The Effect of Iron Overload on the Long-term Toxicological Effects of Fumonisin B_1 in Rat Liver

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Dedicated to my rats

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List of abbreviations

2-AAF	2-acetylaminofluorene
ABC	avidin-biotin-peroxidase complex
AFB_1	aflatoxin B_1
AIN-93M diet	American Institute of Nutrition-93 Maintenance diet
ALP	alkaline phosphatase
ALT	alanine transaminase
Anti-BrdU	monoclonal anti-5-bromo-2’-deoxy-uridine antibody
AST	aspartate transaminase
ATP	adenosine triphosphatase
BHT	butylated hydroxytoluene
BrdU	5-bromo-2’-deoxy-uridine
BSA	bovine serum albumin
BW	body weight
CCl_4	carbon tetrachloride
CD	conjugated dienes
CMS	chloroform-methanol-saline
ddH_2O	double distilled water
DEN	N-nitroso diethylamine
DMT-1	divalent metal transporter
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid disodium salt
ELEM	equine leukoencephalomalacia
F344	Fisher 344 rat
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FA_1	fumonisin A_1
FA_2	fumonisin A_2
Fe	dietary carbonyl iron
GGT	γ-glutamyl transpeptidase
GH	genetic haemochromatosis

GSTP	placental form of glutathione-*S*-transferase
$GSTP^+$	glutathione-*S*-transferase positive
h	hour
Hb	haemoglobin
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCT	haematocrit = packed cell volume
HCV	hepatitis C virus
HFE	classical hereditary haemochromatosis gene
HPLC	high performance liquid chromatography
H&E	haematoxylin and eosin
Ig	immunoglobulin
i.p.	intraperitoneal
i.v.	intravenous
Ireg	transport protein for iron
IRP	iron-regulatory protein
KCl	potassium chloride
LDH	lactate dehydrogenase
LW	liver weight
LW/BW	liver to body weight ratio
MDA	malondialdehyde
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MRC	Medical Research Council
NaOH	sodium hydroxide
nd	not determined
NTD	neural tube defects
OC	oesophageal cancer
$PbNO_3$	lead nitrate
PBS	phosphate buffered saline
PCT	porphyria cutanea tarda
PH	partial hepatectomy
PL	platelet count

PPE	porcine pulmonary edema syndrome
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
RBC	red blood cell count
RDW	red blood cell distribution width
RE	reticuloendothelial
Sa	sphinganine
Sa/So ratio	sphinganine to sphingosine ratio
SL	sphingolipid
So	sphingosine
STDEV	standard deviation
T½	half-life
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reacting substances
tBWG	total body weight gain
TCA	trichloroacetic acid
TfR	transferrin receptor
URO-D	uroporphyrinogen decarboxylase
WBC	white blood cell count

Chapter 1
Introduction

Fumonisin B_1 (FB_1), a mycotoxin produced by the fungus *Fusarium verticillioides*, is a natural contaminant of maize world-wide (Gelderblom *et al.*, 1988b; Sydenham *et al.*, 1990; Marasas, 1996). It has been shown to cause a variety of toxic effects in animals, including equine leukoencephalomalacia (Marasas *et al.*, 1988b; Kellerman *et al.*, 1990; Ross *et al.*, 1990, Wilson *et al.*, 1990) and porcine pulmonary oedema syndrome (Harrison *et al.*, 1990; Ross *et al.*, 1990). Human dietary consumption of *Fusarium*-contaminated maize products has been linked epidemiologically to increased rates of oesophageal cancer in areas of the world where the staple diet consists of maize. In the Transkei region of South Africa (Rheeder *et al.*, 1992; Sydenham *et al.*, 1990), maize forms a major part of the diet and is also used to make traditional beer in rural areas (Bothwell *et al.*, 1964; Gordeuk *et al.*, 1992).

FB_1 is hepatotoxic and hepatocarcinogenic in rats (Gelderblom *et al.*, 1996b). The mycotoxin has been shown in short-term studies to cause progressive toxic hepatitis characterised by hepatocellular necrosis, bile duct proliferation and fibrosis (Gelderblom *et al.*, 1988a). Long-term feeding has resulted in chronic toxic hepatitis that progresses to cirrhosis, cholangiofibrosis, and eventually hepatocellular carcinoma and cholangiocarcinoma (Gelderblom *et al.*, 1991).

Both hepatocellular carcinoma (HCC) and iron overload are important health problems in sub-Saharan Africa (Gordeuk *et al.*, 1992). Chronic hepatitis B virus (HBV) infection is recognised as a major risk factor for HCC; in addition, patients with alcohol-or HBV-induced liver cirrhosis have an increased risk of developing HCC in the presence of hepatic iron overload compared to those with normal body iron stores (Deugnier *et al.*, 1998; Deugnier and Loréal, 2000). The role of hepatic iron overload in black Africans is not fully elucidated. It has recently been proposed that African iron overload might also be risk factor for HCC (Gangaidzo and Gordeuk, 1995; Mandishona *et al.*, 1998). Certain African populations appear to have a genetic predisposition to increased iron absorption, which is augmented by an additional high dietary intake resulting

from the home-brewing of traditional beer in iron pots (Gordeuk *et al.*, 1992). Extreme iron overload is ten times more common in sub-Saharan Africans than in homozygous genetic haemochromatosis in Caucasians (Gordeuk, 1992). HCC is an important complication of genetic haemochromatosis (GH), (Bacon *et al.*, 1999) and the occurrence of HCC in humans with iron overload has been attributed to the progression from iron-induced cirrhosis (Niederau *et al.*, 1985). The direct role which iron plays in the development of HCC is, however, not clear.

The FB_1 mycotoxin could play a role in the development of human HCC in high incidence areas of South Africa, possibly acting as a co-factor with other known contributing factors such as a chronic HBV and HCV infection, excess hepatic iron, and the carcinogenic mycotoxin aflatoxin B_1 (AFB_1). It has recently been shown in rainbow trout and rats that synergism between AFB_1 and FB_1 could contribute to the development of HCC (Carlson *et al.*, 2001, Gelderblom *et al.*, 2002).

Iron could enhance the cytotoxic and oxidative effect of FB_1 since both substances induce free radical production and resulting cell membrane damage. Hepatocytes exposed to FB_1 are more susceptible to chemically induced oxidative stress (Abel and Gelderblom, 1998). By inducing hepatocyte regeneration (Stål *et al.*, 1995), iron acts as a mitogen (Deugnier *et al.*, 1992) and the synergistic effect of iron in studies of cancer promotion can perhaps be attributed to this mitostimulatory effect (Smith *et al.*, 1990). Proliferating cells also appear to be more sensitive to the toxic effects of fumonisins (Li *et al.*, 2000) and the mitotoxic effect of iron on hepatocytes could provide favourable conditions for enhanced FB_1 toxicity.

A pilot study was first conducted to establish a model of iron loading for the subsequent long-term carcinogenesis study. By evaluating the effect of different dietary iron levels on hepatic iron overload over time, and the effect of reducing this dosage after 10 weeks, optimum conditions for iron loading in the absence of adverse side effects could be determined.

The aim of the long-term study was to examine the interaction between FB_1 and increased hepatic iron levels, utilising the stop model of chemical carcinogenesis, and in particular determine whether excess iron could potentiate the hepatocarcinogenic effects of FB_1.

Chapter 2
Literature review

2.1. Toxicological effects of FB_1

2.1.1. Introduction

The discovery and characterisation of the fumonisin B (FB) mycotoxins was the result of the dedicated work of the South African PROMEC Unit of the Medical Research Council (MRC) in collaboration with the Veterinary Research Institute, Onderstepoort, and the Council for Scientific and Industrial Research (CSIR), Pretoria (Gelderblom *et al.*, 1988a; Bezuidenhout *et al.*, 1988). The fumonisins are toxic secondary metabolites produced mainly by *Fusarium verticillioides* (Sacc.) Nirenberg (*Fusarium moniliforme* Sheldon) as well as *Fusarium proliferatum* (Marasas *et al.*, 1984; Ross *et al.*, 1990). *F. verticillioides* is a common fungal contaminant of maize intended for human and animal consumption world-wide (Marasas *et al.*, 1984) and is prevalent in corn screenings and commercial rations (Wilson *et al.*, 1985b).

Fumonisins have been implicated in numerous diseases affecting domestic animals such as horses and pigs (Haliburton *et al.*, 1986). Long-term feeding of FB_1 in rats induces hepatocarcinogenic and nephrocarcinogenic effects in 2 different rats species. (Gelderblom *et al.*, 1991, 2001c; Howard *et al.*, 2001). The fumonisins are epidemiologically linked to the high incidence of oesophageal cancer (OC) in certain regions of the world where the staple diet consists of maize (Kmet and Mahboubi, 1972; Hormozdiari *et al.*, 1975; Rheeder *et al.*, 1992; Sydenham *et al.*, 1990; Chu and Li, 1994). Recently, attention has also been drawn to the high incident rate of neural tube defects (NTD) in areas of high maize consumption, possibly due to fumonisin contamination (Ncayiyana, 1986; Venter *et al.*, 1995).

FB_1 is not mutagenic in the *Salmonella* mutagenicity test (Gelderblom and Snyman, 1991; Park *et al.*, 1992), does not induce unscheduled DNA synthesis in rat hepatocytes (Norred *et al.*, 1992), and is generally regarded as a non-genotoxic carcinogen (Gelderblom *et al.*, 1992). A recent study indicated that

FB_1 induces chromosomal aberrations in primary hepatocytes (Knasmuller *et al.*, 1996) while different *in vitro* and *in vivo* studies reported on the induction of oxidative damage (Gelderblom *et al.*, 2001a).

2.1.2. Chemical structure of the fumonisins

In total, 6 fumonisins have been isolated and characterised. Of these, fumonisin B_{1-3} (FB_1, FB_2, and FB_3) are the major fumonisins occurring naturally on maize, while fumonisin B_4 (FB_4) and the N-acetyl derivatives fumonisins A_1 (FA_1) and A_2 (FA_2) are produced in less significant levels. The fumonisins are diesters of propane-1, 2,3-tricarboxylic acid and 2-amino-12, 16-polyhydroxy-icosanes. The chemical structures of the 3 major fumonisins (FB_1, FB_2, and FB_3) are illustrated in Figure 2.1 (Bezuidenhout *et al.,* 1988).

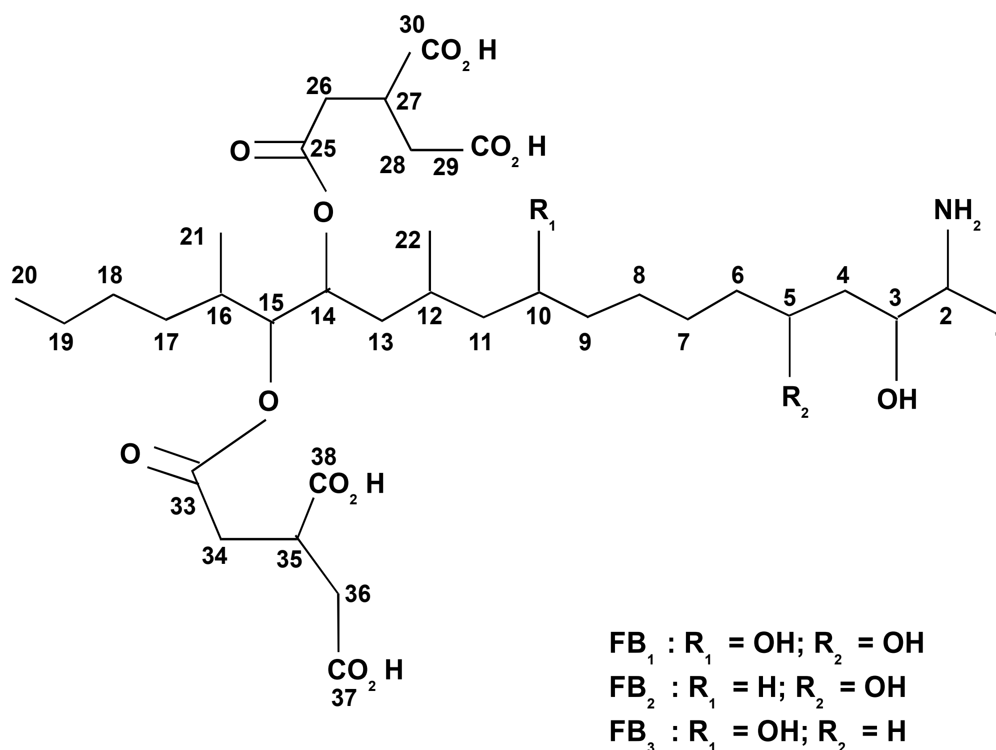

Fig 2.1. Structure of the 3 major fumonisins (FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3).

Amongst the different analytical methods that have been used to determine the fumonisins, high performance liquid chromatography (HPLC) has proven to be most reproducible and sensitive, and is used by many laboratories (Shephard *et al.,* 1996a).

2.1.3. Biological activity and pathogenesis

Sydenham *et al.* (1993) suggested that the toxicological effects of FB_1 result from extremely low levels retained within the organism or by secondary reactions initiated by the toxin. FB_1 has been shown to be poorly absorbed from the alimentary tract; it is cleared rapidly from circulation in plasma (half-life T½ = 20min) and is excreted primarily in faeces (90%), even after intravenous administration (Shephard *et al.*, 1992; Norred *et al.*, 1993; Prelusky *et al.*, 1994). Small quantities are retained in liver and kidney and excreted via bile and urine respectively.

FB_1 bears considerable structural similarity to the long-chain (sphingoid) base backbones of sphingolipids (SL). The compound is known to disrupt the *de novo* pathway of sphingolipid biosynthesis by inhibiting the activity of sphingosine N-acyltransferase (ceramide synthase) (Merrill *et al.*, 1993b). This in turn results in a reduction in the conversion of [^{3}H] sphingosine (So) to [^{3}H] ceramide and an increased amount of sphinganine (Sa). Subsequently, the ratio of free Sa to free So (Sa/So) in serum and tissues increases. This was found when rats, ponies and pigs were exposed to fumonisins in their feed (Riley *et al.*, 1994).

Sphingolipids are thought to be involved in the regulation of cell growth, differentiation, and neoplastic transformation through participation in cell-to-cell communication, cell-substratum interactions, and possible interactions with cell receptors and signalling systems (Hannun *et al.*, 1986; Ballou *et al.*, 1992; Schroeder *et al.*, 1994). Interference with these normal cell activities could be the basis of the toxicity of FB_1. Tolleson *et al.* (1999) suggested that accumulation of excess Sa and depletion of ceramide or complex SL derived from ceramide could be SL-dependant mechanisms for FB_1-induced in vitro apoptosis of cells. The most sensitive target organs would presumably be less tolerant of sphingolipid dysregulation (Bucci and Howard, 1996b).

It has recently also been shown that other mechanisms of hepatotoxicity are involved. These include effect of FB_1 on lipid biosynthesis (Gelderblom *et al*, 1996a), fatty acid accumulation (Gelderblom *et al*, 1996a), and oxidative stress

(Abel and Gelderblom, 1998). Lipid peroxidation has been shown in cell membrane preparations (Yin *et al.* 1998) and isolated rat liver nuclei (Sahu *et al.*, 1998) and in primary rat hepatocytes and rat liver *in vivo* (Abel and Gelderblom, 1998). FB_1 disrupts membrane structure and permeability through elevation of the oxidation rate, free radical production, and lipid peroxidation (Yin *et al.* 1998). *In vitro* studies have shown that lipid peroxidation is dose dependant and corresponds to the cytotoxic effect of FB_1 (Abel and Gelderblom, 1998). However, it appears to be secondary to the FB_1-induced hepatoxicity. Cellular membranes consist largely of phospholipids, which contain fatty acids (Horribin, 1990). Disruption of the phospholipid and n-6 fatty acid metabolic pathways, occurring at low dietary levels of FB_1, needs to be further investigated, especially in regard to cancer promotion (Gelderblom *et al.*, 2001a).

2.1.4. Cancer initiation and promotion

Fumonisins are non-genotoxic, and appear to have weak cancer initiating properties, but function mainly as tumour promoting agents (Gelderblom *et al.*, 1994). This was demonstrated in the short-term study by Gelderblom *et al.*, (1988a) in which FB_1 was also isolated. Studies based on the “resistant hepatocyte” model developed in the liver by Solt and Farber (1976) have also been conducted to show the cancer initiating and promoting potential of FB_1 (Gelderblom *et al.*, 1994). Carcinogenesis is a multi-step process, comprising of initiation, promotion, and progression (Farber and Sarma, 1987). The resistant hepatocyte model encompasses the induction of hepatocytes resistant to the growth-inhibitory or toxic effects of certain carcinogens (for example, genotoxic carcinogens induce this phenotype very rapidly). The stimulatory effect of cell proliferation on cancer initiation was proposed by Cayama *et al.*, (1978). During cancer promotion, the outgrowth of the initiated cell is stimulated, while the growth of normal hepatocytes is retarded (Farber, 1991). In the progression phase, cell populations with a malignant phenotype and growth pattern are established. In the case of FB_1, the balance between the mitoinhibitory effect on the proliferation of normal cells and the stimulation of hepatocyte regeneration due to the hepatotoxicity will determine if cell proliferation will reach a critical point to support the cancer initiating event. Gelderblom *et al.* (1994) showed

that 29.7 mg FB_1/100g body weight (BW) for 21 days effected initiation. The same dose for 7 days did not, suggesting that the inhibitory effect on cell proliferation was dominant. Also, FB_1 has been shown to induce apoptosis (Lemmer *et al.*, 1999), which is known to reduce the number of initiated cells (Bursch *et al.,* 1992). FB_1 has been shown to effect cancer promotion at relatively low doses (50mg/kg diet) without inducing excessive hepatotoxicity (Gelderblom *et al.*, 1996c). This mechanism can be attributed to the mitoinhibitory effect of FB_1 on normal hepatocytes presumably via the disruption of different cell regulatory processes (Gelderblom *et al.*, 1995).

2.1.5. Toxicity in animals

Comparative toxicity studies of culture material of *F. verticillioides* indicated that the target organs vary in different animal species, whereas the liver and kidneys seem to be constantly affected.

2.1.5.1. Rodents

Hepatotoxicity and carcinogenicity in rats and mice

In a chronic feeding study, freeze-dried corn cultures (batch MRC 826B) fed to BD IX rats at different levels (2-4%) induced liver cancer in 80% and ductular carcinoma in 63% of surviving rats after 450 days (Marasas *et al.*, 1984). At a lower dose of 0.5%, the same culture material produced neoplastic nodules, ductular hyperplasia, cholangiocarcinomas, and metastasising hepatocellular cancer (HCC) in 2 rats (Jaskiewicz *et al.*, 1987). Unlike the first study, little fibrosis was found. Basal cell hyperplasia was observed in the oesophagus of 12 of the 21 rats and oesophageal papilloma in 1 animal. Wilson *et al.* (1985a) fed a corn-only diet (deficient in vitamins and lipotropes) to male Fisher 344 (F344) rats and induced nodules, adenofibrosis, and cholangiocarcinomas in the liver after 4 to 6 months. The corn was naturally contaminated with *F. verticillioides* and was associated with an ELEM outbreak.

After the isolation of FB_1 by Gelderblom *et al.* in 1988 (a), many medium and short-term studies were conducted with known levels of the toxin. FB_1 has since been demonstrated to be *hepatotoxic* and *hepatocarcinogenic* in rats (Gelderblom, 1996b; Lemmer *et al.*, 1999). Short-term studies induce the

proliferation of bile ductules, fibrosis, nodular regeneration, and hepatocellular necrosis (Gelderblom *et al.*, 1988a). Early findings also include small rounded, eosinophilic hepatocytes with irregular chromatin, apoptosis, minimal inflammatory reaction, necrotic cells, and an elevation in parameters such as alanine transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), cholesterol, and triglycerides (Voss *et al.*, 2001). With time, an increase in apoptotic and necrotic cells, mitotic figures, vacuolisation, cytomegaly, and pleomorphism is seen. Advanced lesions include bile duct and oval cell proliferation, foci of cellular alteration, cholangiomatous lesions, and fibrosis (Gelderblom *et al.*, 1991). Hyperplastic foci staining positively for gamma glutamyl transpeptidase (GGT) and the placental form of glutathione S-transferase (GSTP) were also reported (Gelderblom *et al.*, 1988a, 1993, and 1996c). Apoptosis of proximal tubule epithelial cells and the resultant cell proliferation in the kidneys have been described, resulting in decreased kidney weights (Voss *et al.*, 2001). An increase in serum creatinine, and GGT, LDH, and protein in urine accompany these lesions.

In a long-term study, male BD IX rats were fed FB_1 at 50 mg FB_1/kg diet (Gelderblom *et al.*, 1991). After 6 months, regenerative nodules and cholangiofibrosis were seen. Between 18-26 months, the liver was characterised by micro- and macronodular cirrhosis with large expansive nodules of cholangiofibrosis. Within nodules, fatty changes, hyaline droplet degeneration, necrosis and areas with a “ground-glass appearance” were seen histologically. HCC was induced in 66% of the rats (metastasising in 4 rats), and 100% of the animals developed cholangiofibrosis. The kidneys showed diffuse interstitial lymphocytic nephritis and mild glomerulonephritis.

In a chronic study of 24 months, low levels of FB_1 (1, 10, 25mg/kg semi-purified diet) were fed to BD IX rats (Gelderblom *et al.*, 2001c). A dose response and the existence of a threshold were thus demonstrated for the induction of hepatocyte nodules. No HCCs or cholangiofibrotic lesions were seen between 18 and 24 months. At the high dose, major lesions included anisokaryosis, neoplastic nodules, oval cell proliferation, bile duct hyperplasia, lobular

distortion, and portal fibrosis while all rats terminated at the end of the study showed foci staining positively for GSTP ($GSTP^+$). At the lower dose, lesser lesions were described and at the lowest level of FB_1 only mild toxic lesions were seen.

In an additional long-term study, FB_1 was fed to female and male F344 rats at various concentrations over a 2-year period (Howard *et al.*, 2001). Female rats received 0, 5, 15, 50 and 100 ppm FB_1 and no occurrence of tumours was noted in any of the organs. Male rats fed 0, 5, 15, 50 and 150 ppm FB_1 exhibited renal tubule adenomas and carcinomas at the 50 and 150 ppm dose levels. Contrary to other studies, no hepatic pathology was noted in either male or female rats.

In addition to liver and kidney toxicity, the immune system is also affected. Bondy *et al.* (1995) described disseminated thymic necrosis, decreased thymic weight and increased serum immunoglobulin M (IgM) concentrations.

Studies in mice have also shown the liver and kidneys to be the target organs, though mice appear to be more resistant to nephrotoxic effects of FB_1 than rats (Voss *et al.*, 1995). They show a similar liver pathology as in rats (Bucci *et al.*, 1998; Sharma *et al.*, 1997; Bondy *et al.*, 1997) and females were shown to be more sensitive than males. In a chronic 2-year study, FB_1 was fed to female and male $B6C3F_1$ mice at various concentrations (Howard *et al.*, 2001). Female mice were fed 0, 5, 15, 50, and 80 ppm FB_1 and the incidence of hepatocellular adenomas was increased at 50 and 80 ppm. Hepatocellular carcinomas were also detected in the two high dose groups. FB_1 did not enhance the incidence of tumours (adenomas and carcinomas) in male mice.

FB_1 does not cross the placenta (Voss *et al.*, 1996) and is not teratogenic (Collins *et al.*, 1998), but may be embryotoxic at maternally toxic doses in mice (Reddy *et al.*, 1996).

Hamsters

The developmental toxicity of fumonisins was tested on pregnant Syrian hamsters (Floss *et al.*, 1994; Penner *et al.*, 1998). At levels up to 18 mg FB_1/kg,

foetal prenatal deaths and delayed foetal development increased in a dose dependant manner, although no clinical signs were observed in the mother animals.

2.1.5.2. Equines

Equine leukoencephalomalacia (ELEM) is a non-contagious, acute, and highly fatal neurological disease affecting horses, donkeys, and mules. It has been referred to as the blind staggers, cerebritis, mouldy corn disease, leukoencephalitis, corn stalk disease, and foraging disease (Wilson *et al.*, 1973). ELEM has been documented in various countries such as the USA (Ross *et al.*, 1990), Egypt (Wilson *et al*, 1971), South Africa (Kellerman *et al*, 1972), New Caledonia (Domenech *et al*, 1985), Argentina (Monina *et al*, 1981), Brazil (Riet-Correa *et al*, 1982), and China (Iwanoff *et al*, 1957), to name a few. Widespread outbreaks in the USA in 1989/1990 and in 1995 claimed the lives of many horses (Ross *et al.*, 1990; House *et al.*, 1995). The disease has been attributed to the consumption of mouldy corn since the beginning of this century (Butler, 1902) and the causative agents were identified as fumonisin B_1 and B_2 (Marasas *et al.*, 1988b) only 80 years later.

Equines are very sensitive to dietary fumonisin exposure, although differences in susceptibility seem to exist (Schwarte *et al.*, 1937; Wilson *et al.*, 1992). The factors that may be important in the appearance of clinical disease include differences in individual resistance, length of exposure, level of contaminant, previous exposure and possibly other conditions concurrently afflicting the animal. Adults also appear to be more susceptible (Wilson *et al*, 1973). The onset of clinical signs after exposure to the toxin varies from a few days to several weeks (Kellerman *et al.*, 1990). The pathognomonic finding is liquefactive necrosis of the white matter of the cerebral hemispheres, though the name is misleading as the grey matter can also be affected, including the cerebellum, brain stem, and spinal cord (Iwanoff *et al.*, 1957; Marasas *et al.*, 1988b). It was once suggested that, in an apparent dose dependent manner, equines could either develop the neurotoxic form or hepatosis without neurological symptoms and in some cases both symptoms simultaneously (Marasas *et al*, 1976). In experimental cases of high doses given over short

periods of time, a hepatic syndrome manifested by icterus, systemic haemorrhages, and oedema was the predominant outcome. Lower doses over longer periods of time produced the classic form of ELEM with neurological symptoms (Marasas *et al.*, 1976). The diagnosis of ELEM is mostly based on gross pathological and/or histological findings. Morbidity is generally 25%, and although mortality usually approaches 100% (Diaz and Boermans, 1994), animals can survive, often with residual neurological deficits (Caramelli *et al.*, 1993; Diaz and Boermans, 1994). Treatment, however, is limited to symptomatic and supportive care aimed at minimising inflammation and self-trauma (Wilkins *et al.*, 1994). It has been suggested by the Mycotoxin Committee of the American Association of Veterinary Laboratory Diagnosticians that the level of FB_1 in the feed should not exceed 5 ppm (Miller *et al.*, 1996).

The pathogenesis of ELEM has not been completely elucidated. The mycotoxins have not been shown to cross the blood brain barrier (Norred *et al.*, 1998). Thus the toxic effect following exposure to FB_1 could be related to secondary effects. Brain tissue is rich in SL, which are important structural components of membranes (Norred *et al.*, 1998), and Wang *et al.* (1992) have demonstrated the inhibition of SL biosynthesis in ponies fed diets containing 15-44 µg FB_1/g feed. Inhibition of brain ceramide synthase by fumonisins, and resultant depletion of SL and increase of cytotoxic Sa and So could be hypothesised to lead to the necrosis. The subsequent elevation of the SL biomarkers in serum also precedes elevation of any other clinical chemistry, especially liver enzymes (Wang *et al.*, 1992; Yoo *et al.*, 1992). However, it has also been demonstrated in a study in which horses consumed FB_1 contaminated feed that the brains of horses, with and without pathological lesions, did not have altered Sa/So ratios, although many other tissues did (Goel *et al.*, 1996). The lesion is grossly similar to that observed after cerebral haemorrhage, suggesting a vascular origin rather than a direct toxic effect on brain tissues (Wilson *et al.*, 1973; Norred *et al.*, 1998). Regions of leukoencephalomalacia (degeneration of the white tissue) are near edges of blood vessels and these areas are strongly infiltrated by inflammatory cells (Xavier *et al.*, 1991). The disruption in the microcirculation of the white matter could result in oedema (Wilkins *et al.*, 1994). Constable *et al.* (2000) has shown

that fumonisin-induced pulmonary oedema in pigs could be due to sphingosine-mediated calcium-channel blockade of the heart and blood vessels. It has thus been proposed that the pathophysiology of ELEM is also related to cardiovascular dysfunction and ELEM perhaps results from an inability to appropriately regulate blood brain flow as the horse lowers its head to eat and drink.

2.1.5.3. Pigs

In a comparative study by Kriek *et al.* (1981), culture material of *F. verticillioides* MRC 826 on autoclaved maize was fed to various animals including pigs. Three doses of 5 g/kg BW were administered and 2 pigs died within 6 days from severe pulmonary oedema. As equine leukoencephalomalacia was also induced with the same culture material, it was suspected that the metabolite causing ELEM and pulmonary oedema would be the same. In 1989, numerous outbreaks of pulmonary oedema and hydrothorax were documented in swine in the USA, concurrently to ELEM outbreaks in horses (Harrison *et al.*, 1990; Osweiler *et al.*, 1992). The disease was linked to the consumption of maize screenings contaminated with *F. verticillioides* and the lesions were identical to those in the study conducted by Kriek *et al.* (1981). Clinical symptoms subsided within a day after removal of the contaminated feed. The affliction received the name “porcine pulmonary edema syndrome” (PPE) (Ross *et al.*, 1990) and it is suspected that the disease has been documented since the 1950’s in Hungary, where it is known as “fattening or unique pulmonary edema of pigs” (Fazekas *et al.*, 1998).

Subsequently, the syndrome was induced experimentally with maize screenings implicated in the 1989 outbreaks (Harrison *et al.*, 1990; Osweiler *et al.*, 1992; Haschek *et al.*, 1992). Pure FB_1 injected intravenously at a dose of 0.4 mg/kg BW/day (d) for 4 days also effected PPE (Harrison *et al.*, 1990). This, however, could not be reproduced in two other studies (Osweiler *et al.*, 1992; Haschek *et al.*, 1992), while PPE has also not been reproduced experimentally by the oral administration of purified FB_1.

Onset of clinical signs can be very abrupt and include intense respiratory distress, decreased heart rate, apathy, cyanosis in the skin and mucous membranes, and recumbency (Osweiler *et al.*, 1992; Smith *et al.*, 1999). Vomiting and diarrhoea have also been reported and recently haemodynamic changes have been measured indicating cardiovascular injury preceding the development of pulmonary oedema (Colvin *et al.*, 1993; Smith *et al.*, 1996 a, b; Gumprecht *et al.*, 1998; Smith *et al.*, 1999; Constable *et al.*, 2000; Smith *et al.*, 2000). Some animals have died before showing any signs. Pigs develop PPE within 4 to 7 days of consumption of feed containing culture material (with fumonisin at levels of >91 ppm or >15 mg/kg BW/d), and within 7 days of daily intravenous doses of FB_1 (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Colvin *et al.*, 1993; Motelin *et al.*, 1994; Gumprecht *et al.*, 1998). Abortions have also been observed after feeding with maize screenings (Harrison *et al.*, 1990). This has, however, not been linked experimentally with fumonisin B_1. It has recently been shown that FB_1 can cause damage to foetuses in utero (Zomborszky-Kovacs *et al.*, 2000). Pulmonary oedema and pathological as well as biochemical changes indicative of liver damage were found in piglets immediately following parturition.

Gross pathologically and histopathologically, an accumulation of clear straw-coloured fluid that clotted upon opening the thorax, and interstitial and interlobular oedema have been found. Accumulation of membranous material (multilamellar bodies) in pulmonary intravascular macrophages in pigs with pulmonary oedema has also been described. FB_1 has also been shown to induce liver injury. Acute changes include disorganisation of hepatic cords, hepatocellular vacuolation, megalocytosis, apoptosis, necrosis, and cell proliferation (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Colvin *et al.*, 1993; Motelin *et al.*, 1994; Gumprecht *et al.*, 1998). Accumulation of membranous material (multilamellar bodies) within hepatocytes and Kupffer cells has also been found (Haschek *et al.*, 1992). In chronic studies early “perilobular fibrosis”, hepatocellular necrosis and nodular hyperplasia is characteristic (Colvin & Harrison, 1992; Casteel *et al.*, 1993; Colvin *et al.*, 1993). Liver pathology has only been found in pigs that survived experimentally

induced PPE (Colvin & Harrison, 1992; Haschek *et al.* 1992; Osweiler *et al.*, 1992; Casteel *et al.*, 1993; Colvin *et al.*, 1993; Motelin *et al.*, 1994).

Pancreatic lesions, hyperplastic oesophageal plaques, gastric ulceration, hypertrophy of the heart and medial hypertrophy of the pulmonary arteries have also been reported (Harrison *et al.*, 1990; Casteel *et al.*, 1993; Casteel *et al.*, 1994; Guzman *et al.*, 1997). In a study conducted by Motelin *et al.*, (1994) using maize screenings containing FB_1 and FB_2 at varying levels, a dose related increase in the serum biochemical parameters ALP, AST, GGT, bilirubin and cholesterol was reported. Haematological values of fumonisin-fed pigs remain within normal limits (Motelin *et al.*, 1994; Rotter *et al.*, 1996).

Levels as low as 5 ppm in the feed have resulted in elevations of the Sa/So ratio (Motelin *et al.*, 1994; Riley *et al.*, 1993) which has led to the recommendation by the Mycotoxin Committee of the American Association of Veterinary Laboratory Diagnosticians that the level of FB_1 in the feed should not exceed 10 ppm (Miller *et al.*, 1996).

In chronic toxicity studies, in addition to hepatotoxicosis, lesions in the oesophageal mucosa and decreases in cardiac output were described (Casteel *et al.*, 1993). Casteel *et al.* (1994) also found medial pulmonary arterial hypertrophy indicative of hypertension. Reduced body weight gain and immuno-suppressive effects resulting from chronic exposure to fumonisin contaminated feed is one of the principal concerns from an economical point of view (USDA/APHIS Veterinary Services Fact Sheet, 1995; Rotter *et al.*, 1996; Smith *et al.*, 1996; Gumprecht *et al.*, 1998). It has been shown that fumonisin residues accumulate in liver and kidneys when fed over extended periods, thus low concentrations are proved to be toxic (Prelusky *et al.*, 1996).

It has recently been hypothesised that PPE in pigs is due to fumonisin induced cardio-vascular impediments (Smith *et al.*, 2000). Pigs treated with 20 mg/kg BW/d for 3 days had lower cardiac outputs and heart rates and showed a marked reduction in cardiac contractility. This decreased cardiac contractility results in an increase in pulmonary artery wedge pressure and subsequent

pulmonary oedema (Haschek *et al.*, 2001). These cardio-vascular effects are consistent with sphingosine mediated L-type Ca^{2+} channel blockade. Pulmonary oedema in pigs that consumed fumonisin is thus primarily due to acute left sided heart failure instead of increased vascular permeability as inhibition of cardiac function occurs without alteration in alveolar/capillary membrane permeability (Constable *et al.*, 2000; Haschek *et al.*, 2001).

2.1.5.4. Non-human primates

Studies with primates were first conducted on baboons (Kriek *et al.*, 1981). Culture material of the *F. verticillioides* strain MRC 602 was fed for 5 months to 2 years at varying dietary concentrations. One animal developed acute congestive heart failure and in another, liver cirrhosis was the principle lesion.

Various amounts of culture material of *F. verticillioides* strain MRC 826 were fed to vervet monkeys (Cercopithecus pygerythrus) in a long-term study over a period of 13.5 years using a low fat carbohydrate diet. The fungal isolate was obtained from home-grown maize in an area in Transkei, South Africa, with a high rate of human OC. Jaskiewicz *et al.* (1987) reported the first results obtained after 180 days. Acute, sub-acute and chronic toxic hepatitis of various degrees was induced in all experimental monkeys, demonstrating close similarity with human viral hepatitis. Disturbance of the trabecular structure, degeneration and necrosis of hepatocytes, mononuclear infiltration and cirrhosis in severe cases were seen. Elevation of serum liver enzymes was also observed.

Fincham *et al.* (1992) demonstrated an atherogenic effect of *F. verticillioides* in the vervet monkeys of the same experiment. The dietary level of less than 0.5% w/w of culture material of strain MRC 826 corresponded to the contamination of a human diet. The finding included an atherogenic plasma lipid profile, increased plasma fibrinogen, and activity of blood coagulation factor VII, which could enhance atherogenesis. It was hypothesised that these alterations were secondary to chronic hepatotoxicity characterised by liver fibrosis and elevated cholesterol, albumin and serum enzymes AST, ALT, LDH, GGT and ALP.

At the termination of the 13.5-year study, the results indicated active chronic toxic hepatitis, as seen histopathologically and in elevated liver function enzymes (Gelderblom *et al.*, 2001d). In addition, cholesterol and total bilirubin were increased. The kidneys were also affected resulting in an increase in serum urea, creatinine, and creatine kinase. A significant decrease in blood parameters such as white and red blood cells was also observed.

In this same long-term study, it was shown that FB_1 disrupts sphingoid metabolism (Shephard *et al.*, 1996b; van der Westhuizen *et al.*, 2001) and the serum Sa/So ratio (van der Westhuizen *et al.*, 2001). It was also shown that the accumulation of the fumonisins in the hair of the vervet monkeys could be used to assess fumonisin exposure (Sewram *et al.*, 2001).

2.1.5.5. Poultry

The effect of fumonisins and fumonisin-containing *F. verticillioides* culture material has been investigated in chickens, ducklings, and turkey poults. Similar results were found in short-term studies on the various species. In a dose response fashion, levels between 75 and 400 mg FB_1/kg feed resulted in a reduction in feed intake, weight gain and feed conversion (Brown *et al.*, 1992; Ledoux *et al.*, 1992; Weibking *et al.*, 1993a; Bermudez *et al.*, 1995). In addition Ledoux *et al.* (1992) reported diarrhoea and rickets. An increase in the weights of organs such as the liver, kidney, proventriculus, and pancreas was also documented (Brown *et al.*, 1992; Ledoux *et al.*, 1992; Weibking *et al.*, 1993a, b; Bermudez *et al.*, 1995). Histopathologically, lesions including hepatic necrosis, biliary hyperplasia, thymic cortical atrophy, and widening of the proliferating cartilage zone in the proximal tibiotarsal epiphyses have been described. Serum calcium, cholesterol and AST were reportedly elevated at higher levels of fumonisin (Ledoux *et al.*, 1992; Weibking *et al.*, 1993a). In addition, culture material containing levels of 75 mg FB_1/kg feed has been shown to increase Sa and Sa/So ratios in young chicks (Weibking *et al.*, 1993a). Chronic studies involving feeding turkey poults 75 mg FB_1/kg feed for 18 weeks also showed a decrease in weight gain, increased liver weights, and also an increase in total white blood cell count, absolute heterophil count, absolute lymphocyte count,

and heterophil:lymphocyte ratio (Bermudez *et al.*, 1996). These FB_1 levels did not result in mortality.

In the various studies, high dietary levels of fumonisins were used to show a toxic effect on poultry. Levels lower than 80 mg/kg feed have been shown not to result in changes of parameters such as body weight, feed efficiency, or water consumption. (Henry *et al.*, 2000). In one recent study, however, levels as low as 5 mg FB_1/kg feed/d for 12 days resulted in liver alterations in ducks (Bailly *et al.*, 2001). In general, though, poultry appear to be relatively resistant to FB_1. As high concentrations do not necessarily reflect contamination levels and consumption by average birds, the significance for the poultry industry can be deemed low (Pittet *et al.*, 1992; Diaz and Boermans, 1994).

2.1.5.6. Ruminants

Ruminants appear to possess a lower susceptibility to fumonisins occurring naturally in feed than horses and pigs. In a feeding study of up to 148 ppm FB_1 for 31 days, no effect on weight gain or feed intake was observed, but impairment of liver and to some extent immune function was described (Osweiler *et al.*, 1993). Administration of 1 mg FB_1/kg/d intravenously to milk-fed calves for 7 days resulted in lethargy and a reduction in appetite (Mathur *et al.*, 2001). Biochemical parameters were assessed and showed indications of liver and renal damage. Pathological lesions in the liver included disorganised hepatic cords, varying severity of hepatocyte apoptosis, hepatocyte proliferation, and proliferation of bile ductular cells. Kidney alterations were characterised by vacuolar change, apoptosis, karyomegaly, and proliferation of proximal renal tubular cells, as well as dilation of proximal renal tubules. Serum Sa concentrations were also shown to be increased. Cardiovascular changes such as those seen in pigs were not found.

Studies with angora goats have also been conducted (Gurung *et al.*, 1998). At levels of 95 mg FB_1/kg for 112 days, no clinical signs were documented. However, in addition to an elevated Sa/So ratio in tissue, a time dependant increase in levels of AST, LDH, GGT, cholesterol and triglycerides showing evidence of mild liver damage and kidney dysfunction were observed.

Acute “nephrosis” and “hepatosis” in sheep fed *F. verticillioides* culture material were recorded in a comparative study performed by Kriek *et al.* (1981). Two sheep administered culture material via a rumen fistula died after 8 doses at 5 g/kg over 10 days (total dose 1920g) and 11 doses over 12 days, respectively. The results were confirmed in 1995 in lambs dosed intraruminally with *F. verticillioides* culture material (Edrington *et al.*, 1995). Fifteen lambs were dosed up to 45 mg FB_1/kg BW total fumonisins for 4 days and terminated on day 11. In this study, feed intake decreased and biochemical parameters were again indicative of liver and kidney damage. Histologically tubular nephrosis and mild hepatopathy was described. The lambs receiving the highest dose died before termination.

A white tailed deer showing neurological symptoms similar to those of ELEM were described in a case in the United States in 1989 (Howerth *et al.*, 1989). The animal had apparently eaten mouldy maize contaminated with *F. verticillioides*. This is the only case of ELEM symptoms documented in ruminants.

In a study where FB_1 was incubated in ruminal fluid for 72 hours (1 mg/l), it was shown that it is poorly metabolised in the rumen (Caloni *et al.*, 2000). FB_1 was not detectable in milk in cows fed an average of 3 mg FB_1/kg BW/d for 14 days, and thus milk does not seem to pose a hazard for human consumption (Richard *et al.*, 1996).

2.1.5.7. Other animals

Rabbits appear to be very sensitive to the toxic effects of purified FB_1 and kidneys, liver and brain have been demonstrated to be the target organs. Multiple intravenous doses of up to 1 mg/kg BW/d for 4 or 5 days caused rabbits to be lethargic and anorectic, and clinical chemistry parameters corresponding to liver and kidney injury and Sa/So ratio in tissues were increased (Gumprecht *et al.*, 1995). However, a single dose of 1.25 mg/kg apparently induces only kidney damage. Histopathologically, the toxicity manifests itself as severe proximal tubular necrosis and mild necrosis, hepatocyte vacuolation, and bile stasis. Doses at levels from 0.5-1.75 mg

FB_1/kg/d have been shown to be lethal for adult pregnant rabbits (Bucci *et al.*, 1996a; LaBorde *et al.*, 1997). FB_1 did not cross the placenta and was not embryotoxic (LaBorde *et al.*, 1997). Two animals that died after a gavage of 1.75 mg/kg/d for respectively 9 and 11 days showed leukoencephalomalacia and haemorrhage in the brain (Bucci *et al.*, 1996).

In the 1960's, aflatoxin B_1 (AFB_1) was shown to cause liver cancer in trout (Halver, 1968). To determine the toxicity of the newly discovered mycotoxin FB_1, feeding studies with *F. verticillioides* culture material containing known levels of FB_1 subsequently were done on channel catfish, *Ictalurus punctatus* (Brown *et al.*, 1994; Lumlertdacha *et al.*, 1995). It was shown that levels of 20 mg/kg and higher are hepatotoxic to year-1 and year-2 channel fish. Carlson *et al.* (2001) also reported the promoting effects of FB_1 on AFB_1 initiated liver tumours. Without a known initiator however, FB_1 at levels up to 104 ppm for 34 weeks did not induce any tumours.

Diets containing *F. verticillioides* culture material contaminated with FB_1, FB_2, and FB_3 have also been fed to adult female minks (Restum *et al.*, 1995). The minks in the treatment group were lethargic and clinical chemistry parameters were altered. No other clinical signs or histopathological lesions were determined. A further study was conducted to determine the effect of dietary fumonisins on the reproductive performance (Powell *et al.*, 1996) of adult female minks. It was shown that the breeding behaviour was not affected, but minks receiving fumonisin had smaller litter sizes, the number of stillborn kits was higher, and the kits had reduced body weights at birth. Hepatocyte vacuolation and alterations in clinical parameters were also noted in the mothers. Fumonisin content in the milk was detected to be 0.7% of the dietary FB_1 concentration, which however did not have an affect on the survival rate of the kits. A similar study also reportedly documented increased free Sa, So and the Sa/So ratio in the urine in mink (Morgan *et al.*, 1997).

2.1.6. Toxicity in humans

Oesophagus

The National Cancer Registry of South Africa has reported oesophageal cancer (OC) to be the most common cause of cancer deaths in black males in South Africa, and the third most common in black females (Sitas *et al.*, 1997). The rate of OC in both sexes in the southern Transkei is the highest in the world, while the frequency in the northern Transkei is comparably low (Jaskiewicz *et al.*, 1987; Makaula *et al.*, 1996). The southern Transkei is an ecological zone that provides ideal conditions for infection of corn ears by *F. verticillioides* and subsequent fumonisin production (Marasas *et al.*, 2001). Home-grown maize is the major dietary staple in southern Africa and is also used in home-brewed beer in the Transkei (Van Rensburg, 1981; Cook, 1971; Marasas *et al.*, 1988a). *F. verticillioides* has been shown to be the most prevalent fungus on maize in the area with highest incidence of OC (Marasas *et al.*, 1981) and the correlation between OC and fumonisins has also been established (Sydenham *et al.*, 1990).

Many factors have been implicated in the development of OC. Alcohol and tobacco smoking are risk factors in OC (Blot, 1994); however a correlation has not been established in the Transkei (Rose, 1973; Sammon *et al.*, 1992). Deficiencies in vitamin A, vitamin B12, vitamin E, selenium, and folic acid were determined in persons from the high incidence area in the Transkei, compared to the low incidence region (Van Helden *et al.*, 1987 Jaskiewicz *et al.*, 1988a). However, levels of zinc, copper, and magnesium were not different between the two population groups. Deficiencies in these nutrients as well as manganese and molybdenum play a role in the development of OC (Van Rensburg, 1985). Exposure to other mycotoxins poses a risk for certain cancers such as OC and liver cancer. Such mycotoxins include AFB_1, the mutagen fusarin C (Gelderblom *et al.*, 1984), and other metabolites produced by *F. verticillioides* (Bever *et al.*, 2000). Conflicting data has been presented in regard to the risk of the consumption of traditional beer. Sammon *et al.* (1992) found that the beer did not play a role in the aetiology of OC in the Transkei, while Segal *et al.* (1988) reported it to be a major risk factor in Soweto, South Africa.

High incident rates of OC have been reported in other areas of the world such as the Linxian County of Henan Province in northern China (Li *et al.*, 1980; Yang, 1980), and the Caspian littoral of Iran (Kmet and Mahboubi, 1972; Hormozdiari *et al.*, 1975). High levels of fumonisin contamination were found in the Linxian County (Chu and Li., 1994), where maize is a large part of the staple diet (Li *et al.*, 1980; Yang, 1980; Zhen, 1984). Fumonisin contamination has been determined in other high incidence areas for OC such as Zimbabwe (Sydenham *et al.*, 1993), the Pordenone Province, Italy (Pascale *et al.*, 1995), and Charleston, South Carolina, USA (Sydenham *et al.*, 1991) where maize is also consumed in high quantities (Rossi *et al.*, 1982; Franceschi *et al.*, 1990; Brown *et al.*, 1988).

A role of fumonisin in the aetiology of OC has however not been conclusively demonstrated and there is thus far no evidence that fumonisins initiate or promote OC in animals. There is also no epidemiological evidence of cancer being linked to fumonisin consumption in animals.

Neural tube

Recent *in-vitro* studies have shown that FB_1 blocks cellular uptake of folate, an important vitamin for cellular physiology (Stevens and Tang, 1997; Wolf, 1998). Folate is also essential in the early stages of pregnancy for organogenesis (Lucock *et al.*, 1998). Recently, the adverse effect of FB_1 on folate uptake by exposure to dietary FB_1 has been implicated in the development of NTD. High rates in blacks in the Transkei region of the Eastern Cape Province, South Africa, have been documented (Ncayiyana, 1986; Venter *et al.*, 1995) and shown to be 5-10 times higher than those residing in Cape Town (Cornell *et al.*, 1983). A number of cases were reported in southern Texas in 1990/1991 (Hendricks, 1999) and in the Hebei Province of China (Moore *et al.*, 1997). These are all areas where high quantities of maize are consumed and where high levels of fumonisins have been determined (Sydenham *et al.*, 1990; Sydenham *et al.*, 1991; Chu and Li., 1994).

Liver

Studies on the possible role of fumonisins in HCC were conducted in China (Ueno *et al.*, 1997). It was demonstrated that in areas of a high incidence of HCC, higher levels of FB_1 contamination were also found. This correlation has not been found in the Transkei however (Makaula *et al.*, 1996). It has been shown in animal studies that synergism between AFB_1 and FB_1 could contribute to the development of HCC. This has been reported in hepatocarcinogenesis studies in rainbow trout and rats (Carlson *et al.*, 2001; Gelderblom *et al.*, 2002) and the findings in animal models, and in the Chinese study, raise the possibility that FB_1 may be a factor in human HCC.

2.2. Dietary iron overload

2.2.1. Iron metabolism

Iron is an essential component of haemoglobin, myoglobin, and many enzymes in the body and thus an important nutrient in a well-balanced diet (Andrews, 1999). An adult can absorb approximately 1-2 mg of dietary iron each day. Ferric iron is enzymatically reduced in the proximal small intestine to ferrous iron by ferrireductase (Riedel *et al.*, 1995). The divalent metal transporter 1 (DMT-1) enables this iron to be transported into the enterocytes of the villus tips of the duodenum, which is the site of major iron absorption (Gunshin *et al.*, 1997). The mechanism by which haeme iron (primarily found in meats) is absorbed is not entirely elucidated (Andrews, 1999). In the enterocytes, iron is then oxidised again to ferric iron by hephaestin and either stored in the enterocyte as ferritin, the major storage form of iron, or transported into the circulation by the transport protein Ireg1 (Vulpe *et al.*, 1999; McKie *et al.*, 2000). In serum, the absorbed ferric iron is bound to transferrin, a molecule that can bind 2 molecules of the oxidised form of iron (Rouault and Klausner, 1997). Usually a third of the circulating transferrin molecules are occupied with iron. The diferric transferrin then binds to a transferrin receptor (TfR) on the cellular plasma membranes of peripheral cells and endocytosis of TfR-transferrin complex takes place. Two types of TfR's have been identified (Kawabata *et al.*, 1999). After acidification, ferric iron is released from the complex and transported into the cytoplasm by DMT-1. Iron is then available for metabolism or for storage as ferritin, and the apo-transferrin is released to bind ferric iron.

The intestinal absorption of iron is controlled by a variety of factors; for example, high dietary intake of iron is self-limiting, as the enterocytes eventually stop absorbing iron due to high intra-cellular levels (Andrews, 1999). The expression of TfR on the cell membrane is regulated by 2 iron-regulatory proteins (IRPs), which in turn are modulated by the level of iron in the cell (Rouault and Klausner, 1997; Harford and Klausner, 1990; Addess *et al.*, 1997; Kim *et al.*, 1995; Iwai *et al.*, 1998). Iron deficiency increases expression of TfR and thus increases iron-uptake and reduces iron storage. Absorption mechanisms also respond to existing body stores and to erythropoiesis (Finch, 1994). The classical genetic haemochromatosis gene (HFE) encodes the protein expressed

in the deep crypt cells of the duodenum, the precursor cells to the enterocytes (Feder *et al.*, 1996). This protein has been shown to be closely associated with TfR at the plasma membrane and regulates the uptake of diferric transferrin in the enterocytes in response to information received by the crypt cells about total body iron stores and the erythrocyte production of the body.

The hepatocytes and the macrophages of the reticuloendothelial (RE) system are the main cells that store iron (Brittenham, 1994; Baker and Morgan, 1994). Iron exceeding the binding capacity of transferrin is taken up by hepatocytes after transport through the portal system and stored as ferritin. Iron deposition is found first in the periportal hepatocytes (zone 1). With increasing iron storage, deposits will also be found in hepatocytes in zones 2 (mid-zonal region) and 3 (perivenular region). Haemosiderin, brown granules visible in haematoxylin and eosin (H&E) stained sections by light microscopy, develops once the capacity for storage of ferritin is surpassed. Iron is utilised mainly in the bone marrow, where it is essential for haeme biosynthesis; haeme is then incorporated into the erythrocytes. RE cells recycle haeme iron by uptake of old erythrocytes and the iron is either stored or released into circulation for re-use (Andrews, 1999).

After absorption into the body, major excretion of excess iron is not physiologically possible, which emphasises the importance of intestinal regulation of iron homeostasis (Andrews, 1999). A healthy adult will not absorb excess dietary iron. An adult excretes approximately 1-2 mg of dietary iron each day, the same amount as absorbed from the diet. The enterocytes storing iron as ferritin are eventually sloughed into the lumen and excreted via the gastro-intestinal tract. Menstruating women also physiologically excrete iron; when blood loss is excessive, iron deficiency anaemia occurs. In addition, skin cell loss and sweating contribute to iron loss, and some iron is also excreted in urine (Bothwell *et al.*, 1979). Iron is also lost during pregnancy, childbirth, and lactation.

There are various methods of assessing iron body stores: measurement of serum ferritin and transferrin saturation; chelation tests using desferrioxamine-induced urinary iron excretion; a liver biopsy with histological grading and/or

biochemical measurement of hepatic the iron concentration (Powell *et al.*, 1994).

2.2.2. Clinical features and pathology of iron overload

Various clinical symptoms and signs occur as a result of iron overload. Depending on the aetiology and degree of iron overload, one can see skin pigmentation, diabetes mellitus, heart failure, hypogonadism and cirrhosis of the liver (Britton *et al.*, 1994). In advanced cases, cardiomyopathy and liver failure can be the cause of death. Men usually develop symptoms earlier than women, who physiologically lose iron via menstruation and pregnancy (Tavill and Bacon, 1990).

Perls' Prussian blue staining is used to illustrate iron distribution and deposition in the parenchyma cells of the affected liver (Britton *et al.*, 1994). Distribution varies, depending on the underlying disorder. In genetic haemochromatosis (GH), deposits are initially seen in hepatocytes in zone 1, the periportal region of the liver, with progressive loading of other hepatocytes throughout the liver lobule (Deugnier *et al.*, 1992). Thus, a gradient extending from zone 1 to zone 3 (perivenular) with maximum deposition in zone 1 develops with increased severity of iron loading. Kupffer cell and portal tract macrophage deposition increases gradually. In other disorders, such as transfusional iron overload, deposition is seen mainly in the macrophages of the RE system (Britton *et al.*, 1994). The most prominent storage form of iron is called haemosiderin, which is seen as tiny granules in the hepatocyte. Haemosiderin can also be seen as brown pigment in the H&E staining.

At high levels of hepatic iron, other histological findings include hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Niederau *et al.*, 1985; Britton *et al.*, 1994). Ramm *et al.* (1997) have correlated high hepatic iron levels in patients with haemochromatosis with the activation of hepatic stellate cells. Activated stellate cells have been shown experimentally to increase collagen production and the authors propose that iron-loaded hepatocytes directly or indirectly stimulate collagen production. Initially, portal areas become expanded with iron-loaded macrophages and collagenous tissue. Liver cirrhosis in

subjects with iron-overload can then be complicated by the development of HCC. It has not been clarified if iron plays a direct role in inducing HCC, or if the increased risk is based on the cirrhotic process (Stål *et al.*, 1995).

2.2.3. Iron overload disorders

2.2.3.1. Primary iron overload

Genetic haemochromatosis

Genetic haemochromatosis (GH) is a common, inherited primary iron overload disorder due to mutations in HFE (Feder *et al.*, 1996). The two most common mutations associated with the HFE gene are the C282Y and H63D mutations (Feder *et al.*, 1996). The C282Y mutation occurs when tyrosine is substituted for cysteine at amino acid 282, and the H63D mutation results from a substitution of aspartate for histidine at amino acid 63. An average of 85% of patients showing clinical signs for GH is homozygous for C282Y. A mutation in the HFE gene influences the TfR-mediated uptake of iron and iron metabolism is disrupted. This results in an abnormal increase in iron absorption in the intestine and increased iron storage in the parenchymal cells of various organs, primarily the liver, but also the heart and pancreas, among others (Sheldon *et al.*, 1935). The iron deposition eventually leads to organ failure. Iron overload based on genetic defects will even take place on diet consisting of a normal amount of iron.

The clinical presentation is usually late in adulthood, due to the time required for iron to accumulate and subsequently cause tissue injury (Cartwright *et al.*, 1979; Niederau *et al.*, 1996; Adams *et al.*, 1997). The disease can present as diabetes mellitus, cirrhosis, and increased skin pigmentation, as first described by Trousseau in 1865. Further clinical features include hepatic dysfunction, hypogonadism, hyperpigmentation, arthritis, cardiomyopathy, and/or fatigue (Bacon *et al.*, 1999). If left untreated, the liver of a GH patient can become cirrhotic, and develop end-stage liver disease and hepatocellular carcinoma (HCC). Investigations may reveal abnormal liver enzyme levels and hyperglycaemia (due to destruction of β-islet cells in the pancreas).

The disease is most common in Caucasian populations, predominantly of northern European descent (Smith *et al.*, 1997). It is not found in African populations (Merryweather-Clarke *et al.* 1997). Morbidity has not been reported in persons under the age of 20 and men develop organ damage earlier than women (Bacon *et al.*, 1999).

Measurement of serum iron, transferrin saturation, or ferritin in association with typical clinical symptoms is suggestive of GH (Bacon *et al.*, 1999). This diagnosis can be confirmed with the help of various other diagnostic methods: 1) family history; 2) abnormal iron studies; 3) histological grading of 3 or 4 for iron on a liver biopsy; 4) hepatic iron concentration; 5) hepatic iron index (iron concentration divided by age) of >1.9; and/or the more recent genotyping for mutations in HFE (Feder *et al.*, 1996; Barton *et al.*, 1997; Bacon *et al.*, 1999).

If diagnosed in the early stages of the disease, phlebotomy with subsequent iron depletion is successful in managing GH (Niederau *et al.*, 1999; Barton and Bottomley, 2000). Phlebotomy can also prevent further complications resulting from iron overload, but does not remove the risk of HCC in cirrhotic patients. Dietary restrictions can be supportive. Conflicting data has been presented in regard to the therapeutic effect of a liver transplant (Pillay *et al.*, 1991; Kilpe *et al.*, 1993; Kowdley *et al.*, 1995; Farrell *et al.*, 1994).

2.2.3.2. Secondary iron overload

Dietary and parental iron overload

Under normal circumstances, a healthy body will not absorb excess dietary iron. Increased iron absorption occurs only in cases of extremely high availability and/or underlying genetic factors resulting in a disruption of iron metabolism (i.e. GH) (Pippard, 1994). A combination of high dietary intake and increased absorption enhances the degree of iron overload at a young age.

Transfusions are life saving therapies used for various afflictions for example β-thalassaemia (Pippard, 1994). As mentioned before, excretion of excess body iron is insufficient, and repeated transfusions, containing 200-250 mg per unit of blood, increase iron stores rapidly, even when stores are ample (Green *et al.*,

1968). Initial loading occurs in macrophages of the RE system, thus posing minimal danger to organs (Andrews, 1999). When the capacity of the macrophages has been exceeded, deposition in parenchymal cells takes place, resulting in organ injury. Because of the underlying disorder, treatment does not include phlebotomy, but rather chelation therapy with deferoxamine.

Chronic liver disease

(i) Alcoholic liver disease

Excessive alcohol intake has been shown to increase iron deposits, resulting in deposition that differs in amount and distribution from that resulting from GH (Valerio *et al.*, 1996). The iron is seen as coarse granules scattered through the hepatocyte cytoplasm, and the hepatocytes that contain iron are randomly distributed in the lobules. The amount of iron deposited is not excessive; the hepatic iron concentration is not significantly elevated in cases of alcohol abuse alone (Bassett *et al.*, 1986; Summers *et al.*, 1990). This contrasts with the deposition of iron in periportal hepatocytes in early GH, the gradual progression to involve all hepatocytes, and the pericanalicular predominance of iron within the cells. Various mechanisms have been proposed for the excess iron deposition in alcoholic liver disease, including iron found in the alcoholic beverages and favourable conditions in the gastrointestinal tract for increased intestinal iron absorption due to an increase in gastric acid secretion (Charlton *et al.*, 1964).

A high proportion of patients with clinically and biochemically diagnosed GH have been reported to consume excessive alcohol; the alcohol is thought to hasten the progression of the liver injury in such patients and thus contribute to manifestation of clinical symptoms (Powell *et al.*, 1994). In patients shown to consume excessive amounts of alcohol and to have co-existent GH, alcohol can contribute to organ injury by enhancing lipid peroxidation and collagen biosynthesis, resulting in the premature onset of fibrosis and cirrhosis (Irving *et al.*, 1988). The combination of excessive alcohol consumption and GH often results in an increase in iron deposits in Kupffer cells and portal tract macrophages. This is thought to be due to release of iron from hepatocytes injured during episodes of alcoholic hepatitis. Similar associations have also

been demonstrated with porphyria cutanea tarda, another iron loading disorder (Powell *et al.*, 1994).

(ii) Porphyria cutanea tarda

Porphyria cutanea tarda (PCT), the most common type of porphyria, is an iron dependent skin disorder resulting in various symptoms such as fragile skin, subepidermal bullae, scarring, and pigment deposition (Kappas *et al.*, 1995). PCT patients can present with liver disease, although cirrhosis is unusual (Lefkowitch and Grossman, 1983). Abnormal liver enzyme values, 'chronic hepatitis' seen histopathologically, and hepatic siderosis are alterations seen in most clinically symptomatic patients (Lefkowitch and Grossman, 1983).

The pathogenesis is based on a decrease in the biological activity of uroporphyrinogen decarboxylase (URO-D), an enzyme essential in the final phase of the biosynthesis of haeme (De Verneuil *et al.*, 1978). The enzyme is present in the liver primarily, but also in erythrocytes. A reduction in enzyme activity results in an accumulation of uroporphyrinogen and other porphyrinogen substrates of URO-D, and in an increased oxidation of porphyrinogen to porphyrin. Symptoms are caused by photosensitization in response to porphyrin deposits in the skin (Kappas *et al.*, 1995).

Iron is believed to play a role in this process though not directly in deactivating URO-D; it is however required (De Verneuil *et al.*, 1978; Elder *et al.*, 1985; Elder and Roberts, 1995). Iron overload can be determined in 60-70% of PCT cases (Fargion *et al.*, 1996) and clinical manifestation seems to appear secondary to this and other factors (Sampietro *et al.*, 1999). Clinical features can be triggered by such elements as excess alcohol consumption, an increase in oestrogen levels, viral infections (in particular the human deficiency and hepatitis viruses), exposure to halogenated hydrocarbons or combination of two or more of these factors (Lundvall *et al.*, 1970; Haberman *et al.*, 1975; Rocchi *et al.*, 1986; Fargion *et al.*, 1992; Kappas *et al.*, 1995). These exogenous factors influence the accumulation of iron in the hepatocytes, which in turn enables PCT to develop and become clinically manifest.

There are two types of PCT: sporadic, the most common form, accounts for 80% of the cases. The reduction in URO-D enzyme activity is restricted to the hepatocytes (Garey *et al.*, 1993). The less common type is familial, in which case the disease in inherited as an autosomal dominant trait. The deactivation of the enzyme occurs in both erythrocytes and the liver (Elder *et al.*, 1985; Garey *et al.*, 1993). A high proportion of PCT patients are C282Y homozygous (Roberts *et al.*, 1997; Elder and Worwood, 1998). It is hypothesised that as with other exogenous factors, mutations in HFE increase susceptibility to PCT due to increased iron absorption.

Depletion of iron through phlebotomy is used in the treatment of the clinical manifestation of PCT, even in cases without acute iron overload, which confirms the role of iron dependent deactivation of URO-D (Kappas *et al.*, 1995). After iron depletion, skin lesions abate and urinary excretion of porphyrins is reduced (Lundvall, 1971).

Haematological diseases

Anaemia results from either abnormal production of erythrocytes or haemoglobin, or depletion of existent red blood cells. Except in cases of blood loss that leads to iron deficiency, anaemia automatically results in an increase in body iron stores (Pippard, 1994).

Thalassaemia major is one example of an iron-loading anaemia (Pippard, 1994). This type of anaemia is caused by haemolysis resulting in release of large amounts of iron into circulation. Thalassaemia major is the homozygous form of β-thalassaemia and is an autosomal hereditary disease. Defective globin synthesis leads to reduced haemoglobin production, which in turn results in severe anaemia and iron overload. The disease becomes clinically apparent at an early age and patients are usually transfusion dependent. Hepatosplenomegaly is a common development, as also skeletal abnormalities. If left untreated, patients die of cardiac dysfunction or cirrhosis, and few survive past the age of 20, even with treatment: Bone marrow transplant is the only possibility of a cure. Splenectomy can be helpful and chelating agents to remove iron can be administered as a supporting therapy.

Sideroblastic anaemia is inherited or acquired, and develops due to defective haeme production (Pippard, 1994). Iron cannot be utilised effectively and accumulates in the mitochondria of developing erythrocytes, producing the diagnostic “ring sideroblasts” (Bottomley, 1982). The inherited form is very rare, and most common in males in childhood or adolescence. The acquired form is more frequent and found more commonly in older patients. The anaemia can remain stable for years; otherwise phlebotomy therapy can be commenced. In more severe cases where blood transfusions are needed, additional chelation therapy may be necessary.

Other haematological disorders resulting in iron-overload are, for example, anaemia associated with chronic renal disorder, in which there is a reduced production of the hormone erythropoietin and erythropoiesis is ineffective; congenital dyserythropoietic anaemia; congenital red cell aplasia; adult hypoplastic anaemia; and haemolytic anaemia.

Neonatal iron overload

Neonatal iron overload is a rare disease of severe hepatic iron overload and subsequent cirrhosis and liver failure affecting babies in the perinatal period (Knisely, 1992). Iron deposits are also seen in the myocardium and pancreatic acinar cells (Goldfischer *et al.*, 1981; Blisard and Bartow, 1986). The affliction is not associated with HFE mutations (Goldfischer *et al.*, 1981) and the cause has not been determined to be primary or secondary (Witzleben and Uri, 1970; Hoogstraten *et al.*, 1990). The only treatment is a liver transplant, which due to technical reasons (the transplant must take place soon after birth), is not usually successful (Rand *et al.*, 1992; Lund *et al.*, 1993; Sigurdsson *et al.*, 1998).

Juvenile haemochromatosis

Juvenile haemochromatosis is a rare iron disorder in persons age 15-30 characterised by a severe rate of iron overload resulting in liver and heart disease (Kaltwasser *et al.*, 1998). Patients die of heart failure before the age of 30. Juvenile haemochromatosis appears to be inherited, but it is not related to mutations in the HFE gene, and the genetic cause remains unknown. The disease has been well documented in several Italian families (Camaschella *et*

al., 1997) and it has been hypothesised that the underlying mechanism of iron overload is the same as in GH (Andrews, 1999).

Sub-Saharan dietary iron overload

African iron overload was first documented in 1929 and was originally called Bantu siderosis (Bothwell *et al.*, 1979; Gordeuk, 1992). Sub-Saharan iron overload has since been described in various populations south of the Sahara and can occur in up to 10% of the population in some rural communities (Gordeuk, 1992). Extreme iron overload is 10 times more common in sub-Saharan Africans than homozygous GH in Caucasian populations.

Until recently, it was believed that the high iron overload found in these populations was due to the high bio-availability of the iron released during the home-brewing of traditional beer made from maize in non-galvanised iron pots (Bothwell *et al.*, 1964; Gordeuk, 1992). The mean value for the total iron concentration in the drink is 15.1 mg/dl (750 times higher than commercially produced beer) and about one third of the iron in the beer is ferrous iron (Pippard, 1994). However, studies have shown that only a small percentage of drinkers develop iron overload, leading to the assumption that genetic factors also play a role in the increased absorption of dietary iron (Gordeuk *et al.*, 1986 Gordeuk *et al.*, 1992; Moyo *et al.*, 1997). These populations appear to have predisposition to increased iron absorption, which is augmented by an additional high dietary intake (Gordeuk *et al.*, 1992). The hypothesis of genetic transmission in African iron overload has recently been confirmed in a study conducted by Moyo *et al.* (1998).

Hepatic iron overload can be mild to severe, with hepatic fibrosis and cirrhosis occurring at high concentrations (Pippard, 1994). It has recently been proposed that African iron overload might also be risk factor for HCC (Gangaidzo and Gordeuk, 1995; Mandishona *et al.*, 1998). Iron levels increase with age, with women accumulating less iron. Other complications seen in cases of severe iron overload without accompanying cirrhosis usually arise from secondary deficiency in ascorbic acid. These include scurvy and osteoporosis, resulting in fractures, attributed to oxidation of ascorbic acid and resulting in decreased

production of collagen and new bone (Seftel *et al.*, 1966). Sub-Saharan iron overload is occasionally linked to diabetes mellitus (Seftel *et al.*, 1961), heart failure, and oesophageal carcinoma (MacPhail *et al.*, 1979).

Iron distribution in the liver differs from that seen in GH, but rather shows a similarity to iron distribution in iron-loading anaemias, namely deposition in Kupffer as well as parenchymal cells (Bothwell and Bradlow, 1960). The typical zonal gradient from periportal to perivenular in GH is not seen (Gordeuk *et al.*, 1992).

A diagnosis is made based on a history of excessive consumption of traditional beer in conjunction with clinical features such as hepatomegaly and hyperpigmentation (Pippard, 1994). High serum iron and ferritin levels, and also transferrin saturation are indicative of iron overload, and analysis of a liver biopsy can confirm the diagnosis (Gordeuk *et al.*, 1986; Pippard, 1994). Phlebotomy can be used remove excess iron (Speight and Cliff, 1974).

2.2.4. Mechanisms of iron toxicity

The mechanism of iron toxicity has not been fully elucidated. It has been proposed that lipid peroxidation is one mechanism of toxicity of iron overload (Bacon *et al.*, 1983). Non-bound ferric iron can be reduced to ferrous iron by superoxide (McCord, 1996). Ferryl ions and hydroxyl radicals are then generated by the reoxidation of ferrous iron. These free radicals can cause extensive damage to cell membranes, proteins, and promote oxidative DNA injury resulting in mutagenesis (McCord, 1996; Andrews, 1999). Subsequent breakdown of polyunsaturated fatty acids takes place in the membrane phospholipids, thus disrupting cell function (Hogberg *et al.*, 1973; Myers *et al.*, 1991). Iron-induced lipid peroxidation in hepatic mitochondria and microsomes has also been observed to impair electron transport and decrease levels of cytochromes P-450 and b5 (Bacon *et al.*, 1985; Bacon *et al.*, 1986).

A recent study linked chronic experimental iron overload and lipid peroxidation to disturbances in plasma lipid transport and hepatobiliary sterol metabolism (Brunet *et al.*, 1999). An elevation of malondialdehyde (MDA) levels was

measured, which is an indication of lipid peroxidation. Young *et al.* (1992) also reported that patients with clinical GH have increased plasma levels of MDA.

In addition to free radical-induced injury, the accumulation of cellular iron in the cell results in low levels of cellular adenosine triphosphatase (ATP), defective cellular calcium homeostasis, and subsequent damage to DNA, which may directly contribute to hepatocellular damage (Britton *et al.*, 1994). Lipid peroxidation of lysosomal membranes has been associated with low membrane fluidity and limited lysosomal acidification (Hogberg *et al.*, 1973).

Long-term iron overload activates hepatic stellate cells and leads to hepatic fibrosis (Britton *et al.*, 1994, Ramm *et al.,* 1997). An inherent association between lipid peroxidation and fibrogenesis due to increased collagen biosynthesis has been made (Parola *et al.*, 1993). It can be theorised that increased collagen gene expression and subsequent fibrosis could be due to iron-induced lipid peroxidation (Britton *et al.*, 1994).

In addition, iron toxicity may be enhanced via impairment of the immune system. Non-transferrin iron and ferritin have been observed *in vitro* to suppress the proliferation of lymphocytes (Matzner *et al.*, 1979; Djeha and Brock, 1992).

2.2.5. Iron and carcinogenesis

An increase in total iron stores has been epidemiologically linked as a risk factor for cancer in humans (Stevens *et al.*, 1988; Selby and Friedman, 1988; Stevens *et al.*, 1994). Iron overload has repeatedly been shown to play a role in the enhancement of tumour cell growth, and iron deficiency and iron chelating agents appear to inhibit carcinogenesis (Deugnier *et al.*, 1998; Deugnier and Loréal, 2000). For example, Chen *et al.* (1999) demonstrated that a high dietary content of iron augments the development of oesophageal adenocarcinoma in rats. Iron has been implicated in the development of HCC via peroxidation and subsequent yield of free radicals (Weinberg, 1983; Perera *et al.*, 1987; Toyokuni 1996). *In vitro* studies have shown that iron can be genotoxic and mutagenic to isolated DNA (Loeb *et al.*, 1988) and chronic iron overload promotes the

unwinding of double-stranded DNA in rat hepatocytes (Edling *et al.*, 1990), supporting the process of cancer initiation.

Transferrin iron may promote tumour cell growth by providing a favourable environment for the tumour cell to proliferate. Cells, including tumour cells, need iron to proliferate, and the presence of iron has been reported to promote DNA synthesis in rat hepatocyte cultures (Chenoufi *et al.*, 1997) and the growth of human hepatoma cells (Hann *et al.*, 1990). It has been shown that nodule hepatocytes are unable to take up iron in a situation of iron overload (Williams and Yamamoto, 1972), in spite of high numbers of diferric transferrin binding sites (Eriksson *et al.,* 1986). The affinity of the receptor to diferric transferrin is not affected. It was suggested that the dissociation of iron from transferrin is disturbed, perhaps due to insufficient acidification in the endosomes, which leads to a slower rate of iron accumulation in hepatocytes within the nodules (Eriksson *et al*., 1986; Andersson *et al*. 1989). Low intracellular iron in the nodules could lead to a reduction in lipid peroxidation, contributing to the cell growth advantage of these hepatocytes in a toxic environment, as described in the resistant hepatocyte model by Solt and Farber (1976). It has been suggested that iron-free foci are proliferative lesions that could be pre-neoplastic/precursors to HCC (Hirota *et al.*, 1982; Deugnier *et al.*, 1993a, b; Blanc *et al.*, 1999). It is not known, however if iron plays a direct role in initiating or promoting HCC, or if HCC results indirectly from iron-induced cirrhosis (Stål *et al.*, 1995).

Many studies support the hypothesis that iron promotes GH by inducing cirrhosis, which is seen in the majority of GH cases (Stål *et al.*, 1995). HCC has been found to be the cause of death in 27.5-45% of patients with clinical GH and concurrent severe iron overload (Niederau *et al.*, 1996; Fargion *et al.*, 1992). The risk in GH patients of developing HCC once cirrhosis has developed has been estimated to be 200-fold higher than in non-cirrhotic GH patients (Bradbear *et al.*, 1985; Niederau *et al.*, 1985). In cases of GH, a risk for HCC still exists even after iron depleting treatment (Fargion *et al.*, 1992). Ludwig *et al.* (1997) also reported continuous iron loading in livers in which cirrhosis is due to causes other than GH. Patients with alcohol-or HBV-induced liver cirrhosis

have an increased risk of developing HCC in the presence of hepatic iron overload compared to those with normal body iron stores (Deugnier *et al.*, 1998; Deugnier and Loréal, 2000).

Elevated liver iron stores have also been found in cases in which HCC developed in non-cirrhotic livers (Turlin *et al.*, 1995). Others have reported an increased risk of various cancers, including HCC, in the presence of iron overload, but in the absence of cirrhosis (Deugnier *et al.*, 1998; Deugnier and Loréal, 2000). Though rare, HCCs have been reported in GH patients with non-cirrhotic livers (Fellows *et al.*, 1988; Blumberg *et al.*, 1988).

Experimentally, disparate results have also been obtained. Yoshiji *et al.* (1991) described the role of iron in the development of pre-neoplastic foci in the livers of rats initiated with diethylnitrosamine (DEN) and promoted by phenobarbital. In an additional study with DEN as initiator, iron dextran administered parenterally did not act as an initiator alone (Carthew *et al.*, 1997). However, the iron dextran did act as a promoter as seen in the increase in the placental form of glutathione-S-transferase (GSTP) expressing foci in the absence of fibrosis. The study by Carthew *et al.* (1997) demonstrates that fibrosis is not necessarily a prerequisite for iron-induced foci in the liver of a rat.

The Solt-Farber animal model of chemical carcinogenesis has been used to examine the effect of iron overload in the development of HCC. In a long-term study supplementing with 2.5%-3% dietary carbonyl iron (Fe), Stål *et al.* (1995) mimicked iron overload as seen in non-cirrhotic GH. To examine the effect as an initiator, iron replaced DEN, partial hepatectomy (PH) or both. The promotive effects were tested on a complete initiating regime consisting of a low-dose DEN and PH, or a necrogenic dose of DEN. The mitostimulatory effect of iron was documented and the mitoinhibitory effect of 2-acetylaminofluorene (2-AAF) was counteracted by iron. However, no promotive effect of iron was noted. In fact, a decrease in volume density of nodules was effected when iron was given with a complete promotive regimen; a decrease in the number of foci in initiated livers was also observed. It was concluded that iron is not genotoxic, does not promote the growth of initiated cells, and has weak mitogenic characteristics.

The same research group conducted a study in 1999 using 1.25-2.5% Fe added to the diet 2 weeks after initiation with DEN and promotion with 2-AAF in combination with PH (Stål *et al.*, 1999). Iron increased the number of pre-neoplastic foci, but no enhancement of progression to HCC was seen. Similar levels of dietary carbonyl iron restricted promotion by carbon tetrachloride (CCl_4) after initiation with DEN (Wang *et al.*, 1999). In a study examining carbonyl iron as a co-factor in the development of HCC, iron enhanced the cytotoxic effect of ethanol in the Solt-Farber model, as measured by elevation in levels of serum aminotransferase. Iron did not however increase the formation of pre-neoplastic GSTP positive ($GSTP^+$) lesions. Lemmer *et al.* (1999) also described an apparent 'protective' effect of carbonyl iron fed for 5 weeks on FB_1-induced cancer initiation and promotion.

In conclusion, dietary iron appears to have a direct and indirect role in the development of HCC in humans and possibly in animal models. The results of the different studies associated with iron overload do not clarify the carcinogenic effect of iron and the mechanism needs to be further elucidated.

2.3. Aims

The aims of the present study were to

(i) to establish a model of iron loading, in which a high baseline hepatic iron level could be maintained at a low dietary iron level;

(ii) to examine the interaction between FB_1 and increased hepatic iron levels, utilising the stop model of chemical carcinogenesis;

(iii) to determine whether excess iron potentiates the hepatocarcinogenic effects of FB_1.

Chapter 3
Materials and methods

3.1. Chemicals

Fumonisin B_1

Fumonisin B_1 (FB_1) was purified from maize cultures of *Fusarium verticillioides* strain MRC 826 according to a method described by Cawood *et al.,* 1991. Extraction of FB_1 was accomplished with CH_3OH/H_2O (3:1) followed by a solvent-partitioning step using $CHCl_3$. The ensuing purification of the aqueous phase was achieved on Amberlite XAD-2, silica gel, and reversed-phase (C_{18}) chromatographic columns. This method yielded FB_1 to the purity of 92.97% as compared to an analytical standard by high performance liquid chromatography (HPLC) (Shephard *et al.,* 1990). The main FB_1 impurities constitute monomethylester derivatives of $FB_{1,}$ which are artefacts of the purification procedure (Cawood *et al.,* 1991).

Carbonyl Iron

Carbonyl iron is manufactured using the carbonyl decomposition process, which yields an extremely pure form of elemental iron (97%) with only traces of carbon, oxygen and nitrogen. In the process, iron reacts with carbon monoxide at high temperatures to form reduced pentacarbonyl iron, $Fe(CO)_5$. The carbonyl iron in this study was purchased from Sigma Chemical Co (St. Louis, Missouri).

5-Bromo-2'-deoxy-uridine

5-bromo-2'-deoxy-uridine (BrdU) is an immunochemically detectable pyrimidine analogue of thymidine (Sigma product information sheet; website http://www.sigma.sial.com/sigma/proddata/b5002.htm). For handling of BrdU, precautions are required. Stocks were stored protected from light at 4^oC. Gloves were worn during preparation, spillage thoroughly cleaned, and syringes and residues incinerated. BrdU was purchased from Roche Products (PTY) Ltd (Roche Diagnostics, Basel, Switzerland).

Monoclonal anti-BrdU

The amount of BrdU incorporated into the cellular DNA was determined immunohistochemically after *in vivo* labelling using a monoclonal anti-BrdU antibody (Immunohistology Grade, mouse IgG1 isotype) (Roche Molecular Biochemicals USA 2000 Biochemicals Catalog website http://biochem-us.comvos.de). The antibody recognises BrdU in the nuclei of formalin-fixed, paraffin-embedded tissue sections and demonstrates the incorporation of BrdU immunohistochemically by an indirect immunoperoxidase method (Sigma Biosciences Information Sheet for Monoclonal Anti-BrdU). The monoclonal anti-BrdU was obtained from Sigma Biosciences (St. Louis, Missouri).

Placental form of glutathione S-transferase

Avidin-biotin-peroxidase complex (ABC) and affinity-purified biotin-labelled goat anti-rabbit IgG (immunoglobulin G) serum (Vector Laboratories, Burlingame, CA) were used in staining paraffin wax sections of liver to detect glutathione-S-transferase (placental form) positive ($GSTP^+$) cells.

Other chemicals

Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), and bovine serum albumin (BSA) were purchased from Sigma Chemicals Co (St. Louis, Missouri) and ethylene diamine tetra-acetic acid disodium salt (EDTA) from Synthon Fine Chemicals PTY Limited.

All other chemicals and solvents for lipid analysis were of analytical grade and obtained from Merck, S.A. Organic solvents (methanol, chloroform and hexane) used for lipid extractions and analyses were glass-distilled prior to use. All glassware was cleaned with a phosphate free soap (Contrad concentrate, Merck, S.A.) and rinsed with glass-distilled methanol.

3.2. Animals

Male Fischer 344 rats were bred in the Primate Unit of the Diabetes Research Group (RIND building) of the South African Medical Research Council (MRC). Rats were housed in a controlled environment at 23 - 24°C and 60% humidity

with a 12 hour (h) artificial light cycle. Water was available *ad libitum*. Food cups were changed as needed and the cages were replaced on a monthly basis.

The Ethics Committee for Research on Animals (ECRA) approved this study and it was conducted in compliance with policies and standards detailed in the MRC's principles and guidelines for the use of animals in biomedical research. These regulations are printed in *GUIDELINES ON ETHICS FOR MEDICAL RESEARCH, REVISED EDITION, 1993* and the *NATIONAL CODE FOR ANIMAL USE IN RESEARCH, EDUCATION, DIAGNOSIS AND TESTING OF DRUGS AND RELATED SUBSTANCES IN SOUTH AFRICA, 1990* published by the Chief Director: Veterinary Services, Private Bag X250, Pretoria 0001.

Pre weaning period

The rat pups were caged with their mothers on corncob bedding. The study commenced on day 3 (day 1=day of birth) and the rats were fed a modified version of the diet developed by the American Institute of Nutrition (AIN-93M) (Reeves *et al.*, 1993) with or without supplemented carbonyl iron twice a day. Food was available *ad libitum*. The mothers were weighed once a week.

Pilot study on iron loading

Sixty-eight male Fischer 344 (F344) rats at the age of 4 weeks (after weaning) were used in the study. The animals were caged 2-3 per cage on corncob bedding and were weighed 3 times a week. Food was available *ad libitum* and during the week the rats were fed twice a day and on the weekends once a day. The bedding of the dietary iron-treated rats was changed on a weekly basis and that of the controls changed every 2nd week as deemed necessary.

Long-term FB_1/Fe interactive study

Eighty male F344 rats at the age of 4 weeks (after weaning) were used in the study. The rats were kept separately on vermiculite bedding, an inert, non-toxic mineral, except for the rats used for averaged feeding, which were kept in wire grid cages, as wastage could not be accounted for in the bedding (see Chapter 3.4.3). Vermiculite bedding was discontinued after 39 days because of the excessive dust build-up caused by vermiculite, making it very impractical. All the

animals were then caged individually in wire grid cages. The cages were rotated every 2 weeks to different shelf levels, starting at week 27. The rats were weighed twice weekly until week 51, after which they were weighed once weekly.

Perspex™ houses were introduced at week 33 to assuage the tendency of ulcerative pododermatitis to develop on grid floors. Perspex™ cast acrylic sheet (polymethyl methacrylate) is an inert substance manufactured from methyl methacrylate, a standard organic chemical produced from crude oil feedstock (www.ineosacrylics.com). The material has been used for many years in medical devices such as in the production of the intra-ocular lens. No detrimental health effects of Perspex™ are known.

3.3. Diets

Each diet was prepared in 6 kg quantities at a time and stored under nitrogen in 3 bags at 2 kg each at 4°C until used.

AIN-93M diet

All the rats received a modified version of the AIN-93M diet, which is recommended as a maintenance diet for rodents. It is formulated to replace the previous version (AIN-76A) to improve animal maintenance. In this study, methionine was substituted for L-Cystine and soybean oil was used as the fat source (Appendix AI). The iron content of the control AIN-93M diet was 35 mg iron per kg diet.

Iron-supplemented diet

The iron-supplemented diet was prepared according to a modification of the method previously described by Plummer *et al.* (1997) by mixing carbonyl iron into the AIN-93M diet to obtain the desired concentrations. The appropriate amount of iron was first mixed into a 200 g sample of the powdered diet after which this sample was diluted to the original concentration and stored.

Fumonisin B_1-containing diet

The required amount of FB_1 stock sample was first dissolved in methanol (50 ml) and mixed into a sample (200 g) of the AIN-93M diet, after which it was dried in a fume cupboard at room temperature for 12h (Gelderblom *et al.,* 1994). Subsequently, the sample was thoroughly mixed into the AIN-93M diet to obtain the desired concentration of FB_1. Gloves and masks were worn for protection while preparing and administering fumonisin-containing diets.

3.4. Experimental design

3.4.1. Pre-weaning iron loading regimen

To achieve iron loading via breast milk, the mothers of the rats allocated to the iron group received pellets (Epol, Ltd, S.A.) on day 1 and 2, and then 1% dietary carbonyl iron (Fe) mixed into the AIN-93M powdered diet starting on day 3. The mothers of the control rats received AIN-93M without iron. The mothers and pups were housed together on corncob bedding. In addition to mother's milk, the pups commenced to consume the mother's AIN diet approximately after 2 weeks and were weaned at an age of 4 weeks.

3.4.2. Pilot study on iron loading

At weaning, 4 rats from the iron supplemented group and 4 rats from the control group were sacrificed to assess body weight (BW), total body weight gain (tBWG), the liver to body weight ratio (LW/BW), liver pathology, the degree of hepatic iron loading (baseline group), and lipid peroxidation.

The remaining 60 rats were then randomly divided into four treatment groups (n=15 each) as outlined in Table 3.1. After 10 weeks, the dietary iron dosage was reduced to 0.5% for all iron-supplemented groups and continued up to 15 weeks after which the experiment was terminated. Subgroups from all four groups were sacrificed on a regular basis during the course of the experiment (Table 3.1) to monitor the various parameters.

Table 3.1. Summary of the treatment regimens for the iron loading trial (pilot study).

Duration of treatment	**Level of dietary iron feeding for week 0-10**			
	1% Fe	**1.5% Fe**	**2% Fe**	**Control**
4 wk	n=4	-	-	n=4
6 wk	n=5	n=5	n=5	n=5
10 wk*	n=5	n=5	n=5	n=5
15 wk	n=5	n=5	n=5	n=5
Total no. of rats	**n=19**	**n=15**	**n=15**	**n=19**

*At week 10, the dietary iron of all the iron-treated rats was reduced to 0.5%.

Termination

The rats that were sacrificed at weeks 4, 6, and 10 were anaesthetised intraperitoneal (i.p.) with sodium pentobarbitone (6%) (0.1 ml/100 g BW) and terminated by cutting the abdominal aorta (exanguination). At 15 weeks, the rats were sacrificed by decapitation 1h after an i.p. injection of BrdU (100 mg/kg BW). The livers were removed, weighed and samples for histopathology prepared. Liver slices (2-3 mm) were taken from the median, left lateral and caudate lobes and fixed in 10% neutral buffered formalin for 24h. The remainder of the liver was snap frozen in liquid nitrogen for biochemical measurement of hepatic iron and the level of lipid peroxidation.

3.4.3. Long-term FB_1/Fe interactive study

Averaged feeding was introduced in the long-term study to reduce the variability of certain parameters, such as BW, tBWG, and the LW/BW ratio, between rats of different treatment groups. This entailed weighing the feed of a specified group every day, calculating the average feed intake, and feeding the other rats accordingly.

Treatment period: weeks 4-10 (iron loading period)

Iron supplemented groups

The rats were subjected to the pre-weaning iron loading treatment regimen as described above. At weaning, 5 rats were sacrificed to determine the degree of

hepatic iron loading (baseline group) and the remaining rats were fed 1% Fe (n=35). The feed intake profiles of 10 rats fed the iron-supplemented diet were monitored once daily and the feed of the other groups adjusted accordingly. At the age of 10 weeks, 5 rats were culled to monitor the extent of iron loading in the liver prior to the FB_1 feeding.

Non iron-supplemented groups

At weaning, 5 rats from the mothers that received the control AIN 93M diet were sacrificed to serve as controls for the iron-treated group, and the remainder of the rats (n=35) were continued on the AIN-93M diet. At the age of 10 weeks, 5 rats were culled as a control for the 1% Fe treated group prior to FB_1 feeding.

Treatment period: weeks 10-35 (FB_1 treatment period)

The iron-treated rats were randomly divided into either the FB_1/Fe (n=15) or Fe (n=15) group, while the AIN-93M control rats were randomly divided into either the FB_1/AIN-93M (n=15) or the AIN-93M control (n=15) group according to a 2x2 factorial design (Table 3.2). At this stage the dietary iron level was reduced to 0.5% to prevent possible toxic effects of the combined FB_1/Fe treatment. The average feed intake of the entire FB_1/Fe group (n=15) was measured while the averaged intake the FB_1/AIN-93M group was occasionally monitored from week 17 onwards for comparison.

Table 3.2. Experimental outlay (2x2 factorial design) of the long-term study (10-35 week period).

Treatment regimens	**FB_1**	**FB_1 control**
0.5% Fe	FB_1/Fe group (n=15)	Fe group (n=15)
Fe control	FB_1/AIN group (n=15)	Control (n=15)

Rats from the different groups were subjected to the following treatment regimens:

FB_1/Fe group (n=15): The rats received FB_1 at a dietary level of 250 mg/kg AIN-93M containing 0.5% Fe for a period of 5 weeks. The dietary FB_1 was lowered to 100 mg/kg AIN-93M diet for the remainder of 20 weeks.

Fe group (n=15): The rats received 0.5% Fe-containing AIN-93M diet for 25 weeks.

FB_1/AIN group (n=15): Rats were fed 250 mg FB_1/kg AIN-93M diet for 5 weeks, followed by a 100 mg/kg diet for 20 weeks in the absence of iron.

***Control (n=15)**:* These control rats received only the AIN-93M diet for 25 weeks.

Treatment period: weeks 35-60 (post FB_1 treatment period)

FB_1-treatment was discontinued at week 35 (Table 3.3.), while the iron treatment protocol was continued up to 60 weeks. The purpose of this was to assess the reversibility of fumonisin-induced liver injury in the fumonisin treated group and to monitor the promoting effects of iron on the FB_1-induced pathological changes of the liver. Five rats from each group were sacrificed at 35 weeks, and at 60 weeks, all remaining rats were terminated to assess iron loading as well as the pathological changes at these time-points.

Termination

A summary of the termination schedule for the rats over the 60-week period is outlined in Table 3.3. At 35 weeks, 10 rats, and at 60 weeks all the rats, received BrdU (100mg/kg BW, i.p.) dissolved in DMSO:Saline (1:3) 1h prior to sacrifice. The animals were anaesthetised with sodium pentobarbitone (6%) (0.1ml/100g BW; i.p.) and subsequently terminated by cutting the abdominal aorta (exanguination) at the different time-points (Table 3.3.). Blood was collected from the abdominal aorta for haematological and biochemical

analyses. The livers were removed and weighed. Slices of liver were taken from the median, left lateral, and caudate lobes, and fixed in 10% neutral buffered formalin for 24hr. The remainder of the liver was snap frozen in liquid nitrogen for biochemical measurement of hepatic iron and the level of lipid peroxidation.

Table 3.3. Summary of the treatment for the long-term study.

Duration of treatment	FB_1/Fe	Fe	FB_1/AIN	Control
4 wk	-	n=5	-	n=5
10 wk*	-	n=5	-	n=5
Total no. of rats		**10**		**10**
35 wk	n=5	n=5	n=5	n=5
60 wk	n=10	n=9[#]	n=10	n=10
Total no. of rats	**n=15**	**n=14[#]**	**n=15**	**n=15**

*At week 10, the dietary iron of all the iron-treated rats was reduced to 0.5% and FB_1 feeding commenced; rats were fed 250 mg FB_1/kg diet for 5 weeks followed by 100 mg/kg for 20 weeks. #One rat died at 60d due to kidney/bladder stones.

3.5. Liver pathology

3.5.1. Light microscopy

Slices of liver (4-5 mm in thickness) were fixed in 10% neutral buffered formalin overnight prior to processing and embedding in paraffin wax. Sections (4 µm) were prepared for light microscopy by following routine processing protocols. 4h processing cycles were used cycle to prevent the tissue from becoming brittle and to preserve antigenicity. Stains included routine haematoxylin and eosin (H&E) and Perls' Prussian blue stain for trivalent iron.

Histopathological evaluation of the liver sections included assessment of hepatocyte necrosis, apoptosis, fatty change, mitoses, architectural distortion, fibrosis and regenerative nodules, and dysplastic nodules (low-grade and high-grade dysplasia). High-grade dysplasia was characterised by a discrete, non-invasive pushing edge into adjacent liver tissue, large cells with clear cytoplasm, mitosis, intranuclear inclusions, large nucleoli, oval cells, apoptosis of occasional dysplastic cells, and moderate nuclear pleomorphism.

Hepatocellular cancer (HCC) was characterised by invasion into surrounding liver tissue and the vascular system, and metastasis to other organs.

Both foci and nodules were evaluated. Lesions up to 10 µm in diameter were called foci, and those larger than 10 µm were termed nodules. The assessment was done ‘blind’ and the numbers on the glass slides were ‘decoded’ after completion of the evaluation.

3.5.2. Immunohistochemistry

3.5.2.1. Hepatocellular proliferation

BrdU can be incorporated into cell DNA at the S-phase of the cell cycle instead of thymidine and with a monoclonal antibody is used to assess cell proliferation (website http://www.sigma.sial.com/sigma/proddata/b5002.htm; Sigma product information sheet). Rats were injected with BrdU 1h prior to termination. Liver sections (2-3 µm thick) were cut from the tissue blocks for the subsequent staining with monoclonal anti-BrdU antibody. BrdU was detected by the ABC method using a monoclonal anti-BrdU antibody (Sigma Chemical Company) and the slides were scored by counting at least 500 cells randomly per liver section (x40), and labelling indices expressed as a percentage. In cases where no staining was seen in 500 cells, the total number of BrdU positive ($BrdU^+$) cells was counted and the labelling was expressed as cells/cm^2. The total area of the liver sections was determined by a computerised image analyser (Kontron Image Analysis Division, Videoplan, Image Processing System).

3.5.2.2. Enzyme altered foci and/or nodules

Glutathione S-transferase (placental form) (GSTP) staining with the avidin-biotin-peroxidase complex (ABC) and affinity-purified biotin-labelled goat anti-rabbit IgG serum (Vector Laboratories, Burlingame, CA) was performed on liver sections (5 µm) to assess enzyme altered cells (Ogawa *et al.* 1980). Sections were washed with petroleum benzene and a graded alcohol series prior to staining with the reagents using the ABC kit. Rabbit GSTP-antiserum (DAKO) was used at a dilution of 1:800. Subsequently, sections were counterstained with Carazzi's haematoxylin to provide blue stained nuclei within the reddish-brown $GSTP^+$ cells. Negative controls, omitting the primary antibody, were

included to test for the specificity of anti-GSTP antibody binding. The number and size of $GSTP^+$ foci were assessed by light microscopy (4-10 X magnification). The $GSTP^+$ stained cells were categorised as singlets, duplets, or minifoci (<10 cells/focus), while larger lesions (>10 cells/focus) were scored according to their internal diameter (largest of the longitudinal or transverse diameter). Lesions up to 10 μm in diameter were called foci, and those larger than 10 μm were termed nodules. The total area of the liver sections was determined by a computerised image analyser (Kontron Image Analysis Division, Videoplan, Image Processing System) and the amount of $GSTP^+$ lesions were expressed as number per cm^2 liver section area.

3.6. Hepatic iron analysis

3.6.1. Biochemical measurement

Iron content was determined using a modified method obtained from the Biochemistry Department, Flinders Medical Centre, Adelaide, South Australia (6D223, methods manual, see Appendix for method and modifications). Liver tissue was dried in a pre-weighed glass test tube overnight at 105°C and then allowed to cool in a dessicator. The tubes were weighed and the dry liver weight calculated. Subsequently, the tissue was digested in 0.4 ml of 35% nitric acid at 70°C for 1h, and then diluted in 0.2 M sodium acetate buffer pH 4.5 and 10N NaOH. If the liver was not totally digested after 1h, then the digestion process was allowed to continue for another 30 minutes (min). Iron concentration, expressed as mmol/kg dry liver, was determined colourimetrically using a Hitachi random access auto-analyser modular (Roche Diagnostic Systems, Basel, Switzerland).

3.6.2. Histological assessment

In addition to the biochemical measurement, the initial intention was to also evaluate iron-loading in the hepatocytes by semi-quantitative grading of Perls' Prussian blue stain for trivalent iron (Williams *et al.,* 1962). Due to a combination of problems, such grading was not feasible on many of the slides from the long-term study. These included problems in processing the tissue sections resulting in poor staining quality; also a shift of iron deposition from

necrotic hepatocytes to Kupffer cells and portal tract macrophages in the FB_1-treated rats rendered the grading of iron in hepatocytes impractical. The degree of the hepatic iron loading obtained in pilot study was nevertheless evaluated to demonstrate the accordance of the biochemical measurement and histological assessment of iron concentration. Stainable iron in hepatocytes was graded 0 to 4, using a modification of the scale devised by Scheuer *et al.* (1962) (Table 3.4.).

Table 3.4. Grading of stainable iron in hepatocytes (modified from Scheuer *et al.,* 1962).

Stainable iron in hepatocytes (%)	**Grade**
Absent	0
less than 25%	1
25% to 50%	2
50% to 75%	3
75% to 100%	4

3.7. Assessment of lipid peroxidation

3.7.1. Homogenate preparation and incubation

Liver samples were homogenised on ice in 19 volumes of 1.15% KCl containing 0.01 M phosphate buffer (pH 7.4). A sample of 200 µl was taken for protein determination and the remaining homogenate was incubated in a closed bottle at 37°C for 1h.

3.7.2. Determination of thiobarbituric acid reacting substances

The concentration of thiobarbituric acid reacting substances (TBARS) measured as malondialdehyde (MDA) was used as an index of the extent of lipid peroxidation. TBARS were measured according to a modified method described by Hu *et al.* (1989). Two ml of the incubated homogenate were vortexed with 2 ml of TCA reagent, consisting of 10% TCA, BHT (12.5 µm BHT/10ml TCA solution) and EDTA (0.372 g EDTA/l TCA solution), and centrifuged at 2000 rpm for 15 min. Addition of EDTA and BHT to the TCA reagent prevents further oxidative damage during the assay procedure. Two ml of the supernatant was

added to 2 ml of a 0.67% 2-TBA solution, vortexed, and heated in capped tubes at 90ºC for 20 min in a water-bath (Kinchington *et al.,* 1993). The mixture was allowed to cool and the absorbency measured at 532 nm (Esterbauer and Cheeseman, 1990) with the Beckman® Spectrophotometer UV 5260. Lipid peroxidation was expressed as nmol MDA equivalents per mg protein, using the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} at 532 nm for MDA (Buege and Aust, 1978).

3.7.3. Determination of conjugated dienes

To confirm the induction of lipid peroxidation, selected tissue samples were also analysed for conjugated dienes (CD) according to the method of Hu *et al.,* (1989). A 2 ml aliquot of the heated homogenate was extracted with 7.5 ml chloroform:methanol (1:2) by shaking for 1 min. Another 2.5 ml of chloroform was added and the solution vortexed for 30 seconds (s). To this mixture, 2.5 ml of saline saturated with chloroform-methanol-saline (CMS) was added and the mixture was vortexed for 30s. After centrifuging at 1500 rpm for 15 min, 2 ml of the bottom chloroform layer was removed and dried under nitrogen at 40^oC. Hexane (1 ml) was used to dissolve the lipids and the absorbency measured at 233nm with the Beckman® Spectrophotometer UV 5260. The conjugated dienes were determined by using the molar extinction coefficient of 2.7×10^4 M^{-1} cm^{-1} and expressed as nmol CD equivalents per mg protein.

3.7.4. Determination of proteins

Proteins in the homogenate were measured for the purpose of lipid peroxidation determination according to the method described by Bradford (1976). The method is based on the observation that the absorbency maximum of Coomassie Brilliant Blue G-250 shifts from 495 nm to 595 nm when the colour reagent reacts with protein.

The reagent is prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol and adding 100 ml 85% (w/v) phosphoric acid to this solution. The stock solution was then diluted 1:5 times with double distilled water (ddH_20) and filtrated with two layers of Whatman paper (Whatman International Ltd, England). Stock solution is kept at 4ºC. A standard solution

(0.2 mg/ml) of bovine serum albumin (BSA) (Sigma, Sigma-Aldrich, Vorna Valley, South Africa) was prepared and kept at -20ºC. Preparation of the standard curve for protein determination is outlined in Table 3.5.

Table 3.5. Preparation of the standard curve using bovine albumin (BSA) as protein standard.

BSA std µl	DdH_2O µl	NaOH (1M) µl	Reagent ml	Final Vol. ml	Protein µg
0	250	250	5	5.5	0
20	230	250	5	5.5	4
40	210	250	5	5.5	8
80	170	250	5	5.5	16
120	130	250	5	5.5	24
160	90	250	5	5.5	32
250	0	250	5	5.5	50

Samples were sonicated, diluted to a 1/10 dilution in phosphate buffered saline (PBS) and kept on ice throughout the procedure. Ten µl of the sample was mixed with 1 M NaOH solution (250 µl), ddH_2O (240 µl) and Bradford reagent (5 ml) (Stoscheck, 1990). The mixture was vortexed and incubated at room temperature for 5 min. Subsequently, the sample was pipetted into a 1.8 ml disposable cuvette and the absorbance measured spectroscopically at 595 nm using a Beckman DU®-62 spectrophotometer. The protein concentration was determined using the Lowry-program in DOS.

3.8. Statistics

The data were analysed by means of the General Linear Model Analysis of Variance. Where applicable, log transformations were performed to achieve either normality or equality of variance. These are the basic assumptions underlying the analysis of variance. When more than two treatment combinations were involved, pair wise comparisons were performed by means of the Newman-Keuls multiple comparison method, and/or the Student t-test. The ANOVA and Tukey *t*-test were used to identify significant differences

between the means of more than two groups. Differences were considered significant when $p<0.05$. The statistical analyses and graphical summaries were performed by means of the Number Cruncher Statistical System (NCSS 2000), Statistical System for Windows (Hintze, JL (1999) *User's Guide*. Number Cruncher Statistical Systems, Kaysville, Utah).

Chapter 4
Results

4.1. Pilot study

The pilot study was conducted to establish a model of iron loading for the subsequent long-term carcinogenesis study. The effect of 1%, 1.5% and 2% dietary carbonyl iron (Fe) on various parameters and the effect of reducing this dosage to 0.5% at 10 weeks was evaluated over a total period of 15 weeks (see Chapter 3.4.2, Experimental design).

4.1.1. Body weight and liver to body weight ratios

Rats fed iron-supplemented feed had softer faeces than control rats. Diarrhoea was seen in only 1 rat from the 1% Fe treatment group.

The mean body weight (BW) of the rats fed the 1% Fe was significantly ($p=0.0002$) lower than the control rats over the treatment period of 15 weeks, despite the reduction of iron to 0.5% after 10 weeks (Fig 4.1a). The mean BW of rats in the 1.5 and 2% Fe groups was also significantly ($p=0.0001$) lower than the controls with no difference between the iron-treated groups (Fig 4.1b). The increase in BW from week 10 to 15 after reduction to 0.5% Fe was significant in the 1.5% ($p=0.002$) and 2% Fe ($p=0.004$) groups, while no significant ($p=0.1$) increase was noticed in the 1% Fe group (Fig 4.1c). The BW of the control rats also significantly ($p=0.0001$) increased during this time period.

The average total body weight gain (tBWG) was significantly ($p<0.05$) lower in the iron-treated rats compared the control rats up to 10 weeks (Fig 4.2). The tBWG of the control group increased until week 10, after which it remained constant up to week 15. At weeks 6 and 10, there was no significant difference ($p>0.05$) in the tBWG between the iron-treated groups, although the 1% Fe group tended to have the highest tBWG. After reducing the iron level to 0.5% at week 10, the tBWG's of the iron-treated groups did not differ significantly ($p>0.05$) from the control group anymore. In this regard, the tBWG of the 2% Fe group surpassed that of the 1% and 1.5% Fe groups, an increase that was

significant (p=0.003). The tBWG of the 1.5% Fe group was also significant (p=0.002) in this time period (week 10-15), while it was only marginally significant in the 1% Fe group (p=0.055). No significant (p=0.4) increase in the tBWG was noticed for the control rats during this period.

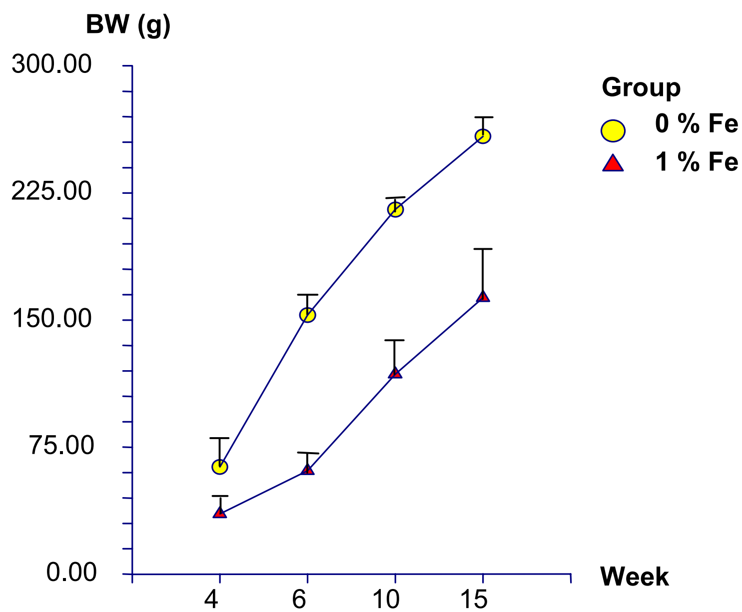

Fig 4.1a

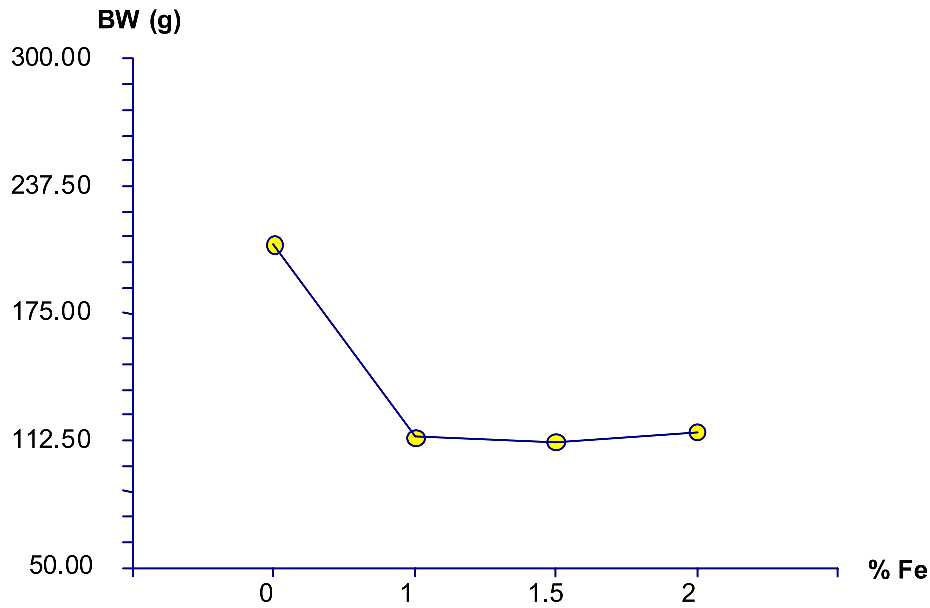

Fig 4.1b

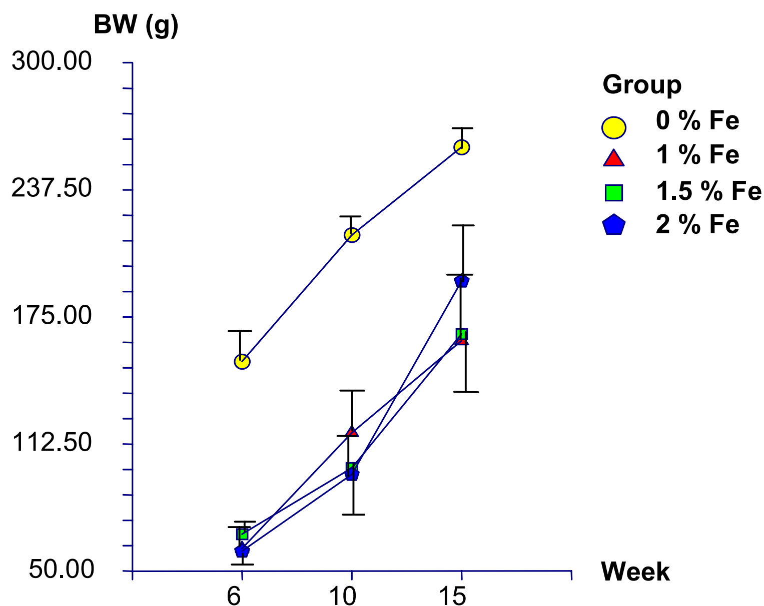

Fig 4.1c

Fig 4.1. a) The change in mean body weight (BW) of the control and 1% Fe group as a function of time. b) The differences between the combined BW of the four treatment groups over the entire treatment period. Standard deviations for the individual mean BW for each group at each time point are shown in the Appendix in Table A.IIIa. c) The effect of the level of dietary iron on the BW as a function of time. Dietary iron levels of all the treatment groups were reduced to 0.5% after 10 weeks.

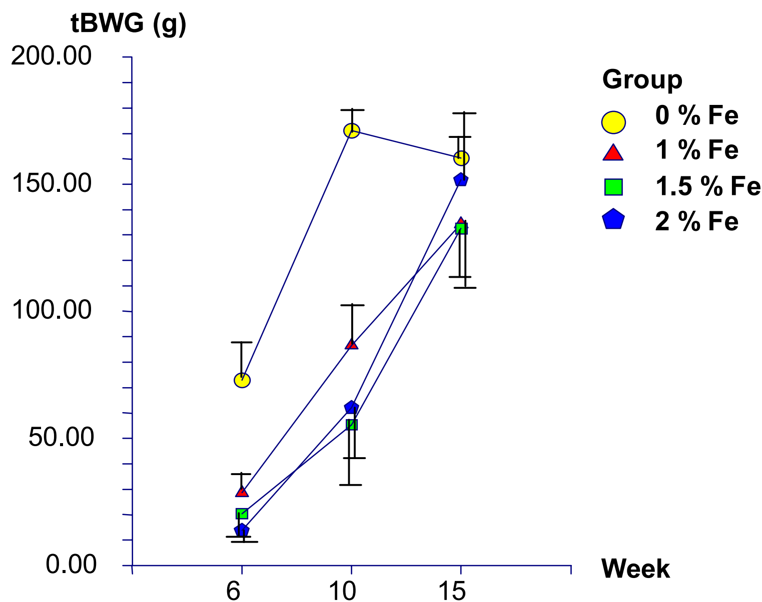

Fig 4.2. The effect of different levels of dietary iron on the total body weight gain (tBWG) over the 15-week study period. The dietary iron level of all the treatment groups was reduced to 0.5% after 10 weeks.

The iron-treated rats exhibited a significantly (p=0.0001) higher mean liver to body weight (LW/BW) ratios than the controls during the entire 15-week study period irrespective the iron treatment regimen used (Fig 4.3a).

At week 4 (pre-weaning treatment period), the difference between LW/BW ratio of the 1% Fe and control group was only marginally significant (p=0.07; data not shown). At week 6, the LW/BW ratio did not differ significantly between the rats of the four treatment groups (Fig 4.3b). The LW/BW ratios significantly (p<0.05) decreased in all treatment groups from week 6 to 10. At week 10, the LW/BW ratio of only the rats fed 1.5% Fe was significantly higher than the control group, while at week 15, all the iron-fed groups (receiving 0.5% Fe for 5 weeks) had significantly higher LW/BW ratios.

The LW/BW ratio of the 2% Fe group was significantly (p<0.05) higher than the 1% Fe group at week 15. The increase in the LW/BW ratio between weeks 10 and 15 was significant (p=0.017) in the 2% Fe group only.

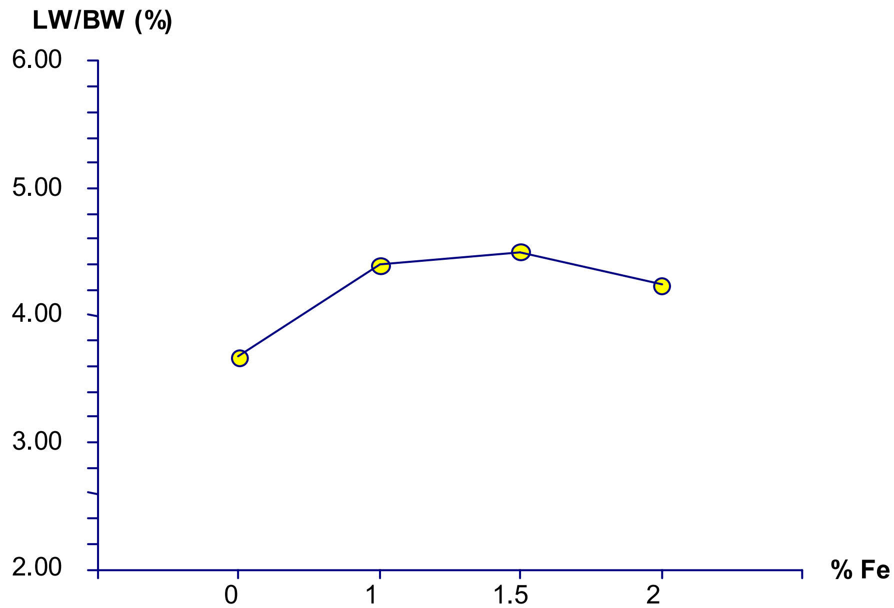

Fig 4.3a

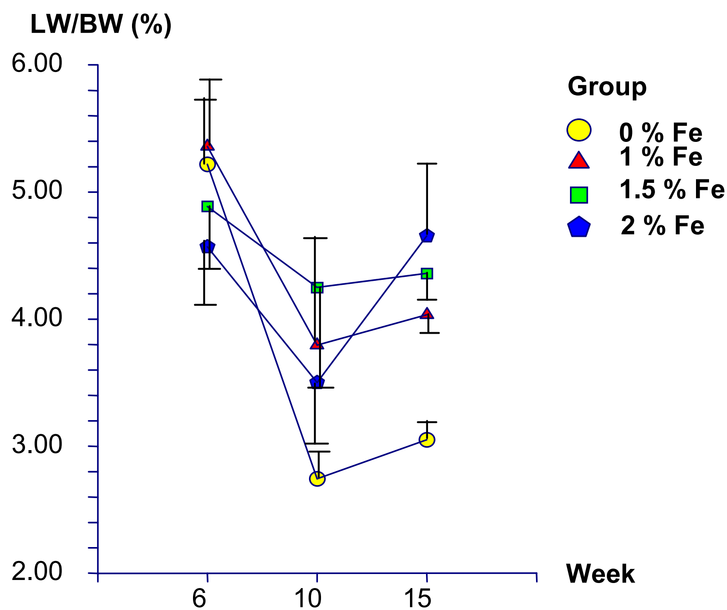

Fig 4.3b

Fig 4.3. a) The effect of varying dietary iron levels on the combined liver to body weight (LW/BW) ratio of each group over the experimental period of 15 weeks. Standard deviations for individual LW/BW ratios at each time point are shown in the Appendix in Table A.IIIa. b) The effect of the dietary iron levels on the LW/BW ratio as a function of time. The dietary iron level of all treatment groups was reduced to 0.5% after 10 weeks.

4.1.2. Liver pathology

4.1.2.1. Macroscopical pathology

The livers of the iron-treated rats had a distinct dark brown colour as compared to the control livers, which were light yellow brown (Fig 4.4a,b). At 15 weeks, the livers of the 2% Fe group were also markedly enlarged in comparison to the controls.

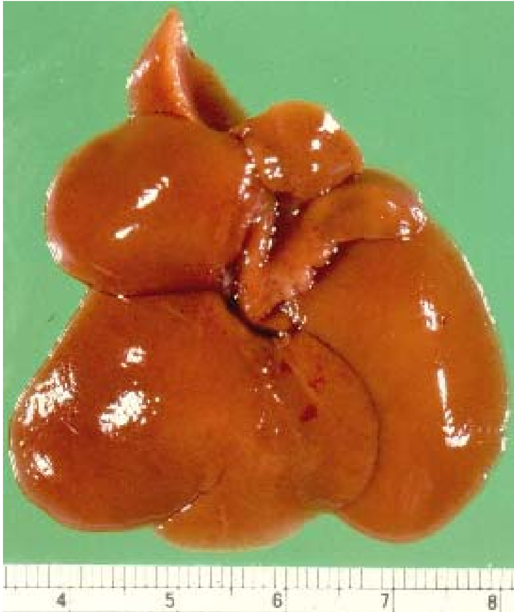

Fig 4.4a

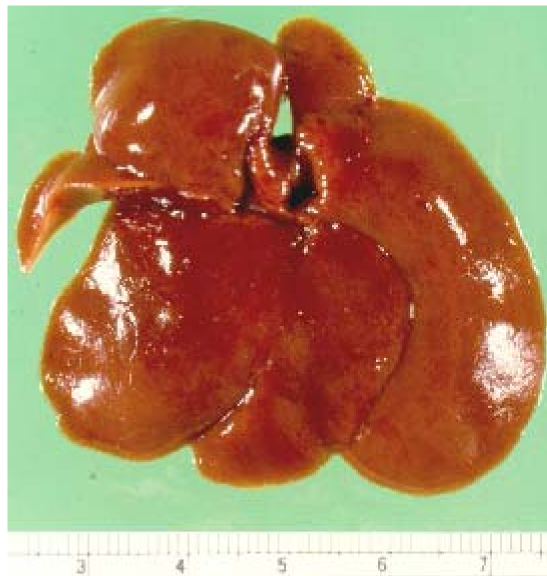

Fig 4.4b

Fig 4.4. Rat livers after 15 weeks of treatment. a) Liver of control rat. b) Liver of 2% Fe-treated rat. The liver from the iron-fed rat is darker and larger.

4.1.2.2. Microscopical pathology

Mild fatty change was seen in 2 rats of iron-treated groups at week 4 and 6. A sparse infiltration of mononuclear cells was seen in the portal tracts and was regarded to be within normal limits. Foci of necroinflammation were seen in a total of 4 iron-treated rats at week 4, 6, and 15. Figure 4.5 shows the normal liver histology of a control rat.

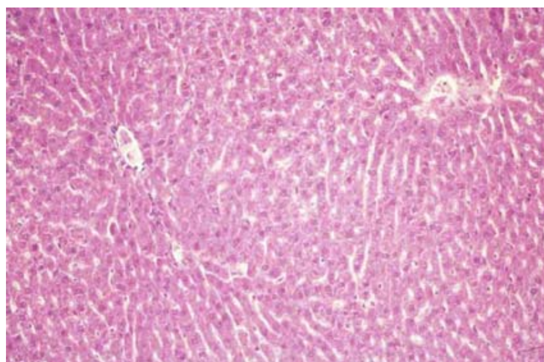

Fig 4.5 Normal histology of a liver from a control rat fed only AIN-93M. H&E staining, objective x10.

Iron deposition was seen more prominently in the hepatocytes in zone 1 (periportal region) of the liver (Fig 4.6). With increasing iron loading, deposits were also found in hepatocytes in zone 2 (mid-zonal region) and 3 (perivenular region), and in Kupffer cells and portal tract macrophages. The deposition was seen as brown granules in the haematoxylin and eosin (H&E) staining and as blue granules in the hepatocytes in the Perls' Prussian blue staining. The semi-quantitative grading of liver sections is described in 4.1.3.2.

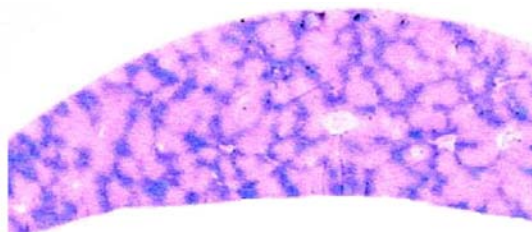

Fig 4.6. Scanned image of a Perls' Prussian blue stained section of liver, mounted on a glass slide, from a rat fed 1.5% Fe for 10 weeks and 0.5% Fe for 5 weeks. The liver shows grade 2 siderosis with maximal deposition in zone 1.

4.1.2.3. Hepatocellular proliferation

A total of 20 rats were injected with 5-bromo-2'-deoxy-uridine (BrdU) (100mg/kg BW, i.p.) dissolved in DMSO:Saline (1:3) 1h prior to termination. No significant difference was seen in the BrdU labelling of hepatocytes in all four groups after the 15-week period (Table 4.1). However, the labelling in the iron-treated group was markedly higher (up to 5-fold in the 1.5% Fe group) as compared to the

controls. No BrdU labelling was performed at the 6 and 10-week termination time points.

Table 4.1. BrdU labelling of hepatocytes in the liver of rats treated with different dietary levels of iron for 15 weeks.

BrdU labelling*	**Treatment group**			
	Control	**1%**	**1.5%**	**2% Fe**
Cells/cm^2	0.77±1.22	1.77±1.55	3.98±2.65	2.44±2.90

*Values represent the means ± standard deviation of 5 rats per group. Dietary iron levels of all the treatment groups were reduced to 0.5% after 10 weeks.

4.1.3. Hepatic iron analysis

4.1.3.1. Biochemical measurement

Over the study period of 15 weeks, the means of the log transformations of the hepatic iron levels of the iron-treated rats were significantly ($p=0.0001$) higher than the controls (Fig 4.7a). At 4 weeks (pre-weaning treatment period), the hepatic iron level of the weaned rats was significantly ($p=0.0004$) higher than that of the controls.

At week 10, the level of hepatic iron in rats in the 2% Fe treatment group was significantly ($p<0.05$) higher as compared to the 1% and 1.5% Fe-treated rats (Fig4.7b). After the reduction in dietary iron to 0.5% for 5 weeks, the hepatic iron concentration significantly decreased in the 1.5 % ($p=0.03$) and 2% Fe ($p=0.012$) groups while no significant ($p=0.55$) change was noticed in the 1% Fe group. There was no significant difference in the hepatic iron levels between the three iron-treated groups after 15 weeks. The hepatic iron level significantly ($p=0.001$) increased in the control group during this treatment period.

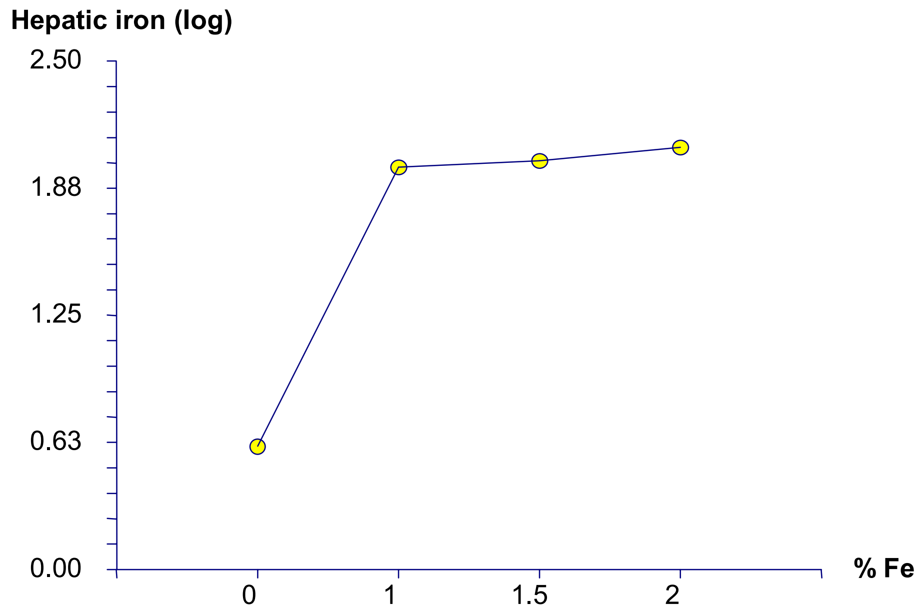

Fig 4.7a

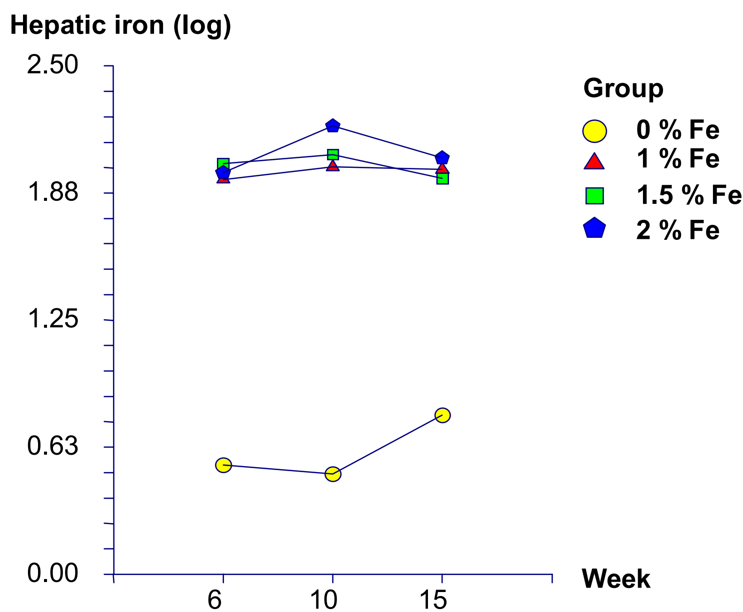

Fig 4.7b

Fig 4.7. a) Level of hepatic iron (log) in rats fed varying dietary iron levels over a period of 15 weeks. b) The effect of dietary iron as a function of time on the hepatic iron level. The dietary iron level of all treatment groups was reduced to 0.5% after 10 weeks. Mean hepatic iron level of 2% Fe-treated rats was significantly ($p<0.05$) higher than in the 1% and 1.5% Fe treatment groups at 10 weeks. Standard deviations for individual hepatic iron concentrations not shown due to the use of log transformations.

4.1.3.2. Histological assessment

The semi-quantitative assessment of the Perls' Prussian blue staining of the liver sections of iron-treated rats demonstrated progressive hepatic iron loading. Grading was performed as described in section 3.6.2. Iron-treated rats were constantly given significantly ($p<0.05$) higher scores than the controls at all time points.

When comparing the different iron-treated groups, a significant increase in hepatic iron was seen between weeks 4 and 6 in the 1.5% ($p=0.002$) and 2% Fe groups ($p=0.019$), while it was markedly increased in the 1% Fe group (Table 4.2.). At 6 weeks, hepatic iron of the 1% Fe group reached a maximum, while that of the 1.5% and 2% Fe treatment groups reached a maximum after 10 weeks. At 10 weeks, the mean score given to the 1% Fe group was significantly lower than the 1.5% Fe group ($p=0.002$) and the 2% group ($p=0.004$). Between weeks 10 and 15, hepatic iron scores decreased significantly ($p=0.001$) in the 1.5% Fe-treated group, and marginally ($p=0.062$) in the 2% Fe group.

Table 4.2. Semi-quantitative hepatic iron grading of liver sections of rats receiving different dietary iron levels for over a period of 15 weeks.

Treatment group	**Weeks**			
(% Fe)	**4**	**6**	**10**	**15**
1%	1.5±0.58	2.7±1.0a	2.7±0.45a	2.4±0.89a
1.5%	nd	3.4±0.89a*	3.8±0.45b#	1.8±0.45a#
2%	nd	2.8±0.27a*	3.7±0.45b	2.7±0.84a

Dietary iron levels of all the treatment groups were reduced to 0.5% after 10 weeks. Values represent the means ± standard deviation of 4 to 5 rats per group. Values followed by the same letter do not differ significantly at the respective time point. *Values significantly increased as compared to the 1% Fe group at 4 weeks. #Significant ($p=0.001$) decrease in hepatic iron score from week 10-15. nd=not determined.

A significant ($p=0.0001$) correlation ($r^2=0.8986$) exists between the quantitative biochemical measurement and the semi-quantitative visual assessment of hepatic iron content (Fig 4.8.).

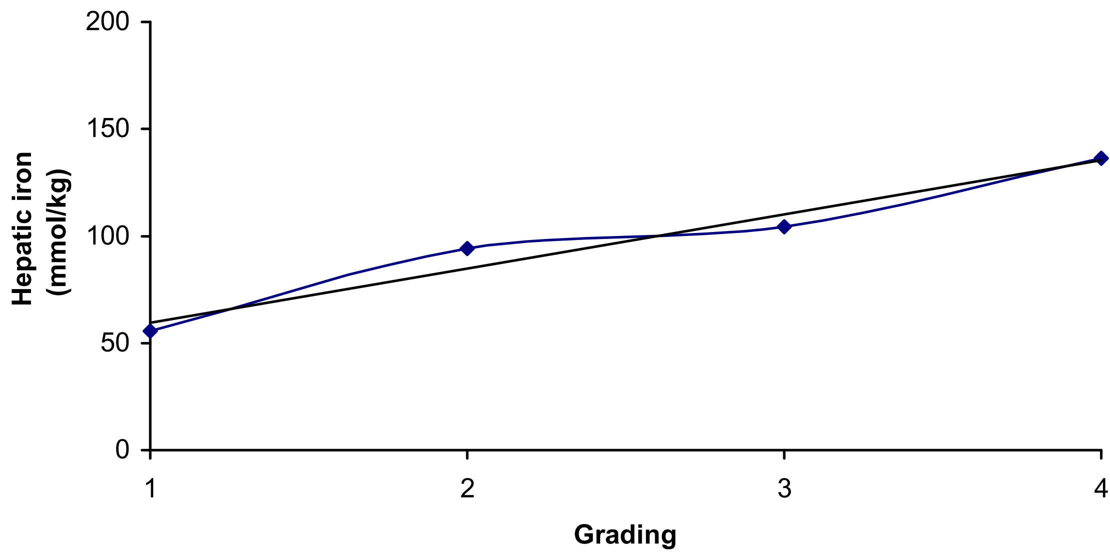

Fig 4.8. The correlation between the semi-quantitative grading and biochemical measurement of hepatic iron content.

4.1.4. Hepatic lipid peroxidation

The mean log malondialdehyde (MDA) levels decreased significantly ($p=0.004$) in all treatment groups as a function of time, irrespective the dietary iron regimen (Fig 4.9a). There was a significant ($p=0.0001$) interaction between the increase in the mean log MDA level and dietary iron level between weeks 6 to 15.

When considering the individual treatment groups, there was a significant ($p=0.01$) increase in the MDA values of the iron-treated group compared to the controls at week 4 (pre-weaning treatment period). At week 6, dietary supplementation with 1.5% and 2% Fe increased the MDA values to significantly ($p<0.05$) higher levels than those of the controls, while the mean MDA of 1% Fe group was markedly (not significantly) higher (Fig 4.9b).

At week 10, only the 1.5% Fe-treated rats had significantly ($p<0.05$) higher MDA levels while the mean levels in the 1% and 2% Fe rats were markedly (not significantly) increased above the control rats.

The MDA levels were significantly decreased in the 1% ($p=0.017$) and 1.5% ($p=0.001$) Fe groups after the reduction of the dietary iron level to 0.5%. However, the MDA levels of the 1.5%, as well as the 2% Fe groups, were still significantly ($p<0.05$) increased above the controls.

Measurement of lipid peroxidation by measuring conjugated dienes (CD) was far less sensitive, however a marginal increase ($p<0.1$) was observed in the iron-treated groups above the controls over the 6 to 15 week experimental period (Fig 4.10).

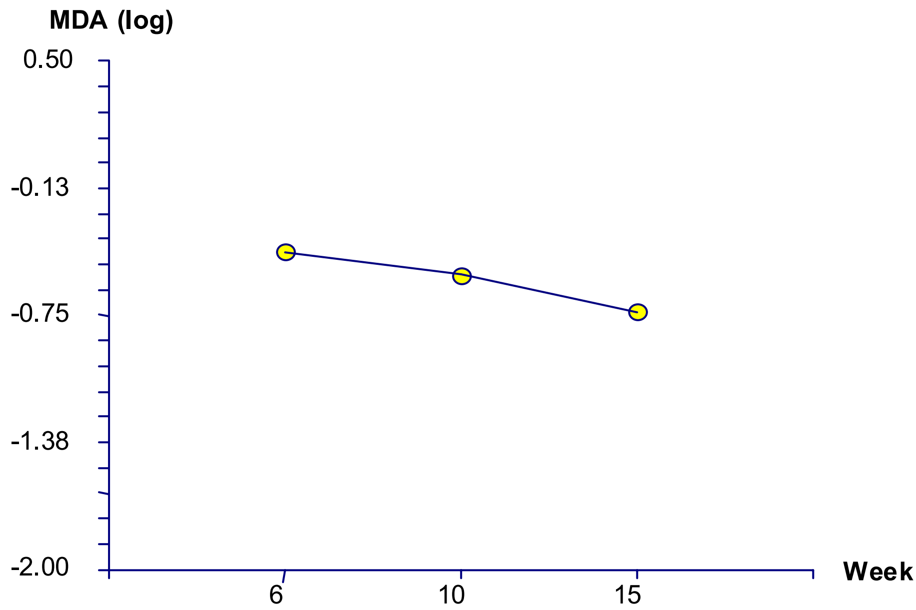

Fig 4.9a

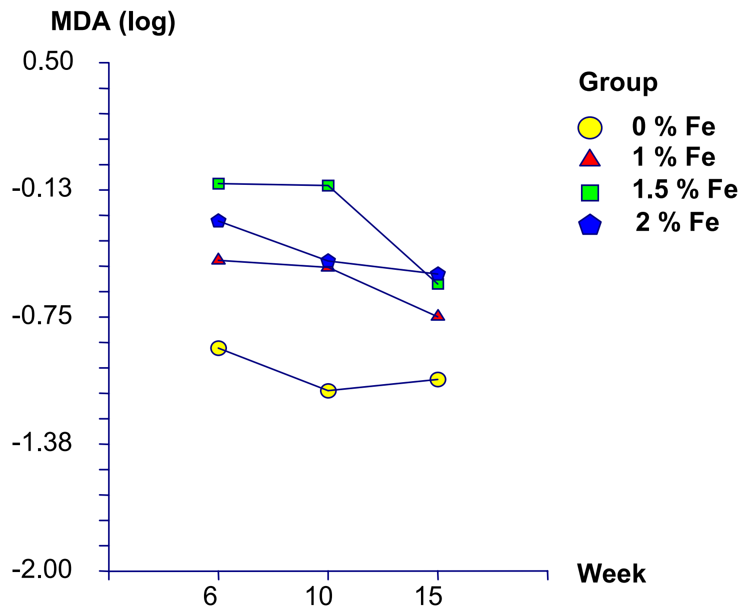

Fig 4.9b

Fig 4.9. a) The combined levels (log) of lipid peroxidation measured as malondialdehyde (MDA) in the liver of all rats over a period of 15 weeks. b) The effect of dietary iron level on MDA as a function of time. The dietary iron level of all the treatment groups was reduced to 0.5% after 10 weeks. Standard deviations for individual MDA levels not shown due to the use of log transformations.

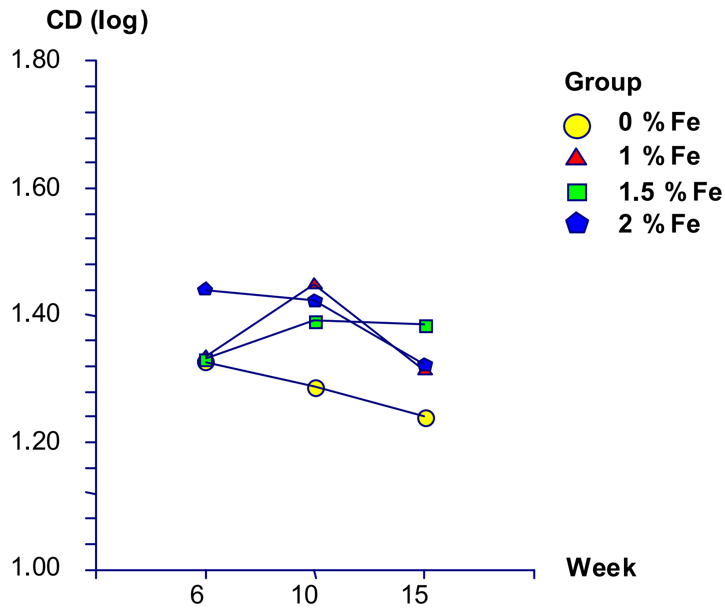

Fig 4.10. The effect of the dietary iron level on lipid peroxidation, measured as conjugated dienes (CD), as a function of time. Dietary iron levels of all the treatment groups were reduced to 0.5% after 10 weeks. Standard deviations for individual CD levels not shown due to the use of log transformations.

4.2. Long-term study

Using a dietary level of 1% Fe for 10 weeks, and a reduction to 0.5% Fe at the time FB_1 feeding commenced, the interaction between FB_1 and increased hepatic iron levels was examined in the long-term study. FB_1 was removed at 35 weeks, while iron feeding continued until week 60 (see Chapter 3.4.3, Experimental design).

4.2.1 Feed intake

The feed intake of rats from the Fe group (4-10 weeks) and FB_1/Fe and FB_1/AIN groups (10-60 weeks) was monitored on a weekly basis and the other groups averaged fed accordingly (see Chapter 3.4.3). Rats fed iron-supplemented feed had softer faeces than non-iron-fed rats; however, no diarrhoea developed in any rats. The profiles of average daily feed, dietary iron, and fumonisin B_1 (FB_1) intake of the rats are presented in Table 4.3, 4.4, and 4.5.

Table 4.3. Average daily feed intake of rats during the experimental period of 60 weeks.

Treatment group	Feed intake (g/100 g BW)		
	4-10 wks	**10-35 wks**	**35-60 wks**
FB_1/Fe	nd	5.2±1.0	4.0±0.9
Fe	10.2±2.1	4.9±0.9	4.3±1.7
FB_1/AIN	nd	5.1±1.0	4.0±0.8
Control	8.5±1.4	4.6±0.8	4.0±1.5

Dietary iron levels of the iron treatment groups were reduced to 0.5% at 10 weeks, at which time FB_1 feeding commenced. Values represent the means ± standard deviation per group. nd: not determined.

Table 4.4. Average daily additional* dietary iron intake of rats during the experimental period of 60 weeks.

Treatment group	Dietary iron intake (mg/100 g BW)*		
	4-10 wks	**10-35 wks**	**35-60 wks**
FB_1/Fe	nd	26.0±1.0	20.0±0.9
Fe	102.0±2.1	24.5±0.9	21.5±1.7

*Iron content in AIN-93M diet (35 mg per kg diet) was not taken into consideration. Dietary iron levels of the iron treatment groups were reduced to 0.5% at 10 weeks. Values represent the means ± standard deviation per group. nd: not determined.

Table 4.5. Average daily FB_1 intake of rats during the experimental period of 10-35 weeks.

Treatment group	FB_1 intake (mg/100 g BW)*	
	Week 10-15	**Week 15-35**
FB_1/Fe	1.67±0.1	0.48±0.1
FB_1/AIN	1.65±0.1	0.47±0.1

*Rats were fed 250 mg FB_1/kg diet for 5 weeks, followed by 100 mg/kg for 20 weeks. Dietary iron levels of the iron treatment groups were reduced to 0.5% at 10 weeks at which time FB_1 feeding commenced. Values represent the means ± standard deviation per group.

4.2.2. Body weight and total body weight gain

The mean body weight (BW) of the rats increased significantly ($p<0.0001$) over the study period, but there was no significant interaction ($p=0.95$) between the BW and treatment regimen. Corresponding to this data, the total body weight (tBWG) between the different time points of measurement was also significant ($p<0.0001$) and did not differ significantly between the groups (Fig 4.11).

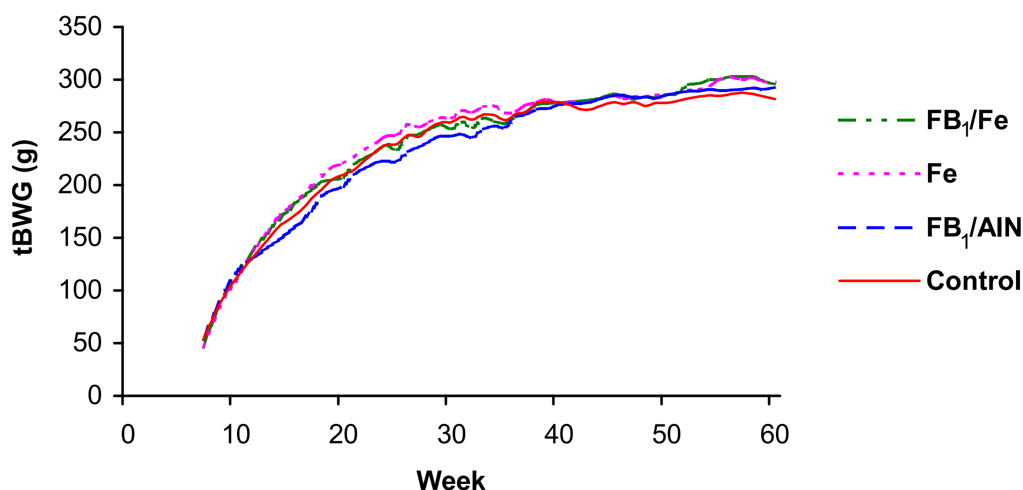

Fig 4.11. The total body weight gain (tBWG) of all treatment groups as a function of time. Dietary iron levels of the iron treatment groups were reduced to 0.5% after 10 weeks. Standard deviations are shown in the Appendix in Table AIIIb.

4.2.3. Liver to body weight ratios

Treatment period: weeks 4-10 (iron loading period)

There was a significant ($p=0.004$) interaction between the increased liver to body weight (LW/BW) ratio and excess iron during the treatment period (Fig 4.12). At week 4, the LW/BW ratios of the controls were higher than the ratios of the iron-treated group, though not significantly. However, at week 10, the means of the LW/BW ratio were significantly ($p<0.05$) higher in the iron-fed group as compared to control rats, increasing significantly in comparison to both groups at week 4. In contrast, the LW/BW ratio of the control rats tended to decrease (not significantly).

Treatment period: weeks 10-35 (FB_1 treatment period)

The mean LW/BW ratios of the rats decreased significantly ($p<0.05$) for all treatment groups from 10 to 35 weeks (Fig 4.12). The Fe group had significantly ($p<0.05$) higher ratios than the other three groups, including the FB_1/Fe group, at 35 weeks.

Treatment period: weeks 35-60 (post FB_1 treatment period)

The LW/BW ratios showed a significant ($p<0.05$) increase over this time period (Fig 4.12). After removal of FB_1 from the diet, the mean LW/BW ratios increased significantly ($p<0.001$) in the FB_1/Fe group and reached a similar level as the Fe group. The increase in the Fe group was marginally significant ($p=0.06$) during the same treatment period. The LW/BW ratios of both iron-treated groups were significantly ($p<0.001$) higher than the non-iron treated rats at 60 weeks. The difference between FB_1-treated and control rats was not significant at this time.

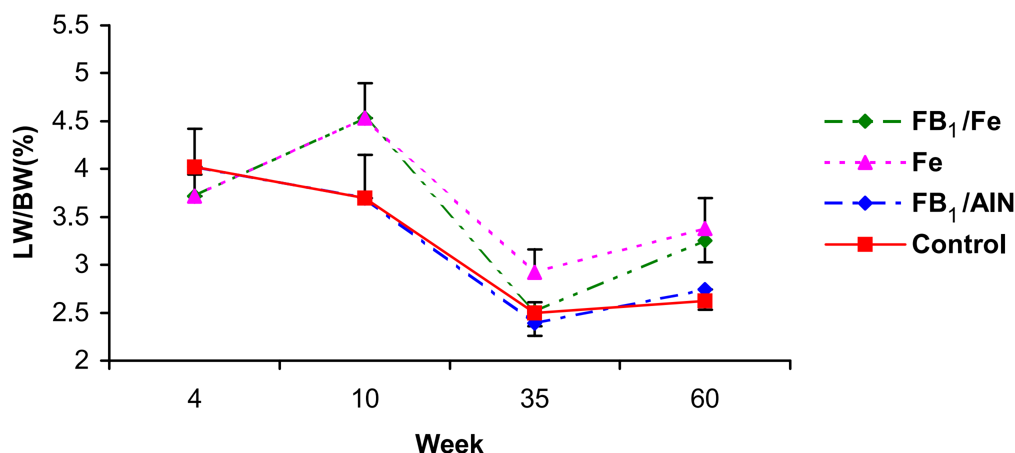

Fig 4.12. The liver to body weight (LW/BW) ratios of all treatment groups as a function of time. Dietary iron levels of the iron-treated groups were reduced to 0.5% after 10 weeks.

4.2.4. Haematological and biochemical parameters

The biochemical parameters of one control rat showing abnormal pathology at 35 weeks was not used in the statistical analysis because of the resulting distortion of the means ± standard deviation.

No significant difference was noticed between the different treatment groups and time points with regard to haematological parameters including white blood cell count (WBC), platelet count (PL), red blood cell distribution width (RDW) and basophilic cells.

The *percentage* of neutrophil cells increased significantly ($p<0.035$) in the Fe group between 35 and 60 weeks. When considering the *quantitative mean neutrophil count* (1/100), the removal of FB_1 increased the count in the FB/Fe group. The increase between 35 and 60 weeks was marginally significant in the FB/Fe ($p=0.056$) and Fe ($p=0.059$) group.

The mean lymphocyte percentage significantly ($p<0.001$) decreased over the time period, irrespective of treatment group. In comparison, the mean percentage of monocytes increased significantly ($p=0.001$) as a function of time with no interaction with Fe or FB_1.

The red blood cell count (RBC) significantly ($p=0.001$) increased with time, irrespective of treatment group. At 35 weeks, FB_1-treated rats had significantly ($p<0.05$) lower values than their counterparts (Fig 4.13a). After the FB_1 was omitted from the diet, the RBC of the rats increased significantly to levels similar to that of the non FB_1-treated rats. In addition, iron-treated rats tended to have lower RBC means than non-treated rats but the difference was not significant. Similar to the RBC levels, the haematocrit (HCT) levels were significantly ($p<0.05$) lower at 35 week in the FB_1-treated rats as compared to the non-treated rats (Fig 4.13b). The mean HCT significantly ($p=0.001$) increased from week 35 to 60. The haemoglobin (Hb) values were also significantly ($p=0.05$) decreased by FB_1 when compared to the other treatment groups (Fig 4.13c). Upon removal of FB_1 at 35 weeks, the Hb increased significantly towards the levels monitored in the untreated control rats.

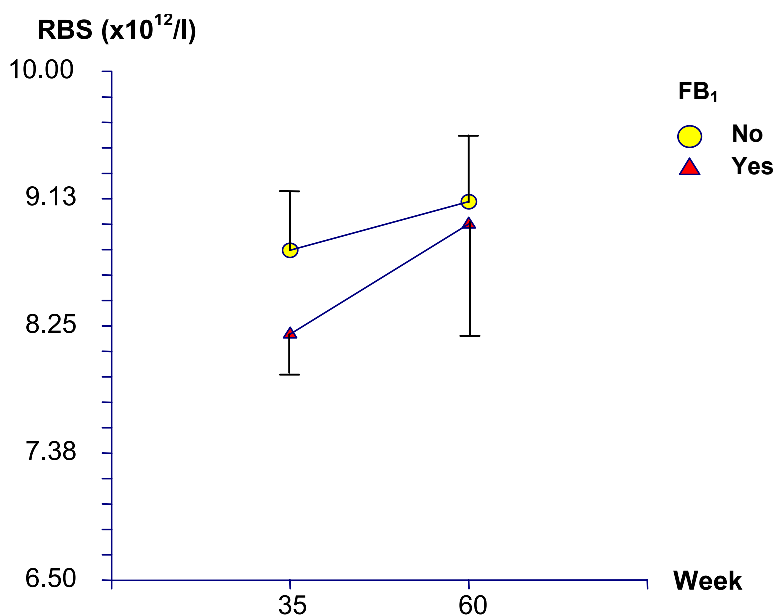

Fig 4.13a

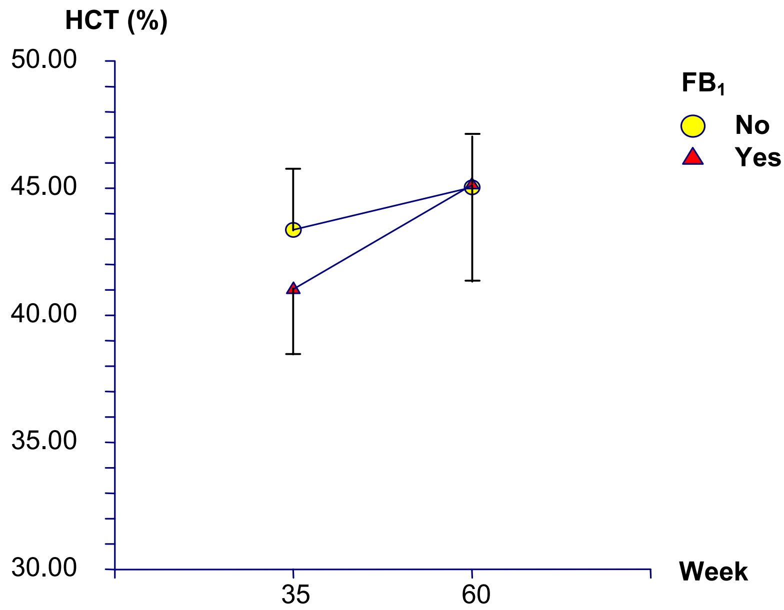

Fig 4.13b

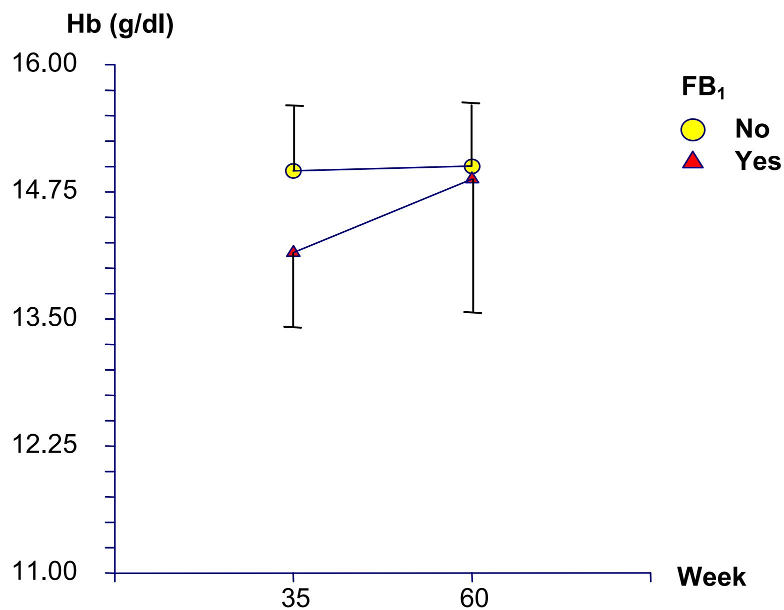

Fig 4.13c

Fig 4.13. The effect of FB_1-treatment (10-35 weeks) and subsequent removal from the diet at 35 weeks on a) red blood cell count (RBC); b) haematocrit (HCT); and c) haemoglobin (Hb). Standard deviations of individual groups are shown in the Appendix in Table AIIIc.

The mean cell volume (MCV) was significantly increased in iron-treated rats at both 35 and 60 weeks ($p=0.03$). The FB_1/Fe group had significantly ($p<0.05$) higher values than the other groups, while values of the Fe group were significantly ($p<0.05$) higher than that of the FB_1/AIN and control groups (Fig 4.14).

The mean cell haemoglobin (MCH) decreased significantly ($p<0.001$) from 35 to 60 weeks, irrespective of group. Iron-treated rats had significantly ($p<0.001$) higher means than non-iron-treated rats (Fig 4.15a). Corresponding to this, the overall mean cell haemoglobin concentration (MCHC) also decreased significantly ($p<0.001$) from 35 to 60 weeks. Again, iron-treated rats had significantly ($p<0.001$) higher means than non-iron-treated rats (Fig 4.15b). No effect was noticed as a result of the FB_1-treatment.

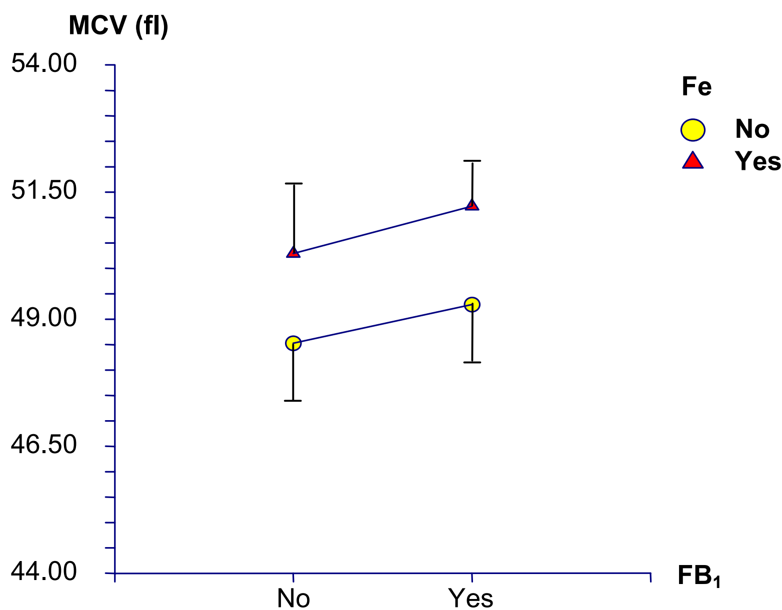

Fig 4.14. The effect of FB_1 and excess iron on the mean cell volume (MCV) during the 35 to 60 week experimental period. Standard deviations of groups at each time point are shown in the Appendix in Table AIIIc.

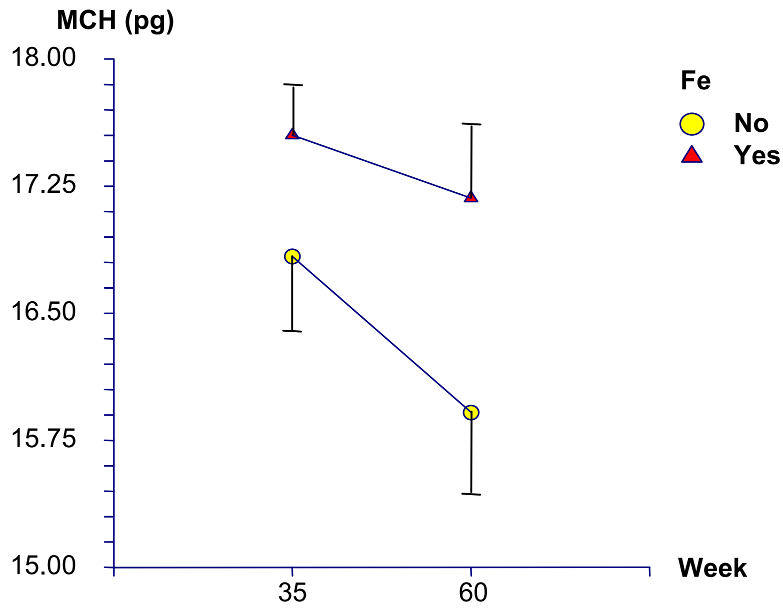

Fig 4.15a

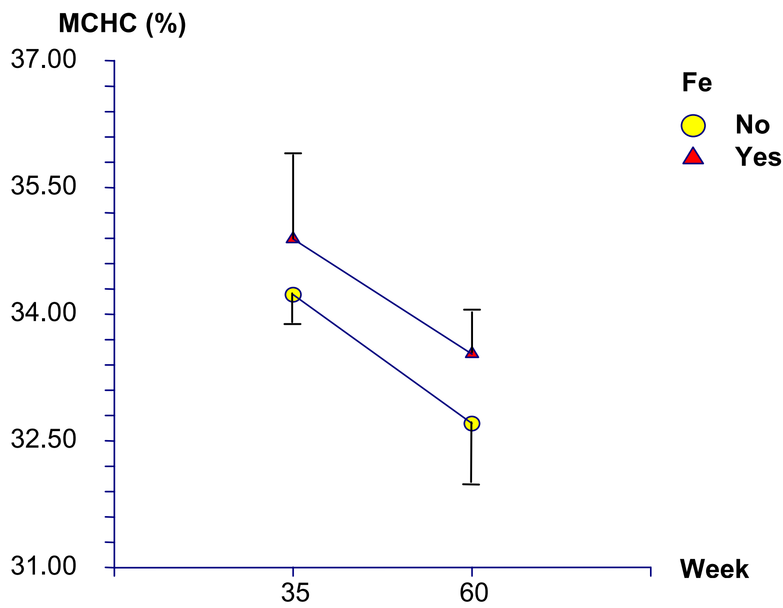

Fig 4.15b

Fig 4.15. The effect of excess iron on a) mean cell haemoglobin (MCH) and b) the mean cell haemoglobin concentration (MCHC) as a function of time (weeks). Standard deviations of individual groups are shown in the Appendix in Table AIIIc.

The biochemical data consisting of serum glucose, γ-glutamyl transpeptidase (GGT), total protein, urea, and total cholesterol were not affected by FB_1 during the treatment period of 25 weeks (10 to 35 weeks) and the post FB_1 period up to 60 weeks.

The mean serum iron was significantly ($p<0.001$) higher in iron-treated rats than in their counterparts (Fig 4.16). Rats from the FB_1/Fe group also tended to have higher iron levels than rats treated with dietary iron only, though the differences were not significant ($p>0.05$).

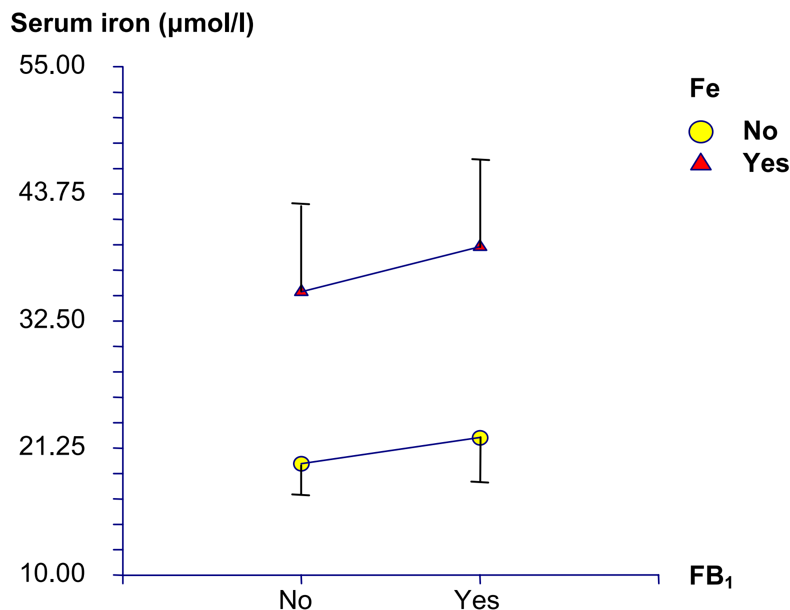

Fig 4.16. The effect of FB_1 and dietary iron on serum iron at 60 weeks.

The mean total and direct bilirubin decreased significantly ($p<0.001$) over time, irrespective of treatment group. At week 35, the rats from the FB_1/AIN group had significantly ($p<0.05$) higher levels than non-treated rats, while Fe significantly ($p=0.05$) decreased the bilirubin parameters (Fig 4.17a,b). However, at 60 weeks the effect of FB_1 was reversed following the removal of the compound from the diet. A similar effect was noticed with respect to the Fe where the total and direct bilirubin were reduced over time to similar levels of the control rats.

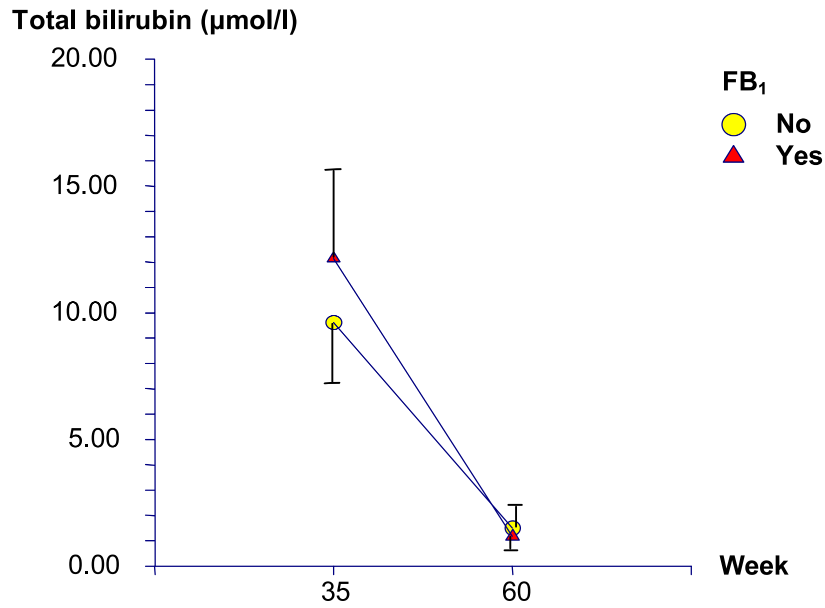

Fig 4.17a

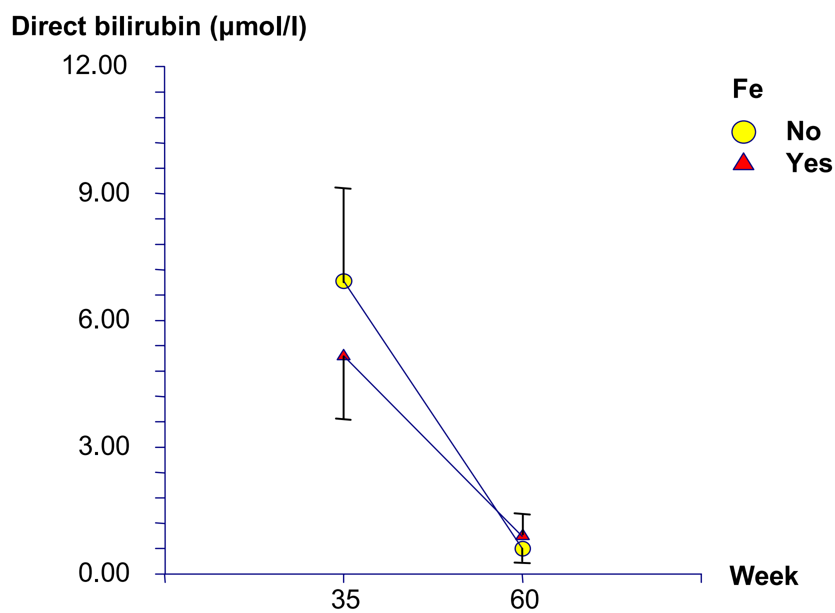

Fig 4.17b

Fig 4.17. a) The effect of FB_1-treatment (10-35 weeks) and subsequent removal from the diet at 35 weeks on total bilirubin. b) The effect of excess iron on direct bilirubin during the 35 to 60 week experimental period. Standard deviations of individual groups are shown in the Appendix in Table AIIIe.

The combined treatment of FB_1/Fe exhibited a marginal effect ($p<0.1$) regarding an increase in the level of the serum enzyme aspartate transferase (AST) (Fig 4.18a). A similar response was noticed with respect to the level of alanine transaminase (ALT), which was significantly ($p=0.03$) increased by FB_1 (Fig 4.18b). In this regard, the combined FB_1/Fe group exhibited ALT levels significantly ($p<0.05$) higher when compared to the control group.

The mean alkaline phosphatase (ALP; $p=0.03$) and creatinine ($p=0.03$) levels significantly) decreased as a function of time while dietary iron further accentuated ($p=0.04$) the decrease in ALP. FB_1 on the other hand significantly ($p=0.001$) increased both the ALP and creatinine levels and rats from the FB_1/AIN group had overall significantly ($p<0.05$) higher ALP values than the other groups (Fig 4.19a). After removal of FB_1, both creatinine and ALP decreased significantly ($p<0.05$) in these rats (Fig 4.19b). At 60 weeks no significant differences were noticed between the FB_1/AIN treated and control rats with respect to these two parameters.

4.2.5. Liver pathology

4.2.5.1. Macroscopical pathology

The livers of the iron treated rats were yellow-brown in colour and were larger than the controls. At 35 weeks, the FB_1/Fe treated animals (5/5) exhibited numerous white foci/nodules on the capsular surface, ranging from 2-8 mm in size and found throughout the liver (Fig 4.20a). A similar appearance was noticed in the liver of the FB_1-treated rats although only 4/5 rats had foci/nodules, and these were noted to be smaller than those of the FB_1/Fe (2-4 mm) (Fig 4.20b).

At 60 weeks, the difference between the two FB_1-treated groups was not as marked. All rats in the two groups had various nodules on the external surface and these ranged in size from small (2 mm) to massive growths over 10 mm (Fig 4.20c,d). All lobes were affected. One rat from the Fe group also had a 3-4 mm nodule in the median lobe.

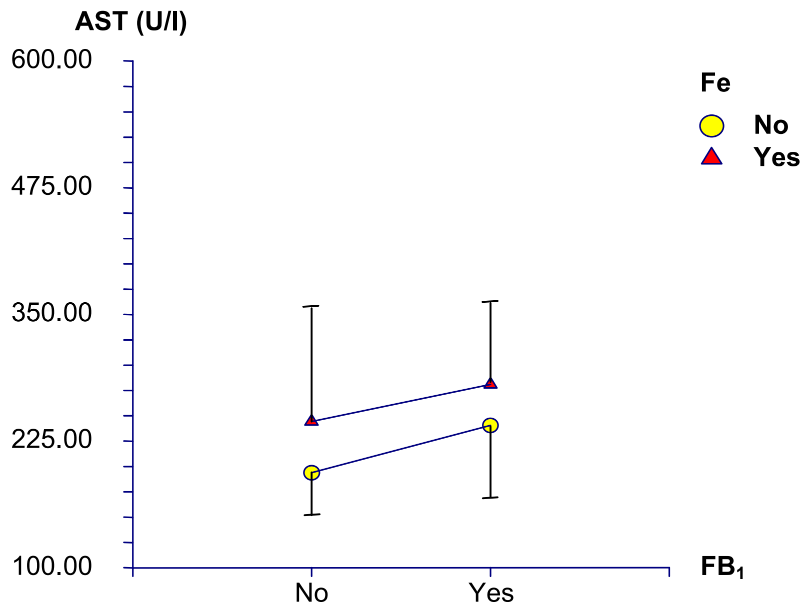

Fig 4.18a

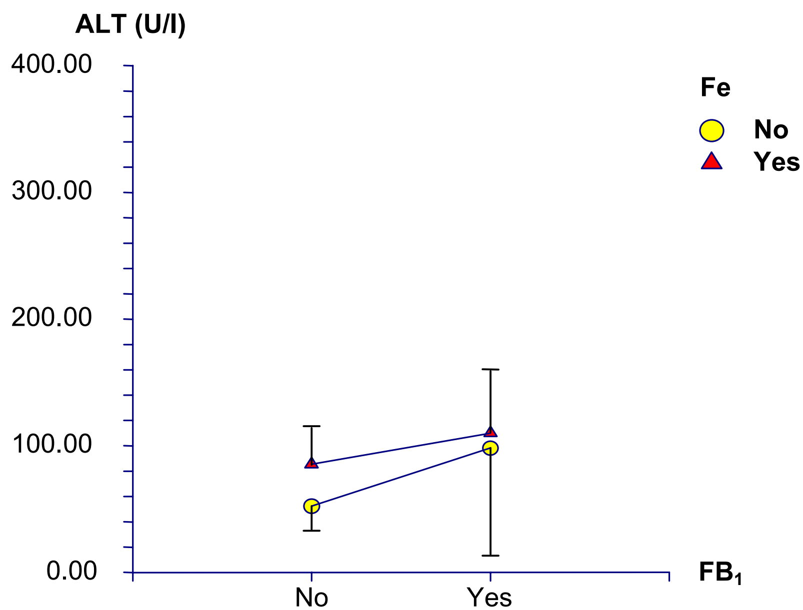

Fig 4.18b

Fig 4.18. The effect of FB_1 and iron overload on a) aspartate transaminase (AST) and b) alanine transaminase (ALT) during the 35 to 60 week experimental period. Standard deviations of groups at each time point are shown in the Appendix in Table AIIIe.

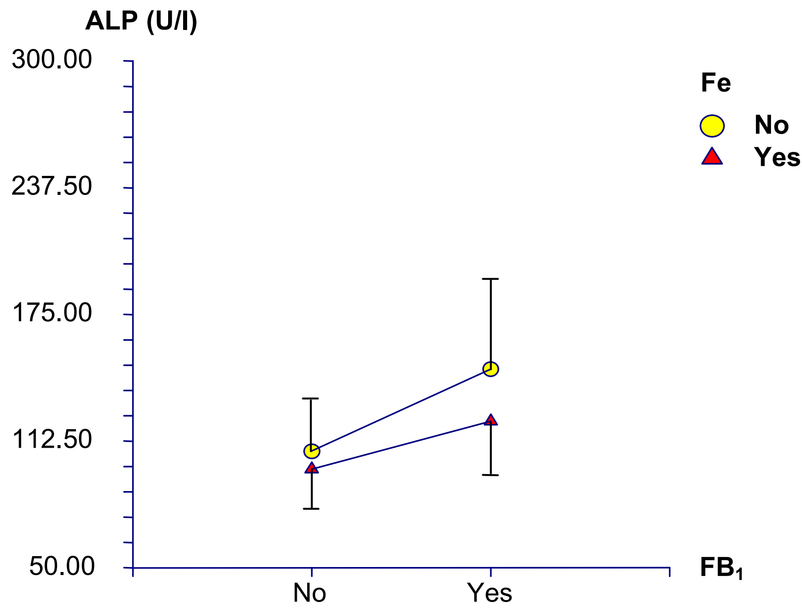

Fig 4.19a

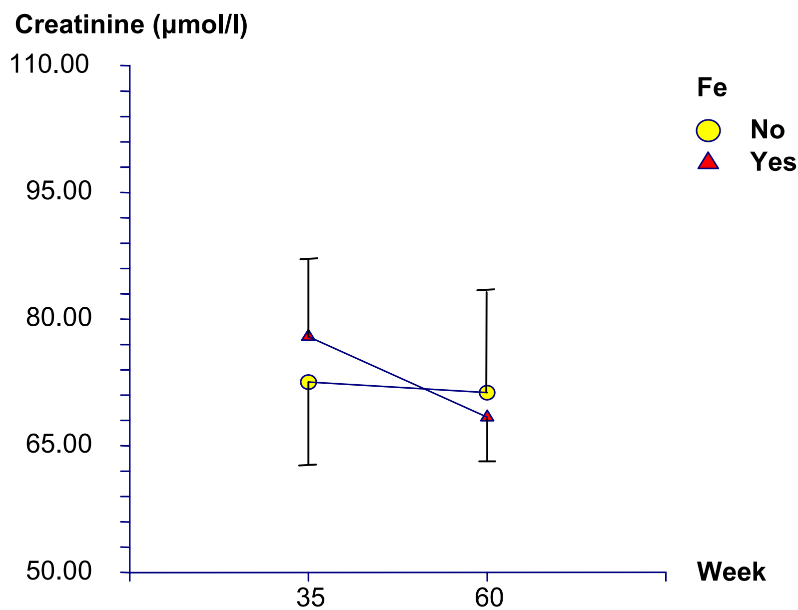

Fig 4.19b

Fig 4.19. a) The effect of FB_1 and dietary iron on alkaline phosphatase (ALP) over the 35 to 60 week experimental period. b) The effect of dietary iron treatment on creatinine during the 35 to 60 week experimental period. Standard deviations of individual groups and at each time point are shown in the Appendix in Table AIIIe.

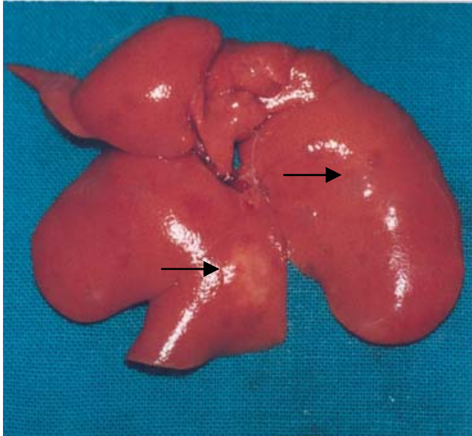

Fig 4.20a

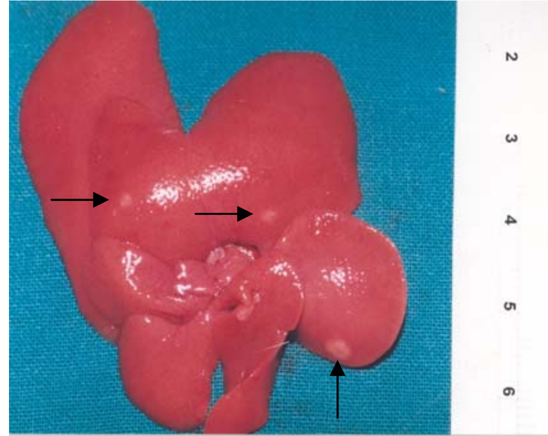

Fig 4.20b

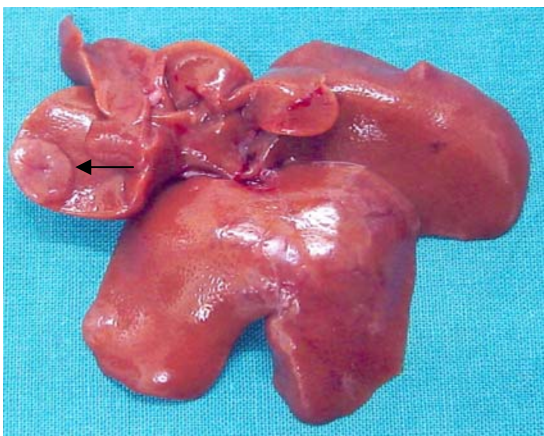

Fig 4.20c

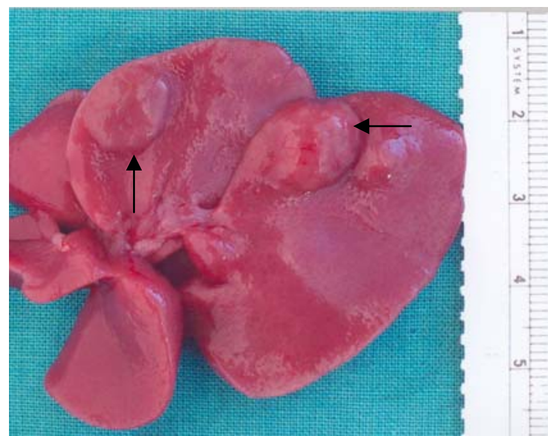

Fig 4.20d

Fig 4.20. Liver pathology of the fumonisin B_1-treated rat. Livers of rats treated with FB_1/Fe at a) 35 weeks and c) 60 weeks. Livers of rats treated with FB_1/AIN at b) 35 weeks and d) 60 weeks. Arrows depict foci/nodules at 35 weeks and larger nodules at 60 weeks.

4.2.5.2. Microscopical pathology

Termination time point: weeks 4 and 10 (iron loading period)

At age 4 and 10 weeks, 5 rats from both groups were terminated in order to assess the 'baseline' hepatic iron loading.

As in the pilot study, iron deposition seen as brown granules in the H&E staining and as blue granules in the Perl's Prussian blue staining were observed most prominently in the hepatocytes in zone 1 (periportal region) of the liver. With increasing iron loading, deposits were also found in hepatocytes in zone 2 (mid-zonal region) and 3 (perivenular region). Minimal iron accumulation was seen in Kupffer cells and portal tract macrophages.

At 10 weeks, a mild non-specific steatohepatitis was observed in 1 iron-treated rat (1/5). Minimal fatty change and foci of necroinflammation were observed in both iron-treated and control rats.

Termination time point: week 35 (25-week FB_1 treatment period)

FB_1/Fe group

After treatment with FB_1, at week 35, all rats (5/5) in FB_1/Fe group exhibited at least 1 nodule displaying high-grade dysplasia, with 4/5 having several nodules (Fig 4.21a; Table 4.6). Three from 5 rats also showed smaller nodules and foci showing mild or no dysplasia. High-grade dysplasia, as defined earlier for the purpose of this study (see Chapter 3.5.1), was characterised by the presence of a non-invasive pushing edge into adjacent liver tissue, large cells with clear cytoplasm, mitosis, intranuclear inclusions, large nucleoli, apoptosis of occasional dysplastic cells, and moderate nuclear pleomorphism (Fig 4.21b). Oval cells were seen inside each of the high-grade dysplastic lesions. No hepatocellular cancers (HCCs), characterised by invasion into surrounding liver tissue and the vascular system, and metastasis to other organs, were seen.

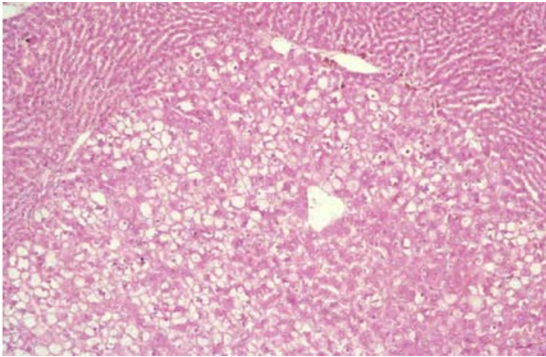

Fig. 4.21a

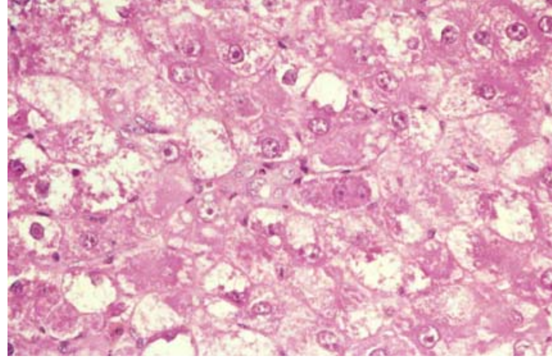

Fig. 4.21b

Fig 4.21. Liver histopathology in FB_1/Fe treated rats. High-grade dysplastic nodule at 35 weeks at a) objective x4 and b) objective x20; H&E staining.

Iron distribution in these rats had changed as compared to iron-loaded rats at 10 weeks. Iron deposition was still seen in hepatocytes, but due to a shift of iron from necrotic hepatocytes, deposits were also seen in Kupffer cells and portal tract macrophages (Fig 4.22). Hepatocytes in the dysplastic nodules did not contain iron, but some iron deposition was seen in Kupffer cells within the nodules.

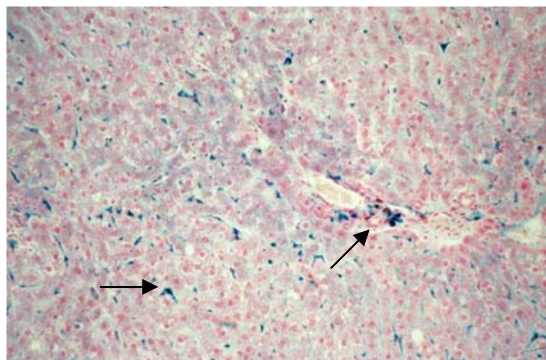

Fig 4.22. Shift in iron distribution in liver section of FB_1/Fe-treated rat. Iron is seen in Kupffer cells (straight arrow) and portal tract macrophages (diagonal arrow) as well as in most of the hepatocytes. Perls' Prussian blue staining, objective x10.

Fe group

The rats in the Fe group showed iron deposition in hepatocytes, Kupffer cells, and portal tract macrophages, as described at week 10, but no evidence of liver injury and no nodules were seen.

FB_1/AIN group

Only 2 rats (2/5) in the FB_1/AIN group had nodules showing high-grade dysplasia (Fig 4.23). The dysplasia showed the same characteristics as described for FB_1/Fe group. In 1 rat (1/5), an area of fatty change, several small low-grade dysplastic nodules and 1 small focus, also displaying low-grade dysplasia, were observed. Two rats (2/5) showed no liver injury at all and were thought to be control animals until the codes on the glass slides were broken. No HCCs were seen.

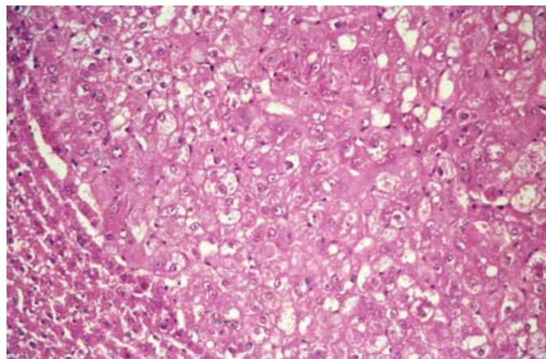

Fig 4.23. High-grade dysplastic nodule in FB_1/AIN treated rat at 35 weeks. H&E staining, objective x4.

Iron deposition in Kupffer cell and portal tract macrophages was also seen in 2 FB_1/AIN animals, which had received no iron supplementation. Hepatocytes, however, had accumulated no iron.

Control group

Some of the control rats showed minor changes: minimal fatty change (2/5), portal tract inflammation (2/5), and tiny foci of necroinflammation (2/5). One control rat, however, showed bile ductular proliferation in one portal tract, polymorphs and ductular cells in the parenchyma, and terminal portal

inflammatory infection. No nodules were seen and the cause of this pathology is not known but it may have been in some way related to respiratory infection that occurred in the final days (see Chapter 4.2.8).

Table 4.6. Summary of hepatic nodules and foci seen histologically in H&E staining at 35 weeks.

Treatment group	Total no. of nodules/foci in H&E sections
FB_1/Fe	33 (in 5/5 rats)
Fe	0
FB_1/AIN	7 (in 3/5 rats)
Control	0

Termination time point: week 60 (25-week post FB_1 treatment period)

FB_1/Fe treated rats

At 60 weeks, 8 animals in the FB_1/Fe group (8/10) showed at least 1 high-grade nodule, and 6 of these 8 rats had several large nodules showing high-grade dysplasia, and also smaller nodules and foci showing varying degrees dysplasia (Table 4.7). Most of the hepatocytes within the nodules had clear cytoplasm, but some ‘mixed’ cell nodules (clear cells and cells with abundant deeply eosinophilic cytoplasm) were also seen. Most of the nodules showing high-grade dysplasia contained mitosis, and showed apoptosis as well as prominent numbers of oval cells. Two rats (2/10) developed only low-grade dysplastic nodules and foci. Also, mild fatty change, slight portal tract inflammation, and foci of necroinflammation in the parenchyma were observed in some rats. No HCCs were seen.

Iron distribution was similar as seen in rats from the same group terminated at 35 weeks. Iron granules were still seen in hepatocytes, but due to a shift of iron from necrotic hepatocytes, increasing deposition was seen in Kupffer cells and portal tract macrophages. Hepatocytes in the dysplastic nodules did not contain iron, but again deposition was seen in Kupffer cells within the nodules (Fig 4.24).

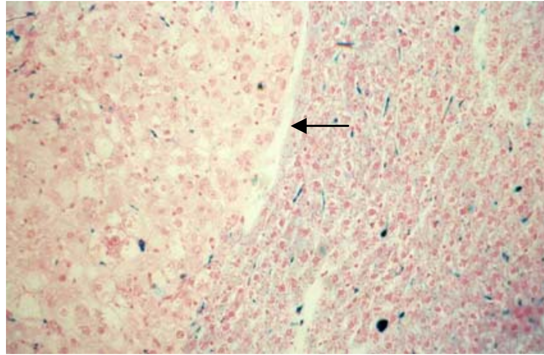

Fig 4.24. Liver section of rat from the FB_1/Fe group at 60 weeks showing grade 3 parenchymal iron deposition. Arrow points to iron-free nodule. Perls' Prussian blue staining, objective x20.

Fe-treated rats

Three rats in the Fe-treated group (3/9) showed mild fatty change, 1 showed portal tract inflammation and 1 rat developed a focus of necroinflammation in the parenchyma. One iron-treated rat developed mild fatty change and surprisingly 1 large, mixed cell nodule. The nodule showed high-grade dysplasia with fatty change within the nodule, mitosis, moderate nuclear pleomorphism, and some cells with multiple nucleoli.

Increased iron accumulation was seen in Kupffer cells and portal tract macrophages, in addition to deposition in hepatocytes. The hepatocytes in the nodule showed no iron deposition (Fig 4.25).

Fig 4.25 Digital image of a Perls' Prussian blue stained section of liver mounted on a glass slide. The nodule did not stain; no iron had accumulated in hepatocytes.

FB_1/AIN –treated rats

Eight rats of 10 in the FB_1/AIN group (8/10) had at least 1 large nodule of high-grade dysplasia (Fig 4.26). The dysplasia showed the same characteristics as described for FB_1/Fe group. In addition, these 8 rats had smaller nodules of varying degree of dysplasia and the remaining 2 rats (2/10) had only foci of low-grade dysplasia. Portal tract inflammation was observed in 1 rat (1/10) and fatty change was described in 3 rats, of which 1 rat showed severe fatty change. No HCCs were seen.

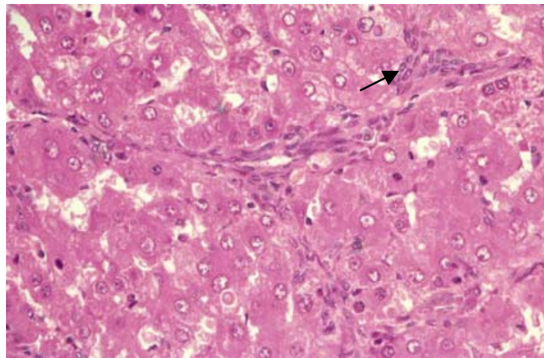

Fig 4.26. High-grade dysplastic nodule in FB_1/AIN-treated rat at 60 weeks. The arrow points to oval cells. H&E staining, objective x20.

Iron deposition in Kupffer cell and portal tract macrophages was also seen in 2 FB_1/AIN animals, which had received no iron supplementation and had no iron deposition in the hepatocytes.

Control rats

Control animals showed mild fatty change (6/10), a focus of necroinflammation (1/10), and portal tract inflammation (1/10). One animal had 2 very small foci of dysplastic cells.

Table 4.7. Summary of hepatic nodules and foci seen histologically in H&E staining at 60 weeks.

Treatment group	Total no. of nodules/foci in H&E sections
FB_1/Fe	> 68* (in 10/10 rats)
Fe	1 (in 1/10 rat)
FB_1/AIN	> 50* (in 10/10 rats)
Control	0

*Exact number of nodules not determined because of poorly defined borders.

4.2.5.3. Immunohistochemistry

4.2.5.3.1. Hepatocellular proliferation

Termination time point: week 35 (25-week FB_1 treatment period)

A total of 10 rats were injected with 5-bromo-2'-deoxy-uridine (BrdU) (100 mg/kg BW, i.p.) dissolved in DMSO:Saline (1:3) 1h prior to termination. Figure 4.27 shows a liver section taken from a rat from the group FB_1/Fe at 35 weeks. Due to the high number of stained hepatocytes in the FB_1/Fe group, two different methods were used to evaluate hepatocellular proliferation. Liver sections of the 3 rats in the FB_1/Fe group were scored by counting at least 500 cells per liver section (x40) and the labelling indices were expressed as a percentage. One section from an FB_1/AIN rat also showed a high labelling index and was scored as a percentage. The sections from the other rats were scored as the number of stained cells/cm^2 (Table 4.8).

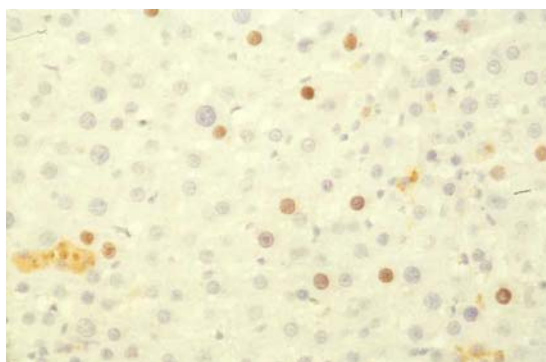

Fig 4.27. Section of liver from FB_1/Fe rat at 35 weeks. The liver shows BrdU staining in the nuclei of hepatocytes. BrdU antibody staining, objective x20.

Liver sections from rats treated with FB_1/Fe had by far the most labelled hepatocytes with a mean of almost 4% of hepatocytes being stained. Rats from the FB_1/AIN group had over twice as many labelled hepatocytes than the controls and a third more than seen in the Fe group. The Fe group also demonstrated twice as much labelling as the controls.

Table 4.8. BrdU labelling of hepatocytes in the liver of rats in the different treatment groups at 35 weeks (FB_1-feeding for 25 weeks).

BrdU labelling*	**Treatment group**		
	Fe	**FB_1/AIN**	**Control**
No. of rats	n=4	n=3	n=1
Cells/cm^2	0.88±1.16	1.02±1.31	0.42

*Values represent the means ± standard deviation per group.

Termination time point: week 60 (25-week post FB_1 treatment period)

The sections from the 35 rats injected with BrdU prior to termination were scored as the number of stained cells/cm^2 and the index is illustrated in Table 4.7. The differences among the groups are not significant. However, the labelling in the all treatment groups was markedly higher (over twice as much) as compared to the controls.

Table 4.9. BrdU labelling of hepatocytes in the liver of rats in the different treatment groups at 60 weeks (FB_1 removal at 35 weeks).

BrdU labelling*	**Treatment group**			
	FB_1/Fe	**Fe**	**FB_1/AIN**	**Control**
No. of rats	n=8	n=8	n=10	n=9
Cells/cm^2	0.71±0.57	0.73±1.09	0.81±0.92	0.34±0.34

*Values represent the means ± standard deviation per group.

4.2.5.3.2. Enzyme altered foci and/or nodules

The number of single cells (‘singlets’) that were positive for the placental form of glutathione S-transferase (GSTP$^+$) was significantly ($p<0.001$) higher in iron-treated rats than in non-iron rats at both 35 and 60 weeks. The FB_1/Fe group tended to have more GSTP$^+$ singlets than those treated with iron alone, though

not significantly ($p>0.05$). A similar result was seen regarding $GSTP^+$ 'duplets' (2 cells together). Iron-treated rats also had significantly ($p<0.001$) more stained duplets than non-iron rats, though in contrast to single cell staining, rats treated with iron alone tended to have higher numbers than the FB_1/Fe. The number of singlets and duplets significantly ($p<0.005$) decreased for iron-treated rats between weeks 35 and 60.

At 35 weeks, groups of more than 2 $GSTP^+$ cells were not reported. At 60 weeks, no significant ($p>0.05$) difference was seen between treatment groups in regard to the number of groups with 4-10 $GSTP^+$ cells.

Foci/nodules were divided into different groups according to the size (in µm) of foci/nodules expressed per cm^2 (Fig 4.29 and 4.30). The four treatment groups did not differ significantly in regard to the number of foci smaller than 1 µm. Only the FB_1/AIN group had marginally ($p=0.075$) higher numbers than the Fe group at 35 weeks.

At 35 weeks, FB_1/Fe rats had significantly ($p<0.033$) more foci (size: 1-10 µm) than control rats and marginally ($p=0.053$) higher than the Fe group (Fig 4.29). The FB_1/AIN group had markedly higher numbers of foci than the Fe and control groups. Both FB_1-treated groups had significantly more foci than the control ($p=0.001$) and Fe groups ($p<0.005$) at 60 weeks (Fig 4.30). The increase in the number of foci was not significant, however.

At both time points, the mean number of 10–20 µm nodules per cm^2 was significantly higher in FB_1/Fe ($p=0.001$) as compared to rats treated with FB_1/AIN, as well as compared to the Fe and control groups (Fig 4.29 and Fig 4.30). At 35 weeks, 1 FB_1/AIN rat developed nodules of this size. This increased to 6 rats having nodules at 60 weeks, and the number of nodules per cm^2 was markedly higher than Fe and control rats, which did not show any nodules at all. There was no significant difference in number of nodules when comparing 35 to 60 weeks.

At 35 weeks, nodules of the size 20-50 µm were found in significantly higher numbers in FB_1/Fe group as compared to the Fe and control groups ($p=0.025$), which did not develop any nodules of this size (Fig 4.29). Fig 4.28 depicts $GSTP^+$ cells of such a nodule from a rat in the FB_1/Fe group. The mean number of nodules per cm^2 in the FB_1/Fe group was marginally higher than the FB_1/AIN ($p=0.083$) rats, of which only 1 rat developed nodules. At 60 weeks, the FB_1/Fe group again had significantly higher numbers of nodules of this size as compared to the Fe ($p=0.001$; 1 rat with 1 nodule) and control group ($p<0.001$; no nodules) (Fig 4.30). The FB_1/AIN group had marginally higher numbers than the Fe group ($p=0.079$) and significantly higher than the controls ($p=0.046$). The number of nodules marginally increased in rats of the FB_1/AIN ($p=0.09$) and FB_1/Fe group ($p=0.085$) from week 35 to 60.

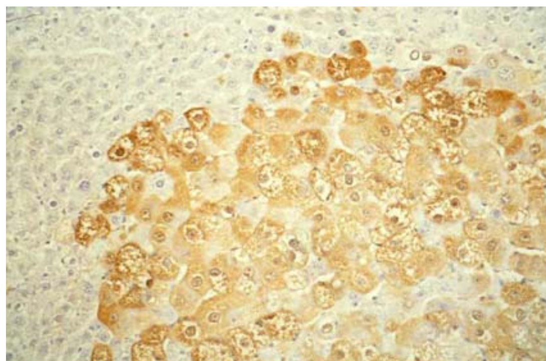

Fig 4.28. $GSTP^+$ cells of a nodule 20-50 µm in a section of liver from an FB_1/Fe treated rat at 35 weeks. GSTP staining, objective x10.

At 35 weeks, only 1 rat from FB_1/Fe group (1/5) had 1 nodule larger than 50 µm. At 60 weeks, both FB_1-treated groups had developed equal numbers of nodules larger than 50 µm/cm^2. No nodules of this size were detected in the Fe and control groups.

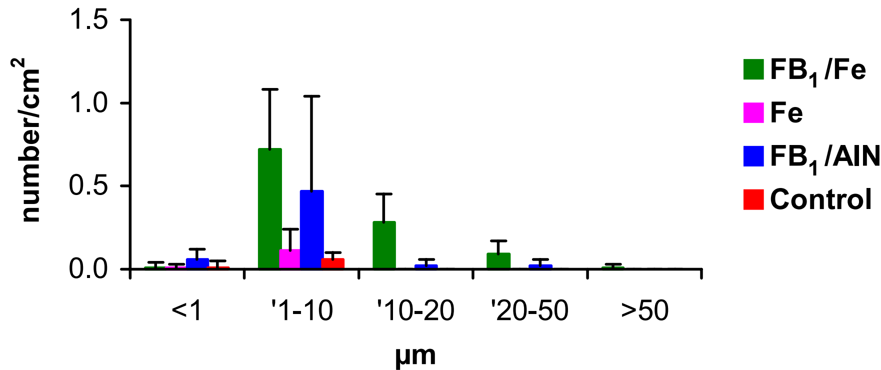

Fig 4.29. The number of foci/nodules seen in the different treatment groups at 35 weeks.

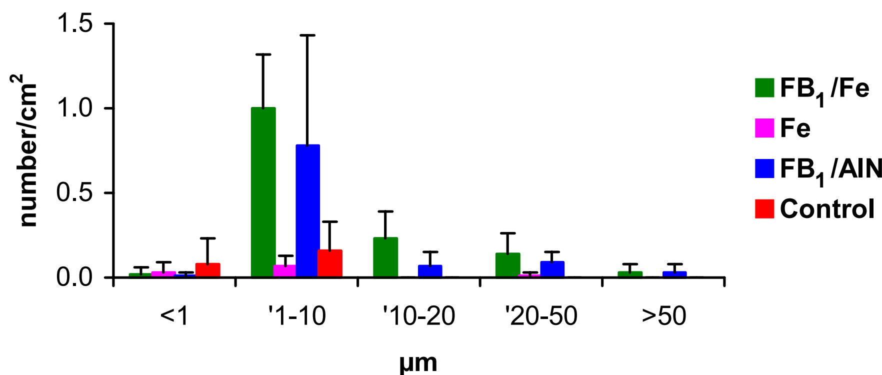

Fig 4.30. The number of foci/nodules seen in the different treatment groups at 60 weeks.

With respect to the total number of foci and nodules,

(i) at 35 weeks, only rats from the FB_1/Fe group had a significantly higher number than rats from the Fe ($p=0.009$) and the control group ($p=0.006$). Though not significant, the FB_1/Fe group had almost twice as many nodules per cm^2 as the FB_1/AIN group. On the other hand, rats from the FB/AIN group had developed markedly higher numbers of nodules than the Fe and control groups;

(ii) at 60 weeks, both FB_1-treated groups had significantly ($p<0.008$) more foci and nodules than the Fe and control groups and did not differ significantly from one another;

(iii) over all (35 and 60 weeks), due to the high numbers per cm^2 at 35 weeks, the FB_1/Fe group developed significantly ($p<0.05$) more foci and nodules than rats treated only with FB_1 (Fig 4.31).

When considering only nodules (lesions larger than 10 µm), the FB_1/Fe group had significantly ($p<0.005$) higher total numbers (0.38/cm^2) when compared to the FB_1/AIN (0.04/cm^2) group at 35 weeks. The Fe and control groups had developed no nodules at this time. This statistical significance in the FB_1/Fe group compared to the other groups was also observed at 60 weeks ($p<0.014$), although there was *no increase* in total numbers of nodules in this group (0.4/cm^2). In contrast, the total number of nodules of this size markedly increased in the FB_1/AIN group (0.19/cm^2) from week 35 to 60 and was significantly higher than the Fe ($p=0.042$; 0.01/cm^2) and control ($p=0.022$; no nodules) groups.

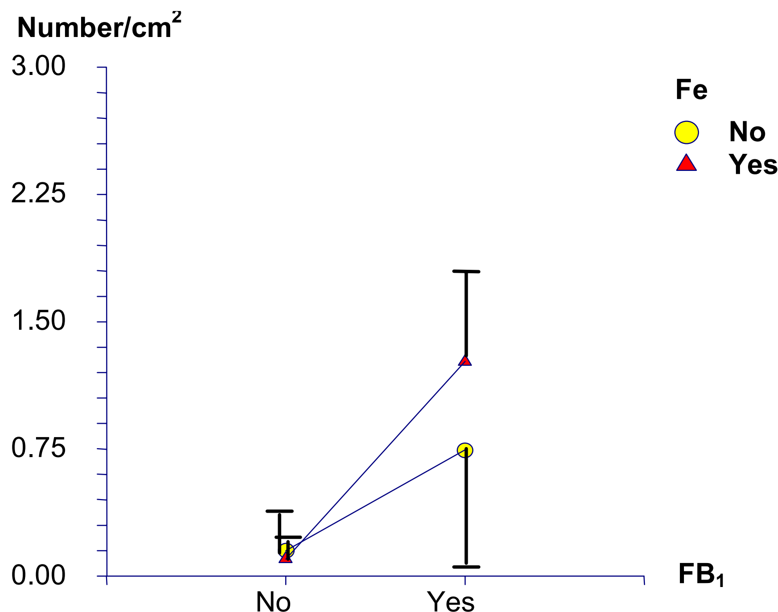

Fig 4.31. The effect of iron overload and FB_1 on the total number of foci/nodules per cm^2 over the period of 35 and 60 weeks. Standard deviations of groups at each time point are shown in the Appendix in Table AIIIh.

4.2.6. Hepatic iron analysis

Biochemical measurement

Treatment period: weeks 4-10 (iron loading period)

The hepatic iron level was significantly ($p<0.001$) higher in the iron-treated group compared to the controls at both time points (Fig 4.32). Hepatic iron concentrations decreased significantly ($p<0.05$) in the iron-treated group as a function of time, while the levels in the livers of the control rats remained the same.

Treatment period: weeks 10-35 (FB_1 treatment period)

There was a significant increase in hepatic iron levels in the FB_1/Fe ($p=0.023$) and control ($p=0.009$) group over this time period (Fig 4.32). At 35 weeks, hepatic iron levels in the FB_1/Fe group were significantly ($p<0.01$) higher than the other three groups, while the Fe group had significantly ($p=0.001$) higher levels than the FB_1/AIN and control groups.

Treatment period: weeks 35-60 (post FB_1 treatment period)

After removal of FB_1, hepatic iron levels decreased significantly in the FB_1/Fe ($p=0.049$) (Fig 4.32). The hepatic iron levels in both iron-treated groups no longer differed significantly ($p=0.2$). These were however significantly ($p<0.001$) higher than the non-iron groups.

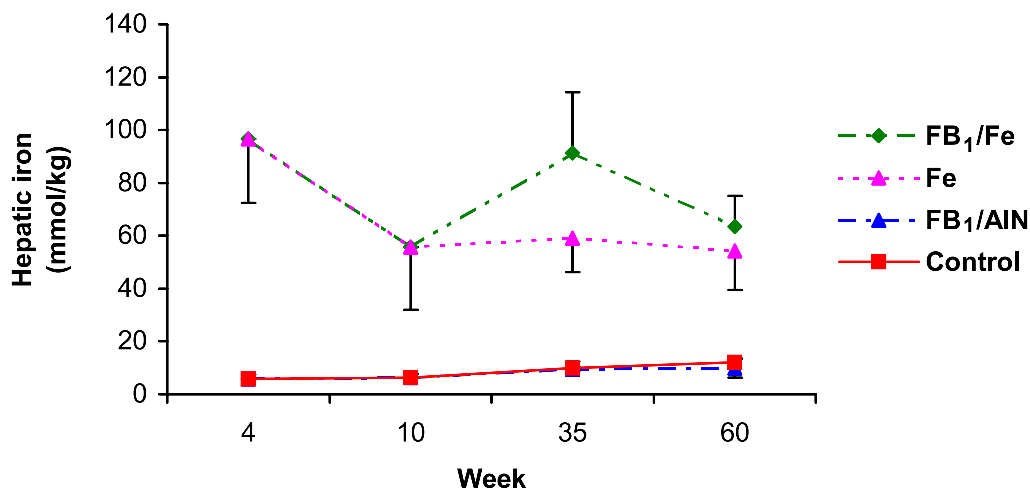

Fig 4.32. The effect of dietary iron on the hepatic iron level as a function of time. Dietary iron levels of the iron-treated groups were reduced to 0.5% after 10 weeks.

4.2.7. Hepatic lipid peroxidation

Treatment period: 4-10 weeks (iron loading period)

There was a significant ($p=0.007$) decrease in the malondialdehyde (MDA) values between 4 and 10 weeks, irrespective of the treatment group. The groups themselves did not significantly differ at either time point (Fig 4.33).

Treatment period: 10-35 weeks (FB_1 treatment period)

A marginal ($p=0.094$) increase in MDA levels was seen only in the FB_1/Fe group between 10 and 35 weeks (Fig 4.33). At 35 weeks, the MDA values of the FB_1/Fe group differed significantly from the FB_1/AIN ($p=0.017$) group and control ($p=0.035$) group and were slightly higher than the Fe group. The Fe group was marginally ($p=0.086$) higher than the FB_1/AIN group, and markedly higher than the control group.

Treatment period: 35-60 weeks (post FB_1 treatment period)

The decrease in MDA values over this time period was significant ($p=0.02$) for the Fe group (Fig 4.33). At 60 weeks, the FB_1/Fe group was significantly higher than all other groups ($p<0.001$).

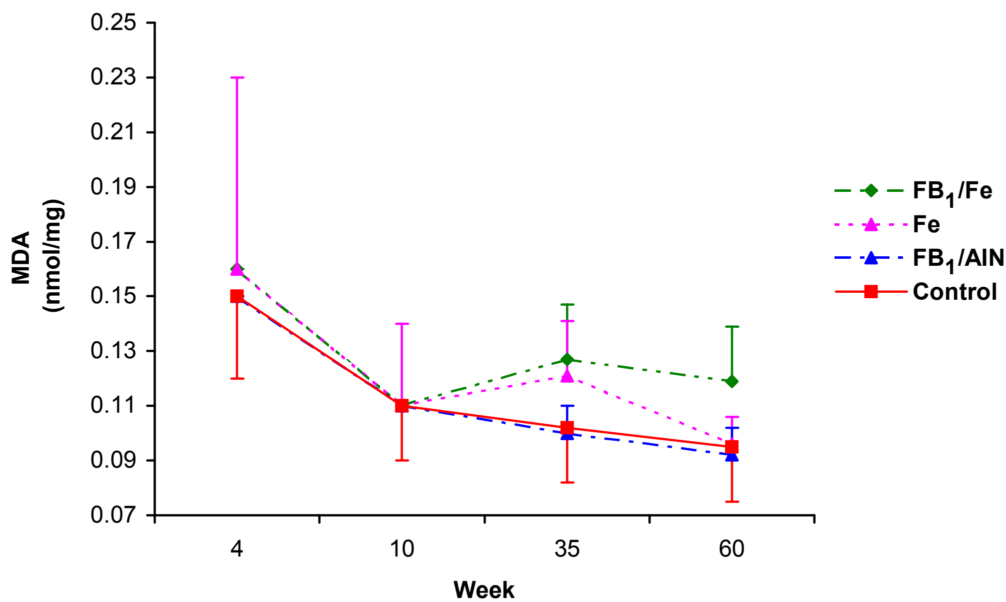

Fig 4.33. The effect of iron overload on the hepatic lipid peroxidation level, measured as MDA, as a function of time. Dietary iron levels of the iron-treated groups were reduced to 0.5% after 10 weeks.

4.2.8. Rat sickness and termination

One rat from the iron-fed group died at 8 ½ weeks. A post mortem revealed bladder and kidney stones and death was not attributed to the dietary iron treatment.

Over the course of the experiment, 19 abscesses in 14 rats were counted (Table 4.8). Eight of these were found in the FB_1/Fe group, 7 in the Fe-group, 2 in the FB_1/AIN group, and 2 in control rats. All but 2 lesions were located in the corner of the mouth and *Escherichia coli* was determined via pus swab. Two control rats had an abscess each, respectively in the umbilical region and on the right hind hock. The abscesses were emptied and flushed with iodine. The effect on feed intake and other biological values were assessed, and it was determined that the abscesses did not influence these parameters.

Mycoplasma pulmonis was isolated from the animal unit and was found to affect all rats, including the controls. The infection expressed itself clinically as head tilts (5 rats) indicative of otitis interna, and respiratory problems (21 cases in 17

rats) (Table 4.8). These were first treated with Penicillin (Peni La Phenix), Amoxycillin (Clamoxyl™), and then with Oxytetracycline (Terravit®). The condition of 3 rats (2 from the FB_1/Fe group, 1 from the Fe group) eventually necessitated early termination at week 53, 54 and 55 respectively. Pneumonia was determined to be the cause of the extreme respiratory distress. Oxytetracycline was introduced after week 55 as prophylactic medicine to further ward off any deaths and no further cases became clinically apparent. Once a unit is infected, the eradication of the organism is nearly impossible and was not feasible in this case.

Table 4.8. Summary of rat sickness and treatments in the long-term study.

	Abscess	**Head tilt**	**Respiratory problems**	**Antibiotics**	**Other**
FB_1/Fe	8(4)	2	6(5)	9(7)	0
Fe	7(6)	0	3	4(3)	1[a]
FB_1/AIN	2	2	8(6)	8(6)	1[b]
Control	2[c]	1	3	5	0

( )=number of animals afflicted; [a]Kidney/bladder stones-->Death at age 60d; [b]Cataract; [c]Abscess in umbilical region (1), rt hind hock (1).

Chapter 5
Discussion

The role of hepatic iron overload in the development of liver cancer in experimental animals, and more specifically rats, has not been clarified mechanistically. Several reports have indicated that iron overload enhances the cancer initiating and/or promoting activity of hepatocarcinogens (Yoshiji *et al.*, 1991; Stål *et al.*, 1999). This has been attributed to the oxidative effect of excess hepatic iron, augmenting cellular damage to membranal structures and macromolecules such as protein and DNA resulting in mutagenesis (McCord, 1996; Andrews, 1999). Increased mutational events in cellular DNA are known to enhance the level of cancer initiating events and the subsequent disruption of cell signalling pathways related to cell proliferation and apoptosis (Cohen and Ellwein, 1990; Cohen, 1998).

In a *pilot study*, the effect of different levels of dietary iron (1%, 1.5%, and 2%) on hepatic iron concentration and various biological parameters was critically evaluated in rats as a function of time. The parameters included pre-weaning iron loading, total body weight gain (tBWG), the liver to body weight (LW/BW) ratio, hepatic iron levels, hepatocellular proliferation, and lipid peroxidation. These parameters were monitored during regular intervals over a period of 15 weeks to obtain optimum conditions of iron loading with minimal adverse effects on the rat. The effect of reducing the dietary iron level to 0.5% after 10 weeks was also evaluated. The aim was to develop a model of iron loading in which a high baseline hepatic iron concentration could be established, and then be maintained, by feeding iron at a relatively low concentration while carrying out a long-term carcinogenesis study with FB_1, without excessive side effects related to iron toxicity. Thus, the investigation into the enhanced and/or protective effects of excess hepatic iron on FB_1-induced hepatocarcinogenesis could be carried out under ideal conditions, in which any synergistic toxicological effects of the combined dietary iron and FB_1 treatment, which could possibly adversely affect the process of hepatocarcinogenesis, were minimised.

The negative effect of dietary iron on tBWG has been documented in rats before and has been attributed to feed refusal and iron toxicity to the gastrointestinal system, resulting in diarrhoea (Mackinnon *et al.*, 1995; Lemmer *et al.*, 1999). Carbonyl iron must be solubilised by gastric acid before intestinal absorption (Huebers *et al.*, 1986); after solubilisation, carbonyl iron is subsequently absorbed similarly to the iron salt ferrous sulphate, a dietary iron supplement that has also been associated with gastro-intestinal side effects (Devasthali *et al*., 1991). Plummer *et al.* (1997) suggested that in addition to diarrhoea per se, carbonyl iron might impair absorption of nutrients that can also contribute to growth retardation in iron-supplemented animals. Additional studies have also suggested that iron supplementation in iron-sufficient children could retard their growth (Idjradinata *et al.*, 1994), and also increase morbidity due to diarrhoea in infants in less-developed countries (Brunser *et al.*, 1993).

The iron-fed rats in this study did not have severe diarrhoea, but only softer faeces than the controls. The tBWG of all the iron-treated rats was however significantly reduced during the treatment period up to 10 weeks (Fig 4.2). This implies that diarrhoea is not the sole cause of reduced weight gain in growing animals, and that feed refusal likely played a prominent role in the growth retardation seen here. The suggestion that carbonyl iron interferes with the uptake of dietary nutrients also needs to be further examined. After the initial 10-week period of iron loading, at which time the dietary iron level was reduced to 0.5% for 5 weeks, the tBWG of the iron-treated rats increased to levels similar to those of the control rats. The tBWG in the control group slightly decreased during this period, reflecting that the BW values of control rats had began to plateau. The 2% dietary carbonyl iron (Fe) group showed the fastest recovery with respect to body weight (BW) (Fig 4.1c). A level of 2% Fe was shown by Lemmer *et al.* (1999) to exhibit excessive toxicity. The dramatic rebound effect in tBWG in the 2% Fe group is clearly related to the reduction in the level of iron feeding, but the mechanisms are not understood. Supplementation with 2% Fe for 10 weeks appeared to adversely affect the rats in regard to tBWG.

At 6 weeks, no significant difference was seen in the LW/BW ratio between the four treatment groups (Fig 4.3b). The LW/BW ratios significantly ($p<0.05$) decreased in all groups from week 6 to 10, corresponding to the significant increase in BW during this time. This decrease was most marked in the control group, due to the additional decrease in the absolute liver weight (LW), while the LW actually increased in the iron-treated groups. After 10 weeks, excess iron was found to increase the LW/BW ratio (Fig 4.3a). At this time point, only the 1.5% Fe group exhibited a significantly higher LW/BW ratio than the control group, while at 15 weeks, after 5 weeks on the lower 0.5% Fe level, the LW/BW ratio was significantly increased in all the iron-fed groups as compared to the controls. The LW/BW ratio dramatically increased between weeks 10 and 15 in the 2% Fe group, surpassing the ratio of the 1% and 1.5% Fe-treated groups. When considering these parameters, it would appear that the 2% Fe level exhibited adverse effects in the liver in addition to on the tBWG.

An increase in absolute liver weights (LW) was reported in a study in which iron dextran was administered parenterally (Carthew *et al.*, 1997). In the current study, an increase in the absolute LW due to iron compared to controls was seen only at the end of the 15-week study period in the 2% group. This was also observed macroscopically; the livers of rats in this group were enlarged in comparison to control livers. This could be attributed to the mitogenic effect of iron.

Corresponding to the data showing an increase in LW/BW ratio, hepatocellular proliferation demonstrated by increased BrdU labelling was up to 5-fold higher in the iron-treated rats than in the control rats (Table 4.1). However, this was not statistically significant, due to the variation between individual rats. Nevertheless, iron appears to have a marked effect on hepatocellular proliferation. Stål *et al.* (1995) also reported increased liver weights and labelling indexes in a long-term study supplementing with 2.5-3% Fe in rat chow.

Macroscopically, the livers of the iron-treated rats had a distinct dark brown colour due to iron deposition, as compared to the control livers, which were light

yellow brown (Fig 4.4a,b). Apart from iron deposition in hepatocytes and Kupffer cells, supplementation with dietary iron over 15 weeks did not alter the normal liver histopathology. Since similar changes were seen in the livers of control rats, the foci of necroinflammation seen in iron-treated rats were attributed to infection with *Mycoplasma pulmonis*. The initial iron deposition observed in the periportal region of the liver is typically seen when supplementing with carbonyl iron (Fig 4.6). This pattern of hepatic iron distribution is similar to hepatic iron overload in genetic haemochromatosis (GH), which is why carbonyl iron is commonly used in animal models of iron overload (Bacon *et al.*, 1983; Park *et al.*, 1987).

As expected, hepatic iron concentration was consistently higher over the study period in iron-fed rats than in the control rats (Fig 4.7a). The 2% Fe level resulted in the highest hepatic iron concentration after 10 weeks and the concentration was significantly higher than the 1% and 1.5% Fe groups (Fig 4.7b). However, after reduction of the dietary iron to 0.5%, the hepatic iron levels were significantly decreased in the 1.5% and 2% Fe groups and at 15 weeks had reached the same hepatic iron level as seen in the 1% Fe group. At the 1% Fe level, hepatic iron concentrations were maintained at a constant level throughout the study, even after reducing the dosage to 0.5% Fe at 10 weeks. Thus, a higher level of dietary iron did not prove to have a long-term advantage over the 1% Fe level.

Throughout the study period, lipid peroxidation, measured as increased levels of malondialdehyde (MDA) (Fig 4.9) and conjugated dienes (CD) (Fig 4.10), was higher in the iron-treated groups than in the controls. Even after reduction of the dietary iron level to 0.5%, the level of oxidative damage was still significantly higher in the iron-treated groups. It has been proposed by Bacon *et al.* (1983) that lipid peroxidation is one mechanism for toxicity of iron overload; chronic iron overload was linked to increased levels of CD as a result of lipid peroxidation. The present study support findings by Stål *et al.* (1996) and Brunet *et al.* (1999) related to the increase in hepatic MDA.

Based on the results obtained in the pilot study, the iron-feeding regimen of 1% Fe was selected during the pre-weaning and pre-carcinogen treatment period of the long-term study. As hepatotoxicity is associated with FB_1-induced cancer induction, a dietary iron level of 0.5% was selected for the FB_1-treatment period, since this level proved effective as a maintenance dose for increased hepatic iron levels with minimal toxic effects to the rat.

The aim of the *long-term study* was to evaluate the effect of iron overload on the toxicological effects of FB_1 in the rat, and in particular to determine whether excess iron could potentiate the hepatocarcinogenic effects of the mycotoxin. The most important finding was seen in the enhanced induction of hepatocellular nodules in rats receiving the combined treatment of FB_1 and dietary iron for 25 weeks. It would appear that excess iron augmented the FB_1 cancer initiating and/or promoting potential. At 35 weeks, the livers of all the rats (5/5) that received the combined FB_1/Fe treatment had developed nodules, as observed macroscopically and microscopically. The macroscopic evaluation of nodules was performed only on the capsular surface. Three slices were taken from the median, left lateral, and caudate lobes from each animal and processed for histopathological evaluation; this was constant for each treatment group. Microscopically, 1 to 14 nodules per animal were seen in the FB_1/Fe group at 35 weeks (Table 4.6; Appendix IIIf). Most of the nodules were classified as high-grade dysplastic lesions (Fig 4.21a,b) while 3/5 rats also had some low-grade dysplastic nodules. Numbers mentioned in the microscopic assessment are only representative of the total liver and are expressed per three tissue sections of liver examined. High-grade dysplasia was assessed according to criteria defined earlier for the purpose of this study (see Chapter 3.5.1) and included large cells with clear cytoplasm, mitosis, intranuclear inclusions, large nucleoli, apoptosis of occasional dysplastic cells, and moderate nuclear pleomorphism. None of the lesions in the study were called hepatocellular cancers (HCCs), which are characterised by invasion into surrounding liver tissue and the vascular system, and metastasis to other organs. Oval cells were seen inside each of the high-grade dysplastic lesions (Fig 4.26). There was much controversy surrounding the origin of oval cells in the past. However, it is now accepted that they are the progeny of liver stem

cells (Sell and Dunsford, 1989; Grisham and Thorgeirsson, 1997). Oval cells are implicated in the genesis of cholangiocarcinomas, but their role in the development of HCCs is uncertain. Short-term feeding with FB_1 has been shown before to induce oval cell proliferation, and these cells were closely related to foci and nodules (Lemmer *et al.,* 1999). Similar results were seen in this study.

Macroscopically, 4/5 rats from the FB_1/AIN group were reported to have nodules on the capsular surface. Only 3/5 rats from this group exhibited nodules in the 3 liver sections examined microscopically, and the result varied from 2 to 3 nodules per slide/rat. The discrepancy between the macroscopic and microscopic observations can be explained by the methodology of assessment. In total, only 7 nodules were recorded in the liver sections from the FB_1/AIN group, of which 4 were high-grade dysplastic (Fig 4.23a; Table 4.6).

At 60 weeks, all the animals of the FB_1/Fe (10/10) and the FB_1/AIN (10/10) groups exhibited nodules as examined macro- and microscopically (Table 4.7). The three liver sections showed nodules varying between 1 to 16 and 1 to 11 in the two groups, respectively (see Appendix IIIg). In 8/10 animals from these two treatment groups, most of the nodules showed high-grade dysplasia (Fig 4.23b), while 2/10 animals of each group only had nodules showing low-grade dysplasia.

It would appear that the progression rate of nodules differed between the FB_1-treated groups at each time point. At 35 weeks, the rate of nodule growth suggests that cancer promotion in the FB_1/Fe group was enhanced as compared with the FB_1/AIN group. However, after removal of FB_1, it seems that the development of nodules in the FB_1/AIN group continued to progress, in contrast to rats treated with the combined treatment FB_1/Fe. The further development of dysplastic nodules appears to in fact be *impaired* by the presence of excess hepatic iron; in addition, there was no progression of these high-grade dysplastic lesions to unequivocal HCCs. It can be hypothesised that if feeding with FB_1, had been continued, the development and progression of

nodules would have continued to be accelerated in the FB_1/Fe group, as compared to the progression rate of nodules in rats treated with FB_1 only.

The observations in the H&E stained liver sections are supported by similar findings in GSTP stained sections. GSTP staining is used to assess enzyme altered hepatic foci and pre-neoplastic nodules, which are considered an estimate of the cancer initiating potency of a hepatocarcinogen (Fig 4.28) (Solt and Farber, 1976; Farber and Sarma, 1987). When considering the total number of $GSTP^+$ foci and nodules after 35 weeks, the number was markedly higher in the combined FB_1/Fe treatment group as compared to the FB_1/AIN group. In regard to the different sizes of the $GSTP^+$ nodules, the number of nodules of the size 20-50 $\mu m/cm^2$ was also significantly higher in the FB_1/Fe group (Fig 4.29). This would imply that the combined treatment not only increased the number of foci and nodules, but also increased the size of the nodules, again indicating that iron overload increases the cancer initiating and/or promoting potency of FB_1.

The effect of excess iron on the progression of FB_1-induced $GSTP^+$ hepatocyte nodules and foci was further evaluated during the post FB_1-treatment period of 25 weeks. There was *no increase* in the development of $GSTP^+$ nodules (>10 um) in the FB_1/Fe group after removal of FB_1 during the 35 to 60 week period (Fig 4.27 and 4.28). Conversely, there was a marked increase in $GSTP^+$ nodule development in the FB_1/AIN group, despite withdrawal of FB_1. This would imply that in the absence of FB_1, continued supplementation with iron did not further support nodule development during the tumour progression phase, but rather delayed the development of $GSTP^+$ nodules, sustaining conclusions drawn with the histopathological results. In contrast, the pre-neoplastic lesions induced in the FB_1/AIN group had acquired the ability of autonomous growth in the absence of FB_1.

Iron significantly increased the number of singlet and duplet $GSTP^+$ cells in the liver when compared to control rats; the cause of this is not clearly understood. At 35 weeks, the FB_1/Fe had more $GSTP^+$ singlets than those treated with iron alone, though this was not statistically significant; the number of the GSTP+

cells decreased in iron-treated rats from 35 to 60 weeks. The FB_1/AIN treatment regimen did not significantly increase the number of $GSTP^+$ singlets or duplets compared to the controls.

At 60 weeks one rat from the Fe group exhibited an iron-free hepatic nodule, which was seen macroscopically and microscopically (Fig 4.25). The GSTP staining of the nodule was very irregular and only some cells in the nodule stained positively. It cannot be ascertained if the nodule developed spontaneously or as a result of iron overload. Single $GSTP^+$ liver cells have been described to be putative initiated hepatocytes, from which the stages of cancer initiation and promotion develop (Moore *et al*., 1987*)*. It can be hypothesised that the nodule of this one rat developed from the $GSTP^+$ singlets induced by excess iron.

Livers of rats from the FB_1/Fe group accumulated more iron than rats in the Fe group, an observation supported by both the biochemical measurements and histological evaluation of hepatic iron (Fig. 4.32). With commencement of FB_1 feeding at 10 weeks, the hepatic iron levels in the FB_1/Fe group increased to significantly higher levels than all other groups. After removing FB_1, these levels decreased to the same level as seen in the Fe group. This indicates an enhancing effect of FB_1 on the accumulation of hepatic iron. Histologically, additional deposition of iron in Kupffer cells and portal tract macrophages was seen in the FB_1/Fe group and attributed to iron release from hepatocytes following hepatic injury and cell death caused by FB_1 (Fig 4.22). In the FB_1/Fe group, new hepatocytes continued to accumulate iron, leading to the higher hepatic iron levels (Fig 4.32). This is in contrast to a study by Deugnier *et al.* (1992), in which it was shown in GH patients that non-iron-related factors (i.e. alcohol) could result in a shift in iron deposition without an increase in the total hepatic iron concentration. After FB_1 removal, cell death was almost certainly reduced, and the shift of iron to Kupffer cells decreased and iron deposition reached levels seen in the Fe group. Liver enzymes decreased during this time, an indication that liver injury was reduced. Serum iron also tended to be higher in the FB_1/Fe group as compared to the Fe group (Fig 4.16). Both iron-treated

groups had significantly higher levels than the FB_1/AIN and control groups in regard to this biochemical parameter.

A marked feature of nodules in the iron-loaded rats was the inability of the intra-nodular hepatocytes to accumulate iron (Fig 4.24 and 4.25). Williams and Yamamoto demonstrated in 1972 the absence of stainable iron in pre-neoplastic and neoplastic lesions in the iron-overloaded rat. Iron-free foci are frequently found in the cirrhotic livers of patients afflicted with GH that has been complicated by HCC (Blanc *et al.*, 1999). It has been suggested that iron-free foci are proliferative lesions that could be pre-neoplastic/precursors to HCC (Hirota *et al.*, 1982; Deugnier *et al.*, 1993a, b; Blanc *et al.*, 1999). The presence of these lesions could be of importance in the screening for early HCC (Deugnier *et al.*, 1993). The absence of iron in these lesions has been documented to be reliable and sensitive indications for hyperplastic lesions (Williams *et al.*, 1976). Eriksson *et al.* (1986) discovered that diferric transferrin binding sites are 60-fold higher on nodule hepatocytes than on cells in the surrounding liver. As the affinity of the receptor to diferric transferrin is not affected, it was proposed that the dissociation of iron from transferrin must be disturbed, due to insufficient acidification in the endosomes (Andersson *et al.*, 1989). This leads to a slower rate of iron accumulation in the hepatocytes within nodules. Low intracellular iron in these cells could imply a lower level of lipid peroxidation in the nodule, contributing to the cell growth advantage of these hepatocytes despite a toxic environment, as described in the resistant hepatocyte model by Solt and Farber (1976).

Averaged feeding was introduced from week 4 in the long-term study, and differences in nutritional intake between the groups were subsequently eliminated (Table 4.3). This entailed weighing the feed of a specified group every day, calculating the average feed intake, and feeding the other rats accordingly. The importance of averaged feeding lies in the reduction in the variability of certain parameters, such as BW, tBWG, and the LW/BW ratio, between rats of different treatment groups. The large differences in tBWG between the different treatment groups in the pilot study were thus avoided in the long-term study (Fig 4.11).

Certain variations observed when comparing the pilot study with the long-term study, despite usage of the same dietary iron level, can be attributed to averaged feeding. The design of the feed cups used for averaged feeding reduces wastage, but also tends to decrease the feed and total dietary iron intake of the rats, which explains lower levels of hepatic iron in the long-term study as compared to the pilot study. The mean hepatic iron level in the pilot study was 100 mmol/kg liver compared to almost 60 mmol/kg during the pre-FB_1-treatment iron loading phase of the long-term study. However, hepatic iron in iron-treated rats of the long-term study was still almost 9-fold that of the control rats (Fig 4.32). Averaged feeding could also explain the decrease in hepatic iron in all groups from week 4 to 10 in the long-term study, as averaged feeding commenced at week 4. In addition to the feed being available *ad libitum* in the pilot study, the rats were fed twice per day, which also appeared to increase feed intake as compared to the long-term study, in which rats were fed once daily. A similar effect of averaged feeding was seen in regard to lipid peroxidation. MDA levels in iron-treated rats were also lower in the long-term study than in the pilot study. In both studies, the MDA levels decreased from week 4 to 10, irrespective of treatment group. The mechanism for this is not understood.

No significant difference could be detected in lipid peroxidation measured as MDA levels between groups up to 10 weeks (Fig 4.33). At the end of the FB_1 treatment period, the level of MDA increased only in the combined FB_1/Fe treatment regimen to levels significantly higher than the levels of the FB_1/AIN and control groups. In a study with a similar protocol, conducted by Lemmer *et al.* (1999), the combined treatment also significantly increased lipid peroxidation in the liver at 5 weeks. However, in this study MDA levels were not investigated at 5 weeks following the high FB_1 dose regimen. The present study indicates that after a further 20 weeks of feeding a decreased level of FB_1 (100 mg/kg), the level of lipid peroxidation was still increased. There was no significant effect noticed in the liver of the rats treated with dietary iron or FB_1/AIN only. Thus, based on relatively low levels of lipid peroxidation effected by dietary iron and FB_1 alone, ideal conditions were created in which to investigate the synergistic

effect the two compounds, as the adverse effect of iron, which could have altered the outcome of the study, was kept to a minimum.

It would appear that elevated hepatic iron levels increased the susceptibility of the liver to undergo lipid peroxidation only in the presence of FB_1. As mentioned before, lipid peroxidation is hypothesised to be one mechanism of toxicity of iron overload (Bacon *et al.*, 1983). FB_1 is known to be a non-genotoxic hepatocarcinogen effecting cancer initiation after feeding of FB_1 at hepatotoxic levels over a period of at least 21 days (Gelderblom *et al.*, 1994). It has been proposed that cancer induction occurs via an oxidative damage mechanism secondary to its hepatotoxic effects (Gelderblom *et al.*, 1996b; Abel and Gelderblom, 1998). According to a recent hypothesis, high oxidative damage should result in an increase in the cancer initiating potency of FB_1 (Abel and Gelderblom, 1998). The high MDA levels resulting from the treatment of FB_1/Fe in this study appear to have resulted from a combined effect of FB_1 and excess iron on lipid peroxidation.

After week 35, MDA levels decreased significantly in the Fe group, which can be attributed to a decline in the average daily feed intake and resulting reduction in dietary iron intake of the rats from 24.5 to 21.5 mg/100 g BW (Table 4.4). This coincides with a decrease in the number of the GSTP+ singlet and duplet cells in rats of the Fe group during this time. MDA levels did not decrease significantly in the FB_1/Fe group, most probably due to the residual combined cytotoxic effect of the FB_1/Fe treatment, even in the absence of the mycotoxin.

In addition to lipid peroxidation, several other factors have been implicated to affect the cancer initiating and promoting phases of FB_1-induced carcinogenesis. A recent study showed that an increase in hepatocyte proliferation induced by partial hepatectomy (PH) or a necrogenic dose of carbon tetrachloride (CCl_4) enhanced the cancer initiating potency of FB_1 in rat liver (Gelderblom *et al.*, 2001d). In the current study, hepatocellular labelling with BrdU in rats from the combined FB_1/Fe treatment group was dramatically higher than the other groups at 35 weeks (Fig 4.27). Hepatocellular proliferation in the Fe and FB_1/AIN groups was also higher than in the control group, though

not significantly (Tables 4.8 and 4.9). Under normal circumstances, hepatocyte turnover rate in the liver is very low, which is why the liver is a good model to investigate cell growth and carcinogenesis. Iron has previously been shown to be mitogenic (Deugnier *et al.*, 1992; Stål *et al.*, 1995); the increase in hepatocellular proliferation in the FB_1/Fe group is attributed to the mitogenic effect of iron, a combined toxicity of FB_1 and iron, and the elevated rate of mitosis in nodules. After removal of FB_1, the enhanced effect of the combined treatment on hepatocellular proliferation was eliminated, and the index of labelling in this group decreased to even lower levels than the Fe and FB_1/AIN groups (Table 4.9). These observations support the hypothesis proposed recently by Li *et al.* (2000) that proliferating cells appear to be more sensitive to the toxic effects of fumonisins.

The results of the present long-term study contrast the findings of Lemmer *et al*. (1999) who described a protective effect of dietary iron when utilising a short-term FB_1 treatment regimen (5 weeks) and higher levels of dietary iron (1%). Iron overload was shown to reduce either the cancer initiating and/or promoting potency of FB_1 in rat liver. The protective effect against cancer promotion was related to the mitogenic properties of iron, which could counteract the mitoinhibitory effect of FB_1 selectively stimulating the outgrowth of initiated cells. However, the relative high dietary iron levels (2% Fe) used in the pre-FB_1 treatment iron loading phase of the study, as well as the 1% Fe level during the FB_1 treatment phase over a period of 5 weeks could have adversely affected the outcome of the FB_1-induced cancer initiating phase. Due to severe growth retardation, it was necessary to discontinue feeding with 2% Fe after 1 week of feeding (for 1 week), after which 1% Fe was supplemented. It is therefore difficult to evaluate the modulating role of iron overload, as the dietary iron levels were associated with toxicity. Carbonyl iron has been reported to enhance apoptosis in CCl_4-induced promotion in chemical hepatocarcinogenesis (Wang *et al.*, 1999). The high level of dietary iron used by Lemmer *et al.* (1999) could have exhibited an inhibitory effect during the combined treatment regimen on the induction of nodules, presumably via the removal of the altered cells via an apoptotic mechanism (Bursch *et al.*, 1992). On the other hand, as the LW/BW ratio was also increased during the iron

treatment, an increased mitogenic response could have reduced the cancer promoting effect of FB_1. Similarly, mitogen-induced cell proliferation effected by carbonyl iron supplementation (Stål *et al.*, 1995) and the mitogen lead nitrate ($PbNO_3$) (Gelderblom *et al.,* 2001d) failed to enhance cancer initiation. Therefore, an increase in apoptosis and cell proliferation could explain the protective effect found by Lemmer *et al.*

Several differences existed between the FB_1 treatment model used by Lemmer *et al.* (1999) and the current study. Although a similar cancer initiating protocol was used, i.e. FB_1 at a dietary level of 250 mg/kg diet over a period of 5 weeks, the reduction in dietary iron to 0.5% at commencement of the FB_1 treatment was new (Table 3.3). Following the first 5 weeks of FB_1 feeding, the current model maintained the FB_1 treatment for a further 20 weeks, but at a dietary level of 100 mg FB_1/kg diet in the absence and presence of dietary iron (0.5% Fe). Cancer promotion has been shown to occur at this non-toxic dietary level, presumably related to the disruption of growth regulatory signals that favours the growth of altered hepatocytes generated during cancer initiation (Gelderblom *et al.*, 1996a). Also different in this study, was the subsequent removal of FB_1 from the diet at 35 weeks to monitor the effect of iron overload on the tumour progression phase of cancer development. Iron increases the LW/BW ratio, which was seen in both studies. FB_1 has been shown to reduce the LW/BW (Gelderblom *et al.*, 1994; 1996b); the effect was also seen in this study. In contrast to the study by Lemmer *et al.*, iron overload could not counteract the mitoinhibitory effect on normal hepatocytes and the resulting selection process of FB_1 in this regard. Iron appears, though, to still enhance the regenerative response to FB_1-induced hepatocyte death, as hepatocellular proliferation was increased, resulting perhaps in the promotion of resistant hepatocytes. Between 10 and 35 weeks, the LW/BW ratio decreased in all groups, due to a significant increase in BW, despite an increase in absolute LW. After 35 weeks, the LW/BW ratio increased in all groups, reflecting the increase in absolute LW and less tBWG. At this time, after removal of FB_1, the LW/BW ratio in the FB_1/Fe group increased to the same level as observed in the Fe group, indicating that the long-term effect of FB_1 on the LW/BW ratio does not persist in the presence of continued iron feeding.

Various iron models involving carcinogenesis cannot necessarily be compared to one another, for example in regard to discrepancies in levels of dietary iron used. In the present study, carbonyl iron was thoroughly mixed into a powdered diet, ensuring exact information of level and intake (see Chapter 3.3). In contrast, other studies have used iron-coated pellets, rendering levels and intake measurements unreliable (Plummer *et al*., 1987). Therefore, conflicting data presented in numerous studies in regard to the role of iron overload in the development of HCC could depend on the specific model utilised, the level of dietary iron, and the length of carcinogen treatment.

The present study suggests a dual role of iron overload in cancer development during FB_1-induced carcinogenesis. During the cancer initiation/promotion phase, iron overload in combination with FB_1 enhances the susceptibility of the liver to the formation of foci and nodules; after removal of FB_1, excess iron impairs the progression of pre-neoplastic hepatic lesions into hepatic tumours.

Further studies are needed into the interaction between prolonged FB_1 feeding and iron overload. We postulate that if FB_1 feeding had continued after 35 weeks with simultaneous iron supplementation, the development of nodules would have been enhanced further and most likely progressed to HCCs. The effect of FB_1 and iron on haematological and biochemical parameters also warrants additional investigation. Certain data support the enhancing effect of excess iron on FB_1-induced hepatotoxicity. The combined treatment FB_1/Fe significantly increased the ALT, AST, and bilirubin serum levels of the rats (Fig 18a,b; Fig 17a,b). Bone marrow also appeared to be affected by this treatment regimen. The mean cell volume (MCV) was notably increased by the combined treatment of FB_1/Fe (Fig 4.14). Dietary iron (in the presence and absence of FB_1) also increased the MCV, though not to such levels as the combined treatment did, as well as mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC). FB_1 (in the presence and absence of dietary iron) significantly ($p=0.001$) increased both the ALP (Fig 4.19a) and creatinine levels. Increased serum creatinine is indicative of kidney injury and has been documented before in FB_1-treated rats (Voss *et al.,* 1993, 1995). Iron did not appear to enhance the effect of FB_1 in this regard. After removal of FB_1,

these serum parameters decreased significantly ($p<0.05$) in rats of the FB_1-treatment groups, and at 60 weeks no significant differences were noticed between the FB_1-treated and control rats with respect to these serum parameters. FB_1 was shown to reduce the red cell blood count, haemoglobin, and haematocrit (Fig 4.13). This appears to be a new observation and the mechanism is not fully understood yet.

Conclusion

1) Excess dietary iron results in hepatic siderosis and augments the cancer initiation and/or promoting potential of FB_1.
2) After removal of FB_1, continued supplementation of iron appears to counteract the progression of hepatocyte nodules induced by FB_1.
3) The combined treatment of dietary iron and FB_1 significantly increased lipid peroxidation and hepatocellular proliferation, both of which are implicated in the pathogenesis of drug/toxin-induced liver injury and HCC.

Chapter 6
Summary

The long-term toxicological effects of hepatic iron overload on the cancer initiating and promoting properties of fumonisin B_1 (FB_1) were investigated in male Fisher 344 rats. An initial pilot (dose response) study over 15 weeks was performed to determine a level of dietary iron that achieves a high hepatic iron concentration in the absence of significant side effects, to be used in the subsequent long-term carcinogenesis study with FB_1. Doses of 1%, 1.5% and 2% dietary carbonyl iron (Fe) were used for 10 weeks, followed by a level of 0.5% Fe for another 5 weeks. After 10 weeks of feeding 1% Fe, the hepatic iron level was 30-fold that of controls. Following the reduction in dietary iron to 0.5%, the hepatic iron level was maintained and side effects were minimal. Irrespective the dose of iron, the iron deposition was initially confined to zone 1 (periportal region), but with increased iron loading, extended to zone 2 (mid-zonal region) and zone 3 (perivenular region). Iron overload was shown to increase hepatocellular proliferation as seen in labelling of cells with 5-bromo-2'-deoxy-uridine (BrdU). Lipid peroxidation, measured as MDA, was also significantly enhanced by excess iron and levels correlated the iron dosage.

In the long-term study, half of the rats were submitted to an iron-loading regimen with 1% Fe for 10 weeks, while the other half received the powdered AIN-93M diet only. The iron was then reduced to 0.5%; the rats were divided into four treatment groups: FB_1/Fe (n=15); Fe (n=14); FB_1/AIN (n=15); and control group (n=15), respectively. FB_1 was fed at 250 mg/kg AIN-93M diet for 5 weeks, followed by 100 mg/kg for 20 weeks. The effect of iron on FB_1-induced initiation and promotion was assessed in 5 rats from each group at week 35. FB_1 was removed from the diet of the remaining rats but iron supplementation continued for 25 weeks. The rats were terminated and the effect of dietary iron on the progression phase of FB_1-induced carcinogenesis was assessed.

The feed intake of rats from the Fe group (weeks 4-10), and FB_1/Fe and FB_1/AIN groups (weeks 10-60) was measured daily and the other groups were

given averaged feeding accordingly; thus, the total body weight gain (tBWG) gain did not differ significantly between groups. Excess iron significantly increased the liver to body weight (LW/BW) ratio; however, iron could not counteract the mitoinhibitory effect of FB_1 on hepatocyte proliferation and the LW/BW ratio of the FB_1/Fe group was significantly lower than the Fe group.

During the 25-week FB_1-treatment period, more foci and nodules of dysplastic cells developed in the FB_1/Fe group than in the FB_1/AIN group. After removal of FB_1, the number of nodules remained constant in the FB_1/Fe group, while increasing in the FB_1/AIN group. Initially, the pattern of iron deposition was the same as in the pilot study. Hepatocyte necrosis caused by FB_1 then resulted in a shift of iron from hepatocytes to Kupffer cells and portal tract macrophages. FB_1/Fe resulted in increased hepatocellular proliferation as demonstrated by increased BrdU labelling at 35 weeks. The withdrawal of FB_1 reversed this effect and the index of BrdU labelling decreased to even lower levels than the Fe and FB_1/AIN groups. Hepatic iron was almost 9-fold that of controls at 10 weeks; a significant increase in the hepatic iron levels in the FB_1/Fe group was noticed as compared to all other groups. However, after removing FB_1, the hepatic iron decreased to the same level as seen in the Fe group. During the FB_1 treatment period, MDA increased in the FB_1/Fe group to levels significantly higher than in the FB_1/AIN and control groups.

The present study suggests a dual role of iron overload in cancer development during FB_1-induced carcinogenesis. During the cancer initiation/promotion phase, iron overload in combination with FB_1 enhanced the susceptibility of the liver to the formation of foci and nodules; but after removal of FB_1, continued supplementation of iron impaired the progression of pre-neoplastic hepatic lesions.

Kapitel 7
Zusammenfassung

Die Auswirkungen von Eisenbelastung auf die toxikologische Langzeitwirkung von Fumonisin B_1 in der Rattenleber

In der vorliegenden Studie wurden die Langzeitwirkungen einer hepatischen Eisenbelastung auf das krebserzeugende bzw. –fördernde Potential von Fumonisin B_1 (FB_1) an männlichen Fisher 344 Ratten untersucht. Vorab wurde in einem 15-wöchigen Vorversuch der für eine Langzeitkarzinogenese-Studie geeignete Eisenanteil im Futter ermittelt, welcher eine möglichst hohe Eisenkonzentration in der Leber, jedoch noch keine signifikanten Nebenwirkungen hervorruft. In den ersten 10 Wochen wurden Konzentrationen von 1%, 1,5% und 2% Karbonyleisen (Fe) im Futter verwendet, um die letzten 5 Wochen mit einem 0,5%-igen Eisengehalt fortzufahren. Nach einer 10-wöchigen Fütterung von 1% Fe wurde gegenüber den Kontrolltieren eine um das 30-fache höhere Eisenkonzentration in der Leber erreicht. Bei der folgenden Reduktion des Futtereisengehaltes auf 0,5% wurde diese Eisenkonzentration in der Leber aufrechterhalten, die Nebenwirkungen waren minimal. Unabhängig von der Höhe der Eisendosierung erfolgte die Eisenablagerung anfänglich in Zone 1 (Läppchenperipherie), erstreckte sich jedoch mit zunehmender Eisensättigung auch auf die Zonen 2 (mittlere Läppchenzone) und 3 (Läppchenzentrum). Mittels Zellmarkierung mit 5-Brom-2'-Desoxy-Uridin (BrdU) wurde eine durch Eisenbelastung induzierte hepatozelluläre Proliferation nachgewiesen. Weiterhin wurde durch den Eisenüberschuss eine dosisabhängige Steigerung der Lipidperoxidation (gemessen als Malondialdehyd (MDA)) festgestellt.

Im Hauptversuch wurde die Hälfte der Tiere 10 Wochen lang entsprechend eines 1%-igen Eisenanreicherungsschema gefüttert, wohingegen der anderen Hälfte ausschliesslich AIN-93M-Futter verabreicht wurde. Anschließend wurde der Eisenanteil auf 0,5 % reduziert. Die Tiere wurden in 4 Gruppen unterteilt: FB_1/Fe (n=15), Fe (n=14), FB_1/AIN (n=15) und Kontrollgruppe (n=15). FB_1

wurde 5 Wochen lang in einer Dosierung von 250 mg/kg AIN-93M-Futter verwendet, gefolgt von 100 mg/kg für weitere 20 Wochen. Die Auswirkung von Eisen auf die FB_1–induzierte Tumorentstehung bzw. -progression wurde an je 5 Ratten pro Versuchsgruppe in der 35. Woche untersucht. Die übrigen Tiere erhielten nun FB_1–freies Futter, während hingegen Eisen über weitere 25 Wochen supplementiert wurde. Anschließend wurden die Ratten getötet und die Beeinflussung der Progressionsphase der FB_1–induzierten Karzinogenese durch Fütterungseisen untersucht.

Um eine auf täglicher Basis gleiche Futtermengenaufnahme aller Ratten zu gewährleisten, wurde von Woche 4-10 entsprechend des Durchschnitts der Fe-Gruppe gefüttert, von Woche 10-60 diente die Menge der FB_1/Fe- und FB_1/AIN-Gruppe als Bezugswert. Es zeigten sich zwischen den einzelnen Gruppen keine signifikanten Unterschiede bezüglich der Körpergewichtszunahme (tBWG). Bei Eisenüberschuß war eine signifikante Erhöhung des relativen Lebergewichtes (LW/BW) festzustellen; der mitoinhibitorischen Wirkung von FB_1 konnte jedoch durch die Eisengabe nicht entgegengewirkt werden, das relative Lebergewicht in der FB_1/Fe-Gruppe war gegenüber der Fe-Gruppe signifikant erniedrigt.

Innerhalb der 25-wöchigen Gabe von FB_1 entwickelten sich in der Leber von Tieren der FB_1/Fe-Gruppe entscheidend mehr Foci und Knötchen von dysplastischen Zellen als in der FB_1/AIN-Gruppe. Während die Anzahl dieser Foci und Knötchen nach Abschluß der FB_1-Gabe in der Gruppe FB_1/Fe unverändert blieb, nahm sie in der FB_1/AIN-Gruppe weiterhin zu. Das Muster der Eisenablagerung entsprach anfänglich dem des Vorversuches; die durch FB_1 hervorgerufenen hepatozytären Nekrosen gingen jedoch mit einer Verlagerung der Eisenspeicherung von Hepatozyten auf Kupffer-Zellen und in den Portalkanälen lokalisierte Makrophagen einher. Wie durch die immunhistochemische Untersuchung mit BrdU in der 35. Woche gezeigt werden konnte, resultierte die Verabreichung von FB_1/Fe in einer erhöhten hepatozellulären Proliferation. Das Absetzen der FB_1-Gabe verursachte einen gegenteiligen Effekt; der Index der BrdU-Markierung sank sogar auf niedrigere Werte als in den Fe- und FB_1/AIN-Gruppen. Die Eisenkonzentration in der Leber betrug nahezu den 9-fachen Wert der Kontrollgruppe in der 10. Woche;

ein signifikanter Anstieg der Eisenkonzentration in der FB_1/Fe-Gruppe im Vergleich zu allen anderen Gruppen war in Woche 35 zu erkennen. Allerdings sanken die Konzentrationen nach Abschluß der FB_1-Gabe auf ähnliche Werte wie in der Fe-Gruppe. Während des Zeitraums der FB_1-Fütterung war eine Steigerung der MDA in der FB_1/Fe-Gruppe auf signifikant höhere Werte als in den FB_1/AIN- und Kontrollgruppen festzustellen.

Die Ergebnisse der vorliegenden Studie lassen erkennen, daß eine Überversorgung mit Eisen nach FB_1-induzierter Karzinogenese die weitere Tumorentwicklung in zweierlei Hinsicht beeinflußt. Während der Tumorinitiation- bzw. Progressionphase ist bei Vorliegen eines Eisenüberschusses in Kombination mit FB_1 die Anfälligkeit der Leber für die Ausbildung dysplastischer Foci erhöht; nach Abschluß der Gabe von FB_1 verzögert eine weitere Eisensupplementation die Progression der prä-neoplastischen Läsionen in der Leber.

Chapter 8
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Table A.I. Dietary composition of the AIN-93M maintenance diet used in both studies.

Typical analysis	
Protein	13.06%
Fat	4.0%
Fibre	5.0%
Carbohydrate	73.8%
Metabolizable Energy, kcal/gm	3.83%

Ingredients	g/kg diet
Corn Starch	465.692
Casein (>or=85% protein)	140.000
Dextrinized Corn Starch (90-94%tetrasaccharides)	155.000
Sucrose	100.000
Soybean Oil (no additives)	40.000
Fibre	50.000
Mineral Mix (AIN-93M-MX)	35.000
Vitamin Mix (AIN-93M-VM)	10.000
Methionine	3.000
Choline Bitartrate (41.1% choline)	2.500
Tert-butylhydroquinone	0.008

AIN-93-VX Vitamin Mix

Ingredients	g/kg diet
Nicotinic Acid	3.000
Ca Pantothenate	1.600
Pyridoxine-HCL	0.700
Thiamine-HCL	0.600
Riboflavin	0.600
Folic Acid	0.200
D-Biotin	0.020

Table A.I. (continued)	
Vitamin B-12 (cyanocobalamin)(0.1% in mannitol)	2.500
Vitamin E (all-rac-alpha-tocopheryl acetate)(500 IU/g)	15.000
Vitamin A (all-trans-retinyl palmitate) (500 000 IU/g)	0.800
Vitamin D-3 (cholecalciferol)(400 000 IU/g)	0.250
Vitamin K (phylloquinone)	0.075
Powdered Sucrose	974.655

AIN-93G-MX Mineral Mix

Ingredients	g/kg diet
Calcium Carbonate, Anhydrous, 40.04% Ca	357.000
Potassium Phosphate, Monobasic, 22.76% P; 28.73% K	250.000
Sodium Chloride, 39.34% Na; 60.66% Cl	74.000
Potassium Sulfate, 44.87% K; 18.39% S	46.600
Potassium Citrate Tri Potassium, Monohydrate, 36.16% K	28.000
Magnesium Oxide, 60.32% Mg	24.000
Ferric Citrate, 16.5% Fe	6.060
Zinc Carbonate, 52.14% Zn	1.650
Manganous Carbonate, 47.79% Mn	0.630
Cupric Carbonate, 57.47% Cu	0.300
Potassium Iodate, 59.3% I	0.010
Sodium Selenate, Anhydrous, 41.79% Se	0.01025
Ammonium Paramolybdate, 4 Hydrate, 54.34% Mo	0.00795
Sodium Meta-silicate, 9 Hydrate, 9.88% Se	1.450
Chromium Potassium Sulfate, 12 Hydrate, 10.42% Cr	0.275
Boric Acid, 17.5% B	0.0815
Sodium Fluoride, 45.24% F	0.0635
Nickel Carbonate, 45% Ni	0.0318
Lithium Chloride, 16.38% Li	0.0174
Ammonium Vanadate, 43.55% V	0.0066
Powdered Sucrose	209.806

A.II. Method for biochemical hepatic iron measurement

1. Purpose and scope

The determination of iron by colorimetric means in acid digests of liver tissue.

2. *Hazards*

Concentrated Nitric Acid – Poisonous, Danger: may be fatal if swallowed. Harmful if inhaled and may cause delayed lung injury. Spillage may cause fire or liberate dangerous gas. First Aid: Do not induce vomiting, if conscious give water. If inhaled take outside for fresh air.

3. Clinical

Hepatic iron measurements were essential for the diagnosis of GH until the advent of genotyping and were useful in differentiating patients with GH from either patients with alcoholic siderosis or heterozygous HG subjects.

4. *Principle*

The liver tissue is dried, weighed, digested in nitric acid and diluted in sodium acetate buffer. The iron concentration is determined as follows: iron is released from transferrin by guanidine hydrochloride and reduced to Fe^{++} by ascorbic acid. Fe^{++} forms a red complex with ferrozine dye and is measured at 570-700nm.

5. Instrument

Hitachi random access auto-analyser modular (Roche Diagnostic Systems, Basel, Switzerland).

6. Specimen

Fresh liver tissue

7. Standards and quality controls

- Standard on Modular is CFAS.
- Control used is $FeCl3$ (M.W. = 270.30g/mol)

  Stock Control (100Mm): weigh 0.2703g FeCl3 and dissolve in 10ml de-ionised water.

  Working Control (1Mm): 50ul stock up to 5ml with water.

  NB! Add 100ul 35% Nitric acid to both the stock and working FeCl3 control as preservative.
- Controls used on the Modular: Precinorm U (Roche) – low control
Precipath U (Roche) – high control

8. *Reagents*

- Nitric Acid: Analytical Grade Nitric Acid (69%)
- Sodium Acetate Buffer 0.2M
- 10N NaOH
- Modular Iron Reagent – Supplied by Main lab.

9. Preparation of reagents

- Working 35% Nitric Acid: Dilute 69% Nitric Acid with an equal volume of water.
- 0.2M Sodium Acetate Buffer: Add 8.2g sodium acetate to 500ml distilled water and dissolve.
- 10N NaOH: 400g NaOH in 1 litre de-ionised water.

10. Stability of reagents

- Working 35% Nitric Acid – stable at room temp.
- Sodium Acetate Buffer – stable at room temp.
- 10N NaOH – stable at room temp.
- Modular Iron Reagent – Stable at $4^{o}C$

11. Test procedure

Weigh a glass tube and record weight. Carefully remove a tiny piece of the frozen liver from Eppendorf tip with tweezers and scalpel blade, and place in the

pre-weighed glass tube. Weigh the tube with the wet liver and record weight. Dry the tissue overnight by placing in an oven at 105^0C. Next day, remove the glass tube from oven and allow to cool to room temp. Carefully weigh the tube plus dried tissue and record weight to 4 decimal places. Calculate the dry weight of the tissue (should at least be 1mg for analysis). Add 0.4ml of 35% nitric acid* to the tube and vortex gently. Prepare a blank tube containing 0.4ml of 35% nitric acid only*. Cap both the tubes with Parafilm and place into a 70^0C water bath for 60 min. If liver not totally digested after 60 min, then leave for another 30 min. Remove digested sample from water bath.

Set up a control as shown below and dilute the samples into clean plastic tubes as follows*:

	Blank	**Control**	**Test 1/10**	**Test 1/30**
35% Nitric Acid	100 µl	50 µl		
Digested liver sample			100 µl	30 µl
1mM FeCl3		50 µl		
10N NaOH	50 µl	25 µl	50 µl	15 µl
Sodium Acetate buffer	850 µl	875 µl	850 µl	955 µl

Do the iron determination on the Modular.

* indicates modifications

12. Calculation

W = dry weight of tissue (mg)

V = vol. of nitric acid extract used (µl)

X = result from Modular (µmol/L)

$$\text{Tissue Fe concentration} = \frac{X * 400}{V * W} \quad \text{(mmol/kg dry weight)}$$

13. Linear range

0 – 179 µmol/L (as on the Modular)

14. Normal range

Results are reported with one of the following AUSLAB coded comments:

1.	<40 mmol/kg	NORMAL (BC17)
2.	40 – 100 mmol/kg	SLIGHT ELEVATION (BC18)
3.	100 – 200 mmol/kg	MODERATE ELEVATION (BC19)
4.	>200 mmol/kg	GROSS ELEVATION (BC20)

15. Interfering substances

The development of the colour of the ferrozine complex is pH-dependent, so effective neutralization of the nitric acid digest is important.

Reference

6D223, methods manual of the Biochemistry Department, Flinders University School of Medicine, Adelaide, Southern Australia

Table A.IIIa. Summary of the data regarding the various parameters measured in the pilot study at each time point.

Group (%Fe)	Week	BW (g)	tBWG (g)	LW (g)	LW/BW (%)	Iron (mmol/kg)	MDA (nmol/mg)	CD (nmol/mg)	BrdU (cells/cm^2)
Ctrl	**4**	63.25±16.17	nd	2.22±0.55	3.53±0.09	6.97±0.85	0.29±0.07	23.39±5.08	nd
1%		35.50±8.10	nd	1.5±0.40	4.22±0.49	70.41±12.95	0.49±0.09	20.44±8.78	nd
Ctrl	**6**	153.00±13.23	73.00±12.96	8.01±1.24	5.22±0.47	3.49±0.57	0.13±0.03	22.15±6.79	nd
1%		61.00±8.69	28.60±6.23	3.25±0.40	5.36±0.52	89.47±22.51	0.37±0.18	22.14±5.12	nd
1.5%		68.20±4.15	20.40±9.45	3.34±0.46	4.89±0.50	105.06±13.28	0.87±0.34	21.97±5.18	nd
2%		59.80±5.54	13.80±5.45	2.73±0.35	4.57±0.46	94.86±11.51	0.69±0.45	30.79±15.65	nd
Ctrl	**10**	215.20±6.80	171.00±8.60	5.91±0.57	2.74±0.19	3.13±0.49	0.10±0.06	20.09±5.63	nd
1%		118.00±19.94	86.60±15.47	4.37±0.58	3.80±0.83	101.85±16.33	0.38±0.25	29.31±9.64	nd
1.5%		100.80±23.79	55.40±25.17	4.26±1.16	4.25±0.79	119.45±30.67	0.97±0.58	25.10±5.23	nd
2%		97.60±19.05	62.00±19.13	3.36±0.44	3.50±0.45	160.62±16.03	0.41±0.25	27.85±9.34	nd
Ctrl	**15**	258.40±8.91	160.20±7.89	7.88±0.40	3.05±0.12	6.12±0.76	0.10±0.04	17.77±4.42	0.77±1.22
1%		163.20±29.88	134.20±27.78	6.55±1.08	4.03±0.15	100.65±28.24	0.21±0.10	20.66±2.44	1.77±1.55
1.5%		166.60±24.85	132.60±17.91	7.30±1.45	4.36±0.21	91.47±23.81	0.26±0.04	24.40±2.32	3.98±2.65
2%		192.60±27.59	151.60±26.75	8.88±0.96	4.65±0.55	112.69±19.53	0.30±0.11	22.11±8.10	2.44±2.90

Data are the means ± standard deviation of 4 to 5 animals per group; Fe: dietary iron; Ctrl: control; BW: body weight; tBWG: total body weight gain; LW: absolute liver weight; LW/BW: liver to body weight ratio; Iron: hepatic iron concentration; MDA: malondialdehyde; BrdU: 5-bromo-2’-deoxy-uridine; nd: not determined.

Table A.IIIb. Summary of data regarding the various parameters measured in the long-term study at the different time points.

Treatment (Group)	Week	BW (g)	tBWG (g)	LW (g)	LW/BW (%)	Iron (mmol/kg)	MDA (nmol/mg)	BrdU cells/cm^2
Fe	**4**	50.40±2.19	nd	1.88±0.19	3.72±0.22	96.66±24.26	0.16±0.07	nd
Control		80.80±7.40	nd	3.19±0.39	4.02±0.40	5.86±1.74	0.15±0.03	nd
Fe	**10**	178.70±27.04	115.80±7.36	8.14±1.66	4.53±0.37	55.64±23.59	0.11±0.03	nd
Control		208.28±7.75	109.00±16.96	7.74±1.24	3.70±0.45	6.24±1.40	0.11±0.02	nd
FB_1/Fe	**35**	316.20±28.23	251.36±28.56	7.92±0.82	2.51±0.15	91.32±22.97	0.13±0.02	3.67±1.62 (%)
Fe		336.80±33.16	266.34±28.01	9.79±0.80	2.92±0.24	59.10±12.78	0.12±0.02	0.88±1.16
FB_1/AIN		333.00±20.40	242.96±19.95	7.95±0.79	2.39±0.13	9.48±0.85	0.10±0.01	1.02±1.31
Control		342.80±32.00	255.40±25.00	8.58±0.91	2.50±0.11	10.02±2.36	0.10±0.02	0.42
FB_1/Fe	**60**	366.38±14.97	295.86±16.29	11.92±1.27	3.25±0.22	63.43±11.63	0.12±0.02	0.71±0.57
Fe		360.63±20.27	297.29±12.15	12.16±1.08	3.38±0.32	54.30±14.81	0.10±0.01	0.73±1.09
FB_1/AIN		373.90±22.18	297.50±18.31	10.14±1.19	2.74±0.21	9.81±3.61	0.09±0.01	0.81±0.92
Control		365.00±28.81	271.88±31.60	9.68±0.91	2.62±0.12	12.10±1.44	0.10±0.02	0.34±0.34

Data are the means ± standard deviation of 5 to 10 animals per group. FB_1: fumonisin B_1; Fe: dietary iron; AIN: American Institute of Nutrition powdered diet-93M; BW: body weight; tBWG: total body weight gain; LW: absolute liver weight; LW/BW: liver to body weight ratio; Iron: hepatic iron concentration; MDA: malondialdehyde; BrdU: 5-bromo-2'-deoxy-uridine; nd: not determined.

Table A.IIIc. Summary of the data in regard to haemotological parameters in the long-term study.

	WBC ($x10^9$/l)	RBC ($x10^{12}$/l)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (%)	RDW	PL ($x10^9$/l)	MPV (μm^3)
35wk										
FB_1/Fe	2.92±0.77	8.23±0.30	14.48±0.53	41.66±2.04	50.64±0.74	17.60±0.23	34.76±0.56	11.12±0.16	637.80±34.62	<5±0.00
Fe	3.40±1.18	8.53±0.43	14.92±0.68	42.68±2.76	50.00±1.57	17.50±0.34	35.02±0.98	11.84±0.44	722.60±82.63	<5±0.00
FB_1/AIN	3.14±1.50	8.16±0.43	13.82±0.71	40.36±2.92	49.46±1.59	16.98±0.38	34.34±0.86	12.52±2.09	637.20±122.87	<5±0.00
Ctrl	2.98±0.79	9.01±0.39	14.98±0.41	44.00±1.10	48.88±0.99	16.68±0.32	34.10±0.32	11.38±0.45	608.50±76.02	<5±0.00

	WBC ($x10^9$/l)	RBC ($x10^{12}$/l)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (%)	RDW	PL ($x10^9$/l)	MPV (μm^3)
60wk										
FB_1/Fe	4.24±1.12	8.96±0.24	15.49±0.42	46.37±1.49	51.79±0.39	17.31±0.25	33.37±0.57	11.41±0.40	679.86±63.89	<5±0.00
Fe	3.46±0.46	8.81±0.49	14.99±0.62	44.50±1.74	50.59±1.22	17.04±0.52	33.69±0.42	11.56±0.36	646.86±187.35	<5±0.00
FB_1/AIN	3.52±0.91	8.90±0.96	14.26±1.60	43.74±4.36	49.18±1.07	16.00±0.54	32.52±0.65	12.48±1.39	636.80±95.96	<5±0.00
Ctrl	3.74±1.87	9.41±0.29	14.91±0.57	45.53±2.20	48.04±1.27	15.74±0.50	32.74±0.73	12.24±0.31	679.13±137.04	<5±0.00

Data are the means ± standard deviation of 5 to 10 animals per group; FB_1: fumonisin B_1; Fe: dietary iron; AIN: American Institute of Nutrition powdered diet-93M; Ctrl: control; WBC: white blood cells; RBC: red blood cells; Hb: haemoglobin; HCT: haematocrit; MCV: mean cell volume; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; RDW: red cell distribution width; PL: platelet count; MPV: mean platelet volume.

Table A.IIId. Summary data in regard to the differential count in the long-term study.

	Neu		Lym		Mono		Eosino		Basoph	
	(%)	(1/100WBC)	(%)	(1/100WBC)	(%)	(1/100WBC)	(%)	(1/100WBC)	(%)	(1/100WBC)
35wk										
FB_1/Fe	38.20±9.78	1.09±0.27	58.40±10.74	1.76±0.75	1.80±0.84	0.05±0.03	1.60±0.89	0.05±0.03	0.00±0.00	0.00±0.01
Fe	29.40±9.50	1.01±0.51	64.60±6.02	2.25±0.85	2.80±1.48	0.10±0.07	3.20±3.11	0.14±0.07	0.00±0.00	0.01±0.01
FB_1/AIN	34.00±7.52	1.03±0.41	62.40±7.96	2.04±1.10	1.40±0.55	0.05±0.03	2.20±1.64	0.07±0.07	0.00±0.00	0.00±0.01
Control	47.40±12.10	1.64±0.90	48.80±13.39	1.57±0.48	1.80±1.30	0.06±0.06	2.00±1.00	0.07±0.05	0.00±0.00	0.00±0.00

	Neu		Lym		Mono		Eosino		Basoph	
	(%)	(1/100WBC)	(%)	(1/100WBC)	(%)	(1/100WBC)	(%)	(1/100WBC)	(%)	(1/100WBC)
60wk										
FB_1/Fe	46.57±8.90	1.97±0.55	47.57±9.11	2.06±0.76	2.86±2.12	0.11±0.13	3.00±1.83	0.13±0.10	nd	nd
Fe	42.14±7.34	1.48±0.40	51.14±9.48	1.74±0.16	4.43±2.76	0.16±0.11	2.29±0.95	0.08±0.04	nd	nd
FB_1/AIN	44.40±10.38	1.55±0.47	47.80±11.12	1.69±0.64	7.25±3.40	0.28±0.18	2.00±1.22	0.08±0.06	nd	nd
Control	40.50±13.29	1.54±0.92	50.63±13.57	1.89±1.06	5.38±2.56	0.20±0.11	3.50±2.14	0.12±0.06	nd	nd

Data are the means ± standard deviation of 5 to 10 animals per group; FB_1: fumonisin B_1; Fe: dietary iron; AIN: American Institute of Nutrition powdered diet-93M; Ctrl: control; Neu: neutrophils; Lym: lymphocytes; Mono: monocytes; Eosino: eosinophils; Basoph: basophils; nd: not determined.

Table A.IIIe. Summary of the data in regard to biochemical parameters in the long-term study.

35wk	Gluc (mmol/l)	GGT (U/l)	T.Prot (g/l)	T. Bili (µmol/l)	AST (U/l)	ALT (U/l)	ALP (U/l)	D. Bili (µmol/l)	Creat (µmol/l)	Urea (mmol/l)
FB_1/Fe	10.40±1.91	5.60±2.07	65.60±2.1	11.04±2.97	307.60±111.37	113.08±47.97	139.20±22.40	5.98±1.79	84.80±3.96	4.72±0.59
Fe	10.76±2.36	3.00±1.00	68.00±4.60	8.54±1.43	228.78±74.08	80.04±23.99	96.60±26.01	4.32±0.51	71.00±6.60	5.38±0.52
FB_1	11.10±0.75	5.40±2.07	65.32±4.28	13.22±3.75	242.58±24.26	89.62±18.29	167.20±68.17	7.74±2.35	78.20±9.01	5.74±0.89
Ctrl	10.00±2.08	4.50±4.12	65.25±1.80	10.73±2.74	199.23±50.29	57.70±14.42	115.50±37.86	6.13±2.05	67.50±8.10	4.55±1.36

60wk	T.Iron (µmol/l)	T.Chol (mmol/l)	T. Bili (µmol/l)	AST (U/l)	ALT (U/l)	ALP (U/l)	D. Bili (µmol/l)	Creat (µmol/l)
FB_1/Fe	39.05±9.39	2.30±0.30	1.14±0.47	258.03±58.20	108.80±49.05	106.13±18.00	0.85±0.44	67.38±3.29
Fe	35.07±8.76	2.52±0.49	1.80±1.05	255.69±134.98	88.79±32.03	100.00±15.12	0.94±0.47	69.38±6.63
FB_1	22.17±4.89	2.11±0.45	1.19±0.35	233.34±87.06	104.13±102.63	127.90±23.54	0.62±0.31	76.60±14.67
Ctrl	19.87±2.59	2.43±0.37	1.21±0.77	196.58±44.38	51.08±20.77	101.00±20.37	0.58±0.30	66.00±5.12

Data are the means ± standard deviation of 5 to 10 animals per group; FB_1: fumonisin B_1; Fe: dietary iron; Ctrl: control; Gluc: glucose; GGT: γ-glutamyltransferase; T Prot: total protein; T. Bili: total bilirubin; AST: aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase; D. Bili: direct bilirubin; Creat: creatinine; T. Iron: serum iron; T. Chol: total cholesterol.

Table A.IIIf. Summary of hepatic nodules seen histologically in H&E staining at 35 weeks.

Rat no.	Treatment group	No. of nodules (H&E)
3	FB_1/Fe	1 (H)
4	FB_1/Fe	7 (L/H)
5	FB_1/Fe	14 (L/H)
11	FB_1/Fe	4 (H)
12	FB_1/Fe	7 (L/H)
17	Fe	0
29	Fe	0
32	Fe	0
34	Fe	0
35	Fe	0
38	FB_1/AIN	
40	FB_1/AIN	3 (H)
41	FB_1/AIN	2 (L)
48	FB_1/AIN	
49	FB_1/AIN	2 (L/H)
57	Control	0
60	Control	0
61	Control	0
68	Control	0
70	Control	0

H = high-grade dysplasia; L= low-grade dysplasia

Table A.IIIg. Summary of hepatic nodules seen histologically in H&E staining at 60 weeks.

Rat no.	Treatment group	No. of nodules (H&E)
1	FB_1/Fe	9 (H) ±*
2	FB_1/Fe	1 (H)
6	FB_1/Fe	8 (L/H) ±
7	FB_1/Fe	8 (L) ±
8	FB_1/Fe	1 (H)
9	FB_1/Fe	12 (L/H) ±
10	FB_1/Fe	16 (L/H) ±
13	FB_1/Fe	8 (L/H) ±
14	FB_1/Fe	3 (H)
15	FB_1/Fe	2 (L)
16	Fe	0
18	Fe	0
19	Fe	0
22	Fe	0
28	Fe	0
30	Fe	1 (H)
31	Fe	0
33	Fe	0
36	FB_1/AIN	1 (L)
37	FB_1/AIN	2 (H) ±
39	FB_1/AIN	7 (L/H) ±
42	FB_1/AIN	1 (L)
50	FB_1/AIN	7 (L/H) ±
51	FB_1/AIN	2 (L/H)
52	FB_1/AIN	7 (L/H) ±
53	FB_1/AIN	4 (L/H) ±
54	FB_1/AIN	11 (L/H) ±
55	FB_1/AIN	8 (L/H) ±

Table A.IIIg. (cont.)		
56	Control	0
58	Control	0
59	Control	0
62	Control	0
63	Control	0
64	Control	0
65	Control	0
66	Control	0
67	Control	0
69	Control	0

H = high-grade dysplasia; L = low-grade dysplasia *exact number of nodules not determined because of poorly defined borders.

Table A.IIIh. Summary of the data of GSTP$^+$ cells and foci/nodules per cm^2 in the long-term study at 35 and 60 weeks.

35wks	No. of cells		nodule size				
	singlets	duplets	<1um	1-10um	10-20um	20-50um	>50um
FB_1/Fe	4.38±3.46	0.26±0.20	0.01±0.03	0.72±0.36	0.28±0.17	0.09±0.08	0.01±0.02
Fe	2.05±2.10	0.35±0.26	0.01±0.02	0.11±0.13	0	0	0
FB_1/AIN	0.37±0.60	0.06±0.08	0.06±0.06	0.47±0.57	0.02±0.04	0.02±0.04	0
Ctrl	0.19±0.08	0.05±0.04	0.01±0.02	0.06±0.04	0	0	0

60wks	cell #				nodule size				
	singlets	duplets	triplet	4-10	<1um	1-10um	10-20um	20-50um	>50um
FB_1/Fe	1.62±1.23	0.10±0.10	0.04±0.05	0.10±0.10	0.02±0.04	1.00±0.32	0.23±0.16	0.14±0.12	0.03±0.05
Fe	2.17±2.07	0.15±0.12	0.06±0.06	0.12±0.10	0.03±0.06	0.07±0.06	0	0.01±0.02	0
FB_1/AIN	0.41±0.29	0.11±0.11	0.08±0.06	0.11±0.12	0.01±0.02	0.78±0.65	0.07±0.08	0.09±0.06	0.03±0.05
Ctrl	0.32±0.26	0.11±0.08	0.06±0.07	0.23±0.20	0.08±0.15	0.16±0.17	0	0	0

Data are the means ± standard deviation of 5 to 10 animals per group; FB_1: fumonisin B_1; Fe: dietary iron; AIN: American Institute of Nutrition powdered diet-93M; Ctrl: control; GSTP: placental form of glutathione S-transferase.

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Curriculum vitae

Name:	Denise Adeline Werner
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Education and Employment

1978-1984	“Elementary School” in Spokane (Grades 1-6)
1984-1986	“Junior High School” (Grades 7 and 8)
1986-1988	“Ferris High School” (Grades 9 and 10)
1988-1992	“Staatliches Eifel Gymnasium“ Neuerburg (German High School and Boarding School, Grades 10-13)
1992-1994	Justus-Liebig-University in Giessen, Germany -Veterinary Medicine
1994-1999	Ludwig-Maximilians-University in München, Germany -Veterinary Medicine

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1999-2002 Completion of the doctoral thesis in question for the Department of Pathology and Neuropathology at the LMU Munich under the direction of Prof. Dr. W. Schmahl and Prof. Dr. P. Hall of the Department of Anatomical Pathology at the University of Cape Town, South Africa.

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08.1999-01.2002	Volunteer work at the Animal Welfare Society in Cape Town, South Africa (1999-2002) →Sterilization program and other operational care
Sept. 1999	Microsurgery course at the University of Cape Town
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01.2000-01.2002	Part-time position as research assistant in the Department of Anatomical Pathology at the University of Cape Town, South Africa
Presentations	"*The effect of different dietary iron levels in the rat*" Presented at the Medical Research Council, Tygerberg, South Africa, June 9th, 2000 *"The effect of iron overload on the long-term toxicological effects of fumonisin B_1 in rat liver"* Presented at (i) The Medical Research Council, Tygerberg, South Africa, December 13th, 2001 (ii) University of Cape Town, South Africa, January 17th, 2002