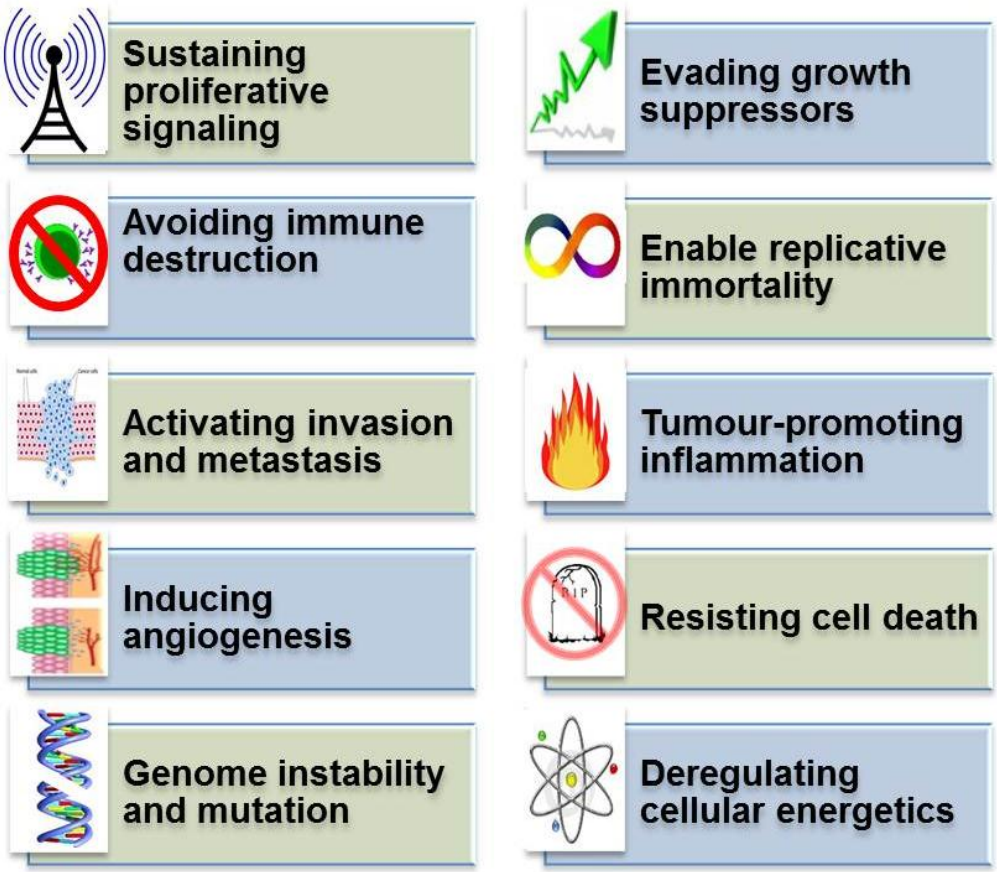

Figure 2.12: The ten hallmarks of cancer
(Adapted from Hanahan and Weinberg, 2011)

2.3.7. The role of the lipid metabolism and cell membrane integrity during carcinogenesis

Carcinogenesis changes the biosynthesis and bioenergetics needs of tumour cells. Disruption of the lipid metabolism is considered to be an early event and a hallmark of carcinogenesis (Milgraum *et al*., 1997; Piyathilake *et al*., 2000; Menendez and Lupu, 2007). A “lipogenic phenotype” arises whereby numerous lipogenic enzymes (such as fatty acid synthase, acetyl-CoA carboxylase and ATP citrate lyase) are overexpressed and activated resulting in an increase *de novo* lipogenesis (Kuhajda, 2006; Swinnen *et al*., 2006; Menendez and Lupu, 2007; Brusselmans and Swinnen, 2009). This has been observed in cancer of the endometrial, ovarian, gastric oesophageal, pulmonary, colon, liver, prostate and breast (Pizer *et al*., 1996; Swinnen *et al*., 2006; DeBerardinis *et al*., 2008, Lisboa *et al*., 2008). Lipid metabolism is associated with numerous oncogenic and tumour suppressor pathways including that of the epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), Myc and p53 (Menendez and Lupu, 2007; Tennant *et al*., 2010). The progressive activation of oncogenic pathways during cancer influences the lipid metabolism at transcriptional, translational and post-translational level (Bhalla *et al*., 2011; Guo *et al*., 2011; Hilvo *et al*., 2011; Zhang, 2012).

The enhanced lipogenesis is thought to provide cancer cells metabolic autonomy, to drive rapid proliferation, activate growth signals and prevent apoptosis (Little *et al*., 2007; DeBerardinis *et al*., 2008, Vazquez-Martin *et al*., 2008). Eukaryotic cells are unable to produce ω-3 and ω-6 FAs and cancer cells therefore have shown an increase *de novo* synthesis of SFAs and/or MUFAs together with an increase in PL saturation (Rysman *et al*., 2010). Due to the decrease in PUFAs, cancer cells are also less susceptible to lipid peroxidation and oxidative stress induced apoptosis (Rysman *et al*., 2010). The disruption in the lipid metabolism will inevitably impact on the cell membrane, its constituents and physical state, functions and this effect has been associated with numerous pathologies (Hendrich and Michalak, 2003; Escribá, 2006). In consequence of the above, damaged, altered, up- or down-regulated membrane protein receptors/enzymes will influence normal transmembrane signal transduction that may also lead to carcinogenesis. Apart from the genetic-driven

carcinogenesis, membrane damage incurred during free radical-induced lipid peroxidation may also lead to DNA alterations and trigger carcinogenesis (Wiseman, 1996).

2.3.7.1. Phospholipids

Changes in membrane PLs are associated with the transformation of cancer cells, tumourigenicity and metastasis (Roos and Choppin 1985; Dahiya *et al*., 1992). Increased PC metabolism where both cellular and membrane levels of PC are increased have been associated with the mitogenic activation of growth factors. PC biosynthesis has been associated with the regulation of cell proliferation in cancer cells whereas in normal cells, the perturbation of this biosynthesis leads to apoptosis (Cui *et al*., 1996; Jackowski, 1996; Baburina and Jackowski, 1998; Finney *et al*., 2000). This increased PC metabolism is thought to be associated with the rapid growth and proliferation of tumour cells (Smith *et al*., 1991; Negendank, 1992; Ruiz-Cabello and Cohen 1992). Aboagye and Bhujwalla (1999) utilising ten cell lines representing different stages of carcinogenesis, indicated a gradual increase in choline metabolism from normal < immortal < oncogene transformed < tumour derived cells. They ascribed the increased choline metabolites in tumour cells to the acquired genetic changes that occur during carcinogenesis and not just the increased metabolic requirements for lipids and/ or FAs. Decreased levels in membrane PC was observed both rat and human hepatocellular carcinoma nodules compared to the surrounding normal cells. This was attributed to a shift to include more PE in tumour membranes (Abel *et al*., 2001, 2009). In addition to PC, increased levels of PE, SM and to lesser extend PI were also seen in tumour cells whereas only changes in the distribution of PS were observed (Kim *et al*., 2002; Ackerstaff *et al*., 2003; Dobrzynska *et al*., 2005; Di Paolo and De Camilli, 2006; Dória *et al*., 2011).

The increased levels of both PLs and SLs with their various metabolites, inevitably contributes to proliferation, survival and migration and angiogenesis during carcinogenesis. The increase in *de novo* lipogenesis and the resultant increase in endogenous levels of SFA and MUFA in tumour cells require an increased expression and activity of stearoyl-CoA desaturase (SCD or fatty acyl-CoA delat-9-desaturase) to convert C16:0 and C18:0 to C16:1 and C18:1, respectively (Igal,

2010; Roongta *et al*., 2011). The newly formed MUFAs in tumour cells are utilised to meet the high demand of acylation reactions and the production of lipids during proliferation. Overexpression of specifically SCD1 and a concomitant increased activity has been observed in colonic and oesophageal carcinomas, hepatocellular adenomas, hepatocarcinomas and chemically induced cancers (Li *et al* 1994; Lu *et al*., 1997; Thai *et al*., 2001; Yahagi *et al.*, 2005).

2.3.7.2. Sphingolipids

Altered SL metabolism has been observed in several cancers. Both SM and ceramide, playing a central role in signaling are associated with apoptosis (inactivation of anti-apoptotic molecules such as Bcl-2); antiproliferative (inactivation of AKT, a protein kinase B and protein kinase C) and proliferation (So-1-phosphate increases the activity of phospholipase C and MAPK) (Hannun and Linardic, 1993; Spiegel and Milstien, 2003; Duan, 2006). Although ceramide is associated with apoptosis, its conversion to So and ultimately So 1-phosphate by acid ceramidase causes cell proliferation. In normal cells the presence of a SL rheostat is seemingly responsible for the mechanistic outcome (Maceyka *et al*., 2002). The cellular levels of SM in tumour cells differ depending on the cell model, experimental conditions and stage of the cancer. Since certain cancers such as colon, liver and prostate cancer showed an increased whereas a decrease are associated with breast, leukemic oesophageal and brain cancers (Dahiya *et al*., 1992, Hendrich and Michalak, 2003). Tumour cells, in an attempt to escape apoptosis via ceramide, seemingly favour the conversation of ceramide to the mitogenic and/or proliferative SL substrates (Ogretmen and Hannun, 2004; Saddough *et al*., 2008; Omotuyi *et al*., 2011). This is accomplished by the overexpression of numerous SL-associated enzymes that reduce ceramide accumulation including, SMS (conversion of ceramide or PC to SM and DAG), acid CDases (ceramide converted to So), SK (conversion of So to So 1-phosphate), CK (conversion of ceramide to ceramide-1-phosphate) and GCS (production of glucosylceramide by adding glucose to ceramide) (Van den Hill *et al*., 1985; Dahiya *et al*., 1992; Elojeimy *et al*., 2007; Ryland *et al*., 2011).

In liver and prostate cancer, the increase in cellular SM cells was linked to the enhanced activity of SMS associated with the rapid proliferation of highly malignant

cells (Van den Hill *et al*., 1985; Dahiya *et al*., 1992). Down-regulation of various ceramide generating enzymes has also been observed in tumour cells such as SPT (first enzyme in the de novo synthesis of ceramide), neutral SMase (SM to Ceramide); CS (So to ceramide), So 1-phosphate lyases (So 1-phosphate to So) and phosphatases (So 1-phosphate degradation) (Kim *et al*., 2008; Beckham *et al*., 2010). The decrease in subcellular site-specific availability of intracellular SM or misdirection of SM have also been linked to malignant cells ability to escape apoptosis (Modrak *et al*., 2000; Ogretmen and Hannun, 2001; Radin, 2001; Andrieu-Abadie and Levade, 2002). The role of membrane SM in LRs and cancer is not clear except the deactivation of SMS resulted in a decrease in LR-associated SM as seen in glioma, leukemia, and lung cultured cancer cells (Lafont *et al*., 2010; Barceló-Coblijn *et al*., 2011). Disruption of LRs during carcinogenesis impairs curtail signaling pathways, for instance in colon cancer it has been shown that LR-associated proteins such as Fas death receptors observed in normal cells, were not present in malignant cells LRs (Michel and Barkovic, 2007). The absence of the Fas apoptotic pathway is known to be inactive in cancer cells with a resultant impaired apoptosis and cancer growth. For this reason LRs are regarded as mediators of cell migration, metastases, cell survival and tumour progression (Ma, 2007; Patra, 2008). From a cancer preventative perspective, LRs potentiates the future of cancer treatment, tumour staging/diagnosis and prognosis.

2.3.7.3. Cholesterol

Cholesterol accumulation due to an altered CHOL metabolism has been indicated in numerous malignancies (Brown *et al*., 1973; Heaffner *et al*., 1984; Kolanjiappan *et al*., 2003; Freeman and Solomon, 2004; Zhuang *et al*., 2005; Li *et al*., 2006). Apparent deregulation of CHOL feedback due to the disruption of transcriptional factors results in an increase in the expression of enzymes associated with the mevalonate/CHOL pathway such as squalene synthase, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, HMG-CoA reductase and farnesyldiphosphate synthase (Siperstein, 1995; Brusselman *et al*., 2007). In addition to this, mavelonate can function as a precursor of various molecules associated with cell cycle regulation including dolichol, geranylpyrophosphate and farnesyl-pyrophosphate (Goldstein and Brown 1990; Duncan *et al*., 2004). Tumour cells require CHOL for growth and can obtain this from either the circulation (increase in LDL receptors and HMG-CoA

reductase activity) or by *de novo* synthesis via the mevalonate/isoprenoid metabolism (Brown *et al*., 1973; Haeffner *et al*., 1984). The increase in membrane CHOL in tumour cells impacts on lipid microdomain structures whereby enhanced segregation of CHOL and SL complexes with specific proteins occur forming new LRs communicating signals whereas the CHOL in non-LR regions remained unaffected (Zhuang *et al*., 2005; Li *et al*., 2006, Brusselmans *et al*., 2007). A caveolae and signaling protein, caveolin-1 is closely associated with membrane CHOL. This protein is known to promote cancer and is a marker for aggressive prostate carcinoma (Cohen *et al*., 2004; Williams and Lisanti, 2004; Zhuang *et al*., 2005; Daniel *et al*., 2006).

2.3.7.4. Fatty acids

Numerous studies have demonstrated that PUFAs play an important role in modulating tumourigenesis. The ω-6 PUFAs are known to promote carcinogenesis whereas ω-3 PUFAs have anticancer properties, hence the importance of the type and ratio of dietary fats that is consumed. However, in the tumour, the levels of PUFAs are lowered, and especially the ω-3 PUFAs with their respective products (Hanai *et al*., 1993). Even the PL species incorporated in to the tumour membrane are more saturated (Rysman *et al*., 2010; Hilvo *et al*., 2011). This remodeling inevitably leads to a more rigid membrane (saturated membrane) that may affect signaling ligand/receptor pathways. Overall the lower FAs environment of tumours is due to an increase in the turnover rates of FAs due their utilisations as a source of energy, building blocks, substrates, and signaling in an altered metabolic environment (Martin *et al*., 1996; Kökoğlu, *et al*., 1998 Reynolds *et al*., 2001; Szachowicz-Petelska *et al*., 2010).

However, in some tumour certain FAs were indeed increased for example C20:4ω-6 and C18:1ω-9, possibly due to an impaired desaturase enzyme (delta-6 desaturase) or an increase in SCD (Gardiner and Duncan, 1991; de Alaniz and Marra, 1994; Hrelia *et al*., 1994). Enzymes associated with the eicosanoid metabolism can also be affected during carcinogenesis; these include the overexpression and activation of COX and LOX enzyme systems (Koki and Masferrer, 2002; Cejas *et al*., 2004; Hull *et al*., 2004; Massoumi and Sjolander, 2007). Various C20:4ω-6-derived eicosanoids

have been associated with cancer promotion metastasis and proliferation by inhibition of apoptosis, angiogenesis, cell adhesion and an increase in DNA synthesis (Sheng *et al*., 1998; Shureiqi and Lippman, 2001; Kimura *et al*., 2000; Larsson *et al*., 2004). If the increase in ω-6 in tumour cells is the problem than incorporation of ω-3 may be the solution. Extensive research has shown the inhibition of cancer promotion by the essential ω-3 FAs such as C20:5ω-3 and C22:6ω-3 by i) the generation of free radicals to stimulate lipid peroxidation and apoptosis, ii) increased incorporation into PLs and membrane whereby lipids are less saturated iii) membrane fluidity increase due to the reduction in CHOL and SM, iv) alteration in eicosanoid precursors and metabolism by reducing ω-6 PUFA derived eicosanoids and v) changes in several signaling molecules/pathways (Schley *et al*., 2007; Corsetto *et al*., 2012). Increased levels of C20:5ω-3 and C22:6ω-3 in tumour cell membranes reduced both CHOL and SM due to the poor interactions between these molecules thereby reducing LRs and the availability and distribution of membrane-associated proteins (Huster *et al*., 1998; Niu and Litman, 2002). The reduction of SM in LRs can also be related to the increased activation of the enzyme SMase and the hydrolysis of SM to ceramide (Wu *et al*., 2005; Corsetto *et al*., 2012). DHA reduced CHOL more than EPA due to its high level of unsaturation (Wassall and Stillwell, 2008).

Disruption of cell membranes encompassing the diverse and complex lipid metabolism is becoming increasingly recognised as a "hallmark of cancer development" (Zhang and Du, 2012). Unraveling the alterations in the lipid metabolism during carcinogenesis will also contribute to new and novel chemotherapeutics strategies. The intricate contribution of lipogenic changes associated with cancer remains inadequate and to some extent overlooked by many cancer researchers even the epigeneticist. Figure 2.13 is an attempt to summarise the most notable changes in the lipid metabolism during cancer.

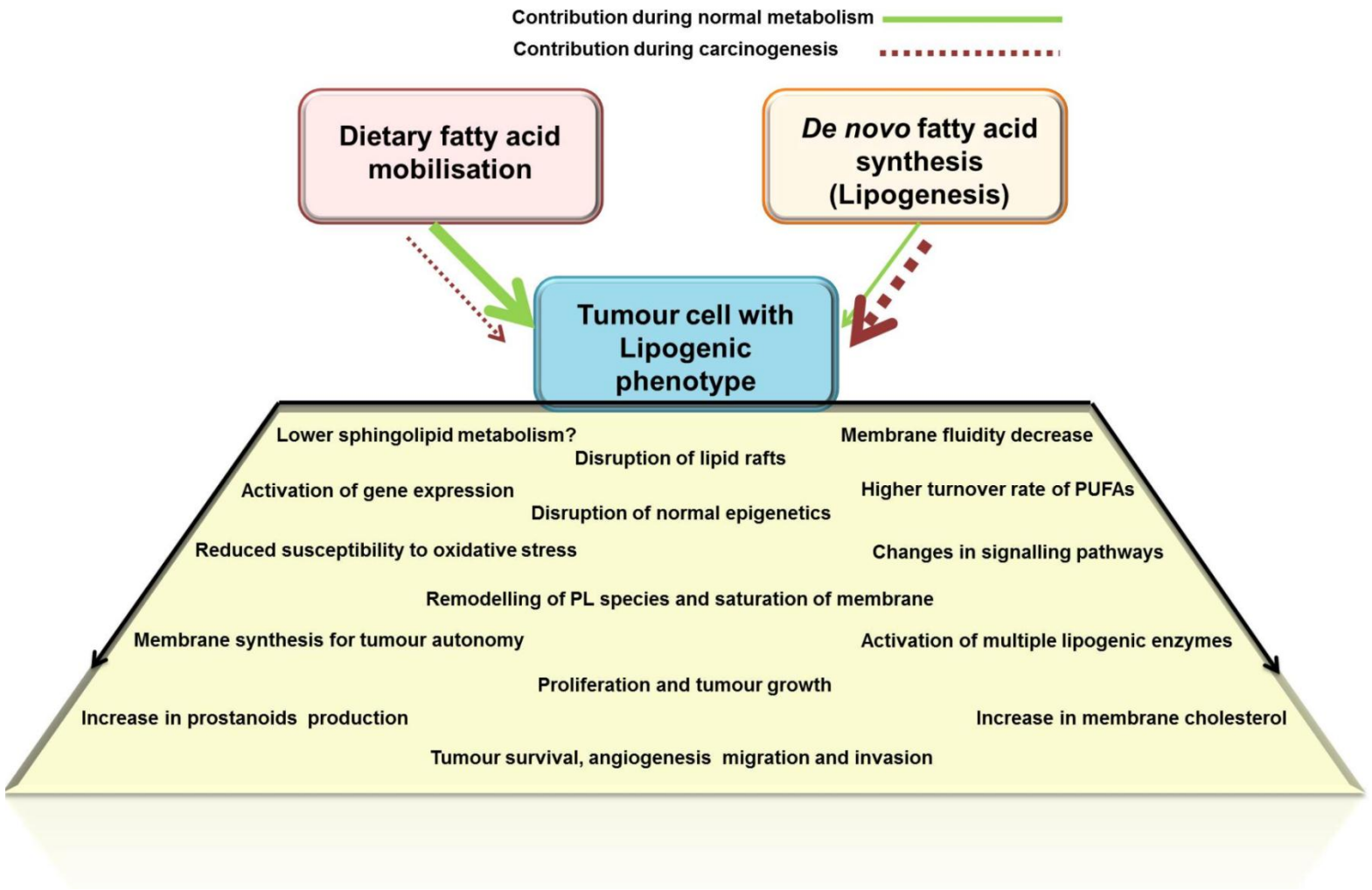

Figure 2.13: Summary of lipogenic effects during cancer

2.4. MYCOTOXINS AS REGULATORS OF MEMBRANE STRUCTURE AND FUNCTION

2.4.1. Introduction

Mycotoxins are secondary metabolites and natural contaminates produced by various food-borne filamentous fungi that can infect food commodities during pre- and post-harvest periods, storage or during food processing. The function of mycotoxins and the reason for their production remains unclear since it plays no role in the normal metabolism or growth of fungi (Moss, 1991, Pitt, 2000). The term *mycotoxin* (mykes, mukos from Greek meaning "fungus" and toxicum from Latin meaning "poison") originates from an incident in 1962 near London, England (Blout, 1961; Forgacs, 1962). A hundred-thousand turkey poults died after consuming aflatoxins contaminated peanut (groundnut) meal and the diseases was referred to as turkey X disease. The farm-to-fork continuum of mycotoxins is a global problem that affects both human and animal health while impacting on the economy, food industry and international trade.

The human and animal diseases caused by mycotoxins are called mycotoxicoses and are grouped under toxicological syndromes or poisoning by natural causes that can be either acute (rapid-onset and observable toxic response) or chronic (low-dose exposure over a long term period culminating in a disease). The mycotoxins considered to be of importance to human health and the agro-economical climate, include: aflatoxin (AF) produced by *Aspergillus spp.*, ochratoxin A (OTA) produced by *Aspergillus spp.* and *Penicillium spp.,* deoxynivalenol (DON), zearalenone (ZEA) and fumonisin B (FB) produced by *Fusarium spp*. (Binder *et al*., 2007). A comprehensive summary of the most important biological characteristics of these mycotoxins are summarised in Table 2.4. Although mycotoxins are regarded as highly toxic and/or carcinogenic, the mechanism involved in causing diseases such as cancer is still unclear (Wild and Gong, 2009; Chassy, 2010).

Table 2.4: Summary of five important mycotoxins and their major biological characteristics

Mycotoxin	Main fungal producing species	Relevant agricultural commodities	Mycotoxin subgroups	Fungal Infection	Associated Human diseases	Provisional Maximum Tolerable Daily Intake	IARC Classification	References
Aflatoxin (AF)	*Aspergillus flavus, Aspergillus parasiticus*	Peanuts Maize Wheat Nuts Rice Cassava Figs Oil seeds Tobacco Milk and diary	AFs: B_1,B_2, G_1,G_2, M_1 and M_2	Pre- and Post-harvest	Acute hepatitis Liver cancer (AFs and pre-hepatitis B virus exposure) Stunting Immune suppressor	None* *Aflatoxin is a genotoxin and has no threshold level. Regulations set by the *South African* Department of Health for AFs: all foodstuffs ready for human consumption should not contain more than 5 µg kg^{-1} for AFB_1 and 10 µg kg^{-1} for total AFs	Group 1 carcinogen	Van Rensburg *et al*., 1985; Van Egmond, 1989; IARC, 1993; Eaton and Groopman, 1994; Pitt, 2000; Li *et al*., 2001; Turner *et al*., 2002; Bennett and Klich, 2003; Marasas *et al*., 2008.
Ochratoxin (OT)	*Aspergillus ochraceus, Aspergillus carbonarius, Penicillium verrucosum*	Wheat Barley Oats Rye Maize Dry-beans Coffee beans Grapes Pork meat Nuts Cocoa Milk	OT: A, B and α	Mainly post-harvest	Possible Balkan endemic nephropathy (BEN)	0.1 µg kg^{-1} bw $week^{-1}$ or ± 14 ng kg^{-1} bw day^{-1}	Group 2B carcinogen	Hamilton *et al*., 1982; Hult *et al*., 1982; Burns and Dwivedi, 1986; Krogh, 1987; Kuiper-Goodman and Scott, 1989; Marquardt and Frohlich, 1992; IARC, 1993; Beardall and Miller, 1994; Creppy, 1999; Fink-Gremmels, 1999; Pitt, 2000; JECFA, 2002; Bennett and Klich, 2003.

Table 4: Summary of five important mycotoxins and their major biological characteristics

Mycotoxin	Main fungal producing species	Relevant agricultural commodities	Mycotoxin subgroups	Fungal Infection	Associated Human diseases	Provisional Maximum Tolerable Daily Intake	IARC Classification	References
Deoxynivalenol (DON) (vomitoxin)	*Fusarium graminearum, Fusarium culmorum*	Maize Wheat Rye Barley	Member of the Trichothecene toxins **Type A:** T-2 toxin; HT-2 toxin; neosolaniol and diacetoxy-scirpenol **Type B:** DON and nivalenol	Pre-harvest	Gastro-intestinal disorders Anorexia Nausea Emesis Headache Chills, Giddiness Convulsions	1 μg kg^{-1} bw day^{-1}	Group 3 carcinogen	Marasas *et al*., 1984; Joffe, 1986; Beasley, 1989; IARC, 1993; JECFA, 2001; Bennett and Klich, 2003; Tritscher and Page, 2004.
Zearalenone (ZEA)	*Fusarium Graminearum, Fusarium culmorum*	Maize Wheat Barley Rice	Member of Resorcyclic acid lactone: ZEA; zearalanone; α- and β-zearalenol	Pre-harvest	Precocious pubertal changes in children Early Menarche Possible reduced fertility (endocrine disrupter)	0.5 μg kg^{-1} bw day^{-1}	Group 3 non classifiable carcinogen (limited evidence in animals)	Kurtz and Mirocha, 1978; El-Nezami *et al*., 2002; Schoental, 1983; Heffron, 1999; IARC, 1999; JECFA, 2000; Pitt, 2000; Bennett and Klich, 2003 Ibánez-Vea *et al*., 2012.
Fumonisin B (FB)	*Fusarium verticillioides, Fusarium proliferatum*	Maize	FB_1;FB_2 and FB_3 (28 known analogues)	Pre-harvest	Possible role in: neural tube defects, stunting, liver and oesophageal cancer	2 μg kg^{-1} bw day^{-1}	Group 2B carcinogen	Bezuidenhout *et al*., 1988; Gelderblom *et al*., 1988; Marasas *et al*., 1988; Harrison *et al*.,1990; Sydenham *et al*., 1991; Rheeder *et al*., 1992; Hendricks *et al*., 1999; Missmer *et al*., 2000; Bolger *et al*., 2001 (JECFA); Marasas, 2001; IARC, 2002; Rheeder *et al*., 2002; Bennett and Klich, 2003; Marasas *et al*., 2004.

2.4.2. Disruption of membrane structure and function: fumonisin B_1 as an example

The FB mycotoxins, ubiquitous contaminants of maize are mainly produced by the fungi *Fusarium verticillioides* and *F. proliferatum* (Rheeder *et al*., 2002) (Table 2.4). Several diseases in animals are associated with FB exposure while in humans; it is implicated with a high incidence of oesophageal and liver cancer including the development of neural tube defects (NTDs) (Rheeder *et al*., 1992; Ueno *et al*., 1997; Marasas *et al*., 2004). Fumonisin B_1 (FB_1) the most abundant mycotoxin, are one of twenty-eight analogues thus far identified (Rheeder *et al.*, 2002). The International Agency for Research on Cancer (IARC) has classified FB_1 as a possibly human carcinogen (group 2B) (IARC, 2002; IARC, 2012).

The disruption of the SL metabolism by FB_1 (inhibition of *de novo* ceramide synthesis enzyme) has been associated with FB_1 toxicity (Riley *et al*., 2001; Soriano *et al*., 2005). Fumonisin B_1 is currently the most studied fumonisin of all consists of a lipophilic backbone that is structurally similar to sphinganine (Sa) and sphingosine (So). This shared structural similarity is responsible for FB_1's ability to disrupt the SL biosynthesis through the inhibition of the *de novo* enzyme, ceramide synthase (sphinganine or sphingosine N-acyl transferase) in the ER (Wang *et al*., 1991; Norred *et al*., 1992) followed by the consequential accumulation of free sphingoid bases, loss of complex SLs and an increase in sphingoid base-1-phosphates (Merrill *et al*., 2001; Riley *et al*., 2001). This disruption of the SL biosynthesis by FB_1 has been suggested to play a pivotal role in the toxic effect that has been observed in both *in vivo* and *in vitro* studies (Soriano *et al*., 2005). The mechanisms by which the inhibition ceramide synthase affects, specifically cancer promotion, is unclear and further complicated by the fact that sphingoid bases are associated with both cell proliferation and apoptosis (Gelderblom *et al*., 1999; Riley *et al.*, 2001; Futerman and Hannun, 2004; Ogretmen, 2006). For instance the accumulation of free sphingoid bases induced by FB_1 may activate apoptosis whereas others, such as So 1-phosphate in addition to the inhibition of ceramide, block apoptosis (Dragan *et al*., 2001; Riley *et al.*, 2001). The disruption of other lipid parameters and the subsequent effect on membrane integrity and function has also been proposed as a possible mechanism for FB_1 induced cancer promotion (Gelderblom *et al*., 2001a; Gelderblom

et al., 2002). A summary of the important lipid modifications included (i) inactivation of *de novo* ceramide synthase enzyme resulting in a decrease in complex ceramide-containing SLs (including SM) and accumulation of free sphingoid bases and metabolites (ii) disruption of the membrane structure by increasing CHOL, affecting the major PLs (iii) impairing delta-6 desaturase enzyme; (iii) modulation of membrane FAs (SFAs and MUFAs increase and PUFAs decrease), (iv) increase in C20:4ω-6 derived prostanoid synthesis of the E2 series (Gelderblom *et al*., 2001a) (Table 2.5).

Cancer promotion by FB_1 is associated to its ability to induce the survival (growth) of initiated cells simultaneous to the removal (apoptosis) of normal cells (Gelderblom *et al*., 2001a). The role of C20:4ω-6 during FB_1-induced lipid metabolism modifications resulting in cancer promotion has become central to its effect, since this may also lead to the survival of initiated cells while normal cells undergo apoptosis (Ramljak *et al*., 2000; Gelderblom *et al*., 2001a, 2008). These changes, together with the disruption of the membrane integrity by FB_1 are likely to affect important signaling processes. Signalling pathways associated with FB_1-cancer promotion as shown by an increased gene expression includes, the α-fetoprotein, hepatocyte growth factor (HGF), tumour or transforming growth factor (specifically TGF-α and TGF-β1) and c-myc, a known oncogenic transcription factor (Lemmer *et al*., 1999). Signalling events such as the induction of cyclin D_1 (a cell cycle regulator), inhibition of the activity of glycogen synthase kinase 3β phosphorylation, activation of Akt and an increase in cyclin dependant kinase 4 complexes with an over stabilisation of cyclin D_1 have been observed (Ramljak *et al*., 2000). These events affected both proliferative and apoptotic pathways that could sustain cancer promotion.

2.5. MYCOTOXIN RISK ANALYSIS

2.5.1. Introduction

According to the Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO) risk analysis is a systematic and disciplined approach defined as “to develop an estimate of risks to human health and safety, to

Tabel 2.5: Summary of the main effects of FB_1 on the lipid metabolism

Parameter	Effect observed	References*
Phospholipid metabolism	• Total PLs increase in rat liver and microsomal membranes • PC increase in rat primary hepatocytes and liver microsomal membranes • PE increase in both rat liver, primary hepatocytes and liver microsomal membranes • PI increase in rat liver microsomal membranes	Gelderblom *et al*., 1996; 2002

Sphingolipid metabolism	• Increase in free Sa in:
	  • rat liver (nodules and surrounding tissue), kidney and primary hepatocytes
	  • mice liver and intestinal cells
	  • porcine serum liver, lung, kidney intestinal epithelial cells (IPEC-1), pulmonary artery endothelial cells and renal epithelial cells ($LLC\text{-}PK_1$)
	  • horse serum
	  • rabbit lung tissue
	  • Swiss 3T3 cells
	  • human colon carcinoma cell line (HT29 cells)
	• Decrease in complex SLs in horse serum
	• Free So increased in rat liver, liver nodules and kidney
	• SM decrease in primary rat hepatocytes
	• Ceramide decrease in primary rat hepatocytes
		Wang *et al*., 1991; 1992
		Haschek et al., 1992
		Schroeder *et al*., 1994b
		Merrill *et al*., 1993; 1995
		Ramasamy *et al*., 1995
		Yoo *et al*., 1996
		Norred *et al*., 1992; 1996
		Gelderblom *et al.,* 1997
		Gumprecht *et al*., 1998
		Riley *et al*., 1993; 1994a; 1994b; 1998
		Schmelz *et al*., 1998
		Encongene *et al*., 2002
		Van der Westhuizen *et al*., 1998; 2004
		Riley and Voss, 2006
		Voss *et al*., 1993; 2006
		Loiseau *et al*., 2007

Parameter	Effect observed	References
Cholesterol	Increase in total cholesterol in rat serum, mice serum, liver and liver microsomal fractions Decrease in free cholesterol in primary rat hepatocytes	Voss *et al*., 1993 Bondy *et al*., 1997 Gelderblom *et al*., 1996, 1997; 2002 Moon *et al*., 2000 Howard *et al*., 2002
Fatty acids	**In the PC fractions** Increase due to FB_1 exposure: • C16:0 in in rat primary hepatocytes and liver microsomal membranes • C18:0 liver microsomal membranes • C16:1 and C18:1 in rat liver • C18:2ω-6 in rat liver and primary hepatocytes • C18:2ω-3 increased in rat primary hepatocytes • C20:4ω-6 in rat liver, primary hepatocytes and liver microsomal membranes • C22:6ω-3 in rat liver microsomal membranes • Total SFAs in rat liver microsomal membranes • Total MUFAs in rat liver microsomal membranes • Total PUFA increased in rat liver microsomal membranes	Gelderblom *et al*., 1996, 1997; 1999, 2002

Parameter	Effect observed	
Lipid-associated enzymes	• Inhibit activity of sphingosine N-acyltransferase (ceramide synthase) in rat liver microsomes • Delta-6-desaturase activity decreased in rat liver microsomal membranes • Delta-5-desaturase activity decreased	Wang *et al*., 1991 Gelderblom *et al*., 2002
Lipid or fatty acid ratios	Overall increase in: • C20:3/C18:0 ratio rat primary hepatocytes: • C20:3ω-6/C20:4ω-6 ratio in rat primary hepatocytes: • C20:4/C20:3 ratio in rat primary hepatocytes: • C20:3ω-6/ C18:2ω-6 ratio in rat primary hepatocytes: • C18:3ω-6/ C20:4ω-6 ratio in rat primary hepatocytes: • PC and PE: increase in C18:3ω-6/C20:4ω-6 and C:20:3ω-6/C20:4ω-6 ratios in rat liver microsomal membranes • PL/CHOL ratio rat primary hepatocytes: • CHOL/total PL ratio in rat liver microsomal membranes • PC and PE: ω-6/ω-3 ratio in rat liver microsomal membranes • Sa/So ratio in porcine serum, liver, lung, kidney, jejunum and pulmonary artery endothelial cells • Sa/So ratio in lung and kidney tissue from rabbits. • Sa/So ratio in rat liver, primary hepatocytes and kidney • Sa/So ratio in human urine Overall decrease observed in: • C20:4ω-6 PC/PE ratio decreased • PC/free CHOL ratio increased in rat liver and primary hepatocytes • PC and PE: ω-6/ω-3 ratio decreased in rat liver • Decrease in PC/PE ratio • PC and PE: ω-6/ω-3 ratio decreased in rat liver • PC: C18:2ω-6/C18:3ω-6 ratio decreased in rat liver microsomal membranes	Voss *et al*., 1993 Gelderblom *et al*., 1996; 1997; 2002 Gumprecht *et al*., 1998 Qiu and Liu 2001 Howard *et al*., 2002 Van der Westhuizen *et al*., 1998; 2004

Parameter	Effect observed	References
Fatty acids	**In the PC fractions** • Total ω-3 FAs in rat liver • Total ω-6 and ω-3 FAs in rat liver microsomal membranes Decrease in levels of: • C16:1 and C18:1 in rat primary hepatocytes • C18:2ω-6, C20:4ω-6 and C22:5ω-6 levels in rat blood • Total PUFAs in rat liver and primary hepatocytes • Total ω-6 FAs in rat liver **In the PE fractions** Increase due to FB_1 exposure: • C16:0 and C18:0 in rat liver microsomal membranes • C18:2ω-6 rat liver- and microsomal membranes • C20:4ω-6 rat liver microsomal membranes • C22:5ω-6 increased in rat liver microsomal membranes • C22:6ω-3 rat liver microsomal membranes • Total MUFAs increased in rat liver microsomal membranes • Total PUFAs increased in rat liver microsomal membranes • Total SATs in rat liver microsomal membranes • Total ω-3 FA increased in rat liver LTS • Total ω-6 and ω-3 FAs in rat liver microsomal membranes	Gelderblom *et al*., 1996; 1997; 2002

Parameter	Effect observed	
Lipid or fatty acid ratios	Overall decrease observed in: • PC: PUFAs/SATs ratio decrease • Total FA/Total PL ratio decreased in rat liver microsomal membranes • PC/PE ratio decreased in rat liver microsomal membranes	
Membrane fluidity	Overall decrease in membrane fluidity	Ferrante *et al*., 2002
	Increased macrophage membrane fluidity	Gelderblom *et al*., 1999; 2002
Other	Prostanoid synthesis inhibit	Abel and Gelderblom, 1998
	PGE_2 and PGA_2 reduced in WHCO3 (human oesophageal cancer cell line)	Sahu *et al*., 1998
	Increased macrophage plasma membrane endocytosis and lipid peroxidation	Gelderblom *et al*., 1999
	Increased lipid peroxidation in rat liver, primary hepatocytes, liver plasma membrane, nuclei membrane and microsomes	Seegers *et al*., 2000 Ferrante *et al*., 2002

*References arranged chronologically.

identify and implement measures to control risks, and to communicate with stakeholders about the risks and measures applied" (FAO and WHO, 2006). The three components of the risk analysis paradigm includes risk assessment, risk management and risk communication.

The paradigm of risk assessment consists of four steps with hazard / risk characterisation and quantification is at the base. A "hazard" can be defined as a biological, chemical or physical entity present in food and/or due to the condition of food, whereas "risk" is the probability of an adverse health effect occurring and includes the severity of the effect due to a specific hazard (European Communities, 2002).

The ultimate aim of risk assessment is to aid scientists and policy-makers to protect consumers from both acute and chronic exposure (Svendsen *et al*., 2008; Dorne, 2010). Data derived from animal exposure studies are used to predict health outcomes in human population at risk of exposure. Only after an upper limit is set can risk be determined among a subpopulation exceeding these levels. Upper limits or control levels are derived from critical health effect observed during animal studies exposed to a certain level and extrapolating this to humans at risk by using a "uncertainty factor" (Dorne, 2010). A standard uncertainty factor or default assumption of 100-fold is used to allow for differences between species and human inter-individual variability (Dorne and Fink-Gremmels, 2012). Importantly the type of risk assessment approach will be influenced by two basic mechanistic differences between hazards, whether it is genotoxic, (deleterious to DNA) or non-genotoxic (DNA-non-reactive or epigenetic carcinogens) (Williams, 1992; Williams and Latropoulos, 2001; Dybing *et al*., 2008). It is generally assumed that a threshold of exposure exists for non-genotoxins whereas genotoxins contains no threshold level (McMicheal and Woodward, 1999; Dybing *et al*., 2002).

2.5.1. Hazard identification

The identification of any biological, chemical or physical agents in a particular food or food group that can induce an adverse health effect(s) is the first step of risk assessment (Codex Alimentarius, 2001). During this process both *in vitro* and *in vivo*

methods are applied to describe the biological characteristics of the hazard, to detect possible targets and toxic endpoints (Renwick, 2004; Trischer and Page, 2004). In this regard the toxicokinetics including absorption, distribution, metabolism and excretion, the target organ/s, toxicological endpoints, the elimination, accumulation of the hazard and its mechanism of action is extensively investigated and described.

2.5.2. Hazard characterisation

The next step is to evaluate all data available on the nature of the effect caused by a hazard, its impact on humans and the dose-response relationship (Dybing *et al*., 2002; Edler *et al*., 2002; Renwick, 2004; Dorne and Fink-Gremmels, 2012). Important observations predicted include the long-term adverse health effects and whether it is acute or chronic. For toxins that elicit their adverse effects at a threshold level, a safe level of intake, or acceptable daily intake (ADI) or tolerable daily intake (TDI) are normally determined. For instance, if a mycotoxin is a non-genotoxin a "practical biological threshold of effect" can be assumed and the no observed adverse levels (NOAEL/NOEL) or lowest observed effects levels (LOAEL/LOEL) are determined (Trischer and Page, 2004; Garćia-Cela *et al*., 2010). This, together with "uncertainty factors" to account for the extrapolation form animals to humans and inter-individual variability are applied to estimate ADIs or TDIs.

2.5.4. Exposure assessment

The potential adverse effect a particular hazard can cause among a specific group (human or animal) at risk is addressed during exposure assessment (Jackson and Jansen, 2010). During this step, valid and quality information on the occurrence (concentrations) and the consumption or intake of a hazard needs to be considered (Renwick, 2004; Trischer and Page, 2004). Quantitative exposure assessment can be conducted using two methods, the deterministic (or point estimate) or probabilistic approach (Kroes *et al*., 2002). During deterministic analysis, a set value (95^{th} percentile, mean, median etc.) are used for consumption multiplied by the occurrence of a mycotoxin. This approach is used for screening purposes and does not measure the exposure of the population. Utilising the whole distribution of hazard

occurrence and consumption data, the probabilistic method, accurate exposure can be measured.

2.5.5. Risk characterisation

The data obtained during hazard identification, hazard characterisation and exposure assessment are integrated to either develop recommendations or to quantify these recommendations (Renwick, 2004, Trischer and Page, 2004). During the quantification process estimates of risk are determined at a specific level of exposure whereby a health effect can be expected. These estimates can be either the ADI or TDI depending if it is an intentional (food additives) or unavoidable hazard.

2.6. CONCEPTUAL FRAMEWORK FOR MYCOTOXIN RISK ANALYSIS IN SOUTH AFRICA: FUMONISIN B_1 AS AN EXAMPLE

2.6.1. Introduction

The risk management process entails the “weighing of policy alternatives” in collaboration with all stakeholders while including risk assessment, human health protection, promotion of fair trade practices, implementation of adequate prevention and control strategies. Risk communication is the continual flow of shared information and ideas among risk assessors, risk managers, consumers, industry, the academic community and other stakeholders. The impact of FB on the lipid metabolism is central to its possible role in human diseases, especially regarding the development cancers such as oesophageal cancer, the development of neural tube defects (NTDs) and growth impairment in children (Gelderblom *et al*., 2008). However, the need for accurate and valid human health risk assessment is imperative, not just in populations residing in developing countries but also in vulnerable subgroups such as children (Sherif *et al*., 2009). Fumonisins occurs naturally and the root of exposure is the consumption of contaminate food commodities such as maize. An integrated approach to assess the risk of FB exposure (or risk assessment), among humans needs to include its toxicological effects, levels of exposure and the mechanisms involved (Gelderblom *et al*., 2008).

The disruption of the lipid metabolism by FB has thus far been ignored in risk assessment. Of particular concern remains the risk of mycotoxin exposure in humans. In this regard, diseases associated with mycotoxin exposure mainly involve the existence of a dose-response relationship before it can be classified as mycotoxicoses and requires human epidemiological investigations (Bennett and Klich, 2003). Human populations at risk of mycotoxin exposure can be defined as subgroups with a chronic dietary exposure to a specific mycotoxin. Subsistence farmers with inadequate farming practices, handling and storage of food, people living in countries with absent or poor food safety legislation and including areas with food insecurity and poverty as well as specific subgroups due to their age, gender, medical history, disease status, lifestyle or dietary- intakes and habits (e.g. unvaried diet) are at risk (Bennett and Klich, 2003; Bryden, 2007). Although ill defined, it is known that a poor nutritional and immune status, existing infectious diseases and alcohol abuse may exacerbate the toxicity of mycotoxins in humans (Bennett and Klich, 2003). Although it is expected that the incidence of human mycotoxicoses is smaller than most other non-infectious and infectious disease, its contribution to burden of disease cannot be ignored in especially developing countries. Although mycotoxin risk analysis is not prioritised in South Africa, the scientists at the Medical Research Council (MRC), the Programme on Mycotoxin and Experimental Carcinogenesis (PROMEC) Unit has developed its own unique multidisciplinary approach spanning over 40 years of science excellence. The following is a comprehensive summary of the “course of action” or conceptual framework applied to address the question in the South African context.

2.6.2. Field and laboratory observations related *Fusarium verticillioides* culture material

Fumonisin research in South Africa started unintentionally with the outbreak of equine leukoencephalomalacia (ELEM) in 1970 resulting in the isolation of *F. verticillioides* and its identification as the pathological fungal toxin in the horses feed (Kellerman and Marasas, 1972; Marasas *et al*., 1967). This was followed investigations into the role of fungal contamination in the development of oesophageal cancer (OC) among people residing in maize subsistence farming communities in high incidence areas in the former Transkei, Eastern Cape Province

(EC) (Jaskiewicz *et al*., 1987; Makaula *et al*., 1996). *F. verticillioides* was shown to be the most prevalent fungal contaminant in their staple diet of maize (Marasas *et al*., 1981). During laboratory observations horses and pigs fed culture isolates of *F. verticillioides* MRC 826, obtained from the former Transkei, developed ELEM and porcine pulmonary oedema, respectively (Kriek *et al*., 1981a). Toxicity was observed in numerous experimental animals including sheep, baboons, ducks and rats fed culture material (Kriek *et al*., 1981a; 1981b; Marasas *et al*., 1984, Gelderblom *et al*., 2001b). These observations made it imperative to characterise this fungus' metabolites or mycotoxins in detail (Marasas, 2001).

2.6.3. Hazard identification and characterisation of fumonisin B mycotoxins

In 1988, FB_1 and B_2 were isolated (Gelderblom *et al*., 1988) from cultures of *F. verticillioides* MRC 826 and the chemical structure elucidated (Bezuidenhout *et al*., 1988). However, to date about 28 FB analogues are known to be produced by this fungus (Rheeder *et al.,* 2002). Studies in animals exposed to FB_1, fed either purified or *F. verticillioides* cultural material, identified the liver and kidneys as target organs (Gelderblom *et al*., 1991; Howard *et al*., 2001). The health outcomes related to toxicity or carcinogenesis observed during FB exposure is also influenced by the animal species, specific strain, gender, exposure duration and levels international response included the evaluation of mycotoxins produced by *F. verticillioides* and the FB mycotoxins by the International Agency for Research on Cancer (IARC) in Lyon, France and classifying FB as a Group 2B carcinogen (i.e. possibly carcinogenic to humans (IARC, 1993; 2002). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) developed a group provisional maximum tolerable daily intake (PMTDI) for FB_1, FB_2 and FB_3 individually or combined of 0.2 μg kg^{-1} bw day^{-1} (JECFA, 2012). These PMTDIs are considered to be adequate for developed countries but lack the necessary refinement and sensitivity to address food safety in developing countries including South Africa (Marasas *et al*., 2008).

2.6.4. Mechanistic research on fumonisin B toxicology

Two fundamental findings rendered the investigations on FB_1 in South Africa into a new direction as well as the research focus into the area of carcinogenesis. The first

finding was the association between high levels of FB contamination in home-grown maize of subsistence farmers resident in the former Transkei area of the EC and the high incidence of OC (Marasas *et al*., 1981, Sydenham *et al*., 1991). The second finding was the development of hepatocellular carcinomas in rats fed culture material containing *Fusarium verticillioides* during long-term feeding studies (Gelderblom *et al*., 1983; Marasas *et al*., 1984; Jaskiewicz *et al*., 1987). The development of a rat hepatocarcinogenesis assay utilising partial hepatectomy, administration of a chemical cancer initiator (e.g. diethylnitrosamine) and cancer promoters (e.g. 2-Acetylaminofluorene and phenobarbital) provided a model to characterised and advance the understanding of the biological effects of FB_1. The hypothesis of cancer initiation by FB was further developed by Gelderblom *et al*. using various experimental rat models using male BD IX, and Fischer 344 rats (Gelderblom *et al*., 1988; 1992; 2001c). Distinct characteristics of FB_1 to affect cancer initiation i) chronic hepatotoxicity ii) no-effect threshold effect for toxicity and carcinogenicity, iii) act as a complete carcinogen in both the liver and kidney; iii) evidently non-genotoxin; v) clastogenic effects that is dose dependent vi) induction of resistant hepatocytes, vii) overexpression of growth stimulating including inhibitory factors and viii) cause oxidative damage via lipid peroxidation (Gelderblom *et al*., 1994; Abel and Gelderblom, 1998; Lemmer *et al*., 1999; Gelderblom *et al*., 2001a). In contrast the ability of FB_1 to promote cancer is associated with an altered lipid metabolism where FB_1 affects a growth differential whereby initiated “resistant” cells proliferate while the growth of normal cells is inhibited (Gelderblom *et al*., 2001a).

Important lipid alterations induced by FB, (Section 2.4.2) include a decrease in SM, depletion of the PUFA content of PC, increase in PE, CHOL, the SFAs and MUFAs content will inevitably affect membrane structure and function. The important changes observed in membrane fluidity parameters such as an increase in the PC/CHOL and decrease in the PC/PE ratios, respectively are indicative of a more rigid membrane structure. The link between cancer, dysregulation of the lipid metabolism (including the cell membrane) and the carcinogenic FB_1 are becoming more evident. This raises the question of the impact this may have on the food safety and human risk assessment of this mycotoxin. However, the lipid-disruptive characteristics of FB_1 have thus far not being included in risk assessment (Gelderblom *et al.,* 2008).

2.6.5. Fumonisin B mycotoxin exposure assessment

In order to investigate the natural occurrence of mycotoxins due to its emerging danger to animals and humans, the development and use of quantitative and sensitive analytical methods were critical. In 1991, the first paper was published on a high-performance liquid chromatography method that simultaneously determines FB_1 and FB_2 (Shepard *et al*., 1991).

The important link between increased levels of FB in home-grown maize from high-OC risk areas in the former Transkei, not only identified the population at risk within South Africa but also set the stage for FB exposure assessment (Shephard *et al*., 1991, Sydenham *et al*., 1991). In 1997, Marasas *et al*. reported the first probable daily intakes (PDIs) for a rural populations living in the former Transkei, that of 345.9 and 46.6 $\mu g\ kg^{-1}$ bw day^{-1} when consuming moldy and healthy maize, respectively. The PDI for urban South African population group was established at 1.2 $\mu g\ kg^{-1}$ bw day^{-1}. Gelderblom *et al*. (2008) and Marasas *et al*. (2008) developed an interactive model illustrating the association between FB contamination and maize intakes (Figure 2.14).

This model clearly demonstrated that in rural population with high home-grown maize intakes are also exposed to higher FB contamination levels. However, due to the lack of anthropometry data, maize dietary intake and practices of the population at risk adequate FB exposure assessment is still elusive. Shephard *et al.* (2005) reported FB exposure due to the consumption of home-brewed traditional Xhosa maize beer in rural areas. During this study per capita consumption data obtained from the Brewers Association of Japan, SABMillers and the South African Food Consumption Study Undertaken amongst Different Population Groups (1983-2000) were used assuming a body weight of 60 kg. The resultant PDIs ranged between 0.2 and 1.0 $\mu g\ kg^{-1}$ bw day^{-1}. When utilising the mean of maize-beer consumers only, in addition to the 97.5^{th} percentile maize beer intake, the calculated PDIs where above the PMTDI and ranged between 6.5 to 25.4 $\mu g\ kg^{-1}$ bw day^{-1} (Shephard *et al*., 2005). In addition, FB exposure among people residing OC-high risk areas, of the magisterial areas of Centane and Bizana respectively, EC was further evaluated

Maize intake (g 60kg^{-1} person day^{-1})

FB (μg kg^{-1})	10	50	100	150	200	400	500
0.2	0.0	0.2	0.3	0.5	0.7	1.4	1.7
0.5	0.1	0.4	0.8	1.3	1.7	3.4	4.2
1	0.2	0.8	1.7	2.5	3.3	6.6	8.3
2	0.3	1.7	3.3	5.0	6.7	13.0	17.0
3	0.5	2.5	5.0	7.5	10.0	20.0	25.0
4	0.7	3.3	6.7	10.0	13.0	26.6	33.0
5	0.8	4.2	8.3	13.0	17.0	33.4	43.0
10	1.7	8.3	17.0	25.0	33.0	66.6	83.0
12	2.0	10.0	20.0	30.0	4.00	80.0	100.0

PDI (μg kg^{-1}bw day^{-1})

PMTDI below 2 μg kg^{-1} bw day^{-1}

PMTDI between 0.8 and 2 μg kg^{-1} bw day^{-1} (nephrotoxicity)

PMTDI equals 0.8 μg kg^{-1} bw day^{-1} (hepatocarcinogenicity)

PMTDI above 2 μg kg^{-1} bw day^{-1}

Figure 2.14: Interactive model illustrating the association between fumonisin B contamination and maize intakes
(Adapted from Marasas *et al*., 2008)

(Shephard *et al*., 2007). During this study weighed food record of uncooked maize consumption was obtained from participants and the PDIs were 3.43 μg kg^{-1} bw day^{-1} and 8.6 μg kg^{-1} bw day^{-1} for people from Bizana and Centane, respectively. This was the first study to using short-term dietary intakes (24-hour recalls) of a high risk population.

Differences in study design and methodology between surveys hinders comparability and their application in exposure assessment (Verger and Fabiansson, 2008). Study design includes: the level of the survey (national, households and individual), what is measured, how it is measured and the observation period. Short-term dietary intakes

are usually assessed using 24-hour recalls, weighed and food records whereas habitual intakes are assessed using food frequency questionnaires (FFQ). Another approached is to use food available as obtained from household budgets surveys, food supply or food balance sheets, the latter being available from the World Health Organisation Global Environmental Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food or Eurostat) (Verger and Fabiansson, 2008). National consumption data are currently available from nineteen countries using standardised food categories (Dorne *et al*., 2009; EFSA, 2011). The Data Food Networking (DAFNE) is another database consisting of household food availability from seventy-five surveys in sixteen European countries (Trichopolou and Lagiou, 1997; Verger and Fabiansson, 2008). No golden standard exist as far as the assessment of dietary intakes are concerned (Verger and Fabiansson, 2008). At best, the long-term dietary surveys using FFQ, food diaries or dietary history will account for longer exposure frequencies and durations whereas the duplicate diet method (duplicate weighing of a cooked/prepared portion dished-up) will provide more relevant portions sizes. These approach have their limitations and also advantages, however, dietary intake at an individual level remains the ideal as far as risk assessment is concerned (Kroes *et al*., 2002; Verger and Fabiansson, 2008).

In South Africa the availability of dietary intake data are limited and the South African national food consumption survey only provides information on children aged 1 to 9. The development and validation of a cultural specific dietary assessment method among black Xhosa-speaking African residing in high-OC and high-FB exposure brought the opportunity to estimate more accurate maize intakes. The Ratio and Portion size Photo (RAPP) tool is a method focused on the estimation of habitual maize intake and cultural practices utilising life-size pictures and quantitative food frequency questionnaire (Lombard *et al*., 2012, Addendum H). This tool provided a valid method to improve FB exposure assessment among the population at risk.

2.6.6. Risk characterisation of fumonisin B mycotoxin exposure

This process within mycotoxin risk assessment includes the estimation of health risks and to summarising risk information within the affected population (Paumgartten, 1993). Unique to South Africa, this process included the

characterisation of the exposed populations by assessing lifestyle, behavioural and environmental factors that will evidently also impact on mycotoxin exposure as well as associated health outcomes (Figure 2.15).

2.6.6.1. Oesophageal cancer

Since the discovery of the FB, this mycotoxin has been associated with a high incidence of OC. This link stems from ecological studies conducted in affected areas in the former Transkei-region of South Africa (Marasas, 2001). While in other parts of the world this "ecological" associated has also been observed, this include the Linxian County of Henan Province in China, the Caspian Littoral of Iran, Kenya and Zimbabwe in Africa, the Veneto region in Italy, South Caroline in the United States of America and Santa Catarina in Brazil (Marasas *et al*., 2012). The mechanism that links FB exposure and OC are not fully understood. In this regard, the multifactorial causes associated with OC imply that FB may be a contributing factor. Squamous cell carcinoma (SCC) of the oesophagus is highly prevalent among a specific ethnic group in South Africa, the black isiXhosa-speaking Africans residing in the EC (Mqoqi *et al*., 2002; Somdyala *et al*., 2010). The age standerdised incidence rates per 100 000 for the EC (1998-2002) was 32.7 and 20.7 for men and women respectively (Somdyala *et al*., 2010). The demographic characteristic of this OC hot spot includes poverty, unemployment (28.3%), low life expectancy (53.7 years for men and 59.3 years for women) and a HIV/AIDS epidemic (Statistic South Africa, 2012; 2013). Risk factor associated with oesophageal SCC includes tobacco use, alcohol intake, genetic factors (allelic imbalance and microsatellite instability in chromosomes, somatic gene mutations and specific polymorphisms) nutrition deficiencies, iron overload, infections with human papilloma virus, cultural practises of self-induce vomiting by emetics (cleansing ritual) and the consumption of mycotoxin contaminated maize and exposure to nitrosamines (MacPhail *et al*., 1979; Groenewald, *et al*., 1981; Isaacson *et al*., 1985; Jaskiewicz *et al*., 1988; Williamson *et al*., 1991; Rheeder *et al*., 1992; Van Rensburg *et al*., 1993; Gamieldien *et al*., 1998; Naidoo *et al*., 1999; Hendricks and Parker, 2002; Matsha *et al*., 2002; Myburgh *et al*., 2002; Pacella-Norman, 2002; Dietzsch and Parker, 2002; Dietzsch *et al*., 2003; Matsha *et al*., 2006a; 2006b). Figure 2.15 summarises the risk factors associated with OC in South Africa. Even with the recent advances in studying these risks, the pathogenesis, epidemiology and behaviour of OC are poorly understood.

2.6.6.2. Neural tube defects

The alteration in the lipid metabolism affected by FB_1 may also contribute to its ability to inhibit the uptake of folate (vitamin B_9) that is associated with the development of NTDs (Marasas *et al*., 2004). Folate receptors are GPI-anchored proteins associated with LRs and seemingly the depletion of sphingolipids during FB_1 exposure has been attributed to this antinutritive effect (Stevens and Tang, 1997; Carratù *et al*., 2003; Sabharanjak and Mayor, 2004). Although of FB has been implicated in the development of NTDs this association has not been studies in South Africa (Hendricks *et al*., 1999; Marasas *et al*., 2004; Missmer *et al*., 2006). Very little data is available on the incidence of NTDs in South Africa due to the lack of a registry. In 1981, Marshall indicated an incidence of 1 in every 1000 birth for blacks Africans, Whites and Indians from three hospitals in Durban, Kwa-Zulu Natal Province. Whereas a hospital-based incidence rate of 6.13 and a Transkei district-wide rate of 6.79 per 1000 births was reported for the period 1980-1984 (Ncayiyana, 1986). Rural areas within South Africa are considerate to have higher NTD rates compared to urban (Ubbink *et al*., 1999; Sayed *et al*., 2008). A possible confounding factor such as the existence of folate micronutrient deficiency among especially the black Africans may also influence NTDs incidence in South Africa (Steyn *et al*., 2006, Vorster, 2010).

2.6.6.3. Growth impairment

The mechanism by which FB causes childhood stunting is still unknown however again the disruption of the sphingolipid has been suggested. Animal and *in vitro* studies have indicated that FB mycotoxins are associated with compromising intestinal tract function, immunity and permeability via the disruption of the sphingolipid metabolism (Enongene *et al*., 2000; Oswald *et al*., 2003; Bouhet *et al*., 2004; Loiseau *et al*., 2007; Smith *et al*., 2012). In this regard the alterations in the intestinal cell viability, proliferation and cytokine production have been indicated. Exposure to FB mycotoxins have implicated in childhood stunting (reduced growth and development) originating from ecological observations of the prevalence of neural tube defects concomitantly to a high FB exposure (Ncayiyana, 1986; Hendricks *et al*., 1999; Missmer *et al*., 2006). Studies conducted in other African countries such as Tanzania indicated that infants aged 6 months exposed to FB-

contaminated maize as a complementary food experienced stunting (Kimanya *et al*., 2010).

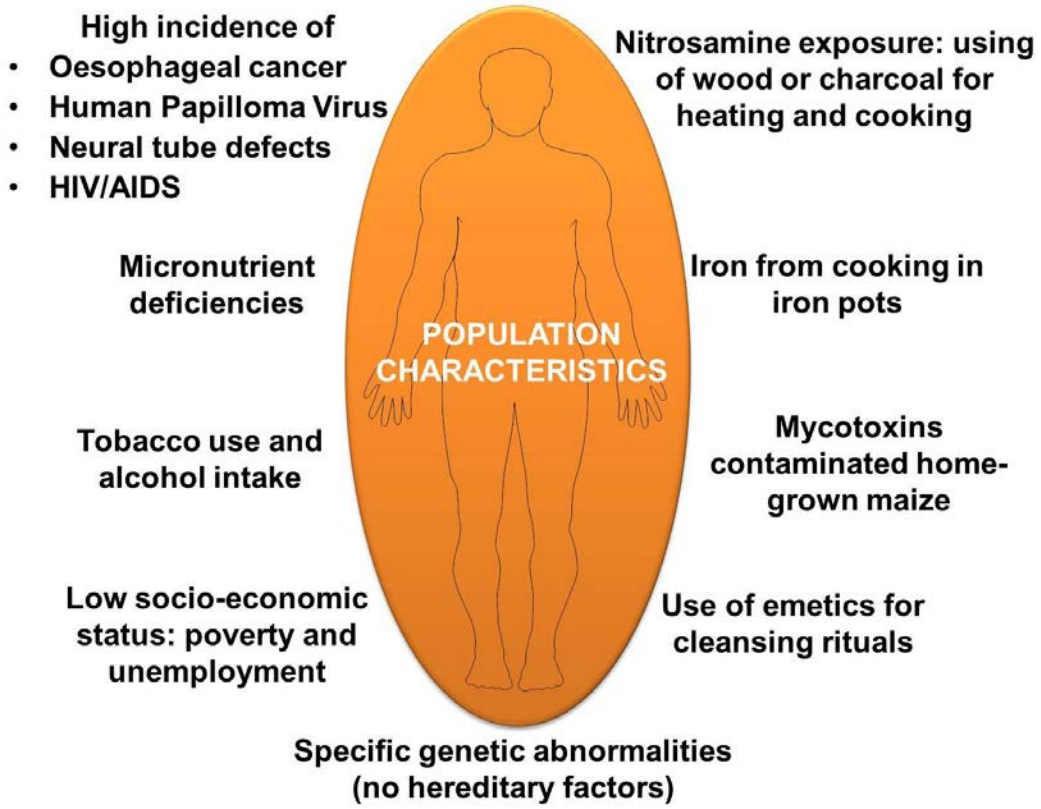

Figure 2.15: Summary of the major risk factors associated of people living in high fumonisin B-exposure areas in South Africa

2.6.7. Risk management and communication in fumonisin B exposure

Risk assessment of the FB mycotoxins contributed greatly the understanding of mechanisms involved in FB-induced carcinogenicity and toxicity, implications in the associated health risks and the development of valid analytical and exposure assessment methods. The recent development and validation of a urinary FB biomarker will improve the accuracy of future exposure assessment among subgroups, specifically the most vulnerable populations at risk (Van der Westhuizen *et al.,* 2011a). A simple method to sort and wash maize kernels to reduce FB contamination prior to cooking was also developed and validated among people living in rural Centane in the EC, a high incidence area of OC. This novel method

reduces FB contamination in maize by 84% and is therefore an important long term nutrition-based management method to ensure safe food (Van der Westhuizen *et al*., 2011b). To communicate this method to the population at risk, a special pamphlet was developed and translated into isiXhosa (Figure 2.16). This pamphlet is distributed after special workshops or training sessions whereby the method is demonstrated by a trained fieldworker. Full-scale deployment of this method among people living in rural areas in the EC is still outstanding. Such food-based solutions where agriculture and nutrition science are intergrated have the advantage of a multidisciplinary approach that will aid the development of tailored and prudent strategies to improve the health of a specific population at risk.

2.7. SUMMARY

Cell membrane biogenesis and homeostasis is inevitably governed by the lipid metabolism. Each membrane lipid constituents seemingly have a dual purpose, structural and functional. The particular arrangement of thousands of lipid species into two bilayers to ensure the coexistence of different fluidity phases (fluid mosaic model) enables a complex platform for numerous cell functions. The intricacy of membrane lipid biophysical interaction is also further challenged to associate with specific integral or peripheral proteins. Although this is regarded as the basics of cell membranes recently the existence of highly-organised microdomains or LR has also been suggested. Disruption of the lipid metabolism will compromise not only the integrity of membranes but also essential cell functions. Numerous diseases including cancer have been associated with lipid metabolism alterations. During carcinogenesis a “lipogenic phenotype” develops due to critical changes in the lipid metabolism. Importantly, these changes are perceived as an early event as well as a hallmark of cancer.

Mechanistic studies have indicated that the carcinogenic FB mycotoxins disrupts lipid metabolism and evidently is also an important regulator of membrane structure homeostasis. This distinct lipid-alteration, induced by FB, is associated with altered growth responses that promote initiated cells into the development of pre-neoplastic lesions that slowly evolve into cancer. In humans the exposure to FB has been associated with the development of liver and oesophageal cancer, including the

development of NTDs and childhood stunting, all of which have been suggested to be due to the lipid-disruptive properties. In South Africa FB exposure is culturally and geographically linked to a high incidence of OC and NTD. However, risk parameters develop internationally are not applicable to rural subsistence farmer communities utilising maize as major dietary staple. In these scenarios exposure exceeded the proposed PMTDI up to 5 to 20 times with children expected to be the most vulnerable subpopulation. Unique risk analysis models therefore need to be developed for the FB mycotoxins by integrating the lipogenic model into specific and applicable risk assessment models in rural subsistent maize consuming communities which is further developed in the current thesis.

Front side **Back side**

Figure 2.16: Educational pamphlet (in isiXhosa) demonstrating the washing and sorting of home-grown maize to reduce fumonisin exposure

(Model: Ms. T. Kulati; Translator: Ms. N. Ndube; Photographer and designer: Ms. H-M. Burger; props and setting: PROMEC Unit, Tygerberg 2010)

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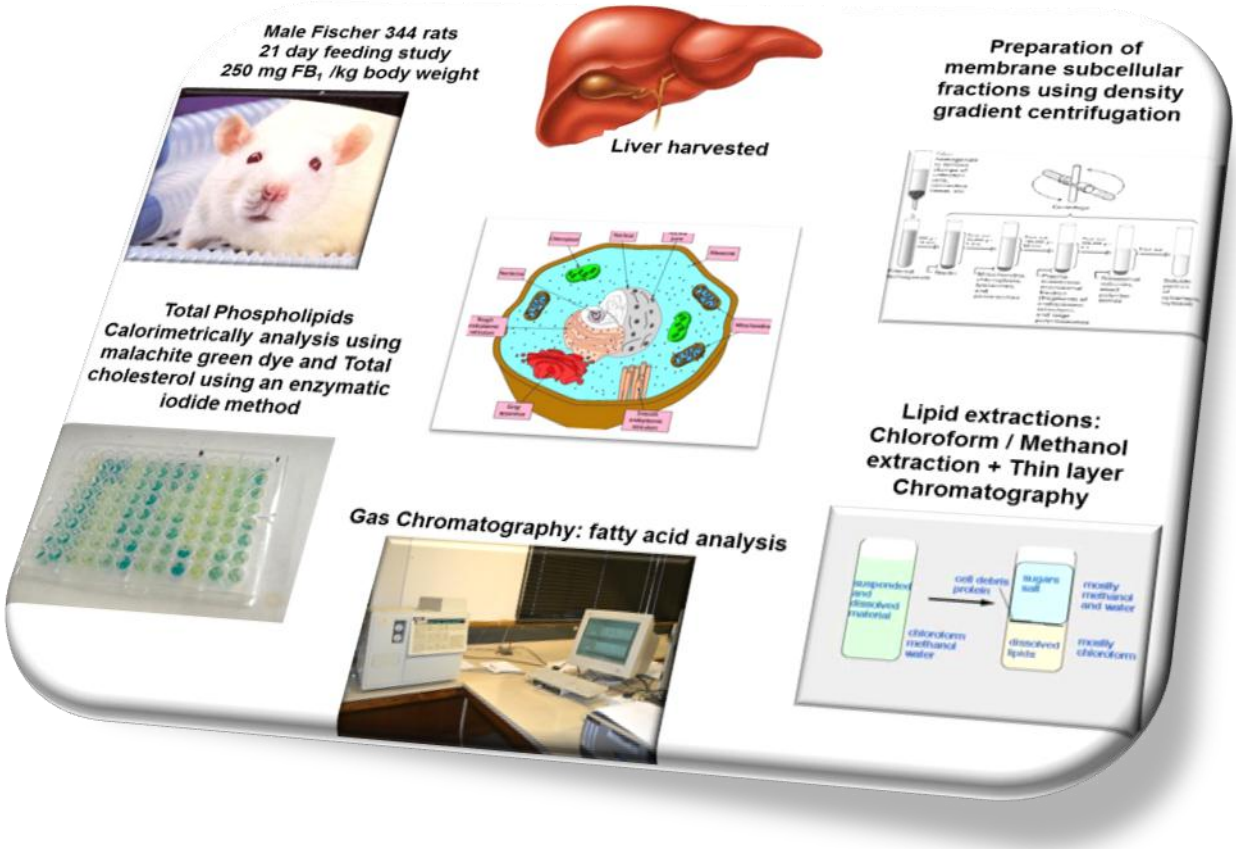

CHAPTER 3

ALTERED LIPID PARAMETERS IN HEPATIC SUBCELLULAR MEMBRANE FRACTIONS INDUCED BY FUMONISIN B_1

Burger, H-M., Abel, S.,Snijman, P.W., Swanevelder, S., Gelderblom, W.C.A. (2007). Altered lipid parameters in hepatic subcellular membrane fractions induced by fumonisin B_1. *Lipids*, 42, 249-261. (Impact factor 2.151)

ABSTRACT

Alteration of lipid constituents of cellular membranes has been proposed as a possible mechanism for cancer promotion by fumonisin B_1 (FB_1). To further investigate this hypothesis a dietary dosage which initiates and promotes liver cancer (250 mg FB_1/kg) was fed to male Fischer rats for 21 days and the lipid composition of plasma, microsomal, mitochondrial and nuclear subcellular fractions determined. The effect of FB_1 on the cholesterol, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as well as sphingomyelin (SM) and the phospholipid associated fatty acid (FA) profiles, were unique for each subcellular membrane fraction. PE was significantly increased in the microsomal, mitochondrial and plasma membrane fractions, whereas cholesterol was increased in both the microsomal and nuclear fraction. In addition SM was decreased and increased in the mitochondrial and nuclear fractions, respectively. The decreased PC/PE and polyunsaturated/saturated (P/S) FA ratios in the different membrane fractions suggest a more rigid membrane structure. The decreased levels in polyunsaturated fatty acids (PUFA) in PC together with a pronounced increase in C18:1ω–9 and C18:2ω–6 were indicative of an impaired delta-6 desaturase. The increased ω6/ω3 ratio and decreased C20:4ω–6 PC/PE ratio due to an increase in C20:4ω–6 in PE relatively to PC in the different subcellular fractions suggests a shift towards prostanoid synthesis of the E2 series. Changes in the PE and C20:4ω–6 parameters in the plasma membrane could alter key growth regulatory and/or other cell receptors in lipid rafts known to be altered by FB_1. An interactive role between C20:4ω–6 and ceramide in the mitochondria, is suggested to regulate the balance between proliferation and apoptosis in altered initiated hepatocytes resulting in their selective outgrowth during cancer promotion effected by FB_1.

INTRODUCTION

The structure, function and integrity of biological membranes are governed by the lipid composition of its bilayer (Gudi *et al*., 1998; Fuller *et al*., 2001; Funari *et al*., 2003). Membranes show an asymmetric bilayer of aminophospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) located in the inner leaflet, whereas phosphatidylcholine (PC) and the sphingolipid, sphingomyelin (SM), are located in the outer leaflet (Nyholm *et al*., 2003; Emoto and Umeda, 2000). The membrane structure and dynamics are important in maintaining cellular function that regulates signalling pathways related to cellular homeostasis (Eriksson and Andersson, 1992; Brown, 1998). The role of cellular membranes during carcinogenesis has become more prominent and forms a part of subtle changes underlying epigenetic events (Weisburger and Wynder, 1984; Stern *et al*., 1999). Isolated membrane fractions from tumour cells demonstrated alterations in their composition, structure and organization and thus in their functional properties (Galeotti *et al*., 1986; Burnes and Spector, 1994).

Fumonisin B_1 (FB_1), a natural occurring mycotoxin with cancer promoting properties is produced by the fungus *Fusarium verticillioides* in maize (Gelderblom *et al*., 1988). FB_1 causes several diseases in animals and is associated with a high incidence of human oesophageal and liver cancer in certain geographical areas in the world (Rheeder *et al*., 1992; Ueno *et al*., 1997), and the development of neural tube defects (Marasas *et al.*, 2004). Fumonisin B_1 is hepatotoxic and hepatocarcinogenic (Gelderblom *et al*., 1991; Lemmer *et al*., 2004) when chronically fed to rats and affects both cancer initiation and promotion properties in a short-term rat liver carcinogenesis model (Gelderblom *et al*., 1994; Gelderblom *et al*., 1996). This model provides an excellent opportunity to study the mechanisms associated with cancer induction by this “apparent” non-genotoxic compound as it lacks genotoxicity in the *Salmonella* mutagen and DNA repair assays (Gelderblom and Snyman, 1991; Norred *et al*., 1992; Knasmüller *et al*., 1997). However, FB_1 induces oxidative damage (Abel and Gelderblom, 1998) and exhibited clastogenic properties (Ehrlich *et al*., 2002), suggesting that the compound could either directly or indirectly, induce DNA damage. Short-term studies utilizing a cancer initiating/promoting model in rat liver indicated that FB_1 closely mimics the characteristics of other genotoxic

carcinogens with respect to initiation (Gelderblom *et al*., 1994). With respect to cancer promotion, evidence supports a hypothesis that FB_1 affects a growth differential, during which initiated hepatocytes proliferate in an environment where the growth of normal cells is inhibited (Gelderblom *et al*., 2001). This became evident as FB_1 inhibits the epidermal growth factor (EGF) stimulatory response in primary hepatocytes *in vitro* (Gelderblom *et al*., 1995) and hepatocyte regeneration following partial hepatectomy *in vivo* (Gelderblom *et al*., 1994) suggesting that FB_1 induces a growth differential similar to most cancer promoters (Tsuda *et al*., 1981; Faber, 1991).

The disruption of lipid metabolism and the subsequent effect on membrane integrity and function has been proposed as a possible mechanism for cancer promotion by fumonisin B_1 (Gelderblom *et al*., 2001; Gelderblom *et al*., 2007; Gelderblom *et al*., 2002). The present study described the effect of FB_1 on the lipid profiles of different rat hepatic subcellular membrane fractions including the microsomes, mitochondria, plasma membrane and the nuclei. The possible role of these changes during the cancer promotion of FB_1 in rat liver is critically evaluated.

METHODS AND MATERIALS

Chemicals and reagents

FB_1 was extracted and purified (>90%) according to the method described by Cawood *et al*. (1991). Fatty acid (FA) analytical standards (C14:0 to C24:1), used for calibration and identification, were obtained from Sigma Chemical Company (St. Louis, Missouri, United States). All the chemical solvents were of analytical grade and glass distilled prior to use.

Animals and diets

Male Fischer rats (150 g body weight) were fed a modified AIN-76 diet after weaning (Gelderblom *et al*., 1994) and housed individually in a controlled environment (23 - 25°C) with a 12 h light/dark cycle with free access to feed and drinking water. FB_1 was dissolved in methanol prior to application of a subsample (200 g) of the AIN-76A

diet and dried overnight. The subsample was mixed with the standard diet to obtain a concentration of 250 mg FB_1 kg^{-1}, a dose that both initiates (Gelderblom *et al*., 1994) and promotes (Gelderblom *et al*., 1996) cancer in rat liver. The diet was stored under nitrogen at 4°C. A control diet was prepared in the same way using an equal volume of methanol. The control (n = 5 rats) and FB_1-containing (n = 5 rats) diets were fed to the rats over a 21-day period. Following the feeding regimen, animals were sacrificed under sagatal aneasthesia after which the livers were harvested and stored in saline at -80°C. The use of experimental animals in this study was in accordance to the requirements of, and approved by the Ethics Committee for Research on Animals (ECRA) of the South African Medical Research Council

Preparation of membrane subcellular fractions

Membrane subcellular fractions were isolated at 4°C according to the method of Bartoli *et al*. (1980) and Loten and Redshaw (1986) with modifications. In short, the livers were homogenized in a buffer containing 250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) and centrifuged at 1 500*g* for 10 min. Both the supernatant (S1) and the pellet (P1) were retained for further isolation of the different subcellular membrane fractions. The supernatant (S1) was centrifuged at 18 000*g* for 10 min to obtain the mitochondrial subcellular pellet (P2) whilst the microsomal fraction remained in the supernatant (S2). The microsomal subfractions were subsequently collected from the S2 fraction by ultracentrifugation at 105 000*g* for 60 min. The mitochondrial and microsomal subcellular fractions were suspended in 10 mM Tris-HCl buffer (pH 7.4) and centrifuged at 18 000*g* for 10 min and 105 000*g* for 30 min, respectively. All the subcellular fractions were stored at -80°C until analysed.

Liver nuclear and plasma membrane subcellular fractions were prepared by fractionating the pellet (P1) on a self-forming Percoll (Sigma Chemical Co., St Louis, Missouri, United States) gradient by centrifugation at 35 000*g* for 20 min. By retaining the top layer and applying another Percoll gradient (45 000*g* for 30 min), the subsequent top and bottom layers, respectively yielded the plasma membrane and nuclear fractions. The collected fractions were centrifuged in 10 mM Tris-HCl

buffer and stored at -80°C prior to analysis. The protein concentration of the different fractions was determined according to the method of Kaushal and Barnes (1986).

Lipid analyses

The different subcellular membrane fractions were subjected to detailed lipid analysis; these included PC, PE, SM, total cholesterol and fatty acid profiles of PC and PE. In short, the different subcellular fractions (1-2 mg protein ml^{-1}) were extracted with chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant (Smuts *et al*., 1994; Folch *et al* 1957) and stored under nitrogen until analysed. The sample extracts were analysed for cholesterol and subsequently for phospholipids by thin layer chromatography (TLC) on 20 X 20 silica plates (Gilfillan *et al*., 1983) using chloroform/methanol/petroleum ether/acetic acid/boric acid (40:20:30:10:1.8, v/v/v/v/w) as developing solvent. Plates were developed for 90 min at room temperature followed by drying under N_2 gas for 30 min, and the respective phospholipid concentrations and fatty acid content determined.

Phospholipids and cholesterol

The phospholipid concentrations were quantified colourimetrically with malachite green dye (Itaya and Ui, 1966) following digestion in saturated perchloric acid at 170°C for approximately 1 h. Total cholesterol was determined on aliquots of the original lipid extract by an enzymatic iodide method (Richmond, 1973) with cholesterinoxidase and cholesterinesterase (Boehringer Mannheim, South Africa).

Fatty acids

The PC and PE fractions were transmethylated with 2.5 ml methanol/18 M sulphuric acid (95:5, v/v) at 70°C for 2 h as described by Tichelaar *et al*. (1989). The resultant fatty acid methyl esters (FAME) were analysed on a Varian 3700 Gas Chromatograph equipped with 30 m fused silica megabore DB-225 columns of 0.53 mm internal diameter (J & W Scientific, Catalogue number. 25-2232). The individual FAME were identified by comparison of the retention times with those of a standard

mixture of free FA C14:0 to C24:1 and quantified with an internal standard (C17:0) as μg FA mg protein^{-1}.

STATISTICAL ANALYSES

For comparisons to test whether two independent diet groups with normal distributions had the same mean, the T-test was used. The Pooled method was used if variances were equal, and the Satterthwaite method for unequal variances. Otherwise the nonparametric Wilcoxon Rank Sum test was used to compare the two independent diet groups with numerical observations that were not normally distributed.

RESULTS

Cholesterol and phospholipid content (Table 3.1)

The fumonisin B_1 treatment significantly increased the cholesterol content in the microsomal ($p = 0.0001$) and nuclear ($p = 0.05$) subcellular fractions, while no significant differences were observed in the mitochondrial and plasma subcellular fractions. The phospholipid, PC was significantly increased in the microsomal ($p = 0.003$) and decreased in the mitochondrial ($p = 0.026$) fractions, respectively. PE was significantly increased in the microsomal ($p = 0.0001$), mitochondrial ($p = 0.026$), plasma ($p = 0.048$) fractions. The SM content was significantly decreased and increased in the mitochondrial ($p = 0.013$), and nuclear ($p = 0.01$) fractions, respectively in the FB_1 treated rats.

Membrane lipid parameters (Table 3.1)

No significant change in the cholesterol/phospholipid (PC + PE) ratio was observed in any of the fractions of the FB_1 treated rats (data not shown). In contrast, a significant decrease in the PC/PE ratio was observed in the microsomal ($p = 0.005$), mitochondrial ($p = 0.005$) subcellular fractions of the FB_1 treated rats, mainly due to an increase in the phospholipid, PE.

The polyunsaturated/saturated (P/S) FA ratio was significantly decreased in PC of the microsomal ($p = 0.039$); mitochondria ($p = 0.024$) and plasma membrane ($p = 0.015$) fraction. In PE, the P/S ratio was only decreased in mitochondrial ($p = 0.035$) and plasma membrane ($p = 0.001$) fractions. Quantitatively (μg mg protein^{-1}), the total PUFA (ω–3 and ω–6 PUFA) levels in PC decreased significantly in the mitochondria ($p = 0.0001$); plasma membrane ($p = 0.05$) and nuclear ($p = 0.04$) fraction. In the microsomal fraction the total PUFA increased significantly ($p = 0.001$) only in PE. Qualitatively, the percentage (%) PUFA (expressed as the % of the total FA) decreased significantly in PC of the microsomal ($p = 0.001$), mitochondrial ($p = 0.0001$) and plasma membrane ($p = 0.002$) subcellular fractions. In PE, the % PUFA was significantly decreased only the mitochondrial ($p = 0.012$) and plasma membrane ($p = 0.001$) subcellular fractions due to FB_1 treatment.

The ω-6/ω-3 PUFA ratios of the treated rats was significantly increased in the PC fraction of the plasma membrane ($p = 0.01$) and marginally in the nuclei ($p = 0.08$). The 20:4ω-6 PC/PE ratio was significantly reduced, quantitatively in the microsomal ($p = 0.005$); mitochondrial ($p = 0.008$) and marginally in the plasma membrane ($p = 0.066$) fractions. Qualitatively, the 20:4ω-6 PC/PE ratio qualitative decreased significantly in the microsomal ($p = 0.005$), mitochondria ($p = 0.008$), plasma membrane ($p = 0.02$) and only marginally ($p = 0.09$) in the nuclear fraction as a result of the FB_1 treatment.

Table 3.1: The effect of fumonisin B_1 treatment on the lipid and fatty acid parameters of different membrane subcellular fractions from rat liver

	Control				Fumonisin B_1 Treated			
Lipid and fatty acid parameters	**Microsomes**	**Mitochondria**	**Plasma membrane**	**Nuclei**	**Microsomes**	**Mitochondria**	**Plasma membrane**	**Nuclei**
Cholesterol (µg mg protein^{-1})	23.53±1.53	30.24±3.22	34.03±5.60	2.76±0.82	**35.37±3.48 (p = 0.0001)**	31.36±4.26	37.99±4.13	**8.31±4.50 (p = 0.05)**
PC (µg mg protein^{-1})	181.00±34.50	276.2±034.10	153.00±25.90	24.89±4.55	**246.20±21.50 (p = 0.003)**	**227.40±30.40 (p = 0.026)**	157.90±38.10	22.94±1.67
PE (µg mg protein^{-1})	53.10±9.40	85.10±9.70	45.60±12.30	9.70±0.97	**122.80±13.10 (p = 0.0001)**	**122.30±9.50 (p = 0.026)**	**70.20±21.20 (p = 0.048)**	10.40±2.31
SM (µg mg protein^{-1})	10.80±2.50	12.90±2.50	7.40±3.10	3.25±0.72	9.80±3.00	**8.8 0±2.20 (p = 0.013)**	8.10±2.90	**7.95±2.94 (p = 0.01)**
PC/PE	3.23±0.40	3.11±0.50	2.80±1.69	2.77±0.58	**1.92±0.30 (p = 0.005)**	**1.76±0.22 (p = 0.005)**	2.24±0.66	3.35±0.18
P/S ratio								
PC	1.25±0.12	1.24±0.06	1.25±0.10	1.12±0.10	**1.12±0.12 (p = 0.039)**	**1.10±0.11 (p = 0.024)**	**1.07±0.09 (p = 0.015)**	1.03±0.07
PE	1.33±0.07	1.55±0.17	1.55±0.05	1.31±0.40	1.40±0.11	**1.35±0.10 (p = 0.035)**	**1.38±0.06 (p = 0.001)**	1.25±0.08
PUFA								
PC (µg mg protein^{-1})	55.27±7.03	66.14±4.00	73.96±18.31	6.40±0.70	55.79±5.02	**49.32±1.50 (p = 0.0001)**	**50.99±10.93 (p = 0.05)**	**5.17±0.87 (p = 0.04)**
PC (% of total FA)	50.81±1.50	50.52±0.95	50.43±2.21	46.60±2.95	**46.21±1.79 (p = 0.001**)	**45.81±1.50 (p = 0.0001)**	**45.70±1.69 (p = 0.002)**	44.99±1.50
PE (µg mg protein^{-1})	22.56±4.60	31.35±6.10	30.31±5.64	2.42±0.70	**36.45±5.18 (p = 0.001)**	35.32±3.02	33.20±8.68	2.61±0.78
PE (% of total FA)	52.71±1.09	56.08±2.40	55.89±1.14	48.10±8.04	53.24±0.79	**52.70±1.23 (p = 0.012)**	**53.09±0.84 (p = 0.001)**	48.30±2.86

Values are means ± standard deviation of 5 determinations. Values in bold, differ significantly ($p < 0.05$) from the corresponding control subcellular fraction. (Actual p-values are indicated in brackets). Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine, SM: sphingomyelin, P or PUFA: polyunsaturated fatty acids, S: saturated fatty acids.

Table 3.1 (continued): The effect of fumonisin B_1 treatment on the lipid and fatty acid parameters of different membrane subcellular fractions from rat liver

Lipid and fatty acid parameters	Control				Fumonisin B_1 Treated			
	Microsomes	Mitochondria	Plasma membrane	Nuclei	Microsomes	Mitochondria	Plasma membrane	Nuclei
ω-6/ω-3 ratio								
PC	23.61±2.16	24.76±2.26	21.51±1.14	24.75±4.47	27.73±5.33	28.16±5.10	**27.25±3.74 (p = 0.01)**	29.96±2.97 (p = 0.08)
PE	11.15±1.33	10.57±1.58	9.86±1.19	15.62±4.10	11.51±2.82	11.12±2.38	11.57±2.13	16.63±3.80
C20:4 ω-6 PC/PE ratio								
(μg mg protein^{-1})	3.01±0.73	2.66±0.52	2.49±1.30	3.20±1.12	**1.64±0.16 (p = 0.005)**	**1.49±0.11 (p = 0.005**)	1.75±0.31 (p = 0.066)	2.10±0.33
(% of total FA)	1.14±0.04	1.10±0.10	1.11±0.06	1.11±1.17	**0.92±0.05 (p = 0.005)**	**0.93±0.06 (p = 0.008)**	**0.96±0.10 (p = 0.02)**	0.96±0.06 (p = 0.09)

Values are means ± standard deviation of 5 determinations. Values in bold, differ significantly ($p < 0.05$) from the corresponding control subcellular fraction. (Actual p-values are indicated in brackets). Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine, SM: sphingomyelin, P or PUFA: polyunsaturated fatty acids, S: saturated fatty acids,

Fatty acids (Tables 3.2.1 to 3.2.4)

Fatty acid (FA) profiles of the PE and PC phospholipids fractions of the different subcellular fractions are summarized in Tables 3.2.1 – 3.2.4. Data from the different subcellular fractions is expressed quantitatively (μg mg protein^{-1}) and qualitatively as a percentage (%) of total FA content.

Saturated FA (SFA): (C16:0 and C18:0)

The FB_1 treatment significantly increased C16:0 ($p = 0.023$) in the PC phospholipid fraction in the microsomes. The total SFA content of PE ($p = 0.002$) increased significantly mainly due the increase in both C16:0 ($p = 0.005$) and C18:0 ($p = 0.002$). In the mitochondria, the SFA levels were decreased in PC ($p = 0.005$) due to a significant decrease in C18:0 ($p = 0.001$). In PE, the SFA levels were significantly increased ($p = 0.001$) due to a significant increase in both C16:0 ($p = 0.0001$) and C18:0 ($p = 0.046$). Similar patterns were also noticed in the plasma membrane PE and PC fractions although differences were not significant. In the nuclear subcellular fraction no effects were observed.

Qualitatively total SFA in the microsomal fraction of the treated rats were not altered despite a significant decrease and increase of the percentage C16:0 in the PC ($p = 0.033$) and PE ($p = 0.015$) fractions, respectively. In the mitochondrial fraction, the percentage total SFA was marginally increased ($p = 0.056$) due to a marginal increase in C16:0, whereas in the plasma membranes it was significantly increased ($p = 0.02$) in the PE fraction. In the nuclei, C16:0 ($p = 0.061$) and the total SFA marginally ($p = 0.065$) increased in PC.

Monounsaturated FA (MUFA): (C16:1ω-1- and C18:1ω-7)

The total MUFA content of PC and PE was significantly elevated in the microsomal fraction ($p = 0.0001$) due to an increase in C16:1ω-7 ($p = 0.008$) and C18:1ω-7 ($p = 0.0001$) in PC and C18:1ω-7 ($p = 0.0001$) in PE.

Table 3.2.1: Effect of fumonisin B_1 on the saturated fatty acid profiles of PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fractions	Control				Fumonisin B_1 Treated			
	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)
Microsomes								
C16:0	22.19±3.06	20.00±1.97	7.59±1.21	17.93±1.48	**27.17±3.36 (p = 0.023)**	**22.42±1.38 (p = 0.033)**	**10.71±1.79 (p = 0.005)**	**15.60±0.57 (p = 0.015)**
C18:0	22.02±4.03	20.13±1.40	9.22±1.75	21.60±1.05	23.11±4.65	18.92±2.11	**15.71±3.46 (p = 0.002)**	22.69±2.03
Total	44.20±4.25	40.13±1.93	16.81±2.76	39.54±1.23	50.28±7.65	41.34±2.67	**26.42±5.20 (p = 0.002)**	38.28±2.20
Mitochondria								
C16:0	25.63±3.12	19.55±1.64	7.61±0.86	13.95±2.70	24.70±1.56	**22.94±1.42 (p = 0.003)**	**10.80±0.54 (p = 0.001)**	16.13±1.01 (p = 0.09)
C18:0	27.70±2.80	21.13±1.50	12.60±2.90	22.53±0.71	**20.22±2.90 (p = 0.001)**	18.75±2.40 (P = 0.066)	**15.53±2.42 (P = 0.046)**	23.07±2.26
Total	53.32±4.63	40.70±1.33	20.20±1.90	36.09±2.30	**44.92±3.53 (p = 0.005)**	41.69±2.75	**26.30±2.75 (p = 0.001)**	39.20±2.06 (p = 0.056)
Plasma membrane								
C16:0	26.46±7.25	8.71±2.98	7.23±2.64	13.30±0.82	23.00±5.26	21.17±1.39 (p = 0.096)	8.94±1.69	14.70±1.90
C18:0	32.93±8.39	22.19±1.67	12.35±1.96	23.35±1.09	24.49±6.28	21.68±1.50	15.20±4.72	23.88±2.22
Total	59.39±14.13	40.90±1.52	19.58±3.51	36.65±1.01	48.17±11.41	42.86±2.20	24.15±6.24	**38.58±1.39 (p = 0.02)**
Nuclei								
C16:0	2.83±0.26	20.75±1.53	0.82±0.27	17.33±7.83	2.61±0.53	22.65±1.00 (p = 0.061)	0.95±0.50	16.88±4.21
C18:0	2.83±0.35	20.69±1.54	1.04±0.19	21.42±3.82	2.44±0.36	21.27±1.25	1.15±0.30	21.73±3.02
Total	5.66±0.52	41.44±1.82	1.86±0.15	38.75±8.47	5.05±0.88	43.92±1.75 (p = 0.065)	2.84±0.70	38.61±2.20

Values are means ± standard deviation of 5 determinations. Values in bold, differ significantly ($p < 0.05$) from the corresponding control subcellular fraction. (Actual p-values are indicated in brackets). Percentage (%) = % of total FA. Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine.

Table 3.2.2: The effect of fumonisin B_1 on the monounsaturated fatty acid profiles of PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fraction	Control				Fumonisin B_1 Treated			
	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)
Microsomes								
C16:1ω-7	0.40±0.09	0.85±0.19	0.23±0.06	0.56±0.17	**1.81±0.81 (p = 0.008)**	1.55±0.89	0.27±0.07	0.40±0.15
C18:1ω-7	8.87±0.78	8.21±0.85	3.09±0.74	7.20±0.58	**13.22±0.66 (p = 0.0001)**	**10.97±0.64 (p = 0.0001)**	**5.41±0.34 (p = 0.001)**	8.07±1.58
Total	9.27±0.77	9.06±0.75	3.32±0.72	7.76±0.45	**15.03±0.55 (p = 0.0001)**	**12.52±1.38 (p = 0.0003)**	**5.67±0.29 (p = 0.0001)**	8.47±1.65
Mitochondria								
C16:1ω-7	0.89±0.20	0.68±0.12	0.19±0.08	0.36±0.16	1.67±1.13	1.55±1.06	0.27±0.11	0.42±0.20
C18:1ω-7	10.60±1.02	8.12±0.90	3.95±0.80	7.10±0.44	**11.77±0.54 (p = 0.033)**	**10.94±0.64 (p = 0.0001)**	**5.11±0.64 (p = 0.019)**	7.68±1.23
Total	11.50±1.10	8.80±0.89	4.15±0.80	7.43±0.50	**13.44±1.60 (p = 0.032)**	**12.49±1.60 (p = 0.0005)**	**5.40±0.70 (p = 0.015)**	8.10±1.40
Plasma membrane								
C16:1ω-7	0.79±0.37	0.50±0.27	0.25±0.18	0.38±0.32	1.03±0.24	**0.98±0.42 (p = 0.037)**	0.16±0.09	0.28±0.15
C18:1ω-7	11.16±2.43	8.17±1.14	3.74±0.24	7.08±0.52	11.62±2.30	**10.46±0.49 (p = 0.001)**	4.89±0.92 (p = 0.08)	8.05±1.19 (p = 0.096)
Total	11.95±2.64	8.67±1.07	3.99±0.92	7.46±0.62	12.65±2.25	**11.44±0.84 (p = 0.0005)**	5.05±0.93	8.33±1.26
Nuclei								
C16:1ω-7	0.23±0.07	1.66±0.53	0.11±0.09	2.11±1.69	0.15±0.03 (p = 0.066)	1.27±0.09	0.14±0.11	3.07±2.56
C18:1ω-7	1.40±0.40	10.32±3.42	0.56±0.31	11.05±4.39	1.13±0.19	9.83±0.20	0.55±0.34	10.01±3.95
Total	1.62±0.40	11.99±3.41	0.66±0.29	13.16±3.71	1.30±0.22	11.09±0.26	0.70±0.28	13.10±4.26

Values are means ± standard deviation of 5 determinations. Values in bold, differ significantly ($p < 0.05$) from the corresponding control subcellular fraction. (Actual p-values are indicated in brackets). Percentage (%) = % of total FA. Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine.

In the mitochondria, the total MUFA were increased in PC ($p = 0.032$) and PE ($p = 0.015$) due to an increase in C18:1ω-7 in PC ($p = 0.033$) and PE ($p = 0.019$) fractions. Only a marginally ($p = 0.066$) decreased of C16:1ω-7 was noticed in the nuclear PC. Except for a marginal ($p = 0.08$) increase in C18:1ω-7 in PE, no changes was noticed in the plasma membrane in the nuclear fraction.

In the PC fractions the qualitative levels of the MUFA was significantly increased in the microsomal ($p = 0.0003$), mitochondrial ($p = 0.0005$) and plasma membrane ($p = 0.0005$) subcellular fractions due to an increase in C18 (between $p < 0.001$ and $p < 0.0001$). In the plasma membrane fraction C16:1 ($p = 0.037$) was increased in PC. In the PE fraction C18:1ω-7 was marginally ($p = 0.096$) increased only in the plasma membrane fraction.

Polyunsaturated FA (PUFA)

ω–6 PUFA, (C18:2ω-6, C18:3ω-6, C20:3ω-6, C20:4ω-6, C22:4ω-6 and C22:5ω-6): In the microsomal subcellular fraction C18:2ω-6 was significantly increased ($p = 0.0004$) in the PC fraction. The total ω-6 PUFA content in the PE fraction was significantly ($p = 0.001$) increased due to a significant increase in the levels of C18:2ω-6 ($p = 0.0001$), C20:4ω-6 ($p = 0.002$), C22:4ω-6 ($p = 0.0001$) and C22.5ω-6 ($p = 0.002$).

In the mitochondria, the total ω6 PUFA content was significantly reduced in PC ($p = 0.0001$) due to a decrease in C20:4ω-6 ($p = 0.0001$), C22:4ω-6 ($p = 0.0001$) and C22:5ω-6 ($p = 0.004$) despite the fact that C18:2ω-6 ($p = 0.014$) increased. Except for a significant increase in C18:2ω-6 ($p = 0.022$), no other changes were noticed with respect to the PE fraction. In the plasma membrane PC, the total ω-6 PUFA content was marginally ($p = 0.05$) decreased due to a decrease in C20:4ω-6 ($p = 0.02$), C22:4ω-6 ($p = 0.02$) and C22:5ω-6 ($P=0.003$). In the nuclei, the total ω-6 FA was decreased significantly in PC ($p = 0.044$) due to a decrease in C20:4ω-6 ($p = 0.022$). In PE, C22:4ω-6 ($p = 0.001$) increased significantly. Due to the low levels of C18:3ω-6 and C20:3ω-6 it was not included in table 2.3 however; they were included in determining the total PUFA values.

Qualitatively the total ω-6 PUFA decreased significantly ($p < 0.005$) in PC in the microsomal, mitochondrial and plasma membrane fractions due to significant ($p < 0.05$) decrease in the long-chain ω-6 PUFA (C20:4ω-6; C22:4ω-6 and C22:5ω-6), in spite of significant increases in C18:2ω-6 in the microsomes ($p = 0.0003$), mitochondria ($p = 0.0002$) and plasma membrane ($p = 0.0002$) fractions. In the nuclei the total ω-6 PUFA only decreased marginally ($p = 0.089$) due to a marginal ($p = 0.054$) and significant ($p = 0.011$) decrease in C20:4ω-6 and C22:5ω-6, respectively while C22:4ω-6 significantly ($p = 0.003$) increased. The total ω-6 PUFA in the PE fraction was decreased significantly in the mitochondrial ($p = 0.02$) and plasma membrane ($p = 0.014$) fractions due to a decrease in the level of C20:4ω-6 ($p = 0.041$) and C22:4ω-6 ($p = 0.003$), respectively. C18:2ω-6 was significantly increased in both the microsomal fraction ($p = 0.033$) as well as the mitochondrial fraction ($p = 0.022$) and marginally in the plasma membrane ($p = 0.056$). In the nuclear fraction, only C22:4ω-6 ($p = 0.002$) was increased.

ω–3 PUFA, (C22:5ω-3, C22:6ω-3): The total ω–3 FA in PC significantly decreased quantitatively in the mitochondrial ($p = 0.0001$) and plasma membrane ($p = 0.012$) due to a decrease in both C22:5ω-3 and C22:6ω-3 ($p < 0.05$). In the microsomes, only C22:5ω-3 was significantly ($p = 0.019$) decreased while in the nuclear fraction, the total ω–3 FA decreased significantly ($p = 0.041$) due to a decrease ($p = 0.046$) in C22:6ω-3. In the microsomal PE the total ω-3 FA ($p = 0.006$) significantly increased due to an increase in C22:5ω-3 ($p = 0.033$) and C22:6ω-3 ($p = 0.01$).

Qualitatively the total ω–3 PUFA decreased significantly in PC in the microsomal ($p = 0.014$), mitochondria ($p = 0.027$) and plasma membrane ($p = 0.002$) fractions. This is due to a significant decrease in both C22:5ω-3 and C22:6ω-3 in the microsomal (C22:5ω-3, $p = 0.004$; C22:6ω-3, $p = 0.018$), mitochondria (C22:6ω-3, $p = 0.038$) and plasma membrane (C22:5ω-3, $p = 0.032$; C22:6ω-3, $p = 0.005$) fractions. The total ω-3 FA in the PE fraction, was marginally decreased ($p = 0.051$) in the plasma membrane subcellular fraction due to a decrease in C22:5ω-3 ($p = 0.012$). No changes were observed in PE for any of the other subcellular fractions.

Table 3.2.3: Fumonisin B_1 modulation of the ω-6 fatty acid profiles of the PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fractions	Fatty Acids	Control				Fumonisin B_1 Treated			
		PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)
Microsomes	C18:2ω-6	9.54±0.40	8.86±0.92	2.84±0.76	6.57±0.64	**15.13±1.77 (p = 0.0004)**	**12.54±1.42 (p = 0.0003)**	**5.20±0.63 (p = 0.0001)**	**7.65±0.85 (p = 0.033)**
	C20:4ω-6	36.76±4.72	33.80±1.20	12.71±2.71	29.66±1.18	32.48±3.60	**26.83±1.35 (p = 0.0001)**	**20.06±3.27 (p = 0.002)**	29.23±0.90
	C22:4ω-6	1.02±0.11	0.94±0.06	1.06±0.19	2.50±0.11	0.93±0.07	**0.78±0.11 (p = 0.009)**	**1.73±0.13 (p = 0.0001)**	2.56±0.36
	C22:5ω-6	4.84±1.40	4.38±0.76	3.80±0.76	8.98±1.37	4.17±0.78	**3.43±0.41 (p = 0.023)**	**6.08±1.10 (P = 0.002)**	8.83±0.05
	Total	53.00±6.65	48.74±1.41	20.68±4.20	48.33±1.26	53.81±5.00	**44.50±1.62 (p = 0.001)**	**33.44±4.92 (p = 0.001)**	48.83±1.06
Mitochondria	C18:2ω-6	11.35±1.0	8.68±0.80	3.71±0.81	6.64±0.70	**13.39±1.35 (p = 0.014)**	**12.45±1.39 (p = 0.0002)**	**4.80±0.17 (p = 0.022)**	**7.21±075 (p = 0.022)**
	C20:4ω-6	44.63±2.96	34.09±1.20	17.33±3.65	30.98±1.88	**28.82±1.60 (p = 0.0001)**	**26.80±1.27 (p = 0.0001)**	19.35±1.32	**28.89±0.06 (p = 0.041)**
	C22:4ω-6	1.20±0.10	0.91±0.05	1.52±0.37	2.72±0.38	**0.83±0.11 (p = 0.0001)**	**0.77±0.11 (p = 0.015)**	1.60±0.20	2.41±0.35
	C22:5ω-6	5.40±1.10	4.11±0.74	5.59±1.10	10.04±1.42	**3.13±0.45 (p = 0.004)**	**3.34±0.36 (p = 0.044)**	6.17±1.24	9.15±1.16
	Total	63.56±3.91	48.55±0.85	28.61±5.62	51.16±2.20	**47.58±1.43 (p = 0.0001)**	**44.19±1.40 (p = 0.0001)**	32.29±2.42	**48.21±0.62 (p = 0.02)**

Values are means ± standard deviation of 5 determinations. Values in bold, differ significantly ($p < 0.05$) from the corresponding control subcellular fraction. (Actual p-values are indicated in brackets). Percentage (%) = % of total FA. Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine.

Table 3.2.3 (continued): Fumonisin B_1 modulation of the ω-6 fatty acid profiles of the PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fractions	Fatty Acids	Control				Fumonisin B_1 Treated			
		PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)
Plasma Membrane	C18:2ω-6	11.95±2.93	8.33±0.56	3.56±0.78	6.66±0.58	12.64±2.86	**11.38±1.19 (p = 0.0002)**	4.56±0.96	7.44±0.68 (p = 0.056)
	C20:4ω-6	49.50±12.98	33.73±1.83	16.38±3.17	30.32±0.76	**31.08±7.04 (p = 0.02)**	**27.78±1.58 (p = 0.0001)**	18.32±5.17	29.18±1.65
	C22:4ω-6	1.47±0.40	0.97±0.10	1.57±0.31	2.91±0.14	**0.90±0.19 (p = 0.02)**	**0.82±0.15 (p = 0.075)**	1.62±0.39	**2.61±0.11 (p = 0.003)**
	C22:5ω-6	6.51±1.29	4.41±0.94	5.50±0.85	10.00±1.28	**3.59±0.85 (p = 0.003)**	**3.21±0.37 (p = 0.016)**	5.77±1.65	9.15±0.79
	Total	70.65±17.42	48.20±2.00	27.48±5.07	50.71±0.77	**49.16±10.55 (p = 0.05)**	**44.05±1.57 (p = 0.002)**	30.52±8.13	**48.76±1.41 (p = 0.014)**
Nuclei	C18:2ω-6	1.36±0.30	10.03±2.21	0.47±0.37	8.96±5.52	1.27±0.20	11.09±1.01	0.54±0.37	9.25±3.60
	C20:4ω-6	4.11±0.70	30.04±4.25	1.40±0.46	27.95±7.40	**2.95±0.50 (p = 0.22)**	25.70±0.35 (p = 0.054)	1.30±0.36	24.51±4.40
	C22:4ω-6	0.08±0.01	0.62±0.11	0.10±0.03	1.79±0.48	0.14±0.06	**1.15±0.29 (p = 0.003)**	**0.20±0.05 (p = 0.001)**	**3.83±1.10 (p = 0.002)**
	C22:5ω-6	0.46±0.11	3.36±0.66	O.30±0.20	5.80±3.75	0.56±0.11	**4.85±0.75 (p = 0.011)**	0.40±0.15	7.34±0.96
	Total	6.11±0.64	44.70±2.70	2.26±0.63	44.99±7.35	**5.01±0.84 (p = 0.044)**	43.52±1.54 (p = 0.089)	2.46±0.76	45.44±3.16

Values are means ± standard deviation of 5 determinations. Values in bold, differ significantly ($p < 0.05$) from the corresponding control subcellular fraction. (Actual p-values are indicated in brackets). Percentage (%) = % of total FA. Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine

Table 3.2.4: Fumonisin B_1 modulation of the ω-3 fatty acid profiles of the PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fractions	Control				Fumonisin B_1 Treated			
	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)	PC (μg mg protein^{-1})	PC (%)	PE (μg mg protein^{-1})	PE (%)
Microsomes								
C22:5ω-3	0.19±0.04	0.17±0.03	0.14±0.06	0.33±0.09	**0.14±0.01** (p = 0.019)	**0.12±0.02** (p = 0.004)	**0.23±0.05** (p = 0.033)	0.34±0.10
C22:6ω-3	1.97±0.40	1.80±0.20	1.68±0.38	3.92±0.40	1.71±0.33	**1.42±0.27** (p = 0.018)	**2.66±0.66** (p = 0.01)	3.90±0.76
Total	2.27±0.42	2.08±0.21	1.88±0.45	4.38±0.46	1.99±0.34	**1.65±0.29** (p = 0.014)	**3.00±0.67** (p = 0.006)	4.41±0.82
Mitochondria								
C22:5ω-3	0.22±0.04	0.17±0.03	0.22±0.07	0.40±0.10	**0.13±0.04** (p = 0.002)	0.12±0.04	0.23±0.04	0.34±0.08
C22:6ω-3	2.22±0.23	1.70±0.20	2.44±0.50	4.39±0.57	**1.48±0.28** (p = 0.0005)	**1.37±0.27** (p = 0.038)	2.70±0.80	4.00±1.00
Total	2.58±0.22	1.97±0.19	2.74±0.60	4.92±0.70	**1.73±0.29** (p = 0.0002)	**1.61±0.28** (p = 0.027)	3.03±0.80	4.50±0.92
Plasma membrane								
C22:5ω-3	0.28±0.10	0.18±0.04	0.22±0.05	0.42±0.08	**0.14±0.03** (p = 0.05)	**0.13±0.03** (p = 0.032)	0.18±0.03	**0.29±0.06** (p = 0.012)
C22:6ω-3	2.73±0.75	1.80±0.27	2.37±0.48	4.31±0.54	**1.46±0.38** (p = 0.009)	**1.31±0.20** (p = 0.005)	2.34±0.66	3.77±0.75
Total	3.31±0.92	2.23±0.23	2.83±0.63	5.17±0.61	**1.83±0.45** (p = 0.012)	**1.65±0.25** (p = 0.002)	2.68±0.70	4.33±0.70 (p = 0.051)
Nuclei								
C22:5ω-3	0.02±0.01	0.12±0.04	0.02±0.02	0.32±0.36	0.01±0.01	0.13±0.05	0.02±0.01	0.32±0.17
C22:6ω-3	0.18±0.03	1.32±0.21	0.12±0.06	2.43.09	**0.13±0.03** (p = 0.046)	1.16±0.07	0.11±0.04	2.05±0.58
Total	0.26±0.06	1.86±0.40	0.16±0.06	3.11±1.11	**0.17±0.04** (p = 0.041)	1.46±0.12	0.15±0.03	2.86±0.67

Values are means ± standard deviation of 5 determinations. Values in bold, differ significantly ($p < 0.05$) from the corresponding control subcellular fraction in controls. (Actual p-values are indicated in brackets). Percentage (%) = % of total FA.

DISCUSSION

FB_1 disrupts sphingolipid, phospholipids, FA and cholesterol metabolism in the liver and kidneys of different animal species. The disruption of the critical balance between proliferation and apoptosis by these different lipid parameters has been associated with cancer promotion by FB_1 in liver and kidney of rats (Gelderblom *et al*., 2001; Riley *et al*., 2001). However, it is unclear how changes in the lipid components of the different subcellular membrane fractions could create a growth differential in the liver that selectively stimulate the outgrowth of initiated cells. The current model and FB_1-dose used promotes cancer in the liver (Gelderblom *et al.,* 1996) while a similar dose over a period of 5 weeks induces hepatocellular carcinoma in Fischer 344 rats (Lemmer *et al*., 2004).

Alterations to lipid components in the liver are associated with changes in membrane fluidity reflected by changes in the cholesterol/phospholipid (PC + PE) ratio, PC/PE and P/S ratios (Mahler *et al*., 1988a, 1998b; Abel *et al*., 2001). In the present study, the PC/PE ratio fluidity indicator, decreased significantly in the microsomal and mitochondrial subcellular fractions, mainly due to a significant increase in PE. The P/S ratio was decreased in PC from the microsomal, mitochondrial and plasma membrane subcellular membrane fractions and in PE in the mitochondrial and plasma membrane subcellular fractions due to a significant decrease and increase in the qualitative levels of PUFA and SFA, respectively. Changes in these lipid parameters, which differ depending on the subcellular fraction, are likely to alter membrane fluidity. A reduction in the PC/PE and P/S ratios is associated with a more rigid membrane structure (Mahler *et al*., 1988a, 1998b), which could adversely affect many critical biological processes occurring at the membrane. Although the cholesterol/phospholipid (PC + PE) ratio was not altered significantly in the present study, the cholesterol was increased in the microsomal and nuclear membrane fraction, which will further increase the rigidity of the membrane structure as it stabilizes the fatty acyl groups (Ohvo-Rekila *et al*., 2002).

Regarding the maintenance of cellular homeostasis, the organisation of lipids and proteins into specialised clusters or microdomains on the outer leaflet in the plasma membrane is of importance. These microdomains, including lipid rafts and caveolae,

are enriched with SM, cholesterol, C20:4ω6-containing plasmanylethanolamine and glycolipids serve as platforms for vesicular trafficking and the initiation and regulation of cell signalling processes (Seghal *et al*., 2002; Pike, 2003). Many different growth factor receptors, including the endothelin, EGF, insulin receptor, insulin-like growth factor, platelet derived growth factor; tumour necrosis factor (TNF) and folic acid receptor are lodged in lipid rafts/caveolae (Cottin *et al*., 2002; Huo *et al*., 2003; Kamen and Smith, 2004; Pike, 2005). Lipid rafts/caveolae-associated signaling events affected by FB_1, include the inhibition of folate receptor-mediated vitamin uptake (Stevens and Tang, 1997), increased expression of TNF-alpha, TNF receptor-1, TNF-related apoptosis-induced ligand (He *et al*., 2005) and the inhibition of the EGF-induced mitogenic response (Gelderblom *et al*., 2001). The effect of FB_1 on these signaling processes has been related to the disruption of membrane integrity involving alterations in the sphingolipid (Stevens and Tang, 1997; He *et al*., 2005), cholesterol, phospholipid and FA metabolism (Gelderblom *et al*., 1999). Although, the plasma membrane concentrations of SM and cholesterol were not altered, the P/S ratio and PUFA were significantly reduced suggesting, as discussed above, a more rigid membrane structure, likely to affect membrane receptor and enzyme responses. The ω6/ω3 ratio was also increased due to a decrease in ω3 PUFA in PC which will impact on the prostaglandin synthesis by directing prostanoids synthesis more towards the E2-series associated with sustained cell proliferation (Bagga *et al*., 2003). In this regard the C20:4ω–6 PC/PE ratio was significantly decreased, possibly due to the FB_1-induced increase in the concentration of PE and the decrease in C20:4ω–6 in PC. The increased level of C20:4ω–6 in PE relative to PC has been implied as an important growth stimulus in hepatocyte nodules (Abel *et al*., 2001). Changes in the content of C20:4ω–6–enriched ethanolamine plasmalogens could also be important during raft mediated receptor responses and should be further investigated.

Changes to the major lipid parameters in the microsomal membrane fraction are similar to that reported previously (Ueno *et al*., 1997). Altered lipid parameters are known to impact on the activity of membrane enzymes relating to the synthesis of proteins, lipids and sterols (Pahl, 1999). The activity of cytochrome P450 isozymes (Spotti *et al*., 2000), ceramide synthase (Wang *et al*., 1991), and the delta-6

desaturase (Ueno *et al*., 1997) has been reported to be inhibited by FB_1. Although FB_1 increased the level of cholesterol in the rat liver microsomal membrane fraction the effect on 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in endogenous cholesterol biosynthesis, has not been elucidated. The level of SM was not altered in the microsomal membrane fraction, which is in agreement with a previous report (Erlich *et al*., 2002).

In the mitochondrial subcellular fraction, the PC/PE ratio was significantly reduced due to a decrease and increase in PC and PE, respectively. Disruption of the typical asymmetric lipid distribution of cell membranes could impact negatively on important physiological processes such as the induction of apoptosis (Bevers *et al.*, 1999). In addition to the changes in PC and PE, SM was also reduced, presumably due to the disruption of the *de novo* sphingolipid biosynthesis by FB_1. In this regard the presence of both ceramide synthase and ceramide has been observed in the mitochondrial membrane fraction (Ardail *et al*., 2001; Bionda *et al*., 2004). Ceramide regulates the generation of reactive oxygen species and the activation of mitochondrial apoptosis via several mechanisms, including glutathione peroxidase depletion and increased lipid peroxidation (Won and Singh, 2006). One of these mechanisms involves the down regulation of Bcl-2, an anti-apoptotic protein, which leads to the opening of the mitochondrial permeability transition pores (PTP) and apoptosis. Resistance to mitochondrial PTP opening is responsible for the promotion of initiated cells and an important event in the 2-acetylaminofluorene-induced hepatocarcinogenesis (Klöhn *et al*., 2003). A moderate increase in lipid peroxidation was noticed in rat liver mitochondria after a dietary exposure of 250 mg FB_1/kg (Norred *et al.*, 1992), which could be related to the disruption of ceramide synthase.

It is unclear whether the depletion of ceramide in the mitochondria of altered hepatocytes is related to the cancer promoting properties of FB_1. However, it was suggested that FB_1-induced apoptosis may be due to the inhibition of ceramide synthase resulting in the depletion of ceramide and other complex sphingolipids and the accumulation of sphinganine and sphingosine (Dragan *et al*., 2001; Riley *et al*., 2001). Cells sensitive to the proliferative effects of decrease ceramide and increased sphingosine 1- phosphate may have a selective growth advantage. Although the liver is not normally a proliferative organ, it seems likely that the disruption of lipid and

sphingolipid metabolism in the mitochondria by FB_1 could result in the impairment of apoptosis in the altered hepatocytes. The differential effect of FB_1 on mitochondrial oxidative damage in normal and initiated hepatocytes, therefore, could play an important role in their altered growth pattern. It has been postulated that, depending on the cell type, the disruptive effect of FB_1 on the sphingolipid metabolism, i.e. a decrease in ceramide and increased sphingosine 1-phosphate, will either favour proliferation or induce cell death (Lemmer *et al*., 1998; Riley *et al*., 2001). A recent study indicated that sphingosine accumulates in FB_1-induced hepatocyte nodules suggesting it may be involved in the enhanced growth characteristics of these lesions via the formation of sphingosine 1-phosphate (Van der Westhuizen *et al*., 2004).

Lipid metabolism in the nuclear membrane fraction is considered to play an important role in signaling events that occur in this cellular compartment (Ledeen and Wu, 2004). The effect of FB_1 on nuclear-associated membrane enzymes regarding lipid metabolism has not been established. In the present study the nuclear membrane fraction behave very similar to the microsomes regarding the increase in cholesterol with the exception that SM also increased. The presence of a SM cycle has been established in the rat liver nuclei (Ledeen and Wu, 2004), however the increase in SM in the nuclei due to FB_1 is unknown. As it was reported that similar levels of cholesterol and SM occur in the rat liver nuclei (Ledeen and Wu, 2004), the increase in SM could resulted from the corresponding increase in cholesterol. This increase and decrease of SM in the nuclei and mitochondria, respectively could also explain why the level of SM was not affected by FB_1-exposured when analyzing whole liver (Gelderblom *et al*., 1997). The decrease in both ω–3 and ω–6 PUFA in the PC fraction could be due to an increase in lipid peroxidation in rat liver nuclei (Abel and Gelderblom, 1998). FB_1-induced peroxidation of the membrane lipids was reported to induce oxidative DNA damage in isolated liver nuclei (Sahu *et al*., 1998).

When considering the FA parameters, the observed changes differ for each subcellular membrane fractions as a result of the FB_1 exposure. Apart from the relative increase and decrease in SFA and PUFA, respectively, the total MUFA were

moderately to significantly increased in PC and PE in the different subcellular fractions, except for the nuclear fraction. Of interest is the increase in C18:1ω–9, the most abundant MUFA in membranes (Pala *et al*., 2001), which is associated with the modulation of the function of membrane-bound proteins in normal cells (Funari *et al*., 2003). In addition, the increased in MUFA, specifically C18:1ω–9 and C18:2ω–6 is associated with the disruption of the delta-6 desaturase enzyme known to be inhibited by FB_1 (Gelderblom *et al*., 2002). The resultant increase in MUFA could also be due to an increase in delta-9 desaturase as observed in tumour growth in mouse mammary carcinoma cells (Lu *et al*., 1997), hepatoma cells (De Alaniz and Marra, 1994), human leukemia and lymphoma cells (Marzo *et al*., 1995). The growth of mammary carcinogenesis *in vitro* was blocked by the addition of an inhibitor of delta-9 desaturase (Khoo *et al*., 1991). It is not known at present whether FB_1 affect the activity of delta-9 desaturase. As C18:1ω–9 is suggested to exhibit anti-oxidative properties (Diplock *et al*., 1988) the accumulation thereof is likely to protect against the increased lipid peroxidation induced by FB_1 in rat liver and subcellular fractions (Diplock *et al*., 1988; Abel *et al*., 2004). The significant reduction in PUFA in most of the subcellular fractions indicated that apart from the inhibition of the delta-6 desaturase, the FB_1-induced lipid peroxidation also impacted on the status of the long chained fatty acids such as C22:5ω–6 and C22:6ω–3. The increase lipid peroxidation and the resultant lipid breakdown products, especially in the nuclei, could be important in the cancer initiating properties of FB_1 and in determining the extent of necrotic and/or apoptotic cell death.

Arachidonic acid (C20:4ω–6) has been associated with the growth regulatory effects of FB_1 in primary hepatocytes and cancer cells (Seegers *et al*., 2000; Ehrlich *et al*., 2002). It was hypothesized that the increase of C20:4ω–6 in the PE phospholipids fraction together with the decrease of long chain PUFA and associated low levels of lipid peroxidation are early events in the neoplastic transformation of hepatic nodules (Abel *et al*., 2001). In the present study the C20:4ω–6 PC/PE ratio was decreased in all the subcellular membrane fractions, due to an increased level of C20:4ω–6 in PE. The importance of C20:4ω–6 is further highlighted by the dual effect it has on cell proliferation or apoptosis via the formation of prostanoids or ceramide, respectively (Cao *et al*., 2000; Tapiero *et al*., 2002; Zhao *et al*., 2002). Recently a member of the

phospholipase A_2 (PLA_2) enzyme-family, the type VI calcium-independent $iPLA_2$, involved in C20:4ω–6-generation and associated with C20:4ω6-induced apoptosis, was detected in close proximity of cyclooxygenase-2 (COX-2) in the mitochondria (Liou *et al*., 2005). It has also been shown that FB_1 stimulates cytoplasmic PLA_2 activity, resulting in an increase in C20:4ω6 and its metabolites (Pinelli *et al*., 1999). The suppression of apoptosis seems to be related to the conversion of C20:4ω–6 to prostanoids by two isoforms of the enzyme cyclooxygenase (COX), shown to be overexpressed in numerous human neoplasms (Shibata *et al*., 2005). In this regard the elevated expression of these COX isoform enzymes could be responsible for reduced availability for ceramide generation via the sphingomyelinase pathway (Zhao *et al*., 2002). The current study indicated that SM synthesis is also impaired by FB_1 in the mitochondria through the inhibition of ceramide synthase.

The interaction between COX-2, PLA_2 and ceramide synthase play a determining role in the regulation of apoptosis and cell proliferation during FB_1 exposure. Therefore, the differential regulation of C20:4ω–6 and ceramide levels in normal and altered hepatocytes are likely to be important determinants in the selective stimulation of preneoplastic lesion in the liver by FB_1.

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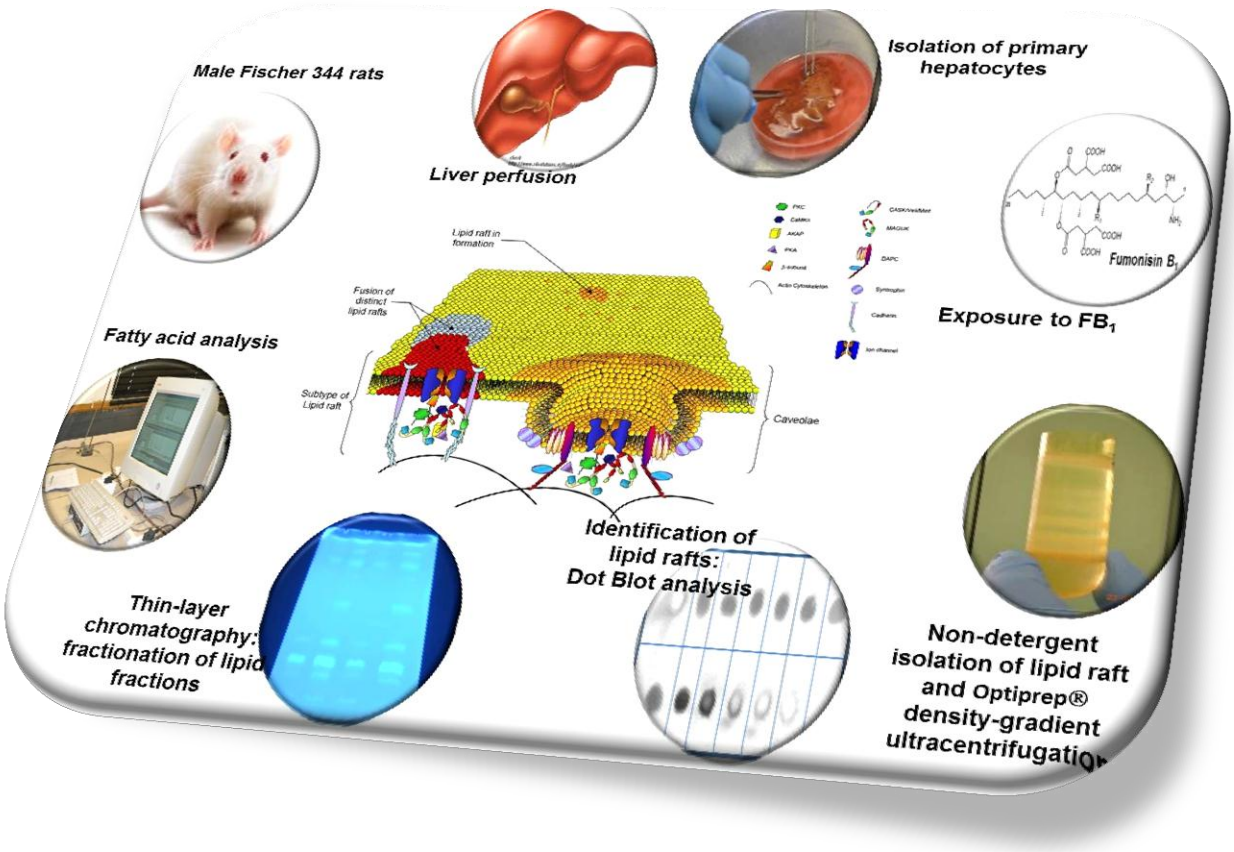

CHAPTER 4

FUMONISIN B_1-INDUCED CHANGES TO THE LIPID COMPOSITION OF MEMBRANE RAFTS IN PRIMARY RAT HEPATOCYTES

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ABSTRACT

Non-detergent isolated lipid rafts from rat primary hepatocytes in short-term exposure to fumonisin B_1 (FB_1) (250 μL) affected the major raft constituents. Cholesterol (CHOL) was significantly increased whereas sphingomyelin (SM) was marginally reduced. Raft-associated phospholipid (PL) phosphatidylcholine (PC) remained unaffected while the inner membrane leaflet PL phosphatidylethanolamine (PE) was increased by FB_1 exposure. Due to the changes observed in the rafts, phospholipid fluidity parameters such PC/PE and PC/(PE+SM) ratio indicated an increase in raft rigidity. The CHOL/PL ratio however was not affected and indicative of a compensatory increase in CHOL due to the increase in PE to maintain raft rigidity and integrity. The increase in PE resulted in a simultaneous increase of its related fatty acid levels, these included total saturated fatty acids (SFAs) and the polyunsaturated fatty acids (PUFAs). The concomitant increases in SFAs and PUFAs are regarded as a counteractive measure to maintain the rafts saturation state. The total ω-3 fatty acids of the PE fraction were noticeably increased, specifically the long chain C22:6ω3 which may further increase CHOL. The perturbation of lipid raft composition by FB_1 suggests the inactivation of ligand-receptor signalling such as the epidermal growth factor pathway or the activation of signals such as those related to Akt. The disruption of the folate receptor and the resultant deficiency suggest impaired lipid raft integrity. The alterations in lipid metabolism as a mechanism for the promotion of cancer by FB_1 via the induction of a growth differential could be related to changes in lipid raft structure and function.

INTRODUCTION

Fumonisin B_1 (FB_1), a group 2B carcinogenic mycotoxin produced by the fungal species *Fusarium verticillioides* has been implicated in several animal and human diseases, presumably via the disruption of lipid metabolism (IARC, 2002). The cell membrane is targeted by FB_1 due to the hydrophobic/hydrophilic characteristics of the molecule during which key lipid parameters are affected. These lipid alterations include an increase in cholesterol (CHOL), phosphatidylethanolamine (PE), arachidonic acid (C20:4ω-6), saturated (SFAs) and monounsaturated (MUFAs) fatty acids and a decrease in polyunsaturated fatty acids (PUFAs) indicative of the disruption of the delta-6 desaturase enzyme. These lipid associated changes result in a more rigid membrane structure (Abel *et al*., 1998; Gelderblom *et al*., 1996a; 2001a; 2001b, 2002). In addition, FB_1 inhibits the production of ceramide and complex sphingoid bases with a resultant accumulation of sphinganine, sphingosine and their 1-phoshate derivatives by disrupting *de novo* ceramide synthase (Wang *et al*., 1991; Riley *et al*., 1996; Merrill *et al*., 2001). A study investigating the effect of FB_1 on lipid parameters of hepatic subcellular membrane fractions yielded a differential pattern including the resultant loss of membrane integrity (Burger *et al*., 2007). Together, these FB_1-induced changes are likely to affect the critical balance between proliferation and apoptosis, suggested to play a key role during liver and kidney carcinogenesis in rats (Gelderblom *et al*., 2001a; Riley *et al*., 2001). Compromised membrane integrity will also impact on membrane associated protein receptors and related signalling events. In this regard FB_1 has been shown to inhibit the folate receptor-mediated uptake and the epidermal growth factor (EGF)-induced mitogenic response (Stevens *et al*., 1997; Gelderblom *et al*., 1995, 1996a; 1999; 2001a; Marasas *et al*., 2004). These responses are associated with membrane microdomains or lipid rafts, and the implications thereof suggest an even more subtle way by which FB_1 induces diseases such as neural tube defects and carcinogenesis.

The presence of nanosized highly structural and functional membrane platforms or lipid rafts, as part of the cell membrane, has been shown to exist by numerous biophysical and biochemical studies (Simons and Sampaio, 2011). Particular lipids such as cholesterol, sphingomyelin and phospholipids containing elongated saturated acyl chains interact to form dynamic lateral mobile liquid-ordered assemblies (McMullen *et al*., 2004; Simons and Vaz, 2004). These lipid rafts are associated with numerous cellular processes such as pathogen invasion, receptor signalling, cholesterol homeostasis, cell adhesion, cell migration, synaptic transmission, cytoskeletal organisation and exo- and endocytosis (Brown and London, 1998; Smart *et al*., 1999; Simon and Toomre, 2000; Harris and Siu, 2002; Tsui-Pierchala *et al*., 2002). Specific proteins associated with lipid rafts include the glycosylphophatidylinositol (GPI)-anchored proteins (Chatterjee and Mayor, 2001), src-family tyrosine kinases (Lck, Fyn and Lyn) (Simons and Toomre, 2000) as well as proteins associated with palmitoylation and myristoylation (Zacharias *et al*., 2002; Smotrys and Linder, 2004; Pike, 2009).

The aim of the current study was to characterise FB_1-induced changes to the major lipid constituents of lipid rafts utilising primary rat hepatocytes.

METHODS AND MATERIALS

Chemicals and reagents

Fumonisin B_1 (FB_1) was extracted and purified (> 90%) according to the method of Cawood *et al* (1991). Cell culture grade reagents were used for the preparation of primary rat hepatocytes. Williams E medium (WE), L-glutamine solution (200 mM), sodium pyruvate solution (100 mM), Insulin (from Bovine pancreas, 20 U/L), chelating agent ethylene glycol tetraacetic acid (EGTA), Hanks Saline Buffer Solution (HBSS), collagen type 1 from rat tail and collagenase type IV were obtained from the Sigma-Aldrich (St Louis, Missouri, United States). The buffering agent hydroxyethyl piperazineethanesulfonic acid

(HEPES, 1 M) and foetal bovine serum (FBS, heat inactivated) were acquired from Gibco® (Life Technologies, Johannesburg, South Africa) whereas Amphotericin B (antibiotic) and MEM non-essential amino acid solution (NEAA, 10 mM) were from Lonza (Whitehead Scientific, Cape Town South Africa). Molecular grade reagents for the isolation of the hepatocyte lipid rafts were used and included sucrose powder, $CaCl_2$, $MgCl_2$, phosphate (10 mM) buffered saline (pH 7.4)**)**, Optiprep®, protease inhibitor cocktail (containing AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A), phosphatase inhibitors (for serine/threonine and alkaline phosphatases) and Calpain inhibitor I (reversibly blocks the calcium-dependent neutral cysteine protease calpain I) from Sigma-Aldrich.

Preparation of primary rat hepatocytes

Male Fischer rats, (150 – 250 g body weight) anaesthetized by intraperitoneal administ**r**ation of sodium pentobarbital (0.22 ml kg^{-1}), were used for the preparation of primary hepatocytes according to the collagenase perfusion technique as described by Berry and Friend (1969) and Hayes *et al* (1984) with minor modifications. Briefly, the rat liver was catheterised via the portal vein and continuously perfused utilising a peristaltic pump system that ensured a flow of 15 ml/min. Blood from the liver was flushed by a 10 min perfusion step using HBSS (pH 7.4) containing 10 mM HEPES and 10 mM EGTA. Digestion of the liver was attained by perfusing the liver for 15 min with a special buffer consisting of WE medium, pH 7.4, 1% HEPES solution, 125 mg collagenase and 180 mg $CaCl_2$. When liver cell dissociation become evident the liver was removed, followed by the detachment of the liver capsule and hepatocytes collected. William's E medium (pH 7.4) containing 10% FBS was added to deactivate the collagenase and cell collected by centrifugation at 800*g*. The cell pellet was suspended in WE medium and viability assessed by the trypan blue dye exclusion method. Cell preparations with a viability less than 80% was rejected. Viable hepatocytes (7×10^6) were plated in collagen type I coated petri dishes

(100 mm^2) supplemented with WE (pH 7.4) containing 10% FBS, 1% HEPES, 1% L-glutamine solution, 0.5% Amphotericin B and 0.01% insulin. Hepatocytes were plated at 37°C at 5% CO_2 for 2 h. The use of experimental rats was approved by the Ethics Committee for Research on Animals (ECRA) of the South African Medical Research Council.

Hepatocyte incubations and isolation of lipid rafts

After the initial 2h attaching period the media were aspirated followed by a washing step (x2) using HBSS, pH 7.4, containing 1% HEPES. Hepatocytes were cultured at 37°C for 24 h in WE medium (pH 7.4) containing 0.5% FBS, 1% HEPES, 1% L-glutamate, 1% NEAA, 5% Amphotericin B, 0.01% insulin, 1% pyruvate and a non-toxic FB_1 level of 250 μM (Gelderblom *et al*., 1996a) while PBS was used as the control treatment. A non-detergent method was used to prepare hepatic lipid rafts (Macdonald and Pike, 2005). Briefly, the primary hepatocytes were collected and washed twice by centrifugation (250*g)* for 2 min using PBS followed by the addition of 1 mL base buffer (20mM Tris-HCL, pH 7.8, 250 mM sucrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, protease- and phosphatase inhibitors). The hepatocytes suspended in the base buffer (1 mL) were lysed by passage (20 times) through a 22 gauge (0.7 x 90 mm) needle, followed by the preparation of a postnuclear supernatant (PNS) by centrifugation (1000*g* for 10 min). This step was repeated with the remaining pellet. The resultant supernatants (2 mL) were combined with an equal volume (2 mL) of base buffer containing 50% Optiprep®. A gradient range of 0 to 20% (4 mL) was applied on top of the PNS plus 50% OptiPrep® solution and centrifuged at 52 000*g* for 90 min using a swing bucket rotor. Twelfth aliquots of equal volumes (2 mL) were collected from the top of the ultracentrifuge tube and subjected to lipid raft analyses.

Lipid raft characterisation

The horseradish peroxidase cholera toxin subunit B (HRP-CTB) conjugated antibody that binds to GM_1 ganglioside receptors, is an outer membrane leaflet marker, to confirm the presence of lipid rafts using Dot Blot analysis (Krager *et al*., 2012). Each gradient was dot blotted (2 μL) on a nitrocellulose membrane (Sigma) and incubated with HRP-CTB (diluted 1: 500 with PBS). The LR-containing blots were enhanced by chemiluminescence peroxidase substrate (KPL LumiGlo Reserve, Whitehead Scientific, Cape Town, South Africa) and the images captured using a ChemiDoc™ imaging system (Bio-Rad Laboratories, Johannesburg, South Africa). The combined gradient fractions of the control and FB_1-treated hepatocytes (fractions 1 to 4 and 5 to 8; total volume 8 mL) were freeze dried, dissolved in 1 ml saline and reserved for the lipid (900 µl) and protein (100 µl) determinations. Total protein content was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Johannesburg, South Africa).

Lipid Analyses

The combined freeze-dried lipid raft fraction (900 µL) was extracted with 24 mL chloroform/methanol (CM, 2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant (Folch *et al*., 1957; Smuts *et al*., 1994). The CM extract was evaporated to dryness and quantitatively transferred to a glass tube with CM saturated with saline (CMS: 86:14:1, v/v/v) containing 0.01% BHT. The CMS extract was washed with saline saturated with CMS and stored at 4°C under N_2. Prior to analyses samples were evaporated to dryness and dissolved in CMS (140 µL). Aliquots (30 µL) of the CMS extract were analysed using thin-layer chromatography (TLC) on 20 x 20 silica plates with a chloroform/methanol/-petroleum ether/acetic acid/boric acid (40:20:30:10:1.8, /v/v/v/w) as the developing solvent (Gilfillan *et al*., 1983). The plates were developed for 90 min at room temperature followed by drying under N_2 gas for 45 min. The respective

lipids (SM, PC and PE) were identified under long wave UV light (320 nm - 400 nm) using 2,5-bis-5'tert-butylbenzoxazoyl-[2']-thiophene (BBOT) for visualisation which was added to the mobile phase prior to TLC analyses. The visible phospholipid spots were scraped off into glass tubes and preserved for FA and phospholipid analyses. Analyses were conducted in duplicate to obtain samples for both FA and phospholipid analyses.

Phospholipid and cholesterol determinations

Prior to the determination of total phosphate concentrations of the respective lipids (SM, PC and PE), the samples were incubated at 170°C in perchloric acid (2 N) for 2 h. The volume of perchloric acid added varied depending on the phospholipid fraction, e.g., SM (200 μL), PC (400 μL) and PE (300 μL). Following the digestion step the samples were diluted with ddH_2O (perchloric acid/ddH_2O ratio 1:5) and the perchloric acid neutralised by adding 6 N KOH (SM - 388 μL, PC - 775 μL and PE - 581 μL) followed by centrifugation of 15 min at 1000 rpm. The Malachite Green Phosphate Assay kit (kit no.: POMG-25H, BioAssay Systems, California, United States) was used to quantify the phosphate concentrations. Aliquots (20 μL) of the original CM lipid extract were used to determine the total cholesterol using a microtiter-adjusted enzymatic iodide method (Richmond, 1973) that included the enzymes, cholesterol oxidase and cholesterol esterase (Calbiochem, MERCK Millipore, South Africa).

Fatty Acid analyses

Phosphatidylcholine and PE silica fractions were transmethylated for 2 h at 70°C with methanol/18 M sulphuric acid (95:5, v/v) using the method described by Tichelaar *et al*. (1989) and Smuts *et al.* (1994). After the various lipid fractions were allowed to cool to room temperature, 4 mL hexane and 1 mL ddH_2O were added and samples vortexed. The top hexane phase were aspirated into a glass tube and evaporated under N_2 gas at 37°C and dissolved in hexane (15 μl) prior to analyses by gas chromatography. The resultant FA methyl esters (FAME) were analysed on a Varian 3300 Gas Chromatograph equipped with a 30 m

fused silica megabore BPX-70 column of 0.32 mm internal diameter (SGE, Cat No 054616). The individual FAME was identified by comparing the retention times with a standard mixture of free FA C14:0 to C24:1 (NU-CHEK-PREP, Elysian, Minnesota, United States). C17:0 (Avanti Polar Lipids, Alabama, United States) was added to the PC (70 μL, 6.3 μg) and 25 μL (2.25 μg) to PE as an internal standard.

Summary of the experimental design

The experimental outlay is detailed in Figure 1. Male Fischer 344 rats were utilized (1) and subjected to the collagenase perfusion technique (2) to prepare and culture primary hepatocytes (3). Hepatocytes exposed to FB_1 (250 μM) for 24 h (4), harvested, lysed and subjected to Optiprep® density-gradient ultracentrifugation (5). The gradients fractions positive for the presence of lipid rafts as indicated by Dot Blot analyses using the HRP-CTB antibody fractions pooled, freeze dried and stored at -80°C (6). Lipid extracts were prepared by chloroform : methanol 2:1, v/v) solvent extraction and extracts subjected to TLC (7) and lipid "spots" subjected to fatty acid (8) and phospholipid analyses (9). Cholesterol analysis was conducted on the original CM lipid extract (9).

STATISTICAL ANALYSES

Data representing 4 experimental repeats were analysed using the NCSS (Hintze, 2007) statistical package Version 8, released July 25, 2012. Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks including normal score tests (Terry-Hoeffding-expected normal scores) was applied to test for statistical differences ($p < 0.05$) and marginal significance ($0.05 < p < 1.0$) between the control and FB_1 treatment. Marginal differences were also considered due to the short non-toxic FB_1 exposure regimen utilised.

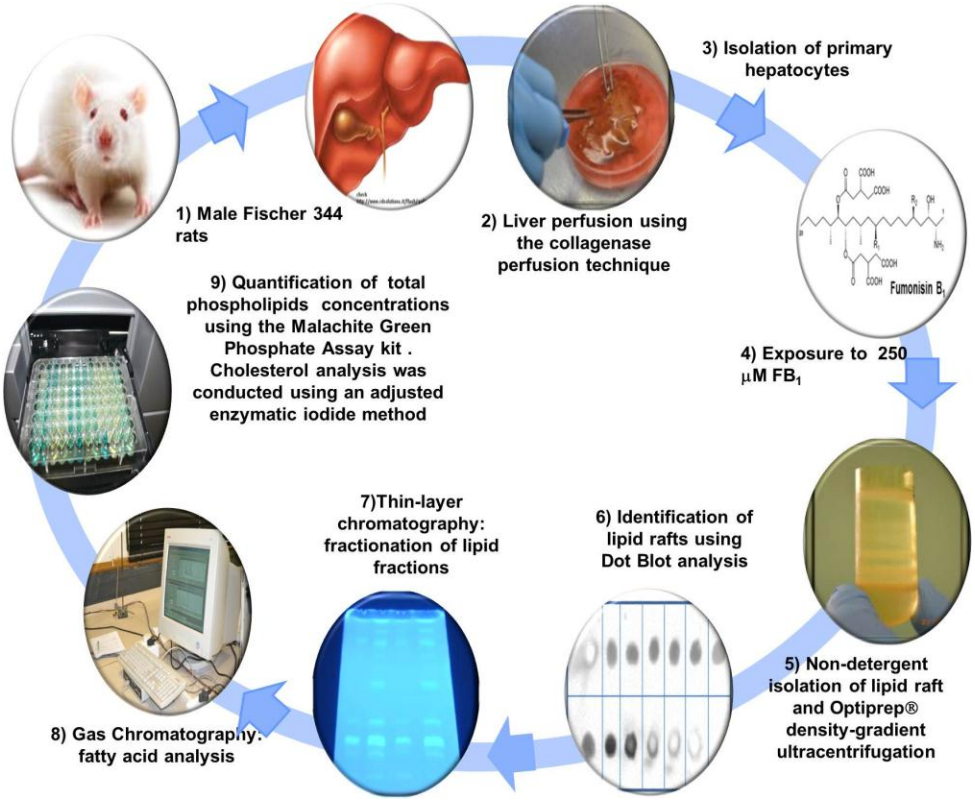

Figure 4.1: Experimental outlay

RESULTS

Identification of lipid raft containing gradient fractions (Figure 4.2)

The isolated lipid rafts were distributed within the 20 to 25% gradient which was in agreement with the method used by Macdonald and Pike (2005). Based on positive stains for HRP-CTB antibodies (Fig 4.2), the lipid raft-rich gradient fractions were pooled i.e., fractions 1 to 4 containing lower (0.3 to 0.9 mg ml^{-1}) and fractions 5 to 8 with higher (1 to 2 mg ml^{-1}) protein concentrations (Table 4.1). Only the major lipid raft fractions (fractions 5 to 8) were used for detailed lipid analyses.

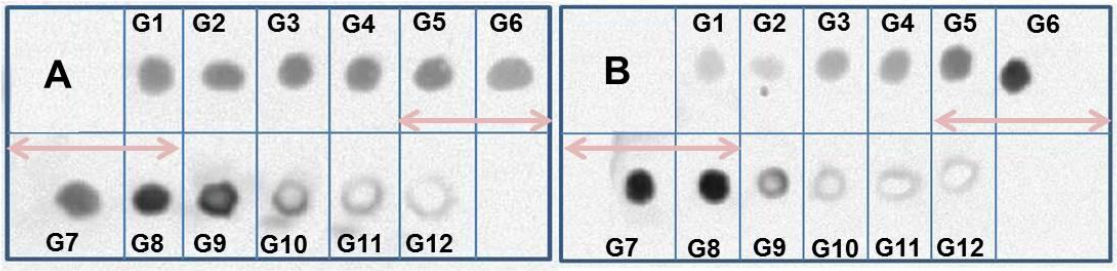

Figure 4.2: Dot Blot analysis of Control (A) and FB_1-treatement (B) identified using the Cholera Toxin B Subunit-Peroxidase conjugated antibody

Abbreviation, FB_1: fumonisin B_1, G: Gradient

Table 4.1: Protein content of the pooled fractions of experimental repeats

Treatment	Protein Concentration (mg mL^{-1})	
	Fractions 1-4	Fractions 5-8
C1	0.3	1.5
F1	0.4	1.5
C2	0.3	1.2
F2	0.4	1.8
C3	0.9	1.0
F3	0.5	1.1
C4	0.6	1.8
F4	0.6	2.1

Abbreviations, C: Control treatment, F: FB_1 treatment. Each sample is a combination of gradient fractions 5 to 8 obtained during ultracentrifugation and stained positively for the Cholera Toxin B Subunit-Peroxidase conjugated antibody

Phospholipid analyses by thin layer chromatography

The major phospholipids separated by TLC were visualised under long wave UV light (365 nm) by the addition of BBOT to the mobile phase (Figure 4.3). Each treatment were subjected to two TLC analyses, one was utilised for total phosphate determinations and the other for fatty acid analysis.

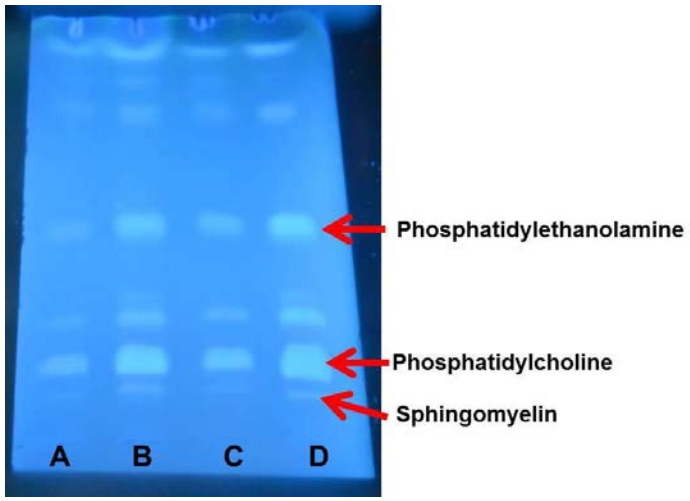

Figure 4.3 Thin-layer chromatography (TLC) of the control (A and B) and FB_1-treatment (C and D) into the various phospholipids

A: Control gradients fractions 1 to 4 combined, B: Control gradients fractions 5 to 8 combined, C: FB_1-treatment gradient fractions 1 to 4 combined, D: FB_1-treatment gradient fractions 5 to 8 combined. Gradients were combined prior to TLC and only B and D where used for total phosphate determinations.

Phospholipid and cholesterol content (Figure 4.4)

The SM content was marginally decreased ($p = 0.08$) by FB_1 while a marked decrease in phosphatidylcholine (PC) was noticed but the change was not significant. Phosphatidylethanolamine (PE) ($p = 0.02$) and cholesterol (CHOL) ($p = 0.006$) were significantly increased by FB_1.

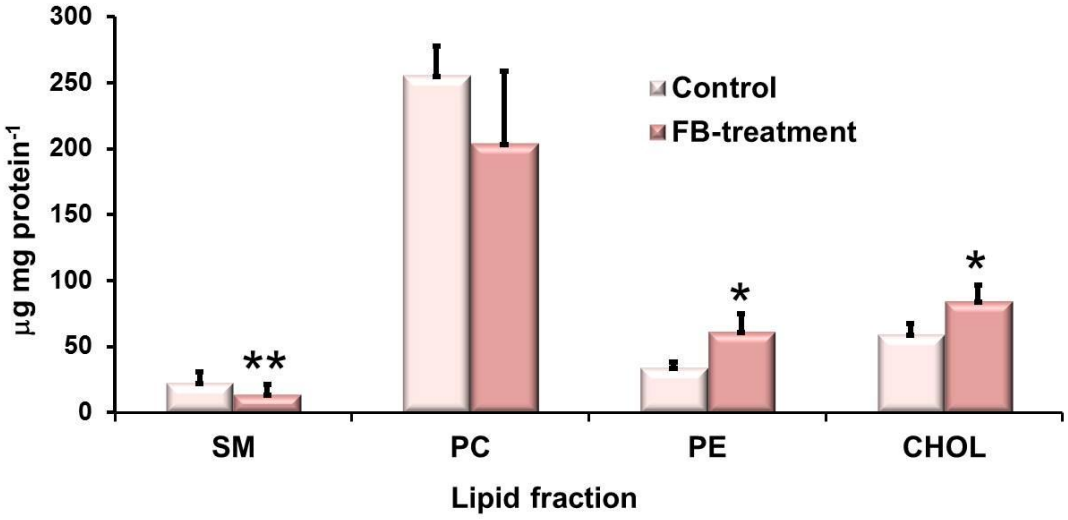

Figure 4.4: The effect of fumonisin B_1 treatment on lipid fractions of membrane lipid rafts isolated from rat primary hepatocytes

Values are numerical mean of 4 determinations. *indicates a significant difference ($p < 0.05$) to control treatment, whereas **shows a marked difference ($0.1 > p > 0.05$). Abbreviations: SM: sphingomyelin, PC: phosphatidylcholine, PE: phosphatidylethanolamine, CHOL: cholesterol.

Membrane fluidity parameters (Figure 4.5)

The PC/PE and the PC/(PE+SM), important phospholipid membrane fluidity ratios were marginally ($0.1 > p > 0.05$) reduced by FB_1 exposure due to the significant increase in PE and marginal decrease in SM. No significant change was noticed in the CHOL/PC+PE ratio due to the increase in both CHOL and PE.

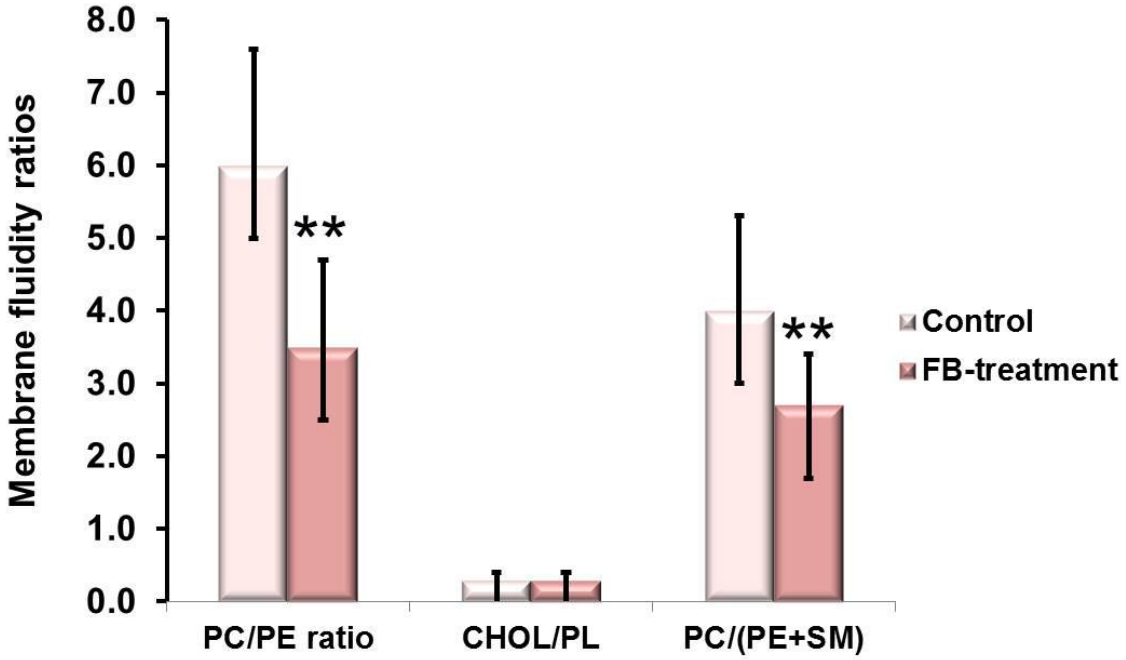

Figure 4.5: The effect of fumonisin B_1 treatment on membrane fluidity ratios of hepatocyte lipid rafts

**Indicates a marginal difference (0.1 > p > 0.05). Abbreviations, CHOL: cholesterol, PL: phospholipid, SM: sphingomyelin, PC: phosphatidylcholine, PE: phosphatidylethanolamine.

Modulation of major lipid raft fatty acids (Table 4.2)

No changes were noticed in the quantitative (μg mg protein^{-1}) fatty acids (FAs) parameters of PC (Data not shown). The saturated fatty acids (SFAs), C16:0 (p = 0.03) increased significantly and C18:0 marginally (p = 0.09) in PE while no noticeable difference was observed in C18:1ω-7 and C18:1ω-9. A significant and marked increase in most of the ω-6 polyunsaturated fatty acids (PUFAs) were noticed in PE, including C18:2ω-6 (p = 0.02), C20:3ω-6 (p = 0.07), C20:4ω-6 (p = 0.09) and C22:4ω-6 (p = 0.04). Of the ω-3 PUFAs, C22:5ω-3 and C22:6ω-3 was marginally (0.09) and significantly (p = 0.006) increased, respectively in the FB_1 treated cells.

Table 4.2: The effect of fumonisin B_1 treatment on ω-6 and ω-3 fatty acids of membrane lipid rafts isolated from rat primary hepatocytes

Treatment	Control		Fumonisin B_1	
	PC (μg mg protein^{-1})	PE (μg mg protein^{-1})	PC (μg mg protein^{-1})	PE (μg mg protein^{-1})
Fatty acid parameter				
C16:0	**53.5 ± 11.4**	**10.8 ± 0.8**	**58.8 ± 7.9**	**16.6 ± 6.7 (p = 0.03)**
C18:0	**42.3 ± 7.9**	**19.6 ± 2.1**	**42.9 ± 10.7**	**25.7 ± 9.2 (p = 0.09)**
C18:1ω-9	11.8 ± 2.2	1.7 ± 0.4	13.9 ± 2.7	2.5 ± 1.7
C18:1ω-7	9.2 ± 3.1	1.8 ± 0.5	9.9 ± 3.5	2.6 ± 1.3
C18:2ω-6	17.8 ± 4.4	2.4 ± 0.6	20.1 ± 0.9	4.1 ± 1.5
C20:3ω-6	**1.4 ± 0.4**	**0.2 ± 0.1**	**1.0 ± 0.7**	**0.4 ± 0.2 *(p = 0.07)***
C20:4ω-6	**53.9 ± 11.4**	**18.3 ± 3.29**	**56.2 ± 8.5**	**25.0 ± 8.3 (p = 0.09)**
C22:4ω-6	**1.0 ± 0.2**	**0.5 ± 0.2**	**1.1 ± 0.2**	**1.0 ± 0.6 (p = 0.04)**
C22:5ω-6	0.4 ± 0.2	0.1 ± 0.2	0.4 ± 0.2	0.2 ± 0.3
C22:5ω-3	**2.2 ± 0.7**	**1.1 ± 0.46**	**2.5 ± 0.3**	**2.3 ± 0.9 (p = 0.09)**
C22:6ω-3	**10.4 ± 2.2**	**6.5 ± 1.2**	**10.9 ± 2.3**	**11.1 ± 3.8 (p = 0.006)**

Values presented as numerical means ± standard deviation of 4 determinations. Values in bold, differ significantly ($p < 0.05$) or marginally ($0.1 > p > 0.05$) from the control treatment. (Actual p-values are indicated in brackets). Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine.

Alterations in lipid raft fatty acid parameters and ratios (Table 4.3)

A marginal difference ($p = 0.09$) was observed in the increase of SFAs, PUFAs and long-chain PUFAs of the PE phospholipid in the FB_1 treated hepatocytes. Whereas total ω-3 in the PE fraction was also noticeably increased ($p = 0.05$) affected by FB_1 exposure. The total monounsaturated fatty acids (MUFAs), total ω-6 and the delta-6-desaturase substrate/product ratio were unaffected by FB_1 in

both PC and PE. Although the C20:4w6 PC/PE ratio was slightly decreased the change was not significant.

Table 4.3: The effect of fumonisin B_1 treatment on additional fatty acid and lipid parameters of membrane lipid rafts isolated from rat primary hepatocytes

Treatment	Control		Fumonisin B_1	
	PC (μg mg protein^{-1})	PE (μg mg protein^{-1})	PC (μg mg protein^{-1})	PE (μg mg protein^{-1})
Fatty acid and lipid parameters				
Total SFAs	**97.3 ± 18.8**	**30.9 ± 2.8**	**90.2 ± 38.5**	**43.0 ± 16.2** ***(p = 0.09)***
Total MUFAs	24.7 ± 5.8	3.9 ± 0.6	23.1 ± 10.5	5.6 ± 2.7
Total PUFAs	**88.9 ± 19.2**	**28.4 ± 4.1**	**83.6 ± 39.5**	**44.7 ± 15.0** ***(p = 0.09)***
Total long-chain PUFAs	**70.7 ± 15.0**	**28.4 ± 4.1**	**64.8 ± 29.6**	**40.6 ± 13.6** ***(p = 0.09)***
Total ω-6 FAs	75.5 ± 16.5	22.3 ± 4.3	71.1 ± 33.8	27.0 ± 13.4
Total ω-3 FAs	**13.4 ± 2.9**	**8.8 ± 1.7**	**12.7 ± 5.8**	**13.8 ± 4.7** ***(p = 0.05)***
Delta-6-desaturase substrate/ product ratio	1.9 ± 0.3	0.8 ± 0.3	2.1 ± 0.3	0.7 ± 0.1
C20:4ω-6 PC/PE ratio	3.4 ±1.2		2.4 ± 0.5	

Values presented as numerical means ± standard deviation of 4 determinations. Values in bold, differ significantly ($p < 0.05$) or marginally ($0.1 > p > 0.05$) from the control treatment. (Actual p-values are indicated in brackets). Abbreviations, PC: phosphatidylcholine, PE: phosphatidylethanolamine, SFAs: saturated fatty acids, MUFAs: monounsaturated fatty acids, PUFAs: polyunsaturated fatty acids.

DISCUSSION

The study of native lipid rafts has been controversial since the reference method of cold detergent isolation has been associated with the production of artifacts (Heerklotz *et al*., 2003; Sengupta *et al*., 2007; Lingwood and Simons, 2010). Similarly, the biophysical nature of lipid rafts in living cells remains a challenge and numerous caveats exist that complicates the determination of the precise lipid composition, size and lifespan (Niemelä *et al*., 2007). Lipid rafts are not only regarded as nanosized assemblies, but are also highly dynamic due the rapid fluctuation of their constituents or coalescence of rafts (Lingwood and Simons, 2010; Shaikh, 2012). To circumvent the limitations of detergent methods, the use of a non-detergent method provides a raft constituent-conserving alternative (Macdonald and Pike, 2005; Persaud-Sawin *et al*., 2009). Despite these complications, detergent and non-detergent methods both concur on the main constituents of lipid rafts including SM, CHOL and PC (Pike *et al*., 2005). In the current study a novel detergent-free method was used to ensure stability of natural lipid species and to allow for quantification thereof (Heffer-Lauc *et al*., 2005; Macdonald and Pike, 2005).

The segregation of specific lipid species and membrane protein to form raft platforms will determine its functional properties. In this regard, the deregulation and acquisition of lipid rafts has been associated with aetiology of numerous diseases including cancer (Pike, 2003; de Laurentiis *et al*., 2007; Patra, 2008; Murai, 2012). Increased levels of lipid rafts have been observed in melanomas, prostate and breast tumours (Hazarika *et al*., 2004; Li *et al*., 2006). Carcinogens such as the mycotoxin $FB_{1,}$ known to alter lipid metabolism and lipid raft associated receptor- and/or mitogenic responses (Gelderblom *et al*., 1999; 2001b), are therefore likely to affect raft constituents. Although FB_1 is regarded as a non-genotoxin lacking mutagenic activity (Gelderblom and Snyman, 1991; Gelderblom *et al*., 1992; Knasmüller *et al*., 1997), long- and short-term exposure studies reported both the cancer initiating and promoting properties (Gelderblom

et al., 1992, 1996b; 2008). Furthermore, the disruption of lipid parameters by FB_1 is closely related to the promotion of cancer involving a growth differential whereby normal hepatocytes are removed by apoptosis and apoptotic-resistant preneoplastic cells allowed to proliferate (Gelderblom *et al*., 2001a, 2008).

In the present study the perturbation of lipid rafts by FB_1 includes the reduction of SM and significant increase in CHOL and PE. These observations have also been noticed *in vivo* in the liver, including the microsomal and mitochondrial subcellular membrane fractions (Gelderblom *et al*., 1996a; 2002; Burger *et al*., 2007). The increased levels of CHOL and PE induced by FB_1 closely mimic those associated with regenerating liver and liver nodules implying the induction of a lipogenic phenotype (Gelderblom *et al*., 2001a; 2001b). However, CHOL was decreased while PC and PE were reported to increase in primary hepatocytes exposed to FB_1 (Gelderblom *et al*., 1996a). In plasma membranes isolated from the liver of rats treated with FB_1, CHOL, SM and PC were unaffected. Phosphatidylethanolamine was significantly increased (Burger *et al*., 2007). The possible dissimilarities observed in the current study may be due to differences in the regulation of membrane integrity with respect to CHOL and SM *in vivo* as compared to the *in vitro* conditions. The active and rapid movement of CHOL to and from lipid rafts may also play a role (Atshaves *et al*., 2007).

In addition the effect of FB_1 on plasma membrane isolated from liver also showed a different pattern regarding the fatty acid (FA) profiles. In the plasma membrane's PC phospholipid fraction the total PUFAs, ω-6 and ω-3 levels decreased and total MUFAs and C18:2ω-6 increased. These changes, especially the decrease in long-chain PUFAs, were related to the impaired delta-6-desaturase enzyme (Gelderblom *et al*., 2002; Burger *et al*., 2007). The differences incurred in plasma membrane exposed to FB vs. lipid rafts in the current study could be related to the different experimental conditions such as *in vivo* vs. *in vitro*, the FB_1 dosages utilised and the duration of exposure. In this regard the dietary study, which utilised 250 mg FB_1 kg bw^{-1} for three weeks, was

associated with a hepatotoxic effecting cancer initiation and promotion of rat liver cancer whereas a in the current study, a non-toxic dose was used over a period of 24 h.

The reduction in lipid raft SM seemingly reflects the FB_1-induced inhibition of the *de novo* ceramide synthase enzyme involved in the production of ceramide and sphingolipids (Wang *et al*., 1991, Merrill *et al*., 2001; Riley *et al*., 2001). In the liver, a concomitant reduction of the pro-apoptotic ceramide and increase in sphingosine 1-phosphate (S1P) associated with cell proliferation has been observed (Pyne and Pyne, 2010; Törnquist, 2013). An increase in the precursor of S1P, sphingosine, in liver nodules following the FB_1 initiation-treatment and promotion by 2-acetylaminofluorene/partial hepatectomy, has also been associated with the altered growth patterns of these lesions (Van der Westhuizen *et al*., 2004). The deregulation of the CHOL metabolism and the resultant cellular increase have also been reported in numerous cancer cells (El-Sohemy and Archer, 2000; Li *et al*., 2003; Duncan *et al*., 2004). Recently, the removal of lipid raft CHOL has been associated with methyl-β-cyclodextrin-induced apoptosis (Onodera *et al*., 2013), whereas, the treatment of several cancer cells with statins stimulated apoptosis due to the depletion of lipid raft CHOL. The reduction in CHOL resulted in an inhibition of growth while the susceptibility to apoptosis was enhanced (Li *et al*., 2006; Glynn *et al*., 2008; Herrero-Martin and Lopez-Rivas, 2008). Therefore, for FB_1 to affect a growth differential during cancer promotion, a dual response is warranted where proliferation is effected in preneoplastic cells while apoptosis is stimulated in the surrounding normal cells. The proliferation of rat liver nodules, as noted in various dietary studies (Gelderblom *et al*., 1996a, 2001b; Van der Westhuizen *et al*., 2004) could therefore be sustained by not only the inhibition of the SM/ceramide–induced pathway apoptosis mechanism, but may also involve the increased lipid raft CHOL with a loss of cell sensitivity to apoptosis.

The exofacial monolayer raft constituents support the existence of lipid rafts exclusively in the latter; however numerous studies have proposed an asymmetric distribution of lipid rafts in the inner cytofacial monolayer (Rajendran and Simons, 2005). Of the main constituents, CHOL has been regarded as the stabilising and driving force behind the formation of rafts in both layers (Quinn, 2010). Cholesterol has a condensing effect on polar lipids and the formation of a liquid-order phase that is important for raft clustering (Leathes, 1925; Recktenwald and McConnell, 1981; Ipsen *et al*., 1987; Quinn and Wolf, 2009). However, CHOL's affinity and interactions with SM surpasses that of CHOL-unsaturated PLs interactions. For this purpose CHOL is considered to be responsible for creating an environment for rafts formation and functionality (Quinn, 2010). Alteration to lipid raft CHOL, whether it be reduction, enrichment or redistribution will affect the raft structure and importantly, the raft-related signalling machinery (Simons and Ehehalt, 2002; Zhuang *et al*., 2005; Sánchez-Wandelmer *et al*., 2009).

The link between FB_1 exposure and the consequential increase in membrane CHOL may therefore alter raft signalling such as down regulation of the EGF-mitogenic response (Gelderblom *et al.*, 1999). The removal of raft CHOL and the subsequent activation of EGF signalling has been demonstrated (Chen and Resh, 2002; Ringerike *et al*., 2002; Li *et al*., 2006). This is achieved by i) increase in EGF binding, ii) augmentation of the formation of EGF-induced receptor dimers and iii) up-regulation of receptor autophosphorylation (Pike *et al*., 2002; Ringerike *et al*., 2002; Westover *et al*., 2003). In contrast to this an increase in CHOL showed opposite effects (Pike, 2005). It would appear that the decreased EGF mitogenic response effected by FB_1 may be a consequence of the increase in lipid raft CHOL. The altered EGF-response in primary hepatocytes exposed to FB_1 were also associated with the modification of the ω-6 fatty acids, specifically by a decrease in C20:4ω-6 and increase in C18:2ω-6 PUFAs indicating a specific role of fatty acid metabolism in the responsiveness of lipid rafts (Gelderblom *et al*., 1999).

Notably the disruption of lipid rafts and the resultant cancer promotion by FB_1 is complex and involves different lipid parameters including sphingolipids, phospholipids, cholesterol and fatty acids. Akt (protein kinase B) is of particular interest from a lipid metabolism, membrane and cancer perspective. Fifty percent of human cancers are associated with the hyper-activation of this pathway that includes its partitioning to detergent resistant membrane microdomains and the stimulating of hepatic lipogenesis (Leavens *et al*., 2009; Gao and Zhang, 2009; Kim *et al*., 2010, Yuan *et al*., 2012). It also displays a plethora of functions involving cell size/growth, proliferation, survival, glucose metabolism, genome stability, and neo-vascularization (Bellacosa *et al.*, 2005). The depletion of CHOL and the subsequent disruption of lipid rafts are associated with a rapid inactivation of Akt signaling (Calay *et al*., 2010). Fumonisin B_1 exposure on the other hand is associated with the increase in CHOL suggesting the activation of Akt as was reported previously (Ramljak *et al*., 2000). The increased CHOL as observed in lipid rafts from hepatocytes exposed to FB_1 may therefore also influence Akt signaling activation.

Of further relevance to Akt activation and FB_1 exposure is the increase in C20:4ω-6 observed the PE phospholipid fraction in liver subcellular membrane fractions (plasma membrane, microsomes and mitochondria) (Burger *et al*., 2007) and marginally in hepatocyte lipid rafts in the current study. C20:4ω-6 can induce Akt phosphorylation possibly via the oxidation and inactivation of tumour suppressor phosphatase and tensin homolog (PTEN) that increase the levels of phosphatidylinositol 3,4,5-triphosphate (PIP_3) and activates Akt by recruiting it to the plasma membrane (Covey *et al*., 2007). The increase in C20:4ω-6 and the proposed increased eicosanoid production (E2 series) via the activation of phospholipase A_2 have been proposed as the important mechanisms responsible for growth modulation in primary hepatocytes and rat liver *in vivo* (Gelderblom *et al*., 1999; Pinelli *et al*., 1999; Burger *et al*., 2007). On the other hand high levels of C16:0 and C18:0 have been implicated in the regulation of apoptosis. These SFAs are known to regulate apoptosis by reducing the mitochondrial membrane

potential and the release of cytochrome C (Hardy *et al*., 2003). The increased levels in lipid rafts as well as the reported increase in microsomes and mitochondria in primary hepatocytes by FB_1 (Burger *et al*., 2007) could be important determinants in effecting apoptosis.

Another lipid raft associated protein complex affected by FB_1 exposure is the folate uptake or transport system (Marasas et al., 2004). The folate receptor (FR) is a GPI-anchored protein found in detergent-resistant lipid rafts (Bonnon *et al*., 2010). The mechanism proposed includes the FB_1-induced alterations of sphingolipid metabolism. Reduction of sphingolipids leads to the increased endocyte trafficking of the GPI-anchored proteins and a possible reduction in the number of FRs (Chatterjee *et al*., 2001; Marasas *et al*., 2004). However, from a lipid raft perspective it is unclear how FR is reduced. While SM is one of the major constituent of lipid rafts and the disruption thereof is expected to impact on raft formation and constituents, it has, however, not the same impact as the removal of raft CHOL (Asano *et al*., 2012). It would appear that CHOL remains the gate-keeper of raft formation and characteristics (Silva *et al*., 2009; Barceló-Coblijn *et al*., 2011). On the other hand, the depletion of SM induced by SM synthase enzyme knockout had no effect on raft CHOL (Li *et al*., 2007). Cholesterol depletion in detergent-resistant lipid rafts by methyl-β-cyclodextrin treatment likewise induced the down-regulation of numerous GPI-associated proteins such as the FR, by disrupting their Golgi-complex oligomerisation (Foster *et al*., 2003). The opposite was observed where increase CHOL induced the oligomerisation and apical sorting of GPI-proteins in rafts (Paladino *et al*., 2008). A study investigating the necessity for raft composition during GPI-anchored protein sorting suggested an indirect effect of FB_1, via the disruption of the ceramide signalling pathways rather than a direct insult on the raft composition (Lipardi *et al*., 2000).

The use of a detergent-free method has been shown to include cytoplasmic leaflet PLs such as PE and PS (Pike *et al*., 2005). Since SM is either absent or

very low in the inner leaflet and CHOL is abundant in both, it has been proposed that PE and PS mixtures, together with CHOL and SM may be able to sustain such ordered rafts (Bakht *et al*., 2007; Mondal *et al*., 2009). Inner leaflet PC/PE/PS domain induced by outer leaflet SM/CHOL–containing rafts has also been observed (Kiessling *et al*., 2006). Hepatocyte lipid rafts display a transbilayer fluidity gradient with the inner leaflet less fluid, consistent with the idea that CHOL is higher in the latter (Atshaves *et al*., 2007; Mondal *et al*., 2009). Due to the significant increase in total lipid raft PE levels of hepatocytes treated with FB_1, the related fatty acids were also increased. Interestingly, was a marginal increase in the total ω-3 fatty acids in the lipid raft PE fraction. The interaction between CHOL and C22:6ω-3-containing PE leads to the induction of raft clustering in model membranes due to the low affinity of CHOL for this phospholipid (Shaikh *et al*., 2004).

Cholesterol, SM and PE are known fluidity modifying factors (Van Meer *et al*., 2008). FB_1 exposure markedly increased raft rigidity as observed by a decrease in two phospholipid fluidity indexes i.e., PC/PE and the PC/(PE+SM) ratio (Abel *et al*., 2001; Fajardo *et al*. 2011). The reduction in raft fluidity is mainly effected by the increase in PE, CHOL and decrease in SM. The fluidity parameter, CHOL/phospholipid ratio was not affected due to the relative high CHOL and PE levels. This increase in raft CHOL could be regarded as a counteractive measure to maintain raft integrity in response to the increase in PE, known to contain high levels of long chained PUFAs. Lipid rafts are known for exhibiting a solid and ordered environment due to the specific inclusion and packaging of saturated lipid constituents. The type of fatty acids incorporated into phospholipid species is also important. PE showed an increase in SFAs as well as long-chain PUFAs possibly also to balance out the increased level of unsaturation in order to maintain lipid raft saturation integrity. An increase in membrane rigidity by FB_1 has also been observed in primary hepatocytes including liver plasma membrane, mitochondrial and microsomal subcellular fractions (Gelderblom *et al*., 1996a, 1999; Burger *et al*., 2007).

The changes in lipid and fatty acid parameters observed in the lipid rafts following FB_1-exposed hepatocytes are likely to be associated with the mitoinhibitory effects induced by FB_1 in primary hepatocytes (Gelderblom *et al*., 1996a). As most of the lipid-associated changes effected by FB_1 closely mimic the lipogenic phenotype prevailing in regenerating liver and proliferation hepatocyte nodules, it provides a working model in explaining the growth differential associated with cancer promotion of cancer in rat liver.

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CHAPTER 5

THE MYCOTOXIN DISTRIBUTION IN MAIZE MILLING FRACTIONS UNDER EXPERIMENTAL CONDITIONS

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ABSTRACT

Mycotoxin contamination of maize and maize-based food and feed products poses a health risk to humans and animals if not adequately controlled and managed. The current study investigates the effect of dry milling on the reduction of fumonisins (FB), deoxynivalenol (DON) and zearalenone (ZEA) in maize. Five composite samples, constructed to represent different mycotoxin contamination levels were degermed yielding degermed maize and the germ. The degermed maize was milled under laboratory conditions and four major milling fractions (SPECIAL, SUPER, semolina (SEM) and milling hominy feed) collected. The whole maize, degermed maize and total hominy feed (germ + milling hominy feed) were reconstructed to ensure homogenous samples for mycotoxin analyses. For comparison, commercial dry milling fractions (whole maize, SPECIAL, SUPER and total hominy feed), collected from three South African industrial mills, were analysed for the same mycotoxins and hence a more accurate assessment of the distribution between the different milling fractions. The distribution of the mycotoxins during the experimental dry milling of the degermed maize differs, with FB mainly concentrated in the SPECIAL, DON in the SEM whereas ZEA was equally distributed between the two milling fractions. Distribution of mycotoxins between the fractions obtained during commercial dry milling generally provided similar results with the total hominy feed containing the highest and the SUPER milling fractions the lowest mycotoxin levels although variations existed. Although milling is an effective way to reduce mycotoxins in maize, kernel characteristics and resultant fungal colonisation may impact on the distribution of specific mycotoxins among the different milling fractions. Differences in industrial dry milling practices and problems encountered in sampling bulk maize remain a large problem in assessing mycotoxin contamination in milling fractions intended for human consumption.

INTRODUCTION

Mycotoxins are secondary metabolites and natural contaminants produced by various food-borne fungi that can infect food commodities during pre- and post-harvest periods, storage or during food processing. Dietary exposure to mycotoxins is a global problem that impacts on human and animal health, as well as the food industry and international trade. The rapid changes encountered in the globalisation of world economies, climate and food sufficiency highlights the need for quality control parameters and safety evaluation of food contaminants that may adversely affect human and animal health (Dimitri and Oberholtzer, 2006).

Some of the mycotoxins considered to be of importance to human health are: aflatoxins (AF) produced by *Aspergillus* spp., ochratoxin A (OTA) produced by *Aspergillus* spp. and *Penicillium* spp*.,* deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FB) produced by *Fusarium* spp. (Binder *et al*., 2007). A large range of agricultural commodities are affected including peanuts, maize, wheat, nuts, beans, cassava, oats, barley and rice. Aflatoxins and OTA are also known to contaminate milk and dairy products (Prandini *et al*., 2009; Pattono *et al*., 2011). Mycotoxins commonly co-occur in agricultural commodities such as maize and can exhibit synergistic, additive, potentiating or antagonistic biological effects upon ingestion (Eaton and Klaassen, 2001; Scudamore and Patel, 2009; Waśkiewicz *et al*., 2012). This has relevance to the livestock industry where the co-occurrence of mycotoxins could exert far greater adverse health effects (Binder *et al*., 2007; Streit *et al*., 2012). Due to their thermal and chemical stability, mycotoxins can only be partly removed by food processing and/or decontamination procedures (Bullerman and Bianchini, 2007; Munkvold, 2003). Complete elimination of mycotoxins is impossible and susceptibility of food commodities to ambient temperatures, rainfall, relative humidity and moisture content plays an important role affecting fungal infestation (Lattanzio *et al*., 2007). The selection, cleaning and grading of unprocessed maize for food or feed production are important initial decontamination steps, although storage conditions can still result in an increase in mycotoxin contamination prior to food processing and production (Bennet and Richard, 1996). Food processing that may reduce mycotoxin contamination

includes sorting, trimming, cleaning, dehulling, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalisation (tortilla process) and extrusion (Bullerman and Bianchini, 2007).

Milling of maize is a physical process regarded as the first step in the production of maize-based products (Castells *et al*., 2008) by removing the outer structures, the hull, pericarp (bran), germ and tip cap to expose the endosperm, which is then utilised to produce various milling fractions such as the grits, germ, meal and flour (Alexander, 1987). The milling fractions mostly utilised as human foodstuffs are the flaking grits and flour, whereas the bran and germ milling fractions are used for animal feed and oil extraction, respectively. In South Africa dry milling is mostly utilised to produce food products such as samp, maize grits, maize rice, unsifted, sifted, coarse, SUPER and SPECIAL maize meal. Apart from the physical breaking of each kernel, the efficiency of the degerming process will also affect the yield and composition of the grits and again impact on the levels of mycotoxins. Inadvertently, differences will exist between whole maize batches and mills, as well as the overlapping of kernel components constituting the different milling fractions. Currently the terminology used for describing the various milling end-products differs globally and restricts comparisons of the corresponding mycotoxin contamination between different countries (Scudamore and Patel, 2009). In this regard utilising particle sizes of each milling fraction could be a simple solution to describe a milling fraction or product (Scudamore and Patel, 2009). Due to the complexity of the milling process the mycotoxins may be distributed, yielding higher or lower levels in the various milled products (Bullerman and Bianchini, 2007).

The level of mycotoxin contamination in whole maize and the distribution thereof between the resultant fractions produced during food processing, remains a food safety challenge. Most studies in this regard are focused on the distribution of mycotoxins in industrial dry milling whereas studies utilising laboratory or small-scale milling to investigate distribution are limited (Slotoff and Dalrymple, 1977; Park, 2002; Broggi *et al*., 2002; Brera *et al*., 2006; Bullerman and Bianchini, 2007; Castells *et al*., 2008; Pietri *et al*., 2009; Vanara *et al*., 2009). Studies investigating the effect of industrial dry milling on FB_1 showed overall a

reduction in contamination levels in the resultant milling fractions intended for human consumption, i.e. milling fractions derived from the endosperm including the maize meal, flour and grits. The milling fractions retaining the hull, pericarp, germ and tip cap intended for animal feed or oil production contained higher levels. (Bennet and Richard, 1996; Saunders *et al*., 2001; Broggi *et al*., 2002; Brera *et al*., 2004; Bullerman and Bianchini, 2007; Scudamore, 2008; Pietri *et al*., 2009; Castells *et al*., 2008; Scudamore and Patel, 2009).

The main purpose of the present study was to investigate the effect of dry milling on the distribution of FB, DON and ZEA mycotoxins between the different milling fractions utilising specific designed composite maize samples containing these mycotoxins. Samples were milled under controlled laboratory conditions and the results were compared to those obtained from South African commercial dry milled maize fractions. The sampling of whole maize and the milling fractions for mycotoxin analysis, even with well-defined legislated sampling protocols in industrial maize milling settings, is still subjected to error and other confounding factors (Blanc, 2006; Scudamore, 2008). The current study included novel and special strategies to overcome some of these errors and ensure homogenous and representative samples for mycotoxin analyses.

METHODS AND MATERIALS

The present study was a collaborative effort between a reputable and prominent South African grain-based manufacturing company, Southern African Grain Laboratory (SAGL) and the South African Medical Research Council (MRC).

Chemicals

Water was obtained from a Milli-Q system (Millipore, Bedford, Massachusetts, United States). Methanol and acetonitrile were HPLC-grade and formic acid was analytical reagent grade, all obtained from MERCK, South Africa. All analytical standards were

sourced from Industrial Analytica and Tega (South Africa) as well as the PROMEC Unit, South African Medical Research Council (MRC).

Experimental composite maize samples

To effectively monitor the influence of the dry milling process on various mycotoxin levels, five experimental composite maize samples were constructed, representing specific contamination levels of the different mycotoxins. Different grade 1 and 2 (according to South African regulations) uncleaned white maize consignments were selected from various maize growing localities within South Africa during the 2010 harvest season. Multiple-mycotoxin analyses were conducted to determine the presence of AFs (B_1, B_2, G_1 and G_2), FB_1, FB_2, OTA, DON, ZEA and T-2 toxin. Following analyses appropriate samples were selected and composite samples (n = 5) of approximately 4 kg each, were prepared for the study (Table 5.1). As no aflatoxins (B_1, B_2, G_1 and G_2), OTA, and T-2 toxin were detected, the selection of samples was based on the levels of FB_1, FB_2, DON and ZEA. The five composite maize samples were screened and cleaned by the removal of non-kernel impurities using a Carter Day Dockage Tester (Carter Day, United States) but were not re-analised for mycotoxin levels prior to the experimental maize processing. All visible foreign material was removed by hand and the cleaned samples were stored in marked and sealed containers at 4°C. As the composite samples were prepared based on maize grading parameters for the current experiment, certain samples would not have been suitable for human consumption. Composite sample 2 contained high levels of DON and ZEA, whereas the remainder of the samples lack the presence of ZEA whereas samples 3 and 5 contained relative high levels of FB and sample 4 a relative high level of DON.

Table 5.1: Composite maize sample design utilising various white maize samples with a specific level of mycotoxin contamination

Composite Samples	Grading of white maize	FB_1 (µg kg^{-1})	FB_2 (µg kg^{-1})	DON (µg kg^{-1})	ZEA (µg kg^{-1})	Percentage (%) of composite sample*	Total mass (g)
1	Grade 2	506	180	ND	ND	51	4500
	Grade 1	ND	ND	917	ND	49	
2	Grade 1	ND	ND	157	102	6	4300
	Grade 1	ND	ND	676	276	4	
	Grade 1	ND	ND	590	183	4	
	Grade 1	ND	ND	697	109	7	
	Grade 1	ND	ND	3016	152	33	
	Grade 2	ND	ND	692	108	5	
	Grade 2	ND	ND	502	96	3	
	Grade 2	ND	ND	341	87	3	
	Grade 2	758	219	511	ND	13	
	Grade 2	657	198	754	42	11	
	Grade 1	650	237	106	ND	3	
	Grade 1	1165	402	ND	ND	8	
3	Grade 1	324	114	594	ND	52	3800
	Grade 2	1082	308	75	ND	48	
4	Grade 1	736	283	730	ND	55	4400
	Grade 1	ND	ND	1845	ND	45	
5	Grade 2	1806	599	264	ND	47	3900
	Grade 2	1229	400	624	ND	53	

ND: not-detected. Abbreviations, FB: fumonisin; DON: deoxynivalenol; ZEA: zearalenone. *Percentage was calculated using the relevant weights (g) obtained from the Southern African Grain Laboratory (SAGL).

Maize conditioning, degerming and dry milling

Conditioning or tempering of each composite sample was conducted in batches to ensure a more consistent sample for degerming and to prevent the pericarp and germ from drying out resulting in insufficient degerming. Prior to the conditioning of the composite samples, each sample was divided into two halves (batch A and B) and treated separately. Conditioning was conducted in two stages; 1) softening of the maize and 2) toughening and loosening of the pericarp for easy removal. For the first stage, the following formula was used to calculate the addition of water to obtain a moisture content of 16.5%.

Mass x (Target moisture % - Actual moisture %) / (100 – Target moisture %)

Each batch was transferred to a sealed bucket and rolled horizontally on an in-house rolling device for an hour and left standing for an additional 2 hours and 45 minutes. For the second conditioning stage, each batch was again subdivided into halves (batches a and b), followed by the addition of water (20 mL /1.0 kg maize, 2%) and rolled horizontally for 5 minutes. Sample division was necessary due to the limited capacity of the degerming procedure and the experimental milling plant.

Degerming of the sample (300 g) was conducted using a modified Grainman rice polisher (Grainman, United States of America) and produced two products, 1) the germ (a mixture of the germ, hull, pericarp and tip cap), 2) the degermed maize consisting mainly of the “endosperm” and remnants of the germ. Finally, for each composite sample the resultant germ and degermed maize samples of all four batches were collected, combined, weighed and stored at 4°C in sealed containers. The conditioning and degerming processes are illustrated in Figure 5.1.

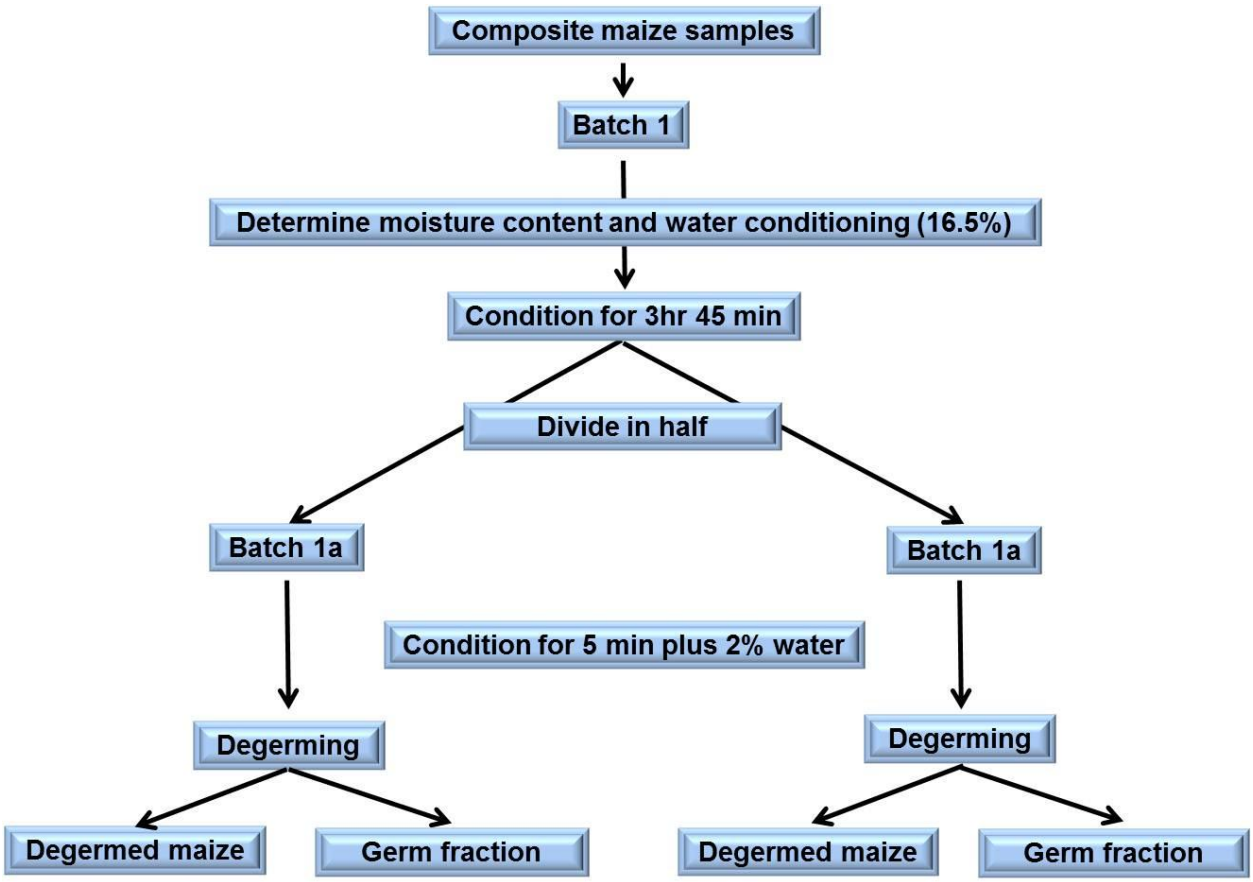

Figure 5.1: Flow diagram of the conditioning and degerming process yielding the germ fraction and degermed maize

The degermed maize from each composite sample was further processed in an experimental dry milling plant utilising a modified Buhler MLU-202 laboratory mill (Buhler, Switzerland). The sieves and mill were thoroughly cleaned with a brush and compressed air following the milling of each sample to eliminate cross contamination. Nine milling sub-fractions (B1-3; S1-3; H1-2 and semolina) that were obtained from each sample were weighed and then combined to give a total of four major milled fractions (Table 5.2): 1) SPECIAL maize meal; 2) SUPER maize meal; 3) Semolina (SEM) and 4) the milling hominy feed (fraction containing large pieces of pericarp and hull with some endosperm attached and clean medium sized pieces of bran and hull as well as the remnants of the tip cap).

Table 5.2: The composition of the major experimental dry milling fractions obtained by combining specific milled subfractions based on the methodology and terminology utilised by a South African milling company

Major dry milling fraction	Milled subfractions	*Particle sizes	Description	Purpose in industry
SPECIAL maize meal	B1 B2 B3 S2 S3	< 0.30 mm	Fine granulated product equivalent to maize flour	Maize-based food product
SUPER maize meal	S1	0.30 mm - 1.40 mm	Coarse granulated product equivalent to maize grits	Maize-based food product
SEM	Semolina	0.71 mm - 2.00 mm	Coarse meal with some pericarp, tip cap and germ fragments	Re-milled to produce maize meals and hominy feed
Germ or milling hominy feed	H1 H2	> 1.00 mm	Product with large to medium sized pieces consisting of germ, hull, pericarp (bran), tip cap and some endosperm	Animal feed or oil production

*Obtained from the Agricultural Product Standards Act of the Republic of South Africa, 1990 (Act no. 119).

The SPECIAL, SUPER and SEM were divided in three portions, the first and second were used to reconstruct whole maize and degermed maize according to the correct mass ratio, while the third portion was retained for mycotoxin analysis. The milling hominy feed was also divided into two portions in order to reconstruct both the degermed maize and the total hominy feed. The total hominy feed represents the fraction containing the germ, hull, pericarp and tip cap relevant to animal feed production. Therefore, the germ and milling hominy feed, obtained from the degerming and milling process, respectively, were combined. This fraction was divided into two portions: 1) for reconstruction of the whole maize and 2) for mycotoxin analysis.

Table 5.3: Percentages (%) of the various milling fractions obtained during experimental dry milling of the degermed maize fraction

Degermed maize	SPECIAL (%)	SUPER (%)	SEM (%)	Milling hominy feed (%)
1	13.7	69.0	10.5	6.8
2	12.9	66.3	13.2	7.6
3	13.5	66.7	12.3	7.5
4	13.6	65.6	12.3	8.5
5	14.1	68.5	11.2	6.2

Reconstruction of the whole maize, degermed maize and total hominy feed

Reconstruction of each composite whole maize sample and the corresponding degermed maize was performed to obtain homogenous samples for mycotoxin analysis. For this purpose the percentage of each fraction produced during either degerming or milling was recorded (Table 5.3). This ensured that the reconstructed whole maize and degermed maize contained representative ratios of each degermed or milling fraction.

The reconstructed degermed maize includes the four major milling fractions, SPECIAL, SUPER, SEM and milling hominy feed while the whole maize consists of the first three milling fractions and total hominy feed. Prior to the reconstruction of the whole maize, the total hominy feed (containing large sized particles) was fractionated into three sizes 1) > 1 mm, 2) > 500 µm and 3) < 500 µm using an Endocotts EFL2000 sifting apparatus (Air and Vacuum Technologies, South Africa) and weighed. The extraction percentages were also noted for each particle size and used to calculate the quantity that had to be added to reconstruct whole maize (Table 5.4).

Table 5.4: Percentages (%) of the various dry milling fractions, including total hominy feed fractions used to reconstruct the whole maize

Composite sample	SPECIAL (%)	SUPER (%)	SEM (%)	*Reconstructed total hominy feed		
				Particle size > 1 mm **(%)**	**Particle size** > 500 µm **(%)**	**Particle size** < 500 µm **(%)**
1	**10.6**	**53.1**	**8.0**	45.1	31.0 ****28.3%**	23.9
2	**9.7**	**49.7**	**9.9**	46.1	33.5 ****30.7%**	20.4
3	**10.6**	**52.2**	**9.6**	40.1	33.7 ****27.6%**	26.2
4	**10.2**	**49.2**	**9.3**	50.4	32.7 ****31.3%**	16.9
5	**10.7**	**52.4**	**8.5**	34.6	34.1 ****28.4%**	31.3

*Reconstructed total hominy feed: germ + milling hominy feed. **The total hominy feed was first fractionated in three particle sizes (> 1 mm; > 500 mm and < 500 mm) and the resultant fraction percentage was noted; thereafter they were combined in the correct ratio to yield the percentage needed to reconstruct the whole maize. Abbreviation, SEM: Semolina.

Sampling of commercial fractions

Commercial maize dry milled fractions were sampled according to standard procedures from three South African industrial maize mills (mills A, B and C) and analysed for the above mentioned mycotoxins. The samples were collected on three consecutive days and included whole maize (10 kg) prior to being conditioned and milled, the SPECIAL and SUPER milling fractions (2.5 kg each) and the total hominy feed (1.5 kg). Uncleaned maize batches (500 g) were randomly withdrawn from the samples for mycotoxin analysis.

Mycotoxin analyses

Preparation of analytical standard solutions

Most of the analytical standards were sourced in solution from the supplier (Industrial Analytical, South Africa, Tega, South Africa and PROMEC Unit, South African MRC). Individual stock standard solutions were prepared as prescribed by the manufacturer. The working dilutions for the calibration curves were prepared using a sample extract from a blank maize sample (matrix matched standard curve).

Sample preparation

To obtain a representative sample for mycotoxin analysis from all the experimental fractions (the reconstructed whole maize, degermed maize, total hominy feed, SPECIAL, SUPER and SEM), each fraction was evenly spread across a clean surface and random portions were sampled (50 g). The reconstructed total hominy feed, whole maize and degermed maize as well as the SEM fractions were subjected to a further milling step using a hammer mill to provide homogenous samples. The finely grounded sample (10 g) was weighed and extracted with 40 ml methanol : H_2O (80:20) by stirring for one minute. The extract was filtered through a glass fibre filter paper and an aliquot filtered through a 0.22 µm syringe tip filter into an autosampler vial, capped and analysed by LC-MS/MS.

LC-MS/MS analyses

The LC-MS/MS system consisted of a Waters Acquity UPLC and TQD mass spectrometer and Mass Lynx software for data acquisition and analysis. A 5 µl aliquot was injected on an UPLC C_{18} BEH 1.7 µm 2.1 x 50 m column at a column temperature of 30°C. The gradient was composed of solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.4 mL min^{-1}. Quantification was done against a matrix-matched calibration curve consisting of at least 5 different mycotoxin concentrations. With each set of samples, a solvent blank, a "blank grain sample" and duplicate recovery samples (known amounts of mycotoxins spiked onto a "blank sample" were prepared and injected).

Average recoveries of 133% and 137% for FB_1 and FB_2, respectively, 141% for DON and 123% for ZEA were recorded. The limit of detection (LOD) for FB_1 and FB_2 was 5 and 1 µg kg^{-1} while for DON and ZEA, 5 and 10 µg kg^{-1}, respectively. Mean mycotoxin levels (numerical mean) and range was determined for each experimental fraction from the 5 composited and 9 commercial samples. Total FB (FB_T) was the sum of FB_1 and FB_2. The mycotoxin distribution, defined as the ratio between the FB's, DON and ZEA mycotoxin levels in the respective experimental and commercial milling fractions, was expressed as a percentage of the corresponding mycotoxin levels in the whole reconstructed and commercial maize samples.

STATISTICAL ANALYSIS

Data presented in Tables are numerical means. To test for statistical differences ($p < 0.05$), the mean mycotoxin levels in the various experimental milling fractions ($n = 5$) were log transformed and expressed as geometric means (GM). Multiple comparisons were conducted on the GM using the standard Tukey-Kramer test. All the analyses were performed using the NCSS (Hintze, 2007) statistical package Version 8, released July 25, 2012. Data was presented in the form of tables and graphs.

RESULTS

Percentage (%) recovery of maize samples

The percentage loss of maize during experimental dry milling was determined by the combined mass of the degermed maize and germ obtained after degerming in relation to the weight obtained of the reconstructed whole maize. The reconstruction efficiency was very high and an average sample loss of 3.12 ± 0.38 % was recorded.

Mycotoxins in the experimental milling fractions (Table 5.5)

The degerming process decreased mycotoxin levels initially by a reduction of between 2.2 and 3.2 times. The germ and milling hominy feed (H1 + H2) were not separately analysed as they were combined to obtain the total hominy feed fraction. Comparison of the mycotoxin geometrical means indicated a significant difference ($p < 0.05$) between the SUPER and total hominy feed fractions for FB_T and ZEA. Levels of DON in the SUPER milling fraction differed ($p < 0.05$) from the reconstructed whole maize, total hominy feed and the SEM milling fractions. All the mycotoxins were noticeably concentrated in the total hominy feed fraction.

The mycotoxin distribution (%) in the experimental fractions (Figure 5.2)

The mycotoxin distribution between the various experimental fractions was calculated for the degermed and milling fractions and expressed as a percentage of the reconstructed whole maize and degermed maize, respectively. During degerming and milling all three mycotoxins (FB_T, DON and ZEA) were concentrated (between 240% and 280%) in the reconstructed total hominy feed fraction whereas they were reduced in the reconstructed degermed maize fraction (between 31% and 38%) containing the milling hominy feed. Milling of the degermed maize yielding the various fractions indicated that the SPECIAL milling fraction contained the highest FB_T, followed by SEM with SUPER containing the lowest concentration.

Table 5.5: Mycotoxin levels for FB_T, DON and ZEA in reconstructed maize sample, degermed maize, total hominy feed and milling fractions under experimental conditions

Fraction	n	FB_T (µg/kg)	DON (µg/kg)	ZEA (µg/kg)
Reconstructed whole maize	5	413.2	329.4 **a**	93.4
		(105 - 1268)	(68 - 787)	(8 - 307)
Reconstructed degermed maize	5	141.8	149.2	29.2
		(22 - 470)	(51 - 337)	(6 - 77)
SPECIAL	5	338.4	127.8	31.0
		(70 - 1161)	(43 - 240)	(0 - 81)
SUPER	5	61.0 **a**	27.2 **a,b,c**	8.6 **a**
		(0 - 221)	(0 - 67)	(0 - 18)
SEM	5	143.0	215.4 **b**	29.8
		(13 - 553)	(55 - 553)	(9 - 92)
Reconstructed total hominy feed	5	1157.0 **a**	941.4 **c**	245.6 **a**
		(236 - 3557)	(240 - 2295)	(30 - 652)

Data presented as numerical means with the range in brackets. Statistical differences in columns (p-values < 0.05) between fractions are based on the geometrical means and are represented by similar bold lowercase letters. Reconstructed fractions: whole maize, degermed maize and total hominy feed according to Table 5.3 and 5.4. Abbreviations, n: number of samples; FB: Fumonisin; DON: Deoxynivalenol; ZEA: Zearalenone; SEM: Semolina. FB_T: Total FB (FB_1 + FB_2).

In contrast, DON tended to be higher in SEM with lower levels in the SPECIAL and the SUPER milling fractions. ZEA was equally distributed between the SPECIAL and SEM (101% and 106%, respectively) milling fractions. The SEM fraction is normally recycled during industrial dry milling to produce the final maize meals (SPECIAL and SUPER) and hominy feed (Table 5.2).

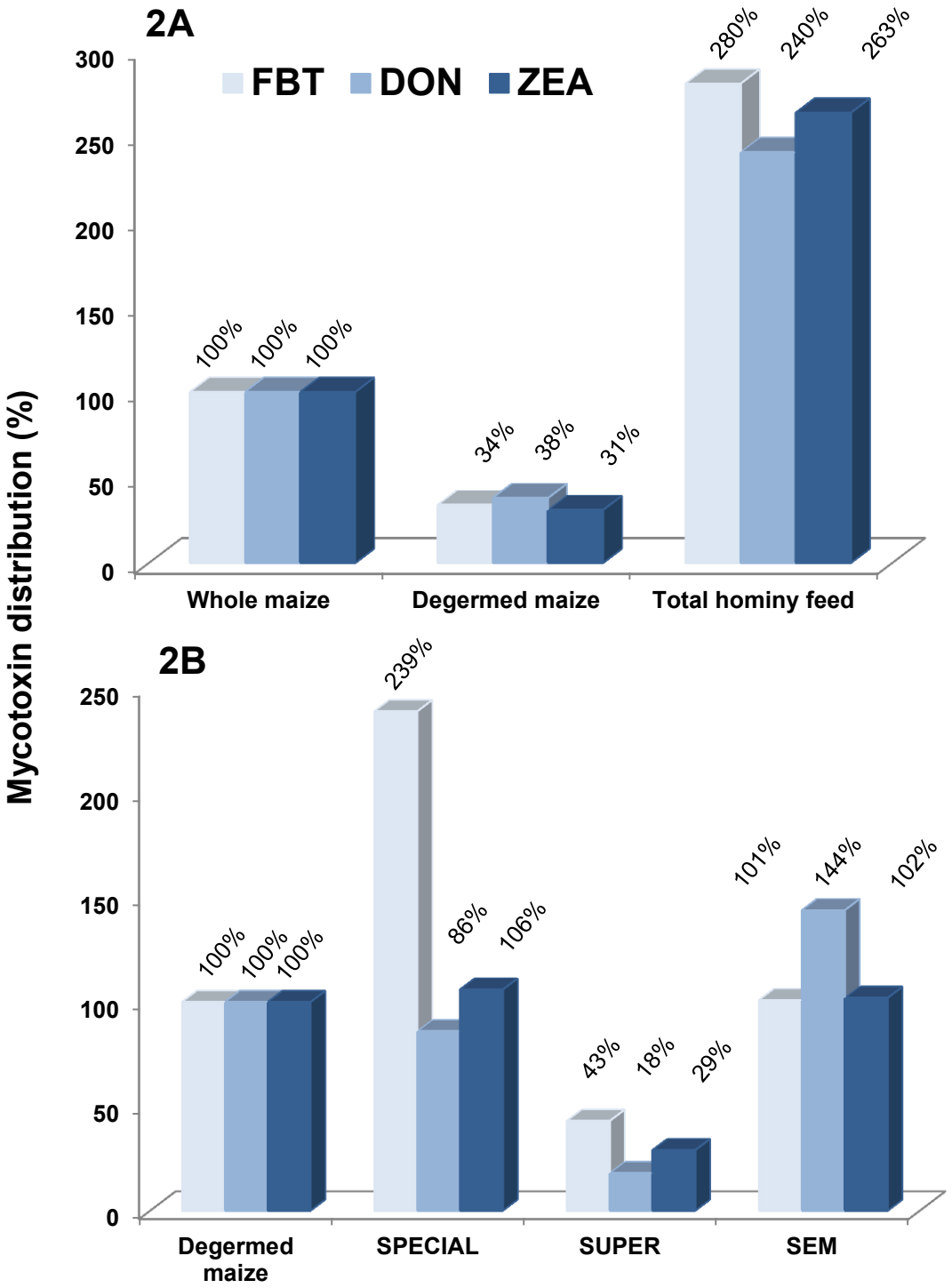

Figure 5.2: **The mycotoxin distribution for the reconstructed total hominy feed and reconstructed degermed maize (2A) are expressed as a percentage (%) of the reconstructed whole maize (100%) whereas the SPECIAL, SUPER and semolina (SEM) are expressed (2B) in relation to the reconstructed degermed maize (100%)**

*Degermed maize contained the milling hominy feed

Mycotoxins in the commercial milling fractions from three South African industrial mills (Table 5.6)

Industrial milling, utilising commercial maize also concentrated FB_T, DON and ZEA in the total hominy feed fraction whereas the SUPER fraction contained the lowest levels. FB_T contamination of the whole maize utilised by mill A was the highest which is also reflected in the higher levels in the SPECIAL and SUPER milling fractions. Although the whole maize utilised by mill A contained the highest level of DON, the SPECIAL and SUPER milling fractions from mill C exhibited the highest content. The levels of ZEA were overall low and negligible in the SUPER fractions. Overall an inconsistent distribution of all the mycotoxins in the milling fractions with respect to the levels in the whole maize is noted. The SEM fraction was not analysed for mycotoxins as it is recycled during industrial milling as described above.

When considering the percentage mycotoxin distribution, the highest % was observed in the total hominy feed fraction. The % distribution for FB_T and DON followed the same pattern in the commercial milling fractions originating from mill A and B with the SPECIAL having a higher mycotoxin level as compared to the SUPER milling fraction. The high % distribution of FB_T in the SPECIAL and total hominy feed fractions of mill C in relation to the relative low level in the whole maize, could be regarded as an error incurred during sampling of the whole maize. The % distribution of ZEA indicates that it was effectively removed into the total hominy feed fraction when considering mills A and C. In contrast, the SUPER fraction from mill B showed a higher % distribution of ZEA compared to the SPECIAL which again could be ascribed to sampling errors.

Table 5.6: Mycotoxin (FB_T DON and ZEA) in commercial dry milling fractions obtained from three South African industrial mills

Fraction	Mycotoxin mean levels and contamination range across various milling fractions (µg kg^{-1})									
		Mill A			Mill B			Mill C		
	n	FB_T	DON	ZEA	FB_T	DON	ZEA	FB_T	DON	ZEA
Whole maize	3	567.3 (69-1171)	112.0 (0-201)	3.7 (0-11)	302.3 (0-907)	35.0 (5-88)	6.3 (0–19)	2.3 (0-7)	90.3 (43-183)	ND
SPECIAL	3	135.3 (92-196)	25.3 (25-26)	ND	20.3 (10-29)	4.3 (0-13)	ND	45.7 (41-50)	26.3 (14-33)	ND
SUPER	3	4.7 (0-7)	4.0 (0-12)	ND	ND	ND	3.2 (0–5)	ND	13.3 (0 -24)	ND
Total hominy feed	3	1584.7 (1101-2197)	633.0 (576–691)	52.7 (32-75)	613.3 (508-750)	307.7 (296-328)	22.0 (19-25)	1183.7 (1129-1153)	527.7 (408-598)	31.3 (19-38)
	Mycotoxin distribution (%) across various milling fractions									
	n	FB_T	DON	ZEA	FB_T	DON	ZEA	FB_T	DON	ZEA
Whole maize	3	100	100	100	100	100	100	100	100	-
SPECIAL	3	24	22	ND	7	12	ND	1987	29	-
SUPER	3	1	4	ND	ND	ND	51	ND	15	-
Total hominy feed	3	279	565	1424	203	879	349	51465	584	-

Data presented as numerical means with the range in brackets. ND: not detected. Abbreviations, n: number of samples; FB: Fumonisin; DON: Deoxynivalenol; ZEA: Zearalenone and FB_T: Total FB (FB_1 + FB_2).

DISCUSSION

Experimental dry milling under laboratory conditions cannot duplicate industrial milling as vast differences exist in consignment scale, operating and manufacturing set-up and mill to mill variations. However, it does provide an opportunity to separate the degerming and milling process while investigating the fate of mycotoxins in the different milling fractions on an amendable laboratory scale. The large variations in mycotoxin levels between the starting raw whole maize and the various final milling products will not only depend on the complexity of the milling process with its incurred differences, but also on the stability and the distribution of the mycotoxins in the kernel matrix (Scudamore, 2008; Scudamore and Patel, 2009). The latter will depend on differences in the fungal colonisation and the resultant mycotoxin production in the maize kernels. Comparison of research findings between different studies conducted using small scale maize dry milling is complicated due to the difference in experimental set-up and the type of products yielded. The experimental degerming and milling made it possible to assess the mycotoxin distribution in the various fractions and to express it as a percentage of the whole maize. The novel concept of reconstructing the whole maize, degermed maize and total hominy feed fractions provided homogenous representative samples for mycotoxin analyses that would otherwise have been subjected to large sampling errors that normally prevail.

Reduction of mycotoxins in the milling fractions was accomplished by a significant removal of FB_T, DON and ZEA in the total hominy feed fraction. This was expected since the total hominy feed fraction contains the germ, hull, pericarp (bran), tip cap and some endosperm, known to be contaminated with mycotoxins (Brera *et al*., 2004; Broggi *et al*., 2002; Scudamore, 2004). The initial experimental degerming process was very effective in concentrating the mycotoxins in the total hominy feed fraction. During the experimental milling of the degermed maize, FB_T was more associated with the SPECIAL fraction, DON with SEM and ZEA was equally distributed between the SEM and SPECIAL milling fractions. Both the SPECIAL and SEM are known to contain less endosperm and some of the pericarp, hull, germ and tip cap not removed in the degerming process. The experimental milling made it possible to obtain the SEM fraction which is normally recycled

during industrial dry milling to yield the final maize meals (SPECIAL and SUPER) and hominy feed fractions. This is of importance due to the specific distribution of mycotoxins within this fraction that will contribute final levels observed within other fractions. On the other hand, the lowest percentage mycotoxin distribution was found in the SUPER milling fractions consisting of the coarse granulated endosperm. Similar results were reported regarding the distribution of FB (FB_1, FB_2 and FB_3), DON and ZEA levels in maize milling fraction obtained during industrial milling with higher levels of FB followed by DON and ZEA in maize flour comparable to the current SPECIAL maize milling fraction (Scudamore, 2008; Scudamore and Patel, 2009). In general the bulk of the three mycotoxins was concentrated in the outer kernel layers and contained in the bran/meal/germ fractions. However, variations do exist depending on the different milling strategies that vary between countries, the designation of the different milling fractions and each consignment of maize.

The low mycotoxin contamination observed in the SUPER milling fraction and total hominy feed containing the highest levels confirms previous reports (Broggi *et al*., 2002; Patey and Gilbert, 1989; Saunders *et al*., 2001, Scudamore, 2008). Mycotoxins are known to be concentrated in the surface layers of the maize kernel and the resultant milling products from these parts such as the germ, hull, pericarp and tip cap fractions are expected to have high levels (Abbas *et al*., 1985; Brera *et al*., 2004; Katta *et al*., 1997; Park, 2002, Scudamore *et al*., 2003). This is in accordance with the fact that fungal colonisation and resultant mycotoxin contamination progresses from the outer layers of the kernel to the inner layer and FB has been shown to be mostly located in the outer layer of the kernel such as the germ, hull and pericarp (Brera *et al*., 2004; Kent and Evans, 1994). Milling fractions derived from the endosperm like the flaking and coarse grits (large particle sizes) have lower levels of mycotoxins, compared to the flour (fine particles) fractions (Scudamore and Patel, 2009). This could be attributed to the bran layer that acts as a physical barrier against the fungal penetration into the endosperm and a reduced transfer of mycotoxins to the inner structure of the kernel (Betchel *et al*., 1985; Castells *et al*., 2008; Siwela *et al*., 2005). The relatively higher mycotoxin levels in the fine flour fraction are attributed to the inclusion of the outer layers, especially the bran areas of the kernels

during milling (Alexander, 1987; Castells *et al*., 2008; Katta *et al*., 1997; Vanara *et al*., 2009).

Numerous studies have shown that when the particle size of the endosperm decreases the mycotoxin levels are known to increase (Scudamore and Patel, 2009, Vanara *et al*., 2009). Also the endosperm texture will be of importance and will affect the quality of the milling product derived from it. Endosperm hardness, defined as the ratio between corneous or vitreous to starchy (soft) endosperm is relevant to maize quality for profitable milling products and further processing (Butrón *et al*., 2009). Hard or flinty endosperm kernels have shown to have lower FB_1 contamination by 50% compared to soft hybrids (Blandino and Reyneri, 2008). Hard kernels are known to be less susceptible to breaking and cracking after harvesting and more inclined to produce coarse milling fractions during the early stages of milling (Stroshine *et al*., 1986). These traits are likely to reduce the level of mycotoxins even during storage and in the final commercial products such as SUPER (Blandino and Reyneri, 2008; Magan *et al*., 2003; Paulsen *et al*., 2003,). Variations in the percentage of each milling fraction produced during the experimental milling of the degermed maize are influenced by the kernel characteristic that exist between the different samples. These kernel differences are likely to be a major determining factor in the observed variations in mycotoxin distribution between the milling fractions. In this regard the resistance to FB contamination is related to the increase in kernel density and a more compact pericarp, functioning as an effective antifungal barrier (Costa *et al*., 2003). Also, a lower surface wax content on the pericarp has been associated with higher FB levels (Sampietro *et al*., 2009). Sydenham *et al.* (1995) found that fumonisin was associated with the outer layers of naturally contaminated kernels and that removal of the pericarps significantly reduced the fumonisin level within the kernels. This supports the findings that *Fusarium verticillioides* was mostly associated with tissue of the upper pedicel and not with the endosperm or embryo (Bacon *et al*., 1992). A recent study suggested that as direct penetration of the intact pericarp by *F. verticillioides* was not observed, the stylar canal would represent the only route to the pericarp cells from outside the kernel (Duncan and Howard, 2010). Less is known about the colonisation habits of *Fusarium graminearum*, but it was found that hyphae grew down the silks towards the cob and infected the developing

kernels by penetrating the ovary directly through the silk attachment point (Miller *et al*., 2007). These differing colonisation patterns would possibly explain the distribution differences between FB and DON in the different milling fractions with FB_T mainly associated in the SPECIAL and DON in the SEM.

Industrial milling, especially mill A and B, also concurred with the mycotoxin distribution pattern observed during the experimental milling with the lowest levels obtained in the SUPER milling fractions while the bulk is removed in the total hominy feed fraction. Recycling of the SEM milling fraction during industrial milling may contribute to higher DON levels in the SPECIAL and SUPER (including the hominy feed) fractions as it tended to be concentrated as compared to FB_T and ZEA, and therefore may increase the risk of mycotoxins. During commercial milling, where tons of whole maize are utilised at a time, the reduction or distribution coefficients could be of value to ensure that the whole kernel complies to a maximum permitted level of mycotoxin contamination prior to milling (Vanara *et al*., 2009). Although large differences existed in the different mycotoxin levels between the experimental and commercial milling fractions, the mycotoxin distribution followed a distinct pattern. Comparing mycotoxin levels in whole maize and corresponding commercial milling fractions intended for human consumption from three mills clearly demonstrates large variations as expected from large commercial production. In this regard the milling fractions and their respective mycotoxin levels obtained from mill C were especially distorted which can be attributed to sampling errors. Numerous other factors are also known to affect the accurate determination of mycotoxins levels and distribution which include variations in milling operations and mycotoxin analysis, inherent kernel characteristics (structure and chemical composition), fungal growth conditions and/or acquired physical damage to kernels. Since the current study indicated that large variations existed in determining mycotoxin distribution during industrial milling, experimental milling provided a novel strategy during which confounding factors such as sampling errors and sample representativeness are minimised. Reconstructing the whole maize, degermed maize and the fractionised total hominy feed produced more homogenous samples for more reliable mycotoxin analysis and accurate mycotoxin distribution.

The mycotoxin distribution factor is an important tool in risk management to establish limits for raw commodities to ensure safe processed foods for human consumption. Characterisation and the manipulation of kernel characteristics and milling practices therefore can become important strategies to further reduce mycotoxin contamination in the resultant milling fractions. This is of relevance as degerming and milling of raw food commodities such as maize remains an effective way to reduce mycotoxin contamination and reduce human exposure.

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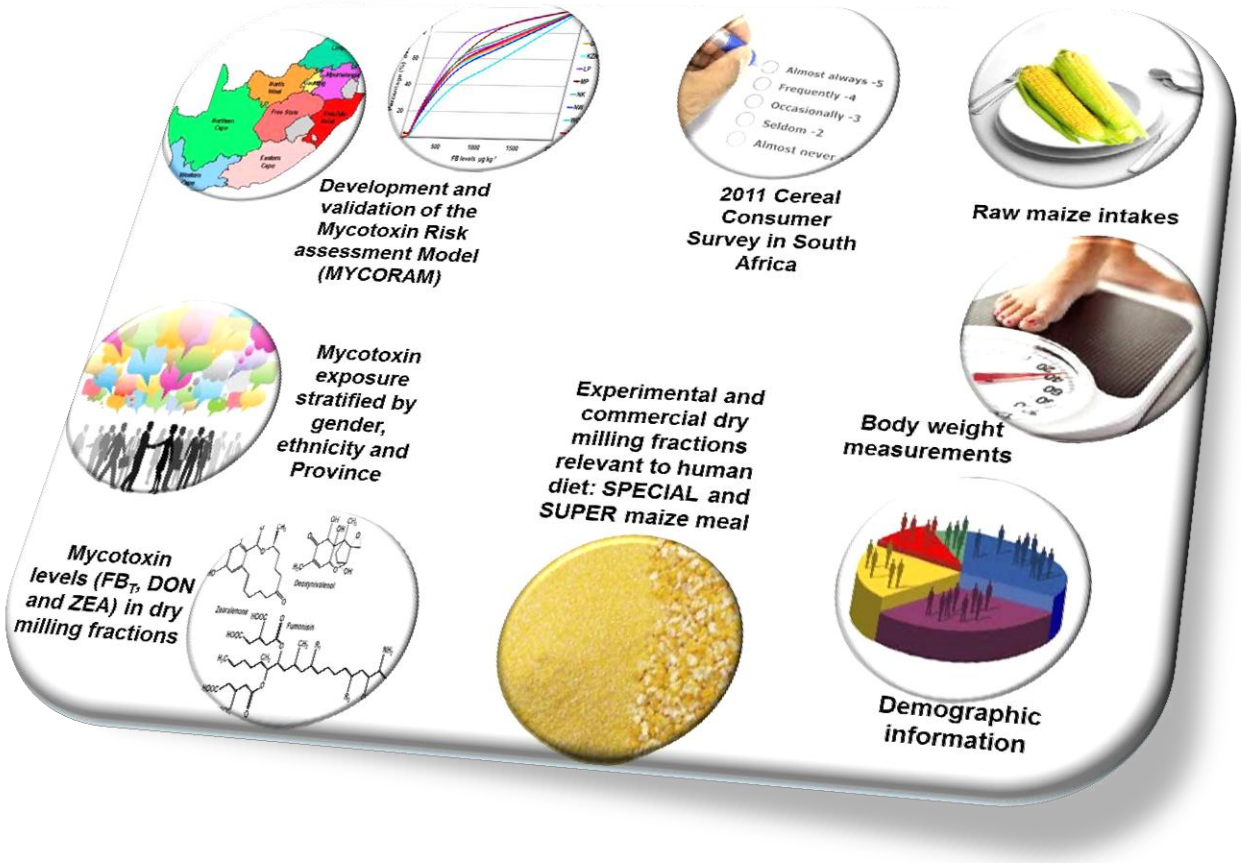

CHAPTER 6

DEVELOPMENT AND EVALUATION OF A SENSITIVE MYCOTOXIN RISK ASSESSMENT MODEL (MYCORAM)

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ABSTRACT

The risk of exposure to fumonisin (FB), deoxynivalenol (DON) and zearalenone (ZEA) mycotoxins in the South African multicultural population, living in nine Provinces, was assessed during a cross-sectional grain consumer survey. The relative per capita maize intake (g day^{-1}) was stratified by gender, ethnicity and Province and the probable daily intake (PDI) for each mycotoxin (µg kg^{-1} body weight day^{-1}) was calculated utilising different model SPECIAL and SUPER dry milled maize fractions representing different exposure scenarios. Men consumed on average more maize (173.0 g day^{-1}) than women (142.0 g day^{-1}) while the black African ethnic group had the highest intake (279.4 g day^{-1}) followed by the Coloured group (169.0 g day^{-1}) with the Asian/Indian and White population groups consuming lower quantities of 101.0 and 80.2 g day^{-1}, respectively. The estimated mean PDIs for each mycotoxin utilising the different dry milled maize fractions were below the provisional maximum tolerable daily intake (PMTDI) for each mycotoxin. Sensitive mycotoxin risk assessment models (MYCORAMs) for exposure was developed utilising specific maize intake increments (g kg^{-1} body weight day^{-1}) and provides information on the percentage of the population exposed above the (PMTDI) for each toxin characterised by ethnicity and Province. Evaluation of the MYCORAM utilising the experimentally-derived SPECIAL milling fraction, maize consumers above the respective PMTDI varied from 7 to 18%; 3 to 9.5% and 0.2 to 4.2% among the Provinces for FB, DON and ZEA respectively. An approximate five-fold lower risk was noticed with the SUPER milling fraction and the milling fractions derived from the commercial maize. In contrast, the dry milling fractions from samples representing mycotoxin levels in home-grown maize of rural subsistence farmers resulted in a higher percentage (50 to 80%) of consumers being exposed above the PMTDI for each mycotoxin. Safety modelling using the MYCORAM stratified by ethnicity to predict maximum tolerated levels will ensure adequate protection for South African maize consumers.

INTRODUCTION

Mycotoxins enter the human food chain via three possible routes i) directly via the diet that includes cereals, such as maize; ii) indirectly via food products derived from fungal contaminated commodities; and iii) consumption of meat or animal products from livestock fed contaminated feed (Turner *et al*., 2009). From a commercial perspective, most food products may contain mycotoxins below the maximum tolerated levels due to good agricultural practices, selective breeding, modern biotechnology strategies, improved storage, food preparation and processing. However, the cumulative exposure to an unvaried diet and/or high intakes of contaminated food commodities is a concern to health authorities and the food industry. South Africa is an agricultural country with maize as an important commodity for both commercial and subsistence farming communities. Maize and maize-based products are consumed by the majority of the population (between 67% and 83%), and the average cooked maize consumption is estimated between 475.6 and 690.1 g person^{-1} day^{-1} (Nel and Steyn, 2002). The population's demographics together with rapid urbanisation impact on dietary patterns and necessitate the assessment of maize intakes in order to determine the risk of mycotoxin exposure. South Africa is known for its population diversity, which is reflected in large differences in social-economic status and cultural traditions.

The mycotoxins relevant to human health include aflatoxins (AFs) produced by *Aspergillus* spp., ochratoxin A (OTA) produced by *Aspergillus* and *Penicillium* spp., deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FB) produced by *Fusarium* spp. (Binder et al., 2007). The adverse health effects in humans include i) hepatitis, liver cancer, stunting and immune suppression by AF (IARC, 1993a; 2002a; Li *et al*., 2001; Turner e*t al*., 2002); ii) nephropathy by OTA, (Hult *et al*., 1982; Marquardt. and Frohlich, 1992; IARC, 1993b); iii) gastro-intestinal disorders, anorexia, nausea, emesis, headache, chills, giddiness and convulsions by DON, (Maresca *et al*., 2002; Amuzie and Pestka, 2010); iv) precocious pubertal changes in children, early menarche and possibly infertility due to the endocrine disruptive effect of ZEA, (Kurtz and Mirocha, 1978; Schoental, 1983) and v) increased risk of oesophageal and liver cancer, neural tube defects and stunting by FB (Marasas *et al*., 2001; 2004; IARC, 2002b; Kimanya *et al*., 2010). The predicted health outcomes

are expected to be further exacerbated by possible additive and/or synergistic effects of mycotoxins due to the co-occurrence of mycotoxins in a particular food commodity and their anti-nutritive effects (Ariño *et al*., 2007). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established, for each mycotoxin, a provisional maximum tolerable daily intake (PMTDI), which includes i) zero for AF since it is a potent genotoxin exhibiting a no threshold level of exposure; ii) 0.1 µg kg^{-1} body weight $week^{-1}$ or ± 14 ng kg^{-1} bw day^{-1} for OTA: (JECFA, 2002), iii) 1 µg kg^{-1} bw day^{-1} for DON (JECFA, 2001), v) 0.5 µg kg^{-1} bw day^{-1} for ZEA (JECFA, 2000) and 2 µg kg^{-1} bw day^{-1} for FB (JECFA, 2001; 2012).

The purpose of this study was to determine the probable daily intakes (PDIs) of FB_T (FB_1 + FB_2), DON and ZEA among a representative sample covering the nine Provinces of the South African population using intake of maize-based food products obtained during a national consumer survey. Aflatoxins (B_1, B_2, G_1 and G_2), ochratoxin A and T-2 toxin are not present in South African commercial maize and were excluded from the present study (Burger *et al,* 2013). Based on defined dietary maize intake categories and relevant mycotoxin exposure levels, two sensitive mycotoxin risk assessment models (MYCORAMs) was developed to determine the percentage of the population exposed above the PMTDI levels for each respective mycotoxin stratified by ethnicity and Province. Different experimental dry milled maize fractions, prepared from specific maize samples containing pre-defined mycotoxin levels as well as commercial fractions intended for human consumption were utilised to evaluate the MYCORAM at provincial level. Maximum tolerated levels (MTLs) for exposure of different ethnic groups were also estimated using the relevant MYCORAM. This is the first study to assess mycotoxin exposure in a broader South African maize consuming population.

METHODS AND MATERIALS

The current study was a collaborative effort between a reputable and leading grain-based South African manufacturing company, the South African Medical Research Council (MRC) and the Nutrition Information Centre, University of Stellenbosch (NICUS), South Africa. Ethical approval was obtained from the Health Research Ethics Committee of Stellenbosch University, South Africa (Addendum C).

Study design

A national consumer survey was conducted by a private South African marketing research company specialising in consumer studies, utilising a cross-sectional study design to ensure a representative sample of South African grain (including maize) consumers. In addition to the structured consumer questionnaire a quantitative food frequency questionnaire (QFFQ) was included. This QFFQ was developed by including maize intake questions originating from a validated questionnaire used during the South African National Food Consumption Survey (Labadarios *et al*., 2008). After informed and signed consent, information on habitual maize intakes and body weight measurements were obtained from each participant in order to assess mycotoxin exposure. Demographic information from the consumer questionnaire was also collected and included ethnicity, age, gender, residential Province, household income, employment and education. South Africa consists of heterogeneous populations with four main ethnic groups: black Africans, (ancestry from the African continent; 79% of total South African population); Coloureds (mixed ancestry, mainly of Khoi origin as well as mixed Caucasian, African Malay and San origins; 8.9% of total population), White (Caucasian descendants from continental Europe and the United Kingdom; 9.6% of total population) and Indian/Asian (originating from India or other Asian countries, 2.5% of total population) (Marais, 1968; Byrnes, 1996; Thomas and Bendixen, 2000). These ethnic groups reside in nine different Provinces, namely the Eastern Cape (EC), Free State (FS), Gauteng (GP), KwaZulu-Natal (KZN), Limpopo (LP), Mpumalanga (MP), North Cape (NC), North West (NW) and Western Cape (WC) Provinces (Blaauw and Gilson, 2001). Professional interviewers were trained on the basics of scientific data collection, the sampling

methodology, completion of the various questionnaires and body weight measurements.

A sample size of 3000 was selected using the South African 2001 Census Household data (Statistics South Africa, 2001) and adult male and female (older than 16 years) consumers of maize from the different ethnic groups within the nine Provinces were eligible to participate. The sample was stratified according to Province, Metro and District Municipality down to the level of suburb/township/village and systematic probability sampling was applied. Each household had an equal chance of being selected to participate in the survey consisting of face-to-face interviews.

Mean raw/uncooked maize intakes

The quantitative food frequency questionnaire, based on usual intakes of maize-based products over a month period and expressed as gram per person per day (g day^{-1}) was used to assess maize intakes. Photographic aids with pictures of various maize-based dishes such as crumbly-, soft- and stiff porridge, samp, combined dishes (samp and beans, spinach and maize meal, pumpkin and maize meal) and non-alcoholic maize fermented beverages, in four different portion sizes were developed to improve the accuracy of maize intake estimates (Addendum D). Individual total raw/uncooked maize intakes were estimated using recipes from FoodFinder 3, a dietary analysis computer software application (*South African* Medical Research Council, PO Box 19070, Tygerberg, South Africa). If a recipe was unavailable a validated culturally specific dietary assessment method, the Ratio And Portion size Photo (RAPP) tool was used to calculate the raw maize intake, (Lombard *et al*., 2012; Addendum E). The RAPP tool was developed to determine the dietary habits and nutrient intakes of rural and urban Xhosa-speaking black Africans living in the EC. Numerical means were calculated for the raw maize intakes and stratified according to the gender, ethnicity and provincial residence

Mycotoxin levels in maize samples for risk evaluation

Five maize samples selected to represent different mycotoxin levels of FB_T (FB_1 + FB_2), DON and ZEA, were dry milled under laboratory conditions and the different fractions normally intended for human consumption (SPECIAL and SUPER maize meal) were collected (Burger *et al*., 2013). The mean mycotoxin levels (n = 5) of FB_T, DON and ZEA of the experimental SPECIAL and SUPER milling fractions were used to determine the exposure to the different mycotoxins. The respective SUPER and SPECIAL fractions of nine commercial maize samples, representing industrial milling, were included for comparative purposes. In addition the SPECIAL and SUPER milling fractions of two selected maize samples (Maize 1 and Maize 2), representing contamination levels of either high total FB_T or low FB_T, high DON and ZEA (DON/ZEA) were used for the evaluation of the mycotoxin risk assessment model (MYCORAM). Multi-mycotoxin analyses of the dry milled fractions utilising a validated and standardised LC-MS/MS method, were conducted by the Southern African Grain Laboratory (SAGL, Pretoria, South Africa) and included FB_1, FB_2, DON and ZEA (Burger *et al*., 2013).

Mycotoxin exposure stratified by gender, ethnicity and Province

The PDIs (expressed as $\mu g\ kg^{-1}$ bw day^{-1}) of the different mycotoxins were calculated using the experimental and commercial SPECIAL and SUPER fractions utilising: i) the total raw/uncooked maize intakes; ii) body weight of the maize consumers and iii) mean mycotoxin (n = 5) levels for FB_T, DON, ZEA. The PDIs for FB_T, DON and ZEA were stratified by gender, ethnicity and Province.

Development and evaluation of the exposure mycotoxin risk assessment model (MYCORAM)

The MYCORAMs for FB_T, DON and ZEA exposure were developed to assess the percentage of maize consumers that will be at risk of mycotoxin exposure above the PMTDI for each mycotoxin stratified by ethnicity and Province. The development of each MYCORAM was conducted in three stages using the data obtained during the cross-sectional maize consumer survey. Firstly the daily individual raw/uncooked

maize intakes were calculated in terms of body weight (g maize kg^{-1} bw day^{-1}). Specific maize intake categories of: i) ≥ 1; ii) ≥ 2; iii) ≥ 4; iv) ≥ 10; v) ≥ 20 g kg^{-1} bw day^{-1} were defined and a specific contamination level for each mycotoxin was selected per increment that will effect a PDIs equal to or above the relevant PMTDIs. These mycotoxin contamination ranges included: 0 to 2000, 0 to 1000, and 0 to 500 μg kg^{-1} for FB_T, DON and ZEA, respectively. To illustrate this, people consuming 1 g kg^{-1} bw day^{-1} at a FB_T contamination level of 2000 μg kg^{-1} will equal the PMTDI of FB (2 μg kg^{-1} bw day^{-1}). In the second stage, the number of maize consumers expressed as a percentage within each maize intake category was stratified either by ethnicity or Provinces. The final stage included the plotting of the percentage of consumers equal or above the PMTDI exposure for a specific mycotoxin against the selected mycotoxin contamination ranges.

The MYCORAM stratified by Province was evaluated using the mean mycotoxin levels of experimental and commercial SPECIAL and SUPER milling fractions. In addition, the corresponding milling fractions of two selected maize samples representing high levels of FB_T (Maize 1) and DON/ZEA (Maize 2), respectively (Burger *et al*., 2013) were included. These contamination levels mimic mycotoxin levels reported in maize samples obtained from rural maize subsistence communities (Sydenham *et al*., 1992; Burger *et al*., 2010).

Safety modelling was also conducted using the MYCORAM stratified by ethnicity to predict maximum tolerated levels (MTLs) guarantee an exposure risk below 1% for maize consumers.

STATISTICAL ANALYSIS

Analysis of covariance (ANCOVA) was used to determine numerical means and to test for normality. As the data failed to be normally distributed, log transformation was used to determine geometric means (GM) which were used for multiple comparisons utilising the Tukey-Kramer test. All statistical analyses and the development of the exposure mycotoxin risk assessment model (MYCORAM) were

performed using the NCSS statistical package Version 8, released July 25, 2012 (Hintze, 2007).

RESULTS

Population characteristics

Study compliance was 94% and comprehensive information on demographics, body weights and raw maize intakes were obtained for 2809 participants. Ninety-nine percent of the total study population (n = 2809) were maize consumers (n = 2778) with only thirty percent of the maize consumer population representing men. According to the 2001 Census Household data gender distribution in South Africa was 48% and 52% for men and women, respectively (Statistics South Africa, 2001). The overall mean population age was 34 (range 16 - 88). Sixty-six % of the population were employed, 9% self-employed, 8% unemployed and the remainder varied between students (6%), housewives (5%) and pensioners (4%) with 2% being non-responders. The household income of the study population (in South African Rand) indicated that 34% had an income of between R500 – R6000, 33% had an income above R8000 and the rest (33%) were non-responders. Twenty-four % the study population had education of grade 1 to grade 11, 45% had a grade 12 or equivalent (National Qualifications Framework, NQF 4 level) education followed by 31% with a Technikon or University degree.

Mycotoxin contamination of maize milling fractions (Table 6.1)

The mycotoxin levels in experimental and commercial (SPECIAL and SUPER) dry milling fractions, intended for human consumption are summarised as numerical means with their respective ranges. Appreciably lower mycotoxin contamination levels were observed in the milling fractions of the commercial samples compared to the pre-selected experimental samples. The mycotoxin levels in the dry milling fractions obtained from the two selected maize samples were much higher with respect to FB_T (Maize 1) and DON/ZEA (Maize 2) which, as mentioned above, reflects contamination levels of raw maize utilised by rural subsistence farmers in South Africa (Burger *et al*., 2010).

Table 6.1: Mycotoxin levels in dry milled maize fractions (SPECIAL and SUPER) from different maize sources* for determining probable daily intake (PDI) and evaluation of the MYCORAM

	FB_T ($\mu g\ kg^{-1}$)	DON ($\mu g\ kg^{-1}$)	ZEA ($\mu g\ kg^{-1}$)
Experimental maize samples (n = 5)			
SPECIAL	338.4 (70.0-1161.0)	127.8 (43.0-240.0)	31.0 (0.0- 81.0)
SUPER	61.0 (0.0-221.0)	27.2 (0.0- 67.0)	8.6 (0.0-18.0)
Commercial maize samples (n = 9)			
SPECIAL	67.1 (20.0-135.0)	18.7 (4.0-26.0)	0.0
SUPER	1.6 (0.0- 5.0)	5.8 (0.0-13.3)	0.6 (0.0-3.2)
Maize 1 (high FB_T) (n = 1)			
SPECIAL	1161.0	195.0	44.0
SUPER	221.0	28.0	13.0
Maize 2 (high DON/ZEA) (n = 1)			
SPECIAL	107.0	240.0	81.0
SUPER	20.0	67.0	18.0

Data expressed as numerical means with ranges in brackets* (Adapted from Burger *et al*, 2013). Abbreviations, n: number of samples; FB: Fumonisin; DON: Deoxynivalenol; ZEA: Zearalenone; FB_T: Total FB (FB_1 + FB_2); MYCORAM: mycotoxin risk assessment model.

Body weight, maize intake profiles and probable mycotoxin intake parameters stratified according to gender, ethnicity and Province

i) Differences between men, women and ethnic groups (Table 6.2)

The mean body weight for men was significantly higher ($p < 0.05$) than the women. The Coloured ethnic group (15% of the maize consumer population) had the highest mean body weight that was statistically higher ($p < 0.05$) compared to the Asian/Indian (4%) and White (18%) groups, whereas the black African group (63%) had a significantly higher mean body weight then the Asian/Indian group. Raw maize intakes ($g\ day^{-1}$) among the men were statistically higher ($p < 0.05$) than the women, though men represented only 30% (n = 776) of the total study population. The mean intake stratified according to ethnicity indicated that the black Africans consumed the

highest amount of maize, which was statistically higher ($p < 0.05$) than all the other ethnic groups. The Coloured group had the second highest intakes that differed ($p < 0.05$) from the black Africans, Indian/Asian and White groups, the latter two groups consuming the lowest but similar amounts. The large variation of maize intakes is indicative of the skewed data distribution. For instance the black African group had significant higher ($p < 0.05$) mean raw maize intake than the Coloured group, although the ranges varied between 0.4 - 2482.7 g day^{-1} and 2.5 - 3055.1 g day^{-1} for the black African and Coloured ethnic groups, respectively.

Probable daily intakes (µg kg^{-1} bw day^{-1}), utilising the mycotoxin contamination levels of the SPECIAL and SUPER milling fractions obtained from the experimental and commercial maize samples (Table 6.1) represented different exposure scenarios. The SPECIAL experimental milling fraction with its overall higher levels of FB_T, DON and ZEA resulted in higher PDIs compared to the SUPER milling fraction. When stratified according to gender, men had a statistically significant ($p < 0.05$) higher PDI compared to the women. Based on ethnicity, the black Africans had the highest PDI that differed significantly ($p < 0.05$) from the other ethnic groups. The Coloured group had a significantly higher PDI compared to the Asian/Indian and White ethnic groups, which did not differ significantly.

Table 6.2: Mean intakes of raw maize, body weights and probable daily intakes for FB_T, DON and ZEA utilising the experimental SPECIAL and SUPER dry milled fractions stratified by gender and ethnicity

Group	n	Percentage of the maize consumer population	Mean body weight (range) (kg)	Mean maize intakes (range) (g day^{-1})	SPECIAL fraction Mean PDI (range) (µg kg^{-1} bw day^{-1})			SUPER fraction Mean PDI (range) (µg kg^{-1} bw day^{-1})		
					FB_T	DON	ZEA	FB_T	DON	ZEA
Maize consumer population	2778	100%	74.0 (36.0-210.0)	157.3 (0.4-3055.1)	0.07 (0.00-2.30)	0.03 (0.00-0.87)	0.010 (0.0-0.21)	0.01 (0.00-0.41)	0.010 (0.000-0.200)	0.002 (0.000-0.010)
Men	776	30%	76.1 **a** (36.0-147.0)	173.0 **a** (0.6-2148.7)	0.08 **a** (0.00-1.10)	0.03 **a** (0.00-0.40)	0.010 **a** (0.000-0.100)	0.01**a** (0.00-0.19)	0.010 **a** (0.000-0.090)	0.002 **a** (0.001-0.027)
Women	2002	70%	72.0 **a** (39.0-210.0)	142.0 **a** (0.4–3055.4)	0.07 **a** (0.00-2.30)	0.03 **a** (0.00-0.90)	0.010 **a** (0.000-0.210)	0.01 **a** (0.00-0.41)	0.005 **a** (0.000-0.185)	0.002 **a** (0.000-0.060)
Asian/ Indian	123	4%	71.0 **c,d** (42.0-104.0)	101.0 **c,d** (0.6–536.7)	0.05 **c,d** (0.00-0.24)	0.02 **c,d** (0.00-0.09)	0.004 **c,d** (0.000-0.022)	0.01 **c,d** (0.00-0.04)	0.004 **c,d** (0.000-0.019)	0.001 **c,d** (0.000-0.006)
black African	1750	63%	75.1 **c** (36.0-210.0)	279.4 **b,c** (0.4-2482.7)	0.13 **b,c** (0.00-1.10)	0.05 **b,c** (0.00-0.40)	0.012 **b,c** (0.000-0.100)	0.02 **b,c** (0.00-0.02)	0.011 **b,c** (0.000-0.100)	0.003 **b,c** (0.000-0.027)
Coloured	407	15%	77.0 **b,d** (39.0-135.0)	169.0 **b,d** (2.5-3055.1)	0.07 **b,d** (0.00-2.30)	0.03 **b,d** (0.00-0.87)	0.010 **b,d** (0.00-0.21)	0.01 **b,d** (0.00-0.41)	0.006 **b,d** (0.000-0.1850)	0.002 **b,d** (0.000-0.058)
White	498	18%	73.7 **b** (40.0-170.0)	80.2 **b** (0.6-1408.4)	0.04 **b** (0.00-0.82)	0.01 **b** (0.00-0.31)	0.003 **b** (0.000-0.075)	0.01 **b** (0.00-0.15)	0.003 **b** (0.000-0.066)	0.001 **b** (0.000-0.021)

Data presented as numerical means with the range in brackets below. Statistical differences ($p < 0.05$) were determined using the log transformed means (geometrical means, GM). Means (within columns) with the same lowercase letter in bold are indicitive of a significantly difference ($p < 0.05$) between the respective groups. Abbreviations, n: sample size; PDI: probable daily intake; FB: Fumonisin; DON: Deoxynivalenol; ZEA: Zearalenone; FB_T: Total FB (FB_1 + FB_2); bw: body weight.

ii) Differences among maize consumers and mycotoxin exposure between the nine South African Provinces (Table 6.3)

The mean body weight of the maize consumers in the Northern Cape (NC) was significantly higher ($p < 0.05$) compared to those from the Gauteng (GP), Limpopo (LP), Western Cape (WC), North West (NW) and Free State (FS) Provinces. Maize consumers from KwaZulu-Natal (KZN) had a higher mean body weight ($p < 0.05$) than those from the NW, whereas those of the consumers of the other Provinces did not differ. When considering the mean raw maize intake profiles, the NC consumers had significantly ($p < 0.05$) higher intakes when compared to those from the GP, FS, Eastern Cape (EC), NW and WC Provinces. Consumers residing in the Mpumalanga (MP), GP, LP, KZN, FS and EC Provinces had similar maize intakes, however the respective intakes for consumers in MP, GP, LP and KZN were significantly higher ($p < 0.05$) when compared to the NW and WC consumers. The raw maize intakes from FS and EC consumers were significantly higher when compared to the WC.

Utilising the various mycotoxin contamination levels obtained from the experimental dry milled fractions (SPECIAL and SUPER), the resultant PDIs across nine Provinces were far below the respective PMTDIs. When considering the SPECIAL milling fraction prepared from the experimental maize samples, maize consumers in the NC and MP Provinces had the highest ($p < 0.05$) mean PDIs (0.09 $\mu g\ kg^{-1}$ bw day^{-1}) for FB_T (338.4 $\mu g\ kg^{-1}$). Except for the NW and WC Provinces, which had the lowest PDIs (0.06 and 0.05 $\mu g\ kg^{-1}$ bw day^{-1}, respectively), the other provinces FS, GP, LP, EC and KZN had similar PDIs ranging between 0.07 and 0.08 $\mu g\ kg^{-1}$ bw day^{-1}. For DON (127.8 $\mu g\ kg^{-1}$) the PDIs were similar in most of the Provinces (0.03 $\mu g\ kg^{-1}$ bw day^{-1}) compared to the NC and WC having the lowest PDIs (0.02 $\mu g\ kg^{-1}$ bw day^{-1}). For ZEA (31.0 $\mu g\ kg^{-1}$), the PDIs (0.01 $\mu g\ kg^{-1}$ bw day^{-1}) were the same for all the Provinces with the lowest (0.004 $\mu g\ kg^{-1}$ bw day^{-1}) observed in the WC. For the experimental SUPER fraction with its low mycotoxin levels, the resultant mean PDIs were far lower (ranging between 0.002 to 0.01 $\mu g\ kg^{-1}$ bw day^{-1}) and did not differ much between the Provinces.

Table 6.3: Mean intakes of raw maize, body weight and probable daily intakes for FB_T, DON and ZEA utilising the experimental SPECIAL and SUPER dry milled fractions stratified by Province

Province	EC	FS	GP	KZN	LP	MP	NC	NW	WC
n	348	189	629	388	346	192	144	241	301
Mean body weight (range) (kg)	75.2 (36.0-170.0)	71.3 **e** (39.0-98.0)	74.0 **a** (40.0-160.0)	76.0 **f** (42.0-170.0)	73.3 **b** (39.0-210.0)	73.8 (49.0-150.0)	79.1**a,b,c,d,e** (50.0-147.0)	71.3 **d,f** (37.0-129.0)	72.5 **c** (49.0-112.0)
Mean maize intake (range) (g day^{-1})	153.0 **c,o** 0.6-1036.7)	156.6 **b,n** (2.5-1119.1)	167.5 **a,h,i** (0.5-3055.1)	163.4 **l,m** (0.6-1262.7)	166.4 **j,k** (2.9-2148.7)	180.5 **f,g** (9.3-2080.5)	201.0 **a,b,c,d,e** (2.3-914.8)	125.0 **d,f,h,j,l** (0.4-1346.6)	103.0 **e,g,i,k,m,n,o** (2.3-830.0)
Experimental SPECIAL milling fraction	**Mean PDI (range) (µg kg^{-1} bw day^{-1})**								
FB_T	0.07 **a,h** (0.00-0.46)	0.08 **i** (0.00-0.54)	0.08 **b,j** (0.00-2.30)	0.07 **e,k** (0.00-0.75)	0.08 **f,l** (0.00-0.97)	0.09 **g,m** (0.00-1.07)	0.09 **a,b,c,d** (0.00-0.37)	0.06 **c,e,f,g** (0.00-0.74)	0.05 **d,h,i,j,k,l.m** (0.00-0.39)
DON	0.03 **a,h** (0.00-0.17)	0.03 **i** (0.00-0.21)	0.03 **b,j** (0.00-0.87)	0.03 **e,k** (0.00-0.28)	0.03 **f,l** (0.00-0.37)	0.03 **g,m** (0.00-0.40)	0.03 **a,b,c,d** (0.00-0.14)	0.02 **c,e,f,g** (0.00-0.28)	0.02 **d,h,i,j,k,l,m** (0.00-0.15)
ZEA	0.01 **a,h** (0.00-0.04)	0.01 **i** (0.00-0.05)	0.01 **b,j** (0.00-0.21)	0.01 **e,k** (0.00-0.07)	0.01 **f,l** (0.00-0.009)	0.01 **g.m** (0.00-0.10)	0.01 **a,b,c,d** (0.00-0.03)	0.01 **c,e,f,g** (0.00-0.07)	0.004 **d,h,i,j,k,l,m** (0.00-0.04)
Experimental SUPER milling fraction	**Mean PDI (range) (µg kg^{-1} bw day^{-1})**								
FB_T	0.01 **a,h** (0.00-0.08)	0.01 **i** (0.00-0.10)	0.01 **b,j** (0.00-0.41)	0.01 **e,k** (0.00-0.14)	0.01 **f,l** (0.00-0.17)	0.02 **g,m** (0.00-0.19)	0.02 **a,b,c,d** (0.00-0.07)	0.01 **c,e,f,g** (0.00-0.13)	0.01 **d,h,i,j,k,l.m** (0.00-0.07)
DON	0.01 **a,h** (0.00-0.04)	0.01 **i** (0.00-0.05)	0.01 **b,j** (0.00-0.18)	0.01 **e,k** (0.00-0.06)	0.01 **f,l** (0.00-0.08)	0.01 **g,m** (0.00-0.09)	0.01 **a,b,c,d** (0.00-0.03)	0.01 **c,e,f,g** (0.00-0.06)	0.004 **d,h,i,j,k,l.m** (0.00-0.03)
ZEA	0.002 **a,h** (0.00-0.01)	0.002 **i** (0.00-0.01)	0.002 **b,j** (0.00-0.06)	0.002 **e,k** (0.00-0.02)	0.002 **f,l** (0.00-0.02)	0.002 **g,m** (0.00-0.03)	0.002 **a,b,c,d** (0.00-0.01)	0.002 **c,e,f,g** (0.00-0.02)	0.001 **d,h,i,j,k,l.m** (0.00-0.01)

Data presented as numerical means with the range in brackets. Statistical differences ($p < 0.05$) were determined using the log transformed means (geometrical means, GM). Means (within rows) with the same lowercase letter in bold are indicitive of a significantly difference ($p < 0.05$) between the different Provinces. Abbreviations: n: sample size, EC: Eastern Cape; FS: Free State; GP: Gauteng Province; KZN: KwaZulu Natal; LP: Limpopo Province; MP: Mpumalanga Province; NC: Northern Cape; NW: North West; WC: Western Cape, PDI: probable daily intake; FB: Fumonisin; DON: Deoxynivalenol; ZEA: Zearalenone; FB_T: Total FB (FB_1 + FB_2); bw: body weight.

MYCORAM development according to ethnicity and Province (Table 6.4, Figure 6.1 A and B)

The percentage of the study population within a specific maize intake category was stratified by ethnicity and across nine South African Provinces. For each maize intake category the equivalent mycotoxin contamination level was selected representing the PDI, the product of which will equal the respective PMTDI of each mycotoxin (Table 6.4). Based on these data, the MYCORAMs for FB_T, DON and ZEA were then developed by predicting the percentages (%) of maize consumers that will be equal or above the respective PMTDI for each mycotoxin, as a function of the selected mycotoxin contamination level: FB_T DON and ZEA according the ethnicity and Province (Figure 6.1 A and B). The percentage of consumers above the limit is affected by the number of people within a specific maize intake category and provides a more informative assessment of exposure compared to the mean PDI.

MYCORAM evaluation stratified by Province

i) Experimental and commercial dry milling fractions (Table 6.5)

The mycotoxin levels associated with (i) the SPECIAL and SUPER fractions obtained from experimental and commercial milling (ii) and two respective maize samples (Maize 1 and Maize 2) with high levels of FB and DON/ZEA (Table 6.1) were utilised to evaluate the MYCORAM. The apparent “consumption” of the SPECIAL milling fraction obtained from the experimental samples [FB (338.4 $\mu g.kg^{-1}$), DON (127.8 $\mu g.kg^{-1}$) and ZEA (31.0 $\mu g.kg^{-1}$)] resulted in higher percentage consumers exposed above the regulated levels. The Province with the highest number of consumers above the limit for FB_T was KZN (18.2%) with the other provinces ranging between 16.0 to 17.0% (LP, MP, FS, and GP), EC, NW and NC ranging between 11.0 to 12.7% with WC (7.0%) having the lowest. For DON the highest % exposure above the limit was observed in the MP (9.5%), KZN (9.1%), GP (9.0%), FS (8.8%) and LP (8.6%) followed by EC (6.0%0, NC (4.8%) and WC (3.3%). The highest percentage above the limit for ZEA was also observed in MP (4.2%) and GP (2.0%), followed by LP (1.4%), KZN (1.2%), NW (1.0%) and FS (0.9%), with the lowest percentages in EC (0.4%), NC (0.2%) and WC (0.2%).

Table 6.4: Development of the Mycotoxin Risk Assessment Models (MYCORAMs): defined maize intake categories and preselected contamination levels of FB_T, DON and ZEA representing exposure equal or above the relevant PMTDI and the percentage (%) of maize consumers within an intake category stratified by ethnicity and Province**

FB_T levels ($\mu g\ kg^{-1}$)	DON levels ($\mu g\ kg^{-1}$)	ZEA levels ($\mu g\ kg^{-1}$)	Intake Categories ($g\ kg^{-1}$ bw day^{-1})	Ethnic groups				
				Asians/ Indians	Black African	Coloured	White	Total
100	50	25	≥ **20**	0	1%	0	0	1%
200	100	50	≥ **10**	0	4%	1%	1%	3%
500	250	125	≥ **4**	10%	35%	11%	65	25%
1000	500	250	≥ **2**	25%	73%	44%	16%	56%
2000	1000	500	≥ **1**	37%	90%	65%	31%	73%
			Up To 1	100%	100%	100%	100%	100%

FB_T levels ($\mu g\ kg^{-1}$)	DON levels ($\mu g\ kg^{-1}$)	ZEA levels ($\mu g\ kg^{-1}$)	Intake Categories ($g\ kg^{-1}$ bw day^{-1})	Provinces									
				EC	FS	GP	KZN	LP	MP	NC	NW	WC	Total
100	50	25	≥ **20**	0%	0%	1%	1%	1%	4%	0%	0%	0%	1%
200	100	50	≥ **10**	2%	4%	4%	3%	3%	5%	1%	2%	1%	3%
500	250	125	≥ **4**	26%	31%	29%	36%	33%	29%	23%	24%	14%	28%
1000	500	250	≥ **2**	60%	62%	59%	67%	76%	64%	66%	56%	48%	62%
2000	1000	500	≥ **1**	79%	78%	76%	83%	91%	92%	83%	76%	74%	81%
			Up To 1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

*Maize intake categories and percentages calculated using individual raw maize intakes and body weights from maize consumers (n = 2522). ** For each maize intake category, the equivalent mycotoxin contamination level was selected such that the PDI produced by their product will equal the respective PMTDI for each mycotoxin. Abbreviations: EC: Eastern Cape; FS: Free State; GP: Gauteng Province; KZN: KwaZulu Natal; LP: Limpopo Province; MP: Mpumalanga Province; NC: Northern Cape; NW: North West; WC: Western Cape, PDI: probable daily intake; FB: Fumonisin; DON: Deoxynivalenol; ZEA: Zearalenone; FB_T: Total FB (FB_1 + FB_2); bw: body weight.

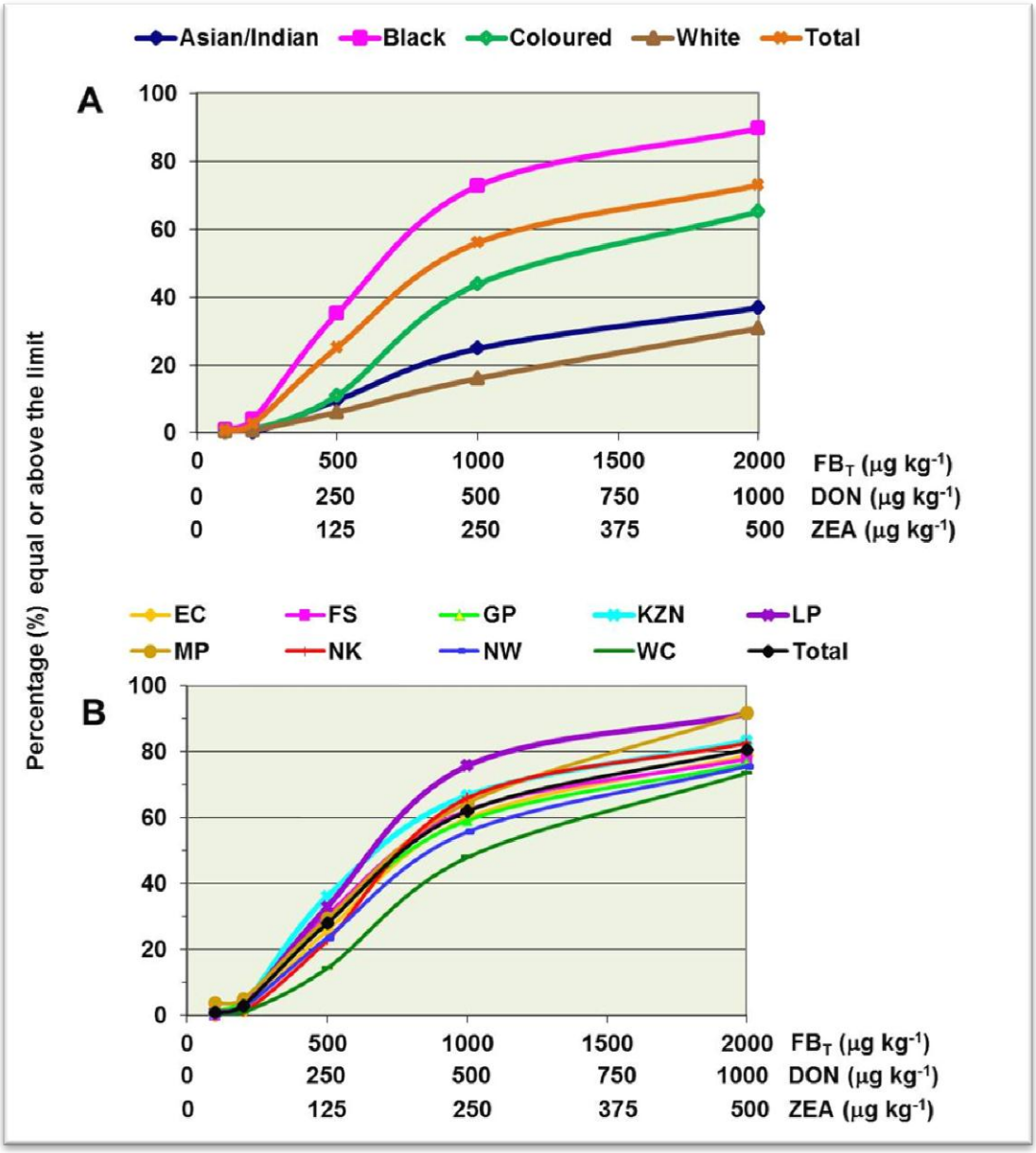

Figure 6.1: MYCORAMs for FB_T DON and ZEA: Percentages of the South African maize consumers stratified by Province (A) and ethnicity (B) equal or above the respective mycotoxin PMTDIs

The percentage of consumers above the limit of exposure using the commercial SPECIAL milling fraction was far less, reflecting lower mycotoxin levels. The highest percentage of consumers above the limit was observed in MP, 2.6% (FB_T) and 1.5% (DON), respectively. None of the maize consumers residing in the different Provinces were exposed above the PMTDI limit for ZEA. The experimental SUPER fractions also produced similar patterns with much lower %, ranging between 0 and 2.4% (MP) for FB_T, 0 to 2.1% (MP) for DON and 0 to 1.3% (MP) for ZEA, respectively.

Table 6.5: Evaluation of the MYCORAM using the relevant experimental and commercial SPECIAL and SUPER dry milled fractions contaminated by FB_T, DON and ZEA, stratified by Province

Maize milling fraction	n	Mycotoxin level (µg kg^{-1})*	% Population above the PMTDI of 2 µg kg^{-1} bw day^{-1} for FB_T								
SPECIAL			**EC**	**FS**	**GP**	**KZN**	**LP**	**MP**	**NK**	**NW**	**WC**
Experimental	5	**338.4**	12.7	16.2	16.0	18.2	17.0	16.3	11.0	12.1	7.0
Commercial	9	**67.1**	0.0	0.0	0.8	0.4	0.6	2.6	0.0	0.3	0.0
SUPER											
Experimental	5	**61.0**	0.0	0.0	0.7	0.4	0.6	2.4	0.0	0.3	0.0
Commercial	9	**1.6**	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
			% Population above the PMTDI of 1 µg kg^{-1} bw day^{-1} for DON								
SPECIAL			**EC**	**FS**	**GP**	**KZN**	**LP**	**MP**	**NK**	**NW**	**WC**
Experimental	5	**127.8**	6.0	8.8	9.0	9.1	8.6	9.5	4.8	6.2	3.3
Commercial	9	**18.7**	0.0	0.0	0.4	0.2	0.4	1.5	0.0	0.2	0.0
SUPER											
Experimental	5	**27.2**	0.0	0.0	0.6	0.3	0.5	2.1	0.0	0.3	0.0
Commercial	9	**5.8**	0.0	0.0	0.1	0.1	0.1	0.5	0.0	0.1	0.0
			% Population above the PMTDI of 0.5 µg kg^{-1} bw day^{-1} for ZEA								
SPECIAL			**EC**	**FS**	**GP**	**KZN**	**LP**	**MP**	**NK**	**NW**	**WC**
Experimental	5	**31.0**	0.4	0.9	2.0	1.2	1.4	4.2	0.2	1.0	0.2
Commercial	9	**0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUPER											
Experimental	5	**8.6**	0.0	0.0	0.4	0.2	0.3	1.3	0.0	0.2	0.0
Commercial	9	**0.6**	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0

Abbreviations, FB: Fumonisin; FB_T: Total FB (FB_1 + FB_2); DON: Deoxynivalenol; ZEA: Zearalenone; PMTDI: Provisional Maximum Tolerable Daily Intake; bw: body weight; EC: Eastern Cape; FS: Free State; GP: Gauteng Province; KZN: KwaZulu Natal; LP: Limpopo Province; MP: Mpumalanga Province; NC: Northern Cape; NW: North West; WC: Western Cape. *Mean mycotoxin levels as published by Burger *et al*., 2013 as summarised in Table 6.1. n = number of samples.

ii) High FB_T (Maize 1) and DON/ZEA (Maize 2) dry milling fractions (Table 6.6): A higher percentage of maize consumers were above the relevant PMTDI when modelling the milling fractions obtained from the high FB_T and DON/ZEA experimental maize samples). When modelling the SPECIAL milling fraction of the high FB_T (1161.0 µg kg^{-1}), exposure levels above the PMTDI ranged between the highest (78.2%) in LP and lowest (52.0%) in the WC. The SPECIAL milling fraction obtained from the high DON (240 µg kg^{-1}) maize sample, resulted in the highest percentage of consumers above the limit (33.8%) in KZN with lowest (13.4%) in WC. The high ZEA (81.0 µg kg^{-1}) containing SPECIAL milling fraction also followed the same pattern as DON with the highest (16.7%) level of exposure in KZN and the lowest in WC (6.4%). For the SUPER milling fraction derived from the high FB_T maize sample (221.0 µg kg^{-1}), the percentage of consumers above the PMTDI ranged from the highest of 6.7% (MP) to the lowest in WC (1.8%). For the high DON (67.0 µg kg^{-1}) containing SUPER milling fraction, consumers above the limit ranged from 0.0% to the highest of 5.2% above the PMTDI in MP, whereas for high ZEA (18.0 µg kg^{-1}) containing SUPER milling fraction, it varied between 0% and 2.8% above the PMTDI in MP.

Predicted safety modelling according to ethnicity (Tale 6.7)

Different maximum tolerable levels (MTLs) for FB_T, DON and ZEA stratified by ethnicity were modelled to effect less than 1% of the consumers at risk of exposure above the PMTDI for each mycotoxin**.** Different MTLs for FB_T, DON and ZEA, were determined for each ethnic group as a function of the level of mycotoxin contamination and maize intake profiles depicted in the MYCORAM (Figure 6.1A). The black African and coloured population groups required far lower MTL's of the different mycotoxins as compared to the Asia/Indian and white population groups within the defined risk paradigm.

Table 6.6: Evaluation of the MYCORAM utilising experimental SPECIAL and SUPER milling fractions obtained from two selected samples reflecting contamination levels of subsistent maize, stratified by Province

Maize milling fraction	Mycotoxin level (µg kg^{-1})*	% Population above the PMTDI of 2 µg kg^{-1} bw day^{-1} for FB_T								
SPECIAL		**EC**	**FS**	**GP**	**KZN**	**LP**	**MP**	**NK**	**NW**	**WC**
Maize 1[H]	1161.0	63.0	64.7	62.0	69.3	78.2	68.8	68.6	58.9	52.0
Maize 2[L]	107.0	0.1	0.3	1.4	0.8	1.1	4.0	0.1	0.6	0.1
SUPER										
Maize 1 [H]	221.0	3.2	5.8	6.2	5.3	5.2	6.7	2.3	3.8	1.8
Maize 2 [L]	20.0	0.0	0.0	0.2	0.1	0.2	0.8	0.0	0.1	0.0
		% Population above the PMTDI of 1 µg kg^{-1} bw day^{-1} for DON								
SPECIAL		**EC**	**FS**	**GP**	**KZN**	**LP**	**MP**	**NK**	**NW**	**WC**
Maize 2 [H]	240.0	24.2	28.8	27.1	33.8	30.9	27.8	21.3	22.1	13.4
Maize 1 [L]	195.0	16.9	20.8	19.8	23.9	22.0	20.5	14.7	15.7	9.4
SUPER										
Maize 2 [H]	67.0	0.0	0.0	1.6	0.8	1.2	5.2	0.0	0.6	0.0
Maize 1 [L]	28.0	0.0	0.0	0.7	0.3	0.5	2.2	0.0	0.3	0.0
		% Population above the PMTDI of 0.5 µg kg^{-1} bw day^{-1} for ZEA								
SPECIAL		**EC**	**FS**	**GP**	**KZN**	**LP**	**MP**	**NK**	**NW**	**WC**
Maize 2 [H]	81.0	11.6	14.9	14.5	16.7	15.4	15.1	9.8	11.1	6.4
Maize 1 [L]	44.0	1.2	3.0	3.7	2.4	2.6	4.7	0.6	1.8	0.6
SUPER										
Maize 2 [H]	18.0	0.0	0.0	0.8	0.4	0.7	2.8	0.0	0.3	0.0
Maize 1 [L]	13.0	0.0	0.0	0.6	0.3	0.5	2.0	0.0	0.2	0.0

Maize 1 [H] contains high FB_T and Maize 1[L]: low DON and ZEA levels whilst Maize 2 [H] contains high DON and ZEA and Maize 2 [L] has low FB_T levels and levels. Abbreviations, FB: Fumonisin; FB_T: Total FB (FB_1 + FB_2); DON: Deoxynivalenol; ZEA: Zearalenone; PMTDI: Provisional Maximum Tolerable Daily Intake; bw: body weight; EC: Eastern Cape; FS: Free State; GP: Gauteng Province; KZN: KwaZulu Natal; LP: Limpopo Province; MP: Mpumalanga Province; NC: Northern Cape; NW: North West; WC: Western Cape. *Mean mycotoxin levels as published by Burger *et al*., 2013 as summarised in Table 1.

Table 6.7: Prediction of maximum tolerated levels using the MYCORAM stratified by ethnicity

Ethnic group	Mycotoxin	Maximum tolerated levels (µg kg^{-1})	Percentage (%) equal or above the PMTDI*
Asian/Indian	FB_T	228	
	DON	114	0.9
	ZEA	57	
black African	FB_T	180	
	DON	90	0.9
	ZEA	45	
Coloured	FB_T	190	
	DON	95	0.9
	ZEA	47	
White	FB_T	210	
	DON	104	0.9
	ZEA	51	

Abbreviations, MYCORAM: Mycotoxin Risk Assessment Model, FB: Fumonisin; FB_T: Total FB (FB_1 + FB_2); DON: Deoxynivalenol; ZEA: Zearalenone; PMTDI: Provisional Maximum Tolerable Daily Intake; bw: body weight. * PMTDI for FB_T, 2 µg kg^{-1} bw day^{-1}; DON, 1 µg kg^{-1} bw day^{-1} and for ZEA, 0.5 µg kg^{-1} bw day^{-1}.

DISCUSSION

The development and implementation of food safety parameters within a specific population requires valid risk assessment based on the estimation of the level of exposure, which requires accurate determination of food consumption data and the contamination levels in the specific food commodities. The process of exposure assessment is considered as a critical phase in risk assessment and relies on utilising an appropriate model (FAO and WHO, 2006, Fryer *et al*., 2006). The choice of an appropriate model is by the aim of the assessment (screening, setting of standards or first-time exposure assessment), the food commodity affected, population characteristics (age, gender, cultural diversity etc.), time frame of the exposure (acute or chronic), the study data available (accuracy and format), the scope of the problem (toxin characteristics) and resources (Parmar *et al*., 1997, Lambe 2002, Fryer *et al*., 2006). Probable daily intakes are computed utilising deterministic modelling based on the simple multiplication of a fixed contamination level (mean or maximum level) observed in the relevant food commodity, by the amount consumed (mean or upper level/95th percentile intake of a population or group). The so-called stochastic (probabilistic) modelling is a distribution-based or population-related approach where mathematical methods are applied to create

probability distributions to represent the input variables. A selected value is then applied to the model algorithms to calculate a “single estimate of exposure” (Fryer *et al*., 2006). The advantage of probabilistic modelling is that it includes variability and uncertainties of data and is regarded as a more accurate and realistic method to assess exposure (Bosgra *et al*., 2009, Cano-Sancho *et al*., 2011). The MYCORAM, developed in the current study, defined the percentage of people at risk of exposure using the full heterogeneity of the data such as maize intakes, body mass, population characteristics, the relevant tolerably daily intake and mycotoxin levels. This model, based on data obtained from South African maize consumers, can then be used to determine MTLs in a specific food commodity to safeguard the affected population. The model could be further refined to develop MTLs for specific subgroups depending on their specific maize consumption profiles.

In most developed countries national food consumption surveys provide detailed information to assess the level of exposure to a specific food contaminant. Food consumption data can also be formatted to provide information on specific food items consumed such as maize e.g., cornflakes, ingredients (i.e. maize meal) or the raw agricultural commodity (Møller and Ireland, 2008; Boon *et al*., 2009). As regular national dietary surveys and expensive dietary recording methods are lacking in South Africa, food frequency questionnaires are valuable and normally used to assess habitual intakes. However, dietary assessment methods are never without their inherent limitations when assessing exposure as the human diet is known for its complexities due to varied food choices and differences in the consumption patterns among individuals. Along with variability in dietary patterns, uncertainties regarding contaminant analyses and sampling methodologies also exist (Hart *et al*., 2003). Most often the association between intake of food contaminants, such as mycotoxins, is compromised due to the use of inadequate dietary assessment methods that are not validated or culturally specific (Kroes *et al*., 2002, Petersen, 2003). It is inevitable, therefore, that the process of risk assessment will differ within and between countries due to the approach followed, history, economy, cultural diversity, policy and infrastructure available.

South African commercial maize is known to contain low levels of mycotoxins compared to home-grown maize cultivated in rural subsistence farming areas (Shephard *et al*., 2005; Shephard *et al*., 2007; Burger *et al*., 2010). However, no regulation exists in South Africa for FB, DON and ZEA, the major mycotoxins occurring in maize, and grain-based companies have to comply with international trading legislation. In the current study the use of standardised methods to estimate maize intake, especially raw/uncooked maize as well as accurate mycotoxin analyses resulted in a valid and reliable outcome to define the risk of mycotoxin exposure. When considering the consumption of commercial maize, consumers are not directly exposed to the raw food commodity but to the various products obtained from maize milling. The low mycotoxin levels of two dry milled fractions relevant to human consumption (the SPECIAL and SUPER fraction) are due to the effective removal of maize kernel constituents vulnerable to fungal colonisation and mycotoxin contamination (Burger *et al*, 2013). The SPECIAL maize milling fraction or maize flour contains maize kernel surface layers known to be more vulnerable to fungal penetration as compared to the SUPER milling fraction consisting of coarse grits, mainly derived from the endosperm and containing far lower levels of mycotoxins (Scudamore and Patel, 2009; Castells *et al*., 2008; Burger *et al*, 2013). The mycotoxin levels associated with these milling fractions resulted in PDIs well below the respective mycotoxin PMTDI for each of the mycotoxins. As expected specific population groups with high maize intakes, such as men and the black African group reflected higher PDIs. However, due to the large variation in the maize consumption profiles, the risk of exposure of some individuals toward the higher end of consumption is masked when considering the mean values within a subgroup or subpopulation.

To address this, the MYCORAM was developed to predict the percentage of consumers that will be at risk considering the respective PMTDIs of FB_T, DON and ZEA. Due to South Africa's heterogenic population a MYCORAM was compiled for the relevant ethnic groups and Provinces. Therefore the percentage of consumers exposed above the PMTDI for the specific MYCORAM will depend on the maize intakes and body weights, including the specific subgroup or population size and characteristics (e.g. gender). During the consumer survey the sampling methodology was based on ethnic distribution according to the 2001 Census Household data and

did not include gender distribution resulting in an under-representation of men. Therefore the percentage of men at risk, normally consuming more maize may be masked within the respective MYCORAMs.

Evaluation of the MYCORAM stratified by Provinces, using experimental and commercial milling fractions, the SPECIAL fractions with higher mycotoxin levels affected a larger percentage (5 to 8 fold) of consumers above the limit compared to the SUPER fractions. The highest percentage of consumers above the limit for FB_T, DON and ZEA were those residing in the MP, LP, GP and KZN. The latter Provinces are known to have larger population sizes and therefore more consumers distributed across the different maize intake categories whereas the NC had the smallest sample size.

Utilising mycotoxin levels from two different experimental SPECIAL and SUPER fractions (Maize 1 and Maize 2), a higher percentage of the population is exposed above the limit for the three mycotoxins. The FB level in the experimental SPECIAL fraction (Maize 1[H], 1161 µg kg^{-1}) was similar to the level reported in home-grown maize (1142 µg kg^{-1}) (Shephard *et al*., 2007). Similarly, the experimental SPECIAL fraction with the higher DON and ZEA (Maize 2 [H])also showed higher percentages above the PMTDI across the nine Provinces. This milling fraction represents a "worst case scenario" relevant to human consumption and is also aligned with situations prevailing in rural subsistence communities. Regarding the DON level, a recent report by Shephard *et al*. (2010) showed similar levels (262.0 µg kg^{-1}) in South African commercial maize meal which favourable compared to the level in the SPECIAL milling fraction (240.0 µg kg^{-1}) of the high DON/ZEA maize sample. The FB exposure levels are in agreement with a study conducted among rural people living in the EC, where both home-grown and commercial maize are consumed in large quantities indicating a larger number of the study population with exposure above the PMTDI (Burger *et al*., 2010).

Based on these MYCORAM analyses, a MTL for each of the different mycotoxins can be projected for the South African population consuming maize and/or processed maize products such as maize meal. Total fumonisin levels of between 50

and 100, 20 and 50 for DON and 20 to 30 µg kg^{-1} for ZEA, provides MTLs that is attainable in the milling industry while lowering the risk (<1%) of maize consumers residing in five of the nine Provinces. These predicted MTLs may still render certain South African maize consumers at risk with between 1.2% to 4.1% of the maize consumers in the four remaining Provinces (GP, KZN, LP and MP) exposed above the relative PMTDI's, depending on the mycotoxin. In contrast, much lower levels of mycotoxins are required in order to ensure that the percentage of consumers at risk is lower than 1% across the nine Provinces. The MTLs based on ethnicity, although realistic from an industry perspective however, global harmonization and international trade may be complicated. In this regard the lowest MTLs for the most vulnerable population (the black Africans) need to be selected and implemented.

Maize consumption remains an important part of the South African diet and ranges from the staple diets in some areas to maize-based snacks and side-dishes in more urban areas. The inclusion of more urban maize consumers, known to consume less maize or maize products in the present survey, is likely modulate the MYCORAM risk profile in a specific Province. Consumption of good quality commercial maize becomes evident; however the unvaried diet of many South Africans consuming high levels of maize may increase the risk even at low levels of mycotoxin contamination. Food safety, being both an integral part as well as a contributor to food security remains a challenge in the context of changing socio-economic realities that hinders basic food sufficiency and access. The MYCORAM provides the opportunity to accurately identify populations groups of different ethnicity that will be at risk as the use of individual data during risk assessment is known to be more accurate than utilising national averages (Kroes *et al*., 2002). This is especially applicable to the current population where the risk of exposure of vulnerable subgroups, is masked due to the inherent diversity in the maize intake of the population. The MYCORAM is, therefore a far more sensitive approach to assess risk and addresses the large variation in the exposure data often encountered during epidemiological surveys. It provides an innovative and interactive way to assess the risk of exposure in maize consumers encompassing the three mycotoxins. The MYCORAM could be useful in setting international standards for inferring risk in specific subpopulations or groups consuming maize. This could be of relevance for population groups consuming traditional diets such as polenta in Northern Italy; tortillas in Mexico and people with

gluten-free diet e.g. celiac disease, dermatitis herpetiformis or an allergy to wheat (Pascale *et al*., 1995, Bolger *et al*., 2001, De Nijs *et al*., 1998)

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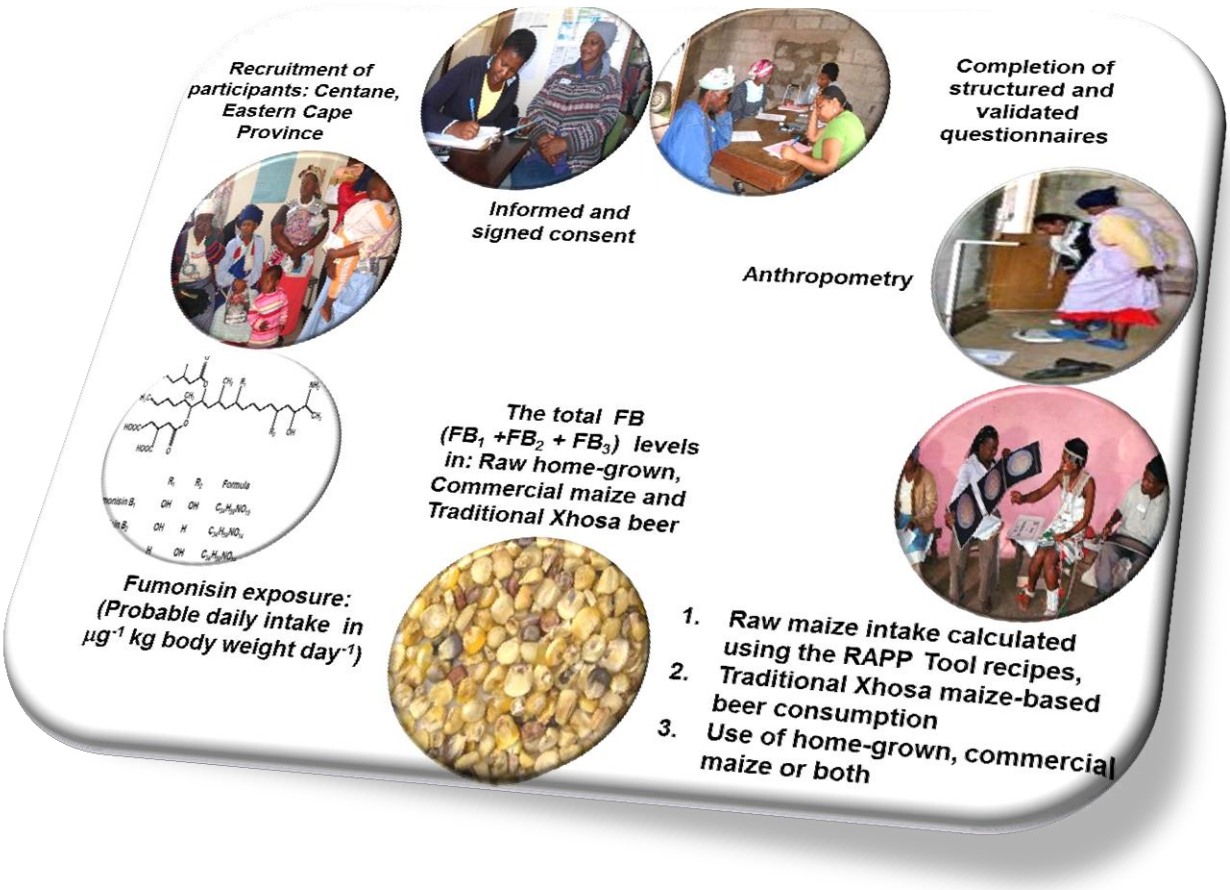

CHAPTER 7

DIETARY FUMONISIN EXPOSURE IN A RURAL POPULATION OF SOUTH AFRICA

Burger, H-M., Lombard, M.J., Shephard, G.S., Rheeder, J.P., Van der Westhuizen, L., Gelderblom, W.C.A. (2010). ***Food and Chemical Toxicology,*** **48, 2103-2108.** (Impact factor 2.321)

ABSTRACT

A validated culturally specific dietary assessment method was used to determine the habitual maize intakes of black Xhosa-speaking Africans living in the Centane region of the Eastern Cape Province to assess their exposure to the carcinogenic fumonisin mycotoxins. The mean total dry weight maize intakes of home-grown, commercial or combined (both maize sources) were 474, 344, 462 g day^{-1}, respectively. When considering the total mean levels of fumonisin in home-grown maize (1142 $\mu g\ kg^{-1}$) and commercial maize (222 $\mu g\ kg^{-1}$), the probable daily intakes (PDIs), expressed as $\mu g\ kg^{-1}$ body weight day^{-1} were 12.1 (95%CI:0.3 - 4926.5) and 1.3 (95%CI: 1.0 - 1.8) for men and 6.7 (95%CI: 1.0 - 457.8) and 1.1 (95%CI: 0.9 - 1.3) for women, consuming home-grown and commercial maize, respectively. Based on the different maize-based beer drinking frequencies the PDIs varied between 6.9 and 12.0 $\mu g\ kg^{-1}$/drinking event. Depending on the maize intake patterns an exposure "window" exists where fumonisin exposure is below the recommended group provisional maximum tolerable daily intake (PMTDI) for fumonisins of 2 $\mu g\ kg^{-1}$ bw day^{-1}. The assessment of fumonisin exposure and development of preventative strategies depend, not only the accurate determination of total fumonisin levels in maize, but also on the distinct dietary patterns of a specific population.

INTRODUCTION

The fumonisin B (FB) mycotoxins are ubiquitous contaminants of maize mainly produced by the fungi *Fusarium verticillioides* and *F. proliferatum* (Rheeder *et al*., 2002). They are known to cause several diseases in animals and are associated with a high incidence of human oesophageal and liver cancer in certain geographical areas of the world (Rheeder *et al*., 1992; Ueno *et al*., 1997), as well as the development of neural tube defects (Marasas *et al*., 2004). To date about twenty-eight fumonisins have been identified (Rheeder *et al*., 2002). Fumonisin B_1, the most abundant occurs together with FB_2 and FB_3 mainly in maize with levels varying with season and geographical location (Shephard *et al*., 1996; Rheeder *et al*., 2002). The fumonisins have been classified as group 2B carcinogens (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2002). A group provisional maximum tolerable daily intake (PMTDI) for fumonisins (B_1, B_2 and B_3) of 2 $\mu g\ kg^{-1}$ bw day^{-1} was established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), using a no observed adverse effect level (NOAEL) of 0.2 mg kg^{-1} bw day^{-1} for nephrotoxicity in rats and a safety factor of 100 (Bolger *et al*., 2001).

The risk of exposure to mycotoxins in human populations residing in developing countries are considered to be far greater where the implementation of legislative control is compromised due to many economic, political and environmental factors (WHO, 2006; Wagacha and Muthomi, 2008). In South Africa, the former Transkei region of the Eastern Cape Province (EC) is a rural area characterised by a high prevalence of poverty and underdevelopment. The majority of the inhabitants are dependent on governmental pensions and grants while migrant labourers provide an additional income (Nel and Davies, 1999; D'Haese and Van Huylenbroeck, 2005). Subsistence farming is a major source of food security where the daily intake of maize is part of a culturally distinct dietary pattern and ethnic tradition. Home-grown maize from these areas is known to be contaminated with high levels of fumonisin and therefore poses an important health risk (Shephard *et al*., 2007). Commercial maize also consumed in these areas, generally contains far lower fumonisin levels, but could still pose a risk if consumed in large quantities. The determination of

fumonisin exposure, which forms an integral part of the human risk assessment process, is therefore of critical importance. However, the lack of any regulations or monitoring of fumonisin levels in South African maize intended for human consumption further contributes to the uncertainty when determining risk (Gelderblom *et al*, 2008; Marasas *et al*., 2008).

The accuracy of assessing mycotoxin exposure is not only dependent on standardised analytical methods to determine the level of contamination in the food matrix, but also on detailed dietary intakes using effective and validated dietary assessment instruments (Kroes *et al*., 2002). In South Africa the national food consumption survey together with a few demographic-specific consumption studies provide limited data on maize consumption in rural areas (National Food Consumption Survey 2000, Nel and Steyn, 2002). In a population at risk to fumonisin exposure, the maize intakes together with the maize-dietary practices will influence exposure. Factors such as the sources of maize, mixture of maize with other food components, seasonal variations of these components, socio-economic status, cultural traditions, food preparations, food availability and accessibility will impact on maize consumption. To address the methodological challenges of estimating dietary mycotoxin intake, a culturally specific dietary assessment tool, the Ratio And Portion size Photo (RAPP) tool was developed and validated among a black Xhosa-speaking African rural population (Lombard *et al*., 2012; Addendum E).

In the present study, the RAPP tool was used to assess maize consumption patterns and consequently fumonisin exposure in Centane, an area in the EC of South Africa where maize is consumed as a major dietary staple. This rural area is known for its high incidence of oesophageal cancer (OC) while the home-grown maize is highly contaminated with the FB mycotoxins (Somdyala *et al*., 2003; Shephard *et al*., 2007).

METHODS AND MATERIALS

Study population

Ethical approval for the study was granted by the South African Medical Research Council Ethics Committee (Addendum F). Three hundred and fifteen apparently healthy volunteers between the ages of 18 and 70 were recruited after informed and signed consent. Volunteers were recruited from 21 rural villages within the magisterial area of Centane, Amathole District Municipality in the EC. A field laboratory was set up at three local trading stores along a central road, providing easy access to participants. Standard anthropometrical measurements were collected in addition to the use of a structured questionnaire to collect information regarding the population's demographics and socio-economic status.

Dietary Assessments

Descriptive information on dietary maize intakes and consumption practices were collected using the validated dietary assessment method.

Dietary assessment tool

The Ratio And Portion size Photo (RAPP) tool is a culturally specific dietary assessment method that determines the dietary habits and nutrient intakes of Xhosa-speaking people living in rural areas in the EC (Addendum E). It consists of life size photographic pictures of the types of foods mostly consumed and a semi-quantitative food frequency questionnaire, focusing on the dietary intake of the past month. The 33 items listed are divided into ten food groups with each food group having two sets of photographs. The first set is a range of portion sizes (small, medium and large) to increase the accuracy of food intake estimation while a second set illustrates different ratios of combined dishes. The maize-based dietary items were divided into three main groups (i) *bread group* which included baked bread, steamed bread, "corn" bread, unonca (baked maize bread), dumplings and "vetkoek" (deep fried pastry); (ii) *cereals and porridge group* included: whole maize kernels, corn-on-the-cob, soft, stiff and crumbly porridge and samp (iii) *combined dish group* which included maize meal combined with spinach, imifino (wild leafy vegetables), pumpkin or beans; samp and

beans; mealie rice combined with spinach, pumpkin or beans. The non-alcoholic maize fermented beverage (also known as mageu, magou, mahewu or amahewu) was also included in the combined dish group. Participant's preference to home-grown, commercial maize or both were determined for each maize-based item. Commercial maize is defined as maize sold by local trading stores/shops supplied nationally by the South African maize industry.

Mean dry/uncooked maize intake patterns

Individual total dry/uncooked maize intakes in grams per day (g day^{-1}) were estimated using the RAPP tool. These intakes were calculated using recipes for each maize-based item and, in the case of combined dishes, the preferred ratio was also taken into account. Thereafter, individuals were grouped according to their consumption of home-grown maize (HG group, n = 5), commercial maize (COM group, n = 173) or both (HG_COM group, n = 137). The total mean dry/uncooked maize intakes for the HG and COM groups were reported as the geometric means with the 95% confidence levels and stratified according to gender. Taking into account that the HG_COM group consists of "home-grown maize" and a "commercial maize" intake, eight subgroups were used to display different percentage dry/uncooked intakes. These eight subgroups, depending on the maize source were 80 - 90%; 70 - 79%; 60 - 69%; 50 - 59; 40 - 49%; 30 - 39%; 20 - 29% and 10 - 19% when considering commercial maize intake. The total geometric mean with the 95% confidence levels were calculated for each of the 8 percentage groups.

Consumption of traditional Xhosa maize-based beer

The consumption of traditional home-brewed Xhosa maize-based beer (also known as mqoboti or umqombothi) was also assessed. The frequency of use and intake in litres (L) on a single drinking event was determined using the RAPP tool. The standard CAGE questions were also used to confirm regular intake and dependency (Ewing, 1984; O'Brian, 2008). The questions were "Have you ever felt you should cut down on your drinking? Yes or No"; "Have people ever annoyed you by criticizing your drinking? Yes or No"; "Have you ever felt bad or guilty about your drinking? Yes or No"; "Have you ever had a drink first thing in the morning? Yes or No". A score out of four was calculated for each participant consuming traditional Xhosa beer.

Total fumonisin in maize and traditional Xhosa maize-based beer

The mean total fumonisin (FB_1 + FB_2 + FB_3) levels were obtained from the analysis of (i) good home-grown (ii) commercial maize and (iii) Xhosa traditional maize-based beer. The mean total fumonisin levels in good home-grown maize were determined in 141 samples from Centane, collected during 1985 - 2004 over various harvesting seasons (Shephard *et al*., 2007). Good home-grown maize is defined as maize separated from visibly mouldy maize by the householders themselves. The fumonisin content of commercial maize meal samples (n = 30) were determined in three South African brands purchased from ten different retail outlets (Shephard *et al*., 2002). The total fumonisin content of the home-grown and commercial maize used in the cooked dishes were assessed and calculated for the dry weight/uncooked maize portion of the samples collected. Levels of fumonisin in traditional Xhosa beer were also determined from 18 samples collected during 1991 - 2003, no distinction were made between beer brewed from home-grown or commercial maize (Shephard *et al*., 2005).

Fumonisin exposure

The probable daily intake (PDI) for fumonisin was calculated and expressed as μg kg^{-1} body weight day^{-1}. PDIs for the groups HG and COM were calculated separately for men and women based on the individual body weight and the total dry/uncooked maize intake. For the HG_COM group, a mean combined PDI was calculated for each of the 8 percentage subgroups. The effect of consuming both home-grown and commercial maize (HG_COM group) on the PDIs of an individual with respect to the amount consumed of each source were also considered separately. Therefore, for each person a PDI was calculated based on the commercial maize, home-grown maize and combined total maize intake. Fumonisin exposure due to the consumption of traditional Xhosa maize-based beer, PDIs were calculated separately for each of the identified frequencies-of-use categories and expressed per single drinking event (μg kg^{-1} bw / drinking event).

STATISTICAL ANALYSIS

Data not normally distributed (asymmetric) were log transformed and described by the geometric mean (GM) and 95% confidence interval (95%CI) using STATA^{tm} version 10.1 (College Station, Texas, United States of America).

RESULTS

Characteristics of the study population

The mean values of the different parameters are the geometric mean with the 95% confidence interval (CI). Eighty-three percent of the study population (n = 315) were women (n = 261). Anthropometric variables included a mean body mass index of 27 kg/m^2 (95% CI: 26 - 28) and a mean weight of 68 kg (95% CI: 67 - 70). The mean age and years of residence in the EC were 42 years (95% CI: 41 - 44) and 40 years (95%CI: 38 - 41), respectively. Socio-economic parameters indicated that a river or dam was the main source of drinking water, whereas wood was used for cooking and heating. The level of education was characterised by either, a primary (37%) or a secondary (40%) level of schooling (2% had a tertiary education whereas 21% had no education), while females headed 60% of the households. Eighty-three percent of the study population was dependent on a social grant and the total mean household income per month was USD109 (95%CI 101 - 119) of which 63% was spent on food (between USD25 and USD75).

Dry/uncooked maize consumption

HG, COM and HG_COM groups (Table 7.1)

Only 2% (n = 5) of the population consumed home-grown maize as the main source of their daily diet, compared to 55% (n = 173) of the study population which consumed commercial maize. The highest maize intake, (474 g day^{-1}, n = 5) were among the HG group and the HG_COM group, with the men having the highest consumption. Men and women consuming only commercial maize (COM group) had lower intakes. The HG_COM group, representing 43% of the study population, mostly used commercial maize for the preparation of maize-based dishes (bread, porridges,

whole kernels and combined dishes) while home-grown maize was used for the preparation of non-alcoholic (mageu) and alcoholic (mqoboti) maize-based beverages.

HG_COM: percentage subgroups (Table 7.2)

Despite the variation in the consumption of home-grown and commercial maize in the different subgroups, the total mean maize intakes were similar and ranged between 400 and 600 g day^{-1}.

Fumonisin B quantification

The mean total fumonisin (FB_1 + FB_2 + FB_3) level determined in home-grown maize was 1142 μg kg^{-1} (range 5 - 10140 μg kg^{-1}) (Shephard *et al*., 2007) and for commercial maize a mean total level of 222 μg kg^{-1} (range 55 - 678 μg kg^{-1}) (Shephard *et al*., 2002).

Fumonisin exposure as a function of the maize source

HG and COM groups (Figure 7.1)

The group PDI of total fumonisin for the HG group was 8.5 μg kg^{-1} body weight day^{-1} (95%CI: 1.7 - 42.2). PDIs for the men (n = 2) and women (n = 3) were 12.1 μg kg^{-1} bw day^{-1} (95%CI: 0.3 - 4926.5) and 6.7 μg kg^{-1} bw day^{-1} (95%CI: 1.0 - 457.8), respectively. The group PDI for the COM group (n = 173) was 1.1 μg kg^{-1} bw day^{-1} (95%CI: 1.0 - 1.3) with gender differences of 1.3 (95%CI: 1.0 - 1.8) and 1.1 μg kg^{-1} bw day^{-1} (95%CI: 0.9 - 1.3) for men (n = 34) and women (n = 139), respectively.

HG _COM group: Percentage subgroups (Table 7.2)

Although the PDIs for fumonisins of all the percentage subgroups were above the PMTDI for fumonisin, the value is almost doubled when home-grown contributed up to 50 % of the maize source.

Table 7.1: Total mean dry/uncooked maize intakes of three groups

Source of maize	n	Percentage of the total population	Dry/uncooked maize intake (g day^{-1})	95%CI
HG group	5	2%	474	-*
Men	2		570	-*
Women	3		420	-*
COM group	173	55%	344	303 - 392
Men	34		383	291 - 505
Women	139		336	290 - 389
HG_COM group	137	43%	462	402 - 530
Men	18		632	462 - 865
Women	119		440	379 - 512

n = number of participants, Data presented as geometric means. 95%CI = 95% confidence interval, * no 95%CI reported due to small sample size (n = 5). HG group = individuals consuming home-grown maize, COM group = individuals consuming commercial maize, HG_COM group = individuals consuming both home-grown and commercial maize.

HG_COM group: Individual maize intakes (Figure 7.2)

For the same individual the relative contribution of home-grown maize to the final PDI was much higher compared to commercial maize. High commercial maize intakes resulted in PDIs well above the PMTDI of 2 μg kg^{-1} bw day^{-1} as was noticed when intakes were between 600 and 700 g day^{-1}. When considering the combined PDIs, exposure to the fumonisins is likely to reach values up to 20 μg kg^{-1} bw day^{-1} which is associated with a very high maize intake consisting of a relative high proportion of home-grown maize.

Table 7.2: Combined fumonisin PDIs of the different percentage subgroups consuming both commercial and homegrown maize (HG_COM-group)

Percentage subgroups	n	Commercial maize		Home-grown maize		Total maize intake	Combined PDI
%		Mean %	Intake (g day^{-1}) GM (95%CI)	Mean %	Intake (g day^{-1}) GM (95%CI)	(g day^{-1}) GM (95%CI)	µg kg^{-1} bw day^{-1} GM (95%CI)
80-90%	31	86%	385 (292 - 507)	14%	61 (46 - 80)	447 (340 - 588)	2.4 (1.8 - 3.2)
70 - 79%	23	77%	328 (201 - 534)	23%	99 (61 - 162)	428 (262 - 697)	2.6 (1.6 - 4.3)
60 - 69%	16	65%	254 (191 - 337)	35%	134 (101 - 177)	388 (294 - 513)	3.0 (2.2 - 4.0)
50 - 59%	17	56%	324 (215 - 490)	44%	255 (169 - 385)	580 (384 - 876)	5.5 (3.5 - 9.0).
40 - 49%	17	46%	242 (187 - 315)	54%	288 (223 - 372)	531 (411 - 687)	6.1 (4.5 - 8.3)
30 - 39%	15	35%	171 (105 - 281)	65%	321(197 - 523)	493 (302 - 805)	5.7 (3.6 - 9.2)
20 - 29%	8	24%	109 (48 - 251)	76%	350 (149 - 821)	460 (197 - 1072)	5.4 (2.2 - 13.3)
10 - 19%	10	15%	56 (31 - 101)	85%	333 (175 - 633)	390 (207 - 735)	5.7 (2.9 - 11.2)

Maize = dry/uncooked maize. GM (95%CI) = geometric means with 95 present confidence interval; PDI = Probable daily intake; bw = body weight, HG_COM group = individuals consuming both home-grown and commercial maize.

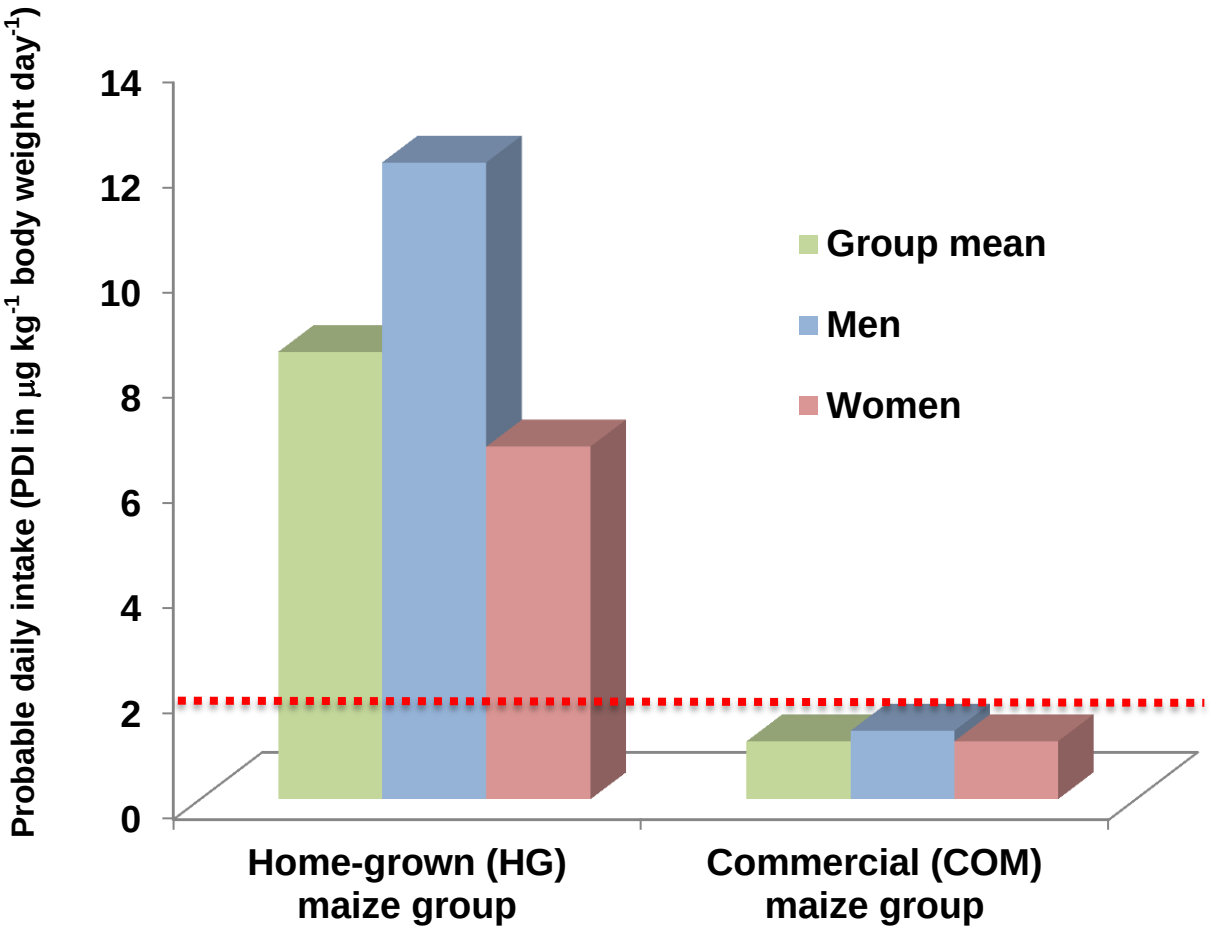

Figure 7.1: Fumonisin exposure of participant consuming home-grown (HG group) or commercial maize (COM group) stratified according to gender. The red dotted line represents the PMTDI of 2 μg kg^{-1} bw day^{-1}

Traditional Xhosa maize-based beer consumption and fumonisin exposure (Table 7.3)

Three distinct drinking frequencies were identified and categorised as those drinking once a month (n = 11), once a week (n = 50) and between 2 and 7 days a week (n = 15), using a communal container. A communal container is typically filled with beer and passed around to those participating in a traditional drinking event or drinking party (Rose, 1972; Matsha *et al*., 2006). The present group commenced drinking traditional beer between the ages of 23 and 27 and have been drinking between 13 and 16 years. No distinction was made between traditional beer made from home-grown or commercial maize and a mean total fumonisin content of 369 ng mL^{-1} (range 43 - 1329) was reported (Shephard *et al*., 2005). Twenty-four percent (n = 76) of the total study population consumed traditional Xhosa beer. In the present study 64% of the beer drinking group uses home-grown maize in the preparation of the beer.

The mean quantity (L) was the highest among those who consumed beer 2 - 7 days a week, which had the highest male presence (60%) with three men consuming beer on a daily basis. The CAGE question score correlated well with the traditional beer drinking patterns. Those drinking beer more frequently (2 - 7 days a week) had a higher score (3/4) compared to those drinking once a week (2/4) and those drinking once a month (0/4). The fumonisin intake per drinking event calculated for these three categories were 6.9 μg kg^{-1} bw for the once a month category, 9.4 μg kg^{-1} bw for the once a week category and 12.0 μg kg^{-1} bw for the 2 - 7 days a week category. The highest probable intake of fumonisin, 12.0 μg kg^{-1} bw per single drinking event, was recorded in the 2 - 7 days a week category. The mean fumonisin intake of the three men consuming 2.3 L day^{-1} was 13.8 μg kg^{-1} bw day^{-1}.

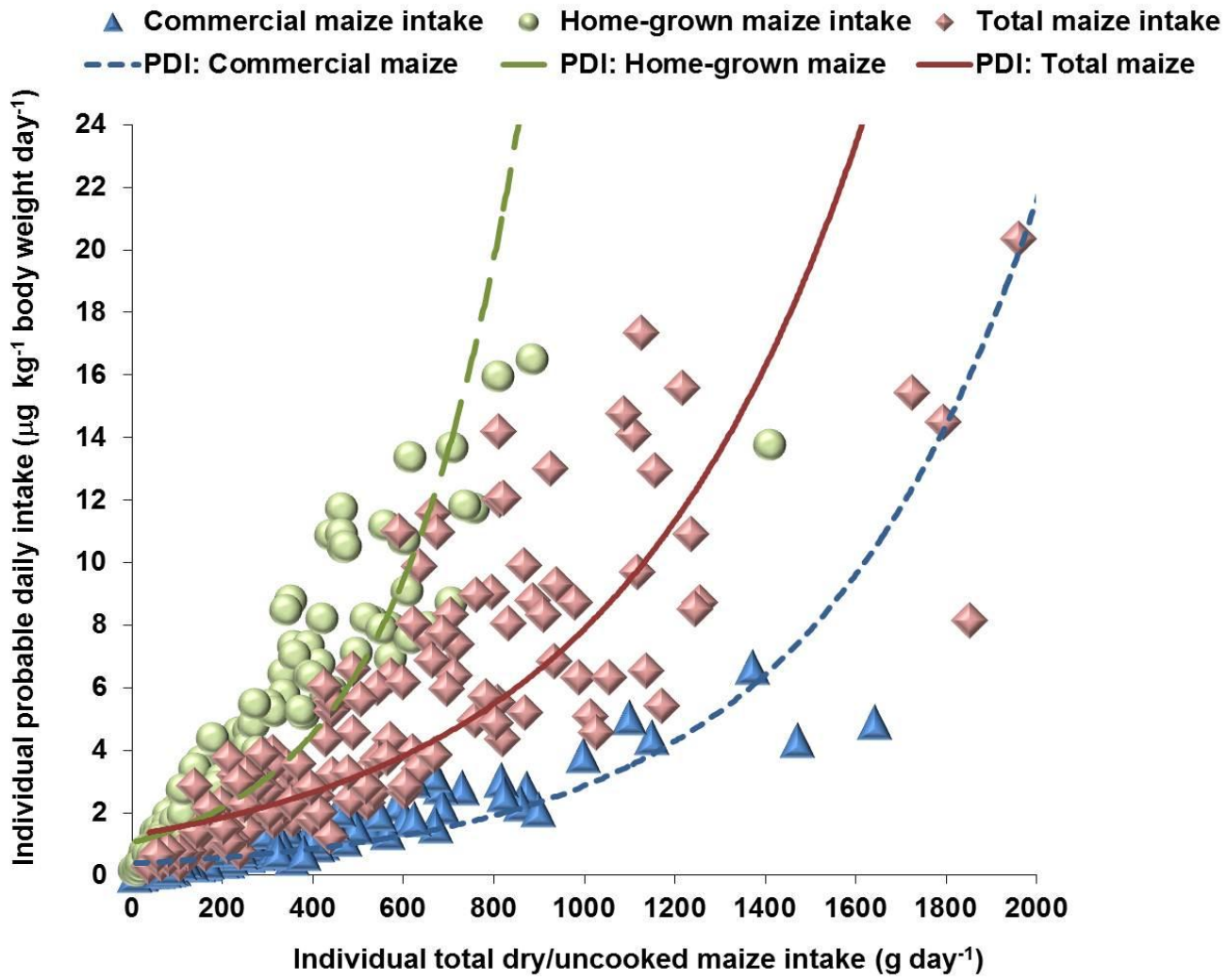

Figure 7.2: Resultant PDIs of individuals consuming both home-grown and commercial maize (HG_COM group). The resultant PDI for home-grown, commercial and the combined maize intakes for each individual in the HG_COM group was calculated. The dotted line represents the PMTDI of 2 µg kg^{-1} bw day^{-1}

Table 7.3: Traditional Xhosa maize-based beer consumption and fumonisin exposure

Parameter	Once a month	Once a week	2 - 7 days a week
Number of participants	11	50	15
Age started drinking Xhosa beer (yrs.)	No data available	27 (23 - 30)	23 (19 - 27)
No of years drinking Xhosa Beer (yrs.)	No data available	13 (9 - 18)	16 (8 - 30)
Weight (kg)	64 (55 - 75)	61 (57 - 64)	64 (57 - 72)
Intake (L)	1.2 (0.8 - 1.7)	1.6 (1.3 - 1.8)	2.1 (1.6 - 3.0)
CAGE Score (score out of 4)	0	2	3
Fumonisin Exposure:			
Probable Intake per single drinking event ($\mu g\ kg^{-1}$ bw/ drinking event)	6.9 (5.0 - 10.0)	9.4 (8.0 - 11.3)	12.0 (8.9 - 16.0)

Values represent geometric means and the 95% confidence interval. CAGE score: four questions to measure dependence and regular intake of alcohol. Abbreviations: bw = body weight, yrs. = years. Data from men and women combined.

DISCUSSION

Human exposure to fumonisins in a rural area of Centane involves comprehensive analysis not only of the level of fumonisin contamination but also of the distinct maize dietary patterns. Typical maize-dietary practises of people living in these rural areas includes hand grinding utilising “grinding” rocks or stamping of home-grown maize to produce maize meal or samp (Rose, 1972; Grobbelaar and Bateman, 1991). The resultant maize meal is either used for the preparation of porridges with different consistencies (soft, crumbly and stiff) or for baked or steamed bread (Rose, 1972; Beyers *et al*., 1979). Both maize meal and samp are usually combined with a variety of seasonal vegetables in different ratios (Beyers *et al*., 1979). Corn-on-the-cob or whole kernels are also included in this distinct Xhosa traditional diet. Twenty-four hour dietary recall analyses of the present study population indicated that two meals are commonly consumed each day consisting of either bread plus a maize-based dish or two maize-based dishes (unpublished data).

To adequately assess fumonisin exposure the source of maize is of importance as home-grown maize has higher levels when compared to commercial maize (Shephard *et al*., 2007). The use of home-grown maize is influenced by seasonal availability, the specific maize-based dish or beverage that is being prepared and gender preferences. The present study population mostly used commercial maize, possibly due to the effects of the rapid urbanisation that is taking place across the rural areas of South Africa and the subsequent changes of rural practices (Byarugaba, 1991; Matsha *et al*., 2006). Many households have changed from traditional agricultural to relying on purchased foods (Byarugaba, 1991). This is evident as 83% of the study population received some type of grant, of which 63% was being spent on food. The lower intake of the COM group as compared that of the HG and HG_COM group could be a reflection of the changes in household expenditure and food security as other food commodities apart from commercial maize are also bought. Dry/uncooked home-grown maize intakes of 570 g day^{-1} and 420 g day^{-1} for men and women, respectively, compared well with the weighed maize intake records of 483 g day^{-1} for men and 428 g day^{-1} for women reported previously in Centane (Shephard *et al*., 2007). These high intakes are similar to those found in other maize-consuming countries in Africa and compared to Western Europe; it is

almost two orders of magnitude higher (Shephard *et al*., 2002; 2007). In the present study population, the heterogeneity and complexity of their maize dietary patterns is further highlighted by those consuming both maize sources. Although those consuming only commercial maize had the highest representation the group consuming either home-grown maize or both had the higher intakes due to higher food diversity of the COM group as discussed above.

The HG group, although not well represented in this group, had the highest PDIs especially among the men who consumed large quantities of maize. This scenario could be the case in many other rural areas within this region. The PDIs for men and women in this group, that of 12.1 and 6.7 μg kg^{-1} bw day^{-1}, were similar to those reported by Shephard *et al*. (2007) for the same area (9.19 and 8.15 μg kg^{-1} bw $day^{-1}$for men and women respectively). However, unlike previous studies conducted in this area utilising weighed dietary records, the use of the validated RAPP tool to estimate individual habitual total intakes of dry/uncooked maize over a one month period from specific sources provided accurate data to calculate group PDIs.

Estimating fumonisin exposure among the group consuming both home-grown and commercial maize is intricate. When using the total fumonisin levels determined in good home-grown maize, the resultant probable daily intakes (PDIs) were higher than the PMTDI of 2 μg kg^{-1} bw day^{-1}. The contributing effect of home-grown maize with its higher fumonisin levels to the combined PDI becomes evident. When the home-grown maize intake increased above 50% the resultant combined PDIs for all the relevant subgroup were doubled (> 5 μg kg^{-1} bw day^{-1}).

When considering the individual total and the separate home-grown and commercial maize intakes, the effect on the resultant PDIs is highlighted even more as a high intake of commercial maize also resulted in an increase above the PMDTI. Fumonisin levels in commercial maze were 222 μg kg^{-1}, while levels between 90 and 320 μg kg^{-1} have also been shown (Van der Westhuizen *et al*., 2011a). Therefore, higher PDI values could increase the vulnerability of this rural population to the adverse biological effects of fumonisins. The risk of exposure by consuming home-grown maize in these areas is a priority for intervention strategies while the consumption of

commercial maize with lower fumonisin contamination levels could be promoted to extend the "window" of exposure, below the PMTDI of 2 μg kg^{-1} bw day^{-1} (Figure 7.2). Other intervention strategies to reduce the fumonisin levels in home-grown maize seem appropriate to further lower the risk of exposure. In this regard the simple intervention procedures introduced recently reduce the level of up to 80% in the home-grown maize in subsistent farmers from this region (Van der Westhuizen *et al*, 2011b)

The consumption of traditional maize-based Xhosa beer plays an important social role and is usually prepared once a week by the women and consumed from a communal container (Rose, 1972; Shephard *et al*., 2005; Matsha *et al*., 2006). In the present study the consumption of Xhosa beer was assessed separately from the maize-based food due to the varying drinking patterns. As beer consumption was not a "per day" event, a probable intake for the fumonisins "per single drinking event" became more appropriate. In this regard the three distinct frequency of use proved to be more comprehensive. The highest intake of 2.1 L was among those drinking 2 - 7 days a week which was supported by the CAGE score (3 out of 4), indicative of frequent use as well as dependency. The low percentage of beer consumption (24%) was due to the mostly female presence (83%) which is known to drink less beer than the men (Matsha *et al*., 2006).

When assessing fumonisin exposure, the probable intake per drinking event to estimate fumonisin exposure compared to the daily intake approach, would be more accurate as the latter would underestimate the risk. The episodic beer drinking events, where large quantities are consumed may have important health consequences as a result of a more acute exposure. In this regard the three men consuming large quantities of beer (2.3 L) on a daily basis had a mean PDI of 13.8 μg kg^{-1} bw day^{-1}. Although good home-grown maize is mostly utilised to prepare beer, the specific use of "visibly mouldy" home-grown maize with higher fumonisin levels is a common practice in rural areas which will further increased the risk of exposure (Shephard *et al*., 2005). In addition, the synergistic interactions of mycotoxins and different dietary constituents including alcohol, are also of relevance (Gelderblom *et al*., 2004; Boffetta and Hashibe, 2006; Peters *et al.*, 2006).

Numerous methodological challenges exist to estimate fumonisin exposure in rural and culturally distinct populations. Using the validated RAPP tool to assess maize and maize beer intakes provides opportunities for future risk assessment studies. This will also benefit the development of a specific biomarker of exposure for fumonisins where the importance of a quantitative relationship between dietary intake and exposure exists. The resultant improved and reliable exposure estimates enable the development and implementation of appropriate intervention measures for a specific high-risk population. The potential health impact of fumonisin exposure on impoverished rural populations in South Africa, in addition to poor nutrition, has not received the necessary attention thus far. In this regard improved regulations of maize by setting realistic regulatory limits, improved risk assessment parameters and continued monitoring programmes for fumonisin are crucial (Marasas *et al*., 2008, Gelderblom *et al.*, 2008).

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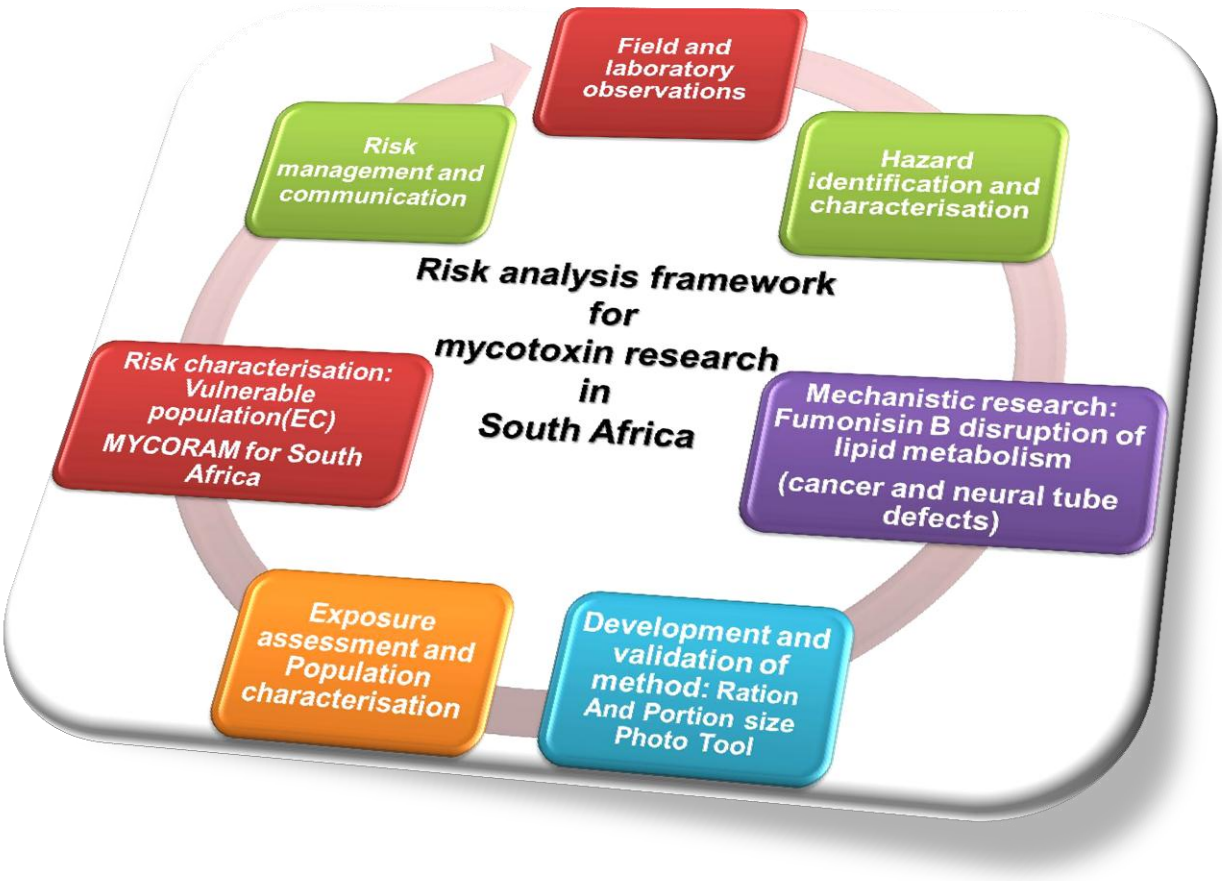

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

The study of environmental cancer risk factors, such as natural-occurring carcinogens, is lacking and more focus is required due to its large contribution to cancer incidence worldwide (Sasco, 2008). The hazard that non-genotoxic carcinogens pose to humans requires thorough evaluation of all possible cellular and molecular mechanisms available. The fundamental reason for this is the plethora of mechanisms by which non-genotoxins can exert their damage (Lima and Van der Laan, 2000). Food commodities containing such carcinogens become an unavoidable and unintentional exposure imparting the need for urgent safety regulations and control strategies (Kroes *et al*., 2002; Zain, 2011). Food safety, interrelated with food security, should include the consumption of safe nutritious food and to circumvent foodborne illnesses (Chassy, 2010; Hanning *et al*., 2012). To contribute to both the mechanistic and risk of exposure, of carcinogenic mycotoxins an integrated approach is essential.

The current study was aimed to confront and contribute to FB_1 risks analysis through a multidisciplinary approach. The two main aspects addressed included studies on the mechanism of action and to better define human risk assessment of this non-genotoxic carcinogen. Cancer promotion by FB_1 through the disruption of lipid metabolism was investigated from a membrane perspective. Dissecting the effects of FB_1 on the architecture of various subcellular membrane fractions of rat liver and primary hepatocytes, including specialised plasma membrane microdomains or lipid rafts, suggested an even more intricate mode of action. To complement this, science-based human risk assessment was evaluated and various aspects relevant to the South Africa population addressed by developing an interactive risk model that may find broader application to describe different risk paradigms. The unique heterogeneity of the South African maize consuming population warrants novel risk assessment models and the establishment of relevant maximum tolerated levels (MTLs) to protect vulnerable subpopulation groups at risk. Validated assessment tools enable a realistic measurement of mycotoxin exposure and impart the magnitude of exposure in vulnerable rural communities. Therefore, the integration of mechanistic mode of action, defining carcinogenesis, and relevant risk assessment approaches are instrumental to the development of mycotoxin control strategies.

Disruption of membrane lipid parameters by fumonisin B_1 as a possible mechanism of cancer promotion

In order for FB_1 to promote cancer, the differential proliferation of pre-neoplastic cells versus the inhibition of the growth of surrounding cells via the induction of apoptotic-cell death is of relevance. The subsequent clonal expansion of initiated cells are achieved either by the up-regulation of proliferation or down-regulation of apoptosis. The circumvention of neoplastic cells from growth control necessities a characteristic and sustainable altered phenotype. Central to the promotion of cancer by FB_1 is the differential effects it elicits on various lipid and fatty acid parameters as observed in normal as compared to neoplastic cells in rat liver (Gelderblom and Marasas, 2012). A typical lipogenic phenotype seems to prevail which have been associated with carcinogenesis in general (Menendez and Lupu, 2007). As membranes of cells are fundamental to the lipid metabolism and vice versa, investigations from this perspective provide opportunities to gain more insight into the mechanisms associated with FB_1–induced cancer promotion. In this regard the following two aspects regarding FB_1-induced cancer promotion need to be considered:

(i) *The differential effect of fumonisin B_1 on various lipid parameters in subcellular membranes isolated from liver could impact on the creation of a growth differential*

(ii) *Disruption of lipid raft composition by fumonisin B_1 in rat primary hepatocytes contributes to changes in key cell signalling pathways of critical growth related responses*

Perspectives

By dissecting the various membranes' lipid and fatty acid parameters related to mechanisms associated with the induction of apoptosis and cell proliferation provides further evidence of the cancer promoting properties of FB_1 in the liver. The differential effects of FB_1 on lipid parameters in the various subcellular membrane fractions in the liver and primary hepatocytes are outlined in Figure 8.1. These changes provide a perspective of how FB_1 may affect different cellular processes related to the altered growth kinetics of normal and altered cell populations.

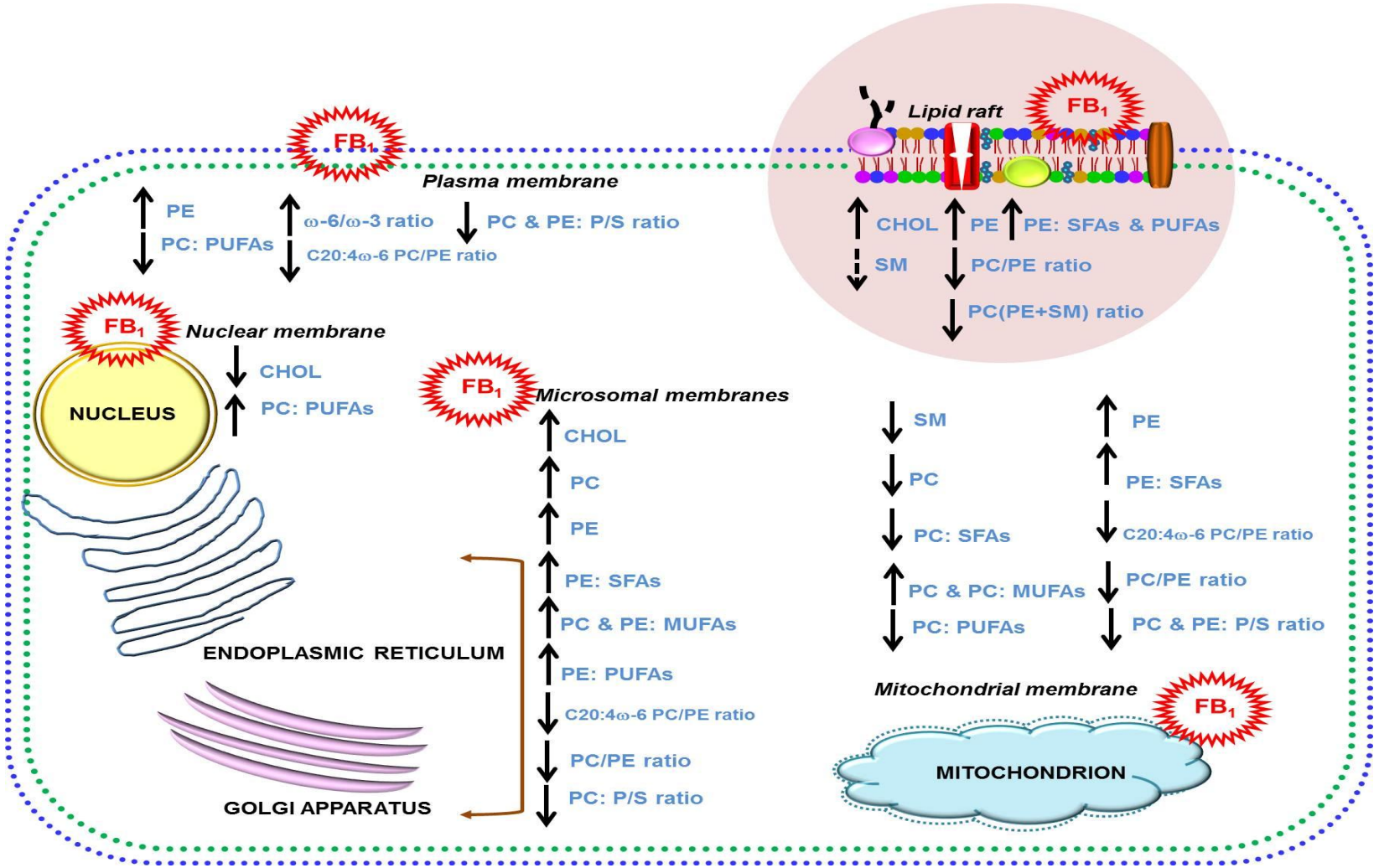

Figure 8.1: The effect of FB_1 on quantitative lipid and fatty acid parameters of various subcellular membrane fractions isolated from chronically exposed rat liver (adapted from Burger *et al*., 2007) including hepatocyte lipid rafts superimposed in pink (adapted from chapter 4)

Abbreviations, CHOL: cholesterol, FB_1: fumonisin B_1, MUFAs: monounsaturated fatty acids, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PUFAs: polyunsaturated fatty acids, SFAs: saturated fatty acids, SM: sphingomyelin, P/S: PUFAs/SFAs

Alterations entails changes to the major membrane constituents include CHOL, the phospholipids PC and PE, their major fatty acid constituents and sphingomyelin. Changes to these lipid parameters could alter the function of subcellular organelles that eventually may lead to the selective growth response of the initiated or pre-neoplastic cells. In this regard several lipid markers have been associated in regulating of apoptosis and cell proliferation underlying cancer promotion.

(i) Disruption of membrane lipid parameters associated with induction of apoptosis (Figure 8.2 A)

Central to the FB_1-induced apoptosis hypothesis is the role of C20:4ω-6. This fatty acid was increased in PE in the microsomal, mitochondrial and plasma membrane as reflected by a decreased C20:4ω-6 PC/PE ratio (Burger *et al*., 2007). C20:4ω-6 is known to play a role in the regulation of SM conversion to ceramide via sphingomyelinase (SMase), with ceramide being a key pro-apoptotic signal (Zhao *et al*., 2002; Darwich *et al*., 2012). In the mitochondria, a decrease in SM was also observed and may imply an increase turnover rate for the production of ceramide. Furthermore, ceramide enhances membrane blebbing and the exposure of inner leaflet phospholipid such as PE to flip-flop to the outer membrane leaflet and apoptosis to commence (Stafford and Thorpe, 2011). The overall increase in PE in the different subcellular fractions, including hepatocyte lipid rafts, could also be related to apoptotic responsiveness.

In the microsomal and nuclear membrane fraction the PUFAs levels of PE and PC was increased, respectively. Therefore, these two membrane fractions are more susceptibility to the induction of apoptosis via lipid peroxidase and oxidative stress. Of interest is also the increase in saturated fatty acids (SFAs) in PE in the microsomal and mitochondrial membrane. Saturated fatty acids are more likely to be esterified and induce endoplasmic reticulum stress and apoptosis (Wang *et al*., 2013). Apoptosis can also be activated by fatty acids by the induction of pro-apoptotic signal, Bax followed by mitochondrial damage (Malhi *et al*., 2006). A decrease in PC as observed in the mitochondrial membrane may also be related to the formation of cardiolipin and its induction of the pro-apoptotic tBid and Bax,

including the formation of diacylglycerol from PC, known to activate protein kinases C and death ligand-induced apoptosis (Wright *et al*., 2001; Esposti *et al*., 2002; Sandra *et al*., 2005). In hepatocyte lipid rafts an increase in both SFAs and PUFAs in PE could also be related to the induction of apoptosis. The increased level of CHOL is associated with the down-regulation of the epidermal growth factors (EGF) receptor-ligand signalling, cellular arrest and apoptosis (Rush *et al*., 2012; Tavolari *et al*., 2012). In addition, the disruption of sphingolipid metabolism and the depletion of ceramide and complex sphingolipids have been associated with the induction of apoptosis in the liver and kidney of rats (Dragan *et al*., 2001; Riley *et al*., 2001). These proposed changes seems to predispose normal cells to undergo cell arrest and resulting in apoptosis in the liver and kidneys of rats chronically exposed to FB_1.

(ii) Disruption of membrane lipid parameters associated with the induction of cell proliferation (Figure 8.2 B)

Increase in CHOL as observed in liver microsomal membranes and hepatocyte lipid rafts effects membrane fluidity (Van der Meer *et al*., 2008). A decrease in the PC and PE: polyunsaturated fatty acid /saturated fatty acid (P/S) ratios of the plasma and mitochondrial membranes and the PC: P/S ratio in the microsomal membrane was also observed. These, together with an increase in CHOL are known to decrease in membrane fluidity. In hepatocyte rafts an additional fluidity marker the PC/(PE+SM) ratio was also decreased (Fajardo *et al*., 2011). The overall more rigid membrane structure of membranes may attenuate the susceptibility of cells to undergo apoptosis (Tavolari *et al*., 2012).

Decreased in SM related to the FB_1-inhibition of ceramide synthase and the increase in anti-apoptotic sphingosine (So) and sphingosine 1-phosphate (S 1-P) could affect proliferation (Riley *et al*., 2001; Van der Westhuizen *et al*., 2004). In this regard levels of SM was reduced in liver mitochondrial membranes and hepatocyte rafts.

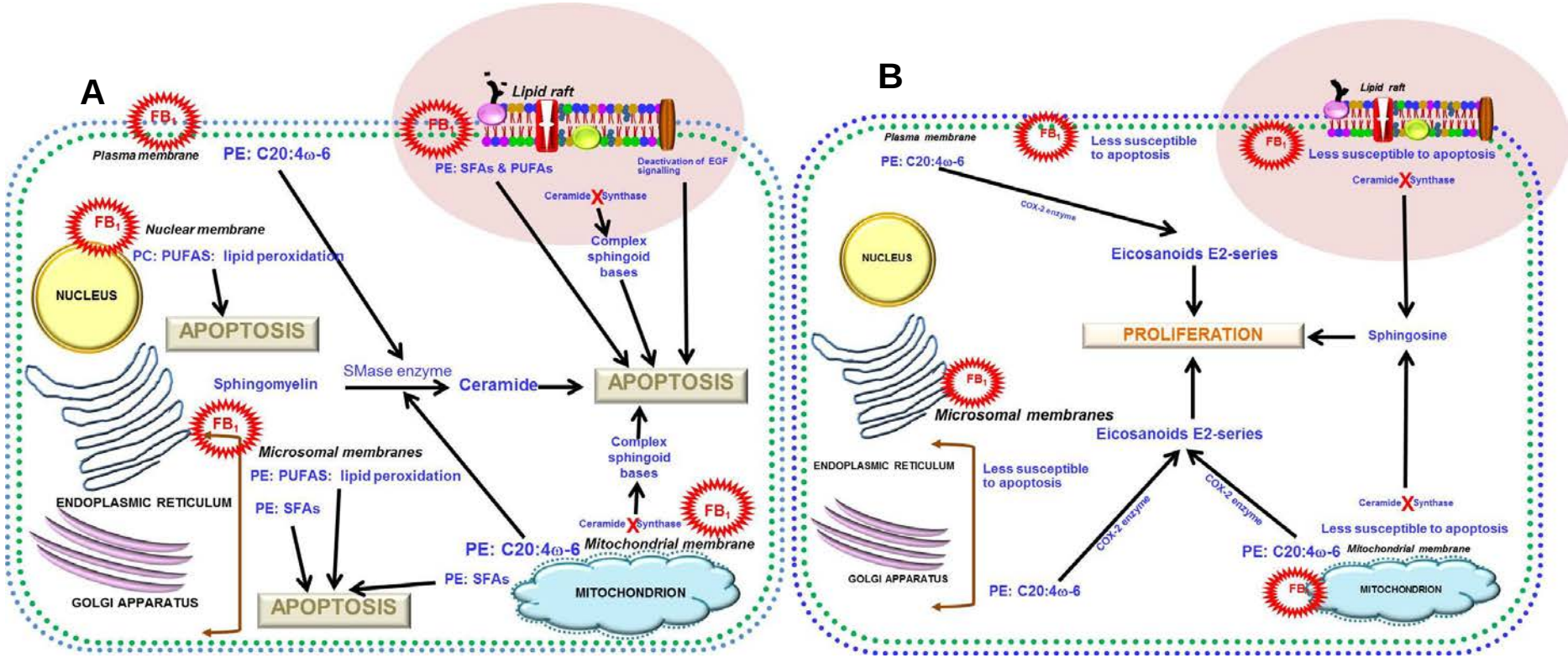

Figure 8.2: Possible mechanism associated with the FB_1-induced alterations of the lipid metabolism that may differentially activate apoptosis (A) or stimulate proliferation (B) during cancer promotion

Abbreviations, COX: cyclooxygenase, EGF: epidermal growth factor, FB_1: fumonisin B_1, PE: phosphatidylethanolamine, PLA_2: phospholipase A_2, S 1-P: sphingosine 1-phosphate, SMase: sphingomyelinase.

The observed decrease in C20:4ω-6 PC/PE ratio, indicative of a respective reduction and increase of C20:4ω-6 in PC and PE, could be attributed to the synthesis of prostanoids (E2 series) via the proposed increased activity of phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX-2) related to cell growth (Gelderblom *et al*., 1999; 2001; 2008; Pinelli *et al*., 1999)

These changes form part of the so-called lipogenic phenotype related to cell proliferation in cancerous lesions and therefore will create the growth differential resulting in the selective outgrowth of genetically altered or "initiated" cells in the liver. Therefore, subtle changes to membrane constituents, manifested as a result of alterations in lipid metabolism by FB_1 are important in determining cellular fate and biological outcome of disease. At present these affects have not been adequately captured in determining risk assessment of the fumonisins and certain paradigms regarding non-genotoxins tend to ignore the possible long-term chronic exposure and epigenetic response that prevail.

FUMONISIN RISK ASSESSMENT IN HUMANS

Although numerous attempts have been made to harmonisation and integrate risk assessment in developed countries it is more complicated in developing countries. This is mainly due to the lack of legislation regarding mycotoxin regulation and the absence of the necessary infrastructure relevant to exposure assessment, continued and adequate risk assessment and management in countries such as South Africa. Despite the magnitude or relevance of mycotoxin exposure, only local grain manufacturing companies institute mycotoxin control to safeguard consumers and to ensure adequate international trade. To address this public health issue in the South African contexts specifically among vulnerable communities, a novel approach is warranted and the following aspects need to be considered.

i) Assessing the distribution of mycotoxins during maize dry milling (experimental and commercial scale) and how it contributes to the risk of exposure

Perspectives

- The distribution of *Fusarium* mycotoxins {FB, deoxynivalenol (DON) and zearalenone (ZEA)} during experimental dry milling differed for each mycotoxin. FB was concentrated in the SPECIAL dry milling fraction, DON in the semolina fraction whereas ZEA was equally distributed between these two milling fractions
- Experimental milling compared to commercial dry milling showed similar mycotoxin distribution with the highest concentrations of mycotoxins in the total hominy feed and the lowest levels in the SUPER milling fraction.
- Dry maize milling, including the degerming is generally considered to be an effective way to reduce mycotoxins.

The quality and processing of the raw food are directly affected by fungal and resultant mycotoxin contamination. The study of the fate of mycotoxins during food processing is therefore important in order to establish a balanced and attainable regulation between the raw food commodity, the intermediate and final products. Numerous studies in this regard have been conducted mostly from an industrial processing perspective (Cheli *et al*., 2013). The reason for investigating industrial processing is that both relevant and practical information can directly be used by the various risk management and regulatory entities. However, there are also various caveats that will impact on the reliability of data obtained. Sampling errors for one incurs large variation in mycotoxin levels obtained from the various milling streams.

The current study evaluate experimental dry milling of maize and the distribution of different mycotoxins, including FB, DON and ZEA in the products intended for human consumption. Reconstruction of specific milling fractions (whole maize, degermed maize and total hominy feed) was introduced to reduce sampling errors to a minimum that will provide a more accurate assessment of the distribution profile of the different mycotoxins. This enabled the homogenisation of samples not possible in sampling large consignments intended for industrial milling. Despite these differences, the

current study indicated the same pattern of mycotoxin distribution in the total hominy feed and SUPER milling fraction reported and concurred with several other studies irrespective the mycotoxin under investigation, the milling scale and milling fractions of interest. The differential distribution of mycotoxins in the various milling fractions and how it was affected by the kernel characteristics and specifications of the fungal colonisation were also highlighted. Detailed comparisons between other studies in this regard are, however, hampered by the lack of international harmonisation of terminology for the various fractions obtained during milling.

The current study concluded that maize milling can effectively reduce mycotoxins while consideration of sampling error, kernel properties and fungal colonisations are warranted. As far as mycotoxin reduction is concerned, a more refined approached should be used by evaluating the degerming process separately from the milling process. It became evident that the physical process prior to milling enables the exclusion of the bulk of mycotoxins due to the removal of contaminated kernels. Whereas during milling, a specific mycotoxin can selectively be concentrated to a finished product, some which may enter the human food chain (Cheli *et al*., 2013). Sorting and washing, like degerming are also categorised as processes that may eliminate mycotoxins prior to food processing (Cheli *et al*., 2013; Van der Westhuizen *et al*., 2011a; 2011b). The hominy feed dry milling fraction, with its high levels of mycotoxins noticed in both the experimental and commercial maize, does have relevance to animal health when mixed into their dietary rations. The co-occurrence of mycotoxins and resultant synergistic and/or additive effects can lead to acute or chronic diseases that will impact on livestock productivity (Binder *et al*., 2007). From a human perspective the current study contributes to the management of mycotoxin contamination in maize intended for consumption and provided practical information to the local maize milling industry. It also provide an opportunity to modelling risk of exposure and providing relative safe MTLs to protect vulnerable subpopulations utilising maize as a main dietary staple.

ii) Mycotoxin risk assessment and evaluation in the South African population

Perspectives

- Mycotoxin exposure, expressed as probable daily intakes (PDIs) using maize milling fractions (SPECIAL and SUPER) intended for human consumption were far below the respective Provisional Tolerable Daily Intake (PMTDI) for FB (2 µg kg bw^{-1} day^{-1}), DON (1 µg kg bw^{-1} day^{-1}) and ZEA (0.5 µg kg bw^{-1} day^{-1}) proposed by JECFA (JECFA, 2000; 2001; 2012).
- The respective PDI's are affected by gender, ethnicity and the province of residence. Depending on the mycotoxin and the relevant dry milled fraction, i) men; ii) the black African and Coloured ethical groups, and iii) people residing in the Northern Cape and Mpumalanga Provinces had higher PDIs.
- Utilising specific maize intake increments (g/bw/day) a sensitive interactive model mycotoxin risk assessment model (MYCORAM) for FB, DON and ZEA was developed. The model provides a far more sensitive approach than considering PDI to identify people at risk to exposure above the established PMTDI of 2 µg/kg bw/day.

Mycotoxin risk assessment is based on hazard identification and characterisation, including dose-response effects, exposure assessment and risk characterisation (Renwick *et al*., 2003). Currently mycotoxin risk assessment is mostly focused on aflatoxins, less is known about the mycotoxins such as FB, DON and ZEA (Pitt *et al*., 2012). The Joint Expert Committee of the Food and Agriculture Organization (FAO) and World Health Organisation (WHO) or JECFA has instituted useful PMTDI based on hazard identification and characterisation. The current study mainly focussed on different aspects of exposure assessment and risk characterisation. South Africa is divided into nine Provinces with each encompassing different cultural (ethnic) groups, demographic and socio-economic parameters which will impact on the maize dietary patterns. The first aspect of risk assessment, that of determining exposure is often regarded as a source of large uncertainty (Kroes *et al*., 2002; Renwick *et al*., 2003; Dybing *et al*., 2008). Apart from methodological challenges in analysis of mycotoxin, exposure assessment also entails epidemiological-based investigations at individual

and population level. The current study utilises a national consumer survey determining maize intake profiles utilising a cross-sectional epidemiological design to ensure representativeness. The inherent biases, measurement errors and limitations of assessment methods ascribed to epidemiological however cannot be excluded from the current study (Lilienfeld, 1983; Kroes *et al*., 2002). From a nutritional epidemiological perceptive the use of a validated questionnaire in addition to photographic aids used in the current study, is regarded as strategies to improve data quality (Labadarios *et al*., 2008; Lombard *et al*., 2013). The next step was to use mycotoxin levels (FB, DON and ZEA) of milling fractions (SPECIAL and SUPER) obtained from experimental and commercial dry maize milling to determine different exposure scenarios. Simple deterministic modelling was applied to calculate PDIs using the numerical means of the respective mycotoxins levels and the maize intake profiles of the different groups and between Provinces. Due to the skewed data distribution, a different modelling approached was developed resulting in the Mycotoxin Risk Assessment Model (MYCORAM) for FB, DON and ZEA which proofed to be more sensitive in determining risk. A different risk pattern emerged when considering the nine South African Provinces that suggest the setting of more specific MTLs to safeguard sensitive population groups. From an international perspective, the MYCORAM can be utilised in modelling risk in specific vulnerable sub-group and/or -populations including to encompass infants, pregnant women, immune-compromised individuals and the aged.

The current study achieved three important aspects of mycotoxin risk assessment in South Africa, i) elucidated mycotoxin distribution in maize milling fractions intended for human consumption ii) to establish, for the first time, national FB, DON and ZEA exposure data and iii) to refine risk assessment by developing the MYCORAM to address the heterogeneity of the South African populations residing in the nine Provinces.

iii) *Dietary fumonisin exposure among a rural maize subsistence farming* community in the Eastern Cape Province, *South Africa*

Perspectives

- Maize is obtained from different sources including home-grown maize, commercial maize and a mixture thereof.
- Men consumed more maize than women when considering all three maize sources with a corresponding higher PDI.
- Dissecting individual PDIs due to the consumption of both home-grown and commercial maize indicated that some individuals, due to a high intake of commercial maize, may also incur a PDI above the PMTDI.
- Considering the consumption of maize-based beer as a source of FB exposure, a novel probable intake per single drinking event was determined.

The integration of nutrition/dietetics and mycotoxicology is also still in its infancy worldwide. During dietary exposure assessment, accurate and reliable data are important yet, as highlighted previously, uncertainty and variability of the data prevail. The development and validation of a cultural specific dietary assessment method, the Ratio and Portion size Photo tool (RAPP tool) provided an opportunity to conduct a more accurate dietary assessment study in determining FB exposure (Lombard *et al*., 2012; 2013). The use of the RAPP tool enabled the dissecting of maize dietary patterns by providing the sources of maize (home-grown, commercial or people consuming both), cultural recipes to calculate raw intake and other sources of FB exposure such as traditional beer. The small sample size of the current study did not lean towards assuming a representative scenario or sophisticated analysis of the intake data and resultant PDIs. However, due to homogeneity of this rural population regarding cultural practises and maize dietary practices, less dissimilarity is expected for other rural areas within the Eastern Cape Province. Although large nutritional epidemiological studies are expensive and numerous logistical challenges (poor infrastructure) exist, it would be of great value to increase the sample size and to expand this study towards other rural areas within this Province and other rural farming regions in South Africa. The ultimate aim of mycotoxin assessment is to develop and implement preventative strategies. Unfortunately, unlike the commercial

industry where MTLs can be implemented, populations depended on home-grown maize remains at risk. The current study using validated assessment method (RAPP tool) provides a relevant FB risk scenario that is expected in high-risk groups and provide the opportunity for the development and validation of simple methods to sort and wash maize kernels to reduce FB contamination prior to cooking (Van der Westhuizen *et al*., 2011a; 2011b).

CONCLUSIONS AND RECOMMODATIONS

(i) *Assessing the link between FB exposure and human cancer, such as oesophageal cancer*

The evidence thus far indicates that FB exerts its effects via non-genotoxic mechanisms including possible epigenetic changes, which are not fully understood at present. With the disruption of the lipid metabolism, a lipogenic phenotype known to be a hallmark of cancer development became evident. The disruption or activation of signal transduction pathways via the modulation of biological membranes can incur the deregulation of growth and cell death responses. In the current study the major lipid "players" orchestrated by FB_1 and the differential effect on cell growth parameters, implies that surrounding normal cells and the genetically altered "initiated" cells will respond differently to the lipogenic phenotype, with the latter selected to clonally expanded during cancer promotion. However, tumour promotion alone by non-genotoxic carcinogens is regarded not sufficient to induce carcinogenesis but additional actions such as cytotoxicity, regenerative hyperplasia and oxidative stress plays a contributing role (Hernández *et al*., 2009).

Ecological studies have proposed an association between FB exposure and the development of OC, due to the lack of epidemiological studies no direct implication is possible. The question that prevails is what type of epidemiological study will be sensitive enough to infer causality. For instance in the rural subsistence farming areas in South Africa a high incidence of OC concomitantly exists with a high intake of home-grown maize contaminated with relative higher levels of FB. Eloquent case-control studies would be required and appropriate individual or group (frequency) matching to prevent maize intake from become a confounder or as a possible risk

factor for OC as was hypothesised by Sammon and Iputo, (2006). Other risk factors for OC including drinking, smoking, cultural habits and poor nutrition also need careful consideration. These include, a monotonous diet of maize resulted in an inadequate and poor nutrient status; a link between FB_1 exposure and the inadequate uptake of vitamins such as folate; the burden of both communicable (HIV/AIDS, tuberculosis and diarrhoeal diseases) and non-communicable diseases (diabetes) (South African National burden of disease study, 2000). The effect of chronic infections and/or immune suppression and chronic exposure to the carcinogenic FB among other mycotoxins (Shephard *et al*., 2013) superimposed on an underlining condition of malnutrition or under-nutrition, however, still needs to be further elucidated.

(ii) *Epigenetic mechanism and FB_1-induced hepatocarcinogenesis*

Evidence for possible epigenetic mechanisms originates from the overexpression of *c-myc* proto-oncogene observed in rat liver exposed to dietary FB_1 as well as the deregulation of folate vitamin uptake observed in *in vitro* and *in vivo* studies (Stevens and Tang, 1997; Lemmer *et al*., 1999; Marasas *et al*., 2004; M.Abdel Nour *et al*., 2007). The mechanism by which FB_1 affects a folate deficiency is still unclear and possible disruption of lipid rafts and/or ceramide signalling may be involved (Chapter 4, Lipardi *et al*., 2000). The association between reduced folate and the overexpression of *c-myc* has to be considered on the basis of events leading to the demethylation of the gene (Gerhäuser, 2012; Lu *et al*., 2008). Reduction in the uptake of folate by FB_1 may therefore incur epigenetic events related to altered growth responses effected during cancer promotion.

The lipogenic phenotype associated with FB_1 could be related to the role of phosphatidylinositol 3-kinase (PI3K)-Akt (protein kinase B) signalling. Exposure of Fischer 344 and BDIX rats to dietary FB_1 indicated the activation of Akt in preneoplastic and neoplastic liver lesions (Ramljak *et al*., 2000). The PI3K-Akt pathway is also known to regulate sterol regulatory element-binding proteins (SREBPs) of which SREBP -1 and -2 are key transcriptional regulators of cholesterol and fatty acid biosynthesis (Horton *et al*., 2002, Krycer *et al*., 2010). Myristoylated Akt also activates the expression of SREBP-associated genes such as 3-hydroxy-3-

methyl-glutaryl-CoA reductase reductase (HMGR) and fatty acid synthase (FAS). Interestingly, FAS have shown to mostly synthesise saturated and mono-unsaturated fatty acids relevant to phospholipids associated with detergent resistant microsomes whereas it had no effect on non-raft phospholipids (Swinnen *et al*., 2003). The overexpression of HMGR and FAS during FB exposure has not yet been elucidated. An enhanced HMGR activity is suggested due to the observed increase in liver, serum, membrane and raft CHOL in *in vivo* and *in vitro* studies following FB_1 exposure (Burger *et al*., 2007, Gelderblom *et al*., 1996; 2001; 2002; Chapter 4).

(iii) *Non-genotoxic mechanisms and risk assessment*

Non-genotoxins such as FB_1 implies a diverse and complex mode of action related to cancer induction, unlike genotoxins that are conceptually unified under their DNA-reactive characteristics (Hernándes *et al*., 2009). The role of non-DNA-reactive, presumably acting via epigenetic mechanism, chemicals are largely underestimated from a risk assessment perspective. To further complicate things, is the poorly-understood interaction between non-genotoxin mechanisms and human heterogeneity such as polymorphisms, hereditary factors and varying lifestyle factors. Therefore, the “full” weight of evidence of the biological effects of non-genotoxins should be considered such as the specific FB_1-induced lipogenic phenotype associated with the disruption of the lipid metabolism. The advantage of studying and understanding the epigenetic phenotype of non-genotoxins not only provides information to establish risk assessment parameters but also create opportunity in devising chemopreventative strategies. The application of “omics” technologies, such as genomics, proteomics and metabolomics in studying the epigenetic mechanism of action associated with FB_1 may provide more clarity. These mechanisms need to evolve in order to better define risk assessment parameters of the fumonisins in humans.

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Addendum A

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Addendum C

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Approval Notice
New Application

13-Jun-2013
Lombard, Martani MJ

Ethics Refernce #: N13/03/042
Title: Annual National Maize Consumption and Fumonisins Exposure

Dear Doctor Martani Lombard,

The **New Application** received on **28-Mar-2013**, was reviewed by members of **Health Research Ethics Committee 1** via Expedited review procedures on **11-Jun-2013** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **11-Jun-2013 -11-Jun-2014**

Please remember to use your **protocol number (N13/03/042)** on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired.
The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389657.

Included Documents:
General Checklist
Investigator declaration
Application Form
Investigator CV
Protocol Synopsis
Research Protocol

Sincerely,

Franklin Weber
HREC Coordinator

Addendum D

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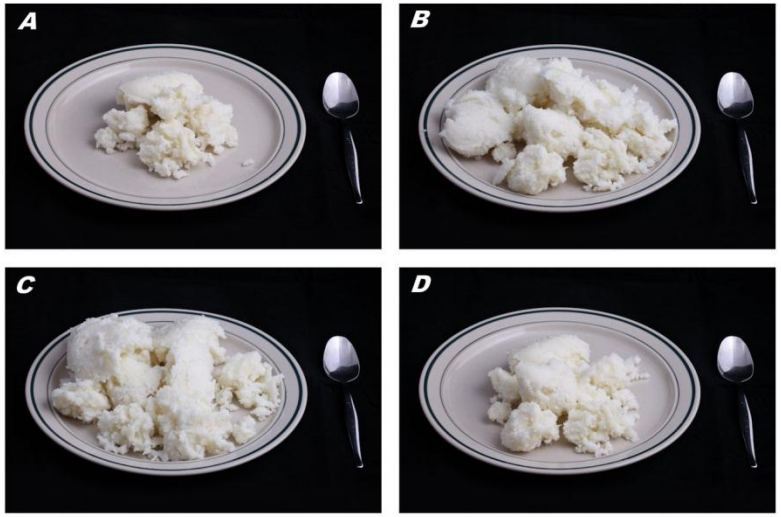

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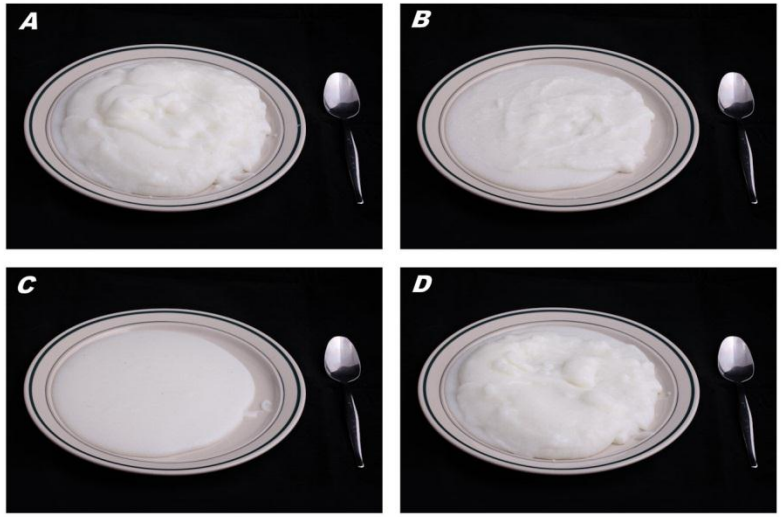

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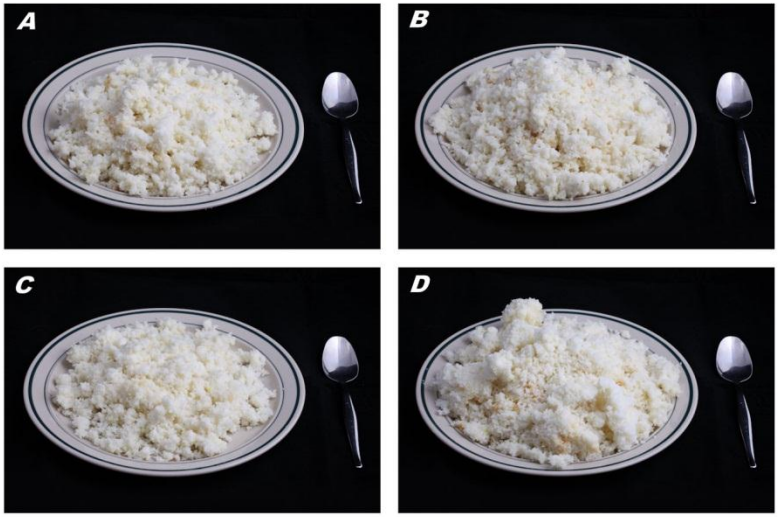

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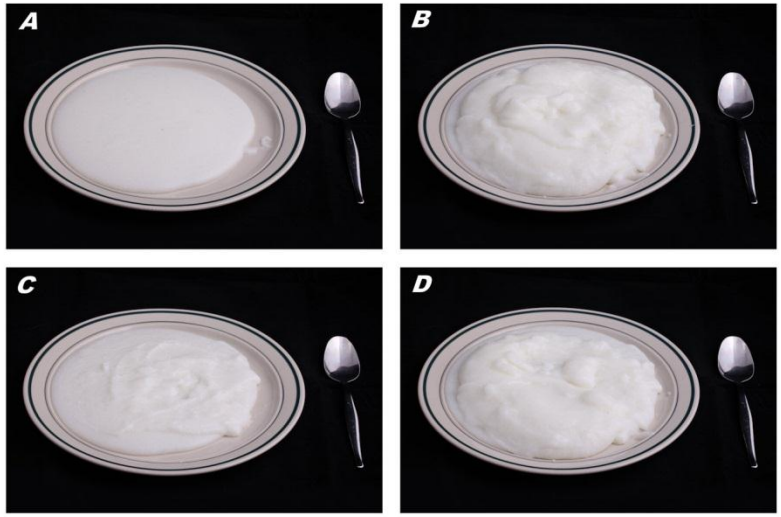

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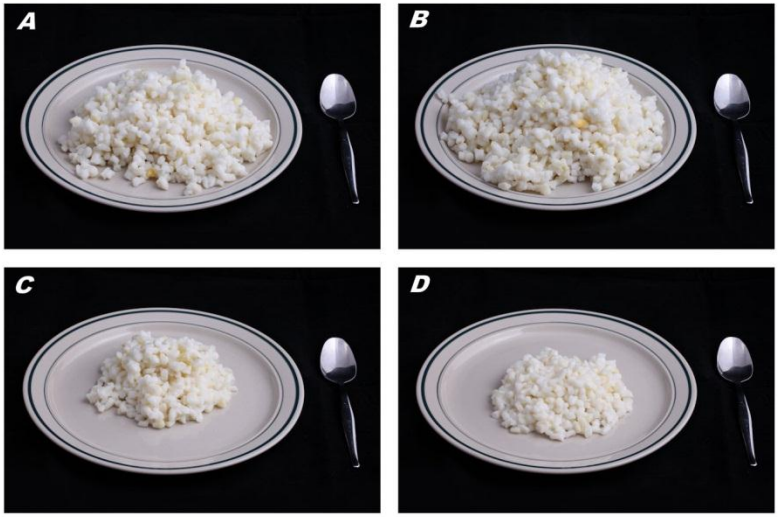

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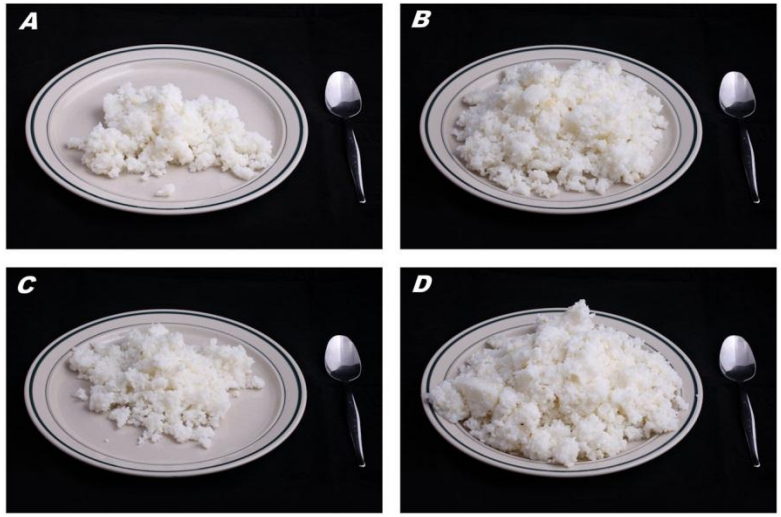

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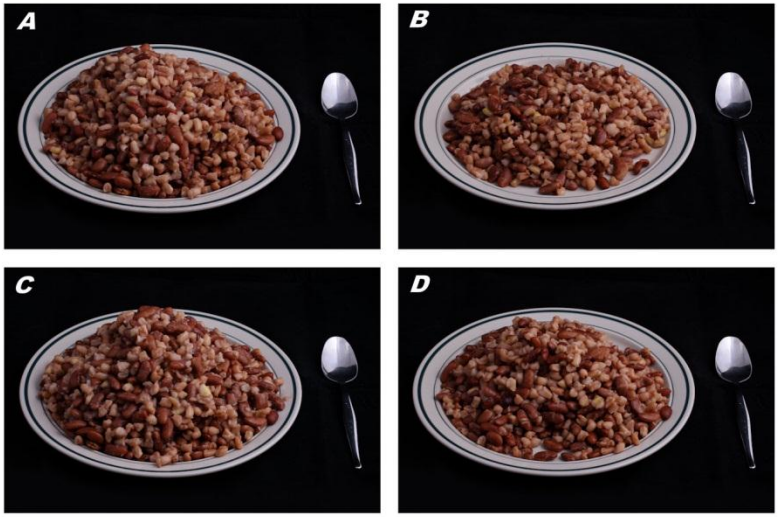

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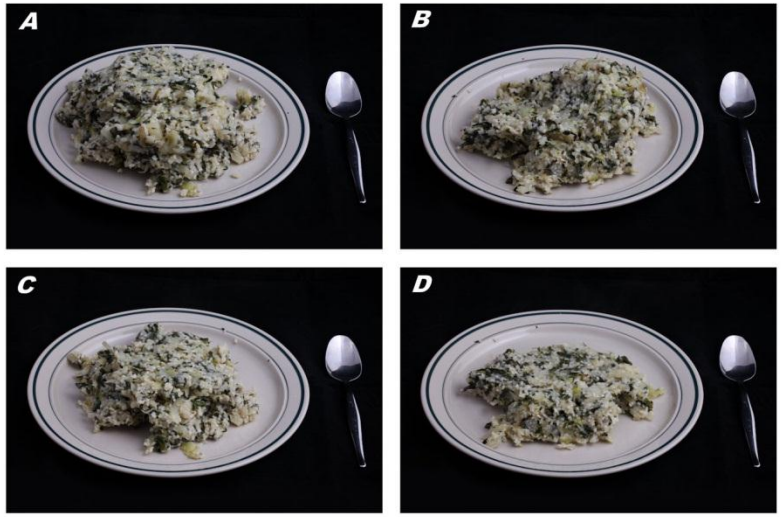

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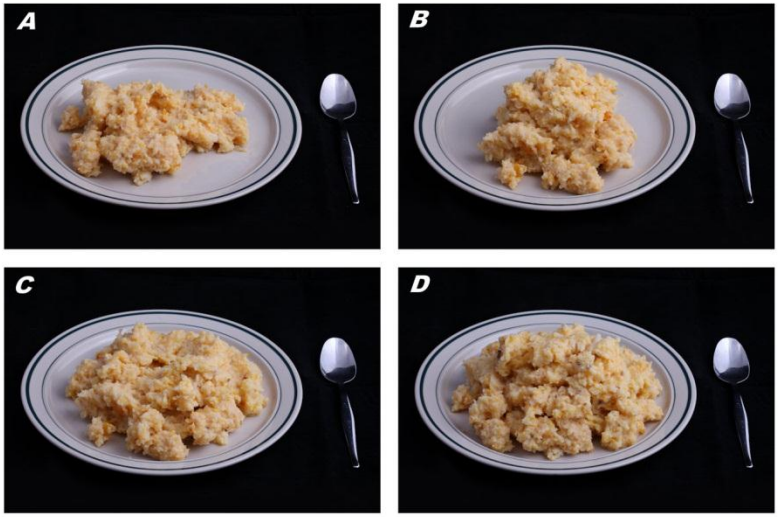

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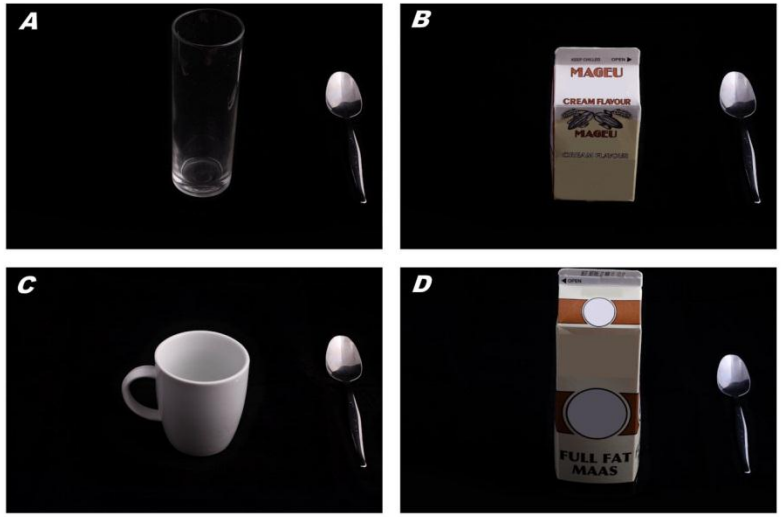
PICTURE CARD 10



PICTURE CARD 10

Consumer Survey: Maize food photographs aids

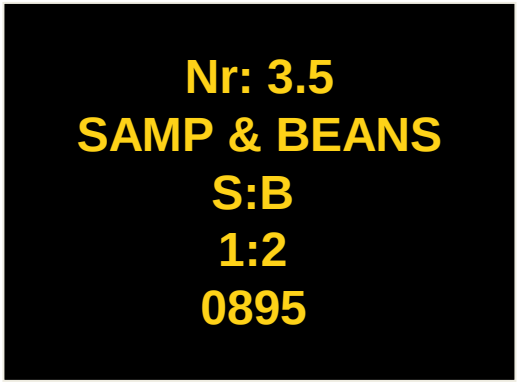
Photograph ID	Food Item	Portion sizes
Picture card 1	Stiff pap	A: 231 g B: 309 g C: 557 g D: 630 g
Picture card 2	Soft pap	A: 269 g B: 308 g C: 478 g D: 640 g
Picture card 3	Grumbly pap	A: 254 g B: 338 g C: 470 g D: 590 g
Picture card 4	Maize fermented with vinegar	A: 231 g B: 308 g C: 478 g D: 640 g
Picture card 5	Samp	A: 155 g B: 207 g C: 414 g D: 622 g
Picture card 6	Maize rice	A: 155 g B: 207 g C: 414 g D: 622 g
Picture card 7	Samp and beans	A: 444 g B: 592 g C: 744 g D: 896 g

Photograph ID	Food Item	Portion sizes
Picture card 8	Maize meal with cabbage and spinach	A: 320 g B: 426 g C: 512 g D: 710 g
Picture card 9	Maize meal and pumpkin	A: 320 g B: 426 g C: 512 g D: 710 g
Picture card 10	Mageu,	A: White coffee mug, 250 mL B: Glass, 250 mL C: Small carton, 500 mL D: Large carton, 1 L

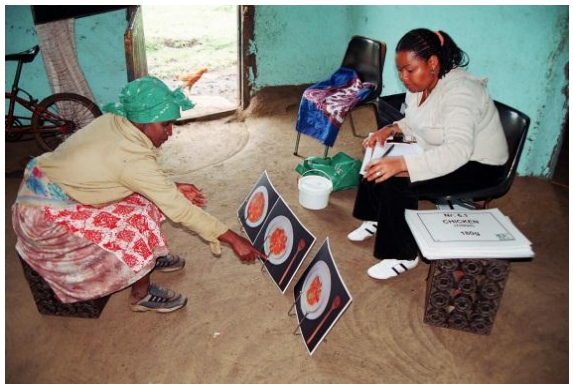
Addendum E

The Ratio And Portion size Photo (RAPP) tool is a culturally specific dietary assessment method to determines the dietary habits and nutrient intakes of Xhosa-speaking people living in rural areas in the Eastern Cape Province, South Africa (Lombard *et al*., 2012; 2013)

Front side of RAPP Tool picture



Backside of RAPP Tool picture



RAPP Tool in action

Figure 1B: RAPP Tool with different portion sizes of crumbly pap (maize porridge), large: L, medium: M and small: S

S

M

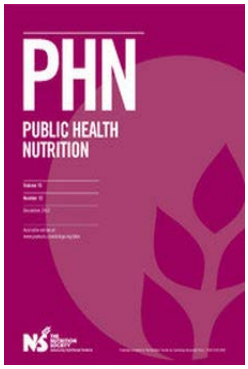
L

Figure 1C:RAPP Tool with different ratio of maize meal and pumpkin

1:2

1:3

3:1



A proposed method to determine fumonisin exposure from maize consumption in a rural South African population using a culturally appropriate FFQ

Martani Lombard, Nelia Steyn, Hester-Mari Burger, Karen Charlton and Wentzel Gelderblom

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A proposed method to determine fumonisin exposure from maize consumption in a rural South African population using a culturally appropriate FFQ

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Abstract

Objective: To develop an FFQ for estimating culture-specific maize intake that can distinguish between home-grown and commercial maize. Home-grown maize is more likely to be contaminated with fumonisins, mycotoxins that are associated with increased risk of oesophageal cancer.

Design: An existing FFQ developed for use in urban Xhosa populations was used as the initial framework for the maize-specific FFQ (M-FFQ). The existing questionnaire contained 126 food items divided into ten food groups (bread, cereals, vegetables, fruit, meat, dairy, snacks, condiments, beverages and fat). The M-FFQ was developed based on additional data obtained from a literature search, 24 h recalls (*n* 159), in-depth interviews (*n* 4), focus group discussions (*n* 56) and expert consultation. Food items available in local shops (*n* 3) were compared with information obtained from focus group discussions.

Setting: Five villages in two rural isiXhosa-speaking areas of the Eastern Cape Province, known to have a high incidence of oesophageal cancer, were randomly selected.

Subjects: Women aged 18–55 years were recruited by snowball sampling and invited to participate.

Results: The final M-FFQ comprised twenty-one maize-based food items, including traditional Xhosa dishes and beverages. The questionnaire focused on maize-specific dishes and distinguished between home-grown maize and commercial maize consumption.

Conclusions: A culturally specific dietary assessment method was designed to determine maize consumption and therefore fumonisin exposure. The questionnaire will be tested against 24 h recalls and other methods to determine its validity, after which it will be used in various epidemiological studies to determine fumonisin exposure.

Keywords
Fumonisin
Mycotoxins
Oesophageal cancer
FFQ
Maize
Dietary intake

Rural areas of the Eastern Cape Province, South Africa have a high incidence of squamous cell oesophageal cancer (OC)[1,2]. Although the aetiology is still unclear, various risk factors have been associated with the disease[3,4]. Exposure to the carcinogenic mycotoxin fumonisin has been associated with a variety of human diseases worldwide including increased oesophageal and liver cancer[5–7], childhood stunting[8], neural tube defects[9] and possible gastrointestinal disorders[10].

Fumonisins, produced by the fungus *Fusarium verticillioides* growing on maize, are found in higher concentrations in home-grown compared with commercially bought maize[11]. isiXhosa-speaking people living in these high OC areas are mostly poor subsistence farmers consuming a staple diet of home-grown maize. Fumonisin contamination of this food source is a major health concern and quantitative assessment of exposure has not been conducted[12]. This is mainly due to the fact that there is no method to determine the intake of maize, especially not one that is able to distinguish between home-grown and commercial maize consumption[11]. What little information is available regarding the eating habits of this population is old and outdated[13,14].

Food safety authorities around the world have conducted risk assessments on the consumption of contaminated

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maize and the International Agency for Research on Cancer declared fumonisins to be a possible human carcinogen (Group 2B)[15]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommends a tolerable daily intake of 2 μg/kg body weight per d[12,16,17].

To assess exposure to fumonisins in Xhosas having maize as a staple food it is necessary to determine the amount of maize consumed daily. Owing to cultural differences, a pre-developed, Western/urban FFQ is not appropriate for use in this population because it does not include the maize-based dishes and beverages common for rural areas. The aim of the present study was to develop a culturally specific FFQ, able to identify maize-based meals and beverages and to distinguish between home-grown maize and commercial maize. The maize-specific FFQ is to be distinguished in name as the M-FFQ.

Methods

Overview

The M-FFQ was developed using mixed methods (Fig. 1). An existing FFQ developed for use with urban Xhosa populations to determine their nutrient intake was used as the starting point for the development of the M-FFQ. The urban FFQ contained 126 food items divided into ten food groups (bread, cereals, vegetables, fruit, meat, dairy, snacks, condiments, beverages and fat). In addition to the basic framework, data were obtained from the literature, a review of 24 h recalls and expert consultation. From this, a draft M-FFQ was developed which was then tested by means of in-depth interviews and focus group discussions (FGD) to develop a final culturally appropriate M-FFQ. Food items available in the local shops were listed and

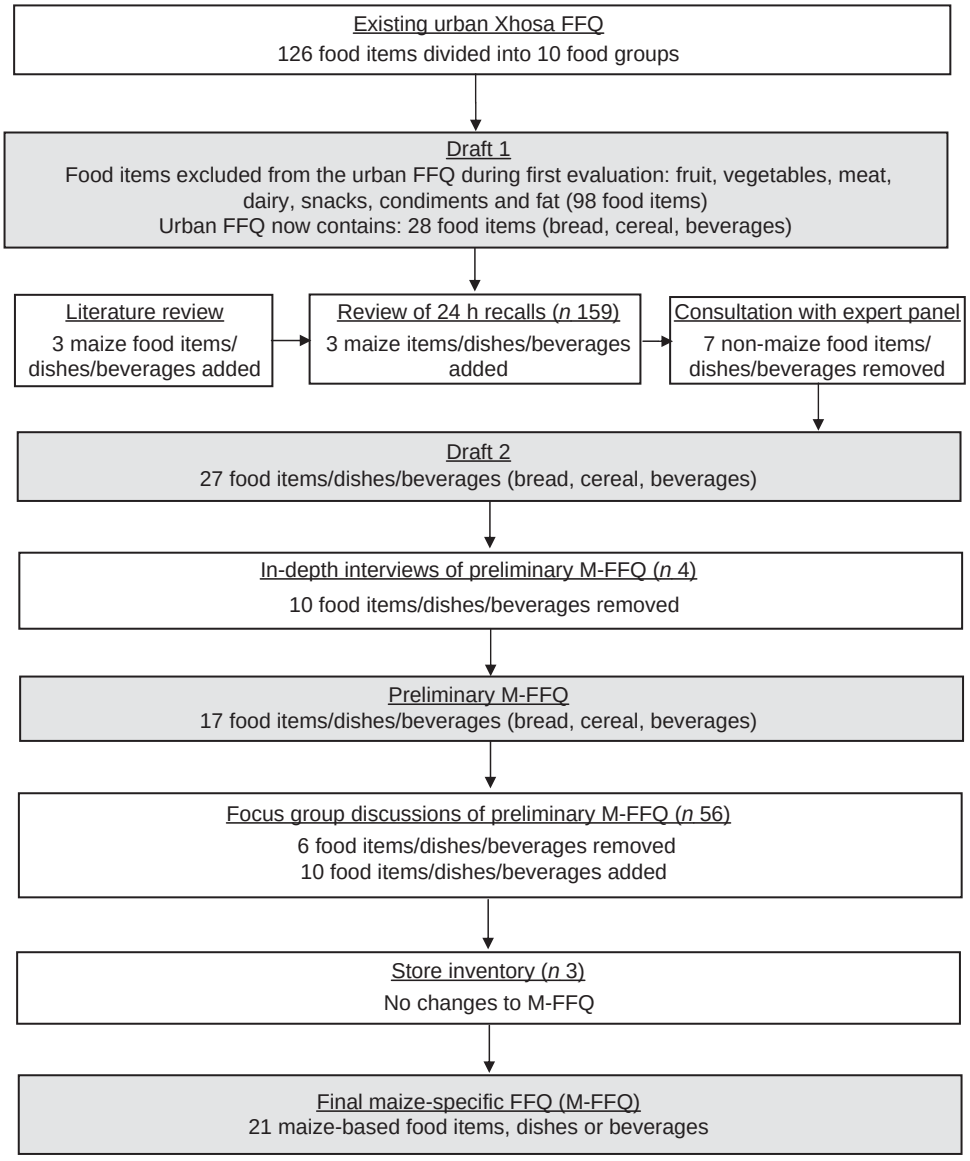

Fig. 1 Steps taken to develop the maize-specific FFQ (M-FFQ)

compared with the information obtained from the FGD. Sociodemographic information was collected by means of a questionnaire.

Participants who participated in developing the M-FFQ

The M-FFQ was developed in two rural areas of the Eastern Cape Province, selected because of the high incidence of OC in these areas. Because of poor infrastructure, villages from each area were randomly selected and female volunteers were recruited by means of snowball sampling. Females were selected because of their traditional role in food selection and preparation[18]. Trained interviewers proficient in isiXhosa conducted the interviews and FGD.

Iterations of the M-FFQ

An expert panel (consisting of three registered dietitians and one nutritionist) evaluated the original urban Xhosa FFQ. Non-maize food items (fruit, vegetables, meat, dairy, snacks, condiments and fat) were excluded. The first draft of the M-FFQ contained twenty-eight food items.

Literature review

Published information regarding the eating habits of people living in high OC areas in the Eastern Cape Province of South Africa was reviewed to provide information on the eating habits of the population[13,14,19]. All relevant articles were obtained from the literature by means of search engines and the Internet. Appropriate keywords ('Transkei', 'rural Eastern Cape', 'eating patterns', 'dietary habits', 'diet', 'Xhosa', 'isiXhosa', 'maize', 'oesophageal cancer', 'esophageal cancer', 'Centani', 'Chentani') were used in the following search engines: MEDLINE PubMed and Google Scholar. Articles were included if they provided information on the specific dietary habits and patterns of isiXhosa-speaking people living in these specific villages.

Review of 24 h recalls

Initial steps to identify food items, dishes and beverages to be included in the M-FFQ were done according to guidelines provided by Teufel[20]. This involved a review of data from a survey previously conducted among the population using single 24 h recalls (n 159; H-M Burger, G Shephard, W Gelderblom *et al.*, unpublished results). Trained fieldworkers conducted recalls. Each participant provided information on the foods consumed the previous day regarding breakfast, lunch and dinner as well as snacks and beverages. During the interview, participants also provided information on food preparation methods and portion sizes. The objective of the review of the recalls was to identify commonly consumed food items and dishes, as well as food preparation methods. For the purpose of the present study, recalls were therefore reviewed to identify eating patterns and not to conduct nutrient analyses.

Consultation with a research expert

A consultation was held with an employee of the Medical Research Council of South Africa (MRC) who was knowledgeable about the eating habits of isiXhosa-speaking people living in the rural areas studied in an attempt to ensure face and content validity of the M-FFQ. The employee has been conducting a population-based cancer registry[2] in these specific areas for many years. Additionally, she was born and raised in one of the study areas. The MRC employee provided valuable information regarding food items, dishes and beverages consumed; also dietary habits and cooking methods of different dishes. Results from the literature review, review of 24 h recalls and the expert consultation were compared with the existing urban Xhosa FFQ to develop a preliminary M-FFQ, which was then evaluated during in-depth interviews and FGD.

In-depth interviews

In-depth interviews were conducted with isiXhosa-speaking women (n 4) who lived in the Eastern Cape Province. Interviews were conducted in isiXhosa, with the help of isiXhosa-speaking interviewers. These women evaluated the preliminary M-FFQ and identified food items, dishes and beverages not frequently consumed by the rural Xhosa population, which were subsequently removed from the M-FFQ. They also identified food items, dishes and beverages which needed to appear on the food list and discussed the most commonly used food preparation techniques and recipes.

Focus group discussions

The preliminary M-FFQ (list of food items, dishes and beverages) was further evaluated by means of FGD in the Eastern Cape Province. Focus group sessions were conducted in two different areas. Women aged 18–55 years who were born and raised in the study areas were invited to participate in the FGD (n 56). Men were initially included in the recruitment, but declined participation because traditionally they are not involved in the cooking process and regard food preparation as being 'women's business'. Because of the low socio-economic status, it was not expected that there would be any differences between the food/dishes/beverages of males and females.

Two nurses from Eastern Cape Province rural areas were trained to facilitate the FGD using a structured interview guide prepared by the research team. Facilitators identified two local women from each area to host the FGD. These hosts were provided with information regarding the number and age of the expected participants. The hosts of the FGD invited participants in the two areas according to the above-mentioned criteria.

Participants in the FGD discussed the M-FFQ food list to ensure inclusion of all traditional and local (Xhosa) foods and exclusion of uncommon food items.

The FGD were audio-recorded, transcribed by a single researcher, and then translated to English by an

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isiXhosa-speaking interviewer to identify food items, dishes and beverages as well as recipes that define the local cuisine.

Information obtained from the various methods described above was integrated to provide a final M-FFQ, comprising commonly eaten food items, dishes and beverages.

Inventory of local shops

Food items and beverages available in three shops of the study areas were inventoried and compared with the M-FFQ list of foods to determine if any more food items needed to be added.

Ethics

The study was approved by the Research Ethics Committees of the University of Cape Town and the MRC (Rec. Ref. 123/2003). Each participant gave informed consent to participate in the study.

Results

Sociodemographic description of participants in the focus group discussions

Table 1 provides a summary of the sociodemographic characteristics of the participants. The mean age of the participants was 44 (SD 16) years. The majority of participants lived in traditional mud houses (*n* 40, 67 %), used river water (*n* 34, 62 %) as a primary water source, made use of the bush for sanitation (*n* 39, 70 %) and cooked their meals outside on a wood fire (*n* 35, 63 %). Of these participants, fifteen (27 %) ended their education while in primary school, between grades 1 and 7. Most households (*n* 52, 78 %) had one wage earner contributing to the household income while 7 % (*n* 4) had two. The remaining households had nobody contributing financially and received only government grants (child or old age). Monthly income per household was mostly between R 500 and R 1000 ($US 63–126), and 13 % (*n* 7) of families earned more than R 1000 ($US 126) per month. Approximately R 200 ($US 25) was spent on food per month.

Table 1 Sociodemographic description of the participants: women aged 18–55 years from two rural isiXhosa-speaking areas, Eastern Cape Province, South Africa

Characteristic	*n*	%
Education		
No formal education	14	25
Primary school (grade 1–7)	15	27
Secondary school (grade 8–12)	27	48
Employment		
Unemployed	28	50
Employed	28	50
Monthly income		
R 500–1000 ($US 63–126)	49	87
>R 1000 (>$US 126)	7	13
No. of people contributing to the household		
1 wage earner	52	78
2 wage earners	4	7
Housing		
Traditional mud house	40	67
Brick house	16	28
Water source		
River water	34	62
Communal tap	15	27
Inside tap	7	12
Fuel for cooking		
Fire	35	63
Electricity	21	37

Urban FFQ

After reviewing the urban FFQ, ninety-eight food items/dishes/beverages were excluded. The majority of these were excluded because they were not maize-based. Twenty-eight (Draft 1) food items/dishes/beverages remained in the questionnaire for further testing.

Literature review

Very little information has been published in peer-reviewed literature on the eating habits of people living in these high OC areas. Three articles written in the early 1970s and 1980s were identified[13,14,19]. Based on the information provided by these articles, three new maize items/dishes/beverages – namely stiff maize porridge and *iminifino* (spinach-like wild leaves), *amarewu* (sour fermented maize beverage) and traditional maize beer – were added to the urban FFQ.

Review of 24 h recalls

Twelve food items, dishes and beverages were consumed in the earlier unpublished study, including bread, *samp* (cracked dry maize kernels), *samp* and beans, soft maize meal porridge, stiff maize meal porridge, pumpkin (mixed with the stiff maize porridge), chicken, rice, eggs, *amarewu*, tea and coffee. The food items, dishes and beverages were checked against the urban FFQ and three more maize dishes were added (*samp*, *samp* and beans, stiff maize porridge mixed with pumpkin).

Consultation with Medical Research Council employee

After consultation with the MRC employee seven non-maize food items/dishes/beverages were excluded from the M-FFQ, including white rolls, brown rolls, whole-wheat rolls, dry biscuits (crackers), Nesquick, Milo and hot chocolate.

Various maize-based dishes are made and consumed at different times of the day. These dishes differ only in the amount of water added. Soft porridge is consumed at breakfast/brunch, stiff porridge at late lunch/dinner, while crumbly porridge is consumed on special occasions. Although these dishes are prepared in the same way, they differ in consistency and are eaten as different dishes at different times. These dishes were therefore included on the initial urban FFQ as three separate maize dishes.

The MRC employee provided information regarding cooking methods and recipes of the different maize-based breads (steamed bread, baked bread, *vetkoek* (maize or flour dough fried in oil) and dumplings (maize or flour dough

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cooked in broth)) and it was decided to include these breads as different dishes on the urban FFQ list. Recipes were also provided and explanations were given for the preparation of different maize-based dishes (*amarewu* and traditional beer). These items were also included as two different beverages.

Information obtained from the literature review, 24 h recall review and expert consultant were evaluated against Draft 1 of the M-FFQ and the result was Draft 2 of the M-FFQ, which included twenty-seven food items, dishes and beverages consumed in these high OC areas.

In-depth interviews

Various food items, dishes and beverages were identified for exclusion from the preliminary M-FFQ list during the in-depth interviews. These items included: whole-wheat bread, fish bread, popcorn, sorghum porridge, sour sorghum porridge, pre-cooked sorghum porridge, sugar-coated cereal, cereals and high-fibre cereals. Maize rice (cracked maize kernels) as a single food item was removed, as it is usually part of a mixed dish. No other dishes were included. The preliminary M-FFQ therefore included seventeen maize-based food items/dishes/beverages.

Focus group discussions

After conducting FGD, six food items/dishes/beverages were excluded: sour maize flour bread, brown rice, wheat, maize meal (bought), maize meal (home-grown) and carbonated drinks.

Ten more maize dishes and beverages were added, including baked bread, whole kernels, maize meal and *imifino*, maize meal and spinach, maize meal and dried sugar beans, soup (whole maize kernels and dried sugar beans), maize rice and *imifino*, maize rice and spinach. The M-FFQ now included twenty-one food items/beverages/dishes.

Inventory of shop foods

No additional food items or beverages were added to the final M-FFQ.

Final M-FFQ

The final M-FFQ (see Appendix) comprised twenty-one items divided into four food groups: bread, cereals (main maize dishes), combined dishes (maize and vegetables) and beverages. Food items and dishes included: baked maize bread, steamed maize bread, dumplings, *vetkoek*, maize on the cob, whole kernels, soft porridge, stiff porridge, crumbly porridge, maize meal cooked with *imifino*, maize meal cooked with spinach, maize meal cooked with pumpkin, maize meal cooked with dried sugar beans, *samp* and dried sugar beans, soup (maize kernels and dried sugar beans), maize rice cooked with *imifino*, maize rice cooked with spinach, maize rice cooked with pumpkin, *amagewu* (maize beverage), *amasi* (sour milk) and traditional maize beer.

The final questionnaire (Appendix) is designed to measure usual intake over a period of 1 month. Participants are asked to report on the maize source used for the different maize-based dishes, namely commercially procured or home-grown. This provides crucial information regarding maize consumption, and thus dietary fumonisin exposure, as well as nutrient intake resulting from mandatory fortification of commercially available maize meal (home-grown maize is not fortified).

Columns include the following options: home-grown/bought ratio, portion size, portions at a time (if the participant consumes more than one portion at a single time), consuming the food item less than once a month, frequency of consumption per week and frequency of consumption per day (if the participant consumes the dish more than once daily).

Discussion

The primary aim of the present study was to develop an FFQ for estimating culture-specific maize intake that can distinguish between home-grown and commercial maize. The newly developed M-FFQ measures frequency, amount and type of maize consumed in a culturally relevant listing of foods, dishes and beverages for Xhosas in rural areas. The M-FFQ estimates total intake of all foods and beverages over a 1-month period and is able to separately quantify exposure to fumosins as it is able to distinguish between home-grown and commercial maize. To our knowledge, this is the first time such a culturally specific questionnaire has been used in the rural areas of the Eastern Cape Province in South Africa.

The final M-FFQ measures habitual dietary intake over a period of 1 month. Because of high levels of illiteracy in the area (25 % received no formal schooling and another 27 % received between 1 and 7 years of schooling) and various Xhosa dialects, the questionnaire is in English and will always be interviewer administered[20]. Interviewers can also help the participants with the more difficult concepts such as portion size estimation[21].

The education levels of people living in these specific rural areas are low, with more illiterate people than in other areas of South Africa. According to the National Food Consumption Survey conducted in 2005, 3·1 % of those living in rural areas and 1·4 % of those in urban areas nationally have no schooling, compared with the 25 % in the present population[22]. Furthermore, poverty rates in these areas are also higher than those in other rural areas. The National Food Consumption Survey reported income between R 500 and R 1000 for 35·9 % of households living in rural areas and for 24·6 % of household living in urban areas nationally, percentages which are much lower than the 87 % reported by households in the present study. The lack of money and employment opportunities, as well as the poor

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infrastructure in these areas, forces people to rely heavily on subsistence farming and this explains their higher fumonisin exposure. This is also the reason for the monotonous diet. Interestingly, poverty and illiteracy are associated with increased risk for OC[3,4]. The national mean age-standardised incidence rate for OC in males and females in South Africa is 11·3 and 5·5/100 000 respectively, while in two selected rural areas of the Eastern Cape Province it is 48·3 and 19·2/100 000 respectively in one area and 37·2 and 14·4/100 000 in the other[1,2].

Participants from randomly selected villages in these two high OC areas in the Eastern Cape Province were included in the present study. These areas were selected because of pre-existing research infrastructure. It is not expected that the eating patterns and dietary habits differ from those of people in low OC risk areas in the Eastern Cape Province. However, it is assumed from previous research that those living in higher OC risk areas may consume more home-grown maize and therefore be more frequently exposed to higher levels of fumonisins[5]. To confirm this, the M-FFQ distinguishes between home-grown and commercially procured maize.

Very little information is available on the toxic levels of fumonisins. Various food safety authorities have undertaken risk assessments on the consumption of contaminated maize and risk assessments. The 56th meeting of the JECFA provided a no observed adverse effect level of 0·2 mg/kg body weight per d and a safety factor of 100, as a group provisional maximum tolerable daily intake for fumonisins of 2 μg/kg body weight per d[17,23].

It was therefore decided to base the M-FFQ on an FFQ design accompanied with portion size photographs, as this provides detail on habitual diet. It is also a feasible method for large studies and is easy to capture.

The newly developed M-FFQ was systematically developed with the use of in-depth interviews and FGD. According to Teufel[20] such FGD shed light on the knowledge, beliefs[24] and attitudes of participants towards their eating habits. Culturally specific food preparation techniques and recipes as well as food availability and farming practices were discussed in the FGD in the present study.

Participants played a major role in the development of the M-FFQ. It is therefore envisioned that this culturally specific M-FFQ will provide accurate information on the dietary habits of those living in rural areas in the Eastern Cape Province of South Africa, especially regarding maize intake and subsequent fumonisins exposure. Although the M-FFQ includes only twenty-one food items, all efforts have been made to ensure that it includes the majority of food items and dishes consumed by the population. Due to the extreme poverty in the area, a lack of access to food (only three stores in the entire area) and because residents are subsistence farmers, their dietary intake is exceptionally limited. The majority of dishes are maize-based and relevant to the exposure study.

The M-FFQ has subsequently undergone further testing to demonstrate validity and reliability and was used further used in a cross-sectional study to determine maize exposure. The questionnaire can also be used in future intervention studies to track changes in exposure.

Conclusion

The M-FFQ was designed to determine the maize intake of people living in rural, high OC areas to contribute to the quantification of fumonisins exposure. This culturally appropriate M-FFQ is a crucial first step in planning and evaluating the impact of future dietary cross-sectional and intervention studies in Xhosa populations.

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Appendix

Newly developed maize-specific FFQ (M-FFQ)

Type of food	Yes/no	Home (H)/bought (B)	Portion size	Portions at a time	Frequency			
					Less than a month	Per month	Per week	Per day
Baked bread								
Steamed bread								
Dumplings								
Vetkoek								
Maize on cob								
Whole kernels								
Soft porridge								
Stiff *pap*								
Crumbly *pap*								
Maize meal+*imifino*								
Maize meal+spinach								
Maize meal+pumpkin								
Maize meal+beans								
Samp+beans								
Soup (kernels+beans)								
Mealie rice+*imifino*								
Mealie rice+spinach								
Mealie rice+pumpkin								
Amagewu								
Amasi								
Traditional beer								

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Article

A Food Photograph Series for Identifying Portion Sizes of Culturally Specific Dishes in Rural Areas with High Incidence of Oesophageal Cancer

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Abstract: Rural areas of the Eastern Cape (EC) Province, South Africa have a high incidence of squamous cell oesophageal cancer (OC) and exposure to mycotoxin fumonisin has been associated with increased OC risk. However, to assess exposure to fumonisin in Xhosas—having maize as a staple food—it is necessary to determine the amount of maize consumed per day. A maize-specific food frequency questionnaire (M-FFQ) has recently been developed. This study developed a food photograph (FP) series to improve portion size estimation of maize dishes. Two sets of photographs were developed to be used alongside the validated M-FFQ. The photographs were designed to assist quantification of intakes (portion size photographs) and to facilitate estimation of maize amounts in various combined dishes (ratio photographs) using data from 24 h recalls (n = 159), dishing-up sessions (n = 35), focus group discussions (FGD) (n = 56) and published literature. Five villages in two rural isiXhosa-speaking areas of the EC Province, known to have a high

incidence of OC, were randomly selected. Women between the ages of 18–55 years were recruited by snowball sampling and invited to participate. The FP series comprised three portion size photographs (S, M, L) of 21 maize dishes and three ratio photographs of nine combined maize-based dishes. A culturally specific FP series was designed to improve portion size estimation when reporting dietary intake using a newly developed M-FFQ.

Keywords: fumonisin; mycotoxins; oesophageal cancer; food photograph series; maize; dietary intake

1. Introduction

A relatively small geographic area in rural areas of the Eastern Cape (EC) Province of South Africa has a high incidence of squamous cell oesophageal cancer (OC) [1]. The aetiology of OC is still unclear; however various risk factors have been associated with the disease including alcohol consumption [2], tobacco use [2], and exposure to the carcinogenic mycotoxin fumonisin [3]. Of particular interest in the South African context is the high exposure to mycotoxins (fumonisin moniliforme) in these areas [3–5]. Past research has shown that fumonisin grow on maize that is stored in suboptimal damp conditions and are found in higher concentrations in home-grown compared to commercially sold maize [5]. Maize is the primary staple food of the black population of South Africa and is consumed in large amounts on a daily basis in the geographical area of interest [3,4]. Fumonisin contamination of this food source is a major health concern and to date no quantitative assessment of exposure has been conducted [6]. Recently, a culturally specific maize-specific food frequency questionnaire (M-FFQ) was developed [7] to determine fumonisin exposure from maize consumption in rural-dwelling residents of the EC Province.

Accurate assessment of fumonisin exposure in isiXhosa-speaking subsistent farmers whose staple food is maize requires determination of maize consumed in various dishes throughout the day. Maize is typically consumed with vegetables, and the ratio of maize to vegetables varies according to availability of vegetables [8,9]. This variation to commonly consumed dishes makes it difficult to estimate the actual amount of maize consumed and requires determination of the ratio of maize to vegetable.

Minimizing measurement error is important when determining diet-disease association. Classification of people based on their food and therefore nutrient intake is determined by the accuracy of the dietary assessment tool used. Therefore, using dietary assessment tools with known measurement errors, the attenuating effects of misclassification can be assessed and interpreted [10].

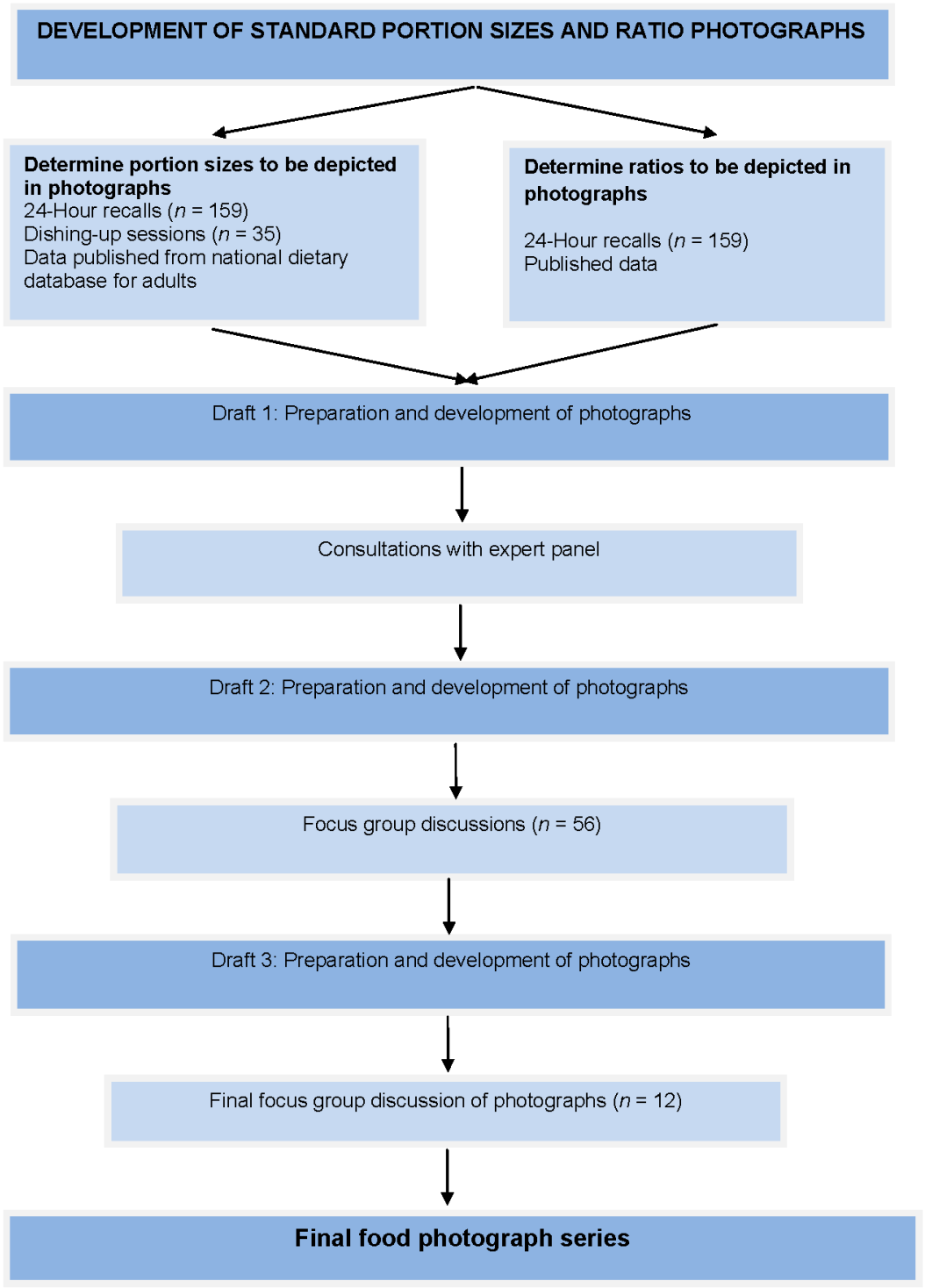
A key error occurring in the measurement of food intake occurs during portion size assessment. The use of scales is often problematic or not appropriate and then the assessment of food intake depends on the participant's ability to remember and describe their usual portion size [10]. Various aids have been developed to improve participants' description of their usual portion size (such as portion size models, food models, photographs and schematic diagrams).

In a study conducted by Nelson *et al.*, (1996) it was concluded that photographs that depicts a range of portion sizes can be used to improve portion size estimation and that the use of such photographs

can further reduce the misclassification of participants. It was however reported that factors such as participant age, gender and body mass index can influence portion size estimation [10].

Owing to culturally specific dietary habits [11], existing food portion size photographs that represent Western, mainly urban-type meals could not be used in this population. The aim of the present study was to develop a culturally specific series of food photographs (FPs) to be used as an aid when conducting dietary interviews in order to improve portion size estimation of maize-based meals and beverages, such as traditional maize-beer.

Figure 1. Process followed for the development of the portion size and ratio photographs.



2. Materials and Methods

2.1. Overview

As shown in Figure 1, the initial FPs on maize, maize-vegetable combined dishes, and maize beverages (n = 21) were developed based on data obtained from (i) a survey using 24 h recalls from EC Province; (ii) women from the study area dishing up their "usual" serving portions and (iii) data on portion sizes obtained from a national database. In addition to the determination of portion size it was also necessary to determine the ratio of maize to vegetables in "combined dishes". This was done by reviewing published data and data from a study using 24 h recalls from the EC Province.

After the initial FPs were finalised (Draft 1) they were shown to an expert group for comment; the FPs were subsequently revised (Draft 2). This was followed by focus group discussions (FGD) with participants recruited from the target study population, who made further recommendations, which resulted in Draft 3. A last FGD resulted in the final set of FPs. Each step is discussed in detail below.

2.2. Determination of Portion Sizes

2.2.1. Use of 24-h Recalls

Standard portion sizes of maize items, dishes and beverages appearing on the M-FFQ were determined using 24 h recall data (n = 159) collected in the same areas [7]. Participants provided information on the maize based food items and dishes consumed the previous day, as well as snacks and beverages to provide information on the dietary habits of the people living in these areas. The objective of the reviews was to identify commonly consumed food items and dishes as well as cooking and food preparation methods.

2.2.2. Dishing up Sessions

Dishing-up (serving) sessions were undertaken in the selected areas to determine the standard portion sizes of these M-FFQ dishes: stiff *pap* (maize meal porridge with a stiff, thick consistency), soft porridge (maize meal porridge having a thin consistency), samp (whole maize kernels) and beans, spinach/*imifino* (a wild leafy vegetable similar to spinach) combined with *pap*, pumpkin combined with *pap*, and soup/*isophi* (watery soup consisting of whole maize kernels and dried sugar beans). Two rural areas of the EC Province were selected because of the high incidence of OC [1]. Because of poor infrastructure; villages from each area were randomly selected. Female volunteers were recruited with snowball sampling. Six women were identified and prepared the six most commonly consumed maize dishes using ingredients provided by the research team.

Thirty five female volunteers (18–55 years) were recruited and asked to dish up a "usual" portion that they would serve an adult male and female living in the household. The served portions were individually weighed on the plate. The weight of the plate subtracted to provide a portion size. Participants were informed that they would not be eating the food so that neither their current state of hunger, nor the fact that the food was free would influence the dishing up process [12].

The mean weight for each dish was determined and the inter quartile (IQ) range (25%, 50% and 75% percentiles) were used to determine S, M, and L portion sizes of each dish.

2.2.3. Data from a National Database

Portion sizes for dishes not included in the dishing-up sessions were determined from a national dietary database on dietary intake compiled from studies that were conducted among different population groups in South Africa from 1983 to 2000 [13]. Two studies conducted in similar rural communities formed part of this data base.

2.3. Determination of Ratios Depicted in Food Photograph Series

Standard ratios of maize to vegetables in nine combined dishes (maize meal + *imifino*; maize meal + spinach; maize meal + pumpkin; maize meals + dried beans; maize meal + dried beans; samp + beans; *isophi*; mealie rice + spinach; mealie rice + pumpkin) were determined using previously unpublished 24 h recall data from our group (n = 159) [7], as well as review of previously published data [8,9].

2.4. Preparation and Development of Initial Photographs

Maize dishes depicted on the initial FPs were prepared by a woman born and raised in the area. Raw ingredients were weighed and cooking methods and preparation steps recorded. An initial FP series (Draft 1) was developed. Photographs were taken at an angle of 42° above the horizon, which is the average angle of viewing when a person is seated at a table) [14]. Different colour backgrounds, reference scales, types of and colour plates were considered in the process (Table 1).

Table 1. Different factors considered in developing the photographs.

Factors considered	Photograph options	Influence of photographs
Colour of the plate	White	Maize dishes are white and therefore there was little contrast between the food and the plate.
	Yellow or cream	Influenced the colour of dishes containing pumpkin.
	Green	Influenced the colour of dishes containing spinach.
	Blue	Influenced some of the photographs containing spinach.
Type of plate used	Bowl	Determining the depth of the dish was difficult when it is presented in a bowl.
	Plate	Determining depth on a plate was easier than that of a bowl.
Background of the photograph	Dark (navy or black)	White maize dishes were more pronounced on a dark background.
Scale	Match box	This was disregarded because smoking is a risk factor for cancer and would send mixed messages.
	Ruler	A ruler is not a known or much used item in this rural area.
	Knife and fork	These utensils are not frequently used in the area.

2.5. Evaluation of Draft 1 of the Food Photograph Series

An expert panel (consisting of two research dieticians and a research nutritionist experienced in the development of dietary assessment methods and the culture of isiXhosa speaking people) evaluated

Draft 1 of the FP series. A second set (Draft 2) of FPs was subsequently developed to address the comments by the expert panel.

2.6. Evaluation of Draft 2 of the Food Photograph Series

Draft 2 of the FP series was evaluated by means of FGD in the area. Xhosa women aged 18–55 years who were born and raised in the area were invited to participate in the FGD (n = 56). Men were excluded from the FGD since food preparation is regarded as "women's business" in these areas [7–9].

Two nurses from the area were trained to facilitate the FGD using a structured interview guide prepared by the research team. Two local women, who were identified by the facilitators to host the discussions, were provided with information regarding the number and age of the required participants. The women invited participants according to the inclusion criteria.

Participants critically reviewed and discussed whether Draft 2 of the FP series truly reflected the various traditional dishes and portion sizes consumed by adults in the area. All discussions were audio-recorded, transcribed and translated to English by an isiXhosa speaking interviewer. A third draft of FP series (Draft 3) was developed to address the relevant comments raised by the FGD participants.

2.7. Evaluation of Draft 3 of the Food Photograph Series

A final FGD was conducted with 12 women aged 18–55 years who had been raised in the area. The participants critically reviewed and discussed the applicability and recognisability of these photographs to the target population and also identified the three most recognisable and commonly consumed ratio photographs. The appropriateness of the actual sizes of the photos was also discussed.

Information obtained from the various methods described above was integrated to provide a final FP series to accompany the newly developed M-FFQ [7].

2.8. Ethics

Ethical approval for the study was obtained from the Research Ethics Committees of the University of Cape Town (UCT) (123/2003) and the Medical Research Council (MRC) of South Africa. Each participant received detailed, easy to understand information (both verbally and written) regarding the study, and written consent was obtained in the participant's first language (isiXhosa).

3. Results

3.1. Socio-Demographic Description of Participants in the Quantitative and Qualitative Assessments

In summary, mean age of the participants was 44 years (±16), most lived in traditional mud houses, used river water as primary water source and had either no, or limited schooling (Table 2).

Table 2. Socio-demographic description of participants.

Socio-demographic characteristic	24 h recalls *n* = 159	Dishing up session *n* = 35	Focus group discussions *n* = 56	Final Focus group discussion *n* = 12
	n (%)	*n* (%)	*n* (%)	*n* (%)
Education				
No formal education	69 (43)	6 (17)	14 (25)	1 (8)
Primary school (grade 1–7)	60 (38)	15 (43)	15 (27)	3 (25)
Secondary school (grade 8–12)	30 (19)	14 (40)	27 (48)	8 (67)
Employment				
Unemployed	87 (55)	25 (71)	28 (50)	4 (33)
Employed	72 (45)	10 (29)	28 (50)	8 (67)
Monthly income				
R 500–R 1000 (63–126 USD)	142 (89)	23 (66)	49 (87)	5 (42)
>R 1000 (126 USD)	17 (11)	12 (34)	7 (13)	7 (58)
No. of people financially contributing to the household				
1 Person	133 (84)	21 (60)	52 (78)	7 (58)
2 Persons	26 (16)	14 (40)	4 (7)	5 (42)
Type of housing				
Traditional mud houses	144 (91)	19 (54)	40 (67)	0 (0)
Brick houses	15 (9)	16 (46)	16 (28)	3 (25)
Informal structures	0 (0)	0 (0)	0 (0)	9 (75)
Water source				
River water	137 (86)	19 (54)	34 (62)	0 (0)
Communal tap	22 (14)	16 (46)	15 (27)	8 (67)
Inside tap	0 (0)	0 (0)	7 (12)	4 (33)
Fuel for cooking				
Fire	140 (88)	21 (60)	35 (63)	5 (42)
Electricity	19 (12)	14 (40)	21 (37)	7 (48)

3.2. Portion Sizes and Ratios Determined by Various Methods

Table 3 shows that portion sizes of maize dishes derived from the 24 h recalls ranged from 490 g (porridge) to 771 g (stiff *pap* and pumpkin). Results from the dishing up sessions are presented in Table 4. Soft porridge had the smallest portion sizes, and samp and beans the largest. Data on ratios are presented in Table 5.

Table 3. Portion sizes derived from 24-h recalls conducted in the Eastern Cape Province (*n* = 159).

Dish	Percentiles						
***n* = 159**	**25% (Small)**	**50%**	**75% (Large)**	**Minimum**	**Maximum**	**Mean**	**SD**
Maize porridge (g)	338	470	590	184	862	490	176
Stiff *pap* * (g)	375	501	703	210	1290	555	234

Table 3. *Cont.*

Stiff *pap* and cabbage (g)	364	526	707	255	930	547	193
Stiff *pap* and pumpkin (g)	656	727	824	524	1250	771	227
Stiff *pap* and *imifino* ** (g)	448	588	770	330	985	600	204
Stiff *pap* and spinach (g)	328	426	530	310	1170	531	323
Stiff *pap* and beans (g)	445	544	765	376	935	613	232
Samp and beans (g)	328	468	646	150	1290	536	261
Amagewu *** (mL)	300	450	1000	200	1000	600	355

50% medium = median; SD = standard deviation; * *Pap* = Porridge; ** *Imfino* = Wild green leafy vegetable; *** *Amagewu* = Maize meal beverage.

Table 4. Portion sizes of six main maize dishes calculated during the dishing-up sessions (n = 35) *.

Dish	**Inter quartile percentiles**						
***n* = 60**	**25% (Small)**	**50%**	**75% (Large)**	**Minimum**	**Maximum**	**Mean**	**SD**
Porridge (g)	358	557	630	146	760	497	174
Stiff *pap* ** (g)	308	478	640	164	1204	505	232
Samp and beans (g)	592	744	896	294	1396	743	217
Stiff *pap* and pumpkin (g)	462	592	794	196	1201	626	237
Stiff *pap* and *imifino* *** (g)	426	512	710	102	1303	561	227
Soup (g)	466	608	726	228	916	587	177

50% medium = Median; SD = standard deviation; * Female volunteers were asked to dish up a portion for herself and a portion for a man/husband (5 women did not have a man/husband thus dished up only one portion; ** *Pap* = Porridge; *** *Imfino* = Wild green leafy vegetable.

Table 5. Ratios depicted on the photographs.

Food type	**Ratios**		
	Cooked maize: Vegetable	**Cooked maize: Vegetable**	**Cooked maize: Vegetable**
Stiff pap + *imifino*	1:1	1:2	2:1
Stiff *pap* * + spinach	1:1	1:2	2:1
Stiff *pap* * + pumpkin	1:3	3:1	1:2
Stiff *pap* * + dried beans	1:2	2:1	1:3
Samp and beans	2:1	3:1	5:1
Soup	1:1	2:1	1:2
Mealie rice + *imifino*	1:3	3:1	1:2
Mealie rice + spinach	1:3	3:1	1:2
Mealie rice + pumpkin	1:3	3:1	1:2

* *Imfino* = Wild green leafy vegetable; ** *Pap* = Porridge.

3.3. Feedback Processes Leading to Development of Final Photographs

The initial photographs were handheld size (10 × 15 cm) and were shot against a black background, on a white plate with a ruler for scale The expert group decided that the black background was too

dark, that the ruler was not a familiar item in the area studied, and that the use of white plates made it difficult to determine portion sizes of the mostly white food items.

Draft 2 of the FP series were taken against a white background with more familiar utensils to illustrate scale (fork, knife and spoon). The food items, prepared by an isiXhosa speaking woman from the area, were dished up onto a light blue enamel plate which is commonly used in households in the area and hence familiar to the participants. Portion size photographs of the traditional beer and other maize based beverages were represented in similar blue enamel mugs. Actual size of the photographs remained the same (10 × 15 cm).

During the FGD, participants commented that the hand-held photographs were too small to identify different portion sizes and that larger photographs were needed. They further commented that it was difficult to see and to determine depth of the portion sizes, especially the white maize dishes on a white background. They also mentioned that the fork, knife and spoon depicted were not acceptable as a scale for indicating dimensions and had to be replaced.

A third draft (Draft 3) of photos was taken using a black background to emphasise the mostly white foods. A light blue plate was used with a wooden spoon (most common dishing up utensil) as a scale (Figure 2). All utensils used in the photographs were procured in the EC Province. Actual size of photographs was increased to 42 × 30 cm to provide life-size images of the dishes, since participants in the FGD concluded that life size photographs would be the easiest to correctly identify portion sizes.

Figure 2. (**a**) Draft 3, portion size photographs for stiff *pap*; (**b**) Draft 3, ratio photographs for maize meal and pumpkin.

(**a**)

(**b**)

Focus group discussion participants found it difficult to estimate the three-dimensional perspective of the brown bread, cabbage, spinach and stiff *pap* from the photographs. This was resolved by reducing the camera angle to allow more depth [14]. Participants also did not recognise the difference between the medium and large portions of brown bread and the combined cabbage and spinach dish. These food items and dishes were repositioned on the plate and re-taken. Lastly, FGD participants indicated that the depicted portion sizes of the traditional beer were too small and suggested it be replaced with larger portion sizes, in plastic jugs.

3.4. Final Food Photograph Series

The final FP series comprised three portion size photographs (S, M, L) of 21 maize food items (Figure 2a), dishes and beverages, including baked maize bread, steamed maize bread, *dumplings*, *vetkoek*, maize on the cob, whole kernels, soft porridge, stiff porridge, crumbly porridge, maize meal cooked with *imifino*, maize meal cooked with spinach, maize meal cooked with pumpkin, maize meal cooked with dried sugar beans, samp and dried sugar beans, soup (maize kernels and dried sugar beans), maize rice cooked with *imifino*, maize rice cooked with spinach, maize rice cooked with pumpkin, *amagewu* (maize beverage) and traditional maize beer.

The FP series also included three ratio photographs of the nine combined dishes (Figure 2b), including maize meal cooked with *imifino*, maize meal cooked with spinach, maize meal cooked with pumpkin, maize meal cooked with dried sugar beans, samp and dried sugar beans, soup (maize kernels and dried sugar beans), maize rice cooked with *imifino*, maize rice cooked with spinach, maize rice cooked with pumpkin.

Coding on the reverse side of the photographs correspond with that on the M-FFQ in order to shorten the dietary interview process and allow easy identification of the relevant portion size of a food that was consumed within the reference reporting period.

4. Discussion

The primary aim of the present study was to develop a FP series to improve portion size estimation when using a newly developed maize-based FFQ (M-FFQ). Both the FP and the M-FFQ were developed specifically for use in rural dwelling South Africans who are exposed to fumonisins through maize consumption [7]. A systematic approach was used to identify culturally appropriate serving sizes for the food items, dishes and beverages, and FPs were taken using these portions.

Measurement error in dietary assessment usually occurs because participants are unable to describe portion sizes accurately [10]. Predetermined portion sizes on FFQs simplify the coding and data entry process and are therefore useful in epidemiological surveys that include large numbers of participants [15]. Due to large potential between-person and within-person variation, particularly seasonally [15], the approach was to include three portion sizes for the most commonly consumed maize-based food items included in the M-FFQ. Most rural people, many of whom have low literacy levels, find it easier to identify portion sizes from local measures (cups, glasses, bundles, heaps or numbers) rather than from measuring units [16]. Two-dimensional models such as photographs and food models have been shown to increase accuracy during portion size estimation in illiterate populations [17].

A strength of the study was the use of qualitative methods, both in-depth interviews and FGD with key informants, to develop photographs as an aid to use with a quantitative dietary assessment tool [18]. Culturally-specific food preparation techniques and recipes were discussed in the FGD, and were confirmed during actual dishing-up (serving) sessions, in which the ingredients were weighed and preparation steps were recorded.

Careful consideration was given to ensure that the final food photograph series was depicted in an easy-to-identify layout with familiar utensils. In this regard, life-size photographs were found to be

more effective than the smaller hand-held cards. This participatory research project involved support from local community representatives, recruited from the geographical area of interest. It is therefore envisioned that this culturally specific FPs will improve portion size estimation to ultimately provide more accurate information on the dietary habits and nutrient intakes of those living in rural areas in the EC Province of South Africa. However, it is of the utmost importance that the food photographs be validated to determine the presence and direction of bias in terms of portion size recalls of these participants [19]. Furthermore, the effect of only three portion size choices needs to be investigated as it is possible that more photograph choices (5–8) would decrease portion size estimation error [19,20]. These additional photographs should ideally represent intermediate values between the current S, M and L to further obtain personalized values.

5. Conclusions

A FP series to improve portion size estimation of people living in rural areas of the EC Province was successfully developed using a comprehensive process of accruing qualitative and quantitative information and expert opinions, as well as very close engagement with the target community.

Acknowledgements

The study was sponsored by the South African Medical Research Council (MRC) as well as the Cancer Association of South Africa (CANSA). The authors further declare that to their knowledge there were no conflicts of interests that could bias information obtained in the paper. Ethical approval was obtained from the Research Ethics Committees of the University of Cape Town (UCT) (123/2003) and the MRC of South Africa.

Conflict of Interest

The authors declare no conflict of interest.

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Addendum F

MRC
SOUTH AFRICA
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Ethics Committee

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25 April 2003

Ms H Burger
PROMEC
MRC

Dear Ms Burger

RE: Diet and other risk factors associated with oesophageal cancer in Transkei

Thank you for your response to the Ethics Committee dated 16 April 2003. The changes have been approved. I am pleased to inform you that ethics approval is now granted for the study.

Wishing you well with your research.

Yours sincerely

PROF. D DU TOIT
CHAIRPERSON: MRC ETHICS COMMITTEE