

The *db* Mouse as a Model for Steatohepatitis

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Table of Contents

List of abbreviations	ix-xiii
Abstract	1
Chapter 1; Introduction – Overview of Nonalcoholic Fatty Liver Disease	
1.1 Introducing Nonalcoholic Fatty Liver Disease	2
1.2 Associated Risk Factors	6
1.2.1 Age	6
1.2.2 Obesity	6
1.2.3 Type 2 Diabetes Mellitus	7
1.2.4 Insulin Resistance (IR)	7
1.2.5 Gender	7
1.2.6 ALT/AST ratio > 1	8
1.2.7 Genetics	8
1.3 Diagnosis	9
1.4 Therapy	10
Chapter 2; Pathogenesis of Fatty Liver Disease	
2.1 Introducing the Pathogenesis of NAFLD and NASH	14
2.2 Insulin Resistance (IR)	15
2.3 Free Fatty Acids (FFA)	18
2.4 β -Oxidation of Fatty Acids	20
2.5 Peroxisome Proliferator Activated Receptors (PPARs)	21
2.6 Cytochrome P450 Enzymes	25
2.7 Leptin	27
2.8 Type II Diabetes Mellitus (T2DM)	29
2.9 Obesity	29
2.10 Hepatic Fibrosis	31
2.11 Cirrhosis	34
2.12 Similarities with Alcoholic Liver Disease (ALD)	36
Chapter 3; Overview of Animal Models in Nonalcoholic Fatty Liver Disease	
3.1 Introducing the Animal Models employed in studying NAFLD	39
3.2 (Ob/Ob) Mouse Model	39
3.3 Tumour Necrosis Factor- α (TNF- α) Knockout Mouse Model	42
3.4 Sprague-Dawley Rat Model	44
3.5 Peroxisome Proliferator Activated Receptors (PPAR) Mouse Model	44
3.6 Methionine and Choline Deficient (MCD) Dietary Model	47
3.7 Lipodystrophic Mouse Model	52
3.8 Leptin Deficient Mouse Model	53
3.9 Fatty Liver Shionogi (FLS) Mouse Model	55
3.10 Zucker (fa/fa) Rat Model	56
3.11 Acyl-CoA Oxidase (AOX) Mouse Model	57
3.12 Human Studies	58
3.13 Summary of Animal Data Derived from studying NAFLD	62
3.13.1 Introduction	62
3.13.2 (Ob/ob) Mouse Model	63
3.13.3 TNF-R1 Knockout Mouse Model	65
3.13.4 Sprague Dawley Mouse Model	66
3.13.5 (Db/db) Mouse Model	67

3.13.6 PPAR- α Mouse Model	68
3.13.7 LepTg Mouse Model	70
3.13.8 Fatty Liver Shionogi (FLS) Mouse Model	71
3.13.9 Zucker (fa/fa) Rat Model	72
3.13.10 A/J Mouse Model	76
3.13.11 C57Bl/J Mouse Model	77
3.13.12 Acyl CoA Oxidase (AOX) Mouse Model	78
Chapter 4; Methods and Materials	
4.1 Introduction	80
4.2 Histology	80
4.2.1 Tissue Processing	80
4.2.2 Stains / Light Microscopy	80
4.3 Biochemistry	81
4.3.1 Lipid Extraction	81
4.3.2 Conjugated Dienes (CD)	82
4.3.3 Lipid Hydroperoxides (LOOH)	83
4.3.4 Thiobarbituric Acid Reactive Substances (TBARS)	84
4.3.5 Total Cholesterol (TC)	85
4.3.6 Hepatic Triglycerides (TG)	86
4.3.7 Bicinchoninic Acid (BCA) Assay of Proteins	87
4.3.8 Measurement of Aminotransferases	88
4.3.9 Measurement of Blood Alcohol Levels	90
4.4 Statistical Analyses	91
4.5 Animal Studies	92
4.5.1 Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	92
4.5.2 Impact of Various Oils, Administered via the Diet (4 Weeks)	93
4.5.3 Study of Short Period of Alcohol Administration (4 Weeks)	94
4.5.4 Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	95
4.5.5 Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	97
Chapter 5; Results of Studies	
5.1 Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	98
5.1.1 Body Mass	99
5.1.2 Liver Mass	100
5.1.3 Liver Proportion of Body Mass	100
5.1.4 Plasma Glucose Concentrations	101
5.1.5 Triglycerides	101
5.1.6 Total Cholesterol	102
5.1.7 Conjugated Dienes as Early Products of Lipid Peroxidation	102
5.1.8 LOOH as Intermediated Products of Lipid Peroxidation	103
5.1.9 TTBARs as Late Products of Lipid Peroxidation	103
5.2 Impact of Various Oils, Administered via the Diet (4 Weeks)	104
5.2.1 Body Mass	104
5.2.2 Gradient Analysis of Body Mass Gain	104
5.2.3 Liver Mass	105
5.2.4 Liver Proportion of Body Mass	105
5.2.5 Plasma Glucose Concentrations	106
5.2.6 Triglycerides	106
5.2.7 Total Cholesterol	107
5.2.8 Conjugated Dienes as Early Products of Lipid Peroxidation	107
5.2.9 LOOH as Intermediated Products of Lipid Peroxidation	108

5.3.10	TTBARS as Late Products of Lipid Peroxidation	108
5.2.11	BCA Protein Levels	109
5.3	Study of Short Period of Alcohol Administration (4 Weeks)	110
5.3.1	Body Mass	110
5.3.2	Gradient Analysis of Body Mass Gain	110
5.3.3	Triglycerides	111
5.3.4	Total Cholesterol	111
5.3.5	Conjugated Dienes as Early Products of Lipid Peroxidation	112
5.3.6	LOOH as Intermediated Products of Lipid Peroxidation	112
5.3.7	TTBARS as Late Products of Lipid Peroxidation	113
5.4	Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	114
5.4.1	Body Mass	114
5.4.2	Gradient Analysis of Body Mass Gain	114
5.4.3	Liver Mass	115
5.4.4	Liver Proportion of Body Mass	115
5.4.5	Plasma Glucose Concentrations	116
5.4.6	Blood / Alcohol Concentrations	116
5.4.7	Plasma Aminotransferases; (ALT and AST)	117
5.4.8	Triglycerides	118
5.4.9	Total Cholesterol	118
5.4.10	Conjugated Dienes as Early Products of Lipid Peroxidation	119
5.4.11	LOOH as Intermediated Products of Lipid Peroxidation	119
5.4.12	TTBARS as Late Products of Lipid Peroxidation	120
5.4.13	BCA Protein Levels	120
5.5	Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	121
5.5.1	Body Mass	121
5.5.2	Gradient Analysis of Body Mass Gain	121
5.5.3	Liver Mass	122
5.5.4	Liver Proportion of Body Mass	122
5.5.5	Plasma Glucose Concentrations	123
5.5.6	Blood / Alcohol Concentrations	123
5.5.7	Plasma Aminotransferases; (ALT and AST)	124
5.5.8	Triglycerides	125
5.5.9	Total Cholesterol	125
5.5.10	Conjugated Dienes as Early Products of Lipid Peroxidation	126
5.5.11	LOOH as Intermediated Products of Lipid Peroxidation	126
5.5.12	TTBARS as Late Products of Lipid Peroxidation	127
5.5.13	BCA Protein Levels	127
5.6	Summary Table of Results	128
5.6.1	Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	128
5.6.2	Impact of Various Oils, Administered via the Diet (4 Weeks)	129
5.6.3	Study of Short Period of Alcohol Administration (4 Weeks)	130
5.6.4	Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	131
5.6.5	Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	132
5.7	Histology	133
5.7.1	Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	133
5.7.2	Impact of Various Oils, Administered via the Diet (4 Weeks)	135
5.7.3	Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	136
5.7.4	Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	140

Chapter 6; Interpretation of Results

6.1 Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	143
6.2 Impact of Various Oils, Administered via the Diet (4 Weeks)	149
6.3 Study of Short Period of Alcohol Administration (4 Weeks)	152
6.4 Impact of Alcohol Administered over 8 and 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	156
6.5 Histopathology Analysis	165
6.5.1 Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	165
6.5.2 Impact of Various Oils, Administered via the Diet (4 Weeks)	165
6.5.3 Impact of Short Period of Alcohol Administration (4 Weeks)	166
6.5.4 Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	166
6.5.5 Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	167

Chapter 7; Discussion and Conclusion

7.1 Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	169
7.2 Impact of Various Oils, Administered via the Diet (4 Weeks)	169
7.3 Impact of a Short Period of Alcohol Administration (4 Weeks)	170
7.4 Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	171
7.5 Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	172
7.6 The Mouse as a Model	173
7.7 An Integrated View of Fatty Liver	174

Chapter 8; Tables – Group Mean Values

8.1 Assessment of the <i>db/db</i> Mouse Model on Regular Chow for 20 Weeks	176
8.1.1 Terminal Body Mass	176
8.1.2 Liver Mass	176
8.1.3 Plasma Glucose Concentrations	177
8.1.4 Triglycerides	177
8.1.5 Total Cholesterol	178
8.1.6 Conjugated Dienes as Early Products of Lipid Peroxidation	178
8.1.7 LOOH as Intermediated Products of Lipid Peroxidation	179
8.1.8 TTBARs as Late Products of Lipid Peroxidation	179
8.2 Impact of Various Oils, Administered via the Diet (4 Weeks)	180
8.2.1 Weekly Body Mass	180
8.2.2 Liver Mass	180
8.2.3 Plasma Glucose Concentrations	181
8.2.4 Triglycerides	181
8.2.5 Total Cholesterol	182
8.2.6 Conjugated Dienes as Early Products of Lipid Peroxidation	182
8.2.7 LOOH as Intermediated Products of Lipid Peroxidation	183
8.2.8 TTBARs as Late Products of Lipid Peroxidation	183
8.2.9 BCA Protein Levels	184
8.3 Study of Short Period of Alcohol Administration (4 Weeks)	185
8.3.1 Weekly Body Mass	185
8.3.2 Triglycerides	186
8.3.3 Total Cholesterol	186
8.3.4 Conjugated Dienes as Early Products of Lipid Peroxidation	187
8.3.5 LOOH as Intermediated Products of Lipid Peroxidation	187
8.3.6 TTBARs as Late Products of Lipid Peroxidation	188
8.4 Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	189
8.4.1 Weekly Body Mass	189

8.4.2 Liver Mass	190
8.4.3 Plasma Glucose Concentrations	190
8.4.4 Alcohol / Blood Levels	190
8.4.5 Aspartine Transaminase Levels	191
8.4.6 Alanine Transaminase Levels	191
8.4.7 Triglycerides	191
8.4.8 Total Cholesterol	192
8.4.9 Conjugated Dienes as Early Products of Lipid Peroxidation	192
8.4.10 LOOH as Intermediated Products of Lipid Peroxidation	192
8.4.11 TTBARs as Late Products of Lipid Peroxidation	193
8.4.12 BCA Protein Levels	193
8.4.13 Fat Change and Inflammation Grading	193
8.5 Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	194
8.5.1 Weekly Body Mass	194
8.5.2 Liver Mass	196
8.5.3 Plasma Glucose Concentrations	196
8.5.4 Alcohol / Blood Levels	196
8.5.5 Aspartine Transaminase Levels	197
8.5.6 Alanine Transaminase Levels	197
8.5.7 Triglycerides	197
8.5.8 Total Cholesterol	198
8.5.9 Conjugated Dienes as Early Products of Lipid Peroxidation	198
8.5.10 LOOH as Intermediated Products of Lipid Peroxidation	198
8.5.11 TTBARs as Late Products of Lipid Peroxidation	199
8.5.12 BCA Protein Levels	199
8.5.13 Fat Change and Inflammation Grading	199

Chapter 9; Appendices – Individual Values

9A Wild Type Mice (<i>wt/wt</i>) on Regular Chow for 20 Weeks	200
A1 Terminal Body Mass	200
A2 Liver Mass	200
A3 Liver Proportion of Body Mass	200
A4 Plasma Glucose Concentrations	201
A5 Triglycerides	201
A6 Total Cholesterol	201
A7 Conjugated Dienes as Early Products of Lipid Peroxidation	202
A8 LOOH as Intermediated Products of Lipid Peroxidation	202
A9 TTBARs as Late Products of Lipid Peroxidation	202
9B Heterozygous Mice (<i>db/wt</i>) on Regular Chow for 20 Weeks	203
B1 Terminal Body Mass	203
B2 Liver Mass	204
B3 Liver Proportion of Body Mass	205
B4 Plasma Glucose Concentrations	206
B5 Triglycerides	207
B6 Total Cholesterol	208
B7 Conjugated Dienes as Early Products of Lipid Peroxidation	209
B8 LOOH as Intermediated Products of Lipid Peroxidation	210
B9 TTBARs as Late Products of Lipid Peroxidation	211
9C Homozygous Mice (<i>db/db</i>) on Regular Chow for 20 Weeks	212
C1 Terminal Body Mass	212
C2 Liver Mass	213
C3 Liver Proportion of Body Mass	214
C4 Plasma Glucose Concentrations	215
C5 Triglycerides	216
C6 Total Cholesterol	217

C7 Conjugated Dienes as Early Products of Lipid Peroxidation	218
C8 LOOH as Intermediated Products of Lipid Peroxidation	219
C9 TTBARs as Late Products of Lipid Peroxidation	220
9D Impact of Various Oils, Administered via the Diet (4 Weeks)	221
D1 Weekly Body Mass	221
D2 Liver Mass	222
D3 Liver Proportion of Body Mass	223
D4 Plasma Glucose Concentrations	224
D5 Triglycerides	225
D6 Total Cholesterol	226
D7 Conjugated Dienes as Early Products of Lipid Peroxidation	227
D8 LOOH as Intermediated Products of Lipid Peroxidation	228
D9 TTBARs as Late Products of Lipid Peroxidation	229
D10 BCA Protein Levels	230
D11 Cage Assignments	230
9E Impact of Short Period of Alcohol Administration (4 Weeks)	231
E1 Weekly Body Mass	231
E2 Triglycerides	232
E3 Total Cholesterol	232
E4 Conjugated Dienes as Early Products of Lipid Peroxidation	233
E5 LOOH as Intermediated Products of Lipid Peroxidation	233
E6 TTBARs as Late Products of Lipid Peroxidation	234
9F Impact of Alcohol Administered over 8 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	235
F1 Weekly Body Mass	235
F2 Liver Mass	237
F3 Liver Proportion of Body Mass	238
F4 Plasma Glucose Concentrations	239
F5 Alcohol / Blood Levels	240
F6 Aspartate Transaminase Levels	241
F7 Alanine Transaminase Levels	242
F8 Triglycerides	243
F9 Total Cholesterol	244
F10 Conjugated Dienes as Early Products of Lipid Peroxidation	245
F11 LOOH as Intermediated Products of Lipid Peroxidation	246
F12 TTBARs as Late Products of Lipid Peroxidation	247
F13 BCA Protein Levels	248
F14 Fat Change and Inflammation Grading	249
F15 Cage Assignments	249
9G Impact of Alcohol Administered over 20 Weeks in <i>db/wt</i> and <i>db/db</i> Mice	250
G1 Weekly Body Mass	250
G2 Liver Mass	254
G3 Liver Proportion of Body Mass	255
G4 Plasma Glucose Concentrations	256
G5 Alcohol / Blood Levels	257
G6 Aspartate Transaminase Levels	258
G7 Alanine Transaminase Levels	259
G8 Triglycerides	260
G9 Total Cholesterol	261
G10 Conjugated Dienes as Early Products of Lipid Peroxidation	262
G11 LOOH as Intermediated Products of Lipid Peroxidation	263
G12 TTBARs as Late Products of Lipid Peroxidation	264
G13 BCA Protein Levels	265
G14 Fat Change and Inflammation Grading	266
G15 Cage Assignments	266
Chapter 10; References	267

List of Abbreviations

ACC: Acetyl-CoA carboxylase

ADH: Alcohol dehydrogenase

ALD: Alcoholic liver disease

ALT: Alanine aminotransferase

ANGPTL3: Angiopoietin-like protein 3

AOX: Acyl-CoA oxidase

AP1: Activator protein-1

ASP: Acylation stimulating protein

AST: Aspartate aminotransferase

BCA: Bicinchoninic acid

BHT: Butylated hydroxyl toluene

BMI: Body mass index

BSA: Bovine serum albumin

CB: Cannabinoid

CCK: Cholecystokinin

CD: Conjugated diene(s)

CE: Cholesterol ester

CLA: Conjugated linoleic acid

CNS: Central nervous system

CPT1: Carnitine palmitoyltransferase-1

CPT2: Carnitine palmitoyltransferase-2

CT: Computerised chromatography

CoA: Coenzyme A

CYP2E1: Cytochrome P450 2E1

CYP450: Cytochrome P450

DCA: Dicarboxylic fatty acids

DHEA: Dehydroepiandrosterone and its sulphate ester (DHEA-S)

DHEA-S: Dehydroepiandrosterone and sulphate ester

DMII: Diabetes mellitus type 2

DMSO: Dimethyl sulfoxide

D/PAS: Periodic acid-Schiff stain with diastase digestion

DPPP: Diphenyl-1-pyrenylphosphine

ECM: Extracellular matrix

EGF: Epidermal growth factor

ER: Endoplasmic reticulum

Erks: Extracellular regulated kinases

FA: Fatty acid

FABP: Fatty acid binding protein(s)

FC: Free cholesterol

FFA: Free fatty acid

FLS: Fatty liver Shionogi

G6P: Glucose-6-phosphate

GK: Glycerol kinase

GLP1: Glucagon-like peptide-1

GLP2: Glucagon-like peptide-2

GLUT: Glucose Transporter(s)

GPO: Glycerol-3-phosphate oxidase

GSH: Glutathione

GTT: Glucose tolerance testing

H&E: Haematoxylin and eosin

HAD: Indiana University high-alcohol-drinking rat line

HCA: Hepatocellular adenoma

HCC: Hepatocellular carcinoma

HGF α : Hepatic growth factor alpha

HMGC α : Hydroxymethyl glutaryl coenzyme A

HNE: 4-hydroxy-2'-nonenal

HSC: Hepatic stellate cell

11 β HSD-I: 11 β hydroxysteroid dehydrogenase-I

IGF2: Insulin growth factor-2

I κ B: Inhibitor kappa kinase beta

IL2: Interleukin-2

IL8: Interleukin-8

i/p: Intraperitoneal

IR: Insulin resistance

ITT: Insulin tolerance testing

JIB: jejuno-ileal bypass

Jnks: Jun N-terminal kinases

ko: Knockout

LCFA: Long chain fatty acids

LDL: Low density lipoprotein

LOOH: Lipid hydroperoxides

LPL: Lipoprotein lipase

LPS: Lipopolysaccharide

MCD: Methionine and choline deficient

MCDD: Methionine and choline deficient diet

MDA: Malondialdehyde

MDH: Malate dehydrogenase

MMP: Matrix metalloproteinases

MnSOD: mitochondrial manganese superoxide dismutase

MTP: Mitochondrial triglyceride transfer protein

MTT: Monotetrazolium dye

MUFA: Monounsaturated fatty acid(s)

NAD/NADH: Nicotinamide adenine dinucleotide, oxidised / reduced forms

NAFLD: Nonalcoholic fatty liver disease

NASH: Nonalcoholic steatohepatitis

NFκB: Nuclear factor kappa beta

8-OHdG: 8-hydroxydeoxyguanosine

O₂⁻: Superoxide anion

Ob-R: Leptin receptor

OCT: Ornithine carbamyl transferase

OPN: Osteopontin

OXN: Oxyntomodulin

P: Indiana University alcohol-preferring rat line

P5P: Pyridoxal-5'-phosphate

PBS: Phosphate buffered saline

PC: Phosphatidylcholine

PCNA: Proliferating cell nuclear antigen

PDGF: Platelet derived growth factor

PH: Partial hepatectomy

PKA: Protein kinase A

PL: Phospholipid

PMN: Polymorphonuclear leucocyte(s)

POD: Peroxidase

PPARα: Peroxisome proliferator-activated receptor alpha

PPARβ: Peroxisome proliferator-activated receptor beta

PPARγ: Peroxisome proliferator-activated receptor gamma

PTX: Pentoxifylline

PUFA: Polyunsaturated fatty acid(s)

PYY: Peptide YY

r-metHuLeptin: Recombinant methionyl human leptin

ROS: Reactive oxygen species

SHRSP: Stroke-prone spontaneously hypertensive rat

sP: Sardinian alcohol-preferring rat line

SR: Sirius red

SREBP1: Sterol regulatory element-binding protein-1

STAT1: Signal transducer and activator of transcription-1

T2DM: Diabetes mellitus type 2

TAG: Triglyceride

TBARS: Thiobarbituric acid reactive substances

tBHP: t-butyl hydroperoxide

TC: Total cholesterol

TG: Triglyceride

TGF β 1: Transforming growth factor beta-1

TNF α : Tumour necrosis factor alpha

TTBARS: Total Thiobarbituric acid reactive substances

UCP-2: Uncoupling protein-2

UCT: University of Cape Town

UDCA: Ursodeoxycholic acid

VLCFA: Very long chain fatty acid

VLDL: Very low density lipoprotein

wk: week

wt: wild type

Abstract

Fatty liver disease is a collective phrase for a spectrum of diseases characterised by increased liver fat content. It ranges from fatty infiltration of the liver to an inflammatory condition, steatohepatitis, which may lead onto cirrhosis. Although not associated with alcohol consumption, nonalcoholic steatohepatitis (NASH) has strong associations with obesity, diabetes and dyslipidaemia. Overlapping pathological mechanisms may be involved. The course of the disease will remain unpredictable, and specific treatment will only be able to be instituted once the pathogenesis is fully understood.

This thesis reviews current understanding of the pathogenesis and explores the suitability of a recently defined obese diabetic mouse model for its value as a model in the heterozygous and homozygous states.

Observations revealed that the *db/wt* phenotype has a larger mass than the *wt/wt* and responds with hyperglycaemia. Lipid accumulation occurs in this model when alcohol is administered and lipid peroxidation occurs but histological changes of steatosis and steatohepatitis do not occur. The *db/db* model is phenotypically distinguished by a large amount of fat storage, diabetes and macrovesicular steatosis that has more lipid peroxidation but no steatohepatitis even when alcohol further increases lipid peroxidation.

The model, as explored, did not reveal steatohepatitis either alone, or with alcohol as a single additional stressor, but both the *db/wt* and *db/db* mouse model could be further investigated to explore whether additional stressors could induce steatohepatitis in this model.

Chapter 1; Overview of Nonalcoholic Fatty Liver Disease

1.1 Introduction

Hepatic steatosis or fatty liver was previously regarded as a benign condition, but this impression has changed since Ludwig *et al.*, (1980) first coined the term nonalcoholic steatohepatitis (NASH) when encountering a group of non-drinkers whose liver biopsies were revealed to be histologically indistinguishable from alcoholic hepatitis. Similarities between nonalcoholic liver disease and alcoholic hepatitis had already been recognised (Miller *et al.*, 1979) but it was not until Ludwig *et al.*, (1980) announced the mimicking of the poorly defined condition that the disease gained true recognition.

The groundbreaking study (Ludwig *et al.*, 1980) was conducted using 20 patients who denied excessive alcohol intake (averaged one drink per week). The liver biopsy specimens were examined and all patients exhibited lobular inflammation and moderate to severe macrovesicular fatty changes which predominantly occurred in central hepatic zones. Alcohol is not the causative factor in the development of these fatty livers, but patient profiles implicated obesity and diabetes as predisposing conditions for NASH.

However, NASH has a prevalence of approximately 2-3% in the general population and is often considered under the denomination of nonalcoholic fatty liver disease (NAFLD) (Yu and Keefe, 2002). This term encompasses a wider range of liver disorders that are beginning to be recognised more frequently in affluent individuals (Haque *et al.*, 2002) with the spectrum ranging from fatty liver alone to cirrhosis and possibly even development of hepatocellular carcinoma (Nanji, 2004). NAFLD is present in approximately 20% of the USA making it the most common form of liver disease within that country (Yu and Keefe, 2002).

NAFLD comprises the complete spectrum of liver degeneration; beginning as fatty liver alone, advancing to steatosis, then steatohepatitis (NASH) before progressing to fibrosis and resulting in cirrhosis which is the final end-point of an inflammatory response in the liver (Alba and Lindor, 2003). The pathology presented by this sequenced chain of events closely resembles that of alcohol-induced liver injury, even though NAFLD patients are exempt from extensive alcohol consumption (Matteoni *et al.*, 1999).

The grouping together of all these multifactorial diseases under the term NAFLD has resulted in a wide range of pathologic and clinical entities. Despite NASH being regarded as potentially the most severe form in this arrangement of diseases, most patients are asymptomatic and a liver biopsy may be needed to determine certain distinguishing features that correlate with the stage of the disease (Fernández-Rodríguez *et al.*, 2003). As a result, Brunt *et al.*, (1999) devised a grading and staging system which reflects the unique pathological features present in this range of diseases (see below for histologic classification). A NASH patient would typically display type 3 or 4 characteristics and therefore only represents a percentage of the NAFLD assemblage.

Type 1: Steatosis

Type 2: Steatosis plus lobular inflammation

Type 3: Steatosis plus lobular inflammation and degenerative ballooning

Type 4: Steatosis plus degenerative ballooning, Mallory bodies ± fibrosis

Matteoni *et al.*, (1999) also created a scoring system based on fat content, necroinflammation and progression to fibrosis within the spectrum of NAFLD. A study undertaken by Matteoni *et al.*, (1999) examined liver biopsy specimens of 132 NASH patients with complete clinical records and established that approximately 29% were suspected of disease progression to cirrhosis. The occurrence of cirrhotic livers results from structural changes along with fibrosis. These changes may be described as a decline in regular lobular architecture and the emergence of fibrous tissue bands formed by regenerative nodules of hepatocytes (Diehl *et al.*, 1988). Brunt *et al.*, (1999), developed a staging system for assessing the extent of liver injury incurred by fibrosis/cirrhosis in NASH patients and is displayed below. Cirrhosis is not reversible and signals the loss of regulatory hepatic function.

Stage 1: Zone 3 perisinusoidal and pericellular fibrosis, focal or extensive

Stage 2: Zone 3 perisinusoidal fibrosis, focal or extensive periportal fibrosis

Stage 3: Perisinusoidal and portal fibrosis, bridging fibrosis

Stage 4: Cirrhosis, with or without perisinusoidal fibrosis

A combination of both of these methods was adapted for the purpose of grading in this study. Fatty cysts, glycogenated nuclei and lipogranulomas can appear at histological examination, but these were not deemed reliable in making a correct prognosis (Brunt, 2002). It is worth noting that a diagnosis usually results from a variation of microscope findings as opposed to the exposure of a single pathological feature. Additional clinical traits which can be used for identification purposes include hepatomegaly, the most common physical sign and evaluation of serum

aminotransferase levels. No specific diagnostic test is currently available (Fernández-Rodríguez *et al.*, 2003).

Liver biopsies are used for histological assessment of NAFLD patients to determine the extent of hepatocyte injury, inflammation and fibrosis. Haematoxylin and eosin (H&E) stains assess hepatic injury and cell disruption, whereas sirius red (SR) preparations can gauge the extent of fibrosis (Brunt, 2002). Periodic-acid Schiff after diastase digestion (D/PAS) is used for evaluation of debris-containing Kupffer cells (Brunt, 2001).

Factors which can promote or cause fatty liver (other than alcohol), include obesity, diabetes, insulin resistance, hyperlipidaemia, rapid weight loss or, more commonly, a combination of these (Fong *et al.*, 2000). Additionally, hepatotoxic drugs, jejunoileal bypass operations, hepatitis C and Wilson's disease are also acknowledged facilitators of fatty liver development (Fong *et al.*, 2000). Rarely, fatty liver is encountered in disorders of lipoprotein assembly: either MTP deficiency or homozygous hypo β lipoproteinaemia.

The occurrence of steatosis is biochemically described as an accumulation of lipids in the liver exceeding 5% of the liver weight (Cairns and Peters, 1983), although this is not universally agreed. Steatosis in NAFLD patients is generally identified to consist of macrovesicular fat (large single lipid droplets) within hepatocyte cytoplasm displacing the nucleus, although mixed steatosis is also recognised to occur, in which microvesicular fat (multiple, small lipid droplets) are additionally present (Brunt and Tiniakos, 2002). Abnormalities in the metabolism and synthesis of lipid and consequent accumulation of triglycerides is believed to result in macrovesicular fat whereas the presence of microvesicular fat is indicative of further progressed liver disease characterised by a diminution of β -oxidation of fatty acids (Brunt and Tiniakos, 2002). The favourable location of fatty injury is located in acinar zone 3 despite the varying severity from patient to patient and is reversible. The potential of ballooning and Mallory bodies being located is dependent on stage of development (Brunt, 2001).

The occurrence and progression of the inflammatory response is coupled with steatosis and is frequently associated in NASH patients, as can be deduced by involvement in the scoring systems for assessment of disease status. The inflammation is mainly lobular and a key feature is the existence of

polymorphonuclear leukocytes (PMN) (Takahashi *et al.*, 1987) which are closely located to ballooned hepatocytes in further advanced cases of NASH (Brunt, 2001). The ensuing inflammation can be either acute or chronic, but caution is advised when examining biopsy samples as steatosis and hepatic inflammation occur under many other circumstances.

The manifestation of hepatocyte injury primarily occurs as degenerative ballooning and is characterised by swelling of the cytoplasm of affected cells (Brunt, 2001), however, whether presence of ballooning degeneration is necessary for diagnostic evaluation of NASH is controversial. The additional appearance of Mallory bodies (dense intra-cytoplasmic inclusions in hepatocytes) provides evidence of further liver deterioration and can be verified by histochemical stains (Brunt, 2001). Megamitochondria are recognized as intracellular structures which display ultrastructural abnormalities and can also be observed in NASH patients as markers of progression to fibrosis, although association with extensive alcohol abuse is more frequent (Caldwell, 2002).

The progression to fibrosis in NASH is not universal as some patients experience a relatively benign pattern of the disease and do not require therapeutic treatment (James and Day, 1998). When fibrosis does develop, it is initially encountered in the centrilobular region zone 3, characterised by pericellular distribution and is referred to as 'chicken-wire' fibrosis that is often seen in alcohol-induced steatohepatitis (Brunt, 2001). Progression of the disease results in fibrosis becoming 'bridged' as a consequence of additional collagen deposition from hepatic stellate cells (HSC), the increased activity of which coincides with steatosis grading advancement (Reeves *et al.*, 1996).

Patients with NAFLD and NASH are predominantly asymptomatic and are usually diagnosed accidentally during alternative investigations. A liver biopsy is usually requested on the grounds of patient history or unexplained clinical findings to clearly determine the presence of disease, although ultrasound and computerised tomography (CT) have also been used in detection with modest results (Yu and Keefe, 2002). Therefore prevalence of NAFLD and NASH is difficult to accurately measure as there will be a speculative number of unreported cases. Observations from various studies have been collated in an attempt at estimating the incidence of NAFLD. Harrison *et al.*, (2002), established a 14% frequency of NAFLD in Japanese

trials whereas Yu and Keefe (2002) approximated the value to be nearer 20% in the USA population.

1.2 Associated Risk Factors

Research performed by Angula *et al.*, (1999), established a link between fibrosis severity and steatohepatitis by examining particular features of patient profiles. The findings implied that body mass index (BMI), older age, diabetes and ratio of liver enzymes aspartate aminotransferase (AST) / alanine aminotransferase (ALT) lower than 1 were helpful markers in providing information for NAFLD or NASH identification. Further studies carried by Chitturi *et al.*, (2002) and more recently by Sorrentino *et al.*, (2004) confirmed that female gender may also be a risk factor to consider when assessing this group of diseases. The additional risk and surrounding complications posed by these menacing factors in relation to NAFLD progression will be discussed individually.

1.2.1 Age

The detection of NAFLD in children has been clearly documented over the years, especially in obese, pubertal adolescence (Baldrige *et al.*, 1995). However, before a correct diagnosis can be established, malnutrition, short gut syndrome and drug and alcohol abuse, especially in older children, must be disproved before the condition can be attributed to NAFLD (Rashid and Roberts, 2000). Despite the prevalence of NAFLD in patients of all ages, individuals between 40-60 years of age seem to be at a higher risk independent of other factors involved (Reid 2001).

1.2.2 Obesity

Obesity is strongly associated with NAFLD patients, but the exact role of obesity within this arrangement of diseases remains undefined as non-obese individuals are also affected, comprising a small proportion of reported cases. Many studies have identified obesity as a possible predictor of NAFLD (Powell *et al.*, 1990; Angulo *et al.*, 1999; García-Monzón *et al.*, 2000) with values as high as 90% in a study undertaken by Ludwig *et al.*, (1980). In contradiction to this consistent observation, Bacon *et al.*, (1994) reported the prevalence of NAFLD in non-obese patients to be higher than 50%. The majority of established NAFLD cases in children is highly associated with obesity (Baldrige *et al.*, 1994).

1.2.3 Type 2 Diabetes Mellitus (T2DM)

Another factor seen to be closely linked with the occurrence of NAFLD is T2DM, though the range of association widely varies from 2-55% (James and Day, 1998). An assortment of studies has found hepatic fibrosis to be more common in obese patients with diabetes (Matteoni *et al.*, 1999; Dixon *et al.*, 2001). One study in particular revealed a 2.6-fold increase risk of progressing to NASH if T2DM was present (Wanless and Lentz, 1990). The added metabolic complications arising from diabetes may contribute to increased oxidative stress and may perpetuate hepatocyte degeneration. (Evan *et al.*, 2002).

1.2.4 Insulin Resistance (IR)

The presence of insulin resistance comes as no surprise since obesity, dyslipidaemia and type 2 diabetes are linked to decreased sensitivity to insulin (Marchesini *et al.*, 1999). The precise role of IR is unclear. Apart from a greater flux of fatty acids from adipose tissue to the liver, IR may aid in activation of tumour necrosis factor- α (TNF- α). Sustained production of this cytokine has been shown to promote the progressive pathogenesis of the disease (Koteish and Diehl, 2002). As a result of the close association between NAFLD and IR, many rodent models such as ob/ob (leptin deficient) and db/db (leptin receptor deficient) rodents are being employed or modified to exhibit the characteristics of obesity, diabetes and insulin resistance in an attempt to gain a better understanding of the pathogenesis of these diseases.

1.2.5 Gender

The recent inclusion of gender as a risk factor in NAFLD development and progression has received some criticism due to some conflicting evidence. Initial studies performed by Ludwig *et al.*, (1980) displayed that an obese, middle-aged female would be a prime candidate for progressive NAFLD. Additional work by Angulo *et al.*, (1999) and Chitturi *et al.*, (2002) corroborated these findings. However, a study of 580 obese patients carried out by Marceau *et al.*, (1999) did not find that gender was an indicator of fibrosis or cirrhosis, though it was statistically significant at predicting inflammation. The debate was continued by the finding, by Bacon *et al.*, (1994), of NASH in young, non-obese males.

As previously mentioned, utilisation of rodent models is becoming more frequent in the exploration of NAFLD causes. A point of interest is the recent development of an animal model that exhibits a relationship between gender and the degree of hepatic injury (Kirsch *et al.*, 2003).

1.2.6 AST/ALT ratio > 1

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are liver specific enzymes. The release of aminotransferases indicates the presence of hepatocyte damage (Teitz, 1987). The enzyme activity of plasma has a strong correlation with the gradation of steatosis (Marceau *et al.*, 1999). The ratio of AST/ALT is advantageous to the physician as a good predictor of NAFLD development and can also guide the decision to perform a liver biopsy.

1.2.7 Genetics

The postulate that certain genes or groups of genes may assume responsibility for inherited conditions which could promote the onset of NAFLD and NASH and their outcome is not yet adequately researched (Day, 2002).

The P450 enzymes present themselves as candidate genes for NASH development. Cytochrome P450 2E1 (CYP2E1) in particular, is capable of producing free radicals which could affect the rate of fatty acid oxidation and thus could contribute to the progressive nature of NASH (Day, 2002). The increased expression of CYP2E1 in a rodent dietary model of steatohepatitis adds further evidence of involvement in complications of NASH (Weltman *et al.*, 1998).

Copper/zinc dismutase and aldehyde oxidase are genes responsible for regulation of mitochondrial manganese superoxide dismutase (MnSOD) which aids the body in combating oxidative stress by activation of uncoupling protein 2 (UCP-2) (Shimoda-Matsubayashi *et al.*, 1996). Alteration of these genes could lead to unregulated UCP-2 activity which could effect lipid metabolism and may itself lead to hepatic damage and necrosis (Pessayre *et al.*, 2001).

Cytokine genes have also been implicated in the pathogenesis of NAFLD and NASH, in particular tumour necrosis factor – α (TNF- α). Kupffer cells are the primary site of TNF- α generation, which, upon release in excess, impedes the normal action of insulin and contributes to oxidative stress through enhanced production of reactive oxygen species (ROS) (Peraldi and Spiegelman, 1998). A study conducted by Hui *et al.*, (2004) confirmed activation of TNF- α in NAFLD patients that correlated with severity of steatosis and fibrosis.

Peroxisome proliferator activator receptors - α and γ (PPAR- α) and (PPAR- γ) are genes which modulate fatty acid oxidation and insulin sensitivity respectively (Day,

2002). PPAR- α is abundantly produced in the liver. Disruption of its function may cause uncontrolled fatty acid accumulation, whereas PPAR- γ has been shown to aggravate steatosis by activating lipogenic enzymes (Tanaka *et al.*, 2005). Recent evidence from rodent and human studies suggests that agonists of PPARs display the ability to reduce triglycerides and lipid peroxidation and are therefore being introduced as a new approach for NAFLD treatment (Reifel-Miller *et al.*, 2005; Kersten, 2002).

Additional candidate genes include: 11 β hydroxysteroid dehydrogenase-1 (11 β HSD-1) which contributes to the magnitude and pattern of obesity (Masuzaki *et al.*, 2001) and Apolipoprotein E, that helps regulate lipid storage and export (Day, 2002). Many classes of genes have gained recognition for NAFLD association. Unless a strong monogenic component is responsible for fatty liver, the modulatory genes linked with NAFLD could hamper the detection of a single causative gene that may unravel the unknown.

Although contributing features have been identified in NAFLD and NASH patients, a solitary, causal factor remains elusive. Additional triggers such as drug abuse, jejunio-ileal bypass operation (JIB), and rapid weight loss are also at fault, but encompass a smaller proportion of patients.

1.3 Diagnosis

NAFLD and NASH patients are typically asymptomatic. Many cases are discovered accidentally or are not even reported. Initial symptoms include; fatigue, lethargy, discomfort in the right-upper-quadrant of the abdomen and slight elevations of aminotransferase levels (Kumar and Malet, 2000). The presence of hepatomegaly and splenomegaly can be seen upon physical examination and provide additional indicators for patient evaluation (Reid, 2001).

The AST/ALT ratio is a good marker of hepatic injury as are the associated risk factors, but the true extent of liver damage remains unresolved until a liver biopsy. This remains the definitive means by which an accurate diagnosis and possible method of treatment can be evaluated (Brunt 2001). A physician will not immediately recommend a liver biopsy as an intervention in lifestyle may bring about a marked improvement in obesity related symptoms.

1.4 Therapy

The management of fatty liver is currently non-specific and involves general health measures with good nutrition and obtaining an ideal body mass, along with avoidance of factors that may promote or cause fatty liver. Healthy eating and exercise continues to be the cheap, safe option of therapy for weight reduction which should subsequently result in an improvement of insulin sensitivity and lowering of liver enzyme levels. Precautionary measures must be adhered to when subjects undertake severely restricted mass reduction diets as rapid weight loss can also induce hepatic injury (Capron *et al.*, 1982). A liver biopsy would be indicated if this therapeutic approach was not successful, and risk factors proceeded to accumulate or the risk of cirrhosis was presented.

Further approaches to weight reduction have included administration of the drug orlistat, but this has produced mixed results. It may be hepatotoxic, thus affecting the risk / benefit ratio adversely (Lau *et al.*, 2002). More extreme measures of losing weight are advertised as gastric banding and jejuno-ileal bypass (JIB) and if patient care is well maintained, the effects of steatosis are lessened and even reversed, although the reoccurrence and worsening of the conditions remains a high possibility (Holzbach, 1977).

Administration of vitamin C has been shown to be beneficial in the alleviation the progress of NAFLD (Oliveria *et al.*, 2003). This antioxidant drug has displayed significant inhibition of steatosis, presumably by reducing oxidative stress levels which are thought to play a key role in facilitating the development and progression of NAFLD.

Additional drugs that have been prescribed as therapeutic agents involve a group of agents known as PPAR- α and PPAR- γ agonists (Desvergne and Wahli, 1999). As previously stated, these agonists possess the ability to modulate lipid transport and oxidation and enhance the actions of insulin, resulting in augmented fatty acid breakdown and control of inflammation. Research undertaken by Ip *et al.*, (2003), exposed Wy-14,643 as a further agonist that shows the capacity to avert hepatic lipid accumulation and should be considered in future use as a preventative treatment.

The insulin-sensitizing thiazolidine-dione (PPAR- γ agonist), troglitazone has been investigated in NASH. Despite reasonable success in reducing ALT levels to within normal limits, post-treatment liver biopsies revealed NASH still to be present. The

drug later proved to be hepatotoxic and was withdrawn from the market (Chitturi and George, 2002).

Metformin is a biguanide, an anti-diabetic drug which has been used to treat T2DM for many years and is especially favoured in obese diabetics. The benefits arise from lowering fasting glucose and subsequent control of lipid production in diabetics. A study performed by Lin *et al.*, (2000), used a diabetic mouse model (*ob/ob*), to examine the effects of metformin and successfully displayed a notable retraction in steatosis development and a normalizing trend of liver function tests, paving the way for future trials.

Clofibrate, a PPAR- α agonist, is an additional consideration for NAFLD management. The action of this drug reduces hepatic triglyceride as opposed to enhancing the effects of insulin (Laurin *et al.*, 1996). It has been surpassed by newer fibric acid derivatives.

Colchicine is an anti-inflammatory and anti-fibrotic drug which can be administered to lessen the effects of cirrhosis in alcoholic and nonalcoholic patients but results to date have been inconsistent. Colchicine is a plant alkaloid that possesses the ability to inhibit the migration of granulocytes into the injured area, thereby inhibiting the metabolic and phagocytic activity of granulocytes (Rambaldi and Gluud, 2005). Furthermore, colchicine has been shown to impede the mediated transport of procollagen (Ehrlich and Bornstein, 1972), improve collagenase activity (Harris and Krane, 1971) and decrease liver fibrosis in rats (Rojkind and Kershenobich, 1975).

A recent review comprised of 1714 patients, randomised in 15 trials, was undertaken to examine the beneficial and harmful effects of colchicine in patients with alcoholic or nonalcoholic fibrosis or cirrhosis (Rambaldi and Gluud, 2005). In the majority of trials, the dosage received was 1mg colchicine, five days a week, although some trials did administer the same dose for 6 and 7 day periods. The median duration of the 15 trials was 18 months. The findings from the assemblage of trials revealed that colchicine had no significant effect on mortality, liver complications, liver biochemistry and liver histology. Colchicine was found to exhibit a positive effect on two serum markers of fibrotic activity (7S collagen and prolidase), however, the levels of collagen-peptidase were unaffected.

Another study investigating the effects of colchicine was carried out by Gong *et al.*, (2004) which examined the outcome in patients being treated for primary biliary cirrhosis. Colchicine is administered to patients with primary biliary cirrhosis because it can delay progression of the disease (Kaplan, 1997) and enhance liver biochemical tests and immunoglobulin levels (Warnes *et al.*, 1987). The study concluded that treatment with colchicine did not significantly improve biochemical or histological parameters such as total cholesterol, ALT, AST, fibrosis, parenchymal inflammation or parenchymal necrosis in comparison to the placebo group which had no intervention. The findings from this study, combined with the data generated by Rambaldi and Gluud, (2005) do not provide any significant evidence that treatment with colchicine successfully ameliorates the effects on liver fibrosis or cirrhosis. Colchicine should only be used in further randomised trials once the detrimental effects and benefits, if any, are clearly defined.

Ursodeoxycholic acid (UDCA) treatment displays membrane-stabilising effects and has few drug interactions. It could play a defensive role in protecting the liver from oxidative stress and the progression of liver disease. The advantage of UDCA administration is the background history of the drug which provides strong evidence for the safe use in the milieu of fatty liver disease (Angulo, 2002).

The development of NAFLD is regarded as a fairly new concept in medical practice and has spurred the development of new and upcoming drugs to combat the outcomes.

Adiponectin is newly established as a possible alleviator of NAFLD. Adiponectin is secreted from adipose tissue and is diminished in obese patients. It is believed to decrease steatosis by increasing insulin sensitivity (Berg *et al.*, 2002). This peptide also exhibits anti-inflammatory effects. It has been cited as a partial activator of TNF- α (Hui *et al.*, 2004). In addition, low levels of adiponectin correlate with higher grades of hepatic steatosis and necroinflammatory and may prove to be an important therapeutic tool (Hui *et al.*, 2004). It is not clear whether low adiponectin concentration merely reflects adipocyte dysfunction or is directly implicated in NASH.

Some therapeutic drug administration is clearly beneficial in treating NAFLD and certain treatments even reverse the outcome of steatosis, although 'knock-on' effects constantly persist and therefore considerate care in management is advised. The influx of experimental rodent work which covers the full range of consequences in

NAFLD is proving to be invaluable and needs to be sustained to gain a full understanding and to achieve effective medical therapies. The pathogenic mechanisms involved in the development and progression of NAFLD require to be clearly defined to establish advantageous treatment options and will be discussed in detail in the proceeding chapter.

Chapter 2; Pathogenesis of Fatty Liver Disease

2.1 Introducing the Pathogenesis of NAFLD/NASH

The pathogenesis of NAFLD and subsequent progression to NASH remains poorly understood, but information gained from studying animal models of hepatic steatosis has been invaluable and has established that the underlying mechanisms of NAFLD and NASH are multifactorial (Koteish and Diehl, 2002).

Steatosis is believed to be a benign condition but its presence facilitates susceptibility to further injury in the liver which has led to the 'two-hit' hypothesis. Normal liver, which has no steatosis, has a better ability to withstand the insults of oxidative stress, lipid peroxidation, abnormal cytokine production and insulin resistance and generally does not result in the development of hepatitis (Day and James, 1998). The presence of steatosis represents the 'first-hit' which is the initial insult but apparently benign to the liver and merely causes the hepatocytes to become fatty. It is suggested that a 'fatty' liver faces greater difficulty in dealing with the complications of a 'second-hit' that results in amplified damage and progressive NAFLD to NASH (Day and James, 1998). The 'two-hit' model is depicted below (Figure 2.1.1).

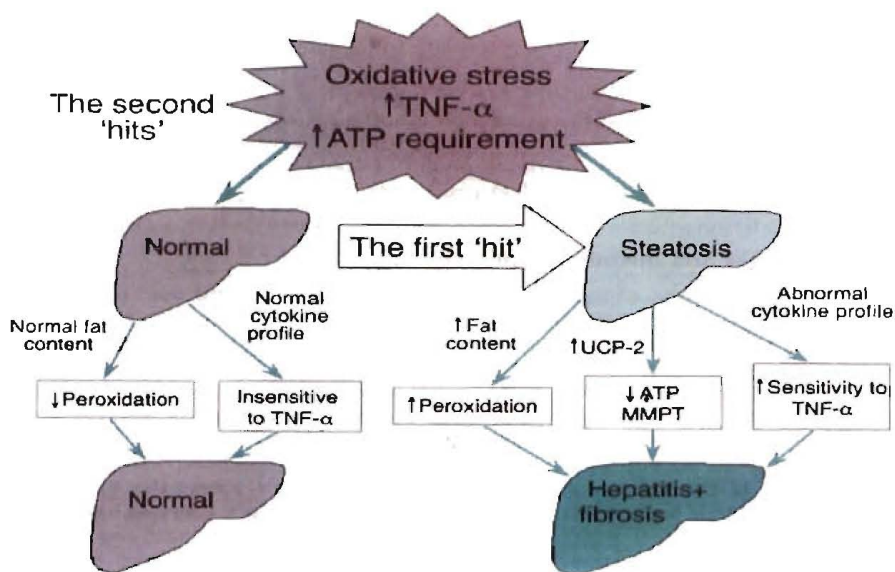


Figure 2.1.1; The 'two-hit' hypothesis of NASH involves the common occurrence of hepatic steatosis as the initial offence (*i.e.* 'first-hit') and following development of steatosis, the liver becomes vulnerable to additional injury (*i.e.* 'second-hits'). These further insults are induced by oxidative stress and other strained situations resulting in progression to steatohepatitis and liver cell death (Day and James 1998).

2.2 Insulin Resistance (IR)

A trademark characteristic of NAFLD is the presence of insulin resistance (IR). The manifestation of obesity, dyslipidaemia and type II diabetes is closely linked to decreased sensitivity to insulin and as sometimes referred to as the 'insulin syndrome' with IR being the common factor, (Marchesini *et al.*, 1999). Recent reports have demonstrated that the majority of NAFLD patients display signs of impaired insulin activity (Pagano *et al.*, 2002; Chitturi *et al.*, 2002) and the close association between IR and the resultant metabolic changes has been a central theme to research.

The association of lowered sensitivity to insulin and obesity is a frequent occurrence. Obese patients display elevated levels of TNF- α expression and activity which coincides with rises in body mass index (BMI) (Kern *et al.*, 1995). The generation of TNF- α rivals the normal functioning of insulin activity and assists in exacerbating insulin sensitivity by facilitating increased reactive oxygen species (ROS) production and oxidative stress (Peraldi and Spiegelman, 1998). This creates further insult and stress to the already vulnerable liver and promotes disease progression.

Additionally, the increased TNF- α also augments the performance of NF- κ B that will amplify TNF- α . The implementation of this loop cycle is detrimental to the patient as NF- κ B, an important transcription factor responsible for contributing to the regulation of inflammation, will promote inflammation. Continuous generation of these two cytokines propagates the activity of other pro-inflammatory cytokines such as interleukin-2 and 8, (IL-2) and (IL-8) which will also incite hepatocyte inflammation (Barnes and Karin, 1997). Studies performed in mice by Uysal *et al.*, (1997) and more recently by Li *et al.*, (2003) have shown that induced mutations of TNF- α receptors significantly improve the sensitisation towards insulin. This in turn, contributes to alleviating the development of fat accumulation leading to severe steatosis and confirms the role of TNF- α as a mediator of the 'second-hit' that worsens NAFLD.

The effects of IR on free fatty acid (FFA) metabolism are also believed to significantly contribute to the development of hepatic steatosis and progression to steatohepatitis. In an insulin-resistant individual, there is a greater flux of FFAs to the liver, causing strain on normal liver function. The increase in lipolytic activity in the adipocytes is part of insulin resistance (Roden *et al.*, 1996). The increased presence of FFA in the hepatocytes inhibits the metabolism in the Krebs cycle, and facilitates fatty acid

oxidation, which endorses oxidative stress in the liver. Elevated hepatic glucose levels coincide with impairment of the Krebs cycle and the resulting sustained stimulation of gluconeogenesis and prolonged inhibition of glycolysis contribute to the accumulation of glucose. An increased production of glucose is met with raised concentrations of insulin in an attempt to improve clearance rates. The capacity of the pancreas to maintain demand for insulin is eventually exhausted and initiates the development of diabetes (Bergman, 1997).

Glucose uptake for cellular function is crucially dependent on insulin. Transporters of glucose (GLUT) gather on cell surfaces in higher numbers allowing glucose-6-phosphate (G-6-P) to accumulate as glycogen, which can be retransformed back to glucose when required for later use (Anthonsen *et al.*, 1998). This reconversion process is modulated by glycogen synthetase and protein phosphatase (Newgard *et al.*, 2000). The phosphorylation status of these enzymes is regulated by protein kinase A (PKA) which becomes inhibited when insulin circulates in high concentrations. Therefore, insulin enhances cellular import and storage of glucose by stimulating glycogen formation and also protects against potential degenerative hepatic glucose accumulation through inhibition of glycogenolysis and gluconeogenesis.

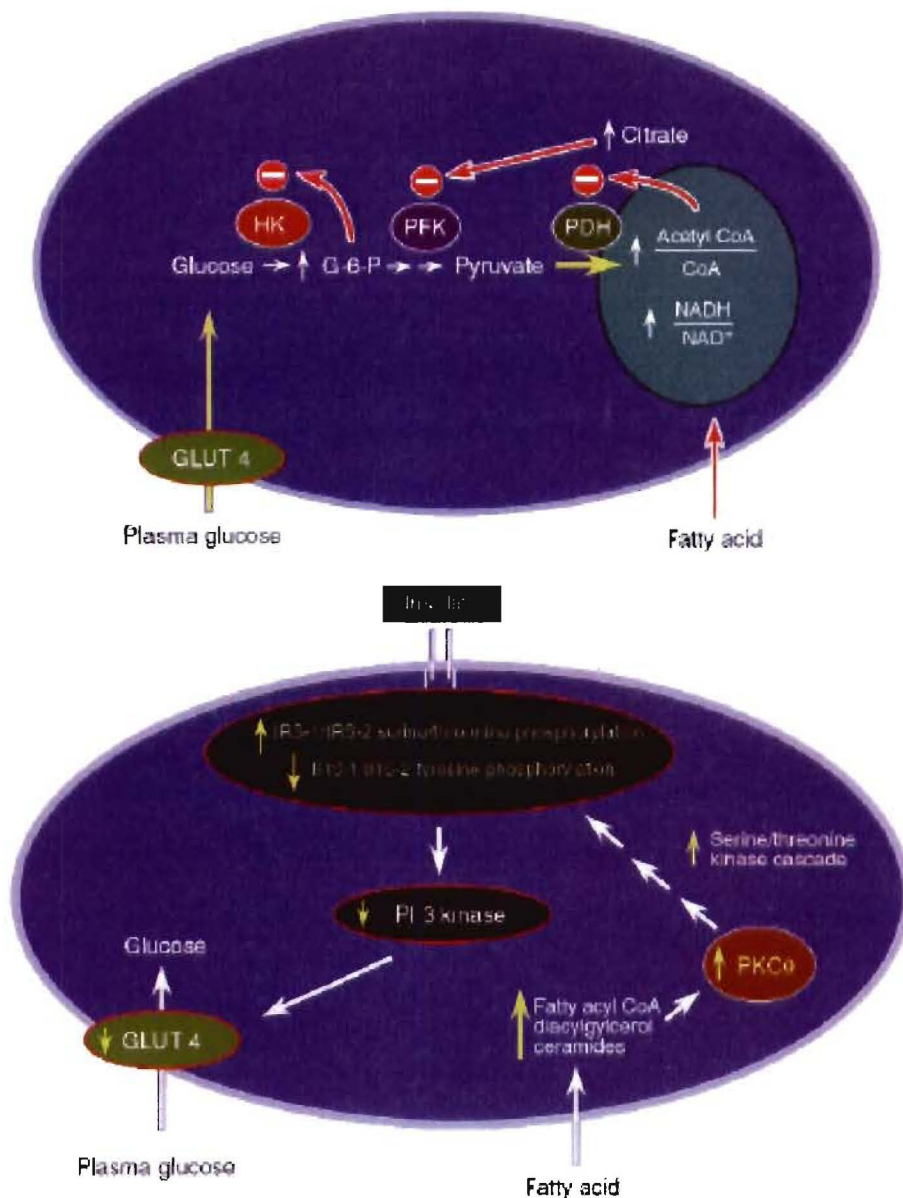


Figure 2.2.1; Top; Mechanism of fatty acid-induced insulin resistance in skeletal muscle. An increase in fatty acid concentration results in an elevation of the intramitochondrial acetyl CoA/CoA and NADH/NAD⁺ ratios, with subsequent inactivation of pyruvate dehydrogenase. This in turn causes citrate concentration to increase, leading to inhibition of phosphofructokinase. Subsequent increases in intracellular glucose-6-phosphatase concentration would inhibit hexokinase II activity, which would result in an increase in intracellular glucose concentration and a decrease in muscle glucose uptake. Bottom; Proposed alternative mechanism for fatty-acid induced insulin resistance in human skeletal muscle. An increase in delivery of fatty acids to muscle or a decrease in intracellular metabolism of fatty acids leads to an increase in intracellular metabolites such as diacyl-glycerol, fatty acyl CoA and ceramides. These metabolites activate a serine/threonine kinase cascade (possibly initiated by protein kinase Cθ) leading to phosphorylation of serine/threonine sites on insulin receptor substrates (IRS-1 and IRS-2), which in turn reduces the ability of the insulin receptor substrates to activate PI 3-kinase. As a consequence, glucose transport activity and other events downstream of insulin receptor signalling and diminished hexokinase II (HK), phosphofructokinase (PFK), pyruvate dehydrogenase (PDH) and protein kinase Cθ (PKCθ) (Schulmann *et al.*, 2000).

A recent study performed by Yesilova *et al.*, (2005), examined the role of acylation stimulating protein (ASP) which is second in abundance behind insulin in adipocytes and partially responsible for triglyceride synthesis. ASP stimulates the uptake of glucose in adipocytes. Mice previously administered ASP were shown to display improved clearance rates of FFA and TG (Saleh, 2001). Diacylglycerol

acyltransferase is an important rate-limiting enzyme involved in TG synthesis and its activity is increased as a direct result of ASP induced glucose uptake, the consequence of which is increased stimulation of FFA re-esterification into triacylglycerol and reduction of FFA endogenous production (Germinario *et al.*, 1993; Faraj *et al.*, 2004).

The work undertaken by Yesilova *et al.*, (2005), could not fully establish a correlation between plasma ASP levels and lipid accumulation in NAFLD patients but did further outline the significance of ASP as a regulatory mediator of FFA uptake and TG synthesis. The dysregulation of the ASP pathway appears to increase the influx of FFAs to the liver which in turn results in hypertriglyceridaemia and a host of additional metabolic changes associated with obesity, and predisposing to coronary heart disease and diabetes (Sniderman *et al.*, 2000; Weyer and Pratley, 1999; Cianflone *et al.*, 1997; Koistinen *et al.*, 2001). ASP and insulin are key factors which determine the rapidity of TG synthesis in the adipocyte, thus playing an important role in controlling FFA plasma concentration and transport and may have noteworthy implications in the metabolic complications of NAFLD and NASH (Sniderman *et al.*, 1998).

A reduction in metabolic clearance is a resulting key feature of NASH patients who fail to successfully dispose of excess glucose. This lessened metabolic capacity of the liver, coupled with impaired lipolysis in adipocytes or disposal from hepatocytes, is a consequence of IR enhancing free fatty acid production. Hepatic steatosis occurs when hepatocytes lose the ability to balance synthesis and exportation of FFA, ultimately resulting in fat accumulation. The additional progression of the disease may depend on the presence or severity of further hits which will include P450, TNF- α and lipid peroxidation (Sanyal, 2005).

2.3 Free Fatty Acid (FFA)

The physiological pathways of FFA are illustrated in figure 2.3.1, but components in this scheme will be discussed and illustrated separately in this section. Free fatty acids (FFA) are the principal metabolic product of adipocytes, and under normal circumstances will not accumulate as triglycerides within hepatocytes (Saleh *et al.*, 1999). FFA synthesis and recycling of FFA and triglycerides to the liver provide for the export of energy from the liver as VLDL. Fatty acids also serve other functions: intracellular signalling, regulation of gene transcription and act as a source of immediate energy when oxidised.

The normal regulation of FFA results in their β -oxidation within mitochondria and peroxisomes, whilst oxidation may also occur within the cytochrome P450 system which responds to increasing demand. FFA may also be re-esterified to triglycerides or may be used for synthesis of other lipids (Fong *et al.*, 2000). The two central mechanisms operated by the liver for providing FFA for cell function are lipogenesis (from acetyl CoA) and recruitment from the circulation by FAT protein (CD36). The FFA are primarily esterified into triglycerides (TG) which are the chief components of fat in the fatty liver (Wanless *et al.*, 1989). Triglycerides accumulate as fat droplets in the cytosol of hepatocytes. For export they are moved by MTP to apolipoproteins B48 in the enterocyte or B100 in the hepatocytes to be secreted as chylomicrons and very low density lipoproteins (VLDL) respectively. These triglyceride-rich lipoproteins are transported in the blood to release FFA under the catalytic influence of lipoprotein lipase, chiefly at muscle and adipose tissue. The remnant particles of chylomicrons and VLDL return to the liver for redistribution within the cell as discussed above. A schematic representation of free fatty acid (FFA) is displayed below (Figure 2.3.1)

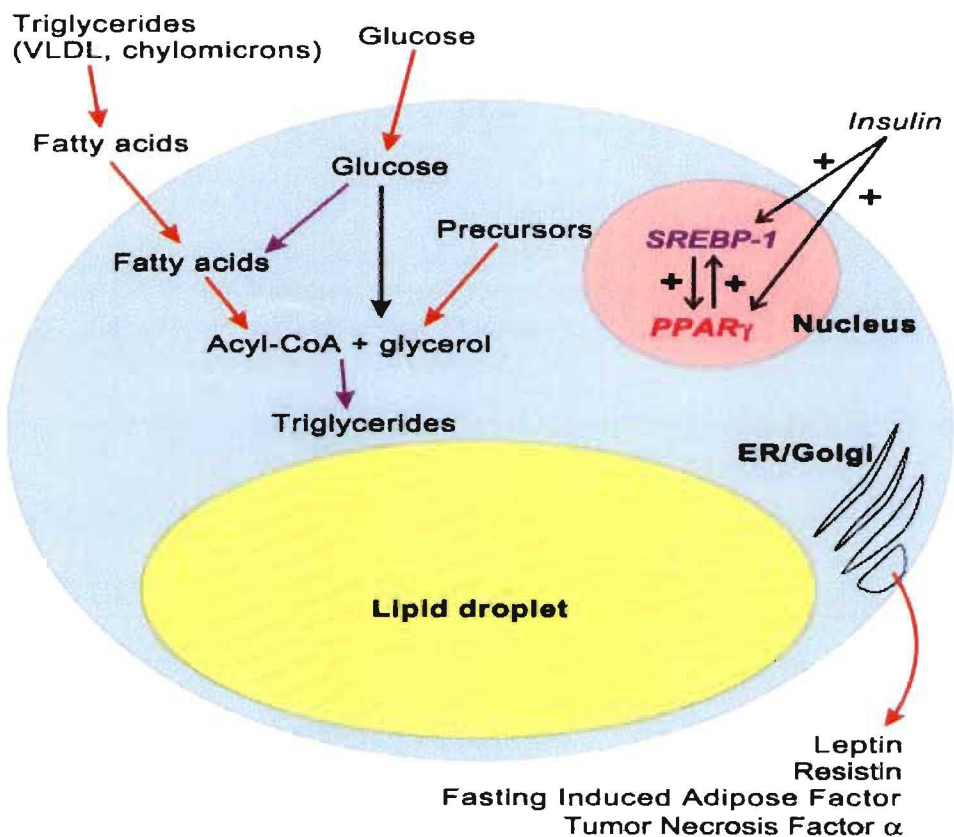


Figure 2.3.1; Diagram illustrating the interrelationship of fatty acid and glucose metabolism (Kersten, 2002).

The hepatocytes adjust to an excess influx of FFA through adaptive modifications whereby secretion of VLDL is increased and the flux of free fatty acids towards β -oxidation is increased. Under normal conditions only a small amount of TG is stored in the liver. Hepatic TG accumulation reflects a discrepancy in triglyceride synthesis and/or fatty acid disposal which may be a result of increased FFA production, reduced capacity of β -oxidation or impairment of VLDL secretion (Fong *et al.*, 2000). These factors all contribute to the production of oxidative stress, accumulation of hepatic fat and the pathogenesis of fatty liver. The consequence of this compensatory system becoming overloaded or being defective is an increase in TG content. The compensatory response and/or the increased TG may contribute to hepatic damage.

2.4 β -Oxidation of Fatty Acids

As mentioned earlier, β -oxidation is a fate of FA which occurs primarily in the mitochondrial inner membrane. Before FA arrive for β -oxidation, they are first transported through the cytoplasm in a combined complex with fatty acid binding proteins (FABP) which delivers the FA to the outer membrane of the mitochondria. Once they arrive at binding sites of the outer mitochondrial membrane, they are transformed into acetyl CoA and the converted derivatives of FA are transported from the outer membrane to inner membrane by carnitine palmitoyltransferase 1 (CPT1) and CPT2 respectively. The consequent β -oxidation of FA derived acetyl CoA generates a high energy yield and has proven to be a very useful pathway for an energy source cleaved from non-esterified FA.

The complexity of the development and progression of fatty liver in NAFLD and NASH remains incomplete but the involvement of β -oxidation is well documented. A very recent study carried out by Nakamura *et al.*, (2005) investigated the expression of various genes known to be involved in β -oxidation of fatty acid metabolism in NAFLD. Target genes included acetyl-CoA carboxylase (ACC) 1, ACC2, fatty acid synthase (FAS), carnitine palmitoyltransferase 1a (CPT1a) and long chain acyl-CoA dehydrogenase (LCAD). The study revealed an increased expression of ACC1 and ACC2 but not FAS using liver biopsies from 12 NAFLD patients. Further findings included a diminished expression of CPT1a which is a rate-limiting enzyme involved in β -oxidation. As a result, β -oxidation is reduced. This provides evidence that dysfunctional β -oxidation maybe partly why there is an accumulation of fat in hepatocytes of patients with NAFLD.

A great deal of attention has been focused on the role of FA in NAFLD and recent research undertaken by Drover *et al.*, (2005) was concentrated on CD36 which is known to be the gene responsible for mediating the transfer of FA across plasma membranes and is therefore regarded as having a very important role in regulating FA metabolism. Impairment of gene CD36 in mice was previously shown to result in irregular uptake of FA and in addition, circulating levels of triacylglycerol (TAG) were increased (Coburn *et al.*, 2000; Febbraio *et al.*, 1999). Conversely, over-expression of the CD36 gene has demonstrated a link to hypertriglyceridaemia, insulin resistance and elevated levels of FA in hypertensive rats (Aitman *et al.*, 1999). In humans, abnormal plasma lipid levels are also often associated with dysfunctional expression of the CD36 gene.

CD36 is referred to as a multi-ligand scavenger receptor and expression favours tissues with a high influx of free fatty acids (Coburn *et al.*, 2000). Drover *et al.*, (2005) investigated the role of CD36 using CD36-null mice and monitoring their responses to acute and chronic feeding in relation to FA utilization and secretion of lipoproteins. The study confirmed the necessity for the CD36 gene through comparing levels of circulating TAG between knockout CD36 mice and their respective wild-type littermates after high fat feeding. The levels of TAG recovered in the knockout mice were 23% which was significantly lower than the 67% of the controls. Furthermore, deficiency of CD36 also resulted in a reduced clearance of lipoproteins and defective lipoprotein lipolysis which enhances the case that an aberration in gene CD36 function could contribute to facilitating fatty liver development and progression.

2.5 Peroxisome proliferator activated receptors – (PPAR)

FFA is required by hepatocytes to maintain cell function. Because FFA supply may exceed the demand, protective pathways are required to prevent stress and liver injury. One mechanism by which the possible cytotoxic effects of FFA have been restrained involves the peroxisome proliferator activated receptors (PPARs). These hormone receptors have gained significant interest due to their orchestrative role of FFA metabolism and their activation in response to a variety of drugs used in treatment of obesity and T2DM (Kersten *et al.*, 2002; Bocher *et al.*, 2002).

Peroxisome proliferator activated receptors (PPARs) display a common structure comprising a central DNA-binding domain structure containing two zinc-fingers (for interaction with DNA) and a large C-terminal domain that binds ligands (Aranda and Pascual, 2001). PPARs are key ligand-activated transcription factors consisting of

isotypes α , β and γ and the different distinguishable forms each permit the liver cells to deal with alternate biological processes (Kersten *et al.*, 2002).

PPAR- α is responsible for regulating fatty acid oxidation, gluconeogenesis, amino acid metabolism and is predominantly operative in the liver (Kersten *et al.*, 2002). A group of ligands which enhances the binding affinity of these receptors is referred to as fibrates and have been in use for the treatment of hypertriglyceridaemia since the 1960s (Kersten *et al.*, 2002). Clofibrate and gemfibrozil are further lipid-lowering drugs that have had limited success in combating the complications of NASH. A further therapeutic approach has included the administration of atorvastatin which has resulted in significant improvements in serum lipid concentrations which correlated with less severe hepatic inflammation and ballooning (Horlander *et al.*, 2001).

PPAR- α is employed by the liver as sensor apparatus to detect elevated levels of FFA. When hepatocytes experience an influx of free fatty acids, PPAR- α relays signals for the expression of acyl CoA oxidase, a key enzyme required for inducement of β -oxidation. This presents a preventative measure against fatty acid cytotoxicity (Kersten *et al.*, 2002). However, despite the rewarding benefits of PPAR- α activation, continuous stimulation by FFA overload may generate ROS and participate in the role of carcinogenesis (Bocher *et al.*, 2002). The role of PPAR- α in regulating nutrient metabolism is shown below; (Figure 2.5.1).

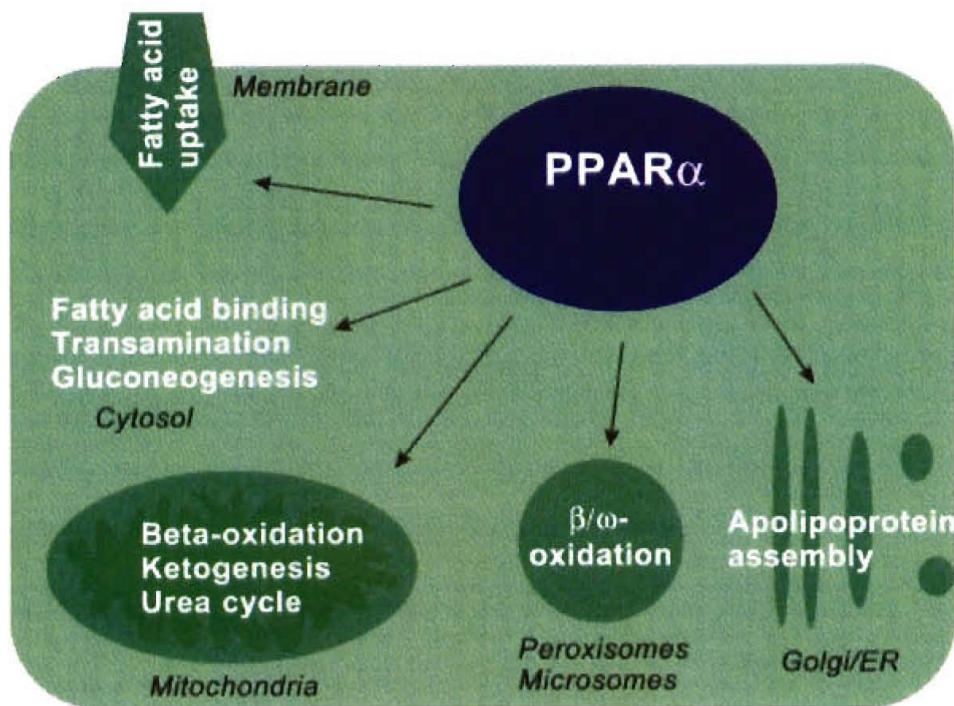


Figure 2.5.1; PPAR- α regulation of nutrient metabolism occurring in hepatocytes. The positive regulatory role PPAR- α in fatty acid metabolism and gluconeogenesis is displayed as well as the negative metabolism of amino acids (Kersten, 2002).

PPAR- α contributes to the control of lipid metabolism, the running of which is also altered by the presence of excess alcohol and products of the inflammatory response. Dysfunctional lipid metabolism may be central in ALD and in NASH as well, explaining the similarities of the two diseases (Kotesih and Diehl, 2002).

Nakajima *et al.*, (2004), investigated the role of PPAR- α in alcoholic liver disease using PPAR- α null mice fed a 4% ethanol diet and assessed for liver injury. The mice administered ethanol displayed hepatic irregularities that included inflammation, mitochondrial swelling and fibrosis, which corresponds with injuries found in alcoholics. Additionally, ethanol-fed PPAR- α null mice were subjected to higher levels of oxidative stress and had markedly increased lipid peroxides after 6 months in comparison to the controls. There was also a reduction of liver mRNA proteins encoding for growth factors (HGF- α , TGF- α and TGF β 1) involved in hepatic proliferation in wild-type mice whose PPAR- α expression was decreased by 60%. This implicates PPAR- α in the pathogenesis of fatty liver as its deficiency can result in altered signalling and stimulation of hepatic cell growth in the presence ethanol. It should strongly be considered that PPAR- α is an important target gene for the modulation of ALD, at least in rodents. PPAR- α is not expressed as highly in humans in comparison to rodents, resulting in the hypothesis that humans may possibly be

more vulnerable to the effects excess alcohol as PPAR- α appears to have a defensive role in ALD.

The knowledge of PPAR- β remains relatively small but previous studies have proposed a partial role in lipid metabolism of nerve cells (Basu-Modak *et al.*, 1999). PPAR- β may also be implicated in adipogenesis, suggesting a link to obesity (Bastie *et al.*, 2000), and hence fatty liver.

In contrast, PPAR- γ has been more extensively studied. It is located in plentiful quantities in white adipose tissue and macrophages (Braissant *et al.*, 1996), although the amounts expressed in the liver are relatively low in comparison (Nanji *et al.*, 2004). Owing to its role in adipocyte differentiation, PPAR- γ is regarded as having a strong connection with obesity (Kersten *et al.*, 2002). Mice that do not exhibit sufficient levels of PPAR- γ become fat intolerant, develop hyperlipidaemia and become insulin resistant (Clarke, 2001). This evidence is suggestive of PPAR- γ being a crucial regulator of triglyceride homeostasis and a potential contributor towards hepatic steatosis development (Gavrilova *et al.*, 2003).

Not only is PPAR- γ central in differentiation of preadipocytes into mature fat cells, a process called adipogenesis, but it is also believed to occupy a regulatory role in lipogenesis (Kubota *et al.*, 1999). PPAR- γ and sterol regulatory element-binding protein-1 (SREBP-1) are thought to be involved in a continuous loop whereby they possess the ability of upregulating each other, thus encouraging lipogenesis in adipose tissue (Kersten *et al.*, 2002). SREBP-1 is a transcription factor responsible for stimulating expression of various genes fundamental in lipogenesis such as acetyl-CoA carboxylase, fatty acid synthase and ATP-citrate lyase. A study carried out by Way *et al.*, (1999) used a PPAR- γ agonist known as GW1929 in Zucker fatty rats and displayed an increased expression of SREBP-1 and genes involved in fatty acid oxidation and confirmed PPAR- γ participation in lipogenesis although it is not yet understood whether it is direct or secondary to up-regulation of SREBP-1.

PPARs have been extensively examined in rodent models and recent work undertaken by Wargent *et al.*, (2005), has implicated the possible involvement of PPAR activation in response to conjugated linoleic acid (CLA). The findings indicated that CLA increased insulin sensitivity in *lep^{ob}/lep^{ob}* mice and that the subsequent activation of PPAR- α and γ may contribute to the improvement of insulin sensitivity in these genetically obese female mice. It is clearly evident that the action of PPARs is

central in controlling fat accumulation and other overlapping pathogenic mechanisms such as lipogenesis, β -oxidation and insulin sensitivity and should remain at the fore for future research into fatty liver development and other surrounding complications.

2.6 Cytochrome P450 Enzymes

The cytochrome P450 proteins (CYP450) are recognized as a group of closely related isoenzymes which are encoded by separate genes and classified according to their molecular structure (Gonzalez, 1990; Nebert *et al.*, 1989). These enzymes perform numerous functions and are located in most human tissue, although the liver has the highest content, in mitochondria and the endoplasmic reticulum. Induction of the CYP450 system is usually in response to drugs, including barbiturates, and toxins such as alcohol and tobacco smoke (Okey, 1989). Once induced, the P450 cytochromes increase the degradation of drugs and toxins. Relentless and unremitting induction may, however, lead to increased generation of oxidative stress and reactive oxygen species (ROS), thus providing an initiating step for liver injury (Lieber, 1997).

CYP2E1 is the most abundant enzyme in the P450 family and is central in the metabolism of alcohol. CYP2E1 is activated as an adaptive response to high blood ethanol levels and catalyses the following reaction (see below).



Although the induction of CYP2E1 accelerates the metabolism of ethanol, this reaction is also closely associated with the release of free radicals whose nature can facilitate lipid peroxidation, mitochondrial damage and oxidative stress which all contribute to liver damage (Lieber, 2004). Furthermore, acetaldehyde (ethanol) production is increased. Ethanal is highly reactive and toxic, adding to the cumulative pathogenic effects (Lieber *et al.*, 1986).

CYP2E1 activity is also exacerbated in response to nutritional states including fasting, diabetes and obesity. This explains the presence of increased CYP2E1 in NASH despite the lack of excess alcohol consumption (Robertson *et al.*, 2001). However, CYP2E1 is not the only factor contributing to the development of fatty liver as products of the inflammatory response and fibrogenesis also combine to the resultant oxidative stress. The many possibilities of CYP2E1 interactions are depicted below in figure 2.6.1 (Lieber, 2004).

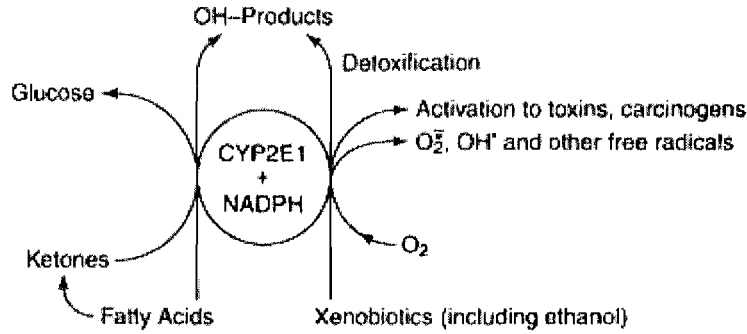


Figure 2.6.1; This diagram displays the many compounds which act as potential substrates for induction of cytochrome P450 CYP2E1 activity that results in detrimental and beneficial effects (Lieber, 2004).

CYP2E1 has been described as a “leaky” enzyme as it is capable of “futile cycling” whereby detrimental oxygen species and hydroxyl radicals are formed in the absence of substrates causing NADPH-dependent lipid peroxidation and cellular damage (Robertson *et al.*, 2001). It is believed that for NASH to occur and progress, additional oxidative stress is necessary as a ‘second-hit’ to establish a certain degree of lipid peroxidation that surmounts regular cell defences resulting in necroinflammation (Day and James, 1998). Common characteristics of NASH such as obesity and diabetes are regarded as mediators of CYP2E1 induction and therefore must be also regarded as ‘second-hit’ candidates capable of causing oxidative stress and hence hepatic damage.

IR is already established as a common characteristic in NAFLD and NASH patients. A further complication of IR is the ability to induce the operation of the cytochrome P450 system CYP2E1. This can also lead to the production of ROS as a result of intra-hepatic metabolic abnormalities which mimics the conditions experienced in alcoholic liver disease (ALD) (Albano *et al.*, 1996). A study performed by Weltman *et al.*, (1998) investigated the presence of hepatic CYP2E1 in a group of 31 patients with NASH that comprised 23% diabetes and 45% obese, both being aetiologic factors closely linked to relative insulin deficiency. The findings revealed that concentrations of CYP2E1 in patients with NASH was increased in comparison to the controls and also very similar to levels identified in patients with alcoholic steatohepatitis. Furthermore the acinar distribution of CYP2E1 seen in NASH patients closely resembles the distribution displayed in early alcoholic liver injury and coincides with the extent of steatosis through acinar zones 3 to 1 as with ALD.

A further source of induction of the CYP2E1 pathway is an elevated concentration of FFA which can occur via peroxisomal β -oxidation providing a supplementary source of oxidative stress. A reduced sensitivity to insulin can also lessen the suppression of HSC and thus cause adipocytes to increase FFA delivery to the hepatocytes, resulting in yet another potential source of liver cell damage (Sniderman *et al.*, 1998).

In addition to CYP2E1, a further P450 cytochrome subfamily, CYP4A, can serve as an alternative microsomal lipid oxidase for catalysing ethanol. The ROS and additional oxidative stress may thus come from other avenues of formation of superoxides and H_2O_2 (Robertson *et al.*, 2001).

Overall, hepatic steatosis is regarded as a benign condition which results when the hepatic triglyceride pool expands as a result of limiting oxidation or export capacity. The development of steatohepatitis requires an additional 'second-hit' in the form of oxidative stress from an array of hepatic pathways such as CYP2E1 that elicits the progressive nature of the disease, thus providing a framework for treatment and disease prevention (Day and James, 1998).

2.7 Leptin

Leptin resistance is yet another potential causative factor in the pathogenesis of the fatty liver diseases. Leptin is a 17 kilodalton protein product derived from adipocytes and is involved in several key biological functions that include the regulation of food intake and energy expenditure (Zang *et al.*, 1994). Furthermore, leptin is known to influence enzymes of fatty acid oxidation and control store levels of body fat thus permitting fluctuations in appetite and metabolism (Zhou *et al.*, 1997).

Leptin is genetically deficient in *ob/ob* mice. This strain of mouse exhibits several characteristics of humans with NAFLD such as obesity, diabetes and development of steatosis. It has become a valuable model for studying the disease mechanisms involved (Unger, 2000). A study by Campfield *et al.*, (1995) revealed that the administration of recombinant leptin suppressed the appetite and consequently reduced the food intake in *ob/ob* mice and had a protective effect. Conversely, when excess caloric intake does occur, leptin aids in stimulating the confinement of extra calories to adipocytes, therefore deflecting potential steatotic damage away from non-adipocytes and exhibiting a defensive role against obesity (Unger, 2002).

In spite of this, when steatotic liver damage is incurred by a NASH patient, dysfunctional leptin expression and signalling is a probable outcome. Leptin is responsible for aiding the regulation of several cytokine receptors but the balance of the leptin effects promotes the initiation of fibrosis. It is known that leptin stimulates the production of the potent profibrotic cytokine transforming growth factor- β 1 (TGF- β 1) (Bissell *et al.*, 2001). Additional proposed profibrotic effects of leptin are that the receptors may activate STAT-3 through ligand-binding, thus increasing collagen gene production and CYP2E1 expression is partly reliant on leptin activity and therefore has the ability to enhance oxidative stress by means of CYP2E1 induction (Saxena *et al.*, 2002; Enriquez *et al.*, 1999). Excess lipids or 'lipotoxicity' due to increased amounts of free fatty acids is also a very common occurrence in NASH and may be attributed to the dysfunctional production of leptin that aims to modulate insulin sensitivity to correct for insulin resistance (Chitturi *et al.*, 2002).

It is not only leptin that has been implicated as a contributing factor in fatty liver development but also leptin receptors. Zucker *fa/fa* rats are diabetic and have leptin resistance as a result of a substitution mutation in the leptin receptor (Ob-R) with an ensuing loss of function (Unger, 2002). After approximately 5 weeks of age, these rats exhibit elevation in FFA plasma levels as well as an accumulation of triglycerides in various organs. Furthermore, Unger (2000) showed that an intravenous injection of cDNA of wild-type OB-Rb consequently restores normal expression of leptin receptors in the *fa/fa* rat, resulting in a regression of hepatic triglyceride levels. Recent research by Cohen *et al.*, (2005) also examined the leptin receptor (Ob-R) and established that the liver unexpectedly contained Ob-R mRNA and therefore could augment circulating levels of leptin when negative energy balance is endured.

Numerous other studies have investigated the role of leptin in genetic rodent models of obesity such as leptin-deficient *ob/ob* mice and Zucker diabetic *fa/fa* rats. This will be discussed later in the NAFLD/NASH animal models chapter. However, it must be noted that increased leptin gene expression has been observed in humans in addition to leptin plasma concentrations and suggests the occurrence of obesity in humans may owe itself to dysfunctional leptin receptors as opposed to leptin itself (Hamilton *et al.*, 1995).

2.8 Type 2 Diabetes Mellitus

It is estimated that 250 million people distributed worldwide will be affected by type 2 diabetes mellitus within 15-20 years as a result of insulin resistance (O'Rahilly, 1997).

A recent advance in the prevention of diabetes and hepatic injury has examined the effects of dehydroepiandrosterone (DHEA) and its sulphate ester (DHEA-S) (Aoki *et al.*, 2003). These adrenal steroids circulate in great numbers in humans and have shown beneficial effects in the treatment of inflammation. The study performed by Aoki *et al.*, (2003) treated diabetic *db/db* mice with DHEA and resulted in reduced TNF- α concentration, inhibition of inflammatory cytokines, an increase in insulin sensitivity and consequently a diminished degree of hepatic injury. A one-year treatment programme with DHEA in post- menopausal women revealed a 30% restoration of insulin sensitivity, but a definitive role of DHEA remains unresolved (Ishizawa *et al.*, 2001).

2.9 Obesity

Although the progression of NAFLD can occur in the lean individual, the presence of obesity is almost a prerequisite. It is now estimated that nearly 1000 people a week are dying prematurely in the United Kingdom alone as a direct effect of obesity (Small and Bloom, 2005). Recent research has been directed at drug-inducing appetite suppression which leads to a lower food intake and the loss of weight. The ingestion of food stimulates the immediate release of numerous gut peptides such as glucagon-like peptide-1 (GLP-1) and -2 (GLP-2), cholecystinin (CCK) and peptide YY (PYY) (Baggio *et al.*, 2004). These peptides amongst others possess the ability to stimulate ascending neural pathways or directly enter the central nervous system (CNS) to signal the termination of food consumption (Korner and Leibel, 2003). They are therefore believed to act as sensitive modulators of feeding behaviour by communicating with CNS satiety centres which govern control of gut motility and glucose homeostasis (Baggio *et al.*, 2004).

Oxyntomodulin (OXM) is a natural digestive hormone consisting of a 37-amino acid peptide which contains the entire sequence of glucagon and is cosecreted with GLP-1 (Dakin *et al.*, 2001). GLP-1 is known to lower blood glucose and to reduce food intake in obese diabetic patients (Drucker, 2002; Zander *et al.*, 2002) whereas GLP-2 is implicated in the inhibition of gastric motility and acid secretion (Drucker, 2001). Less is understood about OXM. Investigative work has examined this peptide and

highlighted a strong association with gastric emptying and stimulation of intestinal glucose uptake in rodents (Jarrousse *et al.*, 1993; Collie *et al.*, 1997). Furthermore, OXM has also displayed capabilities of inhibiting food intake and lowering weight gain in rats and more recently in humans (Dakin *et al.*, 2002; Cohen *et al.*, 2003).

The latest development in oxyntomodulin research is a 4-week trial (Bloom – Argus, 2005), conducted in 26 overweight or obese patients. They received three injections a day, each administered 30 minutes before eating. The results revealed an average weight loss of 2.3 kg from treatment with OXM in comparison to a placebo group which lost approximately 0.5 kg during the same period.

The exact mechanisms by which OXM inhibits food intake in rodents and humans is unclear but it is hoped that the drug will be available on the market as a therapeutic approach for obesity within the next 5 years. Despite OXM being primarily developed as a means of weight reduction, the drug could also have a significant impact in halting the progression of NAFLD.

Rimonabant is an anti-obesity drug which is under current investigation as it is believed to increase satiety and promote weight reduction (Bays, 2004). Cannabinoid (CB) receptors are the molecular targets for the active ingredient in cannabis; Δ^9 -tetrahydrocannabinol, which are triggered by a small family of endogenous lipids (Giuffrida *et al.*, 2001). Cannabis smokers may experience an enhanced appetite when this reaction occurs which has led to rimonabant being examined. Rimonabant is a CB antagonist and functions by blocking the CB-1 receptor (Bays, 2004). It is therefore hypothesised, that inhibition of the receptor responsible for regulating appetite would reduce caloric intake and consequently lower body mass. Rimonabant, at present, is being studied in clinical trials of over 6000 patients, including those with type II diabetes (SPG Media Limited). Initial findings are encouraging, revealing advantageous effects on lipids such as triglycerides and high-density lipoprotein-cholesterol levels (Anthenelli and Despres, 2004).

The epidemic of obesity is still relatively new, but despite public health education highlighting the surrounding complications, it is estimated that over 1 billion adults worldwide are overweight (Bays, 2004). Studies in weight-reducing drugs like rimonabant are still in early development and it remains unclear whether a single treatment target or a multiple of treatment targets provides the most effective

approach. Further research is required to find a suitable treatment that will effectively modulate fatty liver by limiting or preventing obesity.

2.10 Hepatic Fibrosis

Fibrosis is not always present in NAFLD patients but is regarded as the key pathological feature of liver damage in NASH. The fibrotic process in steatohepatitis is believed to be nearly identical to other modes of chronic liver damage such as ALD (Friedman, 2000). The common trait of fibrosis in steatohepatitis is often referred to as pericellular 'chicken-wire' fibrosis due to its histological appearance within hepatocytes and is initially located in acinar zone 3 as with ALD (Brunt and Tiniakos, 2002). The progression to fibrosis has been attributed to various underlying factors including oxidative stress, lipid peroxidation, insulin resistance, and upregulation of cytokines which are all potentially able to be the 'second-hit' after the metabolic stress. The adaptive responses of the liver to further injury help explain the underlying pathogenesis of NASH and contribute to the knowledge of fibrogenic mechanisms. Although the risk factors for fibrosis development and progression in NASH remain unclear, the morphological changes that occur are clearly defined and are outlined below (Figure 2.10.1).

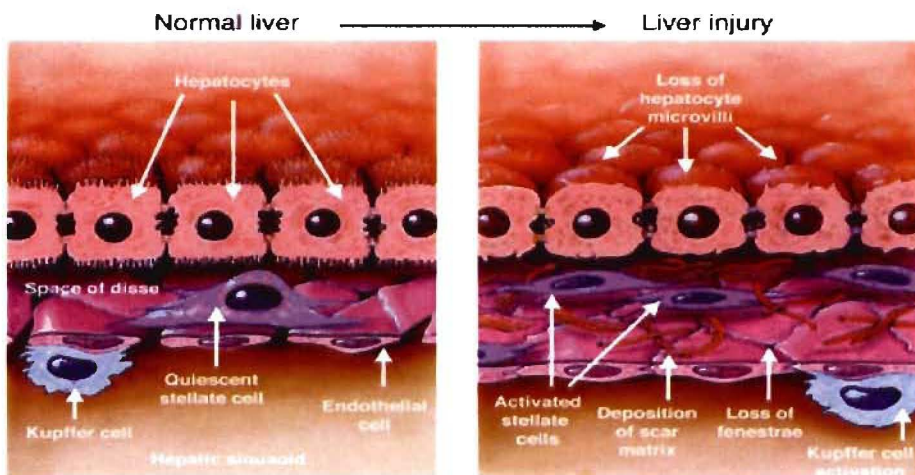


Figure 2.10.1; Changes in cellular structure during fibrotic liver injury; In a normal functioning liver, the hepatocytes possess microvilli and endothelial fenestrae are still present. During liver damage, HSC proliferate and are consequently surrounded by fibrillar matrix whilst hepatocyte microvilli and endothelial fenestrae are lost resulting in fibrosis (Friedman, 2003).

Fibrosis is generally regarded as a defensive repair mechanism in response to cell injury. Fibrogenesis mechanisms can be activated without cell death or inflammation. The trademark characteristic of hepatic fibrosis is the deposition of extracellular matrix (ECM) proteins within the space of Disse (Geerts, 2001). In the normal functioning liver, the ECM provides support for cellular structure, permits growth-

factor transport and is also relied upon for transmitting signals to surrounding cells in order to maintain their differentiated functions (Friedman, 2003). Conversely, an injured liver exhibits scarred tissue with amassing ECM which encompasses macromolecules from three main families: collagens, glycoproteins and proteoglycans. As the liver develops fibrosis, changes in the arrangement of the ECM occur due to an influx of fibril forming collagens (types I and III), fibronectin and laminin (Friedman, 2000; Shuppan *et al.*, 2001). Furthermore, the expression of matrix metalloproteinases (MMP) and their inhibitors appear to be in an imbalance, causing disorder in hepatic compartments because excessive matrix deposition is not being compensated by increased degradation (Friedman, 2000; Shuppan *et al.*, 2001).

Hepatic stellate cells (HSC), among other major cell types including Kupffer cells and endothelial cells are activated in the fibrotic liver. HSC in particular have been viewed as a causative factor for enhancing ECM production during fibrogenesis (Eng *et al.*, 2000). HSC are present in both normal and damaged livers, and can be distinguished by their vitamin A content and location. HSC are located beneath endothelial cells in the space of Disse and in normal functioning liver they connect with other stellate cells via gap junctions (Eng *et al.*, 2000). However, during injury, HSC are converted into myofibroblast-like cells that now contain little vitamin A and facilitate collagen assemblage (Friedman, 2000; Eng *et al.*, 2000). The 'activation' of HSC at the onset of injury is therefore regarded as being a central event in the detrimental progressive nature of hepatic fibrosis which entails the conversion of quiescent vitamin-A storing cells into proliferative, fibrogenic and contractile myofibroblasts (Friedman, 2003) (see figure 2.10.2).

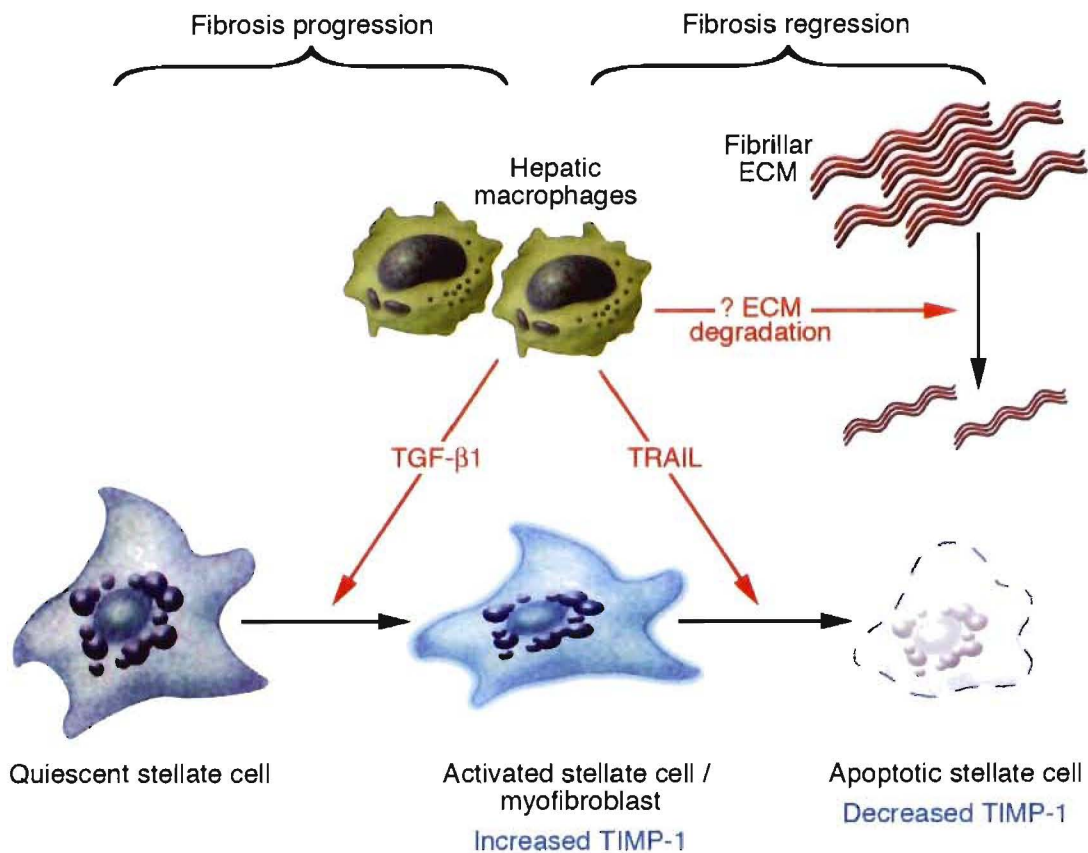


Figure 2.10.2; HSC activation is a response to liver injury by a two-step process of initiation and perpetuation. Resolution of fibrosis may occur by reduction of HSC number by apoptosis and reversal of activated HSC to quiescent phenotype (Friedman, 2003).

The potential disadvantage arising from HSC activation include alterations in ECM components, and the loss of vitamin A. The cells enlarge and continued injury will exacerbate the fibrosis with activation being propagated by increased expression of cytokines such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor- β 1 (TGF- β 1) (Maher, 2000). Transcription factors that include nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1) and signal transducer and activator of transcription-1 (STAT-1) also play a supporting role in activating HSC which coincides in synchrony with accumulation of ECM, remodelling of the matrix and modification of fibrotic activity.

Studies which are primarily concerned with the fibrotic response in NASH patients are limited. A study undertaken by Baldrige *et al.*, (1995) discovered that 14 children with NASH all displayed signs of portal fibrosis and postulated that fibrosis may be more prominent in children than adults. Additional research has also proposed that

old age, obesity, female gender and diabetes mellitus are further independent determinants of fibrosis (Wanless *et al.*, 1990; Angula *et al.*, 1999).

A clear, singular, causative factor for generating fibrotic stimuli in NAFLD and NASH remains elusive. Several inter-related discrepancies in lipid metabolism, inflammatory response, resistance to insulin or a combination play a role. Further work must be aimed at gaining a better understanding of the processes involved in a liver becoming fibrotic in order to channel resources towards identifying possible therapeutic targets for treatment purposes of these at highest risk for serious complications of fatty liver, especially cirrhosis.

2.11 Cirrhosis

Although liver cirrhosis is not evident in every patient with progressive fatty liver disease, clinical studies reveal that approximately 20% of patients with NASH proceed in developing a cirrhotic liver (Diehl *et al.*, 1988; Matteoni *et al.*, 1999). Whether a patient is developing a cirrhotic liver or not is difficult to determine clinically and biochemically and has resulted in the endorsed use of needle biopsy as the 'gold standard' for correct diagnosis (Brunt, 2001). When cirrhosis is fully established in the liver, the regular lobular structure is completely lost and is replaced by regenerative nodules that vary significantly in size, and is bounded by bands of fibrous tissue (Diehl *et al.*, 1998). Cirrhosis can be classified as micronodular, macronodular or more commonly a combination of both. Micronodular cirrhosis consists of small uniform nodules and is frequently associated with alcoholics. It often results in yellow coloration of the liver due to severe fatty change. Macronodular cirrhosis is often revealed after cessation of heavy drinking and comprises large protuberant nodules separated by fibrous bands.

The rate at which cirrhosis proceeds is dependent on the extent of injury incurred by the individual and factors that trigger cell-death, fibrosis and regeneration. A toxin such as alcohol will effect concentration-dependent cell death and damage. Cirrhosis greatly enhances the likelihood of developing hepatocellular carcinoma (HCC), cirrhosis may also result in the loss of liver function and often portal hypertension arises due to the increased resistance to flow. Research carried out long ago by Heston and Vlhakis, (1962) indicated that ageing mice develop hepatic tumours but it remains unclear whether the occurrence of hepatocellular carcinoma is a genuine event in the advancement of NASH. Increases in oxidative stress and lipid peroxidation have been reported in numerous studies involving NASH patients

(Weltman *et al.*, 1996; Esterbauer *et al.*, 1992) suggesting a possible link to arising hepatocarcinogenesis.

A study conducted by Seki *et al.*, (2002) investigated the hepatic expression of 4-hydroxy-2'-nonenal (HNE) and 8-hydroxydeoxyguanosine (8-OHdG) as HNE is a reliable marker of lipid peroxidation and 8-OHdG is regarded as a target of free radicals in cellular DNA that demonstrates signs of oxidative DNA damage. The findings revealed that expression of HNE and 8-OHdG were markedly increased in NAFLD patients in correlation with the stage of fibrosis when compared to patients with normal liver. HNE is a strong chemical attractant for neutrophils and oxidative stress-induced NF- κ B activation (Schaur *et al.*, 1994; Jaeschke *et al.*, 1996). It may therefore contribute towards initiating the activation of HSC which are a primary source of ECM proteins central to the progression of fibrogenesis (Friedman, 1993).

In addition, the accumulation of 8-OHdG in cellular DNA of NAFLD patients resulted in mispairing and provides evidence that this oxidative modification is mutagenic and carcinogenic (Kasai *et al.*, 1991). The probability that oxidative DNA damage or increased levels of oxidative stress is involved in developing HCC is high, but more studies need to be undertaken to confirm the hypothesis. Further mechanisms which have been proposed for facilitating the development of hepatic carcinogenesis include alterations in fatty acid metabolism (Ockner *et al.*, 1993), activation of PPAR- α (Gonzalez *et al.*, 1998), insulin stimulation of TNF- α (Bertrand *et al.*, 1998) and overexpression of insulin growth factor-2 (IGF-2) (Rogler *et al.*, 1994). Other tumour-forming pathways exist but whether cirrhosis is a prerequisite for HCC to transpire remains ambiguous.

The pathogenesis of NAFLD remains unclear despite the advances which have been made in understanding the progressive nature of the disease and the complexity of the process. The 'two-hit' model described by Day and James (1998) summarises the process of events that occurs in this multifactorial disease. The 'first-hit' results in the development of steatosis as a consequence of several metabolic features that include insulin resistance and altered lipid metabolism leading to an increase in esterified fatty acid in the liver. The 'second-hit' occurs as a follow-up to the development of steatosis whereby the liver is more susceptible to disease through confrontation with detrimental factors such as increased lipid peroxidation, P450 CYP activity, insulin, leptin and the inflammatory infiltrate which all significantly contribute to the escalation of the disease.

The important causative features of NAFLD and NASH which facilitate the development and progression of the disease are predominantly researched in rodent models that lends itself to specific manipulation action and the evaluation of the liver biochemically, genetically and histologically. Work undertaken in this thesis aims at investigating whether the (*db/db*) strain is suitable for additional research and describes some biochemical changes along with the histology of the liver.

2.12 Similarities with Alcoholic Liver Disease (ALD)

The occurrence of NAFLD and the subsequent progression from simple fatty liver to fibrotic liver and cirrhosis is similar to the pathogenesis arising in alcoholic liver disease (ALD). A major difference between the two disorders is that NAFLD patients do not consume what is considered to be an excess amount of alcohol whereas the consumption of the ALD patients is significant to cause an effect which results in the same extent of liver injury. The proposed doses of alcohol required to facilitate ALD is 40-80 g/day for males and 20-40 g/day for females over a 10-12 year period (Becker *et al.*, 1996; Grant *et al.*, 1988). However, Ludwig *et al.*, (1980) carried out a study and found patients who denied any alcohol intake or averaged one drink per week still exhibited comparable liver damage as revealed in ALD patients. This contrasting data suggests that a safe alcohol intake limit cannot really be applied in forecasting whether an individual develops fatty liver as some people are more sensitive to the effects, although a low intake coupled with a healthy diet and exercise would certainly decrease the possibility.

Potential pathways by which fatty liver can develop and progress include fatty acid oxidation, increased ROS production by the cytochrome P450 system and effects of IR on oxidative stress (Haque and Sanyal, 2002). The fact that complete abstinence from alcohol does not guarantee avoidance of fatty liver development suggests that certain biochemical pathways involved in metabolizing alcohol and generating fatty liver in ALD must also be operational in NAFLD.

The metabolism of alcohol primarily occurs by alcohol dehydrogenases (ADH), although CYP2E1 and catalases are also regarded as having important roles (Arteel *et al.*, 2003). CYP2E1 is believed to be of significant importance as it is localized in hepatic lobules when liver injury is endured from excess alcohol intake. In addition, CYP2E1 induction has the potential to be a source of oxidative stress but the mechanisms involved remain unclear (Arteel *et al.*, 2003). Possible mechanisms by

which alcohol consumption may lead to liver injury are depicted below (Figure 2.12.1).

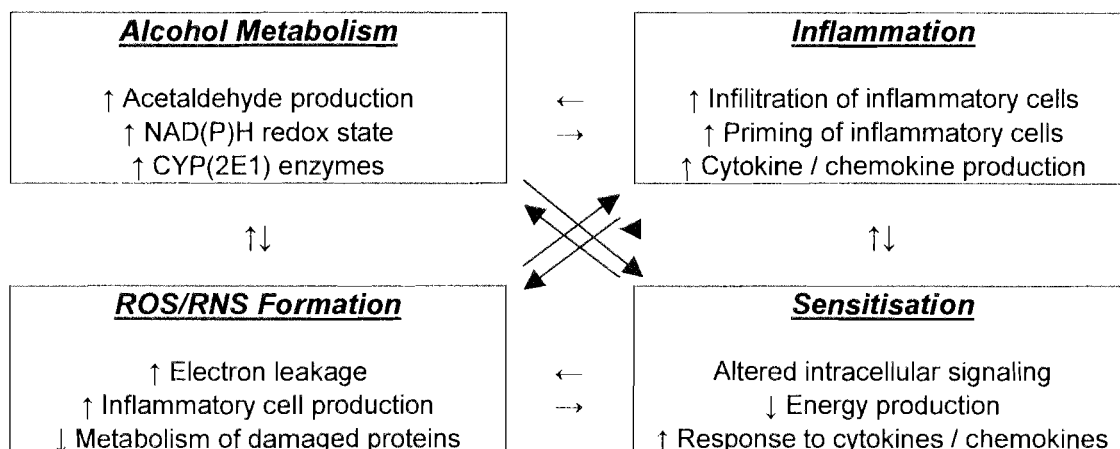


Figure 2.12.1; Mechanisms by which ethanol consumption may lead to liver damage. Ethanol causes a number of physiological and biochemical changes to liver that may lead to initiation and progression of alcoholic liver disease (ALD). These include the following; alcohol metabolism, via the formation of toxic byproducts (e.g. CYP2E1). Inflammation coupled with priming of these immune cells and an increase in proinflammatory cytokines and chemokines. Sensitisation of hepatic cells, via alteration of intracellular signaling, depletion of energy reserves and an increased response to external stimuli (e.g. cytokines/chemokines) alters/enhances the response of the cells during the course of alcohol administration. Reactive oxygen species / reactive nitrogen species (ROS/RNS) formation, via electron leakage from enzyme systems (e.g. mitochondria and CYP2E1), inflammatory cells and by decreased degradation of oxidatively-modified proteins is proposed to play a key role in mediating the effects of ethanol. These events are not mutually exclusive and tend to enhance the effects of the other pathways. The net result is a vicious cycle of damage and progression in the disease. Polymorphisms in key genes involved in these events are also thought to cause the sensitivity of some individuals to chronic alcohol abuse (Arteel *et al.*, 2003).

There are several animal models that are employed to study the effects of alcohol such as the Sardinian alcohol-preferring (sP) rat line which has been purposely bred for over 20 years for high alcohol consumption (Colombo *et al.*, 1997) and the Indiana University alcohol-preferring (P) and high-alcohol-drinking (HAD) lines (Li *et al.*, 1993). However research undertaken in these model poses limitation problems as sucrose must be added to concentrations (>6%) to create a 'sweet taste' otherwise the rodents will behave adversely towards the solution. This sugar supplement consequently decreases the rate of alcohol intake.

Further problems arising from working with rodent models is creating an accurate representation of the conditions that humans endure in developing fatty liver through excess alcohol consumption. The majority of people whose alcohol intake is in excess of 60 g/day will develop fatty liver whilst those who have a severe mixed micro/macrovesicular pattern are more likely to develop fibrosis and resultantly cirrhosis (Teli *et al.*, 1995). However, due to the difference in body mass between humans and rodents, it is difficult to gauge a concentration of alcohol that would

correspond accurately. A study undertaken by Yin *et al.*, (1999) subjected wild-type TNF-R1 knockout mice to ethanol concentrations of $28 \text{ g/kg}^{-1}/\text{day}^{-1}$ to induce fatty liver. The treatment lasted 4 weeks and also included an acclimatization period in which the initial dose was $16 \text{ g/kg}^{-1}/\text{day}^{-1}$ and incremented until the desired dosage was reached. It takes approximately 10-12 years of alcohol abuse to develop fatty liver in humans (Becker *et al.*, 1996; Grant *et al.*, 1988) whereas the wild-type TNF-R1 mice showed significant steatosis and inflammation after only 4 weeks. Additionally, many rodent models do not live beyond 2 years and further confirms the difficulty in trying to bridge the gap between rodent and human data. Research in animal models remains the best option to successfully manage and possibly eliminate the conditions arising in ALD and similarly in NAFLD. Additional work should possibly be aimed at determining the overlapping pathways involved in provoking a fatty liver in ALD and NAFLD and therefore establish why this type of liver injury occurs in NAFLD patients despite alcohol not being an applicable factor.

Chapter 3; Animal Models of NASH and NAFLD

3.1 Introduction

This chapter illustrates the extensive assortment of rodent models that are employed to investigate the pathogenesis and surrounding complications of NAFLD and NASH. From hepatic steatosis being originally regarded as a benign condition, there are now several animal models which have been specifically modified to induce key features considered to be risk factors in development and progression of fatty liver. NAFLD is an asymptomatic disease and detection by liver biopsy remains an invasive, yet the most effective technique by which correct diagnosis can be reached. Obesity, insulin resistance, type 2 diabetes mellitus and dyslipidaemia are almost a prerequisite for facilitation of the disease in human patients (Koteish and Diehl, 2002) and it is these characteristics which are purposely activated in rodent models to replicate the conditions observed in a 'typical' human profile.

The findings generated from animal models of steatosis and steatohepatitis have been invaluable and greatly improved our understanding of the pathogenesis of NAFLD and NASH. Additional studies in animal models will hopefully identify a causative factor of these disorders, therefore allowing successful treatment and even amelioration of the disease. Although, care must be taken when interpreting the results as no animal model to date has been shown to exactly mirror the biochemical and metabolic pathways known to exist in humans with fatty liver.

3.2 (Ob/ob) Mouse Model

A number of animal models has been used in recent years to help advance the understanding of NAFLD. The *ob/ob* mouse has been widely studied as this particular model of genetic leptin deficiency owing to a mutation in the *ob* gene, manifests key traits seen in the human clinical profile of NAFLD. These characteristics include obesity, hyperinsulinaemia, insulin resistance, type II diabetes and hepatic steatosis (Lin *et al.*, 2000).

Many different approaches have been employed to combat the development and progression of NAFLD in the *ob/ob* mouse model with no real success. One study investigated aminosterol 1436, which is a spermine-cholesterol metabolite originally isolated from dog shark and has been found to repress appetite and resultantly reduce weight (Ahima *et al.*, 2002). The action of 1436 suppresses SREBP1 and its target genes (GPAT and SCD-1), which are involved in hepatic lipid metabolism, and subsequently ameliorates the development of fatty liver (Yahagi *et al.*, 2002). A

study undertaken by Takahashi *et al.*, (2004), examined the effects of 1436, in an attempt to decrease body weight and resultantly defuse surrounding complications in Lep^{ob/ob} mice.

Lep^{ob/ob} mice treated with aminosterol 1436 displayed significantly reduced daily food intake and consequent weight loss in comparison to the non-treated animals. Triglyceride, cholesterol and ALT concentrations were also significantly reduced in association with decreased insulin levels. Although further studies are required to establish if 1436 can also reverse other abnormalities of NAFLD such as necrosis and fibrosis, aminosterol 1436 still serves as an effective treatment for insulin resistance and hepatic steatosis by decreasing hepatic lipid synthesis.

Another approach used to examine the abnormalities of NAFLD was to investigate the formation of reactive oxygen species (ROS). ROS are produced in liver mitochondria and there are multiple mechanisms that can promote their generation in NAFLD. The production of ROS may be a result of increased fatty acid oxidation, elevated cytochrome P450 activity or a metabolic consequence of insulin resistance to name a few, all of which typically occur in a NAFLD patient (Haque *et al.*, 2002).

Research performed by Yang *et al.*, (1999) documented an increased expression of mitochondrial uncoupling protein-2 (UCP-2) in fat laden hepatocytes of ob/ob mice. The raised activity of UCP-2 is believed to increase the risk of collapse of membrane structure by partially depolarising the inner mitochondrial cell walls and may explain the vulnerability of fatty hepatocytes to necrosis.

A further study carried out by Yang *et al.*, (2000) used the ob/ob model again to establish whether increased production of ROS and the resultant oxidative stress contributed to the development of fatty hepatocytes and promotion of necrosis through mediation of UCP-2. The study demonstrated a significant increase in the release of H₂O₂ from mitochondria of fatty liver and confirms an increased production of reactive oxygen species compared to the controls. In addition, the mitochondria from fatty livers displayed an elevated rate of O₂ consumption or production which conforms with the idea that mitochondria from ob/ob mice may contain more UCP-2 when compared to the lean wild-type littermates. The increase of UCP-2 and ROS may or may not directly result in necrosis, but in concert with several dispositional factors within fatty livers may promote necrosis that does not occur in normal livers.

Another strategy for identifying the underlying factors involved in the pathogenesis of NAFLD proposes that impaired regeneration in fatty liver may promote progression of the disease. Additional work undertaken by Yang *et al.*, (2001) was aimed at recognizing the altered mechanisms which may contribute to impaired regenerative responses of a fatty liver in NAFLD. Obese (*ob/ob*) mice and normal wild-type controls were subjected to a 70% partial hepatectomy (PH), after which the animals were allowed to recover. The regenerative responses of both groups of animals were recorded. Within the first 36 hours after the PH procedure 7/25 *ob/ob* mice with fatty livers died in comparison to 0/26 lean controls, instantly suggesting an abnormality in the hepatic compensation and/or regeneration ability of obese mice. Further findings revealed substantial decreases of hepatocyte proliferation in surviving *ob/ob* mice by monitoring induction of proliferating cell nuclear antigen (PCNA), the action of which is typically increased as hepatocytes leave the pre-replicative phase of the cell cycle (Michalopoulos *et al.*, 1997). Oxidant-sensitive, growth regulatory protein kinase cascade systems are believed to help regulate hepatocyte proliferation in response to liver injury. Jun N-terminal kinases (Jnks) and extracellular regulated kinases (Erks) are two such systems which are normally activated by cytokines and growth factors (Westwick *et al.*, 1995). However, signalling pathways become altered in fatty livers as an outcome of sustained oxidative stress and result in abnormal function of these systems, hence the poor proliferative response to liver damage in obese animals.

Although a single causative factor of NAFLD remains unidentified, the observations from the study confirmed that fatty livers of *ob/ob* mice had less efficient regenerative responses when compared to normal livers of wild type controls. Zucker *fa/fa* rats have also been shown to display inhibited liver regenerative responses (Selzner *et al.*, 2000) adding further speculation that impaired regeneration may assist in the progressive nature of the disease by inhibiting hepatic proliferation and increasing liver injury.

Obesity and diabetes are fully established risk factors implicated in the development and progression of NAFLD. The exact mechanisms involved in the disease process remain unclear, but as these components appear to be central in amplification of NAFLD, their roles are becoming more scrutinized in the search for a true cause and a possible treatment. Glucocorticoids have been closely examined of late as they are believed to play a significant role in the manifestation of obesity and insulin

resistance and may therefore contribute to the materialisation of the adverse outcomes of the disease (Bjorntop *et al.*, 2000).

A study performed by Liu *et al.*, (2003) attempted to determine the role of glucocorticoids through measuring the activity of 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1). The synthesis of corticosterone is predominantly regulated by liver-specific 11 β -HSD1 and is an essential reaction in facilitating further generation of active glucocorticoids (Voice *et al.*, 1996). Female ob/ob mice were treated with leptin and compared to non-treated ob/ob animals. Fatty livers of leptin-treated mice revealed significantly elevated 11 β -HSD1 activity and increased levels of corticosterone. In addition, body weight was significantly reduced (27.6%) when compared to the untreated obese mice. However, despite the leptin-linked improvements, 11 β -HSD1 activity, corticosterone levels and body weight never returned to normal levels as indicated by the lean littermate controls. The findings implicate increased insulin sensitivity in response to leptin treatment and that the obese nature of ob/ob animals is associated with the impaired activity of 11 β -HSD1. The altered hepatic activity of 11 β -HSD1 in fatty liver may therefore become a very useful tool in understanding the development of obesity and diabetes.

Clearly, much time and resources have been directed at unravelling the pathogenesis of fatty liver of the *ob/ob* mouse model. The characteristics of this particular model closely resemble the conditions seen in humans with NAFLD and it is therefore hoped that a better understanding of ob/ob mice will serve as guide for treatment and the *ob/ob* model remains a fundamental component for future research.

3.3 Tumour Necrosis Factor- α (TNF- α) Knockout Mouse Model and Human Studies of TNF- α

Increased levels of TNF- α are already well-documented to mediate liver damage (Diehl, 2000). Patients suffering from NAFLD and other complications of fatty liver continuously display elevated TNF- α in plasma (Winkler *et al.*, 1998), hence the great interest surrounding this cytokine. A study performed by Ji *et al.*, (2004) investigated the role of TNF- α in ethanol-induced liver injury using TNFR1 knockout mice. TNF- α is judged to permit the incidence of steatosis occurring in part by impeding normal insulin signalling pathways, thus acting with proinflammatory effects (Crespo *et al.*, 2001). The mice lacking the receptor for TNF- α expression displayed minor

reductions in triglyceride levels and grading of steatosis. Furthermore, ALT and necroinflammatory foci were lowered by 50% in comparison but no significant changes were noted in endoplasmic reticulum (ER) stress. These observations implicate the involvement of TNF- α in ethanol-induced liver damage.

Yin *et al.*, (1999) also used TNFR1 knockout mice to establish a role of TNF- α in alcohol-induced liver injury. The mice were exposed to a high-fat ethanol diet for 4 weeks to induce fatty livers before being subsequently terminated. The study showed liver/body weight ratios to be affected by ethanol feeding in comparison to the control diet, as expected. But this finding was not replicated when comparing TNFR1 animals. However, assessment of steatosis, inflammation, necrosis and ALT levels after ethanol feeding revealed all parameters to be significantly increased in the presence of TNF- α . The biochemical and pathological differences arising from evaluation of TNFR1 knockout mice and those capable of expressing TNF- α confirm that TNF- α plays a role in alcohol-induced liver injury.

There is further speculation that TNF- α acts as a second hit in the already discussed "two-hit" hypothesis (Day, 2002). TNF- α is accepted as a 'second-hit' as it is one of the predominant cytokines produced in response to increased reactive oxygen species (ROS) (Yang *et al.*, 2000) and has a major influence in progressive NAFLD. One method by which TNF- α is believed to promote hepatic oxidative stress is through inhibitor kappa beta kinase beta (IKK β) (Yuan *et al.*, 2001). IKK β is partly responsible for the inactivation of nuclear factor kappa beta (NF- κ B) which initiates the transcription of TNF- α . However, because TNF- α is also responsible for stimulation of IKK β , a positive feedback loop is generated that results in insulin resistance, further production of TNF- α and a chronic inflammatory state (Yuan *et al.*, 2001).

Work undertaken by Hui *et al.*, examined TNF- α activity in NASH and NAFLD patients and attempted to establish markers for differentiating histology. There were 109 subjects with varying degrees of liver damage in the study, standardised in order to eliminate confounding factors of age, sex and body weight. Hypoadiponectinemia and TNF- α activation were associated with NASH which was coupled with higher grades of steatosis and necroinflammation. Adiponectin has been shown to inhibit the activity of NF- κ B (Ouchi *et al.*, 2000) which consequently reduced the self-reinforcing flow of TNF- α and effectively lowered hepatic inflammation. Thus

adiponectin may have a potential therapeutic application for alcohol and nonalcohol liver disease.

As illustrated above, TNF- α is implicated in NAFLD and alcohol induced fatty liver damage and the histopathological similarities which exist between the different diseases reinforces the idea that mutual pathways must be involved (Ludwig *et al.*, 1980).

3.4 Sprague-Dawley Rat Model

A study carried out by Lieber (2004) fed male Sprague-Dawley rats a high-fat, liquid diet in an attempt to produce a suitable experimental model which replicates all the key characteristics seen in human patients with NASH. The high-fat diet consisted of 71% of fat-derived energy compared to 35% in the standard diet fed to the control animals. Both diets were fed *ad libitum*.

After 3 weeks, panlobular steatosis and inflammation were evident in the high-fat diet fed rats. This was coupled with elevated plasma lipid concentrations and increased levels of hepatic TNF- α in comparison to the control group. Furthermore, increased plasma insulin was indicative of insulin resistance and CYP2E1 induction was also documented to add to oxidative stress.

However, the rats fed the high-fat diet did not become obese and comparison of the mean body weights against the standard diet fed animals revealed no statistical significance. Restriction of the high-fat diet substantially reduced the hepatic changes observed in these animals, but the undesirable development of mild steatosis still occurred. This provides evidence that restricted, food components can play a strong role in deranging liver function.

Despite the increasing knowledge of NASH and associated liver diseases, an exact model remains elusive as all models to date lack one or more pathogenic factors. The subsequent non-development of obesity in Sprague Dawley rats raises doubt on the suitability of this model for studying NASH, although targeting food components is still regarded as a possible strategy for studying the disease process and modulation of its outcome.

3.5 Peroxisome Proliferator-Activated Receptors (PPAR) Mouse Model

PPARs comprise a superfamily of nuclear hormone receptors which are capable of facilitating the effects of fatty acids and oxidised phospholipids on DNA (Kersten,

2002). Three subtypes of PPARs exist (denoted α , β and γ) and each has a distinct tissue distribution pattern (Braissant *et al.*, 1996). PPAR- α is mainly present in liver, PPAR- β is ubiquitously expressed and PPAR- γ is predominantly located in adipose tissue (Memon *et al.*, 2000).

PPAR- α is believed to be central in the homeostasis of lipid metabolism and is thus likely to participate in the pathogenesis of fatty liver (Desvergne *et al.*, 2004). The altered expression or function of PPAR- α may result in abnormal lipid accumulation and ROS which are key mediators of fatty liver disease. Cytochromes CYP2E1 and CYP4A are possible hepatic sources of ROS (Weltman *et al.*, 1996) as their crucial role in fatty acid production pathways may potentiate the development of steatohepatitis if PPAR- α regulation becomes augmented.

Ip *et al.*, (2003) investigated the role of PPAR- α activation by analysing ROS and lipid peroxidation parameters. Fatty livers were induced by administration of the MCD diet for 5 weeks in PPAR- α deficient mice and compared against animals fed a control diet. Wy-14,643 is an agonist specific for PPAR- α and was administered in addition to the MCD to a further group of animals in an attempt to resolve the function of PPAR- α in steatohepatitis development.

The MCD diet induced significant weight loss as was expected from previous findings, but was not normalised by the Wy-14,643 agonist. Administration of the MCD diet also induced macrovesicular steatosis which was more severe in PPAR- α deficient animals. However, Wy-14,643 administration prevented the onset of steatohepatitis development in wild-type MCD diet-fed mice through cessation of hepatic triglyceride accumulation and even lowered the concentration to levels below those observed in control-diet mice. Thus, activation of PPAR- α significantly reduces hepatic triglyceride levels by stimulating hepatic fatty acid disposal which consequently exhausts the liver of potential material capable of facilitating lipid peroxidation reactions. Additionally, CYP2E1 and CYP4A activity was not increased in MCD diet-fed PPAR- α deficient mice, whereas MCD diet-fed animals treated with Wy-14,643 displayed a huge increase of CYP4A activity despite the absence of steatohepatitis. This information suggests that CYP proteins are unlikely to be a main source of oxidative stress as originally postulated before the commencement of the study.

The activation of pathways involved for hepatic fatty acid turnover are regulated in part by PPAR- α expression which increases fatty acid uptake and β -oxidation and is therefore regarded as an important trigger in provoking the development of steatosis (Fan *et al.*, 1996). However, PPAR- α hepatic expression in humans is significantly reduced in comparison to rodents (Hertz *et al.*, 1998), proposing the possibility of alternative mechanisms being in force for control of lipid accumulation and fatty acid oxidation.

Recent work performed by Reifel-Miller *et al.*, (2005), examined LSN862, an agonist of PPAR- α and PPAR- γ . Rodent models known to exhibit type II diabetes were administered with LSN862 which resulted in a noteworthy reduction of glucose and triglyceride levels. The lowering of triglycerides confirms the earlier work of Ip *et al.*, (2003), while the decrease in glucose displayed an improvement in antidiabetic efficiency and provides strong evidence for using this dual agonist in future therapeutic treatment.

A further study concentrating on the role of PPAR- α was carried out by Crabb *et al.*, (2004). Here the focus was whether PPAR- α mediated responses are altered in the presence of sustained ethanol metabolism. C57BL/6J mice were administered an ethanol-rich diet for 4 weeks and sacrificed. The livers were removed and examined for fatty oxidation, lipid content and DNA binding of PPAR- α /RXR- α complexes in comparison to untreated animals. The findings revealed that ethanol-feeding had induced fatty livers coupled with a 4-fold increase in triglycerides. Ethanol had no effect on PPAR- α mRNA, although RXR- α mRNA displayed a 20% reduction, possibly resulting from increased LPS levels (Uesugi *et al.*, 2002). Ethanol-fed mice also experienced a reduction in expression of genes influenced by PPAR- α , including long chain acyl-CoA and acyl-CoA dehydrogenase and mirrors the fact that β -oxidation of fatty acids was not induced in these animals.

The PPAR- α agonist Wy-14,643 was administered to an additional group of ethanol-fed mice and again proved to lessen the accumulation of fat. The animals receiving Wy-14,643 also displayed a significant increase in free fatty acid oxidation and this coincided with a massive 50% elevation of PPAR- α expression, despite RXR- α levels being unaffected. Overall, functioning of PPAR- α appears to be central in facilitating the development of fatty liver enduring the strain of alcohol. Treatment with Wy-14,643 unblocked the ethanol-induced restriction of PPAR- α which stimulated fatty acid removal and consequently averted fatty liver. In conclusion, Wy-14,643

promotion of PPAR- α activities should be considered as a possible approach for alleviation of liver injury or fibrosis.

The resultant success achieved from work done with PPARs and relevant agonists in rodent models has proved to be critical in identifying components important to fatty liver disease development. However, this success has not been repeated in human studies and further work is required in adapting the findings in rodents to a positive outcome in human treatment (Kersten *et al.*, 2002).

3.6 Methionine and Choline Deficient (MCD) Dietary Model

This section of the thesis has previously examined specialised mouse and rat types that have been specifically generated for studying NAFLD and the associated outcomes. Information generated from these models has played a significant role in understanding the mechanisms involved with the development and progression of the disease. However, several groups have been investigating the pathogenesis of NAFLD via the effects of a particular diet as opposed to a certain rodent model.

The MCD diet is deficient of methionine and choline and has been administered to a wide variation of animal models to induce the characteristics seen in humans with NAFLD. Previous studies fed animals with diets deficient in only choline which had partial success as steatosis did develop (Lombardi *et al.*, 1968) but without the key features of inflammation and fibrosis regularly seen in NAFLD. A study carried out by Leclercq *et al.*, (2000), displayed that MCD diet fed animals developed fibrosing steatohepatitis, and therefore set the trend for further work.

Methionine is an amino acid that is also involved in the transfer of methyl groups, including the formation of phospholipids. Choline is the trimethyl polar moiety of phosphatidylcholine. The synthesis of phosphatidylcholine is extremely important in the liver (Nanji, 2004) not only for membrane properties but also for export of lipoproteins and has potential to be adversely effected by MCDD.

Work undertaken by George *et al.*, (2003) was aimed at studying the cell types and products of lipid peroxidation in high-fat, MCD diet-fed rats that acquire steatohepatitis and fibrosis. Male Sprague-dawley rats were fed the MCD diet *ad libitum* for up to 17 weeks and compared against a control, supplemented diet. The MCD rats displayed a 32% loss in body weight in comparison to the untreated animals. Livers from rats administered the MCD diet displayed signs of steatosis by

week 2, and by week 12, hepatic fibrosis had developed, prominently in zone 3 hepatocytes. Furthermore, glutathione (GSH) levels were significantly reduced and correlated with an increase in TBARS (47-fold by week 17), when compared to the control animals.

The histological features that appear in humans with NAFLD were replicated in the MCD diet-fed rats. In addition, the occurrence of lipid peroxidation in hepatocytes coupled with the development of liver injury and subsequent fibrosis is consistent with the chain of events in humans. This further enhances the theory that lipid peroxidation may be a primary stimulator of the liver damage and fibrosis or at least could be a strong marker for the process.

As previously stated, the formation of reactive oxygen species (ROS) is thought to be closely connected to the underlying mechanisms of NAFLD development by facilitating lipid peroxidation and consequently fibrosis (Yang *et al.*, 2000). Vitamins C and E are antioxidant drugs which are known to react with ROS, intercepting their detrimental effects and therefore reduce the levels of oxidative stress and lipid peroxidation occurring in the liver.

A study performed by Oliveria *et al.*, (2003), investigated the role of these antioxidant vitamins with regards to prevention of NAFLD. Wistar rats were administered the MCD diet for 4 weeks to induce fatty livers and subjected to daily doses of either vitamin C, vitamin E or the vehicle for control animals. The findings from the study revealed slight increases of AST and triglyceride levels which were comparable across all groups. In addition, the induction of macro- and microvesicular fat was noted in rats treated with the vehicle and vitamin E whereas vitamin C treated animals did not develop liver steatosis. It is therefore reasonable to claim that vitamin C may reduce oxidative stress, thus preventing the development of steatosis and associated outcomes. Interestingly, vitamin C is a watersoluble anti-oxidant that is involved in the recycling of tocopherol that can protect lipids against oxidation.

The effect of vitamin C supports the theory of antioxidants being able to defuse the outcomes normally brought about by ROS. However, vitamin E did not prevent the onset of steatosis, possibly owing to the inability to diminish oxidative stress, presumed to occur primarily in an aqueous environment. Further research must be undertaken in to evaluate whether anti-oxidant drugs can be therapeutic agents in combating the disease.

Drug-induced reversal of NAFLD is a further approach which has been taken in an attempt at treating the disease. Pentoxifylline (PTX) is regularly used for peripheral vascular disease but also displays antioxidant characteristics by reducing ROS (Bhat *et al.*, 2001). PTX has also previously shown to decrease TNF- α expression after being treated with LPS (Streiter *et al.*, 1988) and to reduce mortality in patients with alcoholic hepatitis (Akriviardis *et al.*, 2000). Tumour necrosis factor – α (TNF- α) is already established as a major instigator of hepatocyte injury in NAFLD (Kugelmas *et al.*, 2003), as are ROS and increased products of lipid peroxidation.

A study carried out by Koppe *et al.*, (2004) investigated the effects of PTX in mice fed the MCD diet to determine whether PTX administration reduced the progression of steatohepatitis. Fatty livers were induced in C57BL/6J mice by administering the MCD diet for 2 weeks. The treated animals were subjected to 5mg/ml PTX, administered i.p. at a dose of 100 μ g/g tid, compared to untreated animals receiving the vehicle. The observations confirmed the anti-inflammatory effects of PTX as serum ALT levels were significantly decreased, as were TNF- α levels, and inflammation was less severe in animals treated with PTX compared to untreated. However, the PTX treated mice illustrated a higher concentration of hepatic triglycerides in accordance with a higher degree of steatosis in comparison to the vehicle group. No specific explanation is available but it is possible that sparing FA from peroxidation could make more available for esterification to TG. In addition, PTX treatment did not make any difference to the weight loss induced by the MCD diet.

PTX demonstrated its usefulness by impeding the development of steatohepatitis in MCD diet-fed mice. An increased level of triglycerides normally results with an increase in inflammation, which did not occur in this study and was unexpected. Although, a single causative factor could not be identified for development and progression of NAFLD, consideration should be given to the use of PTX as a future therapeutic agent. Further work is required in assessing the pathogenesis applicable to lipid metabolism and the effects of administering PTX in the context of a fatty liver.

Another factor that is highly regarded as a major contributor in the disease process of NAFLD is insulin. The presence of insulin resistance is a common trait in NAFLD and is considered a fundamental metabolic abnormality. Despite the exact functions of insulin remaining unknown, they are presumed to be considerably influenced by TNF- α and free fatty acids (FFA) (Hotamisligi *et al.*, 1999). Research performed by

Rinella *et al.*, (2004), attempted to uncover the role of insulin in steatohepatitis, induced by the MCD diet.

Male FVB/NJ mice were fed the MCD diet for 10 days and 28 days in addition to a control group administered a methionine and choline supplemented diet. Steatohepatitis and elevated ALT levels were brought about by MCD diet administration regardless of the duration of feeding. Insulin sensitivity was assessed by insulin tolerance testing (ITT) and glucose tolerance testing (GTT) and revealed that MCD diet-fed mice displayed no indication of insulin resistance and was comparable with the controls.

Although evidence exists for a strong association between NAFLD and insulin resistance, a definitive relationship has yet to be proven. The experiment conducted by Rinella *et al.*, (2004) indicates that insulin resistance does not appear to feature in animals developing NAFLD when fed the MCD diet. Insulin resistance may not be as critical to NAFLD development as previously thought but the role of insulin resistance in the complex pathogenesis of the disease may be to provide a metabolic stress.

A further probable contributor to NAFLD development may be cytochrome P450 2E1 (CYP2E1), which is believed to initiate lipid peroxidation through the production of oxidative stress (Lieber *et al.*, 1997). A study embarked on by Leclercq *et al.*, (2000), was aimed at ascertaining the role of hepatic CYP2E1 in the development of steatohepatitis. Female C57BL6/J mice were fed the MCD diet for 10 weeks to induce hepatic steatosis and were compared to animals receiving the control diet for the same period. The purpose of this was firstly to determine if CYP2E1 expression was affected by administration of the MCD diet and secondly to confirm lipid peroxidation in MCD diet- induced steatohepatitis. In addition to significant body weight loss and increased ALT levels, as was expected in MCD diet-fed mice, the findings indicated an increase in CYP2E1 enzyme activity when compared to the control group. Furthermore, lipid peroxidation was immensely enhanced in MCD diet-fed animals as verified by the rise in TBARs and 100-fold increase in lipid peroxides.

These results imply that increased CYP2E1 activity may assist in the accumulation of lipid peroxidation products. With this hypothesis, the next logical step was to feed the MCD diet to CYP2E1 deficient mice and their non-deficient heterozygous littermates as a comparison. Hepatic steatosis and necroinflammation were induced by the MCD diet in both sets of mice as expected, but, the CYP2E1 deficient animals displayed a

larger degree of fat accumulation which corresponded to elevated ALT levels and a major increase of lipid peroxides in comparison to the control group.

The data provided presents strong evidence that the enzymatic action of CYP2E1 is involved in the development of NAFLD. This enzyme is not crucial for the instigation of NAFLD but may influence the course of the disease by modulating the flux of TG and permitting increases in lipid peroxidation. It is concluded that further work is necessary to define the exact properties of CYP2E1 within the MCD model as well as the mechanisms promoting the development and progression of NAFLD and the associated outcomes.

The role of osteopontin (OPN) as a mediator of steatohepatitis development has also been investigated. OPN is a Th1 cytokine which is believed to be involved in the inflammatory process of fibrotic diseases such as atherosclerosis (Chiba *et al.*, 2002). The synthesis and secretion of this cytokine occurs in numerous immune cells and is considered to play an important role in the recruitment of macrophages during inflammation (Mazzali *et al.*, 2002). The definitive role of OPN in liver diseases is yet to be established, but a study performed by Sahai *et al.*, (2004) attempted to identify the function of this cytokine from an NAFLD perspective. Female A/J mice were used out of a selection of 8 different strains as these were found to be more sensitive to the effects of the MCD diet. The diet was administered for up to 12 weeks to induce steatohepatitis and other complications surrounding the disease before comparing these animals to that receiving a control diet. The histological examination results revealed the presence of macrovesicular steatosis and progressive hepatic portal inflammation in MCD diet-fed mice. The biochemical analysis confirmed these findings which revealed an increase in CYP2E1 enzyme activity and ALT levels as previously confirmed. Additionally, the concentrations of triglycerides and TBARS were also significantly enhanced, as was TNF- α , in comparison to the controls up to 12 weeks. The elevated level of CYP2E1 is a probable cause of increased lipid peroxidation but consideration should be given to the increase in TBARS merely reflecting an increase in TG.

These results are again suggestive of elevated CYP2E1 activity being implicated in the production of lipid peroxidation and oxidative stress. With the liver histology and biochemistry results further outlining the effects of the MCD diet, the subsequent part of the study was to examine the effects of OPN within the MCD diet framework. OPN knockout mice were fed the MCD diet and compared to the wild type littermates who

were also administered the diet. The animals deficient in OPN displayed a decrease in liver damage and fibrosis. Furthermore, TNF- α expression was also lower in mice lacking the Th1 cytokine and lipid peroxidation and oxidative stress were reduced as well. OPN expression or at least macrophage activation, must not be ignored in defining the underlying mechanisms of NAFLD as it is regarded as an important signal messenger in the development of steatohepatitis.

Despite the relevance of findings generated from administration of the MCD diet, this approach still receives much criticism as it induces significant weight loss and does not involve insulin resistance in contrast to humans who gain mass and develop insulin resistance. Nevertheless many important revelations have appeared from using the MCD diet and it may remain a useful tool to evaluate the development or treatment of NAFLD.

3.7 Lipodystrophic Mouse Model

Lipodystrophic mice strains have genetic defects that affect the development and/or function of adipose tissue, leading to lipoatrophy or adipose tissue excess. Since adipose tissue is primarily responsible for leptin production some of these models are leptin-deficient or deficient for leptin receptors (Koteish and Diehl, 2002). Phenotypic characteristics displayed in the leptin-deficient *ob/ob* mouse model such as insulin resistance, elevated TNF- α levels and the occurrence of fatty liver disease are concordant with human NAFLD (Reitman *et al.*, 1999).

Lipodystrophic model complication of lipoatrophy is the over-expression of sterol regulatory element binding protein-1 (SREBP-1) (Shimano *et al.*, 1996). Hydroxymethyl glutaryl coenzyme A (HMG CoA), low density lipoprotein (LDL)-receptor and acetyl CoA carboxylase are genes heavily involved in fatty acid and cholesterol synthesis and are controlled by the important transcriptional regulator SREBP-1 (Koteish and Diehl, 2002). Resultantly, the enhanced expression of SREBP-1 can facilitate hepatic steatosis and increased ALT levels (Reitman *et al.*, 1999).

The means by which lipid metabolism is disrupted and cytokine functioning is altered in this model remain to be clearly defined, but it is hypothesised that they are very similar to the pathogenic mechanisms in *ob/ob* mice which are also leptin-deficient. However, an important feature of the lipoatrophic mouse model is that the complication of obesity does not arise. The fact that progressive NAFLD and NASH

occur in lean individuals is truly reflected by the presence of fatty liver disease in these non-obese mice and stresses the importance of these models in understanding the pathogenic factors involved.

3.8 Leptin Deficient Mouse Model

The existence of leptin, first discovered in 1994, created a wealth of investigative work to identify the key processes of energy metabolism (Zhang *et al.*, 1994). Leptin is a hormone product of white adipose tissue and appears primarily responsible for suppression of appetite and raised expenditure of energy (Campfield *et al.*, 1996). Leptin is deficient in *ob/ob* mice, aptly named because of a genetic deficiency in the *ob* gene which results in these rodents becoming hyperphagic and severely obese. Most importantly, these rodents develop fatty livers (Nanji *et al.*, 2004). Leptin has a significant role in controlling weight levels, and acts in a preventative measure against obesity. Recent research has also shown that leptin plays a crucial role in alterations of appetite and the relaying of information on fat reserves by orchestrating the storage of body fat in adipocytes (Unger, 2002).

Ogus *et al.*, (2003) examined leptin-related functions in recently developed transgenic mice (LepTg). The genetic nature of LepTg mice prompts the over production of leptin resulting in these animals becoming hyperleptinaemic. The animals were administered a high-fat diet for 20 weeks to induce obesity and to determine if over-expressed leptin aided the responses of the liver to combat the onset of steatohepatitis. The findings from the study established that lean littermates remain in their morphologic state because of an increased production of leptin on a normal control diet. However, the subjection of the LepTg model to a high-fat diet resulted in near immediate development of obesity and suggests that exposure to high fat diets facilitates profuse production of adipose tissue which becomes differentiated causing accumulation of hepatic fat in the organism, including the adipose tissue and liver.

Hyperleptinaemia or partial lipodystrophy is an unusual condition characterised by an insufficiency of adipose tissue and a concerning inadequate supply of leptin (Peterson *et al.*, 2002). As a result, the liver becomes fatty and develops NASH through accumulation of triglycerides. Javor *et al.*, (2005) explored the possibility of reversing the progressive nature of fatty liver in lipodystrophic patients by leptin replacement therapy. The study contained 26 patients with known lipodystrophy but only 10 met the criteria for treatment. The patients received twice daily injections of

recombinant methionyl human leptin (r-metHuLeptin) for a mean duration of 6.6 months. Liver biopsies were obtained before and after intervention to evaluate the effects of therapy. Considerable reductions in transaminases, triglyceride levels and glucose concentrations were noted as well as lowered fat content. Leptin treatment thus ameliorated NASH. Despite the encouraging results with r-metHuLeptin, raised ALT and AST levels were not found in all test subjects and it is not known if the group of patients suffering from lipodystrophy are truly representative of NASH patients. The true action of leptin is not yet fully understood and additional work is required to investigate pathways relevant to fatty liver development, although Lee *et al.*, (2002) has shown leptin to increase PPAR- α expression which has already been described as a possible modulator of fat accumulation.

Angiopoietin-like protein 3 (ANGPTL3) is a liver-specific secretory product. It was recently reported to considerably enhance plasma triglyceride concentrations as a result of inhibiting free fatty acid (FFA) and lipoprotein lipase (LPL) in humans and mice (Koishi *et al.*, 2002). In an attempt to further investigate leptin function, a study undertaken by Shimamura *et al.*, (2004) was aimed at trying to identify leptin as a responsible regulator of (ANGPTL3). Obese, leptin-deficient (*ob/ob*) mice were analysed and were found to exhibit increased values of ANGPTL3 in comparison to the control group. The elevations in ANGPTL3 were accompanied with rises in triglycerides and FFA. Additionally, *in vitro*, leptin and insulin presented the ability to decrease ANGPTL3 expression in hepatocytes. These findings indicate that obesity is correlated with a deficiency in leptin and elevated triglyceride levels and corroborate previous work of Unger *et al.*, (2002). Leptin does not appear solely responsible for regulation of ANGPTL3 as insulin also clearly displays an effect. Variability of ANGPTL3 mRNA expression may influence lipid metabolism and thus contribute to the pathogenesis of the disease.

A study performed by Angulo *et al.*, (2004), examined the effects of leptin in relation to liver fibrosis in human NAFLD. A total of 88 patients with liver biopsy proven NAFLD were enrolled in the study to determine the relationship between levels of leptin and (progression to) fibrosis of the liver. The experimental liver samples were graded for fibrosis. The advance of fibrosis was significantly correlated with increases in leptin concentration. However, patients with more severe fibrosis were also shown to be older, more insulin resistant and more commonly diabetic. Once these variabilities were accounted for, no direct relationship could be formed with leptin concentration and degree of fibrosis. It is therefore likely that increases in leptin

levels are representative of other confounding factors such as age, diabetic state and insulin resistance as opposed to stage of fibrosis, although additional work is required to fully define the role of leptin.

The evidence to date is suggestive of leptin being an enhancement factor for progression of fatty liver disease. TNF- α is also believed to be a causative agent in NASH patients and recent work performed by Shen *et al.*, (2005) hypothesised that a further function of leptin was to increase TNF- α production. Observations in male zucker (*fa/fa*) rats revealed that leptin increases TNF- α in isolated kupffer cells in a dose-dependent manner, but only in the presence of lipopolysaacharide (LPS). Kupffer cells are the primary source of hepatic TNF- α when under LPS inducement (Ulevitch and Tobias, 1995) and because leptin administration boosts the production, it is resultantly regarded as a potential aggravator of the inflammatory response.

3.9 Fatty Liver Shionogi (FLS) Mouse Model

The FLS model is currently also employed to study fatty liver disease. FLS mice have been derived from inbreeding and develop spontaneous fatty liver without obesity, diabetes or hyperphagia (Soga *et al.*, 1999). At birth, this particular model exhibits an abnormal elevation of triglyceride levels and by the age of 15 weeks, macrovesicular fat (large lipid droplets) occupy the hepatocytes, which is accompanied by hepatic cell disorder (Soga *et al.*, 2003). Further evidence of liver injury was confirmed by elevated serum alanine aminotransferase (ALT) levels.

From their fatty livers, the FLS mice develop steatohepatitis and a high incidence of these animals (especially males) progress to develop tumours, including hepatocellular adenoma (HCA) and carcinoma (HCC). Hepatocarcinogenesis was not detected in the non-obese sister strain dd Shionogi (DS) and supports the theory that the tumours observed in the FLS mice are a consequence of chronic fatty liver (Soga *et al.*, 2003).

Although obesity is normally a major factor contributing to the occurrence of NASH in humans, fatty liver also appears in non-obese individuals as with the FLS mice.

The FLS model could prove to be beneficial in improving our understanding the occurrence of hepatocarcinogenesis and NASH, although the exact mechanisms involved are unclear at present. Further studies are required in the pathogenesis of

this model as despite the appearance of liver tumours, liver cirrhosis was not evident in any of the animals and is normally a key finding in a NASH patient.

3.10 Zucker (*fa/fa*) Rat Models

Zucker *fa/fa* rats are similar to several other models being examined for NAFLD as they also possess the characteristics of obesity, leptin receptor deficiency, type II diabetes, hyperinsulinaemia and hepatic steatosis (Chua *et al.*, 1996). A study performed by Nagao (2004) used the Zucker *fa/fa* rats as a dietary model by feeding them conjugated linoleic acid (CLA) for 8 weeks compared with control fed rats. CLA is a mixture of positional and geometric isomers of linoleic acid and is believed to alleviate liver injury through increased production of adiponectin (Nagao *et al.*, 2004). Adiponectin is present in abundance in adipose tissue in rodents and humans. Its release under the influence of CLA has been shown to improve insulin action in vitro and in vivo, therefore indicating that adiponectin may play a protective role against insulin resistance which is one of the first pathological processes in the development of NAFLD (Yamauchi *et al.*, 2001).

The findings in the study carried out by Nagao (2004) revealed that hepatic triglyceride (TG) levels and hepatic injury markers ALT and AST were significantly reduced in the CLA-fed animals in comparison with the controls. Furthermore, the mRNA expression of TNF- α (an inflammatory cytokine) was also suppressed when compared to the control group. It is therefore proposed that enhanced production of adiponectin may alleviate the development and progression of NAFLD in CLA-fed Zucker *fa/fa* rats.

Another study that was carried out using Zucker *fa/fa* rats was undertaken by Yang (1997) who proposed that the process of steatohepatitis development is accelerated when the animals are exposed to low doses of lipopolysaccharide (LPS). The release of several cytokines, (including IL-10, IL-12 and IFN γ), from macrophages and Kupffer cells is promoted by LPS stimulation (Tracey *et al.*, 1993). Once unleashed, the cytokines proceed to stimulate the biological activity of TNF- α which has a significant influence on endotoxin liver injury. The observations from the study revealed obese *fa/fa* rats had a marked accumulation of liver injury markers AST and ALT in contrast to their lean littermates when subjected to LPS exposure. In addition, histological examination revealed all surviving obese *fa/fa* rats to have significantly developed steatohepatitis in comparison to the non-obese controls (Yang *et al.*, 1997). This evidence displays that LPS and/or TNF- α stimulated endotoxins may play

a vital role in contributing to progressive liver damage in obese individuals which could have important clinical consequences.

An additional strain of *fa/fa* Zucker rats currently used to study metabolic disorders is the stroke-prone spontaneously hypertensive rat (SHRSP). SHRSP fatty (*fa/fa*) rats are derived by crossing a segment of the mutant leptin receptor gene from Zucker fatty (*fa/fa*) rats with the genetic background of SHRSP/Izm rats (Yamamoto *et al.*, 2004). The new animal model develops obesity and hypertension simultaneously as well as displaying significantly elevated levels of total cholesterol, triglycerides, glucose and insulin in comparison with the SHRSP/Izm control rats. SHRSP fatty (*fa/fa*) rats are distinct as they are not only associated with metabolic abnormalities but also encounter cardiovascular disease.

Overall, the *fa/fa* model along with the subsequent strains, exhibit several key characteristics relevant for studying NAFLD and other associated conditions. However further work is required to determine the pathological processes linking obesity and liver disease.

3.11 Acyl-CoA Oxidase (AOX) Mouse Model

The AOX mouse model is deficient of acyl-CoA oxidase (AOX) and was generated by inactivation of the AOX encoding gene. In the normal liver, fatty acids are oxidised by mitochondria and peroxisomes via β -oxidation after activation to acyl-CoA. The acyl-CoA that remain unmetabolised in the absence of AOX may serve as a trigger for uncontrolled peroxisome propagation (Hashimoto *et al.*, 1999) as these appear to be powerful agonists for the peroxisomal proliferator activator receptor α .

A study performed by Fan (1998), investigated the AOX mouse model and produced some interesting observations. The livers of mice deficient of AOX displayed severe microvesicular steatosis in liver parenchymal cells and by the age of 15 months, hepatic carcinomas had appeared. Acyl-CoA enzymes, which are abnormally regulated in AOX deficient mice, are believed to sustain proliferation of peroxisomes by acting as ligands for peroxisome proliferator-activated receptors (PPARs). The continued induction of these receptors by acyl-CoA enzymes may be partially responsible for the resultant tumour development (Fan *et al.*, 1998).

The findings provide sufficient evidence to suggest that AOX plays a significant role in hepatic lipid metabolism and may contribute to developing fatty liver, although further studies are required to establish the true pathogenesis.

3.12 Human Studies

As discussed, many animal models have been tried and tested in order to generate a model suitable for studying NAFLD. The various experimental strains being investigated are susceptible to disease development because genetic factors and expression genes are purposely manipulated to manifest the adverse outcomes of NAFLD (Koteish *et al.*, 2002). Although the arising pathology in rodent models is near identical to that in humans, caution should be taken when interpreting the findings because of the species difference which may result in alternative biological pathways being engaged.

Since research is ultimately directed at amelioration therapy or complete elimination of NAFLD in humans, the models discussed above must therefore serve as ideal test models. Additional complications arise in humans, including variation between patients and the practicality problems of sampling; this explains the efforts consumed in animal model experimentation. Many different components have been recognized in work carried out in animal models which has been invaluable in understanding development and progression of liver disease. However, various human studies have also been undertaken in an attempt to eliminate species variability and identify an ever elusive single causative factor.

Obesity is a significant feature being constantly associated with fatty liver due to links with surrounding complications such as diabetes, dyslipidaemia and hypertension (Kissebah *et al.*, 1994). Appearance of obesity and the consequent escalation of these correlated outcomes have been collectively labelled as metabolic syndrome X in the context of a fatty liver.

Work orchestrated by Marceau *et al.*, (1999), examined metabolic syndrome X using 580 patients over a 9 year period. Despite the varied range of disease-associated complications within the group, severe obesity remained a central feature. The study including males and females presented a mean age of 36 years, 95% had endured routine liver biopsies and no patient had any history of hepatitis, nor was it clinically suspected.

The study revealed a significant association between the degree of fat present in liver samples and the components of metabolic syndrome X; fat distribution, impaired glucose tolerance, hypertension and dislipidaemia. The results indicated a close link between elevated AST and ALT levels and the existence of steatosis, which was present in 86% of patients. In addition, the presence of diabetes was recorded as the statistically strongest predictor of cirrhosis ($P < 0.001$), although the occurrence and subsequent progression of steatosis was also a reliable indicator. Furthermore, a strong correlation existed between fasting blood sugar, steatosis and the development of fibrosis. Unfortunately, a significant relationship was not established between any of the variables (including steatosis and fibrosis) and the presence of inflammation. It is therefore hypothesised that steatosis resulting from metabolic syndrome X related obesity is a separate assemblage of reactions to that taking place in non-alcoholic steatohepatitis (Bacon *et al.*, 1994).

The pathogenesis of obesity and the surrounding associated effects are difficult to separate from the outcomes of fatty liver. The exact involvement of insulin, target cytokines and leptin are unclear, but overlapping pathways must exist due to major similarities. The variables of metabolic syndrome X contribute independently as predictors of fatty liver mechanisms, but their collective label of metabolic syndrome X allows the effects of fatty liver disease to be investigated by outlining the risk factors involved.

In addition to obesity, alcohol is a further extension to the growing list of reasons to which fatty liver can be attributed. Further investigative work carried out by Bellentani *et al.*, (2000) examined the prevalence of obesity, alcohol and other risk factors involved in developing hepatic steatosis. Two hundred and fifty-seven people, 19-70 years of age, from Northern Italy partook in the study which comprised 4 different groups; controls, obese, heavy-drinkers and obese heavy-drinkers.

Steatosis was present in 58.3% of the cohort with raised levels of ALT, γ -glutamyltransferase and plasma triglycerides acting as positive indicators. Also detected by comparison with the controls, steatosis was found to be 2.8-fold more common in heavy drinkers whereas a 4.6-fold increase of development was discovered in the obese group. The group consisting of overweight heavy drinkers had a 5.8-fold increase when compared to the controls. Thus, steatosis appears to be more closely associated with obesity than excess alcohol intake, but obese heavy-drinkers are almost guaranteed to develop some form of steatosis. An

immediate reduction in caloric and alcohol intake is recommended in over-weight, high-alcohol consumption patients to decrease the chance of fibrosis and to lessen the effects of fatty liver.

There are many complications surrounding fatty liver disease and they are due to the multi-factorial pathways including steps acting as initiators and modulators of progression. Markers that can detect the extent of liver malfunction are of vital importance in determining if a patient has a fatty liver. Sorrentino *et al.*, (2004) studied the liver enzymatic activity in plasma from 58 patients known to exhibit varying degrees of NASH and compared these to a control group known to display abnormal liver enzymes. The observations of the study revealed the severity of steatosis to be similar between NASH patients and those with irregular metabolism but hepatic mallery ballooning and glycogenated-nuclei were more prominent in patients presented with NASH. Although, age, presence of diabetes and period of being obese were typically linked with fatty liver development and was subsequently shown to significantly enhance the severity of liver damage independent of abnormal enzymatic activity. Despite the inability of liver enzymes to act in the capacity as a marker of NASH detection, the presence of ballooning degeneration may be essential to progression of fibrosis.

Additional human work undertaken by Seki *et al.*, (2002) is of particular interest as it describes lipid peroxidation and oxidative DNA damage in NAFLD patients. 4-hydroxy-2'-nonenal (HNE) is a key metabolite involved in lipid peroxidation and is also regarded as a very reliable marker for this process (Esterbauer *et al.*, 1992). 8-hydroxydeoxyguanosine (8-OHdG) is also believed to be a reliable marker (Kasai *et al.*, 1997), but in the context of oxidative stress as this compound is predominantly produced by free radicals (Shibutani *et al.*, 1991). The research that was carried out concentrated on the hepatic expression of these biomarkers to determine lipid peroxidation and oxidative damage.

The study was conducted using fatty liver samples from 40 patients. Non-alcoholic fatty liver disease (NAFLD) was present in 23 patients whereas non-alcohol steatohepatitis (NASH) had developed in the remaining 17 patients. The criteria for inclusion in the study were macrovesicular steatosis without necro-inflammation in the first group of 23 patients and the presence of macrovesicular steatosis without hepatocyte necrosis for the remainder. Samples from 7 histologically normal livers were used as a control group.

The study revealed a high expression of HNE adducts in hepatocyte cytoplasm in both sets of patients with fatty livers, although slightly higher levels were detected in the NASH group by comparison. Despite the differences, these elevated levels are still strongly indicative of lipid peroxidation. In addition, HNE may also provide a source capable of activating hepatic stellate cells (HSC), these are deemed precursors for collagen type 1 production and could therefore have another significant part in developing fibrogenesis of the liver (Friedman, 1993).

Another interesting observation of the study revealed 8-OHdG expression in only 2/23 NAFLD patients whereas 11/17 patients with NASH were found to exhibit 8-OHdG activity, mainly in hepatocytes. The expression of 8-OHdG is representative of oxidative DNA damage as it causes mispairing which can result in the occurrence of mutagenic and carcinogenic oxidative modification (Kasai *et al.*, 1991). Although hepatocarcinogenesis has been reported in human NASH (Welltman *et al.*, 1998), no defining link has been established between oxidative stress and hepatocellular carcinoma although the possibility still exists.

To concur, lipid peroxidation and oxidative damage are again closely associated with the clinical outcomes of fatty liver disease, but the varied pathological pathways involved in NASH and NAFLD still need to be accurately identified.

3.13 Overview of Various Rodent Models In NAFLD Research

3.13.1 Introduction

As stated previously, research undertaken in a wide array of rodent models has been invaluable in gaining a better understanding of the pathogenesis of NAFLD and the surrounding complications. The employment of rodent models to investigate the effects of NAFLD is not always ideal as it is widely documented that this disease is multifactorial and therefore many of the biological systems involved are not identical to those exhibited in humans. As a result, the facilitation and development of fatty liver and fibrosis in humans may be induced by alternative pathway in rodents and vice versa. For example, PPAR- α is present in large quantities in rodents but levels are considerably lower in humans who also do not experience peroxisome proliferation in response to treatment with fibrates (Crabb *et al.*, (2004). Nonetheless, the information gained from rodent work remains the key at attempts to manage and ameliorate the wide spectrum of effects of NAFLD and NASH.

The data presented in the following tables further illustrates the importance of information acquired from studying various rodent models in the context of NAFLD and NASH. Some of the findings have already been discussed, but the additional information allows a more detailed comparison between the different models. The tables display many fields of omitted data which are parameters that were not recorded for the respective studies. The omissions reveal that too many different potential factors are involved in development and progression of the disease and as a result not all these parameters can be investigated in every study. The lack of a collective research protocol and difficulty in cross-referencing study data confirms the complexity of the disease and problem in finding a single causative factor.

3.13.2 (Ob/ob) Mouse Model

Takahashi <i>et al.</i> , (2004)	(Ob/ob) Mice			
Treatment / Phenotype	(wt/wt)	<i>Lep^{ob/ob}</i> Vehicle	<i>Lep^{ob/ob}</i> 1436 aminosterol	<i>Lep^{ob/ob}</i> FR
Duration of Treatment	-	4 doses at 4 day intervals	4 doses at 4 day intervals	4 doses at 4 day intervals
Sex	Female	Female	Female	Female
Age	12 wks	12 wks	12 wks	12 wks
No. of Animals	4	4	4	4
Terminal Body Mass (g)	-	22.8 ± 0.2	21.0 ± 0.2 [^]	21.8 ± 0.1 [^]
Liver Mass (g)	-	-	-	-
Liver Mass / Body Mass x 100%	-	3.6 ± 0.2	3.3 ± 0.3	3.3 ± 0.1 [^]
Conjugated Dienes (µmol/g.liver)	-	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-	-
TTBARS (nmol/g.liver)	-	-	-	-
Cholesterol (mg/dl)	80.5 ± 2 [*]	137 ± 5 [§]	82.3 ± 1 [*]	125 ± 5 [§]
Triglycerides (mg/g. liver)	78.2 ± 4 [*]	155 ± 6 [§]	95.4 ± 7 [*]	137 ± 3 [§]
Glucose Concentration (mg/dl)	83 ± 6 [*]	381 ± 22 [§]	117 ± 7 [*]	264 ± 7 [§]
CYP2E1 Conc. (arbitrary units)	-	-	-	-
AST (U/l)	-	-	-	-
ALT (U/l)	13.2 ± 1 [*]	87.5 ± 4 [§]	12.8 ± 1 [*]	68.7 ± 6 [§]
Insulin (ng/ml)	1.1 ± 0.2 [*]	24.3 ± 5 [§]	1.82 ± 0.2 [*]	18.4 ± 2 [§]
Histology / Additional Observations; Aminosterol 1436 administration resulted in decreased bwt, inhibited food intake, lowered circulatory lipids, reversed hepatic steatosis, decreased glucose and normalized ALT. Adiponectin and insulin levels were also elevated in response to 1436 which resulted in inhibition of TG synthesis and increased fatty acid oxidation. Treatment of 1436 is recommended to increase insulin sensitivity and may even reverse steatosis to a certain extent with good care and management.				

[^]P<0.01 vs. vehicle. FR = Food Restricted

^{*}P<0.001 vs. vehicle treated *Lep^{ob/ob}* mice. [§]P<0.001 vs. wild-type. FR = Food Restricted

3.13.2 (Ob/ob) Mouse Model (continued)

Memon <i>et al.</i> , (2000)	(Ob/ob) Mice			
Treatment / Phenotype	(ob/wt) Control	(ob/wt) Tro	(ob/ob)	(ob/ob) Tro
Duration of Treatment	10 days	10 days	10 days	10 days
Sex	Male	Male	Male	Male
Age	5-10 wks	5-10 wks	5-10 wks	5-10 wks
No. of Animals	10	10	10	10
Terminal Body Mass (g)	-	-	-	-
Liver Mass (g)	-	-	-	-
Liver Mass / Body Mass x 100%	-	-	-	-
Conjugated Dienes (µmol/g.liver)	-	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-	-
TTBARS (nmol/g.liver)	-	-	-	-
Cholesterol (mg/dl)	1.96 ± 0.1	1.99 ± 0.1	4.45 ± 0.2 **	4.12 ± 0.2
Triglycerides (mg/g. liver)	1.2 ± 0.06	0.1 ± 0.02 *	1.5 ± 0.06 ^	0.7 ± 0.03 **
Glucose Concentration (mg/dl)	8.04 ± 0.3	7.77 ± 0.4	20.6 ± 1.4 ^	11.0 ± 0.9 **
CYP2E1 Conc. (arbitrary units)	-	-	-	-
AST (U/l)	36.3 ± 4.3	34.1 ± 3.8	88.0 ± 8.9 **	74.8 ± 9.4
ALT (U/l)	44.8 ± 3.1	36.4 ± 2.7	99.9 ± 8.0 **	101.4 ± 9.9
Serum FFA (mmol/l)	1.2 ± 0.07	1.2 ± 0.05	1.7 ± 0.07 ^	1.0 ± 0.05 **
<p>Histology / Additional Observations; Troglitazone (Tro) is ligand for activating PPAR-α and -γ. TG and FFA were lowered following Tro admin but had no effect on ALT and AST levels in both lean and obese mice. PPAR-γ activators (stimulated by Tro) induced expression of aP2, FAT/CD36 and UCP2 which is believed to have contributed to the significant decrease of total hepatic content in <i>ob/ob</i> mice. PPAR activation therefore improves lipid utilization in obese diabetic mice and provides mechanism whereby serum lipid levels can be decreased.</p>				

*P<0.01 relative to the control

**P<0.001 relative to the control, ^P<0.001 relative to *ob/ob*, (Tro) = Troglitazone.

3.13.3 TNF-R1 Knockout Mouse Model

Yin <i>et al.</i> , (1999)	TNF-R1 (knockout) Mice			
Treatment / Phenotype	(<i>wt/wt</i>) Control	(<i>wt/wt</i>) Alcohol	TNF-R1 KO Control	TNF-R1 KO Alcohol
Duration of Treatment	4 wks	4 wks	4 wks	4 wks
Sex	Male	Male	Male	Male
Age	-	-	-	-
No. of Animals	6-7	6-7	6-7	6-7
Terminal Body Mass (g)	27.5 ± 0.2	26.8 ± 0.3	26.0 ± 1.5	24.7 ± 0.5
Liver Mass (g)	-	-	-	-
Liver Mass / Body Mass x 100%	5.5 ± 0.2	8.3 ± 0.8*	5.0 ± 0.7	6.7 ± 0.3
Conjugated Dienes (µmol/g.liver)	-	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-	-
TTBARS (nmol/g.liver)	-	-	-	-
Cholesterol (mg/dl)	-	-	-	-
Triglycerides (mg/g. liver)	-	-	-	-
Glucose Concentration (mg/dl)	-	-	-	-
CYP2E1 Conc. (arbitrary units)	-	-	-	-
AST (U/l)	-	-	-	-
ALT (U/l)	30 ± 4	90 ± 6*	42 ± 10	38 ± 9
Pathology Score	0.0 ± 0.0	0.0 ± 0.0	5.5 ± 0.6	0.8 ± 0.4*

Histology / Additional Findings;

No fatty accumulation was present in control mice, *wt/wt* or TNF-R1 but 4 weeks of ethanol administration caused steatosis in 13% of midzonal area in *wt/wt* animals but TNF-R1 displayed less steatosis (3.9%; $p < 0.001$).

TNF- α is proinflammatory cytokine and its absence in knockout mice results in only moderate fatty accumulation with no inflammation or necrosis after ethanol exposure whereas the *wt/wt* which had micro- and macrovesicular steatosis present.

* $P < 0.05$ vs. mice of the same genetic background fed a control diet

3.13.4 Sprague Dawley Rats

Lieber <i>et al.</i> , (2004)	Sprague Dawley Rats	
Treatment / Phenotype	Control	High Fat Diet
Duration of Treatment	3 Weeks	3 Weeks
Sex	Male	Male
Age	-	-
No. of Animals	22	22
Terminal Body Mass (g)	311 ± 9.8	306 ± 5.8
Liver Mass (g)	11.8 ± 0.51	12.1 ± 0.38
Liver Mass / Body Mass x 100%	3.79 ± 0.09	3.94 ± 0.07
Conjugated Dienes (µmol/g.liver)	-	-
LOOH (nmol LOOH/g.liver)	-	-
TTBARS (nmol/g.liver)	-	-
Cholesterol (mg/dl)	-	-
Triglycerides (mg/dl)	-	-
Glucose Concentration (mg/dl)	-	-
CYP2E1 Conc. (arbitrary units)	0.80 ± 0.07	1.31 ± 0.08 ***
AST (U/l)	-	-
ALT (U/l)	-	-
Tumour Development	-	-
<p>Histology / Additional Observations; Control; displayed minimal fat accumulation and few inflammatory cells. High-Fat Diet; showed pronounced hepatic steatosis and abundant mononuclear inflammatory cells, also revealed abnormal mitochondria with degenerative changes.</p>		

Significantly different from the Control; *P<0.05, **P<0.01, P<0.001

3.13.5 (Db/db) Mouse Model

Sahai <i>et al.</i> , (2004)	(Db/db) Mice			
	(db/wt) Control	(db/wt) MCD	(db/db) Control	(db/db) MCD
Treatment / Phenotype				
Duration of Treatment	4 wks	4 wks	4 wks	4 wks
Sex	Female	Female	Female	Female
Age	10-12 wks	10-12 wks	10-12 wks	10-12 wks
No. of Animals	6	6	6	6
Terminal Body Mass (g)	24.7 ± 1.0	15.1 ± 0.1 *	49.6 ± 3.0	42.4 ± 2.0
Liver Mass (g)	1.16 ± 0.1	1.17 ± 0.1	2.10 ± 0.1	3.0 ± 0.1
Liver Mass / Body Mass x 100%	46.7 ± 0.1	7.75 ± 1.0 *	4.16 ± 0.3 *	7.16 ± 0.2 *
Conjugated Dienes (µmol/g.liver)	-	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-	-
TTBARS (nmol/g.liver)	-	-	-	-
Cholesterol (mg/dl)	-	-	-	-
Triglycerides (mg/g. liver)	30 ± 6	121 ± 7 *	46 ± 3	91 ± 7 *
Glucose Concentration (mg/dl)	-	-	-	-
CYP2E1 Conc. (arbitrary units)	3.89 ± 0.3	6.73 ± 0.2 *	18.9 ± 1.0	52.7 ± 3.0 *
AST (U/l)	-	-	-	-
ALT (U/l)	25 ± 5	154 ± 15 *	-	260 ± 20
Insulin (mg/dl)	-	-	-	-
<p>Histology / Additional Observations; Histology revealed hepatic steatosis and lobular inflammation in animal being fed MCD diet. The levels of TNF-α were also raised which corresponded with elevated ALT and worsening steatosis. The MCD exhibited stimulated expression of OB-Ra (leptin receptor) and enhanced signaling which may contribute to the increase in hepatic fibrosis. Levels of osteopontin (OPN) were also raised and are thought to play an important role in the pathogenesis of NASH through its part in increasing OB-Ra expression and leptin signalling.</p>				

Significantly different from the Control; *P<0.01

3.13.6 PPAR- α Mouse Model

Ip <i>et al.</i> , (2003)	PPAR- α Mice					
	Wt Control	Wt MCD	Wt MCD/Wy-14,643	PPAR α ^{-/-} Control	PPAR α ^{-/-} MCD	PPAR α ^{-/-} MCD/Wy-14,643
Duration of Treatment	5 wks	5 wks	5 wks	5 wks	5 wks	5 wks
Sex	Male	Male	Male	Male	Male	Male-
Age	8-10 wks	8-10 wks	8-10 wks	8-10 wks	8-10 wks	8-10 wks
No. of Animals	4	5	4	5	6	3
Mass Change (%)	+ 21	- 34	- 39	+ 23	- 35	- 34
Liver Mass (g)	-	-	-	-	-	-
Liver Mass / Body Mass x 100%	4.0 \pm 0.8	4.1 \pm 0.1	8.0 \pm 1.4*	3.7 \pm 0.5	5.5 \pm 1.1	5.8 \pm 1.4
Conjugated Dienes (μ mol/g.liver)	-	-	-	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-	-	-	-
TTBARS (μ mol/g.liver)	0.10 \pm 0.1	5.3 \pm 1.8**	0.31 \pm 0.1	0.60 \pm 0.2	6.4 \pm 3.1***	4.1 \pm 2.3

* $P < 0.05$ relative to same genotype fed the MCD diet, ^s $P < 0.05$ relative to same genotype fed the control diet

** $P < 0.01$ relative to same genotype fed the MCD diet, * $P < 0.01$ relative to same genotype fed the control diet

*** $P < 0.001$ relative to same genotype fed the MCD diet, ^t $P < 0.001$ relative to same genotype fed the control diet

3.13.6 PPAR- α Mouse Model (continued)

Cholesterol Plasma; (mmol/l)	2.05 \pm 0.55	0.70 \pm 0.16 ^s	2.53 \pm 0.90	2.92 \pm 1.07	0.85 \pm 0.34 ^s	0.87 \pm 0.21
Triglycerides Plasma; (mmol/l)	0.88 \pm 0.11	0.69 \pm 0.15 ^s	0.21 \pm 0.05	1.20 \pm 0.68	0.49 \pm 0.19 ^s	0.47 \pm 0.07
Glucose Concentration (mg/dl)	-	-	-	-	-	-
CYP4a10 Conc. (arbitrary units)	1.0 \pm 0.09	0.97 \pm 0.19	18 \pm 7.4	0.68 \pm 0.34	0.31 \pm 0.18	0.17 \pm 0.06
AST (U/l)	-	-	-	-	-	-
ALT (U/l)	20 \pm 4	720 \pm 100 [†]	175 \pm 83 ^{***}	310 \pm 200	790 \pm 50 [‡]	890 \pm 225
Steatosis Grading (scale = 0-3)	0.5 \pm 1.0	2.3 \pm 0.3 ^s	0.0 \pm 0.0 [‡]	2.1 \pm 1.5	3.0 \pm 0.0 ^z	3.0 \pm 0.0
Histology / Additional Observations	<p>Administration of MCD diet caused weight loss as expected and the addition of Wy-14,643 (PPAR-α agonist) did not modify weight loss in either group.</p> <p>The wild-type mice fed MCD diet developed moderate steatohepatitis and macrovesicular steatosis with neutrophil polymorphs. Administration of Wy-14,643 almost completely prevented accumulation of lipid TBARS although slight elevations in CYP4a10 and 14 were noted.</p> <p>Wy-14,643 did not affect liver histology in PPARα^{-/-} mice and serum ALT and lipid levels were unchanged compared with PPARα^{-/-} mice fed the MCD diet alone confirming requirement for PPARα to provoke these effects.</p>					

^{*}P<0.05 relative to same genotype fed the MCD diet, ^sP<0.05 relative to same genotype fed the control diet

^{**}P<0.01 relative to same genotype fed the MCD diet, ^{*}P<0.01 relative to same genotype fed the control diet

^{***}P<0.001 relative to same genotype fed the MCD diet, [†]P<0.001 relative to same genotype fed the control diet

^zP<0.05 compared to wt mice fed the same diet

3.13.7 LepTg Mouse Model

Ogus <i>et al.</i> , (2004)	LepTg Mice			
Treatment / Phenotype	Wt Chow	LepTg Chow	Wt High Fat	LepTg High Fat
Duration of Treatment	20 wks	20 wks	20 wks	20 wks
Sex	Male	Male	Male	Male
Age	9 wks	9 wks	9 wks	9 wks
No. of Animals	10	10	10	10
Terminal Body Mass (g)	-	-	-	-
Liver Mass (g)	-	-	-	-
Liver Mass / Body Mass x 100%	-	-	-	-
Conjugated Dienes ($\mu\text{mol/g.liver}$)	-	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-	-
TTBARS (nmol/g.liver)	-	-	-	-
Cholesterol (mmol/l)	1.7 \pm 0.1	1.5 \pm 0.1	3.7 \pm 0.3	3.9 \pm 0.2
Triglycerides (mmol/l)	0.5 \pm 0.06	0.1 \pm 0.01 **	0.4 \pm 0.05	0.4 \pm 0.05
Glucose Concentration (mmol/l)	11.7 \pm 0.5	7.8 \pm 0.6 **	15.2 \pm 1.4	15.7 \pm 0.8
CYP2E1 Conc. (arbitrary units)	-	-	-	-
AST (U/l)	-	-	-	-
ALT (U/l)	-	-	-	-
Tumour Development	-	-	-	-
<p>Histology / Additional Observations; Chow fed LepTg mice adipocytes 76% smaller than normal mice. Chow does not cause fatty liver. Alterations in insulin levels were also noted between chow fed and high fat fed diets; 2.2-fold increase in normal mice and 10.7-fold increase in LepTg mice. In addition, LepTg mice fed a high fat diet had an increase in bwt gain 1.6-fold faster than lean littermates in same group. The small adipose quantities in LepTg mice may be caused by altered differentiation of preadipocytes and accumulation of triacylglycerols.</p>				

Significantly different from the Control; * $P < 0.05$, ** $P < 0.01$, $P < 0.001$

3.13.8 Fatty Liver Shionogi (FLS) Mouse Model

Soga <i>et al.</i> , (2004)	Fatty Liver Shionogi (FLS) Mice		
Treatment / Phenotype	-	-	-
Duration of Treatment	-	-	-
Sex	Male	Female	Female
Age	13-16 mths	13-16 mths	20-24 mths
No. of Animals	54	36	42
Terminal Body Mass (g)	-	-	-
Liver Mass (g)	-	-	-
Liver Mass / Body Mass x 100%	-	-	-
Conjugated Dienes ($\mu\text{mol/g.liver}$)	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-
TTBARS (nmol/g.liver)	-	-	-
Cholesterol (mg/dl)	-	-	-
Triglycerides (mg/dl)	-	-	-
Glucose Concentration (mg/dl)	-	-	-
CYP2E1 Conc. (arbitrary units)	-	-	-
AST (U/l)	-	-	-
ALT (U/l)	-	-	-
Tumour Development	HCA = 10 HCC = 12	HCA = 0 HCC = 0	HCA = 7 HCC = 4
<p>Histology / Additional Observations; None of these mice exhibit hyperphagia nor obesity but develop abnormal TG from birth and develop hepatic steatosis. After 2-4 months, mononuclear cell infiltration and clusters of foamy cells appear in addition to fatty liver and corresponds with raised ALT levels. These mice develop tumours following steatohepatitis but unclear whether this is definitive route for NASH patients.</p>			

3.13.9 Zucker (fa/fa) Rats

Yang <i>et al.</i> , (1997)	Zucker (fa/fa) Rats	
Treatment / Phenotype	(fa/wt)	(fa/fa)
Duration of Treatment	-	-
Sex	-	-
Age	-	-
No. of Animals	6-18	6-18
Terminal Body Mass (g)	304 ± 55	496 ± 89*
Liver Mass (g)	9.9 ± 1.6	15.2 ± 2.6*
Liver Mass / Body Mass x 100%	3.26 ± 0.3	3.17 ± 0.4
Conjugated Dienes (µmol/g.liver)	-	-
LOOH (nmol LOOH/g.liver)	-	-
TTBARS (nmol/g.liver)	-	-
Cholesterol (mg/dl)	70 ± 26	140 ± 10**
Triglycerides (mg/dl)	104 ± 19	287 ± 17**
Glucose Concentration (mg/dl)	185 ± 10	259 ± 26*
CYP2E1 Conc. (arbitrary units)	-	-
AST (U/l)	72 ± 2	74 ± 4
ALT (U/l)	56 ± 2	86 ± 6*
Tumour Development	-	-
Histology / Additional Observations; (fa/wt); livers appeared normal, hepatic steatosis was not observed. (fa/fa); grade 3-4 macro- & microvesicular steatosis in addition to hepatocyte apoptosis was evident in all animals.		

Significantly different from the Control; *P<0.05, **P<0.01, P<0.001

3.13.9 Zucker (fa/fa) Rats (continued)

Hiraoka-Yamamoto <i>et al.</i> , (2004)	Zucker (fa/fa) Rats		
Treatment / Phenotype	SHRSP/lzm	SHRSP/(fa/fa)	(fa/fa)
Duration of Treatment	-	-	-
Sex	-	-	-
Age	-	-	-
No. of Animals	-	-	-
Terminal Body Mass (g)	251 ± 3	428 ± 6**	472 ± 4**
Liver Mass (g)	7.7 ± 0.1	12.2 ± 0.8**	10.9 ± 0.2**
Liver Mass / Body Mass x 100%	-	-	-
Conjugated Dienes (µmol/g.liver)	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-
TTBARS (nmol/g.liver)	-	-	-
Cholesterol (mg/dl)	71 ± 3	98 ± 6**	102 ± 2**
Triglycerides (mg/dl)	55 ± 3	296 ± 24***^	193 ± 11**
Glucose Concentration (mg/dl)	98 ± 1	155 ± 14**	148 ± 7**
CYP2E1 Conc. (arbitrary units)	-	-	-
AST (U/l)	-	-	-
ALT (U/l)	-	-	-
Tumour Development	-	-	-
<p>Histology / Additional Observations; Plasma leptin levels in SHRSP/(fa/fa) and (fa/fa) rats elevated 70- and 50- fold higher than SHRSP/lzm rats which explains noted abnormalities in metabolism. SHRSP (fa/fa) rats also display obesity, hypertension and hyperinsulaemia and although beneficial for fatty liver development, strain more concerned with cardiovascular abnormalities.</p>			

Significantly different from the Control; *P<0.05, **P<0.01, P<0.001

^ = P<0.05 compared with Zucker (fa/fa) rats.

3.13.9 Zucker (fa/fa) Rats (continued)

Tomita <i>et al.</i> , (2004)	Zucker (fa/fa) Rats			
Treatment / Phenotype	(fa/wt) Control	(fa/fa) Control	(fa/wt) Alcohol	(fa/fa) Alcohol
Duration of Treatment	6 wks	6 wks	6 wks	6 wks
Sex	Male	Male	Male	Male
Age	4 wks	4 wks	4 wks	4 wks
No. of Animals	10	10	10	10
Terminal Body Mass (g)	-	-	-	-
Liver Mass (g)	-	-	-	-
Liver Mass / Body Mass x 100%	2.3 ± 0.1	2.4 ± 0.1	3.0 ± 0.2 **	4.0 ± 0.3 *
Conjugated Dienes (µmol/g.liver)	-	-	-	-
LOOH (nmol LOOH/g.liver)	-	-	-	-
TTBARS (nmol/g.liver)	-	-	-	-
Cholesterol (mg/dl)	-	-	-	-
Triglycerides (mg/g. liver)	15 ± 3	40 ± 9 **	60 ± 10	125 ± 15 *
Glucose Concentration (mg/dl)	-	-	-	-
CYP2E1 Conc. (arbitrary units)	-	-	-	-
AST (U/l)	-	-	-	-
ALT (U/l)	50 ± 8	60 ± 10	105 ± 12 **	495 ± 65 *
Tumour Development	-	-	-	-
Histology / Additional Observations; Alcohol-fed mice in both groups were shown to have increased plasma FFA and increased fat accumulation with moderate inflammation. Furthermore, the genetic disposition of leptin deficiency in these rats suppresses hepatic metallothionein (MT-1) and increases TNF-α which both enhance sensitivity to steatohepatitis. The data suggests that leptin deficiency causes a worse, more severe steatohepatitis following chronic ethanol exposure.				

*P<0.05 vs. the other groups, **P<0.05 vs. the control groups

3.13.9 Zucker (fa/fa) Rats (continued)

Nagao <i>et al.</i> , (2004)	Zucker (fa/fa) Rats	
Treatment / Phenotype	(fa/fa) Control	(fa/fa) CLA
Duration of Treatment	8 wks	8 wks
Sex	Male	Male
Age	6 wks	6 wks
No. of Animals	6	6
Terminal Body Mass (g)	-	-
Liver Mass (g)	-	-
Liver Mass / Body Mass x 100%	4.74 ± 0.36	3.50 ± 0.17*
Conjugated Dienes (µmol/g.liver)	-	-
LOOH (nmol LOOH/g.liver)	-	-
TTBARS (nmol/g.liver)	-	-
Cholesterol (mg/dl)	-	-
Triglycerides Plasma; (mmol/l)	2.08 ± 0.15	5.40 ± 0.31*
Glucose Concentration (mg/dl)	-	-
CYP2E1 Conc. (arbitrary units)	-	-
AST (U/l)	66 ± 13	24 ± 2*
ALT (U/l)	48 ± 7	19 ± 2*
MTP (nmol/mg. protein)	1.65 ± 0.10	1.91 ± 0.05*
Histology / Additional Observations; Administration of CLA (conjugated linoleic acid) was shown to protect against hepatic injury through raising levels of adiponectin production. CLA also improved insulin sensitivity and suppressed expression of TNF-α.		

Significantly different from the Control; *P<0.05, **P<0.01, P<0.001

3.13.10 A/J Mouse Model

Sahai <i>et al.</i> , (2004)	A/J Mice	
Treatment / Phenotype	A/J Mice Control	A/J Mice MCD
Duration of Treatment	12 wks	12 wks
Sex	Female	Female
Age	7-8 wks	7-8 wks
No. of Animals	3-5	3-5
Terminal Body Mass (g)	-	-
Liver Mass (g)	-	-
Liver Mass / Body Mass x 100%	3.63 ± 0.10	6.84 ± 0.25*
Conjugated Dienes (µmol/g.liver)	-	-
LOOH (nmol LOOH/g.liver)	-	-
TTBARS (nmol/g.liver)	0.67 ± 0.08	1.20 ± 0.08*
Cholesterol (mg/dl)	-	-
Triglycerides (mg/dl)	10 ± 2.5	78 ± 8.0*
Glucose Concentration (mg/dl)	-	-
CYP2E1 Conc. (arbitrary units)	1.00 ± 0.10	2.55 ± 0.18*
AST (U/l)	-	-
ALT (U/l)	27 ± 5.2	250 ± 14*
Tumour Development	-	-
Histology / Additional Observations; Macrovesicular steatosis in zones 2 and 3 after only 1 wk of the MCD diet with portal inflammation resulting after 4 wks. OPN expression was elevated early in development of NASH and preceded increases in TNF-α and oxidative stress.		

Significantly different from the Control; *P<0.01

3.13.11 C57Bl6/J Mouse Model

Leclercq <i>et al.</i> , (2000)	C57Bl6/J Mice	
Treatment / Phenotype	C57Bl6/J Control	C57Bl6/J MCD
Duration of Treatment	10 wks	10 wks
Sex	Female	Female
Age	8-10 wks	8-10 wks
No. of Animals	4	4
Terminal Body Mass (g)	24 ± 1.1	12 ± 0.1 **
Liver Mass (g)	1.01 ± 0.08	0.5 ± 0.01 ***
Liver Mass / Body Mass x 100%	4.22 ± 0.16	4.14 ± 0.05
Conjugated Dienes (µmol/g.liver)	-	-
LOOH (nmol LOOH/g.liver)	56 ± 8	100 ± 19 *
TTBARS (nmol/g.liver)	-	-
Cholesterol (mg/dl)	-	-
Triglycerides (mg/g. wet liver)	24 ± 9	79 ± 6 *
Glucose Concentration (mg/dl)	-	-
CYP2E1 Conc. (arbitrary units)	57 ± 18	167 ± 16 **
AST (U/l)	-	-
ALT (U/l)	22 ± 3	637 ± 128 **
Serum FFA (mmol/l)	-	-
Histology / Additional Observations; Control mice displayed no fat accumulation whereas the MCD diet induced steatosis mainly in zone 2 with large areas of inflammatory infiltrate. Collagen fibre deposits were also noted and confirmed pericellular fibrosis.		

Significantly different from the Control; *P<0.05, **P<0.01, P<0.001

3.13.12 Acyl CoA Oxidase Deficiency (AOX) Mouse Model

Unfortunately, results generated from work undertaken by Hashimoto *et al.*, (1999) are presented in such a manner that many fields of data are not applicable to the parameters being examined and contrasted in the other studies. This further illustrates the difficulty in comparing findings from different research papers. However, the data generated from the study conducted by Hashimoto *et al.*, (1999) was deemed relevant to the parameters being examined in this thesis and therefore has been included despite the alternative mode of presentation.

The work carried out by Hashimoto *et al.*, (1999) used PPAR α ^{-/-}, AOX^{-/-}, and PPAR α ^{-/-}/AOX^{-/-} mouse genotypes in an attempt to demonstrate the role of PPAR α and fatty acid oxidation in the pathogenesis of fatty liver. The schematic representation depicted below (figure 3.13.12.1) reveals the different extent of liver injury exerted on each model and the different steps involved in getting hepatic damage.

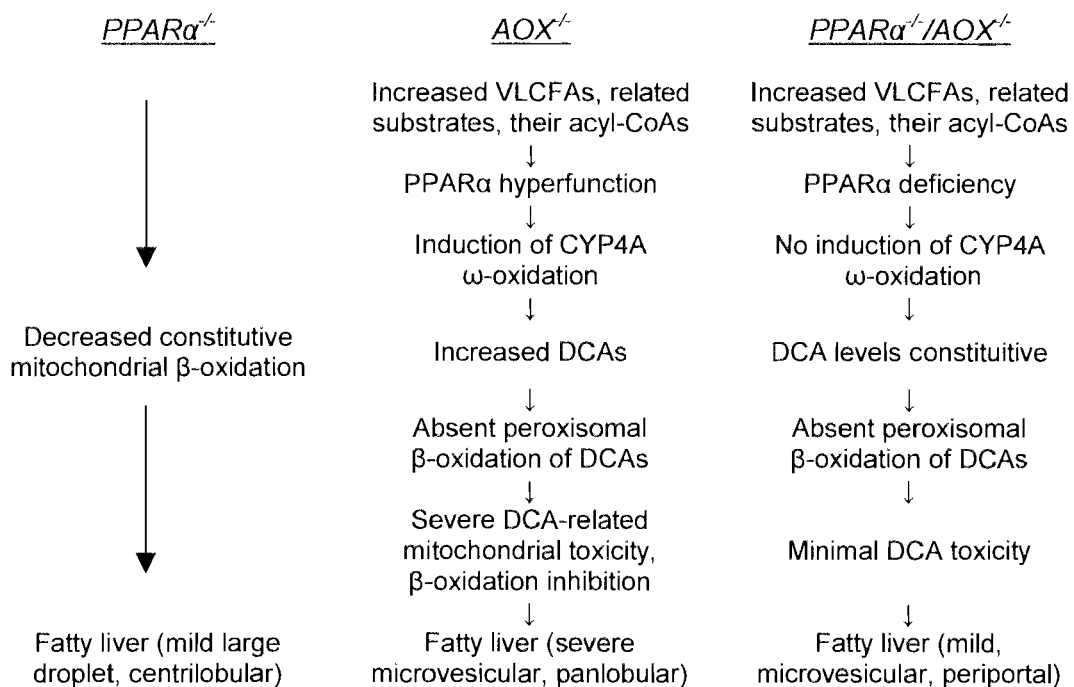


Figure 3.13.12.1 PPAR α ^{-/-}, AOX^{-/-}, and PPAR α ^{-/-}/AOX^{-/-} represent the mouse genotypes. In PPAR α ^{-/-} mice, constitutive mitochondrial β -oxidation is reduced and leads to slow progression of large droplet centrilobular steatosis in liver. The constitutive extra-mitochondrial fatty acid metabolism (peroxisomal and CYP4A metabolism of LCFAs and VLCFAs) is not adversely affected in the absence of PPAR α . In AOX^{-/-} mice, the sustained hyperactivity of PPAR α because of ligands that are not metabolized because of AOX deficiency results in overexpression of CYP4A enzymes and the LCFAs and VLCFAs not metabolised by peroxisomal β -oxidation because of lack of AOX are converted to DCAs by CYP4A ω -oxidation. Extensive microvesicular steatosis is considered a reflection of DCA induced inhibition of mitochondrial β -oxidation. In mice nullizygous for both PPAR α and AOX, the absence of PPAR α and lack of CYP4A induction resulted in the blunting of microvesicular steatosis (Hashimoto *et al.*, 1999).

In conclusion, alterations in PPAR α and AOX can effect lipid homeostasis and it is proposed that VLC and LC Acyl CoAs may play an important role in provoking

spontaneous peroxisomal proliferation by functioning as PPAR α ligands in the facilitation of microvesicular steatosis. Considering all the results from this study, it is proposed that both PPAR α and AOX are considerable factors in hepatic lipid metabolism and in the pathogenesis of fatty liver development and progression.

Chapter 4; Methods and Materials

4.1 Introduction

The research being performed in this thesis is of dual nature as the livers were examined for histology and biochemistry, spanning 2 different laboratories. The different aspects being investigated are from separate scientific backgrounds, but it was hoped that the biochemistry data would correlate with the histology findings. Despite significant biochemistry data being strongly indicative of hepatic damage in several instances, histology findings often failed to reveal the extensive injury that was anticipated. Therefore, histology and biochemistry methods and results have been described separately for clarity.

4.2 Histology

4.2.1 Tissue Processing

Immediately after sacrifice, fresh liver slices were obtained from the left lobe of the liver and stored in 10% formalin at room temperature until they could be processed. The processing begins with washing the tissue in 70% alcohol, before immersion in two changes of 10% neutral formalin for 2h and 1h respectively. The tissue was then dehydrated in two changes of 96% ethanol (10 mins each), followed by four changes of 100% ethanol (15 mins each). Once dehydrated, the tissue was immersed in two changes of xylol (15 mins each). Finally, the tissue was subjected to four changes of embedding wax (two changes at 20 mins each and two changes at 30 mins each) before being embedded in paraffin wax.

4.2.2 Stains / Light Microscopy

Sections were cut 4-6 μ m thick with a micrometer and stains were performed by standard laboratory techniques before being viewed under a light microscope.

These included;

(1) (H&E) - haematoxylin and eosin stain. This is the most widely used stain as it clearly illustrates a wide variety of different cell and tissue structures, is cost effective and not time-consuming. Haematoxylin stains the cell nuclei a blue-black colour and provides good, clearly visible intra-nuclear detail whereas the eosin component stains the cell cytoplasm and connective tissue fibres in shades of pink, orange and red (Bancroft and Stevens, 1990). This stain is used for overall assessment of hepatic injury and cell disruption.

(2) (SR) sirius red stain. This stain uses the cotton dye Sirius red F3B for the detection of amyloid. This is used to gauge the extent of fibrosis and is dependent on the linearity of the dye molecule and β -pleated sheet configuration (Bancroft and Stevens, 1990).

(3) (D/PAS) periodic-acid Schiff after diastase digestion stain. This reaction is useful for detecting tissue carbohydrates, specifically for glycogen when a diastase digestion stage is incorporated into the stain (Bancroft and Stevens, 1990). Periodic acid promotes an oxidative cleavage of carbon-to-carbon bonds or amino derivatives to form dialdehydes which react with fuschin-sulphurous acid and forms a magenta colour upon binding with paraosanine (Stoward, 1967). This stain is of particular use in evaluation of debris-containing Kupffer cells.

4.3 Biochemistry

4.3.1 Lipid Extraction

The original method for extracting lipids described by Folch (1957) is now commonly referred to as the Folch technique. There were however, practicality problems with the original process as it was time consuming and used large volumes of solvent making it in-appropriate for lipid peroxidation studies. An improved modification to the Folch technique was made by Bligh and Dyer (1959) making the method simpler, less time consuming and as a result is well adapted for routine lipid analyses.

The essential concept underlying the technique is that homogenised tissue in a mixture of chloroform and methanol creates a monophasic solution which then separates into a biphasic solution when diluted with chloroform and an aqueous solvent. The lipid fraction remains in the chloroform layer (non-aqueous phase) while the non-lipid impurities are contained in the methanol and water layer (aqueous phase). Chloroform, being the denser liquid is below the aqueous phase.

Freshly frozen liver samples were thawed on ice and approximately 70mg of each liver sample was homogenised in 2ml chilled SEAP (0.15M NaCl, 0.02M NaH₂PO₄, 0.01g % NaN₃, 0.001M EDTA) using a Virtis Handishear®. The resulting solution was sonicated with twenty 1 sec bursts at an output power of 4-6 watts using a Virtis Virsonic®. Sonication was carried out to completely obliterate any remaining subcellular structure. The homogenate from each liver sample was aliquoted (0.8ml per tube) and utilised for measurement of lipid peroxidation. The remaining homogenate of each sample (1.2ml) was stored at -80°C.

To 0.8ml chilled homogenised liver, 1ml chilled chloroform and 2ml chilled methanol were added and vortexed for ten seconds. The solution was then centrifuged at 3000 rpm for 15 mins at 4°C in a Beckman GS-6R centrifuge. The resulting supernatant was then transferred to a clean tube on ice. The remaining pellet was washed by addition of 1ml chloroform, 2ml methanol, 0.8ml SEAP and vortexed for ten seconds. The solution was subjected to further centrifugation as described above. To remove non-lipid impurities from the collected supernatant, a phase split was necessary and was achieved by addition of 2ml chloroform, 2ml SEAP, vortexing for ten seconds and then centrifugation at 3000 rpm for 10 mins at 4°C. The upper aqueous phase, containing the impurities, was removed by suction, leaving the lower non-aqueous phase that contains the lipids. The lower phase was dried in a Pierce Reacti-therm module under nitrogen gas at room temperature. The resultant dried extract was then re-suspended in 200µl chloroform and 20µl aliquots were placed in microreaction vials (Eppendorf tubes). The Eppendorf tubes were then placed in the Pierce Reacti-therm module as described above, to completely dry the solvent. The dried lipid extractions were stored in the dark at -80°C freezer until required for biochemical assessment.

Owing to small sample volumes, plasma lipid studies were not practicable. The plasma was used where possible for glucose and alcohol, as well as transaminase alterations.

4.3.2 Conjugated Dienes (CD)

Conjugated dienes manifest in unsaturated fatty acids as an early indicator of lipid peroxidation reactions (Vasankari *et al.*, 1995). Their appearance is due to a rearrangement of double bonds following free radical attack (Recknagel and Glende, 1984). The molecules containing CD are characterized by an intense absorption (K band) which may range from 215 to 250 nm, depending on the presence of nearby substituent groups (Recknagel and Glende, 1984). The spectra of lipids that contain CD are characterised by an intense K band near 233 nm with a lesser secondary absorption in the region of 260-280 nm due to ketone dienes.

Conjugated dienes were measured using a GBC UV/VIS 916 spectrophotometer. The baseline was established using distilled water and the absorbance of a cyclohexane blank was measured to verify purity. At 234 nm, unoxidised cyclohexane has an optical density of 0.049.

The CD content of each sample was measured by adding 1ml cyclohexane to the 20µl dried lipid extract and vortexing for ten seconds. The mixture containing cyclohexane and the experimental lipid sample were transferred to a quartz cuvette for reading in the spectrophotometer. The absorbance of each sample (in duplicate) was read at 234 nm immediately after mixing. The concentration of conjugated dienes from each sample was calculated using a molar extinction coefficient (Σ) of $2.95 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

4.3.3 Lipid Hydroperoxides (LOOH)

Lipid peroxidation reactions are complex and involve alterations of biosynthetic unsaturated fatty acids. These reactions yield conjugated dienes as previously described, lipid hydroperoxides (LOOH) and other fragmentation products including aldehydes and carboxylic acids. Jiang *et al.* (1991) modified a method, initially described by Gupta (1973) which resultantly measures the LOOH indirectly through the oxidation of $\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$ by peroxides. This occurs in the presence of a ferric-complexing dye, xylenol orange, which has maximal absorption at 560 nm. The method will detect Fe^{3+} , H_2O and lipid hydroperoxides but lipid extracts will by definition only contain lipid hydroperoxides. The determination of these lipid hydroperoxides will assist in the evaluation of the oxidative stress occurring in the liver (Jiang *et al.*, 1991).

To measure the LOOH concentration, several reaction components were added to the dried lipid extract. Firstly, addition of 100µl chloroform and vortexing for ten seconds was necessary to re-dissolve the lipid. Secondly, 350µl of methanol was added and vortexed for ten seconds to generate a monophasic mixture. The aqueous component of the assay is subsequently added, 50µl "nanopure" H_2O , and vortexed for ten seconds. Finally, 600µl of FOX 2 Mix* was added and vortexed for ten seconds. Standard reactions were prepared as described above, except 50µl of the relevant standard was used as a replacement for nanopure H_2O . The tubes were left at room temperature for 20 mins for the reaction to occur and the absorbance was read immediately after at 560 nm. The concentration of lipid hydroperoxides in each experimental sample (in duplicate) was determined from a non-linear regression curve of the t-butyl hydroperoxide (t-BHP) standards. The reliability of the assay was assessed by the calculating the ratio of t-BHP to FeCl_3 at an absorbance of 1.00. The recorded value was 6.79 ± 0.23 .

***Fox 2 Mix:** 1 part FOX 2A (2.5mM ferrous ammonium sulphate, 2mM Xylenol Orange in 0.25mM H₂SO₄)

4.5 part FOX 2B (0.88mM BHT in methanol), always prepared fresh.

FeCl₃ Standards

FeCl ₃ [4mM] Stock Solution (μl)	Volume of Water (μl)	Concentration of Working Solution [mM]	Quantity of Ferric Chloride [nMol]
25	975	0.1	5
50	950	0.2	10
100	900	0.4	20
200	800	0.8	40
400	600	1.6	80
500	500	2.0	100
800	200	3.2	160

t-BHP Standards

t-BHP [0.696mM] Stock Solution (μl)	Volume of Water (μl)	Concentration of Working Solution [mM]	Quantity of Butylated Hydroperoxides [nMol]
5	995	0.0042	0.2
10	990	0.0075	0.35
20	980	0.015	0.75
40	960	0.03	1.5
200	800	0.14	7
400	600	0.28	14
800	200	0.56	28

4.3.4 Thiobarbituric Acid Reactive Substances (TBARS)

TBARS are intermediate to late products in the process of lipid peroxidation damage. The process by which TBARS produce a secondary product red dye, after reaction with malondialdehyde (MDA) has been widely used as a sensitive assay method for lipid peroxidation in animal tissues (Ohkawa *et al.*, 1978). The assay measures the red chromogen, formed as a result of MDA reacting with two molecules of thiobarbituric acid and was carried out according to the method of Asakawa and Matasushita (1979), with slight modifications.

To determine the concentrations of TBARS from experimental samples, 50μl chloroform was initially added to each dried extract and vortexed for ten seconds to re-dissolve the lipid. This was transferred to a tube, to which 50μl 0.27% FeCl₃ was added and also vortexed for ten seconds. The next step was to add either 100μl ethanol for measurement of “total” TBARS (i.e. the amount present at time of death and those generated during tissue handling) or 100μl 0.22% butylated hydroxytoluene (BHT), an antioxidant for measurement of “free” TBARS (i.e. the amount of TBARS present in the sample at time of death) and vortexed for ten seconds. These may be lost in the process of extraction. To all tubes, 750μl glycine buffer, pH 3.6 (0.2M glycine HCl) was added and vortexed for ten seconds, after which 750μl TBA reagent (0.5% thiobarbituric acid, 0.3% SDS) was added and

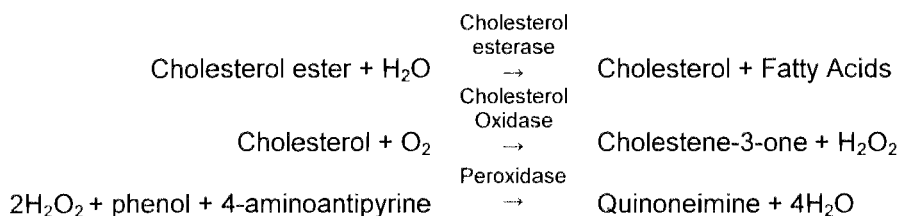
vortexed again. Chloroform and water blanks were prepared by the same method, except that 50µl chloroform and 50µl were added in place of the re-suspended dried extract respectively. A glass bead was inserted to each tube, which was then capped by marbles and boiled at 100°C on a Techne® DB-3A dri-block. After 20 mins, the tubes were removed and allowed to cool to room temperature.

After cooling, 250µl of the pink aqueous phase from each experimental lipid sample was transferred to a microtitre plate. The absorbance of each sample (in duplicate) was read at 540 nm. The concentration of total TBARS and free TBARS was calculated using a molar extinction coefficient (ϵ) of $1.09 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Free TBARS can be measured but were not viewed as significant in extracts because of potential losses of volatile compounds and were therefore not analysed in the studies presented in this thesis.

4.3.5 Total Cholesterol (TC)

Total cholesterol concentration was measured using a commercial kit (KAT™) (No. AD704CH). The enzymatic method used in the kit is derived from the procedure described by Allain et al., (1974). Cholesterol esters are hydrolysed using cholesterol esterase as a catalyst to produce cholesterol and fatty acids. The resultant cholesterol is oxidised with cholesterol oxidase and forms cholestene-3-one and hydrogen peroxide. Under the catalytic influence of peroxidase and phenol, the hydrogen peroxide reacts with 4-aminoantipyrine to produce the chromogenic red indicator quinoneimine (see below).



The supplied commercial standard was not used as more accurate standard curves were being produced from the supply of cholesterol in the laboratory. However, the provided enzyme reagent was still used for TC determination.

A series of standards were prepared by dissolving cholesterol (1µg/µl) in measured volumes of ethanol and mixing, as described in table (4.3.5.1). The standards were dried in the fume cupboard after mixing. To the dried cholesterol standards, 50µl

ethanol, 15µl 1% Triton X-100 and 500µl 0.9% NaCl were added and vortexed for ten seconds after each. Experimental samples were prepared by adding 50µl ethanol, 15µl 1% Triton X-100 and 500µl 0.9% NaCl to 20µl dried extract and vortexing for ten seconds after each. From the prepared standards and sample mixtures, 125µl was transferred to a microtitre plate, to which 125µl of the provided enzyme reagent was added and mixed. The plate was then left at room temperature for 30 mins for the reaction to occur. The absorbance was read at 500nm with the quantity [µg] of TC of each sample (in duplicate) being calculated from a linear regression curve of the standards.

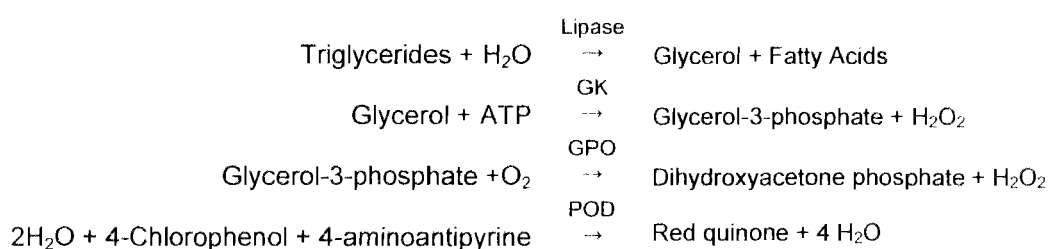
4.3.5.1 Cholesterol Standards

Weight of Cholesterol (µg)	Volume of Ethanol (µl)	Cholesterol Content (µg)
0	2.5	0
2.5	2.5	2.5
5	5	5
7.5	7.5	7.5
10	10	10
15	15	15
20	20	20
25	25	25
30	30	30
40	40	40
50	50	50

Precinorm® U, a commercially provided standard with a TG range of 111-153 mgdl⁻¹ was used as a calibrator.

4.3.6 Hepatic Triglycerides (TG)

Hepatic triglyceride concentration was measured using a commercial kit (KAT™) (No. AD801T). The method derived from Werner (1981), Triglycerides are hydrolysed using lipases to produce glycerol and fatty acids. The resultant glycerol is phosphorylated with the assistance of glycerol kinase (GK) and consequent oxidation with glycerol-3-phosphate oxidase (GPO) forms dihydroxyacetone phosphate and hydrogen peroxide. Under the catalytic influence of peroxidase (POD), the hydrogen peroxide reacts with 4-aminoantipyrine and 4-chlorophenol to produce the chromogenic indicator red quinone (see below).



A series of standards was prepared, using the supplied commercial standard, by mixing measured volumes with 0.9% NaCl, as described in table (4.3.6.1). The standards were then mixed with 50µl ethanol and 15µl 1% Triton X-100. Experimental samples were prepared by adding 50µl ethanol, 15µl 1% Triton X-100 and 500µl 0.9% NaCl to 20µl dried extract and vortexing for ten seconds after each. From the prepared standards and sample mixtures, 125µl was transferred to a microtitre plate, to which 125µl of the provided enzyme reagent was added and mixed. The plate was then placed in an oven at 37°C for 5 mins for the reaction to occur. The absorbance was read at 500nm with the quantity [µg] of TG in each sample (in duplicate) being calculated from a linear regression curve of the standards.

4.3.6.1 Triglyceride Standards

Volume of TG Standard (µl)	Volume of 0.9% NaCl (µl)	Triglyceride Content (µg)
0	500	0
1	499	2
2.5	497.5	5
5	495	10
10	490	20
20	480	40
25	475	50
30	470	60
40	460	80
50	450	100

Precinorm® U, a commercially provided standard with a TG range of 111-153 mgdl⁻¹ was used as a calibrator

4.3.7 Bicinchoninic Acid (BCA) Assay of Proteins

BCA is a water-soluble compound that is a highly specific reagent for cuprous ions (Cu⁺). The biuret reaction of BCA and Cu⁺ yields an intense purple colour that is very stable and displays maximal absorption at 562nm. Protein quantitation by BCA was measured according to the method of Smith *et al.*, (1985). The method of Lowry *et al.*, (1951) is more commonly used for measurement of proteins and is very similar in context to that of Smith *et al.*, (1985) as both techniques are dependent on the formation of a colour complex produced from a biuret reaction. However the quantitative method of Smith *et al.*, (1985) was preferred as it is regarded as being simpler, sensitive and more tolerant to interfering substances (Brown *et al.*, 1989).

To determine the protein concentration, homogenate was firstly diluted 1:20 with nanopure H₂O. 10µl of this diluted mixture was transferred to a microtitre plate and brought to a final volume of 100µl, also using nanopure H₂O, then shaken for one

minute. 100µl of BCA-µD (see BCA solution list below) was added and shaken for a further minute and repeated again. The addition of BCA-µD was carried out in two stages to ensure thorough mixing of the homogenate and the BCA solution. After the addition of BCA-µD and shaking, the microtitre plate was floated on the surface of a waterbath at 60°C for 1 hr for the reaction to occur. The microtitre plate was then briefly cooled and shaken for 1 min before the absorbance was read at 560 nm which has an optical density between 0.2 and 1.6. The concentration of proteins in each experimental sample (in duplicate) was determined from a non-linear regression curve of the bovine serum albumin (BSA) standards. Standard reactions were prepared by diluting BSA in nanopure H₂O as detailed in the table below (4.3.7.1).

BCA Solutions: BCA-µA (8% Na₂CO₃, 1.6% NaOH, 1.6% Na₂ tartate and NaHCO₃, pH 11.25)
 BCA-µB (4% BCA in nanopure H₂O)
 BCA-B (4g CuSO₄ in 100ml nanopure H₂O)
 BCA-µC (100 parts BCA-µB +4 parts BCA-B), unstable and should be made fresh.
 BCA-µD (equal parts of BCA-µA + BCA-µC), unstable and should be made fresh.

4.3.7.1 Albumin Standards

Volume of Stock Solution (µl)	Concentration of Stock Solution (µg/µl)	Volume of Water (µl)	Quantity of Albumin [µg]
0	0	100	0
2	0.25	98	0.5
4	0.25	96	1
10	0.25	90	2.5
20	0.25	80	5
40	0.25	60	10
4	5	96	20
8	5	92	40
16	5	84	80

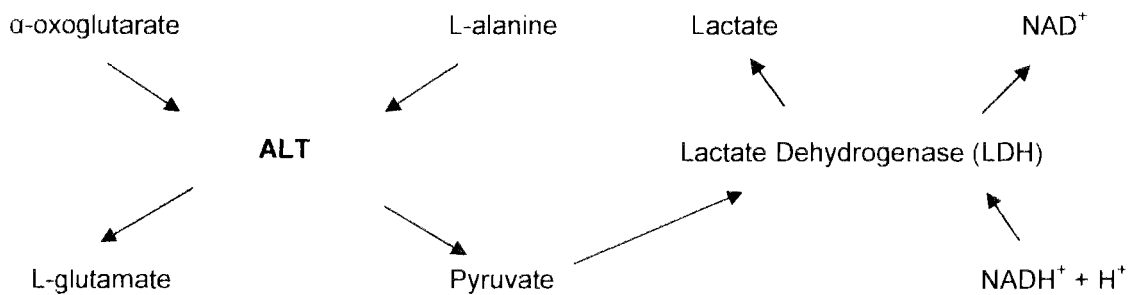
4.3.8 Measurement of Aminotransferases

Aminotransferases are identified as a family of enzymes that operate as catalysts in the interconversions of amino acids and α-oxoacids through transfer of amino groups. The release of aminotransferases indicates the presence of hepatic injury, however, these enzymes may be released before the clinical manifestations of liver disease become evident (Tietz, 1987). The measurement of aminotransferases was determined as described by Tietz (1987).

Blood samples were taken at time of termination of the animals and were immediately subjected to centrifugation in a Labnet Spectrafuge 16M at 14,000 rpm for 10 mins to produce serum. The serum was stored into 30µl aliquots at -70°C until required for biochemical assessment.

4.3.8.1 Alanine aminotransferase (ALT)

ALT is a liver-specific enzyme which catalyses an amino transfer reaction. Under the catalytic influence of ALT, an amino group is transferred from L-alanine to α -oxoglutarate with the assistance of pyridoxal-5'-phosphate (P-5-P), to generate pyruvate and L-glutamate. However, the direct measurement of aminotransferase activity is difficult and has resulted in the activity being determined indirectly by assessing pyruvate production via lactate, (as displayed below).

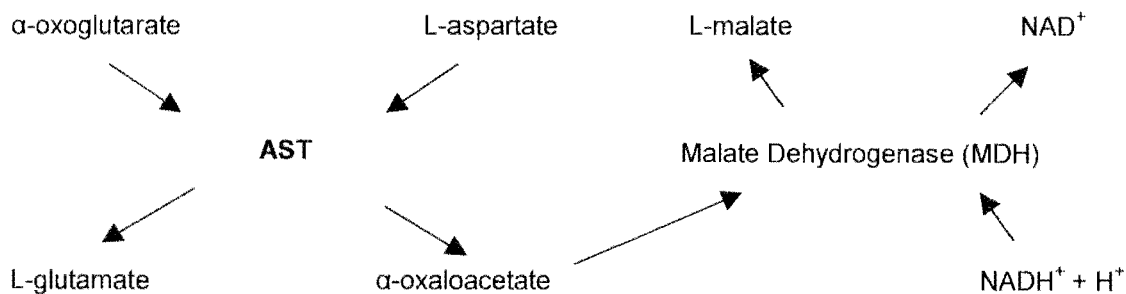


Aminotransferase buffer (13.6g KH_2PO_4 , 3.3g NaOH in 1 litre distilled H_2O , pH 7.4) and oxaloacetic acid (0.73g oxaloacetic acid in 30ml buffer, pH 7.4, adjusted to a final volume of 50ml) were prepared and stored at 4°C and -20°C , respectively.

In addition, a mixture containing 36mg NADH, 19ml buffer, $50\mu\text{l}$ lactate dehydrogenase (25mg/ $2.5\text{ml H}_2\text{O}$) and L-alanine (7.6g L-alanine in 100ml buffer, pH 7.4) was freshly prepared, of which $650\mu\text{l}$ was added to $50\mu\text{l}$ serum and mixed. The solution remained at room temperature for 15 mins to permit the NADH-dependent reduction of endogenous oxoacids. Finally, $50\mu\text{l}$ oxaloacetic acid was added to all samples and vortexed for ten seconds. The absorbance was read at 340nm every 30 secs for 5 mins and the catalytic activity (quantity of enzyme that catalyzes reaction of $1\mu\text{ml}$ substrate per min) was represented as U/L.

4.3.8.2 Aspartate aminotransferase (AST)

Elevated AST activity signifies a disruption in liver cell integrity and the key concept for determination of AST levels is similar to that for ALT (Tietz, 1987). The notable difference being that, under the catalytic influence of AST, amino groups are transferred from L-aspartate to α -oxoglutarate and resultantly generate α -oxaloacetate and L-glutamate. As mentioned previously, the direct measurement of aminotransferase activity is difficult and has also resulted in AST activity being determined indirectly by measuring α -oxaloacetate production via L-malate (as displayed below).

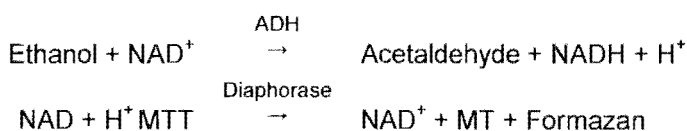


Aspartic acid solution (pH 7.4) was prepared by mixing 2.66mg aspartic acid in 100ml aminotransferase buffer. Malate dehydrogenase (MDH) was prepared in distilled water to a concentration of 5mgmg^{-1} . Oxaloacetic acid solution was prepared by dissolving 0.735g oxaloacetic acid in 50ml buffer and was stored at -20°C in 5ml aliquots.

In addition, a reaction mixture containing 36mg NADH, 19ml buffer, 4.5ml aspartic acid solution and $50\mu\text{l}$ MDH was freshly prepared, of which $650\mu\text{l}$ was added to $50\mu\text{l}$ serum and mixed. The solution remained at room temperature for 15 mins to permit the NADH-dependent reduction of endogenous oxoacids. Finally, $50\mu\text{l}$ oxaloacetic acid was added to all samples and vortexed for ten seconds. The absorbance was read at 340nm every 30 secs for 5 mins and the catalytic activity was represented as U/L.

4.3.9 Measurement of Blood Alcohol Levels

Ethanol concentration was measured using a quantitative assay for gauging levels of alcohol in mouse serum. A commercial kit (AxSYM REA Ethanol Reagent Pack) (Ref 3B32-20) was based on the enzymatic method described by Shaffar and Stroupe (1983) using an Abbot AxSYM® Analyser. The oxidation of ethanol is catalysed by alcohol dehydrogenase (ADH) which produces acetaldehyde and reduction of the coenzyme nicotinamide adenine dinucleotide NAD (Tietz, 1970). The resultant NAD reacts with monotetrazolium dye (MTT) in the presence of diaphorase to generate a colour change by which ethanol concentration can be determined (see below).



The assay of ethanol concentration used $200\mu\text{l}$ of mouse serum which was stored frozen at 4°C prior to use. The samples, along with ethanol calibrators were loaded

onto reaction wells in the Abbot AxSYM® Analyser and mixed with the 3 ethanol reagents from the commercial kit to determine the concentration of ethanol present. Low, medium and high dose calibrators were commercially provided (X Systems® REA Ethanol Calibrators) and displayed the range values as described in table (4.3.9.1). The reagents supplied included 5% nicotinamide adenine dinucleotide (NAD) in sodium citrate buffer, 5% yeast alcohol dehydrogenase and <1% diaphorase in a protein stabiliser solution and <1% monotetrazolium dye and <0.01% fluorescein in solvent. Once the reactions were complete, values of experimental samples were calculated for ethanol concentration using a calibrated curve derived from the ethanol calibrators.

4.3.9.1 Ethanol Standards

Dose Level	Ethanol Concentration (mmol/l)	Range (mmol/l)
Low	10.85	7.38 – 14.32
Medium	21.71	16.94 – 26.47
High	54.27	43.50 – 65.03

AxSYM® system specific for this assay was used to calibrate controls. The values falling below the minimum range were calculated using a linear curve.

4.4 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 4 Software. Comparisons were conducted between 2 treatment groups (*e.g.* heterozygous mice (db/wt) and homozygous mice (db/db)) using non-parametric, two-sided tests. To compare the effect of one treatment within groups (*e.g.* effects of alcohol in (db/wt) or (db/db) groups), non-parametric single variable analyses of variance (Kruskal-Wallis test) were conducted. Differences achieved statistical significance when $p < 0.05$. Linear regression was available on the same software.

The majority of values being presented for statistical analyses will be displayed with 3 decimal places unless more are required.

4.5 Animal Studies

4.5.1 Assessment of the db/db Mouse Model on Regular Chow over 20 Weeks.

Male and female 20 week old wild type (wt/wt), heterozygous (db/wt) and homozygous (db/db) mice were received from an in-house breeding programme at the UCT Animal Facility. However, due to poor littering numbers, this study could not be conducted concurrently and as a result the animals were placed on the protocol and sacrificed as they became available over a 15 month period. The animal ethics approval reference for this study is 03/042.

The mice were allocated into 3 different groups dependent on their genotype and assigned an animal number prior to termination. The resultant 3 groups based on the limited number of animals that did become available were made up as follows;

6 Male and 6 Female wild type mice (wt/wt)

16 Male, 8 Female heterozygous mice (db/wt)

10 Male, 14 Female homozygous mice (db/db)

During the study and prior to termination, the mice were housed by genotype, sex and litter parentage to minimise fighting. The animals were fed on a diet of regular chow (EPOL mice cubes reg no. V1787) over the 20 week period which consisted of the following; protein – 180g/kg, moisture – 120g/kg, fibre – 60g/kg, fat – 25g/kg, calcium – 18g/kg and phosphorous – 7g/kg. Sterilised wood shavings were used as bedding and suitable nesting material was also provided. The shavings and nesting were changed at least once a week. Temperature and humidity conditions were constantly controlled and a 12 hour light/dark cycle in operation. The mice were housed in Room 132 of the Rodent Accommodation Section at Chris Barnard Building, Cape Town South Africa.

Body weights and glucose concentration were recorded at termination.

The scheduled terminations occurred when the animals were 20 weeks old. The animals were killed by cardiac puncture, followed by exsanguination under a combination anaesthetic of ketamine and xylazine. The anaesthetic drugs are obtained from the suppliers with the following concentrations; 100mg/ml and 20 mg/ml respectively. 1.2 ml of ketamine is mixed with 0.8 ml of xylazine and 8 ml of saline is added to give a final volume of 10 ml. Each mouse is injected with a dose of 0.2 ml (i/p).

Blood was collected in eppendorf tubes for protein and enzyme determination. Livers were removed after dissection and weighed before being wrapped in tin foil and snap-frozen in liquid nitrogen. The liver samples were stored at -80 °C until required for biochemical analysis. Another portion of the liver was processed for histological examination.

4.5.2 Impact of Various Oils, Administered via the Diet (4 Weeks)

Male and female 12 week old heterozygous (db/+) mice were received from an in-house breeding programme at the UCT Animal Facility on 24 January 2005. The animal ethics approval reference for this study is 04/029.

The mice were randomly allocated into 4 treatment groups, assigned an animal number and allowed to acclimatise for a 1 week period prior to commencement of treatment. The 4 groups were made up as follows;

3 Male and 3 Female heterozygous mice (db/wt) = Control; chow only

3 Male and 3 Female heterozygous mice (db/wt) = Sunflower Oil

3 Male and 3 Female heterozygous mice (db/wt) = Canola Oil

3 Male and 3 Female heterozygous mice (db/wt) = Sunflower Oil and Cholesterol

The mice were housed by sex and dose group. Sterilised wood shavings were used as bedding and suitable nesting material was also provided. The shavings and nesting were changed at least once a week. Temperature and humidity conditions were constantly controlled and a 12 hour light/dark cycle in operation. The study was conducted in Room 132 of the Rodent Accommodation Section at Chris Barnard Building, Cape Town South Africa.

The feed used during this study was EPOL mice cubes reg no. V1787 (same nutritional contents as described previously 4.5.1) and was mixed with the various oils at 5g oil per 100g chow. The cholesterol was added at 60mg per 100g chow in addition to sunflower oil for the respective treatment group. The specially formulated diets were made available *ad libitum* to the animals. The diets were analysed for early, intermediate and late lipid peroxidation contents in an attempt to gauge the differences between the diets being administered to the animals (figure 4.5.2.1). Water was also made *ad libitum* to the mice.

4.5.2.1 Lipid Peroxidation Contents of Diet

	CD ($\mu\text{mol/g}$. liver)	LOOH ($\mu\text{mol/g}$. liver)	TBARS ($\mu\text{mol/g}$. liver)
Regular Chow	3.4	0.261	1.865
Sunflower Oil	27	24.4	62.3
Canola Oil	17	20.0	43.2

The animals were dosed continuously *via* their diet for 4 consecutive weeks. Body weights were recorded weekly and at termination. The scheduled terminations occurred after completion of 4 weeks of treatment. The animals were killed by cardiac puncture, followed by exsanguination under ketamine and xylazine anaesthesia (as described in 4.5.1). Blood was collected in eppendorf tubes for protein determination. Livers were dissected and weighed before being wrapped in tin foil and snap frozen in liquid nitrogen. The liver samples were stored at $-80\text{ }^{\circ}\text{C}$ until required for biochemical analysis. Another portion of the liver was processed for histological examination.

4.5.3 Study of Short Period of Alcohol Administration (4 Weeks)

Male and female 14 week old wild type (wt/wt), heterozygous (db/wt) and homozygous (db/db) mice were received from an in-house breeding programme at the UCT Animal Facility. However, due to poor littering numbers, this study could not be conducted concurrently and as a result the animals were added as they became available over a 7 month period. The animal ethics approval reference for this study is 03/042.

The mice were allocated into 6 treatment groups, assigned an animal number and allowed to acclimatise for a 1 week period prior to commencement of treatment. The resultant 6 groups based on the limited number of animals that did become available were made up as follows; (please note that saccharine was used to disguise the taste of alcohol to ensure the mice drunk from their water bottles).

1M, 1F wild type mice (wt/wt) = Water and 0.025% (w/v) Saccharine

1M, 1F wild type mice (wt/wt) = 20% Ethanol and 0.025% (w/v) Saccharine

2M, 2F heterozygous mice (db/wt) = Water and 0.025% (w/v) Saccharine

2M, 2F heterozygous mice (db/wt) = 20% Ethanol and 0.025% (w/v) Saccharine

2M, 2F homozygous mice (db/db) = Water and 0.025% (w/v) Saccharine

1M, 3F homozygous mice (db/db) = 20% Ethanol and 0.025% (w/v) Saccharine

The mice were housed by genotype, dose group and litter parentage to minimise fighting. Sterilised wood shavings were used as bedding and suitable nesting material was also provided. The shavings and nesting were changed at least twice a week. Temperature and humidity conditions were constantly controlled and a 12 hour light/dark cycle in operation. The water-ethanol mixes were prepared twice weekly and stored under a seal to prevent evaporation of alcohol. The study was conducted in Room 132 of the Rodent Accommodation Section at Chris Barnard Building, Cape Town South Africa.

The animals being treated with 20% ethanol and 0.025% (w/v) saacharine, were given a 2 week adaptation period to reach the required concentration of ethanol. At the start of treatment, the ethanol concentration was 3% and increased by 3% intervals every 2 days until the required 20% concentration was achieved. These animals were then treated with the desired 20% concentration for 4 weeks. The ethanol and water for their respective treatment groups were made available *ad libitum* to the animals. The feed used during this study was EPOL mice cubes and was also made *ad libitum*.

All animals were dosed continuously *via* their water bottles for 6 consecutive weeks. The animals being treated with ethanol had 2 weeks adaptation and 4 weeks at 20% concentration. Body weights were recorded weekly and at termination.

The scheduled terminations occurred when the animals were 20 weeks old. The animals were killed by cardiac puncture, followed by exsanguination under ketamine and xylazine anaesthesia (as described in 4.5.1). Blood was collected in eppendorf tubes for protein and enzyme determination. Livers were dissected and were unfortunately not weighed before being wrapped in tin foil and snap frozen in liquid nitrogen due to a technical error. The liver samples were stored at -80 °C until required for biochemical analysis. Another portion of the liver was processed for histological examination.

4.5.4 Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice

Male 5 week old heterozygous (db/wt) mice and homozygous (db/db) mice were received from Jackson Laboratory, Bar Harbour, Maine, USA on 28 January 2005 and were immediately housed in the UCT Animal Facility for a 4 week acclimatisation period prior to commencement of treatment. The animal ethics approval reference for this study is 03/042.

Following the acclimatisation period, the mice (all male) were randomly allocated into 4 treatment groups and assigned an animal number. The 4 groups were made up as follows; (please note that saccharine was used to disguise the taste of alcohol to ensure the mice drunk from their water bottles).

7 heterozygous mice (db/wt) = Water and 0.025% (w/v) Saccharine

6 heterozygous mice (db/wt) = 20% Ethanol and 0.025% (w/v) Saccharine

7 homozygous mice (db/db) = Water and 0.025% (w/v) Saccharine

5 homozygous mice (db/db) = 20% Ethanol and 0.025% (w/v) Saccharine

The mice were housed by genotype, dose group and litter parentage to minimise fighting. Sterilised wood shavings were used as bedding and suitable nesting material was also provided. The shavings and nesting were changed at least twice a week.

Temperature and humidity conditions were constantly controlled and a 12 hour light/dark cycle in operation. The water-ethanol mixes were prepared twice weekly and stored under a seal to prevent evaporation of alcohol. The study was conducted in Room 132 of the Rodent Accommodation Section at Chris Barnard Building, Cape Town South Africa.

The animals being treated with 20% ethanol and 0.025% (w/v) saccharine were given a 3 week adaptation period to reach the required concentration of ethanol. At the start of treatment, the ethanol concentration was 2% and increased by 2% intervals every 2 days until the required 20% concentration was achieved. These animals were then treated with the desired 20% concentration for 8 weeks. The ethanol and water for their respective treatment groups were made available *ad libitum* to the animals. The feed used during this study was EPOL mice cubes and was also made *ad libitum*.

All animals were dosed continuously *via* their water bottles for 11 consecutive weeks. The animals being treated with ethanol had 3 weeks adaptation and 8 weeks at 20% concentration. Body weights were recorded weekly and at termination.

The scheduled terminations occurred when the animals were 20 weeks old. The animals were killed by cardiac puncture, followed by exsanguination under ketamine and xylazine anaesthesia (as described in 4.5.1). Blood was collected in eppendorf

tubes for protein and enzyme determination. Livers were dissected and weighed before being wrapped in tin foil and snap frozen in liquid nitrogen. The liver samples were stored at -80 °C until required for biochemical analysis. Another portion of the liver was processed for histological examination.

4.5.5 Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice

Male 5 week old heterozygous (db/wt) mice and homozygous (db/db) mice were received from Jackson Laboratory, Bar Harbour, Maine, USA on 28 January 2005 and were immediately housed in the UCT Animal Facility for a 4 week acclimatisation period prior to commencement of treatment. The animal ethics approval reference for this study is 03/042.

Following the acclimatisation period, the mice (all male) were randomly allocated into 4 treatment groups and assigned an animal number. The 4 groups were made up as follows; (please note that saccharine was used to disguise the taste of alcohol to ensure the mice drunk from their water bottles).

7 heterozygous mice (db/wt) = Water and 0.025% (w/v) Saccharine

7 heterozygous mice (db/wt) = 20% Ethanol and 0.025% (w/v) Saacharine

7 homozygous mice (db/db) = Water and 0.025% (w/v) Saccharine

7 homozygous mice (db/db) = 20% Ethanol and 0.025% (w/v) Saacharine

The mice were housed by genotype, dose group and litter parentage to minimise fighting. Sterilised wood shavings were used as bedding and suitable nesting material was also provided. The shavings and nesting were changed at least twice a week. Temperature and humidity conditions were constantly controlled and a 12 hour light/dark cycle in operation. The water-ethanol mixes were prepared twice weekly and stored under a seal to prevent evaporation of alcohol. The study was conducted in Room 132 of the Rodent Accommodation Section at Chris Barnard Building, Cape Town South Africa.

The animals being treated with 20% ethanol and 0.025% (w/v) saacharine, were given a 3 week adaptation period to reach the required concentration of ethanol. At the start of treatment, the ethanol concentration was 2% and increased by 2% intervals every 2 days until the required 20% concentration was achieved. These animals were then treated with the desired 20% concentration for 20 weeks. The ethanol and water for their respective treatment groups were made available *ad*

libitum to the animals. The feed used during this study was EPOL mice cubes and was also made *ad libitum*.

All animals were dosed continuously *via* their water bottles for 20 consecutive weeks. The animals being treated with ethanol had 3 weeks adaptation and 20 weeks at 20% concentration. Body weights were recorded weekly and at termination.

The scheduled terminations occurred when the animals were 32 weeks old. The animals were killed by cardiac puncture, followed by exsanguination under ketamine and xylazine anaesthesia (as described in 4.5.1). Blood was collected in eppendorf tubes for protein and enzyme determination. Livers were dissected and weighed before being wrapped in tin foil and snap frozen in liquid nitrogen. The liver samples were stored at -80 °C until required for biochemical analysis. Another portion of the liver was processed for histological examination.

Chapter 5; Results of Studies

The results are presented for both biochemical and histological studies. The raw data are tabulated and appended (chapters 8&9 respectively) and the process data shall be presented in this chapter. The anthropometric data of each study will be presented before liver composition and health related parameters (glucose, alcohol and transaminases). The lipid peroxidation having to be presented in this context will be presented last. Although some comment is made in this chapter, the interpretation of the information will take place in the next chapter. The emphasis is to give an account of all the data generated in each study. This will take the format mainly of histograms. The aim is to examine macroscopic, microscopic and biochemical features of the liver as well as morphometric aspects of animal on regular chow for 20 wks in *wt/wt*, *db/wt* and *db/db* animals.

5.1 Assessment of the *db/db* Mouse Model on Regular Chow for 20 Weeks

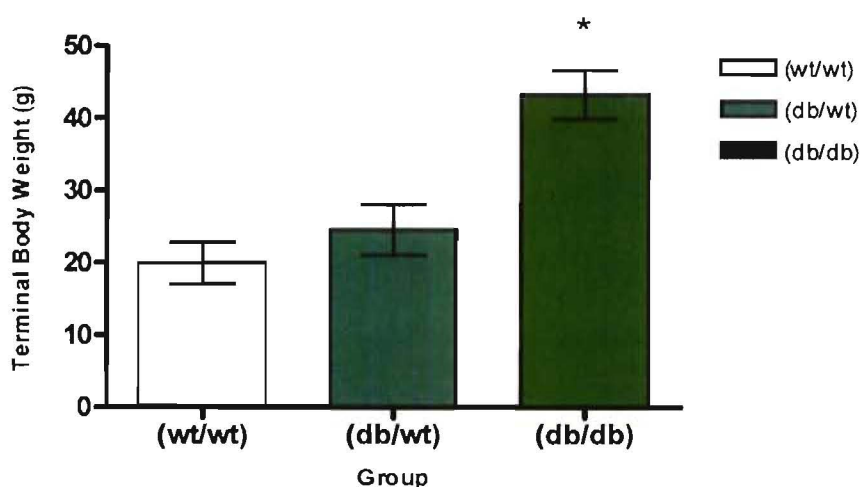


Figure 5.1.1: Body mass of *wt/wt* (n=12), *db/wt* (n=24) and *db/db* (n=24) mice on regular chow for 20 weeks. The mass in *db/db* animals was significantly elevated in comparison to the *wt/wt* ($p<0.0001$) and *db/wt* animals ($p<0.0001$). The *db/wt* group displayed a slightly increased mean body mass when compared to the *wt/wt* group ($p=0.0003$). Data represented as mean \pm SD.

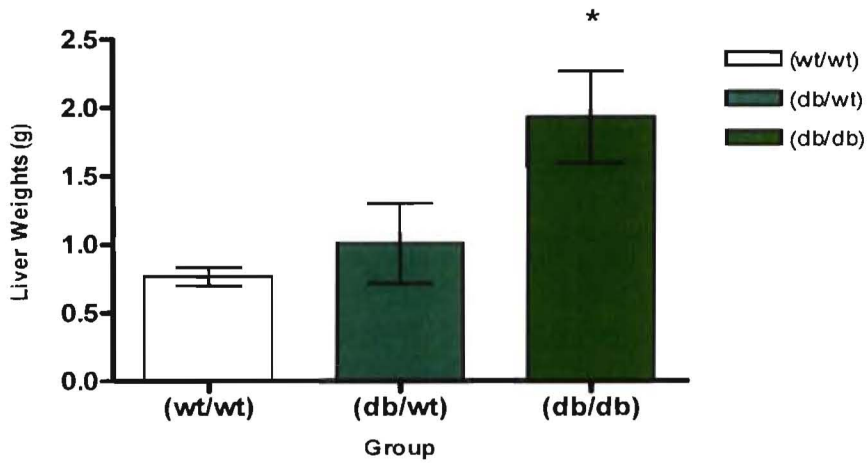


Figure 5.1.2: Liver mass of *wt/wt* (n=12), *db/wt* (n=12) and *db/db* (n=12) mice on regular chow for 20 weeks. The increase in liver mass of *db/db* mice is significant when compared to *wt/wt* and *db/wt* mice ($p < 0.0001$ for both). The *db/wt* group revealed a slight increase relative to the *wt/wt* group ($p = 0.011$). Data represented as mean \pm SD.

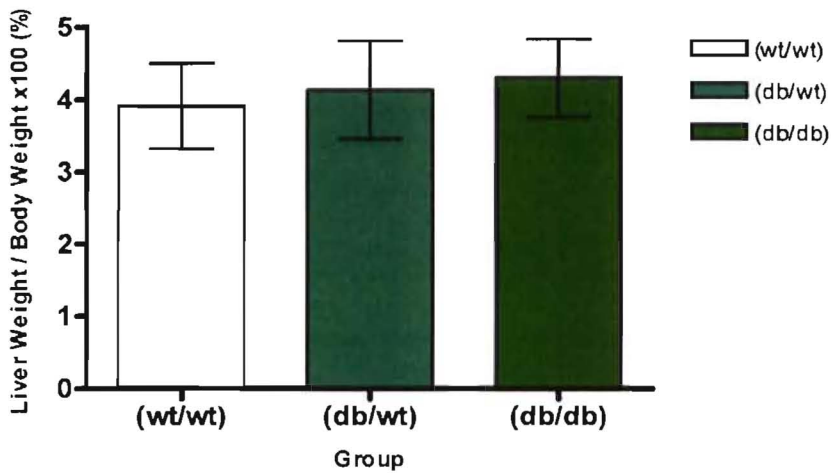


Figure 5.1.3: Liver proportion of body mass of *wt/wt* (n=12), *db/wt* (n=12) and *db/db* (n=12) mice on regular chow for 20 weeks. The resultant liver mass/body mass ratios were not statistically significant. Data represented as mean \pm SD.

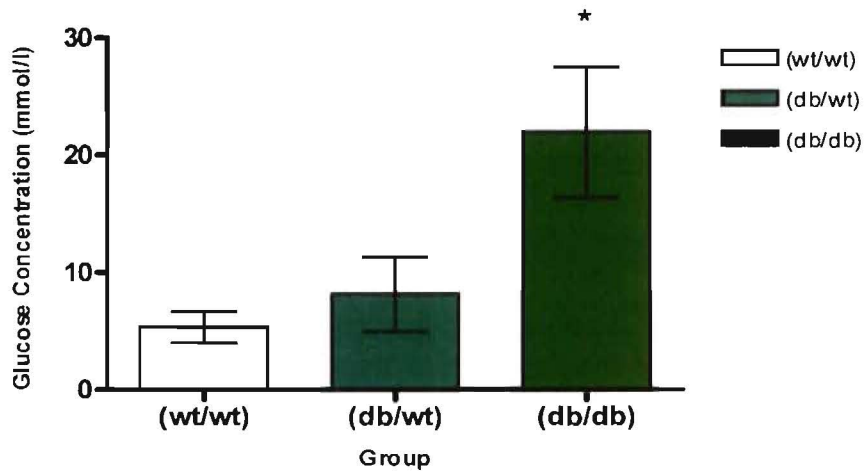


Figure 5.1.4: Plasma glucose concentration in livers from *wt/wt* (n=12), *db/wt* (n=24) and *db/db* (n=24) mice on regular chow for 20 weeks. Glucose levels were significantly increased in *db/db* animals when compared to the *wt/wt* and *db/wt* groups; ($p < 0.0001$ for both). The slight increase in the average glucose *db/wt* group in contrast to the *wt/wt* group was not statistically significant. Data represented as mean \pm SD.

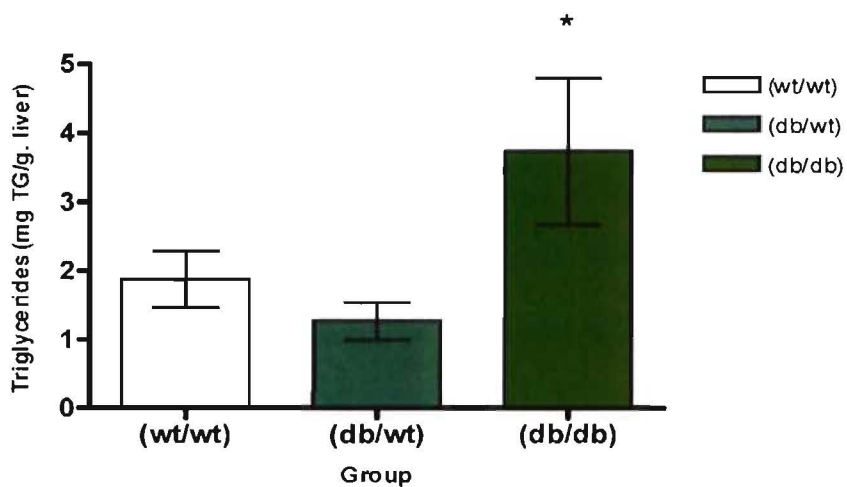


Figure 5.1.5: Triglycerides in livers from *wt/wt* (n=12), *db/wt* (n=24) and *db/db* (n=24) mice on regular chow for 20 weeks. TG were significantly raised in *db/db* mice when compared to *wt/wt* mice ($p < 0.0001$) and *db/wt* mice ($p < 0.0001$), but there was no significant differences when comparing *db/wt* and *wt/wt* groups. Data represented as mean \pm SD.

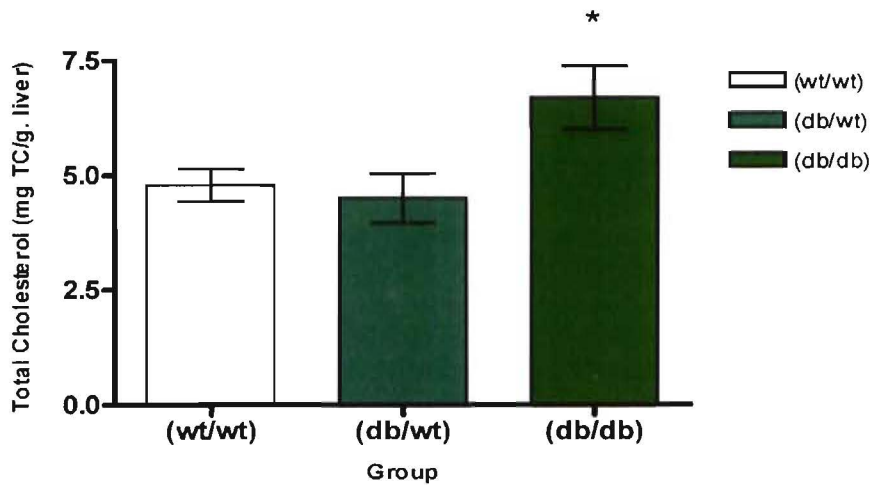


Figure 5.1.6: Total cholesterol concentrations in livers from *wt/wt* (n=12), *db/wt* (n=24) and *db/db* (n=24) mice on regular chow for 20 weeks. TC was significantly elevated in *db/db* mice in contrast to *wt/wt* and *db/wt* mice ($p < 0.0001$ for both). Concentrations for *wt/wt* and *db/wt* groups were similar. Data represented as mean \pm SD.

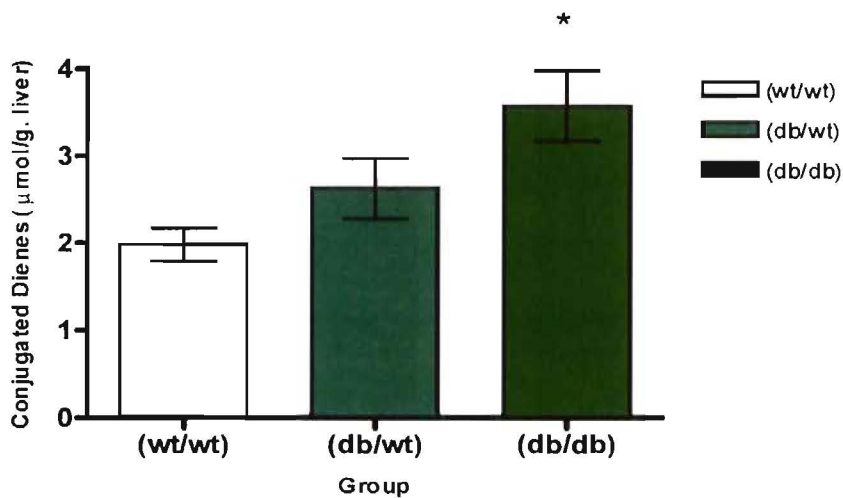


Figure 5.1.7: Conjugated dienes as early lipid peroxidation products in livers from *wt/wt* (n=12), *db/wt* (n=24) and *db/db* (n=24) mice on regular chow for 20 weeks. Conjugated Dienes were significantly higher in *db/db* animals ($p < 0.0001$) in comparison to *wt/wt* and *db/wt* respectively. A statistically significant increase is also present in the *db/wt* group when contrasted to the *wt/wt* group ($p < 0.0001$). Data represented as mean \pm SD.

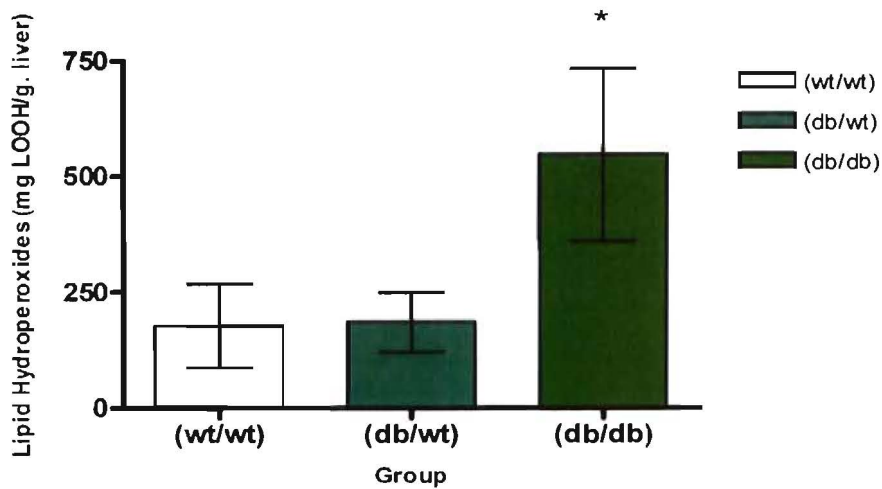


Figure 5.1.8: LOOH as intermediate lipid peroxidation products mean in livers from *wt/wt* (n=12), *db/wt* (n=24) and *db/db* (n=24) mice on regular chow for 20 weeks. Statistical significance pertained when *db/db* mice were compared to the *wt/wt* and *db/wt* animals ($p < 0.0001$ for both). Data represented as mean \pm SD.

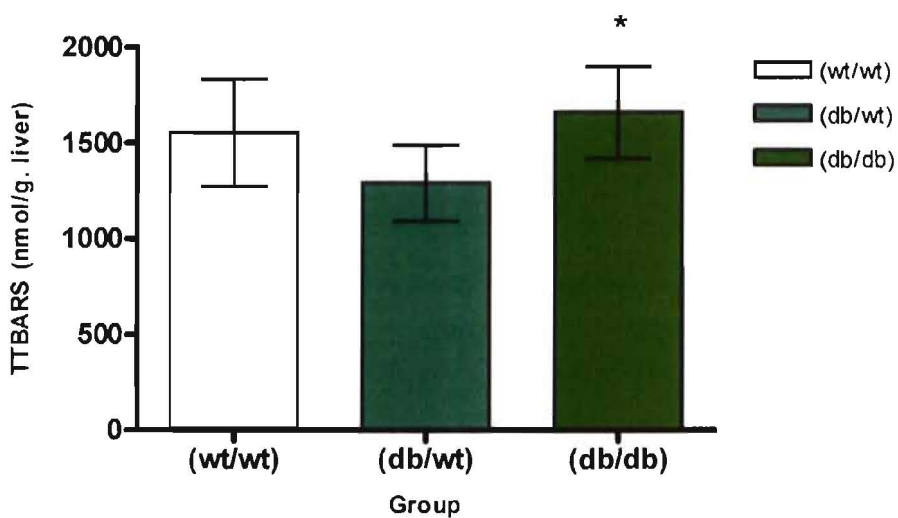


Figure 5.1.9: TTBARS as late lipid peroxidation products in livers from *wt/wt* (n=12), *db/wt* (n=24) and *db/db* (n=24) mice on regular chow for 20 weeks. Concentrations of TTBARS in *db/db* mice vs *wt/wt* mice was not statistically significant ($p = 0.247$) whereas *db/db* vs *db/wt* did attain statistical significance ($p < 0.0001$). Data represented as mean \pm SD.

5.2 Impact of Various Oils, Administered via the Diet (4 Weeks)

The aim was to evaluate the impact on lipid and lipid peroxide parameters in the livers of *db/wt* mice fed diets enriched with edible oils and cholesterol over 4 weeks subsequent to a 2 wk acclimatisation period. Canola oil provides MUFA and sunflower oil provides PUFA in addition to dietary fatty acids. The mouse model is *db/wt*.

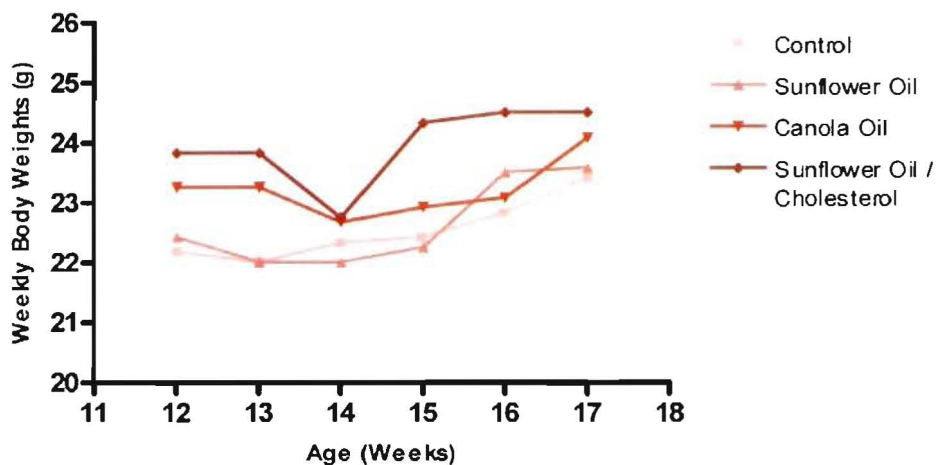


Figure 5.2.1: Body mass in control (chow only) ($n=6$) and 4 week supplemented sunflower oil ($n=6$), canola oil ($n=6$) or sunflower oil and cholesterol ($n=6$) in *db/wt* mice aged 17 weeks. The body mass was recorded for 1 week acclimatisation prior to the 4 week treatment period. There is slight variation in body weight gain and loss over the treatment period but the mean weight gain of all groups is reasonably comparable, controls; +1.2g, sunflower oil; +1.2g; canola oil; +0.8g, sunflower oil and cholesterol; +0.7g. Data represented as mean \pm SD.

Group / Treatment	M	C (y when x=0)	r^2
(a) Control	0.252 ± 0.06	18.9 ± 0.84	0.8283
(b) Sunflower Oil	0.301 ± 0.12	18.3 ± 1.79	0.6019
(c) Canola Oil	0.111 ± 0.12	21.6 ± 1.69	0.1875
(d) Sunflower Oil / Cholesterol	0.198 ± 0.15	21.1 ± 2.16	0.3102

a v b; ($p=0.73$) not statistically significant

a v c; ($p=0.31$) not statistically significant

a v d; ($p=0.74$) not statistically significant

Figure 5.2.2: The gradients of mean weight gain were contrasted for the alcohol-supplemented and control groups for control (chow only) ($n=6$) and 4 week supplemented sunflower oil ($n=6$), canola oil ($n=6$) or sunflower oil and cholesterol ($n=6$) in *db/wt* mice aged 17 weeks. Statistical significance was not attained in either treatment group relative to the controls. Data represented as mean \pm SD.

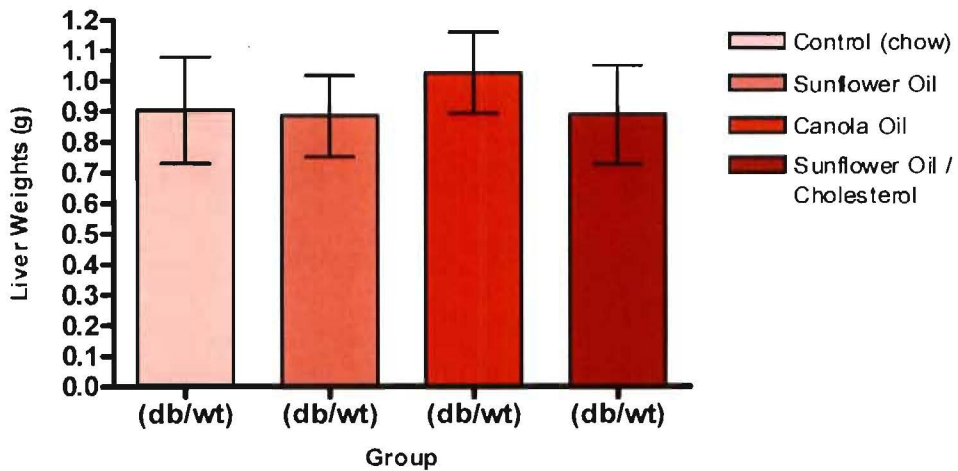


Figure 5.2.3: Liver mass in control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. The groups were comparable, no statistically significant change was achieved in any group and specific comparison to the controls revealed no differences: sunflower oil; ($p=0.82$), canola oil; ($p=0.21$), sunflower oil and cholesterol; ($p=0.87$). Data represented as mean \pm SD.

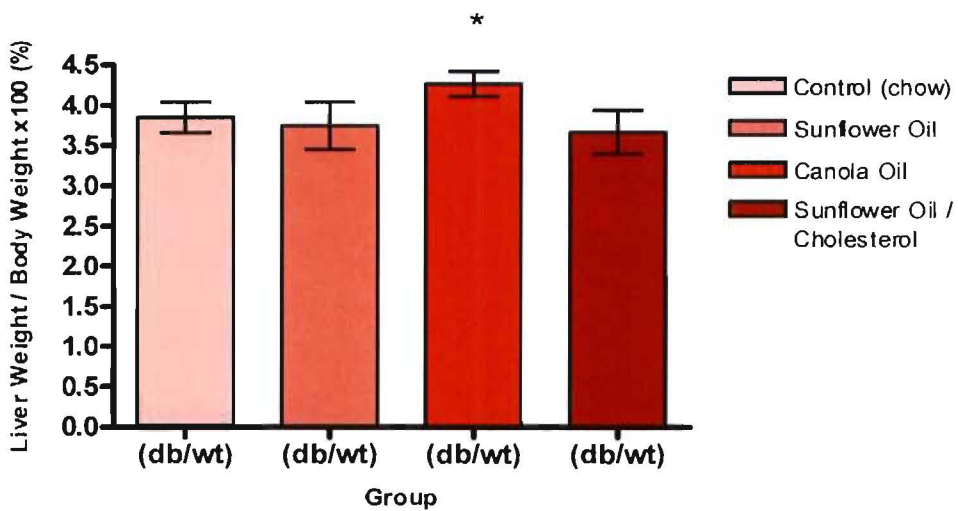


Figure 5.2.4: Liver proportion of body mass in control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. The canola oil group had a statistically significant difference from controls; ($p=0.0019$), whereas this was not achieved in the other groups, sunflower oil; ($p=0.48$), sunflower oil and cholesterol; ($p=0.20$). Data represented as mean \pm SD.

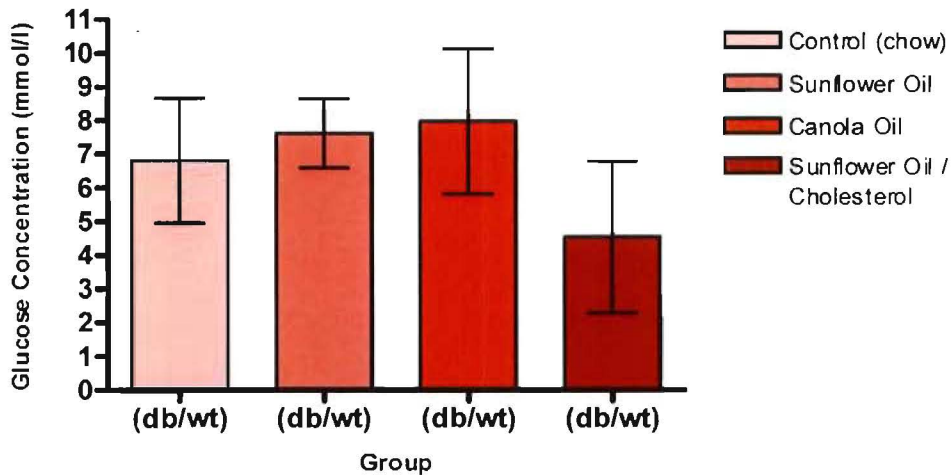


Figure 5.2.5: Plasma glucose concentration in control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. Statistical significance was not achieved in any treatment group, sunflower oil; ($p=0.38$), canola oil; ($p=0.34$), sunflower oil and cholesterol; ($p=0.083$). Data represented as mean \pm SD.

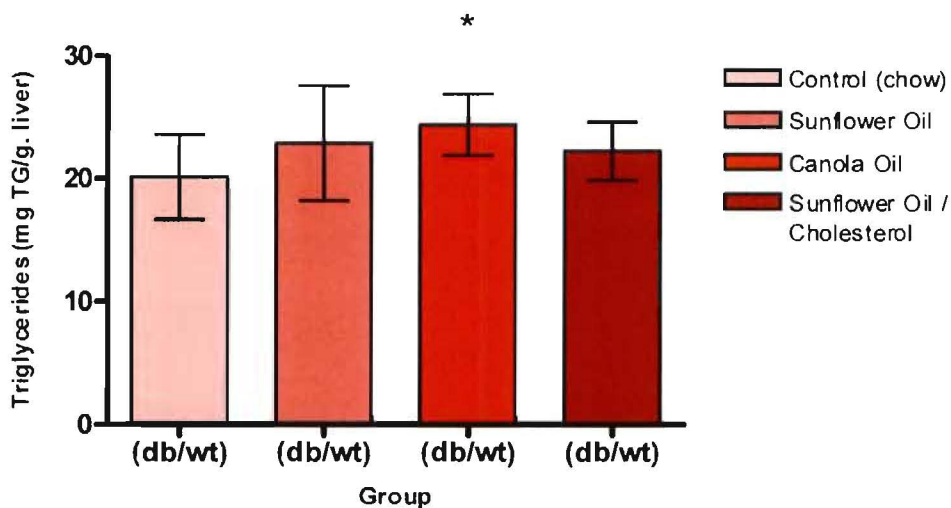


Figure 5.2.6: Triglycerides in livers from control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. Statistical significance was only reached in canola oil treated animals; ($p=0.036$) whereas sunflower oil; ($p=0.28$) and sunflower oil and cholesterol; ($p=0.25$) did not differ significantly. Data represented as mean \pm SD.

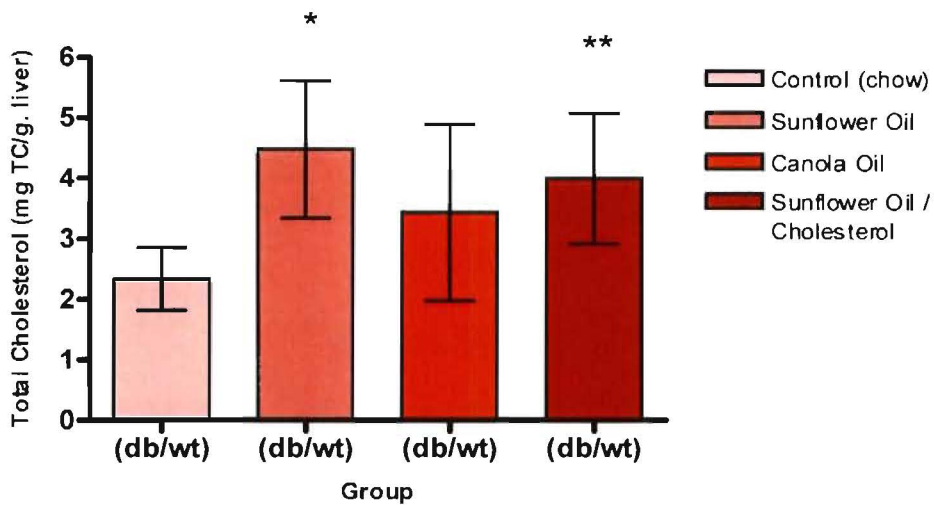


Figure 5.2.7: Total cholesterol concentrations in livers from control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. The levels of total cholesterol were increased in the treatment groups in contrast to control animals, reaching statistical significance in mice treated with sunflower oil; ($p=0.0018$) and with sunflower oil and cholesterol; ($p=0.007$), but not for the canola oil group; ($p=0.11$). Data represented as mean \pm SD.

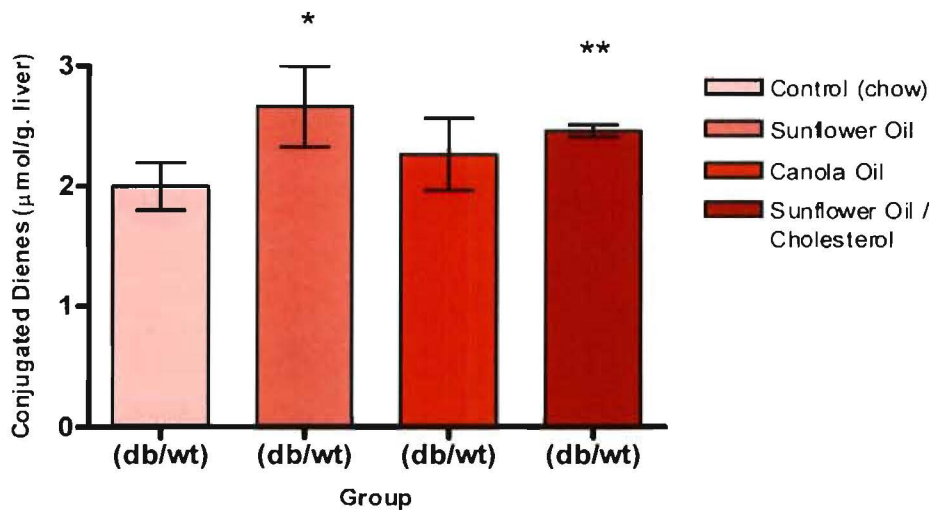


Figure 5.2.8: Conjugated dienes as early lipid peroxidation products in livers from control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. Statistical significance was achieved in the sunflower oil treated mice; ($p=0.0019$) and the sunflower oil and cholesterol treated mice; ($p=0.0002$). The canola oil treated animals did not reach statistical significance; ($p=0.101$). Data represented as mean \pm SD.

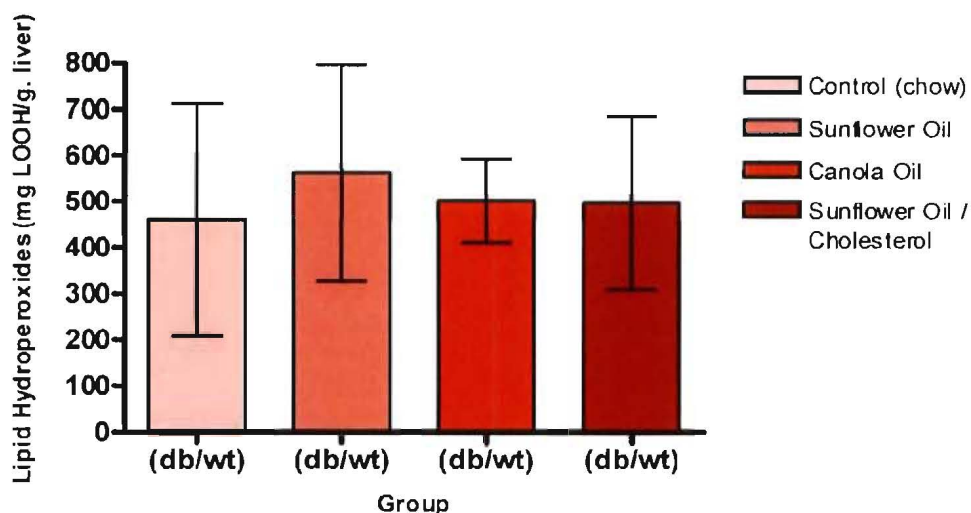


Figure 5.2.9: LOOH as intermediate lipid peroxidation products in livers from control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. In comparison to the control animals, statistical significance was not displayed in any group, sunflower oil; ($p=0.49$), canola oil; ($p=0.71$), sunflower oil and cholesterol; ($p=0.77$). Data represented as mean \pm SD.

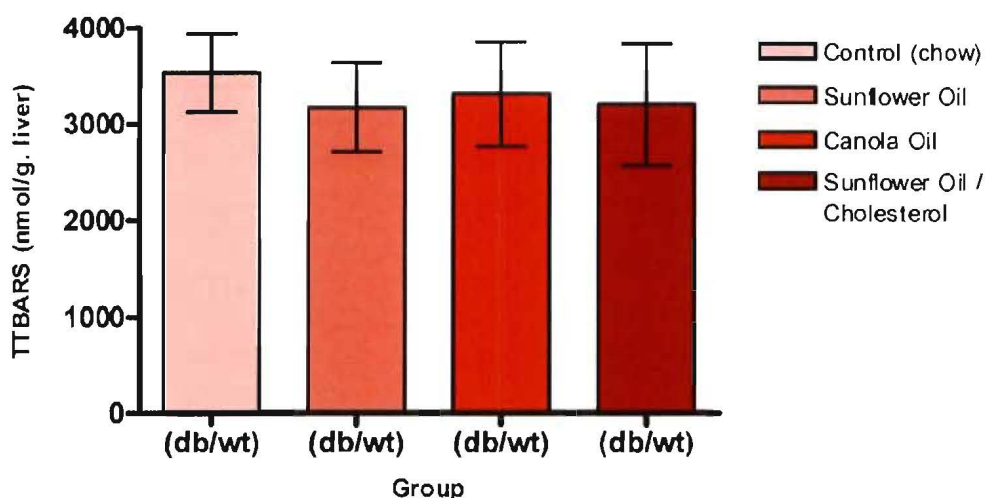


Figure 5.2.10: TTbars as late lipid peroxidation products in livers from control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. No statistical significance was found, sunflower oil; ($p=0.18$), canola oil; ($p=0.45$), sunflower oil and cholesterol; ($p=0.31$). Data represented as mean \pm SD.

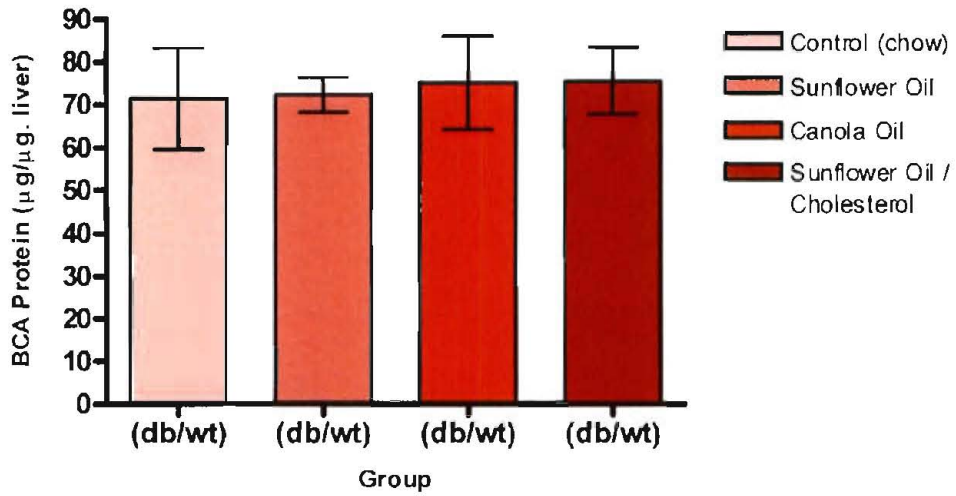


Figure 5.2.11: BCA protein in homogenate from control (chow only) (n=6) and 4 week supplemented sunflower oil (n=6), canola oil (n=6) or sunflower oil and cholesterol (n=6) in *db/wt* mice aged 17 weeks. No statistically significant difference was found when comparing the control group with those consuming: sunflower oil; ($p=0.86$), canola oil; ($p=0.58$), sunflower oil and cholesterol; ($p=0.48$). Data represented as mean \pm SD.

5.3 Study of Short Period of Alcohol Administration (4 Weeks)

The aim is to examine macroscopic, microscopic and biochemical features of the liver as well as morphometric aspects of animal on 20% alcohol for 4 weeks in *wt/wt*, *db/wt* and *db/db* animals.

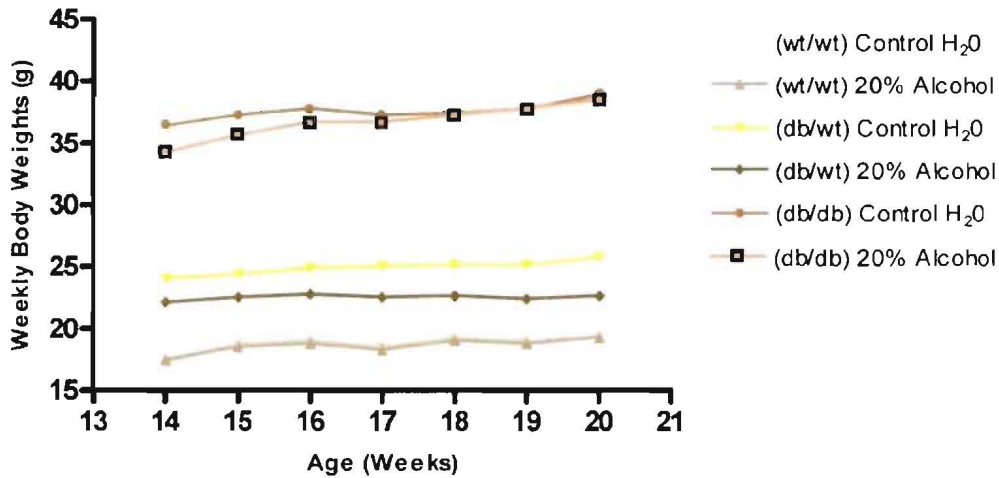


Figure 5.3.1: Body mass in control and 4 weeks alcohol-supplemented *wt/wt* (n=4), *db/wt* (n=8) and *db/db* (n=8) mice aged 20 weeks. The body mass was recorded for 2 weeks prior to the 4 weeks alcohol administration during which the alcohol-treated animals were allowed to acclimatise to increasing concentrations of alcohol until the full 20% concentration was achieved. The mean weight gain for alcohol vs. control animals in *wt/wt*, *db/wt* and *db/db* groups were comparative. Data represented as mean \pm SD.

Group / Treatment	m	C (y when x=0)	r ²
(a) <i>wt/wt</i> – Control (H ₂ O)	0.268 \pm 0.09	14.2 \pm 1.61	0.6181
(b) <i>wt/wt</i> – Alcohol (20%)	0.214 \pm 0.07	14.9 \pm 1.19	0.6545
(c) <i>db/wt</i> – Control (H ₂ O)	0.250 \pm 0.03	20.7 \pm 0.59	0.9119
(d) <i>db/wt</i> – Alcohol (20%)	0.041 \pm 0.04	21.8 \pm 0.65	0.1875
(e) <i>db/db</i> – Control (H ₂ O)	0.291 \pm 0.09	32.6 \pm 1.50	0.6898
(f) <i>db/db</i> – Alcohol (20%)	0.629 \pm 0.07	26.0 \pm 1.23	0.9392

a v b; ($p=0.657$) not statistically significant

c v d; ($p=0.002$) statistically significant

e v f; ($p=0.014$) statistically significant

Figure 5.3.2: The gradients of mean weight gain were contrasted for the alcohol-supplemented and control groups for *wt/wt* (n=4), *db/wt* (n=8) and *db/db* (n=8) mice aged 20 weeks. The difference between *wt/wt* animals was not statistically significant ($p=0.66$) whereas statistical significance was attained when comparing control and alcohol-supplemented animals for *db/wt* and *db/db* groups ($p=0.002$ and $p=0.014$ respectively). Data represented as mean \pm SD.

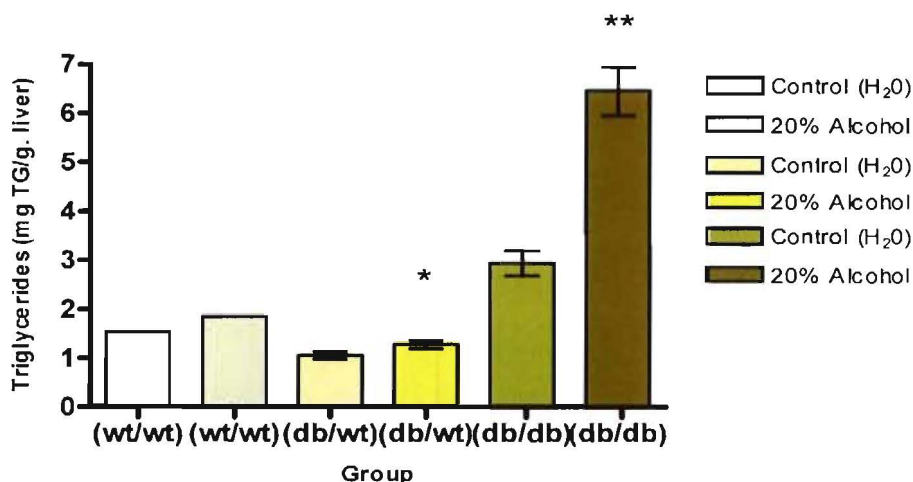


Figure 5.3.3: Triglycerides in livers from control and 4 weeks alcohol-supplemented *wt/wt* (n=4), *db/wt* (n=8) and *db/db* (n=8) mice aged 20 weeks. Triglyceride concentration was increased in alcohol-treated *db/db* mice relative to the controls ($p < 0.0001$). The triglyceride content was also increased in the other groups, although statistical significance could only be evaluated in the alcohol-treated and control *db/wt* animals ($p = 0.009$). Data represented as mean \pm SD.

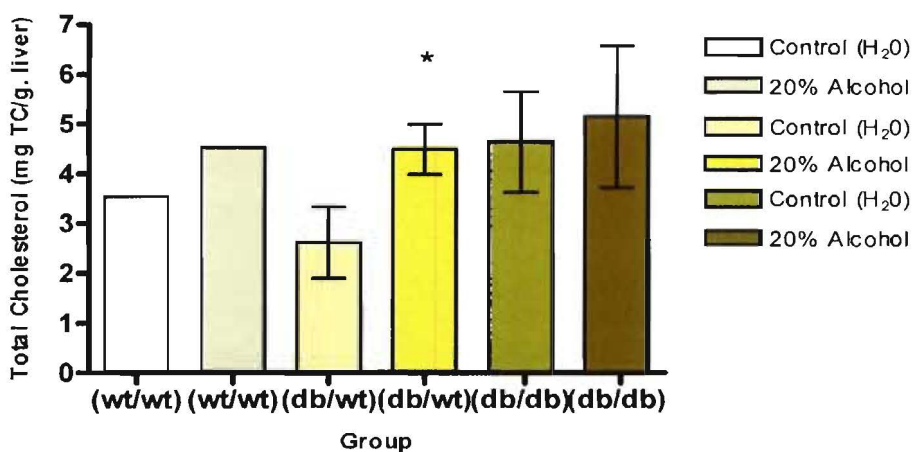


Figure 5.3.4: Total cholesterol concentration in livers from control and 4 weeks alcohol-supplemented *wt/wt* (n=4), *db/wt* (n=8) and *db/db* (n=8) mice aged 20 weeks. Alcohol-treated mice again exhibited slightly higher values than controls but only the elevation in *db/wt* alcohol-treated mice was of statistical significance against their respective controls ($p = 0.005$). Data represented as mean \pm SD.

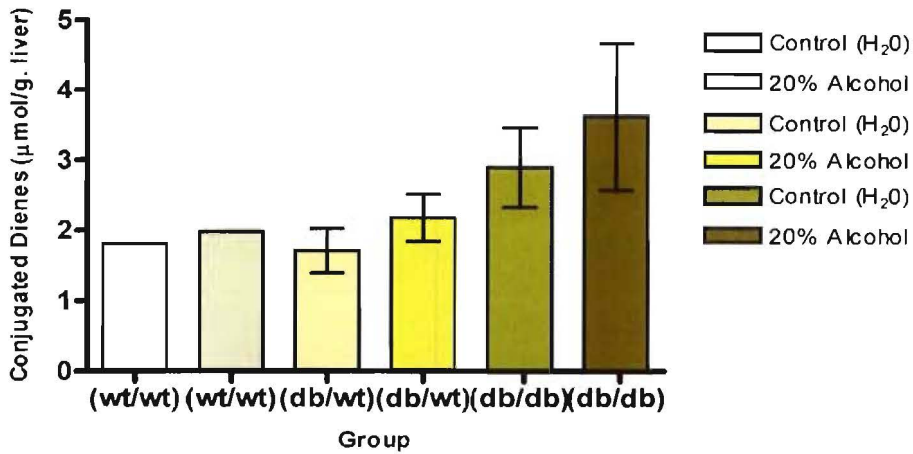


Figure 5.3.5: Conjugated dienes as early lipid peroxidation products in livers from control and 4 weeks alcohol-supplemented *wt/wt* (n=4), *db/wt* (n=8) and *db/db* (n=8) mice aged 20 weeks. Conjugated dienes were slightly raised in alcohol-treated animals when compared to the controls of their respective *wt/wt*, *db/wt*, and *db/db* groups. Data represented as mean \pm SD.

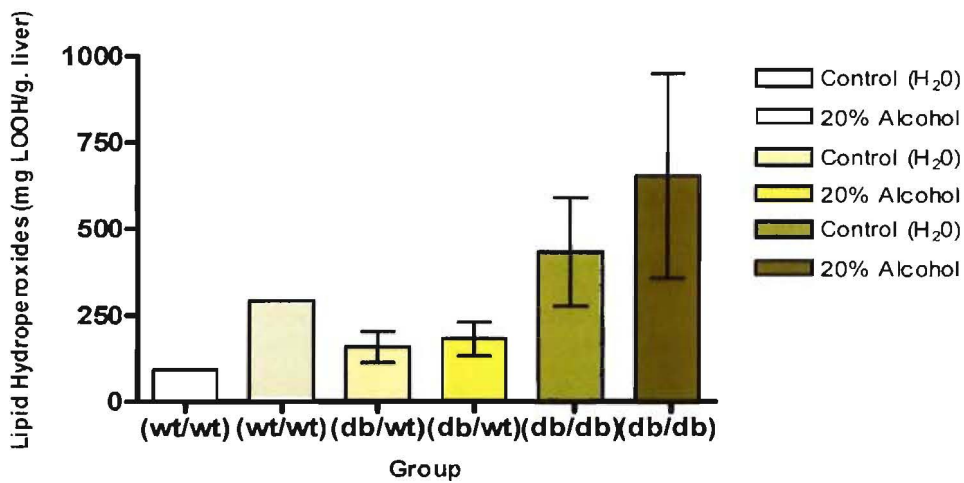


Figure 5.3.6: LOOH as intermediate lipid peroxidation products in livers from control and 4 weeks alcohol-supplemented *wt/wt* (n=4), *db/wt* (n=8) and *db/db* (n=8) mice aged 20 weeks. All alcohol-treated groups in comparison with their respective water-treated control animals, appeared to have small increases of average LOOH but statistical significance was not achieved ($p > 0.10$). Data represented as mean \pm SD.

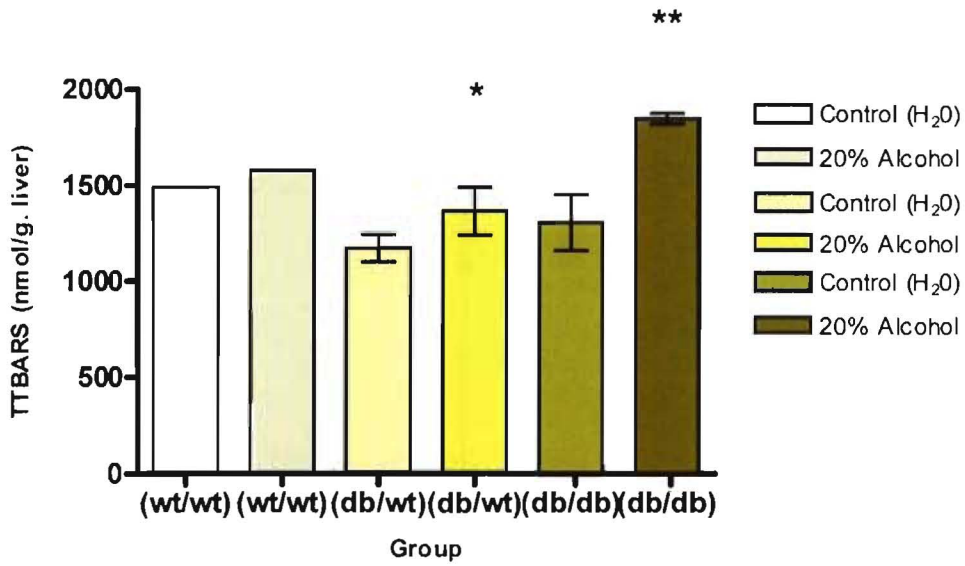


Figure 5.3.7: TTBARs as late lipid peroxidation products in livers from control and 4 weeks alcohol-supplemented *wt/wt* (n=4), *db/wt* (n=8) and *db/db* (n=8) mice aged 20 weeks. All alcohol-treated groups displayed increases when compared to their respective control groups. The levels of TTBARs were shown to be statistically significant for *db/wt* alcohol-treated vs. control ($p=0.037$) and *db/db* alcohol-treated vs. control ($p=0.0003$). Data represented as mean \pm SD.

5.4 Impact of Alcohol Administered over 8 Weeks in *db/wt* and *db/db* Mice

The aim is to examine macroscopic, microscopic and biochemical features of the liver as well as morphometric aspects of animal on 20% alcohol for 8 weeks in *db/wt* and *db/db* animals.

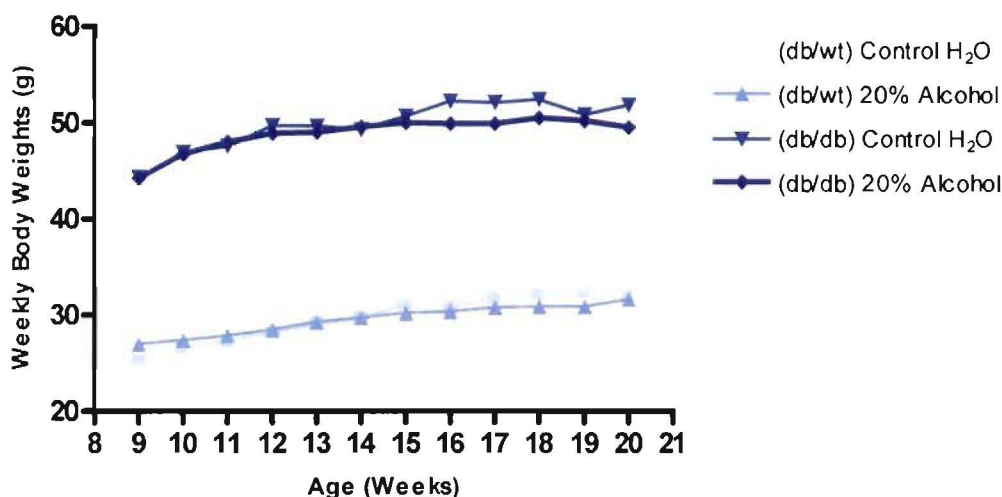


Figure 5.4.1: Body mass in control and 8 week alcohol-supplemented *db/wt* (n=7, n=6, respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. The body mass was recorded for 3 weeks prior to the 8 weeks alcohol administration of 20% ethanol concentration during which the alcohol-treated animals were allowed to acclimatise to increasing concentrations of alcohol until the full 20% concentration was achieved. The mean body mass of alcohol-treated *db/wt* mice was comparable to their respective controls throughout the entire treatment period, however the mean weight gain over the same duration was slightly decreased for the alcohol-treated group; +4.5g compared with +6.8g for the controls. The body mass for the *db/db* animals were comparable for the first half of the treatment period but notable changes became apparent soon after and also resulted in a decreased weight gain for the alcohol treated *db/db* mice; +5.2g compared with +7.4g for the controls. Data represented as mean \pm SD.

Group / Treatment (8wk)	m	C (y when x=0)	r ²
(a) <i>db/wt</i> – Control (H ₂ O)	0.655 \pm 0.06	20.2 \pm 0.83	0.9324
(b) <i>db/wt</i> – Alcohol (20%)	0.414 \pm 0.28	23.6 \pm 0.41	0.9577
(c) <i>db/db</i> – Control (H ₂ O)	0.601 \pm 0.10	41.1 \pm 1.54	0.7728
(d) <i>db/db</i> – Alcohol (20%)	0.397 \pm 0.09	43.1 \pm 1.38	0.6480

a v b; ($p=0.0009$) statistically significant
c v d; ($p=0.155$) not statistically significant

Figure 5.4.2: The gradients of mean weight gain were contrasted for 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. The difference between *db/db* animals was not statistically significant ($p=0.155$) whereas statistical significance was attained when comparing control and alcohol-supplemented animals for *db/wt* groups ($p=0.0009$). Data represented as mean \pm SD.

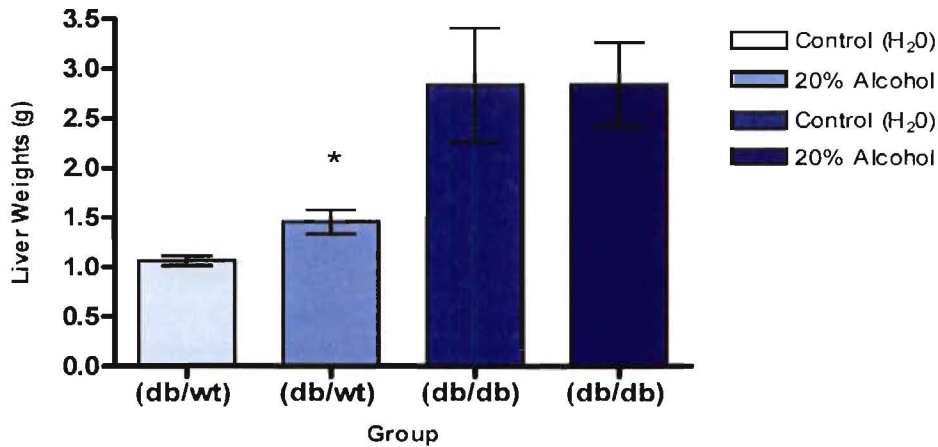


Figure 5.4.3: Liver mass in control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. The increases in alcohol-treated *db/wt* mice compared with the controls was found to be statistically significant ($p < 0.0001$), but not for the *db/db* mice ($p = 0.99$). Data represented as mean \pm SD.

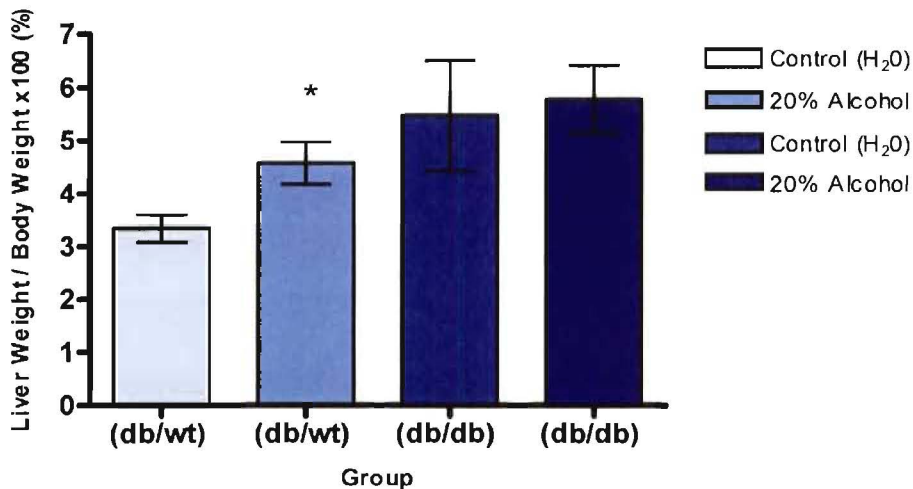


Figure 5.4.4: Liver proportion of body mass in control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. The liver mass/body mass ratios demonstrated a significant increase in alcohol-treated *db/wt* mice when compared to controls ($p < 0.0001$) but not for the *db/db* animals ($p = 0.57$). Data represented as mean \pm SD.

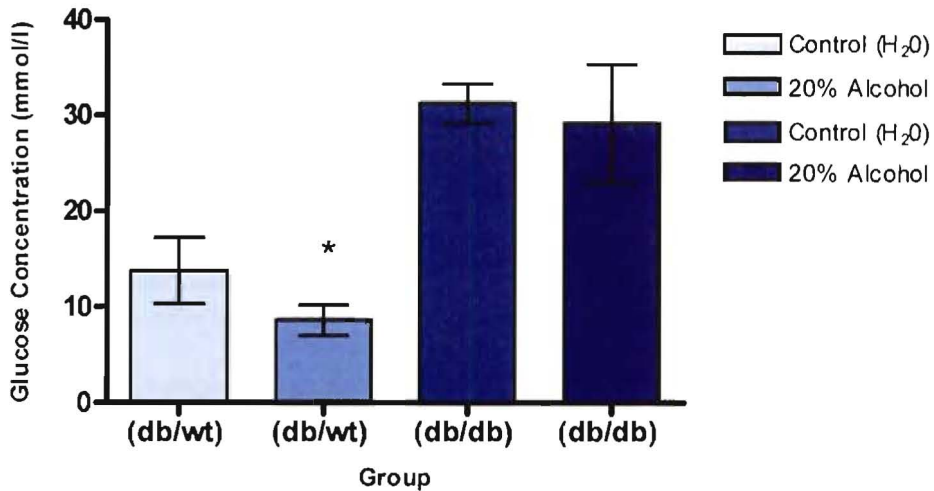


Figure 5.4.5: Plasma glucose concentrations in blood from control and 8 week alcohol-supplemented *db/wt* ($n=7$, $n=6$ respectively) and *db/db* ($n=6$, $n=5$) mice aged 20 weeks. Glucose concentrations were decreased in alcohol-treated animals, attaining statistical significance in *db/wt* mice; ($p=0.006$) but not in *db/db* mice; ($p=0.54$). Data represented as mean \pm SD.

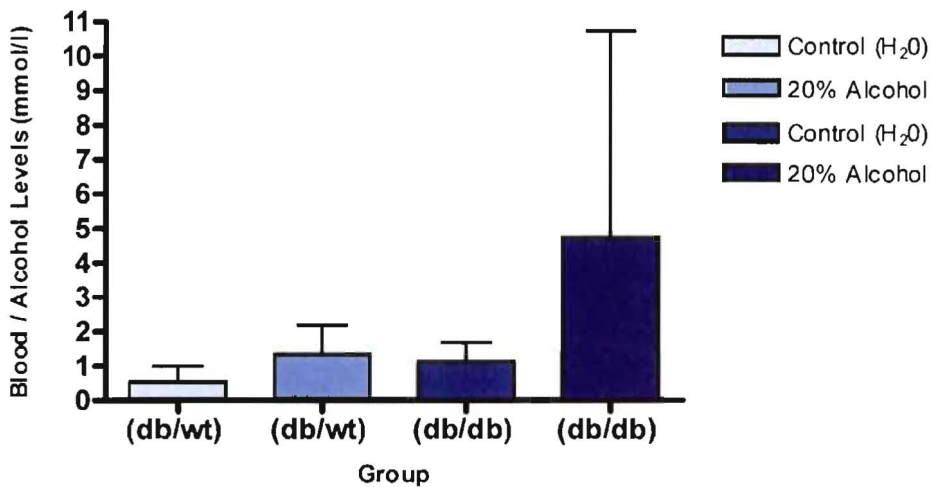


Figure 5.4.6: Alcohol concentrations in blood in serum from control and 8 week alcohol-supplemented *db/wt* ($n=7$, $n=6$ respectively) and *db/db* ($n=6$, $n=5$) mice aged 20 weeks. The alcohol-treated animals displayed higher levels of ethanol than their respective controls but statistical significance was not achieved in either group, *db/wt* mice; ($p=0.055$), *db/db* mice; ($p=0.17$). Data represented as mean \pm SD.

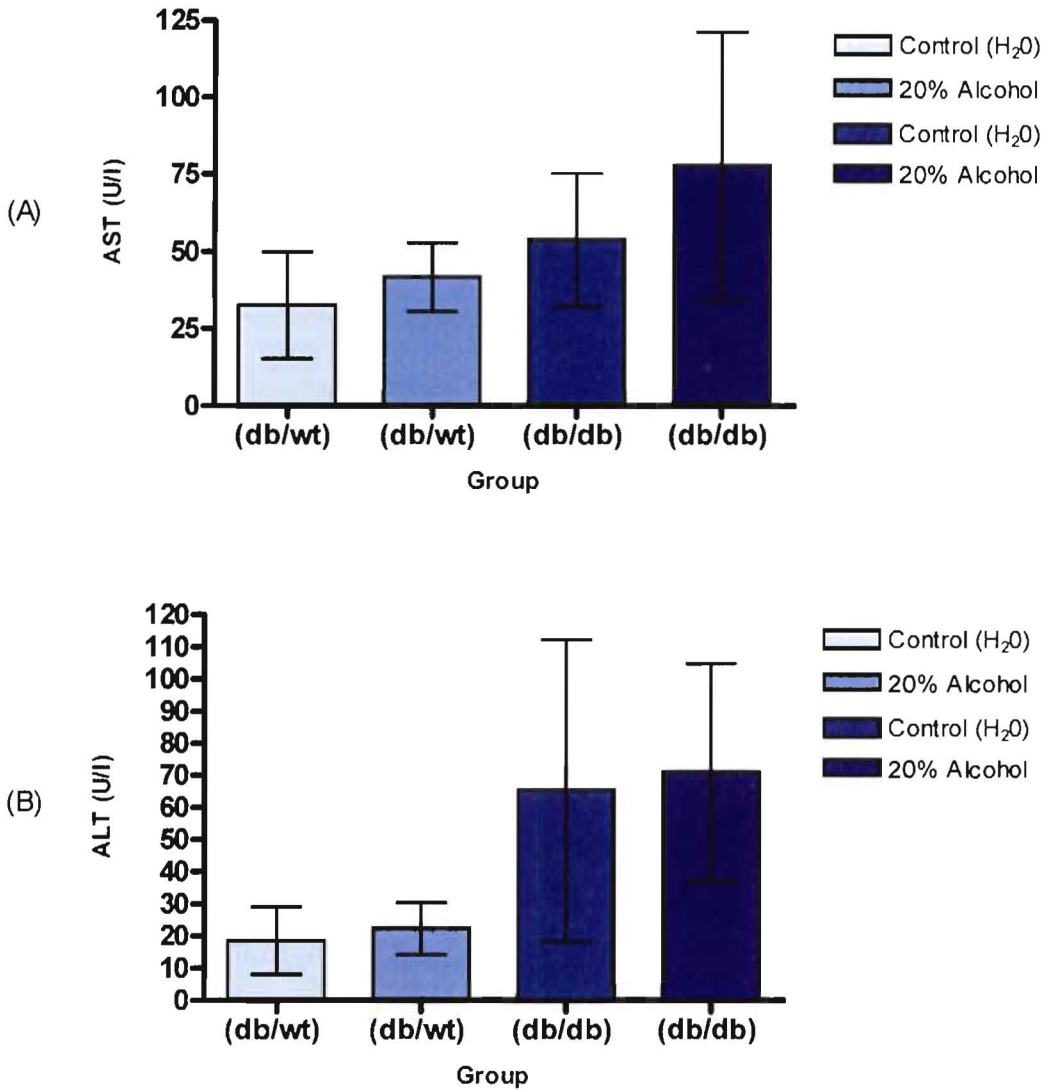


Figure 5.4.7: Plasma AST and ALT levels in serum from control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. (A) Mice treated with alcohol displayed no significant increase in AST in either group, *db/wt* mice; ($p=0.29$), *db/db* mice ($p=0.26$). (B) ALT levels were not significantly different between controls and alcohol-supplemented groups, *db/wt* mice ($p=0.49$), *db/db* mice ($p=0.83$). Data represented as mean \pm SD.

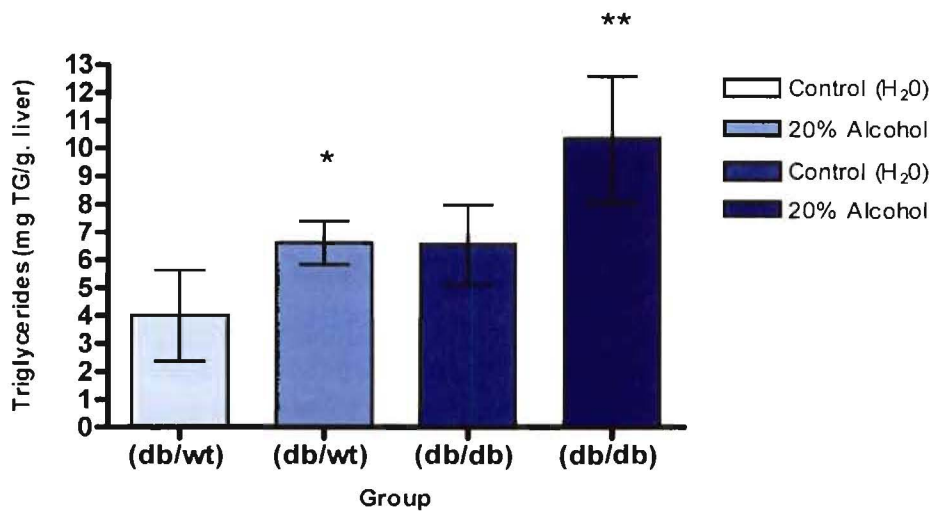


Figure 5.4.8: Triglycerides in livers from control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. Triglycerides had a statistically significant increase in alcohol-treated groups in contrast to their controls, *db/wt* mice; ($p=0.004$) and *db/db* mice; ($p=0.008$) Furthermore the elevated levels in *db/wt* alcohol treated mice were marginally raised in contrast to the *db/db* controls but was not statistically significant. Data represented as mean \pm SD.

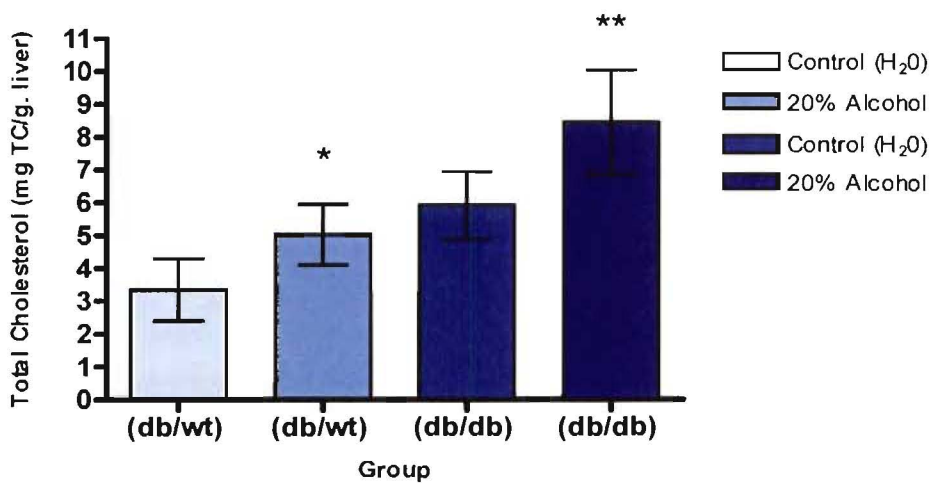


Figure 5.4.9: Mean total cholesterol concentrations in livers from control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. Total cholesterol attained statistically significant increases in alcohol-treated animals when compared to their respective controls, *db/wt* mice; ($p=0.008$) and *db/db* mice; ($p=0.010$). Data represented as mean \pm SD.

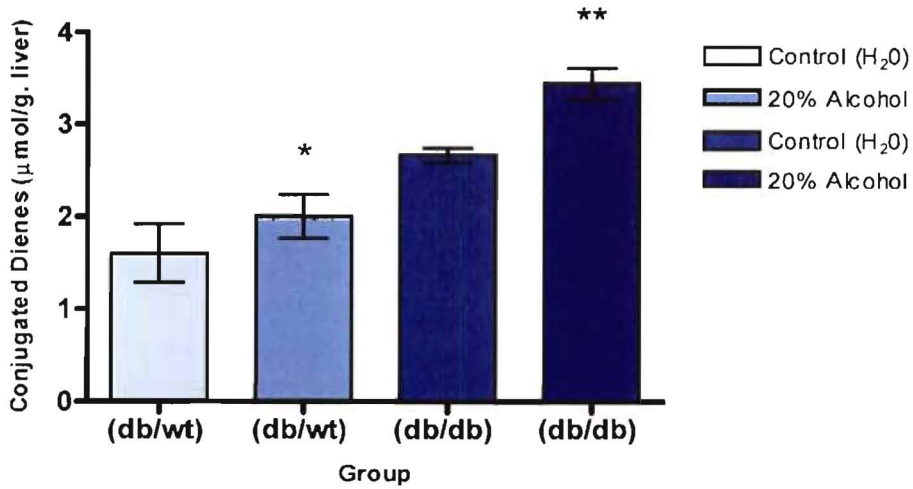


Figure 5.4.10: Conjugated dienes as early lipid peroxidation products in livers from control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. Conjugated dienes were significantly increased in alcohol-treated animals for *db/wt* and *db/db* groups; ($p=0.027$) and ($p<0.0001$) respectively. Data represented as mean \pm SD.

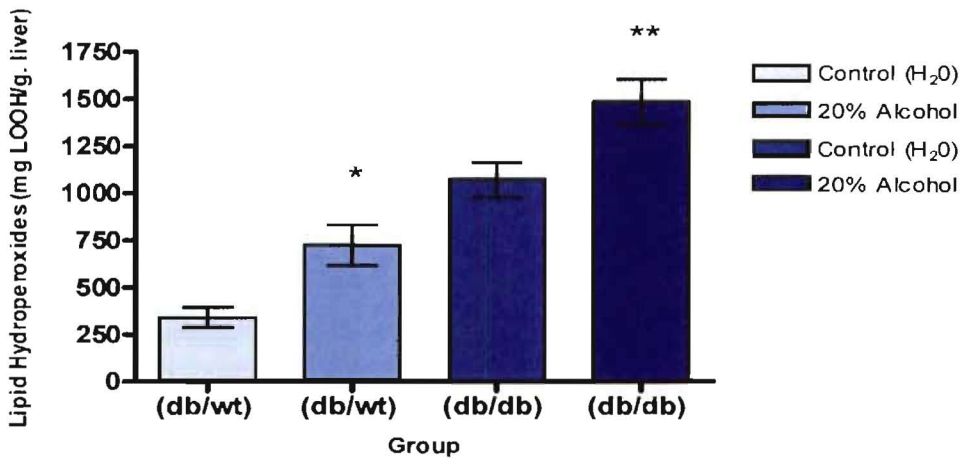


Figure 5.4.11: LOOH as intermediate lipid peroxidation products in livers from control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. The concentrations of lipid hydroperoxides displayed statistically significant increases in alcohol-treated groups for *db/wt* and *db/db* animals; ($p<0.0001$ for both groups). Data represented as mean \pm SD.

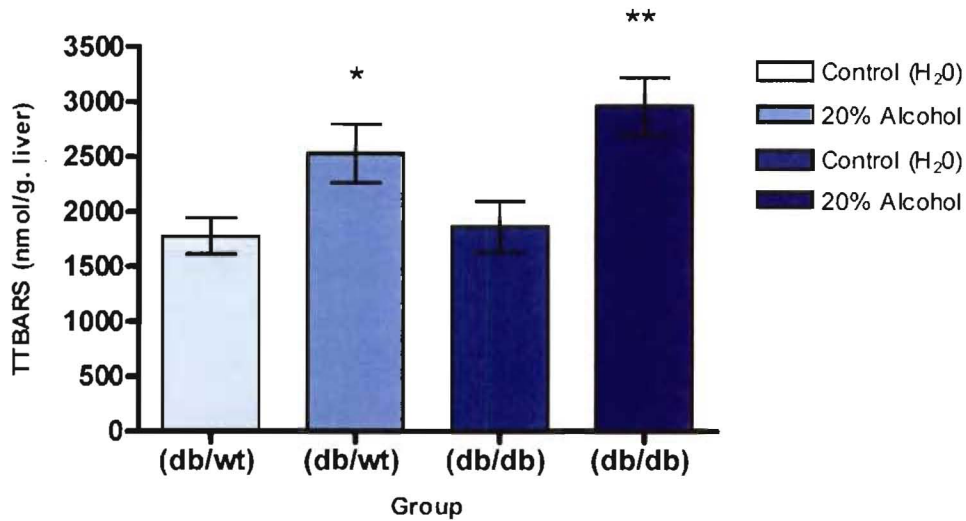


Figure 5.4.12: TTBARS as late lipid peroxidation products in livers from control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. Increases in TTBARS were statistically significant for alcohol-treated groups in *db/wt* mice; ($p < 0.0001$) and *db/db* mice; ($p < 0.0001$) when compared to respective controls. Data represented as mean \pm SD.

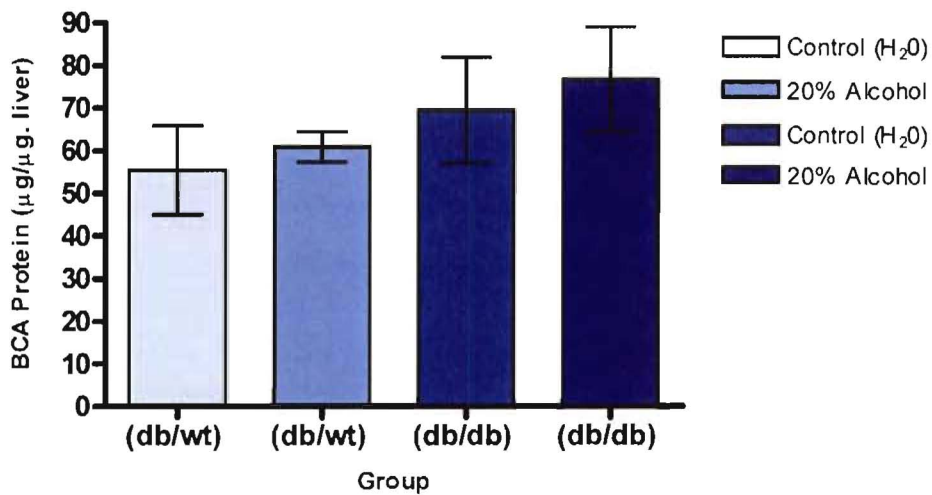


Figure 5.4.13: BCA protein in livers from control and 8 week alcohol-supplemented *db/wt* (n=7, n=6 respectively) and *db/db* (n=6, n=5) mice aged 20 weeks. BCA concentrations were determined for all groups and despite small increased changes being recorded for alcohol-treated animals, statistical significance was not attained in *db/wt* mice; ($p = 0.26$) or *db/db* mice ($p = 0.35$). Data represented as mean \pm SD.

5.5 Impact of Alcohol Administered over 20 Weeks in *db/wt* and *db/db* Mice

The aim is to examine macroscopic, microscopic and biochemical features of the liver as well as morphometric aspects of animal on 20% alcohol for 20 weeks in *db/wt* and *db/db* animals.

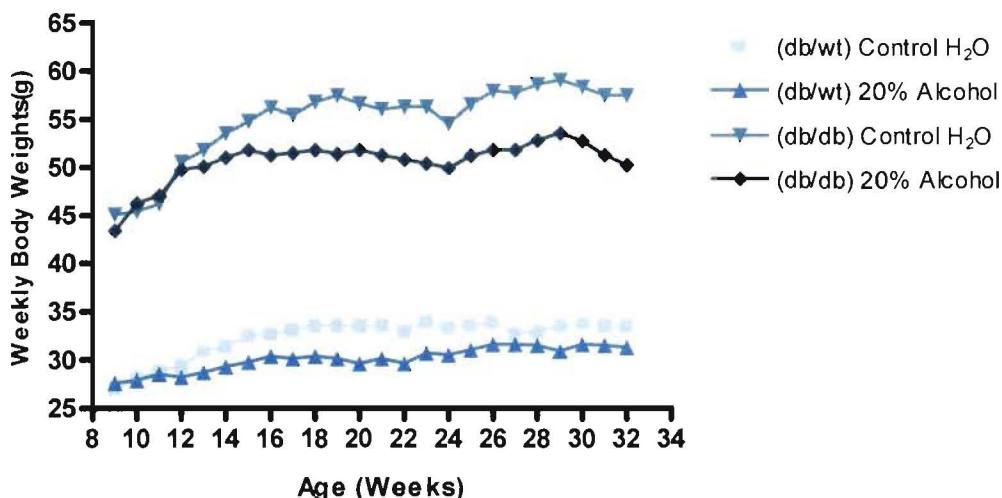


Figure 5.5.1: Body mass in control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. The body mass was recorded for 3 weeks prior to the 20 weeks alcohol administration of 20% ethanol concentration during which the alcohol-treated animals were allowed to acclimatise to increasing concentrations of alcohol until the full 20% concentration was achieved. The body mass of alcohol-treated animals and their respective controls were comparable at the start of the treatment period for *db/wt* and *db/db* mice. However after approximately 4-5 weeks of treatment, both sets of controls appear to have a slightly increased rate of weight gain when compared to the alcohol-treated mice. This lower degree of weight gain is never recovered and is demonstrated by the resulting differences between the groups over the treatment period. In *db/wt* mice, the mean weight gain for the controls is +6.4g compared with 1.2g for alcohol-treated animals whereas the respective gains for *db/db* mice are +12.3g and +6.8g. Data represented as mean \pm SD.

Group / Treatment (20wk)	m	C (y when x=0)	r ²
(a) <i>db/wt</i> – Control (H ₂ O)	0.215 \pm 0.04	29.9 \pm 0.82	0.5942
(b) <i>db/wt</i> – Alcohol (20%)	0.159 \pm 0.02	26.8 \pm 0.32	0.8385
(c) <i>db/db</i> – Control (H ₂ O)	0.473 \pm 0.07	45.1 \pm 1.54	0.6673
(d) <i>db/db</i> – Alcohol (20%)	0.200 \pm 0.05	46.5 \pm 1.11	0.4093

a v b; ($p=0.18$) not statistically significant

c v d; ($p=0.003$) statistically significant

Figure 5.5.2: Slope analysis mass in control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. The body mass was recorded for 3 weeks prior to the 20 weeks alcohol administration of 20% ethanol concentration. Results from the gradient analysis were contrary to 8wk study as difference between *db/wt* animals was not statistically significant ($p=0.18$) whereas statistical significance was attained when comparing control and alcohol-supplemented animals for *db/db* groups ($p=0.003$). Data represented as mean \pm SD.

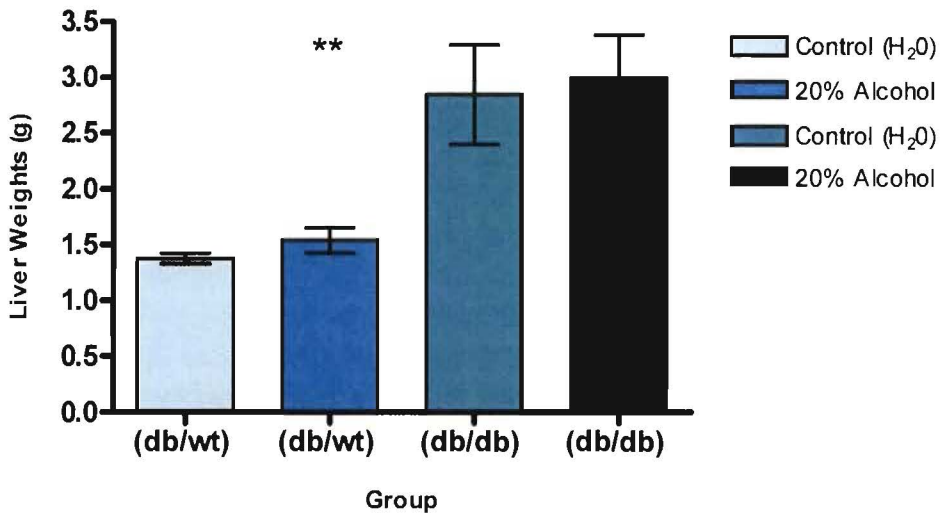


Figure 5.5.3: Liver mass in control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. There was no statistical significance reached in *db/db* mice; ($p=0.55$). In *db/wt* mice; statistical significance was achieved; ($p=0.007$). Data represented as mean \pm SD.

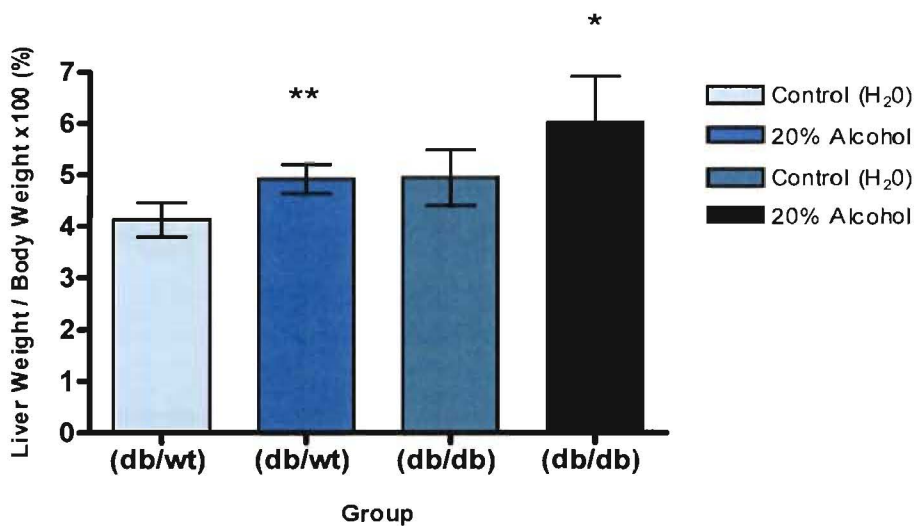


Figure 5.5.4: Liver proportion of body weight in control and 20 week alcohol supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. Liver mass/body mass ratios from alcohol-treated animals, the differences between the *db/db* groups achieved statistical significance ($p=0.033$) as did *db/wt* mice ratios, ($p=0.003$).

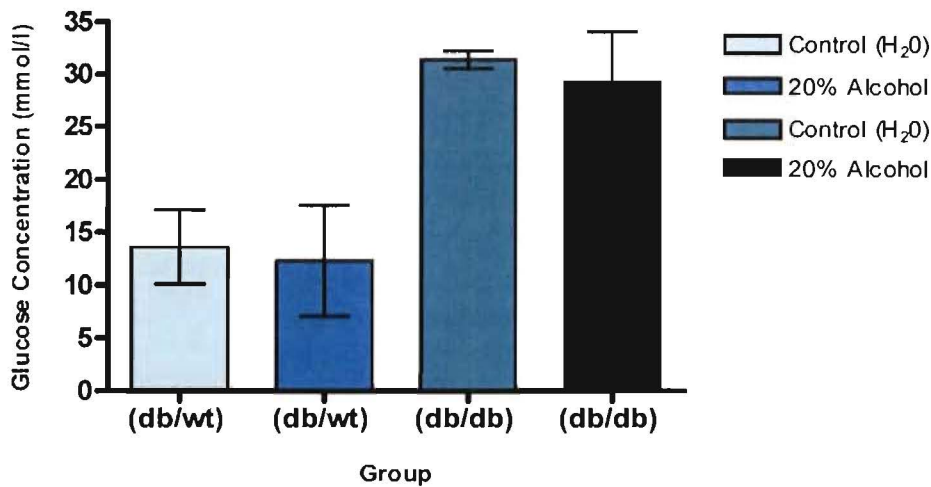


Figure 5.5.5: Plasma glucose concentrations in blood from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. The glucose concentrations were lower for both groups in comparison to the controls, but the results did not show statistical significance for either *db/wt* mice; ($p=0.63$) or *db/db* mice; ($p=0.29$). Data represented as mean \pm SD.

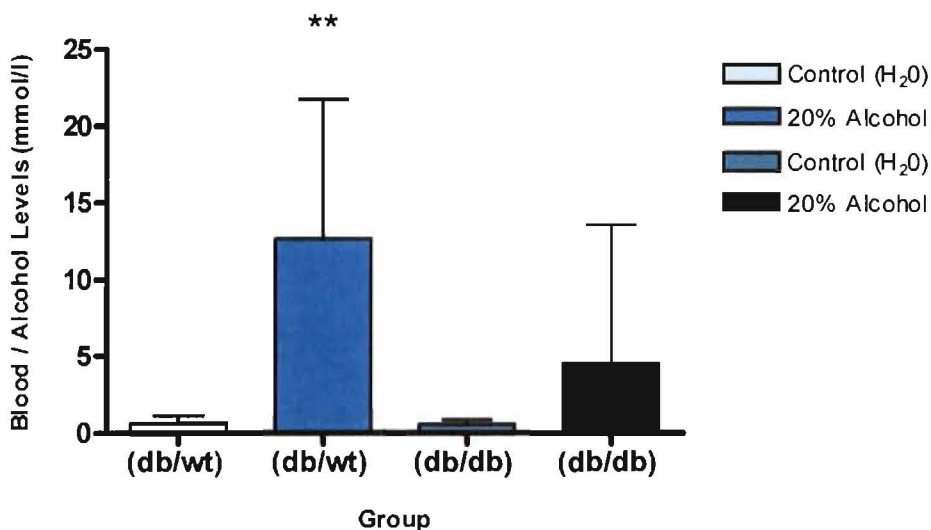


Figure 5.5.6: Alcohol concentrations in blood from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. The increase in *db/wt* alcohol-treated mice was shown to be statistically significant in comparison to the controls; ($p=0.0054$) but not in alcohol-treated *db/db* mice; ($p=0.31$). Data represented as mean \pm SD.

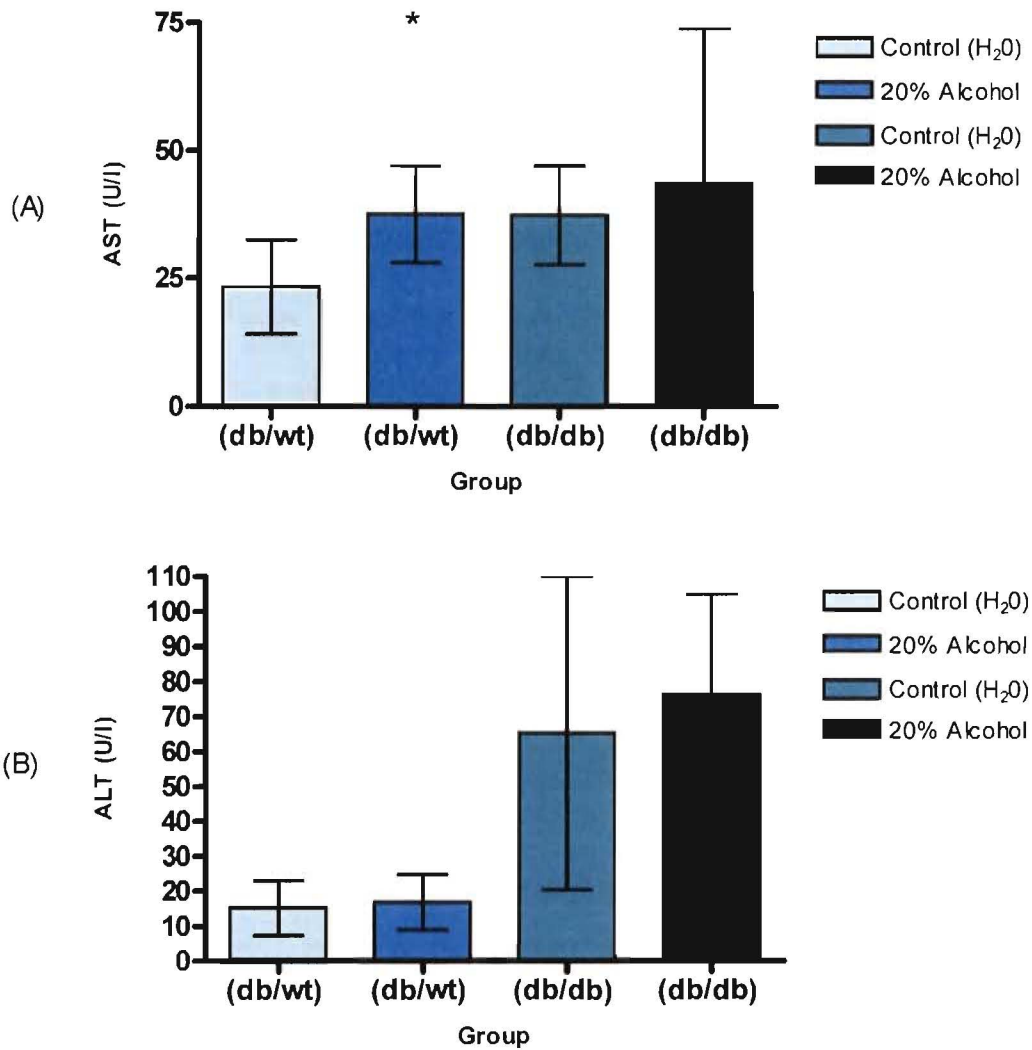


Figure 5.5.7: AST and ALT levels in serum from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. (A) The AST levels gained statistical significance for *db/wt* mice; ($p=0.037$) but not for *db/db* mice; ($p=0.64$). (B) The levels of ALT between alcohol-treated and control animals of their respective groups were not statistically significantly different for *db/wt* mice; ($p=0.75$) and *db/db* mice; ($p=0.62$). Data represented as mean \pm SD.

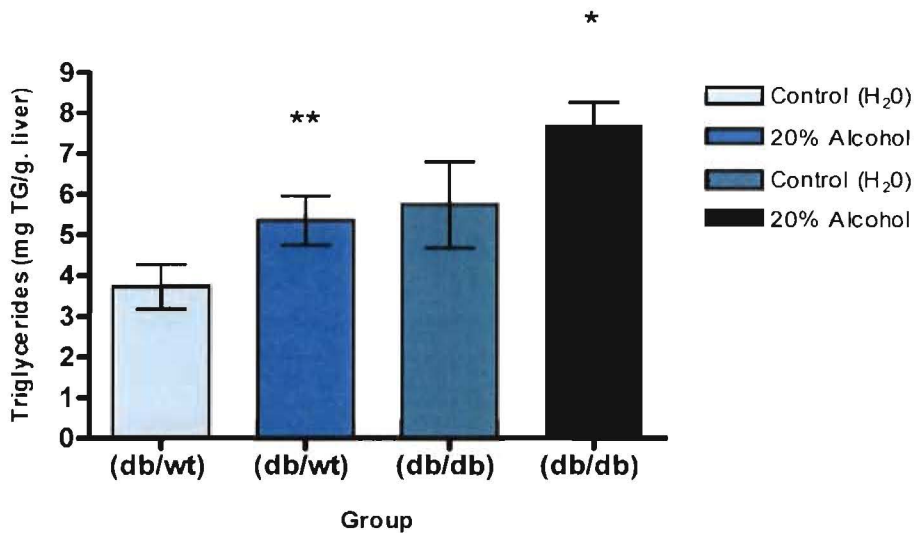


Figure 5.5.8: Triglycerides in livers from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. Triglycerides were increased in alcohol-treated groups in contrast to their respective controls for *db/wt* mice; ($p=0.0012$) and *db/db* mice; ($p=0.0030$). Data represented as mean \pm SD.

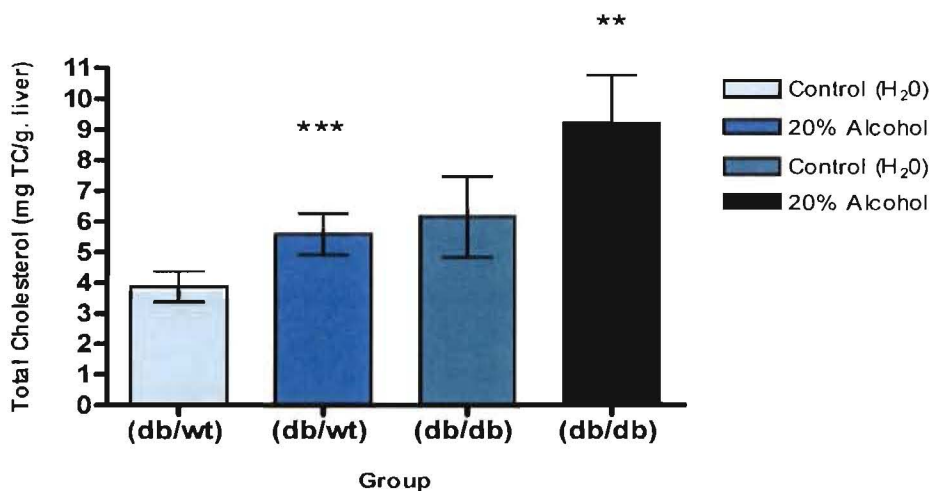


Figure 5.5.9: Total cholesterol concentration in livers from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. The increases of total cholesterol detected in alcohol-treated animals were statistically significant when compared to the control groups for *db/wt* mice and *db/db* mice; ($p=0.0010$) and ($p=0.0046$) respectively. Data represented as mean \pm SD.

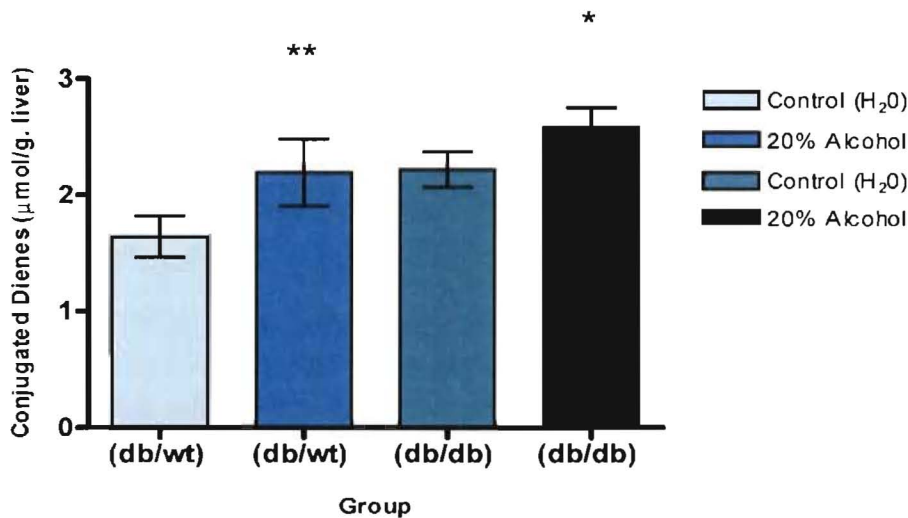


Figure 5.5.10: Conjugated dienes as early lipid peroxidation products in livers from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. Conjugated dienes of alcohol-treated animals displayed significantly elevated levels in comparison to the control groups, *db/wt* mice; ($p=0.031$) and *db/db* mice ($p=0.0025$). Data represented as mean \pm SD.

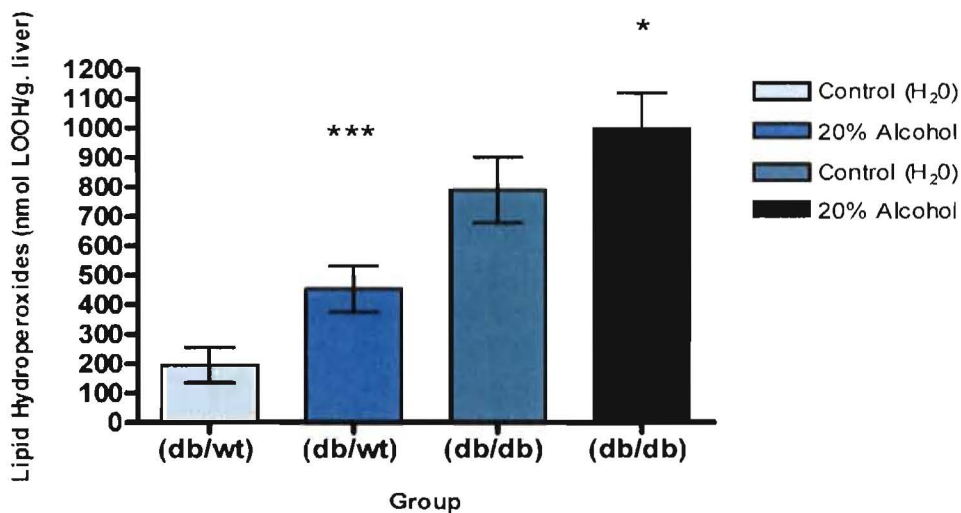


Figure 5.5.11: LOOH as intermediate lipid peroxidation products in livers from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. Increased concentrations of lipid hydroperoxides were shown in *db/wt* and *db/db* alcohol-treated animals when contrasted against the controls, *db/wt* mice; ($p=0.0002$) and *db/db* mice; ($p=0.0109$) respectively. Data represented as mean \pm SD.

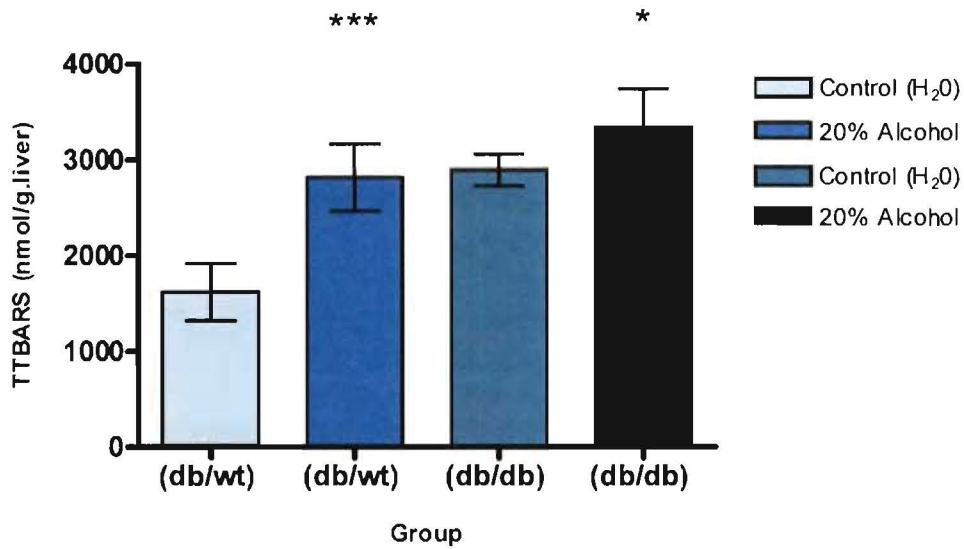


Figure 5.5.12: TTBARs as late lipid peroxidation products in livers from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. The TTBARs concentrations gained statistical significance in *db/wt* mice; ($p=0.0002$) and *db/db* mice; ($p=0.030$), Data represented as mean \pm SD. Data represented as mean \pm SD.

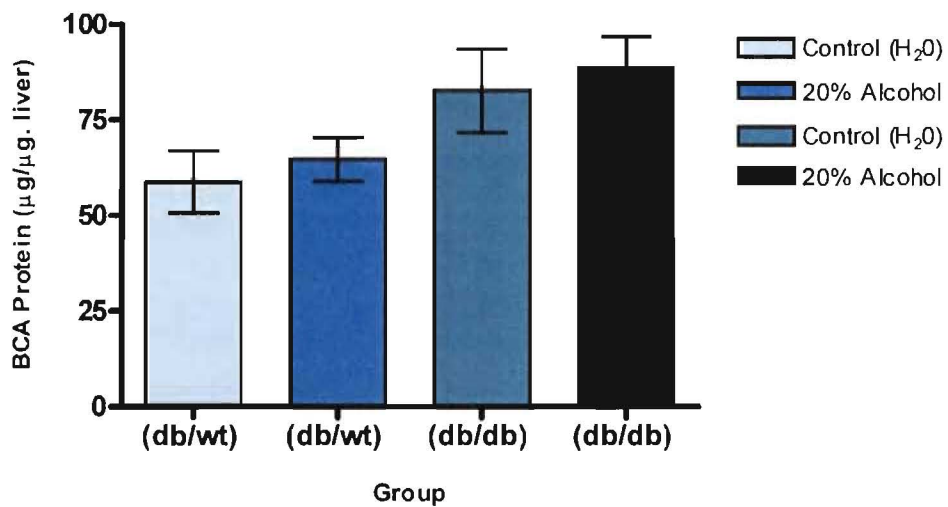


Figure 5.5.13: BCA protein in homogenate from control and 20 week alcohol-supplemented *db/wt* (n=7, n=4 respectively) and *db/db* (n=6, n=6) mice aged 32 weeks. No statistical significance was attained when compared to respective controls, *db/wt* mice; ($p=0.24$) and *db/db* mice; ($p=0.30$). Data represented as mean \pm SD.

5.6.1 Summary of Results for the db/db Mouse Model on Regular Chow for 20 Weeks

	Wild Type (<i>wt/wt</i>)		Heterozygous (<i>db/wt</i>)		Homozygous (<i>db/db</i>)	
	M	F	M	F	M	F
Sex						
No. of Animals	6	6	16	8	10	14
Terminal Body Mass (g)	22.4 ± 0.8	17.3 ± 1.6	26.4 ± 2.4	20.8 ± 1.7	44.8 ± 2.4	42.2 ± 3.9
Liver Mass (g)	0.78 ± 0.06	0.75 ± 0.08	1.27 ± 0.13	0.75 ± 0.09	1.77 ± 0.18	2.09 ± 0.39
Conjugated Dienes (µmol/g.liver)	2.07 ± 0.21	1.89 ± 0.12	2.58 ± 0.35	2.70 ± 0.34	3.48 ± 0.23	3.63 ± 0.47
Lipid Hydroperoxides (nmol LOOH/g.liver)	217 ± 76	137 ± 91	176 ± 71	216 ± 49	704 ± 151	433 ± 111
TTBARS (nmol/g.liver)	1795 ± 87	1309 ± 149	1264 ± 225	1337 ± 127	1559 ± 162	1731 ± 260
Cholesterol (mg TC/g.liver)	4.71 ± 0.39	4.85 ± 0.33	4.47 ± 0.43	4.54 ± 0.73	6.67 ± 0.80	6.68 ± 0.62
Triglycerides (mg TG/g.liver)	1.94 ± 0.44	1.81 ± 0.39	1.19 ± 0.22	1.41 ± 0.49	3.16 ± 1.13	4.14 ± 0.81
Glucose Concentration (mmol/l)	5.5 ± 1.7	5.1 ± 1.0	9.1 ± 3.4	6.0 ± 1.0	23.0 ± 2.9	20.4 ± 8.0

5.6.2 Summary of Results from the Impact of Various Oils, Administered via the Diet (4 Weeks)

	All animals in this study, both male and female were Heterozygous (<i>db/wt</i>)			
Sex / No. Animals	3M + 3F	3M + 3F	3M + 3F	3M + 3F
Treatment	Chow Only (control)	Sunflower Oil	Canola Oil	Sunflower Oil / Cholesterol
Terminal Body Mass (g)	23.4 ± 3.6	23.6 ± 2.7	24.1 ± 3.2	24.5 ± 5.0
Mean Mass Gain (g) Weeks 12-17	+ 1.2	+ 1.2	+ 0.8	+ 0.7
Liver Mass (g)	0.91 ± 0.17	0.88 ± 0.13	1.03 ± 0.13	0.89 ± 0.16
Conjugated Dienes (µmol/g.liver)	2.00 ± 0.20	2.66 ± 0.33	2.26 ± 0.30	2.46 ± 0.05
Lipid Hydroperoxides (nmol LOOH/g.liver)	400 ± 251	561 ± 235	500 ± 90	497 ± 188
TTBARS (nmol/g.liver)	3532 ± 410	3173 ± 461	3314 ± 546	3204 ± 632
Cholesterol (mg TC/g.liver)	2.33 ± 0.52	4.47 ± 1.13	3.43 ± 1.46	3.99 ± 1.08
Triglycerides (mg TG/g.liver)	20.1 ± 3.5	22.8 ± 4.7	24.4 ± 2.8	22.2 ± 2.4
BCA Protein (µg/µg. liver)	71.3 ± 11.9	72.2 ± 4.0	75.0 ± 10.8	75.5 ± 17.7
Glucose Concentration (mmol/l)	6.8 ± 1.9	7.6 ± 1.0	8.0 ± 2.2	4.5 ± 2.2

5.6.3 Summary of Results from the Impact of a Short Period of Alcohol Administration (4 Weeks)

	Wild Type (<i>wt/wt</i>)		Heterozygous (<i>db/wt</i>)		Homozygous (<i>db/db</i>)	
	1M + 1F	1M + 1F	2M + 2F	2M + 2F	2M + 2F	1M + 3F
Treatment	H ₂ O	20% alcohol	H ₂ O	20% alcohol	H ₂ O	20% alcohol
Terminal Body Mass (g)	22.4 ± 0.8	17.3 ± 1.6	26.4 ± 2.4	20.8 ± 1.7	44.8 ± 2.4	42.2 ± 3.9
Mean Mass Gain (g) Weeks 14-20	+ 2.2	+ 1.8	+ 1.8	+ 0.5	+ 3.5	+ 4.2
Conjugated Dienes (µmol/g.liver)	1.81	1.98	1.71 ± 0.31	2.18 ± 0.34	2.90 ± 0.57	3.62 ± 1.04
Lipid Hydroperoxides (nmol LOOH/g.liver)	93	291	158 ± 44	182 ± 49	434 ± 156	654 ± 296
TTBARS (nmol/g.liver)	1490	1577	1175 ± 72	1367 ± 125	1307 ± 144	1849 ± 26
Cholesterol (mg TC/g.liver)	3.54	4.53	2.62 ± 0.72	4.49 ± 0.51	4.65 ± 1.02	5.15 ± 1.42
Triglycerides (mg TG/g.liver)	1.53	1.84	1.05 ± 0.08	1.28 ± 0.09	2.93 ± 0.26	6.44 ± 0.48

Please note that a standard deviation value could not be calculated for wild type (*wt/wt*) animals as the numbers were too small.

5.6.4 Summary of Results from the Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice

	Heterozygous (<i>db/wt</i>)		Homozygous (<i>db/db</i>)	
	7M	6M	6M	5M
Sex / No. Animals	7M	6M	6M	5M
Treatment	H ₂ O	20% alcohol	H ₂ O	20% alcohol
Terminal Body Mass (g)	32.0 ± 2.7	31.8 ± 1.1	51.8 ± 5.3	49.5 ± 8.7
Mean Mass Gain (g) Weeks 9-20	+ 6.8	+ 4.5	+ 7.4	+ 5.2
Liver Mass (g)	1.06 ± 0.06	1.45 ± 0.12	2.83 ± 0.57	2.84 ± 0.42
Conjugated Dienes (µmol/g.liver)	1.60 ± 0.31	2.00 ± 0.23	2.66 ± 0.07	3.45 ± 0.16
Lipid Hydroperoxides (nmol LOOH/g.liver)	339 ± 54	724 ± 107	1073 ± 92	1486 ± 118
TTBARS (nmol/g.liver)	1762 ± 154	2529 ± 266	1862 ± 230	2962 ± 257
Cholesterol (mg TC/g.liver)	3.35 ± 0.95	5.03 ± 0.91	5.91 ± 1.02	8.46 ± 1.58
Triglycerides (mg TG/g.liver)	4.00 ± 1.63	6.61 ± 0.78	6.55 ± 1.41	10.33 ± 2.25
BCA Protein (µg/µg.liver)	55.3 ± 10.5	60.7 ± 3.6	69.4 ± 12.4	76.7 ± 12.2
AST Levels (U/l)	32.6 ± 17.3	41.7 ± 11.1	53.8 ± 21.4	77.8 ± 43.4
ALT Levels (U/l)	18.6 ± 10.5	22.3 ± 8.0	65.3 ± 46.9	71.0 ± 33.9
Blood / Alcohol Levels (mmol/l)	0.53 ± 0.46	1.33 ± 0.86	1.12 ± 0.56	4.73 ± 6.0
Glucose Concentration (mmol/l)	13.8 ± 3.4	8.6 ± 1.6	31.2 ± 2.0	29.2 ± 2.8

5.6.5 Summary of Results from the Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice

Sex / No. Animals	Heterozygous (<i>db/wt</i>)		Homozygous (<i>db/db</i>)	
	7M	4M	6M	6M
Treatment	H ₂ O	20% alcohol	H ₂ O	20% alcohol
Terminal Body Mass (g)	33.4 ± 2.7	31.3 ± 2.1	57.4 ± 5.5	50.2 ± 5.7
Mean Mass Gain (g) Weeks 9-32	+ 6.4	+ 1.2	+ 12.3	+ 6.8
Liver Mass (g)	1.37 ± 0.05	1.53 ± 0.11	2.84 ± 0.44	2.99 ± 0.38
Conjugated Dienes (µmol/g.liver)	1.64 ± 0.18	2.19 ± 0.29	2.22 ± 0.15	2.58 ± 0.17
Lipid Hydroperoxides (nmol LOOH/g.liver)	195 ± 60	452 ± 78	789 ± 10	98 ± 121
TTBARS (nmol/g.liver)	1617 ± 30	2816 ± 349	2891 ± 164	3336 ± 399
Cholesterol (mg TC/g.liver)	3.88 ± 0.50	5.58 ± 0.67	6.14 ± 1.31	9.17 ± 1.58
Triglycerides (mg TG/g.liver)	3.72 ± 0.54	5.35 ± 0.59	5.74 ± 1.06	7.67 ± 0.60
BCA Protein (µg/µg. liver)	58.6 ± 8.0	64.8 ± 6.0	82.5 ± 10.9	88.5 ± 8.0
AST Levels (U/l)	23.3 ± 9.2	37.5 ± 9.5	37.3 ± 9.6	43.5 ± 30.3
ALT Levels (U/l)	15.1 ± 7.8	16.8 ± 7.9	65.2 ± 44.8	76.3 ± 28.6
Blood / Alcohol Levels (mmol/l)	0.64 ± 0.49	12.65 ± 9.09	0.56 ± 0.27	4.49 ± 9.09
Glucose Concentration (mmol/l)	13.6 ± 3.5	12.3 ± 5.2	31.4 ± 0.8	29.2 ± 4.8

5.7 Histology Findings

5.7.1 Results for the *db/db* Mouse Model on Regular Chow for 20 Weeks

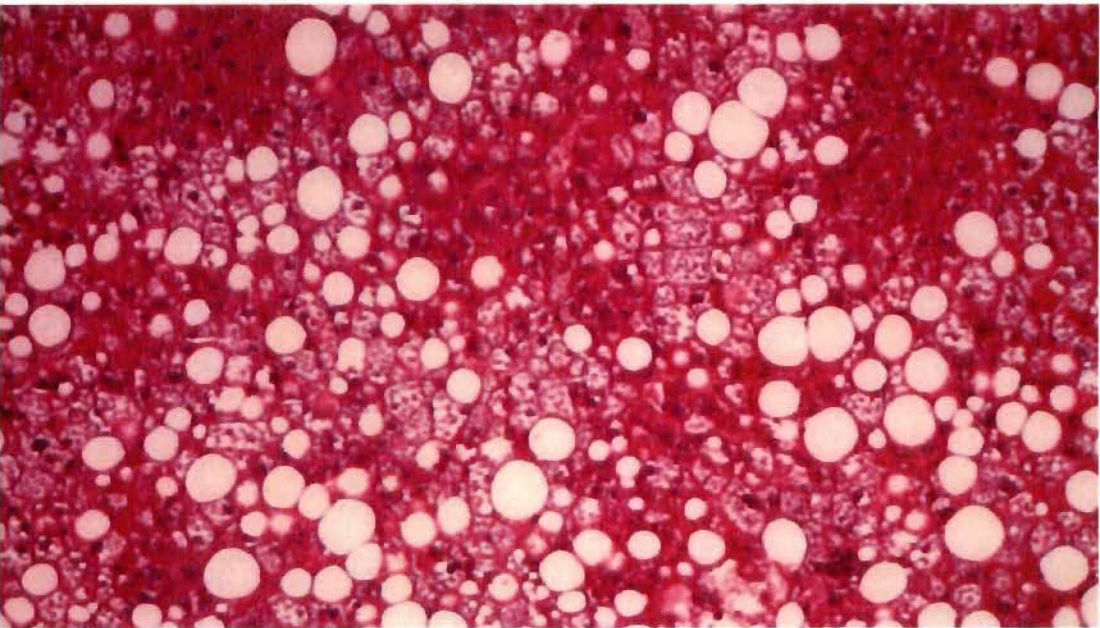
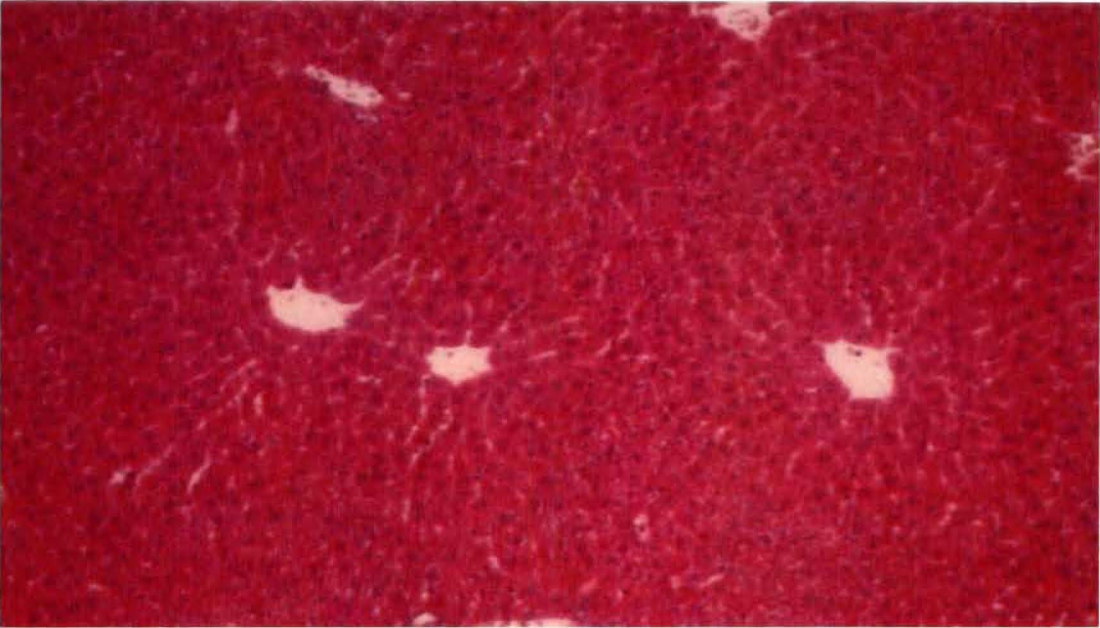
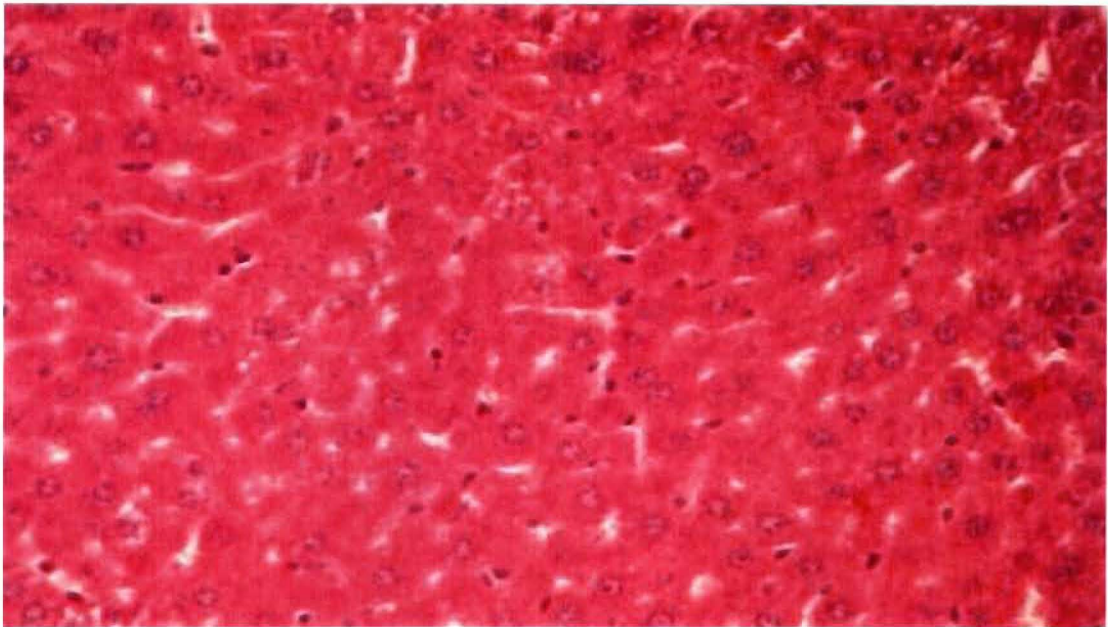
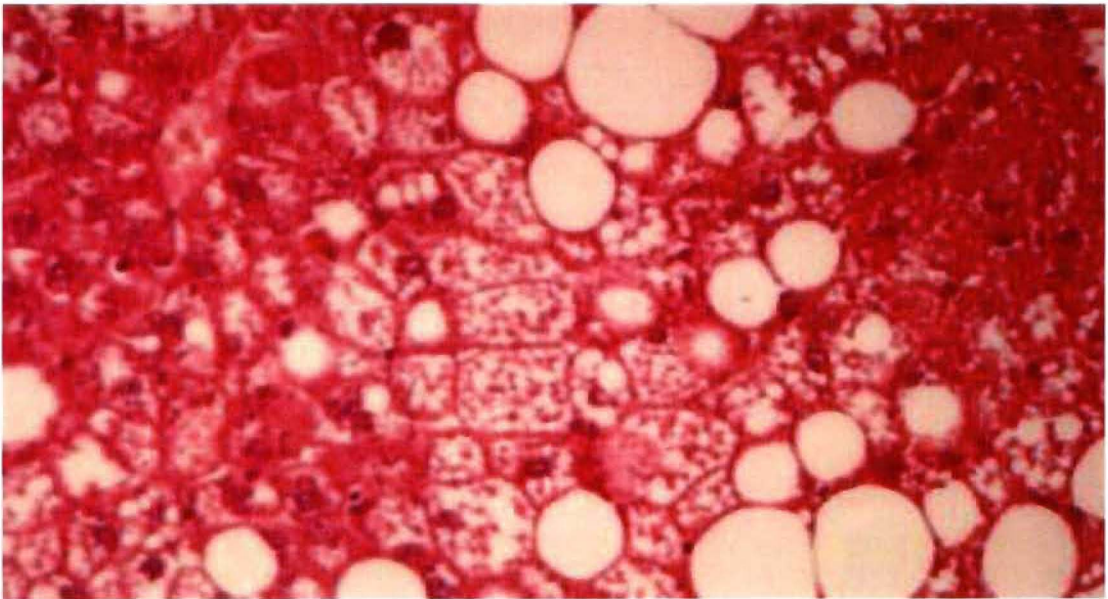


Figure 5.7.1; Histological liver sections stained with H&E from (A) heterozygous (*db/wt*) mouse on regular chow for 20wks (B) homozygous (*db/db*) mouse on regular chow for 20wks.



(C)

20x



(D)

40x

Figure 5.7.1; Histological liver sections stained with H&E from (C) heterozygous (*db/wt*) mouse on regular chow for 20wks (D) homozygous (*db/db*) mouse on regular chow for 20 wks.

5.7.2 Results from the Impact of Various Oils, Administered via the Diet (4 Weeks)

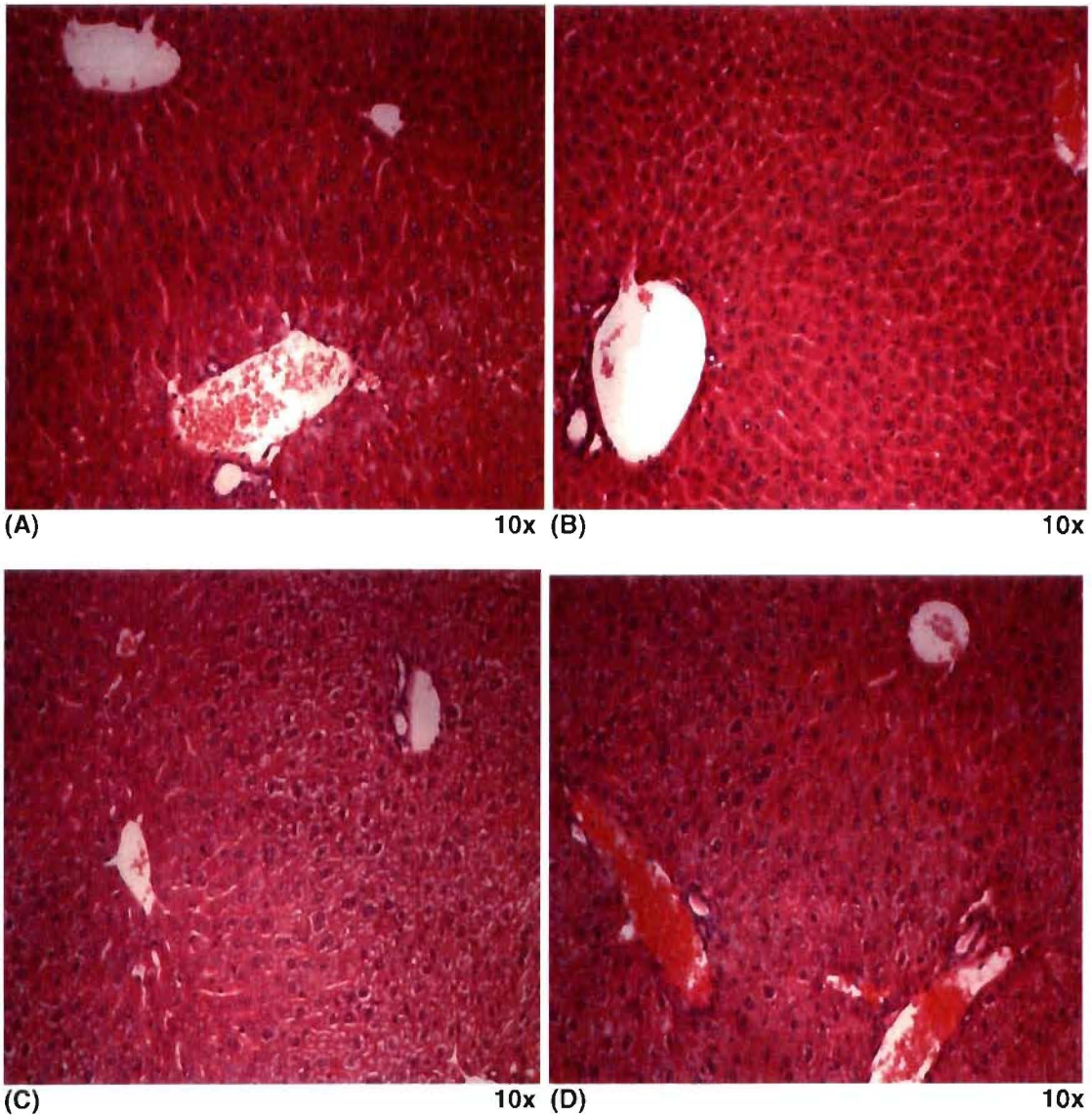


Figure 5.7.2; Histological liver sections stained with H&E from heterozygous mice (*db/wt*) fed the following diets; (A) Control; regular chow for 4wks (B) regular chow supplemented with sunflower oil for 4wks (C) regular chow supplemented with canola oil for 4wks (D) regular chow supplemented with sunflower oil and cholesterol for 4wks. The histology was normal for all groups.

5.7.3 Results from the Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice

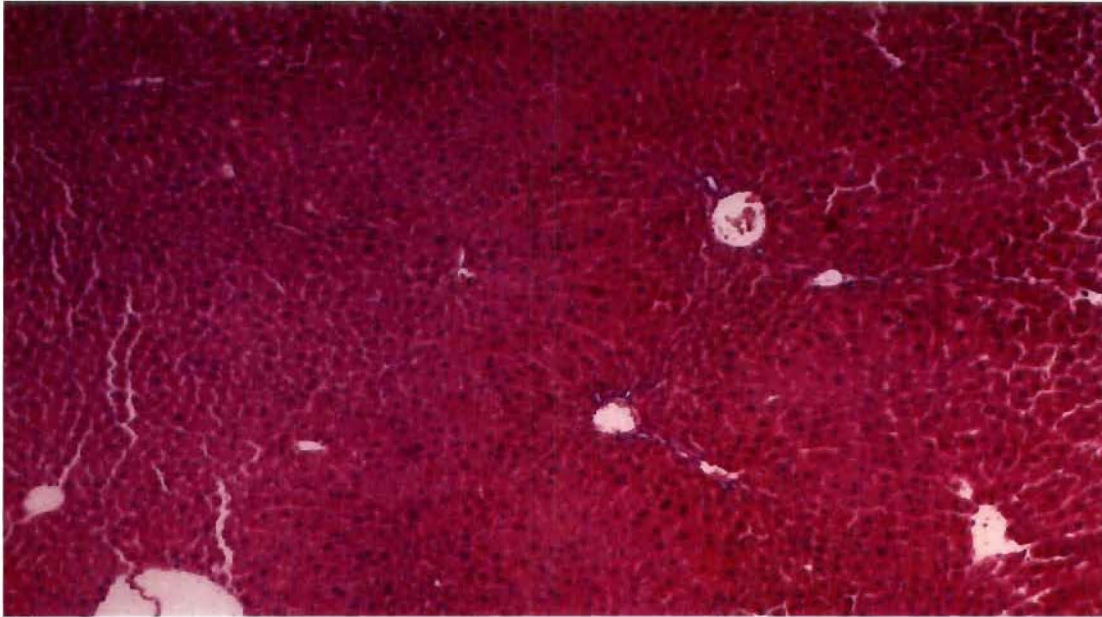
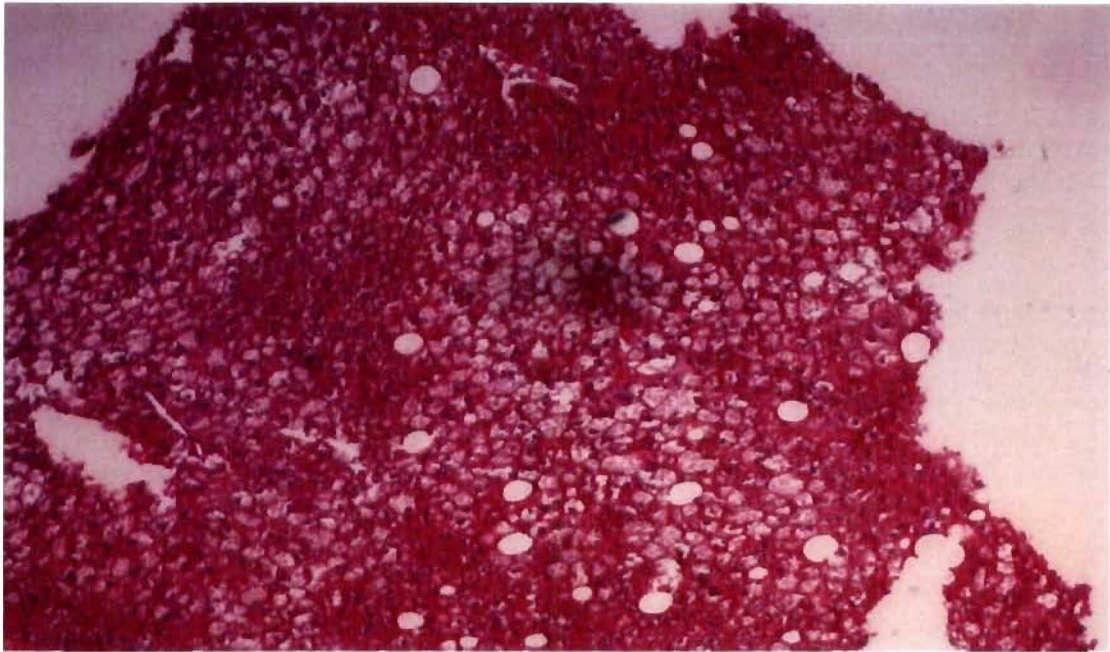


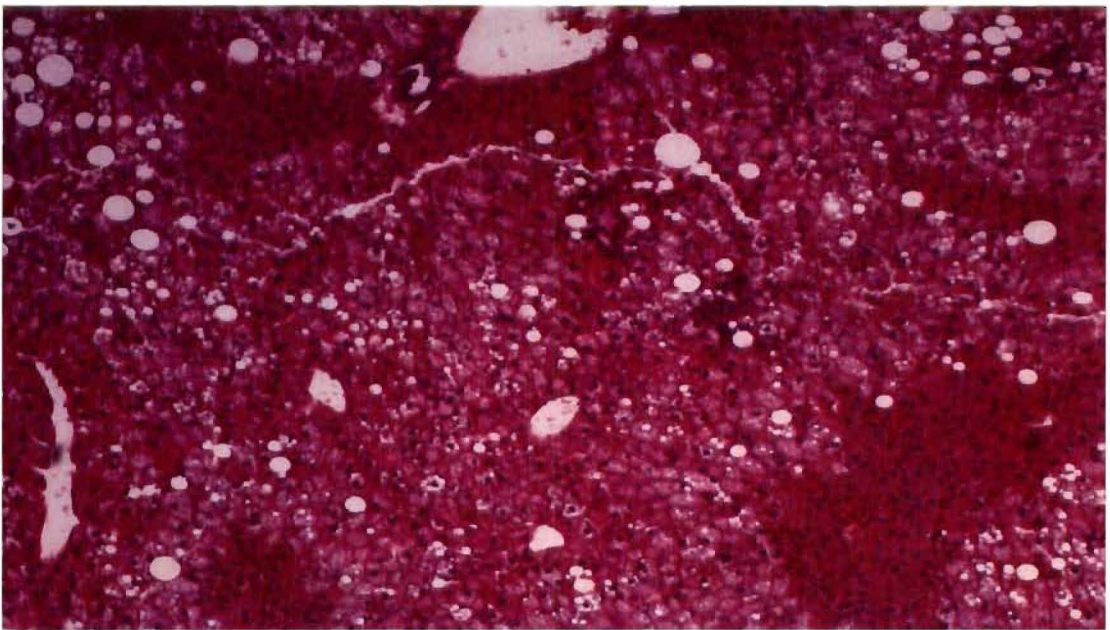
Figure 5.7.3 Histological liver section stained with H&E from (A) heterozygous (*db/wt*) mouse on 20% alcohol + 0.025% (w/v) saccharine for 8wks.

*Please note that there were slight processing problems with the H&E staining for Control; heterozygous (*db/wt*) mice on water + 0.025% (w/v) saccharine for 8wks which is why the liver section is not presented here.*



(B)

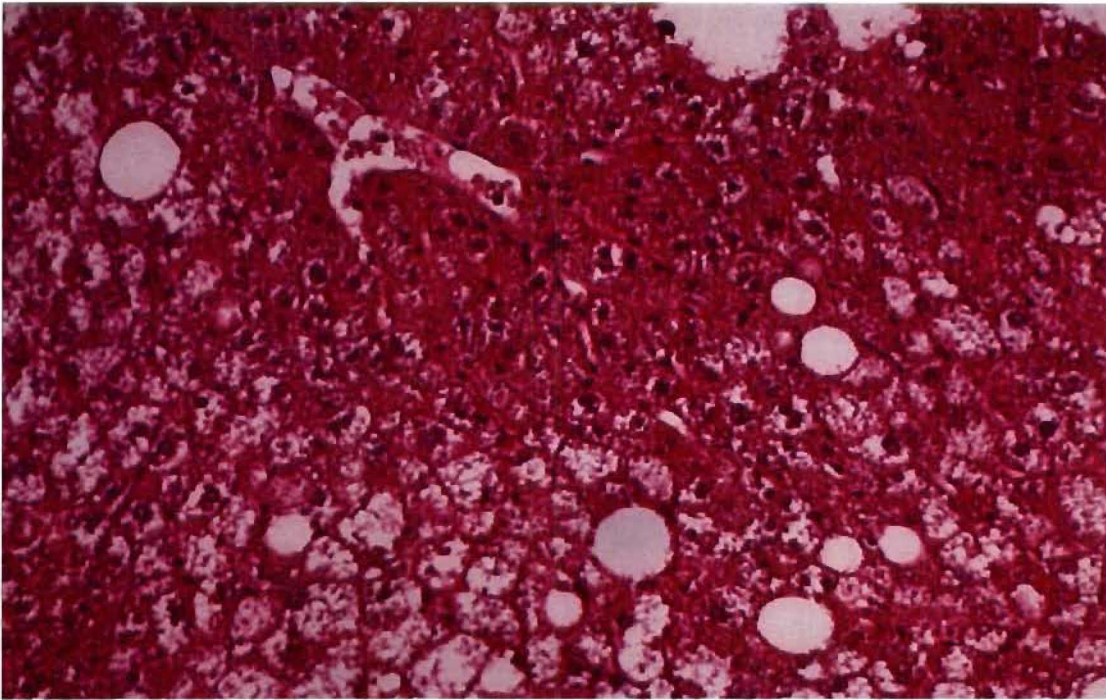
4x



(C)

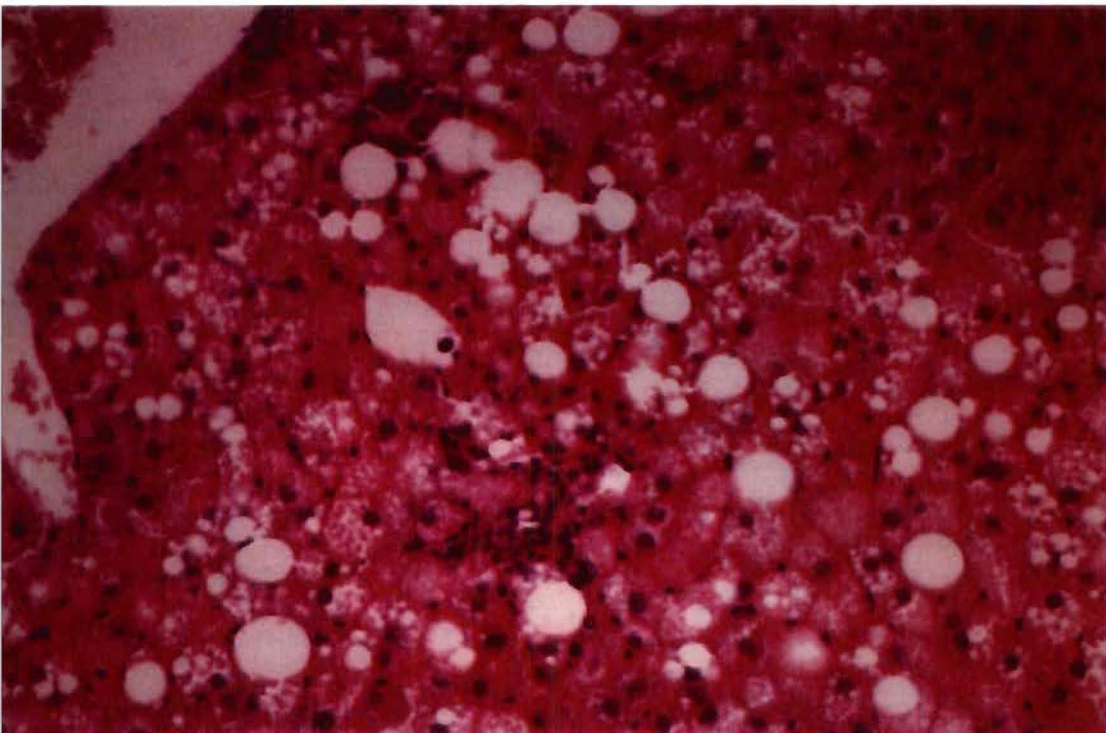
4x

Figure 5.7.3 Histological liver sections stained with H&E from (B) Control; homozygous (*db/db*) mouse on water + 0.025% (w/v) saccharine for 8wks (C) homozygous (*db/db*) mouse on 20% alcohol + 0.025% (w/v) saccharine for 8wks.



(D)

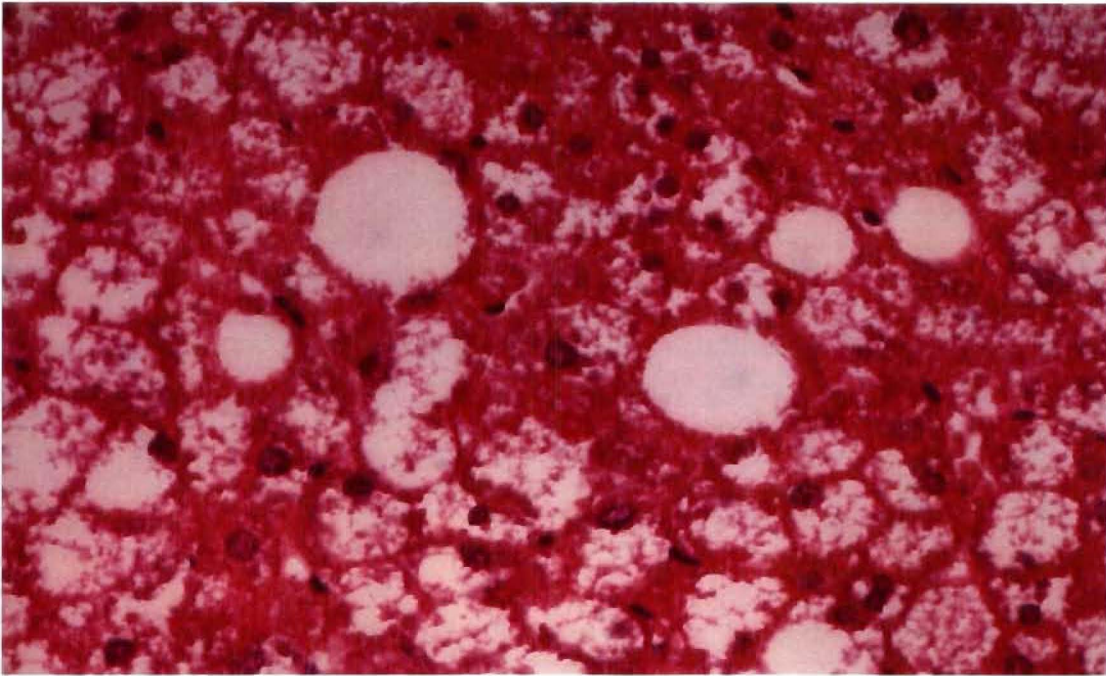
10x



(E)

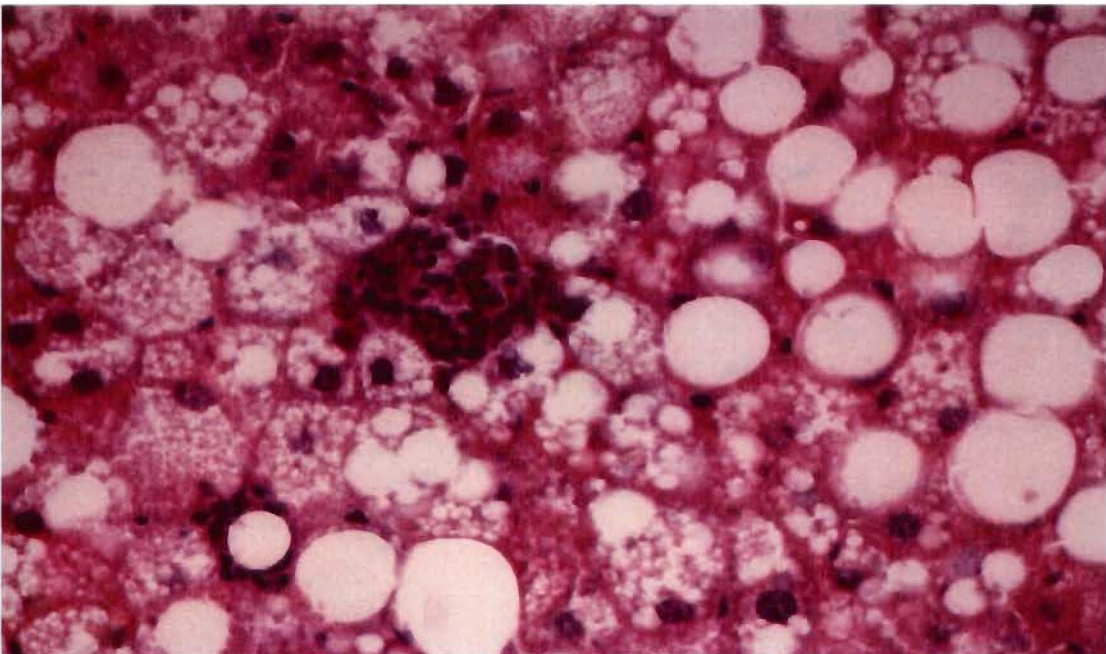
10x

Figure 5.7.3 Histological liver sections stained with H&E from (D) Control; homozygous (*db/db*) mouse on water + 0.025% (w/v) saccharine for 8wks (E) homozygous (*db/db*) mouse on 20% alcohol + 0.025% (w/v) saccharine for 8wks.



(F)

20x

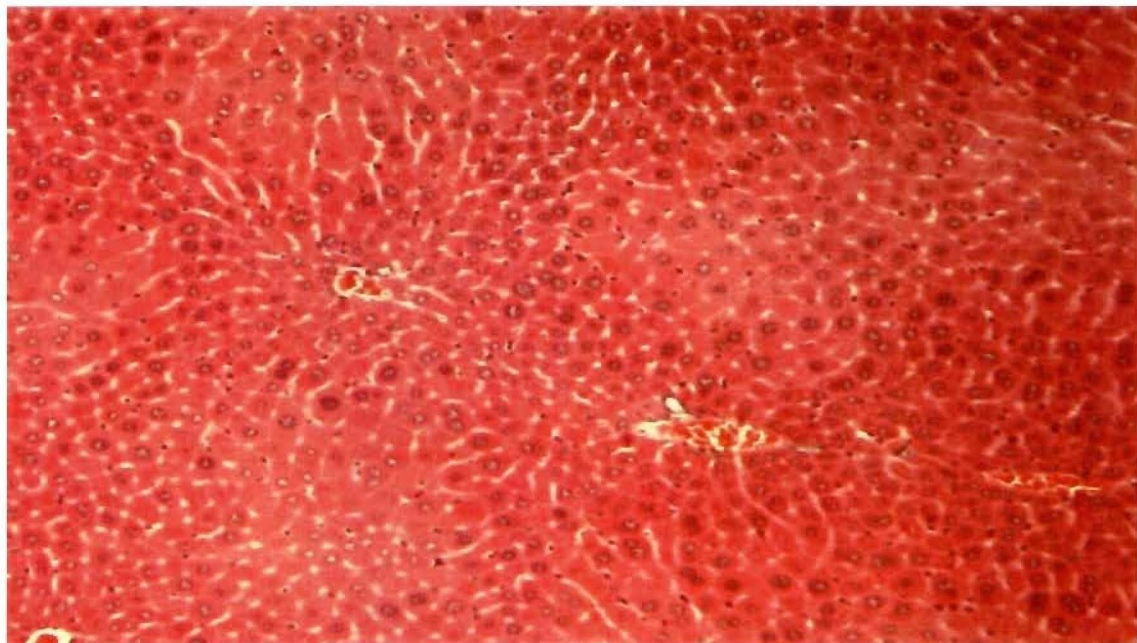


(G)

20x

Figure 5.7.3 Histological liver sections stained with H&E from (F) Control; homozygous (*db/db*) mouse on water + 0.025% (w/v) saccharine for 8wks (G) homozygous (*db/db*) mouse on 20% alcohol + 0.025% (w/v) saccharine for 8wks.

5.7.4 Results from the Impact of Alcohol Administered over 20 Weeks in *db/wt* and *db/db* Mice



(A)

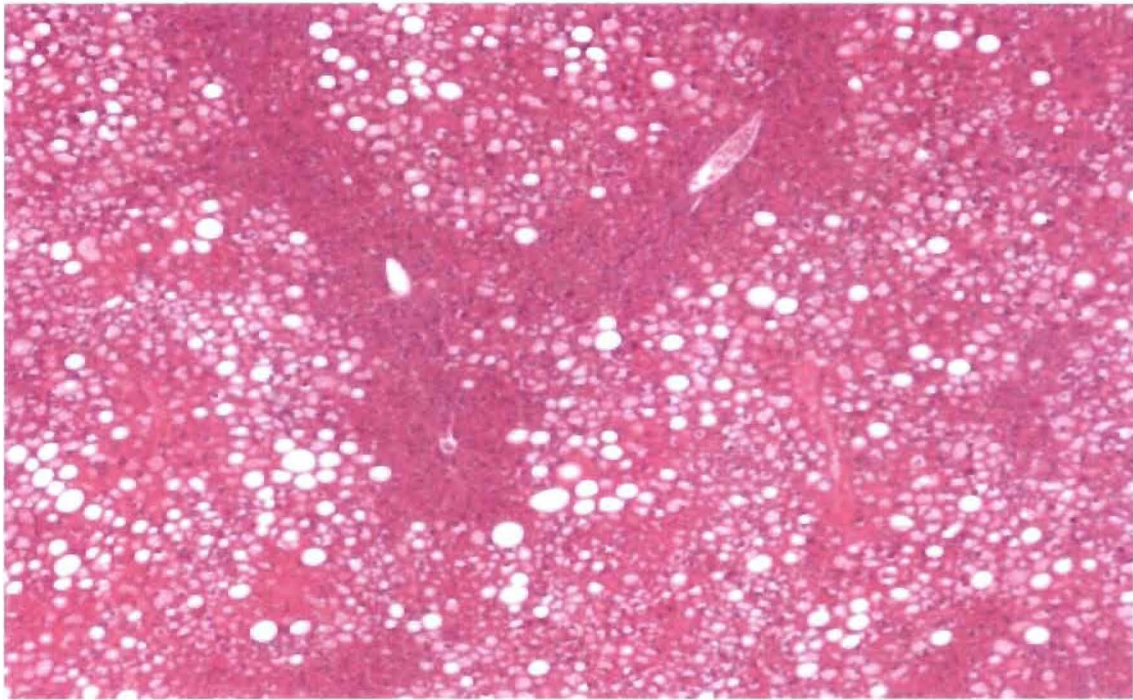
20x



(B)

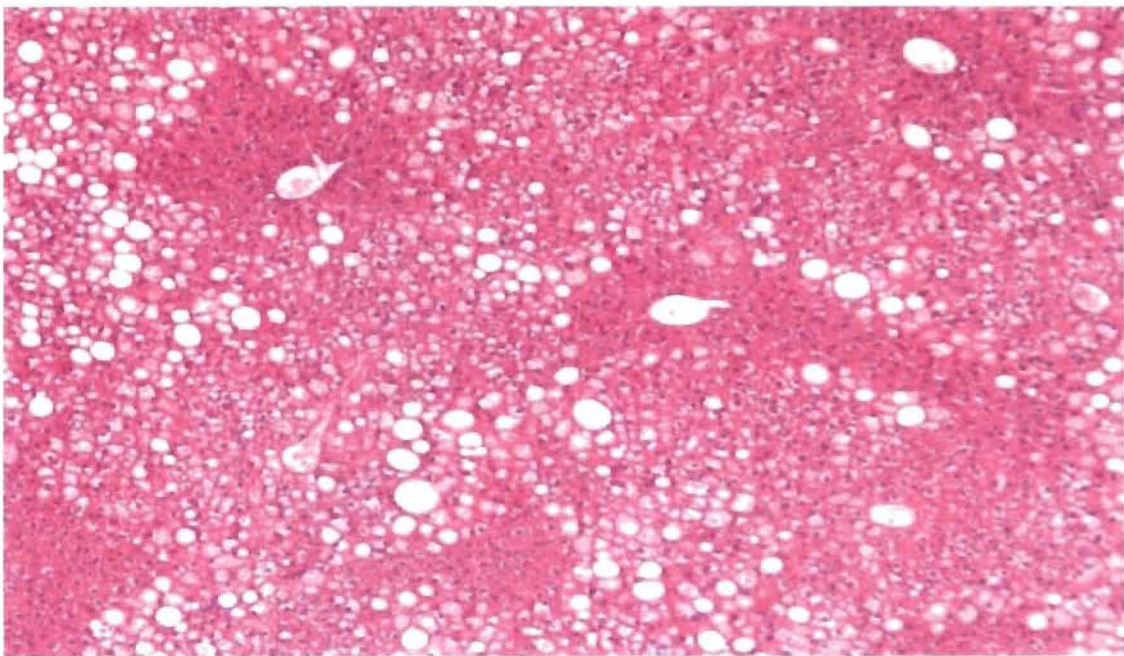
10x

Figure 5.7.4 Histological liver sections stained with H&E from (A) Control; heterozygous (*db/wt*) mouse on water + 0.025% (w/v) saccharine for 20wks (B) heterozygous (*db/wt*) mouse on 20% alcohol + 0.025% (w/v) saccharine for 20wks.



(C)

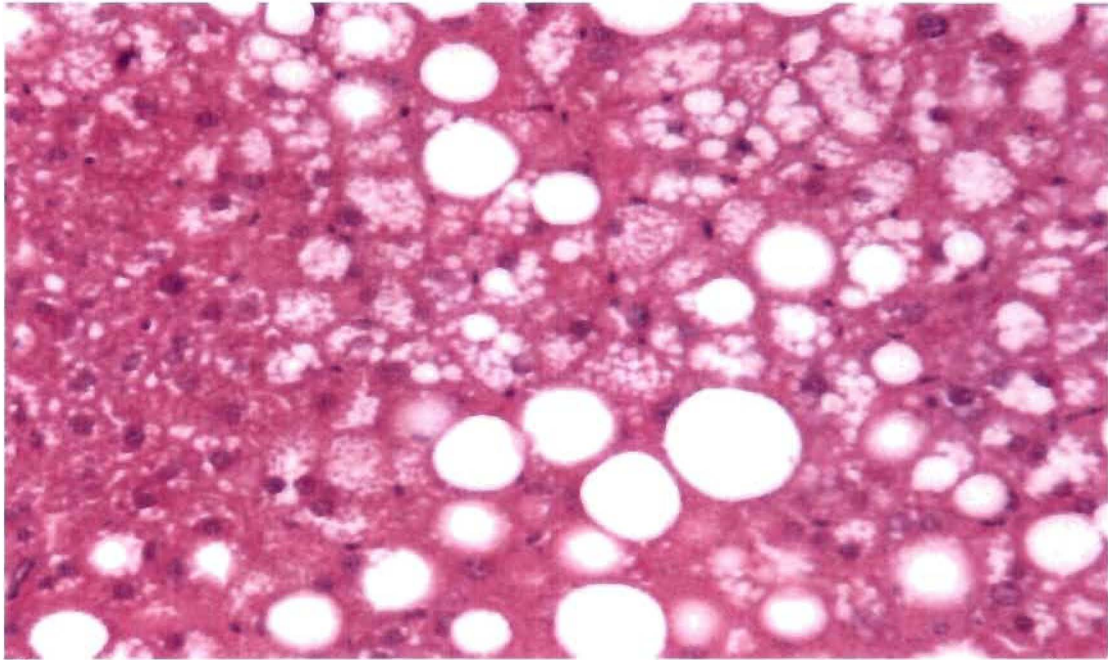
20x



(D)

20x

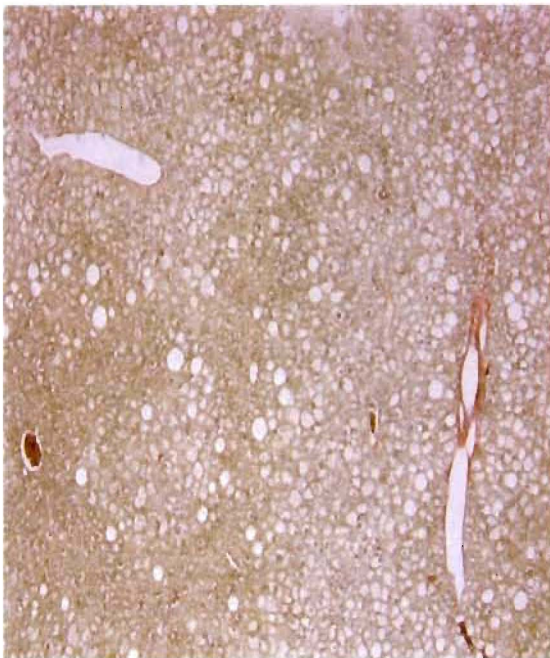
Figure 5.7.4 (C) Control; homozygous (*db/db*) mouse on water + 0.025% (w/v) saccharine for 20wks (D) homozygous (*db/db*) mouse on 20% alcohol + 0.025% (w/v) saccharine for 20wks.



(E)

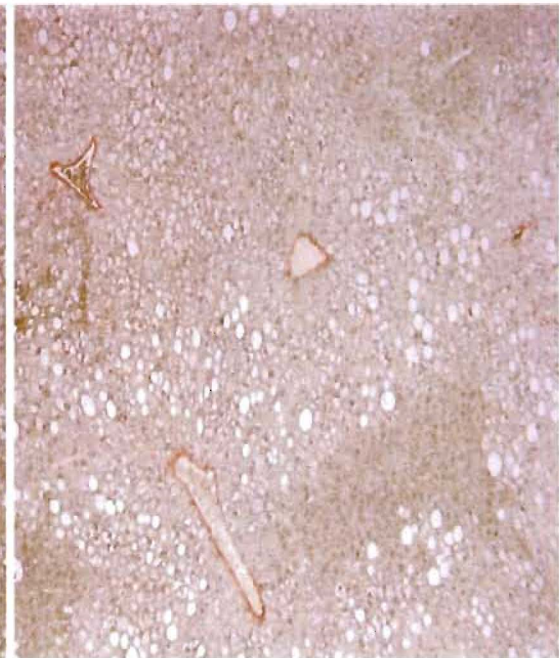
40x

Figure 5.7.4 Histological liver section stained with H&E from (E) Control; homozygous (*db/db*) mouse on water + 0.025% (w/v) saccharine for 20wks.



(F)

10x



(G)

10x

Figure 5.7.4 Histological liver section stained with sirius red for collagen; (F) Control; homozygous (*db/db*) mouse on water + 0.025% (w/v) saccharine for 20wks (G) homozygous (*db/db*) mouse on 20% alcohol + 0.025% (w/v) saccharine for 20wks.

Chapter 6; Interpretation of Results

6.1 Assessment of the *db/db* Mouse Model on Regular Chow for 20 Weeks

The *db/db* mouse was investigated to test its suitability as an appropriate model to study the development of fatty liver in the context of NAFLD and NASH. Alcohol was used as a possible trigger factor for NASH as it is known to induce enzymes and to cause changes similar to NASH in humans. Based on physical appearance alone, it was apparent from the outset that there could be considerable biochemical differences between the homozygous, heterozygous and wild-type strains of the *db/db* mouse model as the pictures in figure 6.1.1 clearly reveal. The assessment of *wt/wt*, *db/wt* and *db/db* animals was undertaken in an attempt to fully characterize the model. Furthermore, the graphs display results for combined sexes, male and female, to assess each genotype as a single constituent but grouped sexes and individual results are presented in the tables and appendix sections respectively.

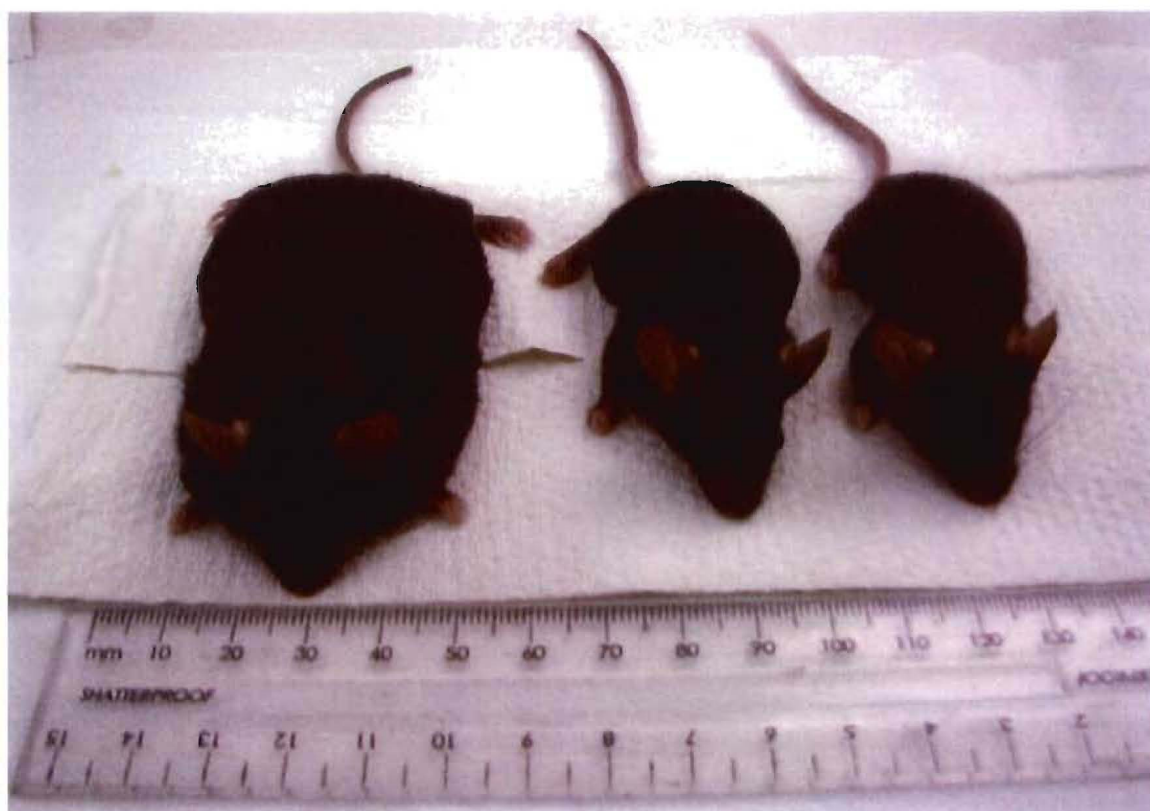


Figure 6.1.1: From left to right, the picture displays the size difference between the homozygous (*db/db*), heterozygous (*db/wt*) and wild-type (*wt/wt*) strains.

The analysis of these animals revealed several characteristics that mirror those typically witnessed in humans and confirms its usefulness as a model to study NAFLD and NASH. Firstly, the *db/db* mice were obese, laden with visceral fat (see

figure 6.1.2) and exhibited body weights which dramatically increased in size in comparison to the lean *wt/wt* and *db/wt* mice; (43.5g vs. 19.9g and 23.6g respectively), and attained statistical significance ($p < 0.0001$ for both). Additionally, the mean difference in body mass between the *wt/wt* and *db/wt* animals is 3.7g which is statistically significant ($p = 0.0003$). Despite this disparity in body mass being small, it could possibly be attributed to an effect of the *db* gene and may influence responses, especially in the setting of hyperglycaemia.

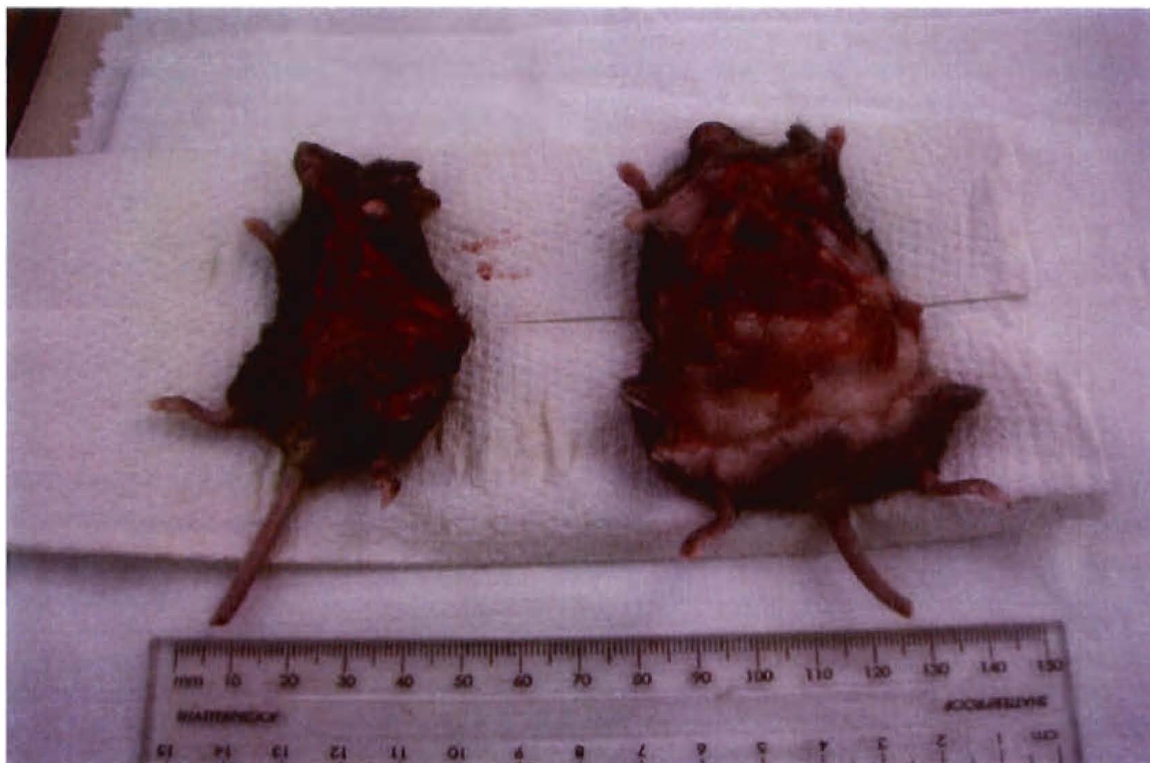


Figure 6.1.2: This clearly depicts the difference in fat present in the heterozygous (*db/wt*) and homozygous (*db/db*) strains.

The mass of liver was also noticeably increased in *db/db* mice in contrast to the *wt/wt* and *db/wt* mice (see figure 6.1.3), achieving statistical significance of $p < 0.0001$ for both which reflects the larger body mass of the homozygous mice. Besides this, liver mass was slightly elevated in the *db/wt* animals when compared to the *wt/wt* animals and again reached statistical significance ($p = 0.011$). This information provides further evidence that the *db* gene is possibly causing an effect, and although it is slight, a definite trend is beginning to occur. Furthermore, the relative liver weight: body weight ratios were assessed for *wt/wt*, *db/wt* and *db/db* animals which resulted in all groups being comparative. Therefore, despite the *db/db* mice having larger livers, they do appear to be in appropriate proportion to their body mass. An additional observation emerging from the *db/db* mice is that, although the mean body mass for

both sexes is comparable, a marginal decrease in female body mass does exist when contrasted against the males; (42.2g vs. 44.8g respectively), yet conversely, female liver mass is increased in comparison to the males (2.09g vs. 1.77g respectively), although statistical significance is not attained ($p=0.103$). It is unclear whether gender may be affecting the discrepancy in liver mass as previous studies have displayed the females to be at higher risk of fatty liver development (Angulo *et al.*, 1999), but there are too few animals in the groups to determine if this is a 'true' causative effect.

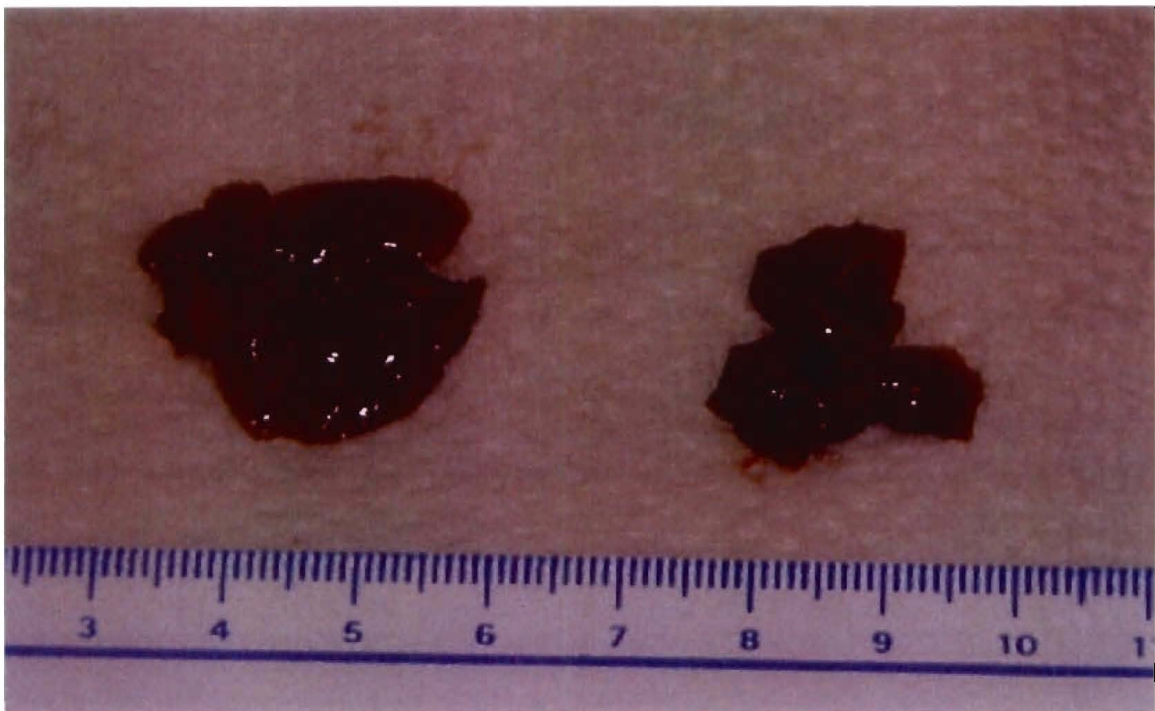


Figure 6.1.3: From left to right, the picture displays the variation in liver size of homozygous (*db/db*) and heterozygous (*db/wt*) mice strains.

Additionally, the livers of *db/db* animals were shown to have yellow coloration and were significantly enlarged in contrast to the *wt/wt* and *db/wt* groups; ($p<0.0001$ for both). Despite the significant increase of liver mass in *db/db* animals, the liver was proportionate to body mass when adjusted for body mass ratios and was found to be comparative to that of *wt/wt* and *db/wt* groups. However, the extent of fat accumulation and the low degree of inflammatory response was not significant enough to facilitate NASH in these mice. As a result the aptness of this model could be slightly restricted, meanwhile posing the question whether some sort of contributing factor is missing in the genetic make-up that prevents the progression to NASH. Alternatively, the inflammatory response of mice may differ. It is also possible

that dietary fat may need to be increased significantly more, possibly with heated fat to resemble humans.

In comparison, histological examination of the lean *wt/wt* and *db/wt* groups displayed hepatocytes which were normal in structure and function with no aggregations of fat droplets present. There were no noteworthy histological differences detected between male and female livers in *wt/wt*, *db/wt* or *db/db* groups respectively. Although marked steatosis was evident in the *db/db* mice, the inflammation and fibrotic processes of NASH were absent in the control and alcohol groups. These findings indicate that histology, though sensitive for qualitative changes such as the presence of inflammation and fibrosis is insensitive in detecting more subtle change.

Biochemical analysis was undertaken to assess early, intermediate and late products of lipid peroxidation and all these parameters were found to be elevated in *db/db* mice. Conjugated dienes are considered to be a reliable marker as early products of lipid peroxidation. The increase in CD in *db/db* mice attained statistical significance when compared to the *wt/wt* and *db/wt* groups ($p < 0.0001$ for both), highlighting the difference in fatty acid metabolism. There was also a slight increase in CDs in *db/wt* mice when compared to the *wt/wt* group which demonstrated statistical significance ($p < 0.0001$) and therefore continues to emphasize the growing trend that the *db* gene may be influential in FA metabolism and consequently the development of fatty liver. Furthermore, CD concentrations of male vs. female were analysed for *wt/wt*, *db/wt* and *db/db* groups and although they were comparative for *wt/wt* and *db/wt* groups; (1.81 vs. 1.98 and 1.71 vs. 2.18 $\mu\text{mol/g}$. liver respectively), the values were slightly elevated in the females. This may show an inherent difference in peroxidation activities or susceptibility between the genders. A superior increase was observed in females when comparing sexes in the *db/db* mice; (2.90 vs. 3.62 $\mu\text{mol/g}$. liver respectively), but this did not achieve statistical significance either ($p = 0.36$). In addition, it must be noted that all increases of LOOH occurred in the female gender and despite statistical significance not being reached in all groups, a slight effect of gender difference could be attributed for the change. A clear effect cannot, however be determined due to the small group sizes.

Lipid hydroperoxides are classified as intermediate products of lipid peroxidation. They were found to be significantly increased in *db/db* animals when evaluated against the *wt/wt* and *db/wt* animals ($p < 0.0001$ for both). In comparison, there was no significant difference between the *wt/wt* and *db/wt* groups and therefore the *db* gene

does not appear to display an effect on LOOH concentration. Additionally, no notable changes were exhibited when comparing sexes for *wt/wt* and *db/wt* groups, but the difference proved to be significant in *db/db* animals ($p < 0.0001$) due to a dramatic increase in male mice. This finding contradicts the present trend of female gender being influential but a reasonable degree of variation did exist within all groups as demonstrated by the standard deviations.

Late products of lipid peroxidation; total TBARS, were analysed and the increases exhibited in *db/db* mice were found to be statistically significant in comparison to the *db/wt* group; ($p = 0.0095$). The *db/db* animals also displayed increases of TTBARS when contrasted against the *wt/wt* group but these were only slight and did not attain statistical significance ($p = 0.79$ and $p = 0.25$ respectively). The values were comparable for *wt/wt* and *db/wt* groups, although levels were marginally elevated in *wt/wt* animals. The methods in the numerous published studies are not consistent, thus making a comprehensive comparison of experiments difficult, especially for products of lipid peroxidation.

Hepatic triglyceride content was also significantly increased in *db/db* mice in comparison to the lean *wt/wt* and *db/wt* groups ($p < 0.0001$ for both). The massive difference could be due to abnormal TG synthesis or decreased oxidation and export (or all these processes) in the *db/db* group. The increase of lipid peroxidation products could, in part, reflect oxidative stress from enhanced oxidation that does not compensate for altered fluxes into the liver of FA or *de novo* synthesis. Furthermore, the *db* gene did not exert any influence on TG concentration in the heterozygous state as the content was slightly higher in *wt/wt* mice in contrast to the *db/wt* group, although the values were relatively comparable. The TG content was comparable between *wt/wt* male and female mice; (1.81 vs. 1.91 mg TG/g. liver) and *db/wt* male and female mice; (1.19 vs. 1.41 mg TG/g. liver respectively). Despite the fact that no statistical significance was reached, values were marginally higher in female animals. Additionally, the increases in female mice of *db/db* group were statistically significant in contrast to the males ($p = 0.019$) and provides evidence of a slight effect which could be attributed to female gender.

Concentrations of total cholesterol were examined and again revealed statistically significant increases in *db/db* mice when contrasted against *wt/wt* and *db/wt*; ($p < 0.0001$ for both). Increases in cholesterol can not be the consequence of the diet as all animals were fed regular chow for 20 weeks. Therefore, the significant increase

must be attributed as a resulting defect of the *db/db* model. TC was comparable between *wt/wt* and *db/wt* groups (4.78 vs. 4.51 mg TC/g. liver respectively), although levels were slightly raised in *wt/wt* animals and therefore demonstrated no influence from the *db* gene. Minor increases were observed in females when compared to males in their respective *wt/wt*, *db/wt* and *db/db* groups but the differences were too small to be successfully attributed to female gender.

Glucose concentrations in plasma were shown to continue the growing trend of increases in *db/db* mice being statistically significant when compared to *wt/wt* and *db/wt* groups ($p < 0.0001$ for both). The prevailing increase in glucose in *db/db* mice is indicative of diabetes being present in this genotype. Slight increases were observed in *db/wt* animals in contrast to the *wt/wt* group values were comparative and no statistical significance was attained. Additionally, glucose concentrations were revealed to be increased in males when compared against the females from *wt/wt*, *db/wt* and *db/db* groups. The difference was insignificant between sexes of *wt/wt* and *db/db* animals but did achieve statistical significance in *db/wt*; ($p = 0.02$). It is possible that in the longer term, more striking differences will emerge in *db/wt* mice as diabetes may progress.

Assessment of the varying genotypes within this mouse model revealed that *db/db* mice were obese in comparison to the lean mice; *wt/wt* and *db/wt*, which was confirmed by exhibiting mean body mass approximately double in size. Increases in liver mass of *db/db* mice were also statistically significant in comparison to the *wt/wt* and *db/wt* animals but after adjusting for liver mass: body mass ratios, liver of *db/db* group was found to be in appropriate proportion to body mass resulting in all 3 groups displaying relatively similar values. CDS, LOOH, TTBARS, TC, and TG were all notably higher in *db/db* mice in comparison to the *wt/wt* and *db/wt* and although statistical significance was not reached in every parameter investigated, evidence does suggest that severely altered fatty acid metabolism is occurring within these homozygous mice. The occurrence of obesity and steatosis are prominent characteristics of NAFLD and could be attributed to the resulting derangement of FA metabolism. The progression from fatty liver to NASH did not transpire in these animals and the reasoning remains undefined. In addition, the significant increase of plasma glucose is strongly indicative of diabetes which is also strongly associated with NAFLD and further enhances the credibility of the *db/db* mouse as a suitable model. Additionally, the presence of the *db* gene did appear to provoke various increases in body mass, liver mass, CDS, LOOH and glucose concentration in

comparison to the *wt/wt* group and potentially may be a slight effect. However, the *db/wt* group displayed slight decreases for TTBARs, TC and TG in comparison to the *wt/wt* animals and resultantly obscures the correct interpretation. The question of female gender being attributed as an effect is also debatable, despite displaying some statistically significant increases, results were very variable and unpredictable and not consistent throughout the groups. Consequently, any small difference cannot be attributed to female gender and further studies with larger group sizes would have to be undertaken to establish an effect.

Overall, the *db/db* mouse mirrors several characteristics often distinguished in NAFLD patients such as obesity, diabetes and fatty livers. However, further stress must be exerted on this model in an attempt to facilitate the progression to NASH and therefore help identify a cause behind this disease.

6.2 Impact of Various Oils, Administered via the Diet (4 Weeks)

In society today, various oils are utilized for cooking and preparation of many foodstuffs. Human evolution might not have been on a continuously high consumption of TG. The purpose of this study was therefore aimed at determining whether the lipids contained within any particular oil were present in significant amounts to produce increased levels of fat accumulation. It could also test whether PUFA, being more prone to peroxidation, significantly influenced these parameters in the short term. In an attempt to provoke hepatic fatty change, lean *db/wt* mice were used as it was thought that any minor change that may occur would be masked in the already obese, diabetic *db/db* mice. Unfortunately, due to limitations with animal numbers, all groups only contained 3 males and 3 females which were pooled together for statistical reasons.

The initial observations that were made comprised the weekly recording of body weights which were comparable over the treatment period. However, after the first week of treatment, the controls were the only group to have an increase in mean body mass gain; (+0.3g). The sunflower oil-treated group displayed no change whereas the canola oil-treated mice and sunflower oil/cholesterol-treated mice were shown to lose weight; (-0.6g and -1.0g respectively). This anomaly can be accredited to the diet modification as the oil-treated animals would have possibly taken a dislike to the new taste and would therefore not have consumed normal quantities in the beginning. This is reflected by all groups displaying an increased mean weight gain in

comparison to the controls the following week; (+0.1g vs. +0.3g, +0.2g and +1.5g respectively). As these changes are negligible and values were comparable over the treatment period it must be concluded that administration of any oil or combination with cholesterol did not have any effect on body weight gain. This is further confirmed by contrasting gradients for the treated groups against the control animals as no statistical significance was attained; ($p=0.73$, $p=0.31$ and $p=0.74$ respectively). In spite of this, there are a small number of individuals who still develop NAFLD and NASH without becoming obese so even though an obvious increase in body mass did not occur, the possibility of developing fatty liver still remains.

Liver mass was also comparable for all groups despite the canola oil treated animals having a slightly higher mean value than the controls; (1.025g vs. 0.905g respectively). However the canola oil group did have a statistically significant increase of liver weight: body weight ratio; ($p=0.002$) and although this change was minor, it could be the outcome of fat accumulation attributed to a slight effect of treatment with canola oil. Histological examination of the liver sections provided conflicting evidence as no significant findings were produced. There was no indication of fat accumulation in either treatment group as all hepatocytes appeared to be normal in structure without the present of steatosis. The slight increase in liver mass of canola oil-treated animals can therefore not be attributed to treatment and again signifies that the surplus of ingested lipids from oil administration does not appear to have an effect in contrast with the control animals.

In light of the insignificant histology findings, livers were analysed for products of lipid peroxidation to assess whether the oil administration affected the livers at a biochemical level. No single treatment group achieved a consistent increase in any of the parameters investigated and the control animals even had slightly elevated TTBARs when compared to the treatment groups. These findings suggest that under near physiologic conditions, dietary fat alone does not stress the liver severely. Conjugated dienes represent one of the early products of lipid peroxidation and were found to be slightly raised in all treatment groups in contrast to the controls. Gaining statistical significance in the sunflower oil and sunflower oil/cholesterol-treated animals; ($p=0.0019$ and $p=0.0002$ respectively), and revealing a possible trend for the canola oil group; ($p=0.10$) is not surprising as oils contain more CD.

The concentrations of LOOH were determined to depict the middle products of lipid peroxidation. Although the treatment groups maintained slight increases when

compared to the control mice, all values were fairly comparable and no statistical significance was attained in any group. The increase in CD thus did not promote LOOH. This is in accordance with the belief that more than one hit is required.

TTBARS were marginally raised in the control group in comparison to the treatment groups but the values were all comparable nonetheless. The significance of this finding is unclear but the low numbers of animals and mixed sex grouping are potentially confounding factors. Although many studies correlate fat accumulation with TBARS concentrations, it is actually the levels of TG which is a more accurate indicator of lipid content. TG levels were increased in all treatment groups in contrast to the control group. Despite, canola oil treated animals being the only group to reach statistical significance; ($p=0.036$), it is possible to speculate that the increases in triglyceride concentration which were incurred were done so through increased fatty acid flux from excess dietary fat in the oils. Bigger differences may be observed on higher fat diets and/or more prolonged exposure.

The concentrations of total cholesterol were also assessed with values being considerably increased in all treatment groups in contrast to the control mice. Statistical significance was achieved in the sunflower oil; ($p=0.002$) and sunflower oil/cholesterol-treated animals; ($p=0.007$). The canola oil-treated animals did not attain statistical significance but this was because the values were unevenly distributed amongst the group which would have possibly been more prominent had the group numbers been larger. High cholesterol can be a contributing factor of fatty liver development but is considered more damaging in the context of cardiovascular problems. It is not only TC that is involved in facilitating the development of a fatty liver as numerous more steps are involved in the complex process which explains why results from TG analysis may be more useful in outlining the underlying causes of NAFLD and NASH.

The levels of circulating protein were shown to be comparable for all treatment groups when compared to the control group and consequently displayed no effect of oil administration. The results of glucose concentration again highlighted the inconsistency of the findings generated from small numbers of study animals. The levels of glucose displayed in the sunflower oil and canola oil groups were slightly higher than the controls whereas the values in the sunflower and cholesterol treated animals was considerably lower but still comparable resulting in statistical significance not being achieved and glucose concentrations not being effected.

Overall, there does not appear to be a significant effect of the oil treatment on the *db/wt* mice, but lack of animal numbers and insufficient loading of dietary lipids could be at fault for not inciting a more definitive outcome. Another point of significance is that humans are the only species that heat food before consumption, so the administration of unheated oil to mice involved in this study is not a true representation of human dietary activities. Resultantly, any future work carried out under the same scope as this experiment should possibly aim at heating oils before administration as this promotes further oxidation of the lipids involved and may be the extra influence required to overload biochemical pathways and influence fatty liver development.

6.3 Study of Short Period of Alcohol Administration (4 Weeks)

NAFLD and NASH by definition asserts that these disorders only develop in patients who consume immaterial quantities of alcohol but the actual amount which is deemed insignificant has come under great debate as Bacon *et al.*, (1994) believed values of $\geq 20\text{g/day}$ to be inappropriate whereas other authors such as Brunt *et al.*, (1999) considered 20g/day to be within acceptable limits. Investigative work on this model has already established the *db/db* mice to be obese and diabetic with mixed steatosis, although liver damage was not severe enough to warrant the diagnosis of NASH. Therefore, the purpose of this study was to determine whether alcohol is a contributing factor in the facilitation of fatty liver disease in the reputed non-drinker or act as a catalyst for the progression to NASH. In addition, due to animal availability and restrictions of pilot study agenda, the animal numbers were kept to a minimum but were in sufficient quantities to obtain histological, biochemical and statistical information in order to gauge whether a main study should occur. Liver mass and glucose concentrations were not taken for these animals in error.

Body mass was recorded over the entire treatment period to monitor whether alcohol administration has any notable effects in either of the 3 treatment groups; *wt/wt*, *db/wt* or *db/db* when compared to their respective controls. The mean gain for alcohol-treated *wt/wt* mice was comparable to their respective controls; despite being marginally decreased; (+2.2g vs. +1.8g). As the difference is too small and there are only 2 animals per group, this potential effect cannot be attributed to treatment for *wt/wt* animals. The alcohol-treated animals in the *db/wt* group displayed a mean gain of +0.5g in contrast to +1.8g of their respective controls. The slopes of growth generated overtime for alcohol-treated and control *db/wt* groups were analysed using the equation ($y = m(x) + c$) to determine if there was any differences in the rate of

mean body mass gain. The gradient of the *db/wt* control animals was shown to be significantly steeper than that of the respective *db/wt* alcohol-treated group; ($p=0.002$) and is therefore concluded that alcohol administration results in a decreased mean body mass gain in *db/wt* treated mice over a period of 4 weeks. Similarly, the control mice in the *db/db* group revealed a decrease in mean body mass gain when compared to the alcohol-treated animals; (+3.5g vs. +4.2g) and when the gradients were contrasted, this difference was also demonstrated to be statistically significant; ($p=0.014$).

Previous findings generated from assessing this mouse model revealed that a certain magnitude of stress pre-exists in the *db/db* mice by virtue of their metabolic deficiency. Results produced from administering alcohol for 4 weeks in *db/db* animals have significantly proven to have a supplementary effect on body mass. Furthermore, the effects of alcohol administration also significantly altered the mean body mass gain of *db/wt* animals in contrast to the respective control group and it could therefore be argued that the *db/wt* animals contain various precursors for fatty change which alcohol manipulates to impose an effect. Gender could not be investigated in this pilot study as both sexes had to be combined to generate sufficient group numbers.

Histological examination of the fresh liver sections displayed alcohol to have no effect in *wt/wt*, *db/wt* or *db/db* groups by comparison with the respective controls. The observations confirmed the presence of NAFLD in *db/db* control mice as confirmed earlier through H&E sections displaying grade 2 and 3 micro- and macrovesicular steatosis. The *db/db* alcohol-treated mice revealed no additional signs of hepatic injury or fat accumulation resulting in the inability of alcohol to provoke the subsequent progression to NASH. Alcohol administration also failed to generate a response in *wt/wt* and *db/wt* mice in contrast to their respective controls. Livers sampled from alcohol-treated mice and controls in *wt/wt* and *db/wt* groups were found to be comparable as all individuals displayed hepatocytes which were normal in structure and function with no steatosis present. Alcohol does not seem to have any substantial effect on the histology of these livers, but it must be noted that the alcohol was made available to the animals *ad libitum* and therefore may not have been consumed in significant quantities to generate a noteworthy change. The period of 4 weeks for which the alcohol was administered may also be insignificant to incur any sizeable injury.

Investigations into the concentrations of CDs displayed slight increases in the 3 alcohol-treated groups in contrast to the control animals but this difference was more pronounced in the *db/db* mice. Furthermore, values were unevenly distributed and not elevated enough to gain statistical significance in either of the treatment groups but does display a trend in that alcohol could be responsible for contributing to an increase in early products of lipid peroxidation. In addition, the increase observed in *db/db* controls when contrasted to *db/wt* control was found to be statistically significant ($p=0.011$) which was expected from previous comparison of the different genotypes. However, the alcohol-treated animals from *db/wt* and *db/db* groups were also compared and although statistical significance was attained ($p=0.039$) the change was more considerable between the controls. Resultantly, despite the stress of alcohol acting as a potential exacerbator of early lipid peroxidation products, it appears as though the presence of the *db* gene is causing a more significant effect.

The results produced from assessment of LOOH revealed increases in the 3 alcohol-treated groups when contrasted to their respective controls. The difference was only minor in the *db/wt* control vs. alcohol; (158 vs. 182 nmol LOOH/g. liver respectively) whereas the increases in the alcohol-treated animals was more definitive in the *wt/wt* and *db/db* groups; (93 vs. 291 and 434 vs. 654 nmol LOOH/g. liver respectively). The discrepancies were not considerable enough to reach statistical significance in either group, but provides further evidence of a growing trend that alcohol may be causing a slightly increased effect on lipid peroxidation products. The control animals were compared from the *db/wt* and *db/db* groups and again the increase in the *db/db* animals was established as being statistically significant ($p=0.015$) as was the increase when comparing the alcohol-treated animals from the same groups ($p=0.21$). Although the administration of alcohol is beginning to display a trend in contrast to the respective controls, the difference between the controls from *db/wt* and *db/db* groups attains more pronounced statistical significance and adds scope to whether the presence of the *db* gene causing a more detrimental effect than the administration of alcohol. There was also an increase in *wt/wt* alcohol-treated animals when contrasted to the *db/wt* alcohol-treated group but this can be explained by inter-group variation and small group numbers.

TTBARS were shown to be elevated in all alcohol-treated groups in comparison to the respective control animals and attained statistical significance for *db/wt* mice; ($p=0.038$) and *db/db* mice ($p=0.0003$). Furthermore, values for *wt/wt* alcohol-treated animals and controls and *db/wt* alcohol treated animals were all exhibited to be

marginally increased in comparison to *db/db* controls, but no statistical significance was achieved. Alcohol could be attributed as an effect but small numbers in the *wt/wt* groups, no accumulation of fat in respective livers and inadequate manifestation of significant statistics provides conflicting evidence and therefore any true causative effect remains unresolved.

The concentrations of total cholesterol were examined for all groups and slight increases were again observed in *wt/wt*, *db/wt* and *db/db* alcohol-treated mice in contrast to the controls. Statistical significance was only reached between the *db/wt* animals; ($p=0.005$), but adds further support that alcohol may be producing a slight effect in these animals.

TG levels were investigated and demonstrated that the content for *db/db* alcohol-treated mice was dramatically increased in comparison with the control group; ($p<0.0001$). Statistical significance was also achieved for an increase in alcohol-treated *db/wt* animals ($p=0.009$) in contrast to the controls. A marginal increase was noted in the alcohol-treated animals of the *wt/wt* group as well and even though these differences are being demonstrated to be small, there is a definite trend developing which may become more distinctive if further hepatic damage was incurred. Triglyceride content is an excellent marker of lipid droplet formation and potential problems of fat accumulation which makes this a powerful finding. The more sensitive detection of quantitative changes gives this biochemistry approach an advantage over the histology. The TG content in the *db/db* controls is significantly raised in comparison to the *wt/wt* and *db/wt* groups without the addition of alcohol as a further stress factor. When alcohol is administered, the *db/db* treated animals display concentrations which are more than double that of the *db/db* controls; (2.93 vs. 6.44 mg TG/g. liver). Therefore, despite *db/db* mice already having disrupted metabolic functions, the addition of alcohol may further aggravate the situation. Despite the gain in TG, and same increase in lipid peroxidation markers, histological examination did not reveal necrosis and inflammatory changes. Alcohol may have a preferred route of being incorporated into fatty acids, producing fatty acid palmitate. This fatty acid is not subject to the lipid peroxidation products being examined in this study.

In conclusion, although alcohol has not made a statistically significant impact on all parameters being investigated, a slight trend is evident. Increasing the period of alcohol administration may cause a significant change that would facilitate the

progression to NASH or enable a noteworthy change in *wt/wt* or *db/wt* mice. If alterations in fatty acid synthesis or any other metabolic pathway which is involved in development and progression of fatty liver were to become induced by alcohol, it is hoped that a main study would be able to highlight when and where the changes occur in attempt at alleviating the disease.

6.4 Impact of Alcohol Administered over 8 and 20 Week Periods in *db/wt* and *db/db* Mice

The results obtained from the alcohol pilot study were deemed significant enough to proceed to a study over longer periods. The period of alcohol administration necessary to cause a more pronounced effect was uncertain so it was decided to split the animals into 2 batches; one batch undertaking 8 weeks of alcohol administration (short-term) whereas the second batch of animals had a duration of 20 weeks alcohol administration (long-term). Alcohol-treated animals were compared against their respective controls and were assessed in an attempt to identify any significant changes that may facilitate or lead to further progression of this disease. For the purposes of reporting and clarity, both 8 week and 20 week alcohol administration studies will be discussed together.

Body mass was recorded over a 3wk adaptation period for all animals prior to the respective 8wk and 20wk administration of alcohol for treated animals, or water for the controls. The 8wk study demonstrated that *db/wt* alcohol-treated animals had a slightly decreased mean body mass gain in contrast to the controls; (+4.5g vs. +6.8g respectively). The 8wk *db/db* alcohol-treated mice also exhibited a decrease when compared to the control group; (+5.2g vs. +7.4g respectively). Despite 8wk alcohol-treated *db/wt* and *db/db* groups displaying a decrease in mean body mass gain when contrasted against the controls, the differences were only significant in *db/wt* animals upon assessing the gradients; ($p=0.0009$). In comparison, the body mass of 20wk *db/wt* and *db/db* alcohol-treated mice was comparable to their respective controls at the start of treatment, however, after 4-5 weeks both sets of controls appear to increase their rate of mean body mass gain and the resultant reduced gain experienced by the *db/wt* and *db/db* alcohol-treated animals is not regained by the time of termination. This is reflected in the mean body mass gain values of 20wk *db/wt* alcohol-treated animals which was decreased in comparison to the control group; (+6.4g vs. +1.2g respectively) as were the *db/db* alcohol-treated mice from the 20wk study when compared to the controls; (+12.3g vs. +6.8g respectively). Nevertheless, statistical significance is only achieved between the *db/db* groups

which arises from analysing the gradients; ($p=0.003$). Overall, all 8wk and 20wk alcohol-treated *db/wt* and *db/db* groups display a reduced mean body mass gain in comparison to the controls, but statistical significance is only reached in 8wk *db/wt* and 20wk *db/db*. This finding is somewhat contradictory, as the trend of alcohol inducing a causative effect continues, but the duration of administration and susceptibility of genotype is somewhat misleading. The 8wk study gives the impression that the *db/wt* treated mice are more prone to the effects of alcohol administration whereas the 20wk study reveals the *db/db* animals having a higher propensity to a reduction in body weight gain. This finding is considered to be spurious. Repeat studies with increased group numbers may give a more accurate indication of whether this observation is real.

Observations made from comparing the 8wk and 20wk studies indicate that the increased duration of alcohol administration may be a causative factor in reducing mean body mass gain. Since food and drink consumption were not specifically monitored, it is not known whether the alcohol affected the amount of food taken nor the amount of liquid taken in. The mean gain for 8wk *db/wt* controls revealed an increased difference of +2.3g in comparison to the respective alcohol-treated animals, whereas the difference in mean gain for 20wk *db/wt* controls was +5.2g when contrasted against the alcohol-treated animals. Similar results were also displayed when comparing the *db/db* mice. The 8wk *db/db* controls were shown to have an elevated mean body mass gain difference of +2.2g in contrast to the 8wk *db/db* alcohol-treated group whilst the difference between 20wk controls and alcohol-treated mice was +5.5g. As a result, although 8wk alcohol administration certainly causes a reduction in weight gain in *db/wt* and *db/db* mice, evidence suggests that the effect is more definitive in animals who have endured administration for 20 wks.

Liver mass was recorded for all animals from 8wk and 20wk groups, but unfortunately could not be compared with the pilot study as this data was not noted in error. Statistically significant increases in mean liver mass were noted for 8wk and 20wk *db/wt* alcohol-treated animals in comparison to the controls; ($p<0.0001$ and $p=0.0068$ respectively). Slight increases were also observed for 8wk and 20wk *db/db* alcohol-treated groups in contrast to the control animals but this was not of noteworthy difference to attain statistical significance; ($p=0.99$ and $p=0.55$ respectively). These findings suggest that although alcohol may be eliciting an effect in all treated groups, the changes in mean liver mass are more noticeable in the *db/wt* groups from the 8wk and 20wk studies in comparison to the *db/db* animals.

This evidence indicates that *db/wt* animals are possibly more prone to metabolic alterations from exposure to alcohol than *db/db* animals. The changes that are causing the liver to increase in size in *db/wt* mice may have already occurred in *db/db* mice through an alternative mechanism of metabolism and that is why the values of *db/db* animals are comparable regardless of 8wk or 20wk alcohol administration. It would be of considerable interest to determine how alcohol results in an increased liver mass in *db/wt* but not *db/db* mice.

When comparing 8wk and 20wk studies, the differing periods of alcohol administration did not appear to have made any significant difference to the mean liver mass despite marginal increments being noted in the *db/wt* and *db/db* 20 wk alcohol-treated animals in contrast to the *db/wt* and *db/db* 8 wk alcohol-treated groups; (1.53g vs. 1.45g and 2.99g vs. 2.84g respectively). The 8wk *db/wt* alcohol-treated mice exhibited a larger increase in liver mass when contrasted against their respective controls in comparison to the difference between 20wk *db/wt* alcohol treated mice and the control group; (+0.39g vs. +0.16g). However, this discrepancy results from 8wk *db/wt* control mice having a significantly decreased mean liver mass in comparison to the 20wk *db/db* controls; (1.06g vs. 1.37g), but since 8wk and 20wk *db/wt* alcohol-treated animals are comparable, this finding was considered to be spurious.

Liver mass / body mass ratios were analysed to determine if liver mass was proportional to body mass. The results demonstrated statistically significant increases for 8wk and 20wk *db/wt* alcohol-treated animals in comparison to the controls; ($p < 0.0001$ and $p = 0.003$ respectively). The liver mass increase from the 8wk and 20wk *db/wt* alcohol-treated groups are considered to be out of proportion and provides support for that the increases in liver mass are a direct result of alcohol consumption. Slight increases were also observed for 8wk and 20wk *db/db* alcohol-treated groups in contrast to the control animals but did not reach statistical significance for 8wk administration; ($p = 0.57$) but was achieved for 20wk; ($p = 0.033$). Therefore, despite any change in liver mass not being significantly apparent between *db/db* controls and alcohol-treated animals from 8wk and 20wk, when adjustments are brought about to bring body mass into consideration, the 20wk alcohol-treated *db/db* animals display liver mass which appears slightly out of proportion from the body mass. Furthermore as 8wk *db/db* alcohol-treated mice still exhibit proportionate liver mass after adjustments, it could again be speculated that the longer duration of alcohol administration has had a causative aspect. In addition it could also be argued

that *db/wt* mice are more susceptible to alterations in metabolism as a direct result of alcohol than *db/db* animals.

Histological examination of the livers did not display any significant differences between 8wk animals and their respective counterparts in the 20wk study. As a result, administering alcohol for longer periods did not induce additional hepatic injury, NASH was not induced and observations were comparable. It appears that the biochemistry v. histology are at a variance about the fat content in the liver when the results of the 8 and 20 wk studies for *db/wt* with alcohol and *db/db* without alcohol are compared. The latter group, though displaying frank macrovesicular steatosis do not achieve the absolute TG concentration specified for fatty liver to be diagnosed. As stated in the review, the TG content should be > 50mg/g. liver. Since calculations were checked and spectrophotometric analysis was excluded as technical reasons for a low reading in the *db/db* mice, the only other biochemical explanation is that losses may occur in processing very fatty liver samples. The fat noticeably sticks to glass tubes and may not be fully suspended by the small amounts of detergent in assays. It is also possible that the distribution of the fat is different, *i.e.* in cytosolic droplets in *db/db* animals and dispersed in membranes and lipoproteins in the other animals.

Results for early lipid peroxidation products were found to be somewhat conflicting. Conjugated dienes were shown to be statistically significantly increased for 8wk and 20wk alcohol-treated *db/wt* animals when contrasted against the controls; ($p=0.027$ and $p=0.031$ respectively). CDS were also demonstrated to be statistically significant for 8wk and 20wk alcohol-treated *db/db* groups in comparison to the controls; ($p<0.0001$ and $p=0.0025$ respectively). This adds further evidence to the developing trend that alcohol administration affects metabolic pathways and leads to altered biochemical functions. However, the inconsistency arises when the 8wk period of alcohol administration is weighed against the 20wk study. The *db/wt* control and alcohol-treated animals from the 8wk study exhibit a CDs content which is slightly decreased in contrast to the *db/wt* control and alcohol-treated animals from the 20wk study; (1.60 vs. 1.64 and 2.00 vs. 2.19 $\mu\text{mol/g}$. liver respectively). On the other hand, *db/db* control and alcohol-treated animals from the 8wk study display statistically significant increases when compared to the *db/wt* control and alcohol-treated animals from the 20wk study; (2.66 vs. 2.22 and 3.45 vs. 2.58 $\mu\text{mol/g}$. liver respectively). The reason behind this is not clear, although it does suggest that despite alcohol triggering a causative effect in *db/wt* and *db/db* animals, the period of administration

does not appear to be relevant. The concentrations of CDs may decrease with age, but there is no data to properly confirm this possibility. It is also possible that different batches of animals or other incidental factors not recognized at the moment are playing a role in the variability of the data.

Lipid hydroperoxides were assessed to monitor intermediate lipid peroxidation products. These were revealed to be dramatically increased in *db/wt* and *db/db* alcohol-treated animals in comparison to the relative controls for 8wk; ($p < 0.0001$ for both) and 20wk studies; ($p = 0.0002$ and $p = 0.0109$ respectively). In addition, the *db/wt* control and alcohol-treated mice from the 8wk study were shown to have statistically significant increases when contrasted against the *db/wt* control and alcohol-treated mice for the 20wk study; ($p = 0.0007$ and $p = 0.0025$ respectively). Increases were evident in *db/db* control and alcohol-treated mice from the 8wk study in comparison to the 20wk groups which also attained statistical significance; ($p = 0.0005$ and $p < 0.0001$ respectively). Again, alcohol administration demonstrates a causative effect by inducing increases in intermediate lipid peroxidation products in *db/wt* and *db/db* mice for both 8wk and 20k periods. The increases encouraged by alcohol administration in the 8wk and 20 wk studies are more definitive than those achieved in the 4wk pilot study as no statistical significance was attained over that period.

Resultantly, it can be concluded that 4 weeks of alcohol administration is not sufficient to produce a significant change in LOOH concentration whereas 8 weeks duration does inflict a noteworthy effect. Additionally, an increased alcohol administration period of 20 weeks does not provoke further change and therefore a treatment period of 8 weeks may be optimal to reveal this effect. The inability of alcohol to stimulate the development of NASH even after a further 12 weeks of alcohol administration means that this process alone is not enough to elicit NASH. Histological findings were also comparable for *db/wt* and *db/db* mice from 8wk and 20 wk studies.

TTBARS also reflected the notable effects of alcohol administration: the increases in 8wk and 20wk *db/wt* alcohol-treated animals were statistically significant in contrast to the controls; ($p < 0.0001$ and $p = 0.0002$ respectively). The raised values in the alcohol-treated groups for 8wk and 20wk *db/db* animals also achieved statistical significance in comparison to the control groups; ($p < 0.0001$ and $p = 0.03$ respectively). It is clearly evident that alcohol is altering metabolism in a way that enhances lipid

peroxidation in the treated mice from the 8wk and the 20wk studies, but whether the longer period of alcohol administration further exacerbates the damage remains unclear. The comparison of TTBARS for 8wk and 20wk studies for *db/wt* and *db/db* alcohol-treated animals does not reveal any differences that are of statistical significance; ($p=0.18$ and $p=0.11$ respectively). Although TTBARS are significantly elevated in the 20wk study for alcohol-treated *db/db* mice when compared to 8wk alcohol-treated *db/db* mice, there is no other evidence to suggest that longer alcohol administration prompts further increases in late lipid peroxidation production. As previously mentioned, histology also indicates that as inflammatory infiltrate does not occur; nor does fibrosis.

Triglycerides were again dramatically increased in 8wk and 20wk *db/wt* animals in contrast to the controls and attained statistical significance; ($p=0.004$ and $p=0.001$ respectively). TG was also shown to be significantly elevated in 8wk and 20wk *db/db* animals when compared against the control groups; ($p=0.008$ and $p=0.003$ respectively). Further evidence of a trend developing in favour of 20wk alcohol administration inciting increased damage was noted through statistically significant values being exhibited in 20wk treated *db/wt* and *db/db* animals in comparison to the 8wk groups; ($p=0.026$ and $p=0.02$ respectively). Again, the TG content observed in the 8wk and 20wk studies was not met through 4wk alcohol administration in the pilot study and casts further speculation of a minimal duration of intervention before eliciting an effect. Alcohol is definitely aggravating the near-normal metabolic functioning of *db/wt* mice and also enhancing the already existing TG accumulation in the *db/db* mice but neither the *db/wt* nor the *db/db* animals are more susceptible to NASH over this period.

The enhanced duration of alcohol administration in the 20wk study also failed to make a more significant impact than 8wk groups when investigating total cholesterol concentrations. The 8wk and 20wk alcohol-treated *db/wt* animals were shown to display statistically significant increases in TC when contrasted against the controls; ($p=0.008$ and $p=0.001$ respectively) as did the *db/db* groups for 8wk and 20wk studies; ($p=0.01$ and $p=0.005$ respectively). However, statistical significance was not attained when comparing 8wk and 20wk *db/wt* alcohol-treated mice; ($p=0.33$) nor *db/db* alcohol-treated mice; ($p=0.47$). Although the TC increases in 20wk *db/wt* and *db/db* alcohol-treated animals did not achieve statistical significance over their respective 8wk counterparts, a slight trend may exist but the group sizes are inadequate to determine a real effect and therefore larger numbers would be required

to confirm or disregard this possibility. Furthermore, the levels of TC observed in *db/wt* and *db/db* animals in the 4wk pilot study did not reach that of *db/wt* and *db/db* groups in 8wk and 20wk studies. This implies that alcohol administration requires a particular time but this point is not known from these studies. It appears as though 4wk administration is insignificant, whereas the disruption caused at 20wk approximates that of the 8wk. Sustained alcohol administration beyond 20wks may or may not further metabolic and liver damage, with the onset of NASH. Further studies would be needed to establish when this occurs and whether there is an optimal duration of alcohol administration that produces a proportionate hepatic effect. Indeed, the human may be prone to NASH on the basis of long term stress.

Fibrosis may increase the liver protein content. The levels of protein in the liver were marginally raised for 8wk alcohol-treated *db/wt* and *db/db* animals in comparison to the controls but these differences were not deemed significant; ($p=0.26$ and $p=0.35$ respectively). Similar minor increases were observed in 20wk alcohol-treated *db/wt* and *db/db* groups and again were found to be statistically insignificant in contrast to the control animals; ($p=0.23$ and $p=0.30$ respectively). Overall, despite the small increases, all groups displayed comparable values and therefore, duration of alcohol administered or genotype had no effect on protein concentrations.

Plasma aspartate transaminase (AST) and alanine transaminase (ALT) were examined because augmentation of these hepatic enzymes is strongly indicative of liver damage. Increases in AST levels were evident in 8wk and 20wk alcohol-treated *db/wt* animals in comparison to the controls, but these differences only attained statistical significance in the 20wk group; ($p=0.29$ and $p=0.037$ respectively). Minor increases were also noted in the 8wk and 20wk alcohol-treated *db/db* mice when contrasted with their controls, however, statistical significance was not reached in either group; ($p=0.26$ and $p=0.64$ respectively). The ALT levels were found to be slightly increased in 8wk and 20wk studies for both *db/wt* and *db/db* alcohol-treated animals in comparison to the control groups, but statistical significance was not achieved. Furthermore, the period of alcohol administration did not provoke further disruption in AST values or ALT values. Differences between the 8wk and 20wk *db/wt* alcohol-treated animals and 8wk and 20wk *db/db* alcohol treated animals were shown to be insignificant for both parameters; AST ($p=0.56$ and $p=0.16$ respectively), ALT ($p=0.31$ and $p=0.78$ respectively).

The previous findings have illustrated that alcohol administration alters hepatocyte metabolism as many of the *db/wt* and *db/db* treated animals have exhibited significant increases in lipid peroxidation products in addition to elevated TC and TG content. However, the extent of liver damage observed at histology suggests that the liver is only in the early stages of fatty development. The progression to steatohepatitis and possibly fibrosis did not occur. The results of AST and ALT levels suggests that significant hepatocyte damage did not occur. The hepatic injury being incurred with alcohol alone has not reached the point of inflammation and fibrosis either because of inadequate timing or severity of stress or because the appropriate additional hit(s) were not present.

Blood/alcohol levels were carried out in an attempt to establish that treated animals had consumed alcohol, how such consumption affected the plasma and whether it reflected the proportionate amount of disruption which was incurred. The alcohol was administered *ad libitum* and it is therefore impossible to guarantee equal intake throughout the different groups. Furthermore, as the alcohol was available to the treated animals up until termination, it is also possible that some animals would have consumed their last drink just before sacrifice whereas others may have had their last intake the night before. The high degree of variation between respective alcohol-treated animals reflected the experimental difficulties with administering the alcohol *ad libitum*.

The 8wk alcohol-treated *db/wt* and *db/db* animals displayed increases in blood alcohol in comparison to the relevant controls but these differences did not attain statistical significance; ($p=0.055$ and $p=0.17$). This suggests that a number of *db/wt* and *db/db* alcohol-treated animals did not consume any alcohol before termination, although *db/db* alcohol-treated mouse 23 exhibited a concentration 15.33 mmol/l. This reading is dramatically increased in contrast to the other alcohol-treated animals (*db/wt* and *db/db*) as evidenced by the mean bar being totally skewed. It could be the result of drinking alcohol just before termination. The reason owing to 8wk *db/db* animals displaying a higher blood/alcohol concentration than 8wk *db/wt* can only be explained by the reasons given previously and any future studies should consider withdrawing the alcohol from the treated animals 24hr prior to sacrifice.

Conversely, the 20wk *db/wt* animals revealed an increased blood/alcohol level in contrast to the 20wk *db/db* animals, although this was not statistically significant due to the high degree of variation. This discrepancy can be part explained by the same

reasoning as before in that these concentrations only reflect how recently the animal in question consumed alcohol. However, it is possible that there is a greater capacity to metabolise alcohol in the *db/db* mice. In addition, *db/wt* alcohol-treated animals 9 and 12 and *db/db* alcohol-treated animal 22 displayed concentrations which were radically raised compared with any other animal; (20.89, 19.32 and 23.05 mmol/l respectively). This demonstrates the unreliability of random blood/alcohol measurements, rather than individual metabolic differences. It raises the possibility of fluctuating alcohol levels. The 20wk *db/wt* and *db/db* alcohol-treated groups did exhibit increases in blood/alcohol levels when compared against the controls as expected, but only the *db/wt* mice were shown to be significant; ($p=0.005$ and $p=0.31$ respectively).

It is already established that *db/db* animals have diabetes, thus, the analysis of glucose concentrations was undertaken to determine whether alcohol administration further exacerbates the metabolic pathways involved or acts as a mechanism whereby similar effects to insulin deficiency are induced in the non-diabetic *db/wt* animals. Glucose concentrations for 8wk and 20wk, *db/wt* and *db/db* alcohol-treated animals are actually decreased in comparison to the control groups. These differences are not significant for the 20wk study; ($p=0.63$ and $p=0.29$ respectively), nor 8wk *db/db* animals; ($p=0.54$) but does attain statistical significance for 8wk *db/wt* mice; ($p=0.006$). The reasoning for this finding is unknown but alcohol may have an anti-gluconeogenic effect or may have induced starvation. When contrasting 8wk *db/wt* and *db/db*, control and alcohol-treated animals against the respective 20wk groups, the findings were revealed to be comparable and again displays that an increased duration of alcohol exposure from 8wks to 20wks does not make a significant difference.

In conclusion, alcohol administration has a definite effect on body and liver mass, products of lipid peroxidation, hepatic TC and TG and central markers of hepatic injury are altered to a limited extent. The pilot study displayed a trend for these changes after only 4 weeks of alcohol administration, whereas the results exhibited from the main 8wk and 20wk studies were more pronounced. However, there was no significant difference between the 8wk and 20wk duration and therefore the extra 12wks of alcohol administration had no additional deleterious effect. It remains unclear whether *db/wt* or *db/db* mice are more susceptible to metabolic stress in response to alcohol administration. Although a trend in *db/wt* mice may be present, the changes are small. Gender may still play a role in determining an individual's

susceptibility but this aspect could not be assessed as the mice were all male. Any future studies should be directed at confirming or rejecting this theory in addition to highlighting changes that are associated with facilitation and development of fatty liver.

6.5 Histopathology Analysis

6.5.1 Results for the *db/db* Mouse Model on Regular Chow for 20 Weeks

Analysis of livers from *db/wt* and *db/db* mice (figure 5.7.1 (A-D)) revealed histopathological differences and further confirmed the diversity between the *db* genotypes. (A) + (C) reveal the liver of heterozygous (*db/wt*) mice to be normal as liver sections showed preservation of normal architecture, with no fibrosis, steatosis, inflammation or necrosis. In contrast, livers from homozygous (*db/db*) mice; (B) + (D) showed grade 2 and 3 micro- and macrovesicular steatosis, predominantly present in zone 3 and is characteristic of this genotype. Necroinflammation was not observed. Despite the presence of significant steatosis, no steatohepatitis was present evidenced by a lack of lobular inflammatory foci. This raises the possibility of additional contributing factors being required for progression to steatohepatitis in the *db/db* model.

Similar to heterozygous (*db/wt*) mice, livers from wild type (*wt/wt*) animals were shown to be normal, displaying hepatocytes which appeared normal over many fields with no aggregations of fat droplets present. There were no histological differences between male and female livers in *wt/wt*, *db/wt* or *db/db* groups respectively.

6.5.2 Impact of Various Oils, Administered via the Diet (4 Weeks)

The histological findings generated from the administration of various oils in *db/wt* mice (figure 5.7.2) uncovered no significant differences in the liver of the control group on regular chow for 4wks and the relevant treatment groups; sunflower oil, canola oil and sunflower oil and cholesterol respectively. The control animals showed normal liver architecture, as expected, with no signs of steatosis. The addition of the various oil supplements did not have any notable effect (figure 5.7.2 (A-D)). This supports the fact that no major differences were observed from the biochemical assessment. Oil supplementation is therefore insufficient for the induction of steatosis in heterozygous mice. One concludes that the oil-supplemented diets with modest amounts of oils in the short term had no effect. There were no differences between male and female mice.

6.5.3 Impact of a Short Period of Alcohol Administration (4 Weeks)

Analysis of liver sections revealed that alcohol administration for 4 wks had no effect on liver architecture for *wt/wt*, *db/wt* and *db/db* animals. Unfortunately, the H&E stained sections of these livers were misplaced after examination and therefore cannot be presented. The *wt/wt* and *db/wt* control mice displayed normal hepatic structure as expected, without the accumulation of fat or infiltration of inflammatory cells. It was anticipated that 4wks of 20% alcohol administration would induce a fatty liver or even steatohepatitis in alcohol-treated *wt/wt* and/or *db/wt* mice, but no significant histological differences were observed between the respective groups.

In contrast, *db/db* mice (with and without alcohol) showed mixed micro- and macrovesicular steatosis but no inflammation or fibrosis was presented and no differences were observed between the *db/db* mice receiving alcohol and *db/db* animals not receiving alcohol with respect to steatosis, inflammation and fibrosis. It is proposed that an increased period of alcohol exposure may still provoke the development and possible progression of fatty liver. The duration of 4wks may not be significant to trigger an effect, but some encouraging data from the biochemistry parameters being investigated was sufficient to proceed to a longer study.

6.5.4 Impact of Alcohol Administered over 8 Weeks in *db/wt* and *db/db* Mice

The H&E liver sections (figure 5.7.3) from control and alcohol-treated *db/wt* animals (A) were comparable as both groups showed normal liver hepatocytes, no inflammatory infiltrate and no steatosis or fibrosis. Control *db/db* animal (B) showed macrovesicular steatosis without inflammatory infiltrate. Moderate macrovesicular steatosis was predominantly located in zones 2 and 3, but also located in zone 1 in the alcohol-treated *db/db* mice (C). There is inflammation present in the portal tract but this is considered to be non-specific.

Upon increasing the magnification of control and alcohol-treated *db/db* animals (figure 5.7.3) the differing levels of inflammation become more apparent. (D) and (F) reveal no signs of inflammation for the *db/db* control animals whereas (E) and (G) illustrate necroinflammatory foci in the hepatic nodules along with polymorphs and lymphocytes in mixed inflammation in alcohol-supplemented *db/db* mice. Fibrosis is not present in the *db/db* genotype, even after exposure to 20% alcohol for 8wks. This raises the possibility that an additional stressor or 'second-hit' is required to promote chronic change over time. It appears as though alcohol may have a slight effect on

liver of *db/db* mice, while there is no effect of alcohol on *db/wt* animals. Longer studies are required to evaluate fibrosis with these inflammatory responses.

The amount of fat present in each liver (table 6.5.4.1) was graded 'blind' by an independent pathologist. Unblindly, the analyses revealed that alcohol had no significant effect on hepatic structure in *db/wt* mice. The change in fat was slightly elevated for alcohol-treated *db/db* animals when compared to the respective controls but this change was not significant. The levels of inflammatory aggregates were also comparable for control and alcohol-treated *db/wt* animals but this was not the case for the *db/db* mice where the amounts detected are higher for the alcohol-supplemented group in contrast to the controls ($p=0.005$), however larger group sizes would determine if this was a 'real' effect or not.

Table 6.5.4.1

Group / Genotype	Treatment	No. Animals	Fat Change (0-3) \pm SD	Inflammatory Aggregates* \pm SD
1 (<i>db/wt</i>)	H ₂ O Control	7	0.0 \pm 0.0	0.0 \pm 0.0
2 (<i>db/wt</i>)	20% Alcohol	6	0.17 \pm 0.41	0.0 \pm 0.0
3 (<i>db/db</i>)	H ₂ O Control	6	2.33 \pm 0.52	1.33 \pm 1.63
4 (<i>db/db</i>)	20% Alcohol	5	2.80 \pm 0.45	6.00 \pm 2.55

* = Inflammatory aggregates; 20 x objective, 20 fields counted

6.5.5 Impact of Alcohol Administered over 20 Weeks in *db/wt* and *db/db* Mice

The administration of alcohol does not appear to affect the histopathology of *db/wt* and *db/db* mice when compared with their respective controls, despite the animals being supplemented for 20wks (an additional exposure of 12wks in comparison to the previous study). The findings (figure 5.7.4) showed *db/wt* mice (with and without alcohol, (A) and (B)) to have normal liver histology with no steatosis, inflammation or fibrosis. Alcohol also failed to induce additional liver damage in *db/db* animals, as zone 2 and 3 steatosis with minimal inflammation and no fibrosis was evident in alcohol-treated *db/db* mice (D), which was comparative to the respective controls (C). Figure 5.7.4 (E) displays unmistakable macrovesicular steatosis in a *db/db* control animals and is also representative of the similar liver morphology observed in the alcohol-treated animals.

Fatty change was also assessed semi quantitatively for the 20wk animals (table 6.5.5.1). Alcohol had no significant effect on liver architecture in *db/wt* mice. The

change in fat was slightly elevated for alcohol-treated *db/db* animals but this was not statistically significant. Inflammatory aggregate values were unexpected for *db/wt* and *db/db* animals as control values were higher than the respective alcohol-treated groups. This cannot be fully explained as it contradicts all previous histology data. Larger group sizes in future studies may be able to prove or disprove this finding.

Table 6.5.5.1

Group / Genotype	Treatment	No. Animals	Fat Change (0-3) ± SD	Inflammatory Aggregates* ± SD
1 (db/wt)	H ₂ O Control	7	0.0 ± 0.0	2.86 ± 3.87
2 (db/wt)	20% Alcohol	4	0.25 ± 0.50	1.25 ± 1.5
3 (db/db)	H ₂ O Control	6	1.83 ± 0.75	3.5 ± 1.64
4 (db/db)	20% Alcohol	6	2.0 ± 0.0	1.5 ± 1.64

* = Inflammatory aggregates; 20 x objective, 20 fields counted

The sirius red stain (figure 5.7.4) displays levels of normal collagen present in *db/db* animals, (F) and (G), and again reveals no significant differences between the control and alcohol-treated mice. The examination of these findings revealed that *db/db* mice develop simple steatosis coupled with the presence of lobular inflammation, but again fibrosis was not evident in these animals. Alcohol is not compounding the already present changes, but they are still there. Though some inflammation is attributable to alcohol, it is not significantly changing the histologic picture after 20 weeks of administration.

Chapter 7; Discussion and Conclusion

This chapter will briefly summarise the studies before considering the model and integrating the pathogenesis of fatty liver.

7.1 Assessment of the *db/db* Mouse Model on Regular Chow for 20 Weeks

Compared with the control (*wt/wt*) animals, the heterozygous (*db/wt*) animals displayed marginal differences in some parameters whereas the homozygous (*db/db*) animals differed greatly in all parameters.

The *db/wt* status does influence body mass and liver mass making the obese mouse not entirely a 'recessive' condition. The liver remains the same proportion of the body as the ratio of liver mass / body mass was not abnormal. Interestingly, the *db/wt* mice liver had high CD concentration, but no increased LOOH or TTBARs, TC, TG and glucose.

The *db/db* mice were strikingly larger, with larger livers but the same liver mass / body mass ratio. In these mice the liver CD and LOOH were prominently higher, as were TC, TG and glucose. The TTBARs were increased but not so markedly so.

From these results it appeared that the *db/wt* mice could have some increased sensitivity towards fatty liver and were preferred as the investigative line along with the obviously highly deranged *db/db* model. These findings also suggest that there may be an increase in early peroxidation products with the increase in body and proportionally increased liver mass, and that the full obese phenotype is not essential for these changes. These changes are also suggestive of early metabolic stress without frank diabetes. Whether there is insulin resistance that could provide a similar stress to diabetes, can only be resolved with more detailed experiments including insulin concentration and glucose disposal studies. Fluxes of fatty acid in plasma, hepatocyte TG, PL and VLDL synthesis or oxidation could be investigated further by studies in liver slices or whole body.

7.2 Impact of Various Oils, Administered via the Diet (4 Weeks)

When the differently enriched diets are compared with the control diet for their effects on the parameters studied in this thesis, only minor differences became apparent. Note that the background diet was found to comprise of 3.4, 0.26 and 1.87 $\mu\text{mol/g}$ liver of CD, LOOH and TTBARs respectively. Additionally, the oils were not heated in contrast to man's fatty diet.

Although small numbers of animals were used, statistical analysis identified some interesting observations. Even though mean body mass did not differ, the livers of canola oil-fed *db/wt* mice (diet high in MUFA) appeared heavier but their ratio to body mass was the same as for control animals. Sunflower oil-fed *db/wt* mice (diet high in PUFA) consumed more CD and had a higher CD content in their livers, with and without additional dietary cholesterol. The LOOH and TTBARs did not differ. Interestingly, the hepatic TC was increased by PUFA (+ or – cholesterol) but liver TG was minimally increased only by the MUFA oil. Glucose was unaffected.

Unlike previous studies, this moderately fat-enriched diet did not elicit a significant fat accumulation. The PUFA dietary fats did however influence the CD content. It must be noted, however, that this supplement did not resemble the human diet because the oils were not heated. If this study was to be repeated it is recommended that oils should be heated before administration to maximize oxidative products and increase stress on the liver in an attempt to induce a noteworthy effect. The period of administration should also be increased.

7.3 Impact of a Short Period of Alcohol Administration (4 Weeks)

The *db/wt* mice were larger than the *wt/wt*, and the *db/db* were larger again, suggesting that there is a gene dose effect on body size even though the *db/wt* was not frankly obese. The administration of 20% alcohol in water to *wt/wt*, *db/wt* and *db/db* mice over a short period of 4 weeks revealed some differences in response in growth.

Whereas alcohol administration did not affect the growth rate of the *wt/wt* mice, it appeared to impair the growth of the *db/wt* mice. Regrettably, there were too few animals in the *wt/wt* cohort to examine for differences by conventional statistics. The starting points of the *db/db* mice destined to be controls or consumers of alcohol were different and could thus influence the response. But the growth rate appeared rapid initially and the animals converged on the same mean body mass with no apparent deleterious effect of alcohol administration.

The impact of short term alcohol administration on lipid peroxidation was different for the 3 animal variants but the small number of *wt/wt* mice results in some lack of confidence. The apparent increases of CD with alcohol administration were not statistically significant, nor were those of LOOH. There was also an increase of TTBARs with alcohol administration in each category of animal but TTBARs were

not very different in absolute concentration in the *db/db* model that distinctly has a fatty liver.

In all instances, alcohol administration resulted in an increase in the hepatic TG content; for *wt/wt*, *db/wt* and *db/db* animals. The statistical analysis could not be done on the *wt/wt* animals to evaluate the apparent increase of TG but increases in *db/wt* and *db/db* animals were shown to be statistically significant ($p=0.009$ and $p<0.0001$ respectively). Interestingly, it appears that there may be some increase in hepatic cholesterol content with the administration of alcohol. It is not clear whether such an increase, if real, reflects a passive phenomenon (storage in same compartment as TG) or active phenomenon (in which alcohol influences cholesterol metabolism).

Taken together, these studies suggest that over a relatively short period (4 weeks), of alcohol administration, liver cells accumulate TG and TC and that *db/db* mice are more vulnerable to the TG accumulation than *db/wt* mice. Despite the fact that there is much more TG in the hepatocyte of the *db/db* mouse on alcohol, the lipid peroxidation products did not increase proportionally; indicating that the 2 processes are not directly coupled, *i.e.* the peroxidation products are not proportionally included in TG. Indeed, they may even be metabolically quite distinct. This is in accordance with publications in the review where liver damage occurred in the absence of TG when fatty acid oxidation was promoted. The suggestion is that inadequate fatty acid disposal (by oxidation) \pm inadequate inhibition of synthesis can be witnessed by TG accumulation while TG accumulation per se does not lead to steatohepatitis (even when stressed by alcohol in this model).

7.4 Impact of Alcohol Administered over 8 Weeks in *db/wt* and *db/db* Mice

Contrary to the impression from the 4 wk study, the administration of alcohol, though still retarding growth, had a smaller impact over 8 wk than over 4 wk. Although alcohol administration increased the liver mass and the proportion of liver / body mass in the *db/wt* mice, the same was not seen for *db/db* mice. Inspection of figure 6.4.1 suggests that around the age of 15 weeks both alcohol-fed groups had lower growth rates. Such alcohol feeding may thus impose malnutrition on these animals, in contrast to the 4 weeks experiment.

Noting that there was a gene dose effect on liver mass in the standard chow, it would have been interesting to see the effect of alcohol in the short term on liver mass but regrettably these studies were not done. At the 8 week exposure, alcohol appears to

have significantly affected only *db/wt* animals although a relatively small increase may be masked in the variability in the current data set for the *db/db* mice.

Liver enzyme release into plasma showed no apparent hepatocyte damage with alcohol. Despite no change in plasma enzymes, alcohol increased the early lipid peroxidation products (figure 6.4.4) in both *db/wt* and *db/db* animals, again suggesting an uncoupling of LOOH products and TG storage, or an independent effect of alcohol to increase oxidative stress. In contrast to the 4 week study, LOOH increased in both gene groups and, like CD, may show an uncoupling of lipid peroxidation from storage and suggests that a period of >4 weeks is necessary for this effect. The same applies to TBARS.

Alcohol did not appear to affect the plasma glucose concentration of the *db/db* mice but, if anything, had an 'antidiabetic' effect on the *db/wt* mice, comparable with an anti-gluconeogenic effect reported for alcohol.

The biochemical findings of alcohol administration over 8 wks confirm a change in fat content and a consistent increase in lipid peroxidation products.

7.5 Impact of Alcohol Administered over 20 Weeks in *db/wt* and *db/db* Mice

Inspection of figure 6.5.1 reveals that the growth slopes of the animals fed alcohol changed soon after the introduction of alcohol and after 20 weeks the alcohol-fed animals weighed less than their controls. The graph also suggests that the animals reach maturity at 15 and 18 weeks. Alcohol increased the liver mass by a small proportion after 20 weeks in the *db/wt* (figure 6.5.3) but increased the proportion the liver makes to the body mass in both *db/wt* and *db/db* animals. Glucose concentration was not effected whilst alcohol concentration was higher in both groups that had alcohol supplemented, possibly more so in the smaller animals (*db/wt*). Whilst AST was mildly increased by alcohol in the *db/wt* group, no significant changes of plasma ALT occurred. Alcohol increased the TG content in both groups and affected protein content. Like the 8 week interpretation, alcohol increased the hepatic TC content in *db/wt* and *db/db* animals.

As in the 8 week group, CD levels were increased by alcohol, as were the LOOH and TTARS. The concentration of the TTARS appeared higher in the 32wk *db/db* control mice than 8wk whereas the CD and LOOH content were higher in 8wk *db/db* control mice when compared with the respective 32wk control animals.

Overall, the deleterious effects of alcohol administration on health are in evidence with a significant reduction in body mass in *db/wt* and *db/db* groups. Consistent with the previous studies the TG content increased together with cholesterol content but protein content changed little. AST increased in the plasma of alcohol-consuming *db/wt*. Plasma ALT, strikingly higher in *db/db* mice did not change with alcohol consumption. Random plasma alcohol was increased in *db/wt* mice, but not significantly in *db/db* mice.

7.6 The Mouse as a Model

Rodents are good animals to study because they are inexpensive, genetic manipulation is possible and interventions can be controlled. However, certain disadvantages arise from using mice and rats in medical research. These include time scale; a process requiring years in humans may not be suitably studied, especially if the process is not equivocally sped up in the rodent model, and, adaptations in metabolism with evolution may not make it susceptible to some processes in man despite the similarity in basic biochemistry and immune systems.

Rodents have regulated energy uptake. Though dietary constituents are varied, energy intake remains similar day to day. In contrast, obesity associated with fatty liver in humans is associated with a decrease in energy expenditure and increase in energy intake, often with much fat that is heated. Potentially, such thermally stressed oils are predisposed to the putative inflammation inciting products such as HNE. In this context insulin resistance, dietary stress and susceptible fatty acids converge on a potentially differently primed hepatocyte and immune systems to rodents to elicit fatty liver. Humans also have different lipoprotein metabolism and store more TG in adipose tissues.

The *db/db* model is an extreme one in which obesity, diabetes and fatty liver mimic human condition but appears not to promote inflammation and fibrosis until possibly the addition of alcohol. More dietary lipid stress together with alcohol may promote a steatohepatitis that approaches the histological and possibly clinical outcome to cirrhosis. Additional oxidative stressors may be required, although such a complete model may not be apt, it could test therapeutic strategies that, if successful, need to be tested in human for verification.

The *db/wt* model may be of interest to pursue in the longer term as the animals appears to gain TG almost to the biochemical point of fatty liver of *db/db* mice or with alcohol.

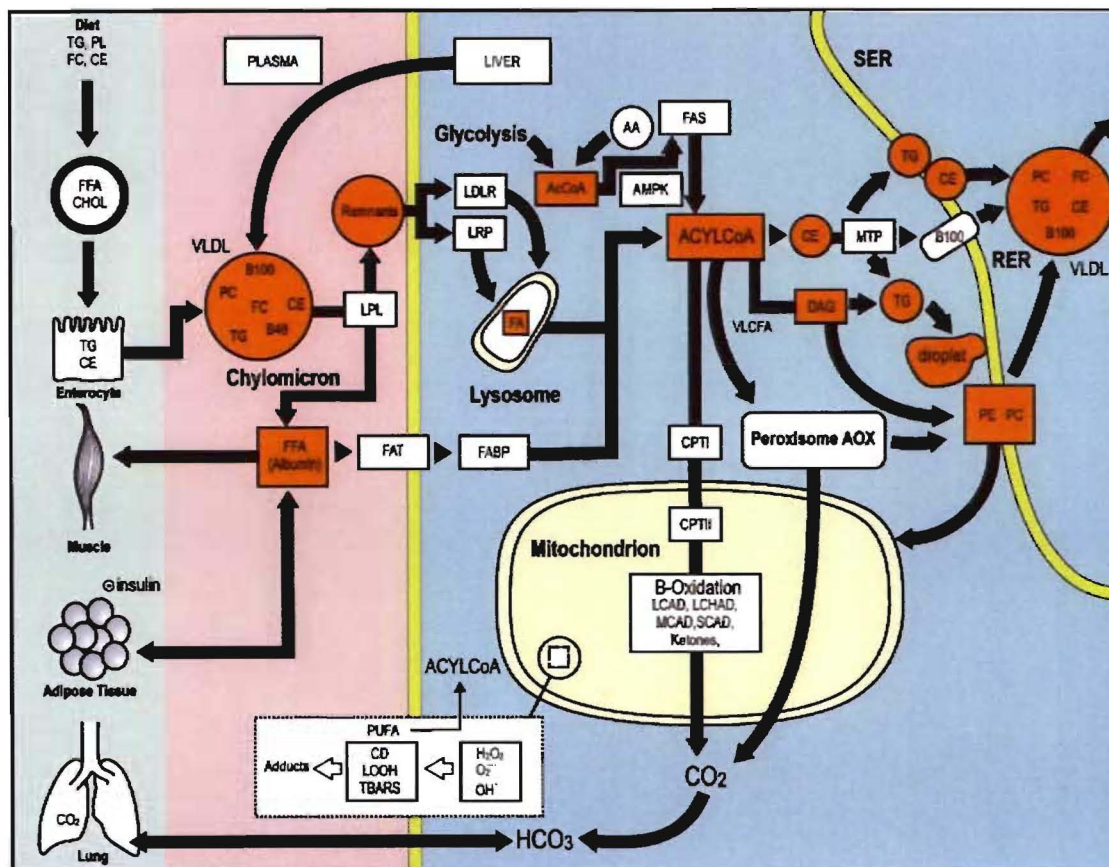
7.7 An Integrated View

A normal liver contains TG in various locations of the cell, including cytosolic droplets, lipoproteins and likely (non polar) membranes. The metabolic processes increasing the fat content can be the result of several stressors, and may or may not be coupled with cellular events that elicit cell damage, accumulation of cytosolic droplets visible on microscopy and an inflammatory and fibrotic response. The diagram (figure 7.7.1) depicts the multiple pathways in the liver that a fatty acid may follow to arrive in the liver and be processed. Numerous enzymes are involved in these pathways and several errors or stressors may induce TG accumulation and/or inflammatory response.

Absence of apo B and absence of MTP, both causing a β -lipoproteinaemia will disrupt export. Complete disruption of fatty acid oxidation through mitochondria and peroxisomes would also dramatically induce fatty liver. If these processes are within the normal activity, then excessive FA entry into the cells through lipid peroxidation and/or fatty acid transport could lead to TG accumulation if no induction of β -oxidation occurs. Increased FA synthesis could further overload the β -oxidation. However, increased β -oxidation can increase lipid peroxidation, causing cell damage, apoptosis and necrosis, eliciting inflammation and fibrosis. Thus for similar metabolic stresses, much variation in response can be attributed to the genetic and environmental factors pertaining in a given individual. The fatty liver relating to alcohol may thus also have a variable contribution from alcohol, especially at lower dosage.

Further studies of fatty liver disease should address the mechanisms involved by determining fluxes of FA in plasma, oxidation (even if indirectly by tracer studies of CO₂ in breath) and lipid peroxidation export. Furthermore, multiple gene micro-array studies could declare metabolic and immune responses and variation within these.

Figure 7.7.1: Overview of FA metabolism; including dietary assimilation, plasma transport as lipoproteins and albumin bound fatty acids, intracellular processes of β -oxidation, lipid peroxidation, lipoprotein formation and storage of TG droplets. Abbreviations – LCAD; long chain acyl dehydrogenase, LCHAD; long chain hydroxyl acyl dehydrogenase, MCAD; medium chain acyl dehydrogenase, SCAD; short chain acyl dehydrogenase, LRP; LDL receptor-related protein, SER; smooth endoplasmic reticulum, RER; rough endoplasmic reticulum, MTP; mitochondrial triglyceride transfer protein



Chapter 8; Tables – Group Mean Values

8.1 *Db Mouse Model – Group Mean Values: Males and Females*
Table 8.1.1 *Terminal Body Mass (g) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	Body Weight (g) ± SD
1	Wild Type (wt/wt)	6M	22.4 ± 0.8
2	Wild Type (wt/wt)	6F	17.3 ± 1.6
3	Heterozygous (db/wt)	16M	26.4 ± 2.4
4	Heterozygous (db/wt)	8F	20.8 ± 1.7
5	Homozygous (db/db)	10M	44.8 ± 2.4
6	Homozygous (db/db)	14F	42.2 ± 3.9

Db Mouse Model – Group Mean Values: Males and Females
Table 8.1.2 *Liver Mass (g) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	Liver Weights (g) ± SD
1	Wild Type (wt/wt)	6M	0.78 ± 0.06
2	Wild Type (wt/wt)	6F	0.75 ± 0.08
3	Heterozygous (db/wt)	6M	1.27 ± 0.13
4	Heterozygous (db/wt)	6F	0.75 ± 0.09
5	Homozygous (db/db)	6M	1.77 ± 0.18
6	Homozygous (db/db)	6F	2.09 ± 0.39

Table 8.1.3 *Db Mouse Model – Group Mean Values: Males and Females
Glucose Concentration (mmol/l) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	Glucose Concentration (µmol/g. liver) ± SD
1	Wild Type (wt/wt)	6M	5.5 ± 1.7
2	Wild Type (wt/wt)	6F	5.1 ± 1.0
3	Heterozygous (db/wt)	16M	9.1 ± 3.4
4	Heterozygous (db/wt)	8F	6.0 ± 1.0
5	Homozygous (db/db)	10M	23.0 ± 2.9
6	Homozygous (db/db)	14F	20.4 ± 8.0

Table 8.1.4 *Db Mouse Model – Group Mean Values: Males and Females
Triglycerides (mg TG/g. liver) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	Triglycerides (mg TG/g. liver) ± SD
1	Wild Type (wt/wt)	6M	1.94 ± 0.44
2	Wild Type (wt/wt)	6F	1.81 ± 0.39
3	Heterozygous (db/wt)	16M	1.19 ± 0.22
4	Heterozygous (db/wt)	8F	1.41 ± 0.49
5	Homozygous (db/db)	10M	3.16 ± 1.13
6	Homozygous (db/db)	14F	4.14 ± 0.81

Table 8.1.5 *Db Mouse Model – Group Mean Values: Males and Females Cholesterol (mg chol/g. liver) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	Cholesterol (mg chol/g. liver) ± SD
1	Wild Type (wt/wt)	6M	4.71 ± 0.39
2	Wild Type (wt/wt)	6F	4.85 ± 0.33
3	Heterozygous (db/wt)	16M	4.47 ± 0.43
4	Heterozygous (db/wt)	8F	4.54 ± 0.73
5	Homozygous (db/db)	10M	6.67 ± 0.80
6	Homozygous (db/db)	14F	6.68 ± 0.62

Table 8.1.6 *Db Mouse Model – Group Mean Values: Males and Females Conjugated Dienes (µmol/g. liver) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	Conjugated Dienes (µmol/g. liver) ± SD
1	Wild Type (wt/wt)	6M	2.07 ± 0.21
2	Wild Type (wt/wt)	6F	1.89 ± 0.12
3	Heterozygous (db/wt)	16M	2.58 ± 0.35
4	Heterozygous (db/wt)	8F	2.70 ± 0.34
5	Homozygous (db/db)	10M	3.48 ± 0.23
6	Homozygous (db/db)	14F	3.63 ± 0.47

Table 8.1.7 *Db Mouse Model – Group Mean Values: Males and Females Lipid Hydroperoxides (nmol LOOH/g. liver) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	Lipid Hydroperoxides (nmol LOOH/g. liver) ± SD
1	Wild Type (wt/wt)	6M	217 ± 76
2	Wild Type (wt/wt)	6F	137 ± 91
3	Heterozygous (db/wt)	16M	176 ± 71
4	Heterozygous (db/wt)	8F	216 ± 49
5	Homozygous (db/db)	10M	704 ± 151
6	Homozygous (db/db)	14F	433 ± 111

Table 8.1.8 *Db Mouse Model – Group Mean Values: Males and Females TTBARS (nmol/g. liver) ± Standard Deviation*

Group	Genotype	No. Animals / Sex	FTBARS (nmol/g. liver) ± SD
1	Wild Type (wt/wt)	6M	1795 ± 87
2	Wild Type (wt/wt)	6F	1309 ± 149
3	Heterozygous (db/wt)	16M	1264 ± 225
4	Heterozygous (db/wt)	8F	1337 ± 127
5	Homozygous (db/db)	10M	1559 ± 162
6	Homozygous (db/db)	14F	1731 ± 260

8.2
Table 8.2.1 *Impact of Various Oils, Administered via the Diet (4 Weeks) – Group Mean Values: Males and Females Combined Weekly Body Mass (g) ± Standard Deviation*

Age (Weeks)	Group / Treatment			
	1 Control	2 Sunflower Oil	3 Canola Oil	4 Sunflower Oil and Cholesterol
12	22.2 ± 3.1	22.4 ± 2.9	23.3 ± 3.7	23.8 ± 4.8
13	22.0 ± 3.4	22.0 ± 2.2	23.3 ± 3.5	23.8 ± 5.8
14	22.3 ± 3.4	22.0 ± 3.0	22.7 ± 3.5	22.8 ± 5.3
15	22.4 ± 3.6	22.3 ± 2.3	22.9 ± 3.1	24.3 ± 5.7
16	22.8 ± 3.2	23.5 ± 2.9	23.1 ± 3.5	24.5 ± 4.9
17	23.4 ± 3.6	23.6 ± 2.7	24.1 ± 3.2	24.5 ± 5.0
Mean Weight Gain (g) Weeks 12-17	1.2	1.2	0.8	0.7

Table 8.2.2 *Impact of Various Oils, Administered via the Diet (4 Weeks) – Group Mean Values: Males and Females Combined Liver Mass (g) ± Standard Deviation*

Group / Genotype	Treatment	No. Animals / Sex	Liver Weights (g) ± SD
1 (db/wt)	Control	3M + 3F	0.905 ± 0.17
2 (db/wt)	Sunflower Oil	3M + 3F	0.884 ± 0.13
3 (db/wt)	Canola Oil	3M + 3F	1.025 ± 0.13
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	0.889 ± 0.16

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Group Mean Values: Males and Females Combined
Glucose Concentration (mmol/l) ± Standard Deviation**

Table 8.2.3

Group / Genotype	Treatment	No. Animals / Sex	Glucose Concentration (mmol/l) ± SD
1 (db/wt)	Control	3M + 3F	6.8 ± 1.9
2 (db/wt)	Sunflower Oil	3M + 3F	7.6 ± 1.0
3 (db/wt)	Canola Oil	3M + 3F	8.0 ± 2.2
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	4.5 ± 2.2

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Group Mean Values: Males and Females Combined
Triglycerides (mg TG/g. liver) ± Standard Deviation**

Table 8.2.4

Group / Genotype	Treatment	No. Animals / Sex	Triglycerides (mg TG/g. liver) ± SD
1 (db/wt)	Control	3M + 3F	20.1 ± 3.5
2 (db/wt)	Sunflower Oil	3M + 3F	22.8 ± 4.7
3 (db/wt)	Canola Oil	3M + 3F	24.4 ± 2.8
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	22.2 ± 2.4

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Group Mean Values: Males and Females Combined
Cholesterol (mg chol/g. liver) ± Standard Deviation**

Table 8.2.5

Group / Genotype	Treatment	No. Animals / Sex	Cholesterol (mg chol/g. liver) ± SD
1 (db/wt)	Control	3M + 3F	2.33 ± 0.52
2 (db/wt)	Sunflower Oil	3M + 3F	4.47 ± 1.13
3 (db/wt)	Canola Oil	3M + 3F	3.43 ± 1.46
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	3.99 ± 1.08

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Group Mean Values: Males and Females Combined
Conjugated Dienes (µmol/g. liver) ± Standard Deviation**

Table 8.2.6

Group / Genotype	Treatment	No. Animals / Sex	Conjugated Dienes (µmol/g. liver) ± SD
1 (db/wt)	Control	3M + 3F	2.00 ± 0.20
2 (db/wt)	Sunflower Oil	3M + 3F	2.66 ± 0.33
3 (db/wt)	Canola Oil	3M + 3F	2.26 ± 0.30
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	2.46 ± 0.05

Table 8.2.7 *Impact of Various Oils, Administered via the Diet (4 Weeks) – Group Mean Values: Males and Females Combined Lipid Hydroperoxides (nmol LOOH/g. liver) ± Standard Deviation*

Group / Genotype	Treatment	No. Animals / Sex	Lipid Hydroperoxides (nmol LOOH/g. liver) ± SD
1 (db/wt)	Control	3M + 3F	400 ± 251
2 (db/wt)	Sunflower Oil	3M + 3F	561 ± 235
3 (db/wt)	Canola Oil	3M + 3F	500 ± 90
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	497 ± 188

Table 8.2.8 *Impact of Various Oils, Administered via the Diet (4 Weeks) – Group Mean Values: Males and Females Combined TTBARS (nmol/g. liver) ± Standard Deviation*

Group / Genotype	Treatment	No. Animals / Sex	FTBARS (nmol/g. liver) ± SD
1 (db/wt)	Control	3M + 3F	3532 ± 410
2 (db/wt)	Sunflower Oil	3M + 3F	3173 ± 461
3 (db/wt)	Canola Oil	3M + 3F	3314 ± 546
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	3204 ± 632

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Group Mean Values: Males and Females Combined
BCA Protein ($\mu\text{g}/\mu\text{g}$. liver) \pm Standard Deviation**

Table 8.2.9

Group / Genotype	Treatment	No. Animals / Sex	BCA Protein ($\mu\text{g}/\mu\text{g}$. liver) \pm SD
1 (db/wt)	Control	3M + 3F	71.3 \pm 11.9
2 (db/wt)	Sunflower Oil	3M + 3F	72.2 \pm 4.0
3 (db/wt)	Canola Oil	3M + 3F	75.0 \pm 10.8
4 (db/wt)	Sunflower Oil and Cholesterol	3M + 3F	75.5 \pm 7.7

8.3

Table 8.3.1

**Study of Short Period of Alcohol Administration (4 Weeks) –
Group Mean Values: Males and Females Combined
Weekly Body Mass (g) ± Standard Deviation**

Age (Weeks)	Group / Treatment					
	1 (wt/wt) H ₂ O Control	2 (wt/wt) 20% Alcohol	3 (db/wt) H ₂ O Control	4 (db/wt) 20% Alcohol	5 (db/db) H ₂ O Control	6 (db/db) 20% Alcohol
14	17.3	17.5	24.0 ± 3.1	22.1 ± 3.3	36.4 ± 7.3	34.3 ± 2.1
15	18.8	18.5	24.4 ± 3.8	22.5 ± 3.5	37.3 ± 7.2	35.6 ± 2.1
16	19.0	18.8	24.9 ± 3.1	22.8 ± 3.8	37.8 ± 7.8	36.6 ± 2.1
17	18.5	18.3	25.0 ± 3.8	22.5 ± 3.9	37.3 ± 8.1	36.6 ± 1.7
18	19.3	19.0	25.1 ± 3.7	22.6 ± 3.3	37.4 ± 8.6	37.3 ± 1.8
19	19.0	18.8	25.1 ± 3.7	22.4 ± 3.2	37.8 ± 8.9	37.8 ± 2.1
20	19.5	19.3	25.8 ± 3.6	22.6 ± 3.3	38.9 ± 9.3	38.5 ± 2.0
Mean Weight Gain (g) Weeks 9-20	+ 2.2	+ 1.8	+ 1.8	+ 0.5	+ 3.5	+ 4.2

Please note that a standard deviation value could not be calculated for groups 1 and 2, as the number of animals was too small.

**Study of Short Period of Alcohol Administration (4 Weeks) –
Group Mean Values: Males and Females Combined
Triglycerides (mg TG/g. liver) ± Standard Deviation**

Table 8.3.2

Group / Genotype	Treatment	No. Animals / Sex	Triglycerides (mg TG/g. liver) ± SD
1 (wt/wt)	H ₂ O Control	1M + 1F	1.53
2 (wt/wt)	20% Alcohol	1M + 1F	1.84
3 (db/wt)	H ₂ O Control	2M + 2F	1.05 ± 0.08
4 (db/wt)	20% Alcohol	2M + 2F	1.28 ± 0.09
5 (db/db)	H ₂ O Control	2M + 2F	2.93 ± 0.26
6 (db/db)	20% Alcohol	1M + 3F	6.44 ± 0.48

Please note that a standard deviation value could not be calculated for groups 1 and 2, as the number of animals was too small.

**Study of Short Period of Alcohol Administration (4 Weeks) –
Group Mean Values: Males and Females Combined
Cholesterol (mg chol/g. liver) ± Standard Deviation**

Table 8.3.3

Group / Genotype	Treatment	No. Animals / Sex	Cholesterol (mg chol/g. liver) ± SD
1 (wt/wt)	H ₂ O Control	1M + 1F	3.54
2 (wt/wt)	20% Alcohol	1M + 1F	4.53
3 (db/wt)	H ₂ O Control	2M + 2F	2.62 ± 0.72
4 (db/wt)	20% Alcohol	2M + 2F	4.49 ± 0.51
5 (db/db)	H ₂ O Control	2M + 2F	4.65 ± 1.02
6 (db/db)	20% Alcohol	1M + 3F	5.15 ± 1.42

Please note that a standard deviation value could not be calculated for groups 1 and 2, as the number of animals was too small.

**Study of Short Period of Alcohol Administration (4 Weeks) –
Group Mean Values: Males and Females Combined
TTBARS (nmol/g. liver) ± Standard Deviation**

Table 8.3.6

Group / Genotype	Treatment	No. Animals / Sex	FTBARS (nmol/g. liver) ± SD
1 (wt/wt)	H ₂ O Control	1M + 1F	1490
2 (wt/wt)	20% Alcohol	1M + 1F	1577
3 (db/wt)	H ₂ O Control	2M + 2F	1175 ± 72
4 (db/wt)	20% Alcohol	2M + 2F	1367 ± 125
5 (db/db)	H ₂ O Control	2M + 2F	1307 ± 144
6 (db/db)	20% Alcohol	1M + 3F	1849 ± 26

Please note that a standard deviation value could not be calculated for groups 1 and 2, as the number of animals was too small.

8.4

Table 8.4.1

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Weekly Body Mass (g) ± Standard Deviation

Age (Weeks)	Group / Treatment			
	1 (db/wt) H ₂ O Control	2 (db/wt) 20% Alcohol	3 (db/db) H ₂ O Control	4 (db/db) 20% Alcohol
9	25.2 ± 1.5	27.3 ± 1.3	44.4 ± 3.1	44.3 ± 4.0
10	26.5 ± 1.7	27.8 ± 1.3	46.9 ± 2.4	46.8 ± 4.4
11	27.3 ± 1.7	28.1 ± 1.1	47.6 ± 2.2	48.0 ± 4.4
12	28.1 ± 1.5	28.7 ± 1.0	49.7 ± 2.4	48.9 ± 4.2
13	29.0 ± 1.8	29.4 ± 1.4	49.6 ± 3.4	49.0 ± 4.7
14	29.6 ± 2.1	29.8 ± 1.5	49.3 ± 4.8	49.6 ± 5.4
15	31.2 ± 2.3	30.3 ± 1.4	50.7 ± 4.4	50.0 ± 6.2
16	30.9 ± 2.4	30.4 ± 1.0	52.3 ± 4.6	49.9 ± 6.4
17	31.7 ± 2.6	30.9 ± 1.1	52.1 ± 5.1	49.9 ± 7.6
18	32.3 ± 3.1	31.0 ± 0.8	52.4 ± 5.4	50.5 ± 8.2
19	32.4 ± 2.6	30.9 ± 1.0	50.8 ± 5.3	50.2 ± 8.7
20	32.0 ± 2.7	31.8 ± 1.1	51.8 ± 5.3	49.5 ± 8.7
Mean Weight Gain (g) Weeks 9-20	6.8	4.5	7.4	5.2

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Liver Mass (g) ± Standard Deviation**

Table 8.4.2

Group / Genotype	Treatment	No. Animals	Liver Weight (g) ± SD
1 (db/wt)	H ₂ O Control	7	1.06 ± 0.06
2 (db/wt)	20% Alcohol	6	1.45 ± 0.12
3 (db/db)	H ₂ O Control	6	2.83 ± 0.57
4 (db/db)	20% Alcohol	5	2.84 ± 0.42

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Glucose Concentration (mmol/l) ± Standard Deviation**

Table 8.4.3

Group / Genotype	Treatment	No. Animals	Glucose Concentration (mmol/l) ± SD
1 (db/wt)	H ₂ O Control	7	13.8 ± 3.4
2 (db/wt)	20% Alcohol	6	8.6 ± 1.6
3 (db/db)	H ₂ O Control	6	31.2 ± 2.0
4 (db/db)	20% Alcohol	5	29.2 ± 2.8

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Blood/Alcohol Levels (mmol/l) ± Standard Deviation**

Table 8.4.4

Group / Genotype	Treatment	No. Animals	Blood/Alcohol Levels (mmol/l) ± SD
1 (db/wt)	H ₂ O Control	7	0.53 ± 0.46
2 (db/wt)	20% Alcohol	6	1.33 ± 0.86
3 (db/db)	H ₂ O Control	6	1.12 ± 0.56
4 (db/db)	20% Alcohol	5	4.73 ± 6.00

Table 8.4.5 *Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males Aspartine Transaminase (AST) Levels (U/l) ± Standard Deviation*

Group / Genotype	Treatment	No. Animals	AST Levels (U/l) ± SD
1 (db/wt)	H ₂ O Control	7	32.6 ± 17.3
2 (db/wt)	20% Alcohol	6	41.7 ± 11.1
3 (db/db)	H ₂ O Control	6	53.8 ± 21.4
4 (db/db)	20% Alcohol	5	77.8 ± 43.4

Table 8.4.6 *Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males Alanine Transaminase (ALT) Levels (U/l) ± Standard Deviation*

Group / Genotype	Treatment	No. Animals	ALT Levels (U/l) ± SD
1 (db/wt)	H ₂ O Control	7	18.6 ± 10.5
2 (db/wt)	20% Alcohol	6	22.3 ± 8.0
3 (db/db)	H ₂ O Control	6	65.3 ± 46.9
4 (db/db)	20% Alcohol	5	71.0 ± 33.9

Table 8.4.7 *Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males Triglycerides (mg TG/g. liver) ± Standard Deviation*

Group / Genotype	Treatment	No. Animals	Triglycerides (mg TG/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	4.00 ± 1.63
2 (db/wt)	20% Alcohol	6	6.61 ± 0.78
3 (db/db)	H ₂ O Control	6	6.55 ± 1.41
4 (db/db)	20% Alcohol	5	10.33 ± 2.25

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Cholesterol (mg chol/g. liver) ± Standard Deviation**

Table 8.4.8

Group / Genotype	Treatment	No. Animals	Cholesterol (mg chol/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	3.35 ± 0.95
2 (db/wt)	20% Alcohol	6	5.03 ± 0.91
3 (db/db)	H ₂ O Control	6	5.91 ± 1.02
4 (db/db)	20% Alcohol	5	8.46 ± 1.58

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Conjugated Dienes (µmol/g. liver) ± Standard Deviation**

Table 8.4.9

Group / Genotype	Treatment	No. Animals	Conjugated Dienes (µmol/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	1.60 ± 0.31
2 (db/wt)	20% Alcohol	6	2.00 ± 0.23
3 (db/db)	H ₂ O Control	6	2.66 ± 0.07
4 (db/db)	20% Alcohol	5	3.45 ± 0.16

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Lipid Hydroperoxides (nmol LOOH/g. liver) ± Standard Deviation**

Table 8.4.10

Group / Genotype	Treatment	No. Animals	Lipid Hydroperoxides (nmol LOOH/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	339 ± 54
2 (db/wt)	20% Alcohol	6	724 ± 107
3 (db/db)	H ₂ O Control	6	1073 ± 92
4 (db/db)	20% Alcohol	5	1486 ± 118

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
TTBARS (nmol/g. liver) ± Standard Deviation**

Table 8.4.11

Group / Genotype	Treatment	No. Animals	FTBARS (nmol/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	1762 ± 154
2 (db/wt)	20% Alcohol	6	2529 ± 266
3 (db/db)	H ₂ O Control	6	1862 ± 230
4 (db/db)	20% Alcohol	5	2962 ± 257

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
BCA Protein Assay (µg/µg. liver) ± Standard Deviation**

Table 8.4.12

Group / Genotype	Treatment	No. Animals	Protein Concentration (µg/µg. Liver) ± SD
1 (db/wt)	H ₂ O Control	7	55.3 ± 10.5
2 (db/wt)	20% Alcohol	6	60.7 ± 3.6
3 (db/db)	H ₂ O Control	6	69.4 ± 12.4
4 (db/db)	20% Alcohol	5	76.7 ± 12.2

**Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Fat Change and Inflammation Grading ± Standard Deviation**

Table 8.4.13

Group / Genotype	Treatment	No. Animals	Fat Change (0-3) ± SD	Inflammatory Aggregates* ± SD
1 (db/wt)	H ₂ O Control	7	0.0 ± 0.0	0.0 ± 0.0
2 (db/wt)	20% Alcohol	6	0.17 ± 0.41	0.0 ± 0.0
3 (db/db)	H ₂ O Control	6	2.33 ± 0.52	1.33 ± 1.63
4 (db/db)	20% Alcohol	5	2.80 ± 0.45	6.00 ± 2.55

* = Inflammatory aggregates; 20 x objective, 20 fields counted

8.5

Table 8.5.1

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Weekly Body Mass (g) ± Standard Deviation

Age (Weeks)	Group / Treatment			
	1 (db/wt) H ₂ O Control	2 (db/wt) 20% Alcohol	3 (db/db) H ₂ O Control	4 (db/db) 20% Alcohol
9	27.0 ± 0.8	27.6 ± 0.5	45.1 ± 1.3	43.4 ± 1.8
10	28.1 ± 1.2	27.9 ± 1.0	45.4 ± 0.9	46.3 ± 1.4
11	28.8 ± 1.1	28.5 ± 0.9	46.2 ± 1.1	47.1 ± 1.0
12	29.4 ± 1.1	28.2 ± 1.2	50.6 ± 3.1	49.8 ± 1.6
13	30.9 ± 1.0	28.7 ± 1.3	51.8 ± 2.2	50.1 ± 2.2
14	31.4 ± 1.3	29.3 ± 1.2	53.5 ± 2.8	51.0 ± 2.9
15	32.5 ± 2.0	29.8 ± 1.0	54.8 ± 3.6	51.8 ± 2.5
16	32.6 ± 1.7	30.4 ± 1.1	56.2 ± 3.6	51.3 ± 2.4
17	33.1 ± 2.1	30.1 ± 1.3	55.4 ± 3.7	51.5 ± 2.9
18	33.5 ± 2.0	30.4 ± 1.2	56.8 ± 4.0	51.8 ± 3.2
19	33.5 ± 2.2	30.1 ± 1.0	57.4 ± 3.8	51.4 ± 3.5
20	33.4 ± 2.8	29.6 ± 0.8	56.6 ± 4.4	51.8 ± 3.3

Table 8.5.1 *Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males*
(continued) *Weekly Body Mass (g) ± Standard Deviation*

Age (weeks)	Group / Treatment			
	1 (db/wt) H ₂ O Control	2 (db/wt) 20% Alcohol	3 (db/db) H ₂ O Control	4 (db/db) 20% Alcohol
21	33.4 ± 2.4	30.1 ± 1.3	56.0 ± 3.7	51.3 ± 2.9
22	32.9 ± 2.6	29.6 ± 1.1	56.3 ± 4.2	50.8 ± 3.2
23	33.9 ± 2.8	30.7 ± 0.8	56.3 ± 5.0	50.4 ± 3.6
24	33.3 ± 2.9	30.5 ± 1.0	54.5 ± 4.4	49.9 ± 3.5
25	33.4 ± 2.8	31.0 ± 1.1	56.5 ± 4.4	51.2 ± 3.8
26	33.9 ± 3.0	31.6 ± 0.9	57.9 ± 4.8	51.8 ± 4.6
27	32.7 ± 2.8	31.6 ± 1.4	57.7 ± 4.5	51.8 ± 4.9
28	32.9 ± 3.2	31.5 ± 1.3	58.6 ± 4.3	52.8 ± 5.0
29	33.4 ± 2.8	30.9 ± 1.5	59.0 ± 5.0	53.5 ± 5.7
30	33.8 ± 2.1	31.6 ± 1.7	58.3 ± 4.5	52.7 ± 5.8
31	33.5 ± 3.3	31.5 ± 2.1	57.4 ± 4.2	51.3 ± 5.7
32	33.4 ± 2.7	31.3 ± 2.1	57.4 ± 5.5	50.2 ± 5.7
Total Weight Gain (g) Weeks 9-32	+ 6.4	+ 1.2	+ 12.3	+ 6.8

**Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Liver Mass (g) ± Standard Deviation**

Table 8.5.2

Group / Genotype	Treatment	No. Animals	Liver Weight (g) ± SD
1 (db/wt)	H ₂ O Control	7	1.37 ± 0.05
2 (db/wt)	20% Alcohol	4	1.53 ± 0.11
3 (db/db)	H ₂ O Control	6	2.84 ± 0.44
4 (db/db)	20% Alcohol	6	2.99 ± 0.38

**Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Glucose Concentration (mmol/l) ± Standard Deviation**

Table 8.5.3

Group / Genotype	Treatment	No. Animals	Glucose Concentration (mmol/l) ± SD
1 (db/wt)	H ₂ O Control	7	13.6 ± 3.5
2 (db/wt)	20% Alcohol	4	12.3 ± 5.2
3 (db/db)	H ₂ O Control	6	31.4 ± 0.8
4 (db/db)	20% Alcohol	6	29.2 ± 4.8

**Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Blood/Alcohol Levels (mmol/l) ± Standard Deviation**

Table 8.5.4

Group / Genotype	Treatment	No. Animals	Blood/Alcohol Levels (mmol/l) ± SD
1 (db/wt)	H ₂ O Control	7	0.64 ± 0.49
2 (db/wt)	20% Alcohol	4	12.65 ± 9.09
3 (db/db)	H ₂ O Control	6	0.56 ± 0.27
4 (db/db)	20% Alcohol	6	4.49 ± 9.09

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Aspartate Transaminase (AST) Levels (U/l) ± Standard Deviation

Table 8.5.5

Group / Genotype	Treatment	No. Animals	AST Levels (U/l) ± SD
1 (db/wt)	H ₂ O Control	7	23.3 ± 9.2
2 (db/wt)	20% Alcohol	4	37.5 ± 9.5
3 (db/db)	H ₂ O Control	6	37.3 ± 9.6
4 (db/db)	20% Alcohol	6	43.5 ± 30.3

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Alanine Transaminase (ALT) Levels (U/l) ± Standard Deviation

Table 8.5.6

Group / Genotype	Treatment	No. Animals	ALT Levels (U/l) ± SD
1 (db/wt)	H ₂ O Control	7	15.1 ± 7.8
2 (db/wt)	20% Alcohol	4	16.8 ± 7.9
3 (db/db)	H ₂ O Control	6	65.2 ± 44.8
4 (db/db)	20% Alcohol	6	76.3 ± 28.6

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Triglycerides (mg TG/g. liver) ± Standard Deviation

Table 8.5.7

Group / Genotype	Treatment	No. Animals	Triglycerides (mg TG/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	3.72 ± 0.54
2 (db/wt)	20% Alcohol	4	5.35 ± 0.59
3 (db/db)	H ₂ O Control	6	5.74 ± 1.06
4 (db/db)	20% Alcohol	6	7.67 ± 0.60

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Cholesterol (mg chol/g. liver) ± Standard Deviation

Table 8.5.8

Group / Genotype	Treatment	No. Animals	Cholesterol (mg chol/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	3.88 ± 0.50
2 (db/wt)	20% Alcohol	4	5.58 ± 0.67
3 (db/db)	H ₂ O Control	6	6.14 ± 1.31
4 (db/db)	20% Alcohol	6	9.17 ± 1.58

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Conjugated Dienes (µmol/g. liver) ± Standard Deviation

Table 8.5.9

Group / Genotype	Treatment	No. Animals	Conjugated Dienes (µmol/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	1.64 ± 0.18
2 (db/wt)	20% Alcohol	4	2.19 ± 0.29
3 (db/db)	H ₂ O Control	6	2.22 ± 0.15
4 (db/db)	20% Alcohol	6	2.58 ± 0.17

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Lipid Hydroperoxides (nmol LOOH/g. liver) ± Standard Deviation

Table 8.5.10

Group / Genotype	Treatment	No. Animals	Lipid Hydroperoxides (nmol LOOH/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	195 ± 60
2 (db/wt)	20% Alcohol	4	452 ± 78
3 (db/db)	H ₂ O Control	6	789 ± 110
4 (db/db)	20% Alcohol	6	998 ± 121

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
TTBARS (nmol/g. liver) ± Standard Deviation

Table 8.5.11

Group / Genotype	Treatment	No. Animals	FTBARS (nmol/g. liver) ± SD
1 (db/wt)	H ₂ O Control	7	1617 ± 300
2 (db/wt)	20% Alcohol	4	2816 ± 349
3 (db/db)	H ₂ O Control	6	2891 ± 164
4 (db/db)	20% Alcohol	6	3336 ± 399

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
BCA Protein Assay (µg/µg. liver) ± Standard Deviation

Table 8.5.12

Group / Genotype	Treatment	No. Animals	Protein Concentration (µg/µg. liver) ± SD
1 (db/wt)	H ₂ O Control	7	58.6 ± 8.0
2 (db/wt)	20% Alcohol	4	64.8 ± 6.0
3 (db/db)	H ₂ O Control	6	82.5 ± 10.9
4 (db/db)	20% Alcohol	6	88.5 ± 8.0

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Group Mean Values: Males
Fat Change and Inflammation Grading ± Standard Deviation

Table 8.5.13

Group / Genotype	Treatment	No. Animals	Fat Change (0-3) ± SD	Inflammatory Aggregates* ± SD
1 (db/wt)	H ₂ O Control	7	0.0 ± 0.0	2.86 ± 3.87
2 (db/wt)	20% Alcohol	4	0.25 ± 0.50	1.25 ± 1.5
3 (db/db)	H ₂ O Control	6	1.83 ± 0.75	3.5 ± 1.64
4 (db/db)	20% Alcohol	6	2.0 ± 0.0	1.5 ± 1.64

* = Inflammatory aggregates; 20 x objective, 20 fields counted

Chapter 9; Appendices – Individual Values

Appendix A1 Wild Type Mice (wt/wt) – Individual Values: Males and Females Terminal Body Mass (g)

Sex	Animal	Body Mass (g)
M	1	22.7
	2	22.5
	3	21.2
	4	22.1
	5	23.5
	6	22.6
F	7	17.7
	8	17.5
	9	17.9
	10	19.6
	11	16.0
	12	15.2

Appendix A2 Wild Type Mice (wt/wt) – Individual Values: Males and Females Liver Mass (g)

Sex	Animal	Liver Mass (g)
M	1	0.8731
	2	0.7377
	3	0.7484
	4	0.7784
	5	0.8145
	6	0.7113
F	7	0.7979
	8	0.8575
	9	0.6443
	10	0.8132
	11	0.6987
	12	0.7158

Appendix A3 Wild Type Mice (wt/wt) – Individual Values: Males and Females Liver Mass / Body Mass x 100 (%)

Sex	Animal	Liver Weight / Mass x 100 (%)
M	1	3.69
	2	3.28
	3	3.53
	4	3.52
	5	3.47
	6	3.15
F	7	4.51
	8	4.9
	9	3.6
	10	4.15
	11	4.37
	12	4.71

**Appendix A4 Wild Type Mice (wt/wt) – Individual Values: Males and Females
Glucose Concentration (mmol/l)**

Sex	Animal	Glucose conc. (mmol/l)
M	1	3.4
	2	5.4
	3	3.7
	4	7.4
	5	7.0
	6	6.3
F	7	4.2
	8	6.0
	9	6.2
	10	3.8
	11	4.9
	12	5.7

**Appendix A5 Wild Type Mice (wt/wt) – Individual Values: Males and Females
Triglycerides (mg TG/g. liver)**

Sex	Animal	Trial 1	Trial 2	Mean
M	1	2.46	2.56	2.51
	2	2.18	2.49	2.34
	3	1.94	1.82	1.88
	4	1.99	2.00	2.00
	5	1.27	1.74	1.51
	6	1.33	1.44	1.39
F	7	2.38	2.19	2.29
	8	2.10	2.40	2.25
	9	1.98	1.76	1.87
	10	1.38	1.73	1.56
	11	1.17	1.49	1.33
	12	1.54	1.59	1.57

**Appendix A6 Wild Type Mice (wt/wt) – Individual Values: Males and Females
Cholesterol (mg chol/g. liver)**

Sex	Animal	Trial 1	Trial 2	Mean
M	1	4.29	4.41	4.35
	2	4.53	4.48	4.51
	3	5.12	4.96	5.04
	4	4.97	5.07	5.02
	5	4.15	4.35	4.25
	6	5.24	4.99	5.12
F	7	5.27	5.16	5.22
	8	5.37	4.75	5.06
	9	4.18	4.80	4.49
	10	5.25	5.02	5.14
	11	4.32	4.68	4.50
	12	4.86	4.51	4.69

**Appendix A7 Wild Type Mice (wt/wt) – Individual Values: Males and Females
Conjugated Dienes ($\mu\text{mol/g. liver}$)**

Sex	Animal	Trial 1	Trial 2	Mean
M	1	1.87	1.62	1.75
	2	2.00	1.99	2.00
	3	2.23	2.09	2.16
	4	2.31	2.46	2.39
	5	2.18	2.07	2.13
	6	1.93	2.06	2.00
F	7	2.11	2.03	2.07
	8	1.71	1.74	1.73
	9	1.67	2.06	1.87
	10	2.07	1.57	1.82
	11	1.95	2.01	1.98
	12	1.58	2.22	1.90

**Appendix A8 Wild Type Mice (wt/wt) – Individual Values: Males and Females
Lipid Hydroperoxides (nmol LOOH/g. liver)**

Sex	Animal	Trial 1	Trial 2	Mean
M	1	42	101	72
	2	232	185	209
	3	283	244	264
	4	258	216	237
	5	219	245	232
	6	280	298	289
F	7	255	203	229
	8	296	256	276
	9	44	85	65
	10	88	59	74
	11	87	109	98
	12	53	114	84

**Appendix A9 Wild Type Mice (wt/wt) – Individual Values: Males and Females
TTBARS (nmol/g. liver)**

Sex	Animal	Trial 1	Trial 2	Mean
M	1	1828	1810	1819
	2	1920	1828	1874
	3	1883	1810	1847
	4	1682	1755	1719
	5	1571	1737	1655
	6	1883	1810	1847
F	7	1243	1426	1335
	8	1188	1353	1271
	9	1225	1060	1143
	10	1463	1499	1481
	11	1517	1426	1472
	12	1079	1225	1152

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females
Terminal Body Mass (g)**

Appendix B1

Sex	Animal	Body Mass (g)
M	1	25.6
	2	25.9
	3	29.3
	4	27.9
	5	29.0
	6	27.2
	7	19.5
	8	27.7
	9	26.7
	10	29.9
	11	25.8
	12	26.2
	13	24.4
	14	24.5
	15	26.2
	16	26.7
F	17	18.6
	18	20.0
	19	23.0
	20	21.5
	21	22.0
	22	19.4
	23	19.2
	24	22.5

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females
Liver Mass (g)**

Appendix B2

Sex	Animal	Liver Mass (g)
M	1	1.1560
	2	1.3540
	3	1.3691
	4	1.4012
	5	1.0999
	6	1.2154
	7*	-
	8*	-
	9*	-
	10*	-
	11*	-
	12*	-
	13*	-
	14*	-
	15*	-
	16*	-
F	17	0.5912
	18	0.7265
	19	0.7806
	20	0.8103
	21	0.7415
	22	0.8272
	23*	-
	24*	-

* = The liver mass of these animals were not taken in error

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females**

Appendix B3 Liver Mass / Body Mass x 100 (%)

Sex	Animal	Liver Mass / Body Mass x 100 (%)
M	1	4.52
	2	5.23
	3	4.67
	4	5.02
	5	4.04
	6	4.47
	7*	-
	8*	-
	9*	-
	10*	-
	11*	-
	12*	-
	13*	-
	14*	-
	15*	-
	16*	-
F	17	3.18
	18	3.63
	19	3.40
	20	3.77
	21	3.37
	22	4.26
	23*	-
	24*	-

* = The liver mass of these animals were not taken in error

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females
Appendix B4 Glucose Concentration (mmol/l)**

Sex	Animal	Glucose conc. (mmol/l)
M	1	8.4
	2	8.3
	3	7.6
	4	6.8
	5	3.4
	6	6.3
	7	7.2
	8	6.6
	9	5.3
	10	14.0
	11	13.9
	12	9.9
	13	9.5
	14	12.7
	15	14.6
	16	11.8
F	17	4.5
	18	5.8
	19	7.2
	20	6.6
	21	7.3
	22	5.3
	23	5.4
	24	6.2

**Heterozygous Mice (db/+) – Individual Values:
Males and Females**

Appendix B5 Triglycerides (mg TG/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	1.17	1.31	1.24
	2	1.06	1.21	1.14
	3	1.42	1.44	1.43
	4	1.75	1.56	1.66
	5	1.04	0.99	1.02
	6	1.03	1.32	1.18
	7	0.91	1.00	0.96
	8	1.10	1.18	1.14
	9	0.94	1.15	1.05
	10	0.97	1.12	1.05
	11	0.93	1.01	0.97
	12	0.86	1.39	1.13
	13	1.26	1.16	1.21
	14	1.16	1.02	1.09
	15	1.02	1.22	1.12
	F	16	1.78	1.57
17		1.38	1.41	1.40
18		1.31	1.58	1.45
19		1.29	1.06	1.18
20		1.69	1.54	1.62
21		2.13	1.99	2.06
22		0.98	1.27	1.13
23		1.05	1.30	1.18
24		0.99	1.61	1.30

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females**

Appendix B6 Cholesterol (mg chol/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	4.73	4.69	4.71
	2	4.31	3.98	4.15
	3	4.60	5.10	4.85
	4	4.77	5.25	5.01
	5	4.65	3.59	4.12
	6	3.6	3.70	3.68
	7	4.20	4.12	4.16
	8	4.27	4.36	4.32
	9	4.27	4.46	4.37
	10	4.54	4.52	4.53
	11	5.15	5.25	5.20
	12	5.41	4.98	5.20
	13	4.23	4.71	4.47
	14	4.19	4.23	4.21
	15	3.99	4.19	4.09
	16	4.65	4.43	4.54
F	17	3.72	3.74	3.73
	18	3.51	3.73	3.62
	19	4.44	3.96	4.20
	20	4.79	4.87	4.83
	21	5.26	5.20	5.23
	22	5.33	5.48	5.41
	23	5.27	5.27	5.27
	24	3.99	4.05	4.02

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females**

Appendix B7 Conjugated Dienes ($\mu\text{mol/g. liver}$)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	2.43	2.59	2.51
	2	2.26	2.31	2.29
	3	2.18	2.09	2.14
	4	2.47	2.58	2.53
	5	2.42	2.42	2.42
	6	2.18	2.20	2.19
	7	3.31	2.99	3.15
	8	2.31	2.57	2.44
	9	2.46	2.45	2.46
	10	2.48	2.36	2.42
	11	2.53	2.50	2.52
	12	2.50	2.50	2.50
	13	2.03	2.88	2.46
	14	3.46	3.00	3.23
	15	3.23	3.29	3.26
	16	2.95	2.76	2.86
F	17	3.02	3.07	3.05
	18	2.61	2.83	2.72
	19	2.97	3.08	3.03
	20	2.94	2.65	2.80
	21	3.01	2.75	2.88
	22	2.72	2.22	2.47
	23	2.71	2.56	2.64
	24	2.02	1.98	2.00

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females**

Appendix B8 Lipid Hydroperoxides (nmol LOOH/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	206	195	201
	2	237	237	254
	3	167	169	168
	4	196	202	199
	5	135	170	153
	6	26	84	55
	7	80	92	86
	8	78	56	67
	9	81	89	85
	10	250	254	252
	11	279	245	262
	12	243	281	262
	13	118	193	156
	14	248	222	235
	15	192	216	204
	16	181	173	177
F	17	219	184	202
	18	296	265	181
	19	240	277	259
	20	246	259	253
	21	235	237	236
	22	154	154	154
	23	178	223	201
	24	113	178	146

**Heterozygous Mice (db/wt) – Individual Values:
Males and Females**

Appendix B9 TTBARS (nmol/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	987	1060	1024
	2	969	1115	1042
	3	1097	969	1033
	4	1079	1115	1097
	5	1426	1536	1481
	6	1426	1609	1518
	7	1371	1389	1380
	8	1335	1389	1362
	9	1554	1572	1563
	10	1481	1390	1436
	11	1609	1463	1536
	12	1408	1517	1463
	13	1134	1225	1180
	14	1134	1280	1207
	15	878	1006	942
	F	16	914	1006
17		1444	1335	1390
18		1335	1371	1353
19		1371	1481	1426
20		1298	1134	1216
21		1371	1316	1344
22		1645	1463	1554
23		1042	1261	1152
24		1207	1316	1262

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C1 Terminal Body Mass (g)

Sex	Animal	Body Mass (g)
M	1	45.3
	2	41.4
	3	46.1
	4	42.7
	5	45.5
	6	43.4
	7	45.2
	8	43.4
	9	45.2
	10	47.3
F	11	44.7
	12	49.6
	13	47.2
	14	43.9
	15	38.0
	16	45.9
	17	36.6
	18	42.2
	19	41.9
	20	42.7
	21	39.8
	22	36.5
	23	41.8
	24	40.1

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C2 Liver Mass (g)

Sex	Animal	Liver Mass (g)
M	1	1.9814
	2	1.4321
	3	1.7746
	4	1.8221
	5	1.8354
	6	1.7866
	7*	-
	8*	-
	9*	-
	10*	-
F	11	1.9867
	12	2.5272
	13	2.0883
	14	2.1109
	15	1.4141
	16	2.3851
	17*	-
	18*	-
	19*	-
	20*	-
	21*	-
	22*	-
	23*	-
	24*	-

* = The liver mass of these animals were not taken in error

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C3 Liver Mass / Body Mass x 100 (%)

Sex	Animal	Liver Mass / Body Mass x 100 (%)
M	1	4.37
	2	3.46
	3	3.85
	4	4.00
	5	4.03
	6	4.17
	7*	-
	8*	-
	9*	-
	10*	-
F	11	4.44
	12	5.09
	13	4.42
	14	4.81
	15	3.72
	16	5.19
	17*	-
	18*	-
	19*	-
	20*	-
	21*	-
	22*	-
	23*	-
	24*	-

* = The liver mass of these animals were not taken in error

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C4 Glucose Concentration (mmol/l)

Sex	Animal	Glucose conc. (mmol/l)
M	1	20.9
	2	23.4
	3	23.4
	4	20.3
	5	25.3
	6	21.9
	7	23.6
	8	29.5
	9	19.9
	10	21.4
F	11	13.6
	12	12.6
	13	16.9
	14	19.5
	15	14.9
	16	10.3
	17	24.8
	18	33.1
	19	28.1
	20	27.2
	21	19.2
	22	25.0
	23	23.3
	24	27.5

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C5 Triglycerides (mg TG/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	2.59	2.83	2.71
	2	2.29	2.76	2.53
	3	2.12	2.41	2.27
	4	2.32	2.12	2.22
	5	3.29	2.95	3.12
	6	5.59	4.68	5.14
	7	5.42	4.83	5.13
	8	2.25	2.90	2.53
	9	3.75	3.55	3.62
	10	1.85	2.62	2.24
F	11	4.90	4.53	4.72
	12	2.50	2.76	2.68
	13	3.03	3.18	3.11
	14	3.76	3.59	3.68
	15	4.24	3.79	4.02
	16	4.48	4.04	4.26
	17	3.53	4.02	3.78
	18	4.45	4.58	4.52
	19	5.09	4.67	4.88
	20	5.48	5.45	5.47
	21	5.19	5.32	5.26
	22	3.74	4.85	4.30
	23	3.98	4.39	4.19
	24	2.77	3.70	3.24

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C6 Cholesterol (mg chol/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	5.41	5.08	5.25
	2	5.72	5.93	5.83
	3	7.32	7.97	7.65
	4	7.36	7.11	7.24
	5	6.48	7.02	6.75
	6	6.44	7.53	6.99
	7	8.27	7.04	7.66
	8	6.56	7.36	6.96
	9	6.71	6.22	6.47
	10	5.95	5.98	5.97
F	11	6.63	6.63	6.63
	12	7.58	7.14	7.36
	13	6.67	6.77	6.72
	14	6.93	7.08	7.01
	15	7.01	6.94	6.98
	16	5.99	6.00	6.00
	17	6.61	6.78	6.70
	18	7.28	7.00	7.14
	19	7.36	7.18	7.27
	20	6.70	6.73	6.72
	21	5.65	5.19	5.42
	22	7.82	7.15	7.49
	23	5.96	5.35	5.65
	24	5.86	7.06	6.46

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C7 Conjugated Dienes ($\mu\text{mol/g. liver}$)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	2.92	3.14	3.03
	2	3.70	3.46	3.53
	3	3.69	3.71	3.70
	4	3.41	3.51	3.46
	5	3.55	3.93	3.74
	6	3.57	3.32	3.45
	7	4.04	3.95	4.00
	8	3.03	3.50	3.27
	9	3.09	3.61	3.35
	10	3.45	3.05	3.25
F	11	3.44	3.22	3.33
	12	3.35	3.47	3.41
	13	3.27	3.19	3.23
	14	4.38	4.26	4.32
	15	4.24	4.13	4.19
	16	4.52	4.44	4.48
	17	3.58	3.90	3.74
	18	3.74	3.56	3.64
	19	3.93	3.59	3.76
	20	4.03	3.81	3.92
	21	3.18	3.57	3.38
	22	3.44	3.53	3.49
	23	3.29	3.10	3.20
	24	2.60	2.99	2.80

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C8 Lipid Hydroperoxides (nmol LOOH/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	586	502	544
	2	967	886	927
	3	609	704	657
	4	566	623	595
	5	887	715	801
	6	783	717	750
	7	686	732	709
	8	656	680	668
	9	995	851	923
	10	417	526	472
F	11	550	411	481
	12	748	627	688
	13	470	538	504
	14	319	402	361
	15	340	353	347
	16	358	356	357
	17	470	388	429
	18	331	313	322
	19	409	407	408
	20	362	329	346
	21	354	347	351
	22	333	362	348
	23	548	516	532
	24	601	573	587

Homozygous Mice (db/db) – Individual Values: Males and Females

Appendix C9 TTBARS (nmol/g. liver)

Sex	Animal	Trial 1	Trial 2	Mean
M	1	1426	1517	1472
	2	1810	1755	1783
	3	1517	1481	1499
	4	1682	1554	1618
	5	1316	1206	1261
	6	1828	1719	1774
	7	1481	1463	1472
	8	1572	1444	1508
	9	1499	1481	1490
	10	1719	1700	1710
F	11	1737	1517	1627
	12	1499	1645	1572
	13	1627	1389	1508
	14	1609	1389	1499
	15	1901	1737	1819
	16	1645	1700	1673
	17	1536	1645	1591
	18	1517	1645	1501
	19	1499	1517	1508
	20	1463	1499	1481
	21	1974	2011	1993
	22	2377	2139	2258
	23	2084	1993	2039
	24	2176	2011	2094

Appendix D1 *Impact of Various Oils, Administered via the Diet (4 Weeks) – Individual Values: Males and Females*
Weekly Body Mass (g)

Group / Genotype	Treatment	Animal / Sex	Age (weeks)					Body Mass Gain / Loss (g) 12-17 weeks	
			12	13	14	15	16		17
1 (db/wt)	Control (chow only)	1M	25.0	25.0	26.5	26.5	26.5	27.0	+2.0
		2M	25.5	25.5	24.5	24.5	25.0	26.5	+1.0
		3M	24.5	24.5	25.0	25.5	25.5	26.5	+2.0
		4F	19.0	19.0	19.5	18.5	20.0	20.5	+1.5
		5F	20.0	20.0	20.5	21.0	21.0	21.0	+1.0
		6F	19.0	18.0	18.0	18.5	19.0	19.0	0
2 (db/wt)	Sunflower Oil	7M	23.5	22.5	23.5	23.5	24.5	24.5	+1.0
		8M	24.5	24.0	24.5	23.5	25.0	24.5	0
		9M	25.0	24.5	25.5	25.0	27.0	26.5	+1.5
		10F	19.0	20.0	19.5	19.5	21.5	20.5	+1.5
		11F	24.0	22.0	21.0	22.5	24.0	25.5	+1.5
		12F	18.5	19.0	18.0	19.5	19.0	20.0	+1.5
3 (db/wt)	Canola Oil	13M	24.5	25.5	24.5	25.0	25.5	26.5	+2.0
		14M	28.0	27.5	26.5	26.0	26.5	27.5	-0.5
		15M	26.0	25.0	25.5	25.0	25.5	26.0	0
		16F	23.0	23.5	22.0	23.0	23.5	24.0	+1.0
		17F	19.5	19.0	20.0	20.5	19.5	21.0	+1.5
		18F	18.5	19.0	17.5	18.0	18.0	19.5	+1.0
4 (db/wt)	Sunflower Oil and Cholesterol	19M	28.5	29.5	27.5	29.0	29.0	28.0	-0.5
		20M	29.0	30.0	29.0	30.5	29.0	29.5	+0.5
		21M	27.0	27.5	26.0	29.0	29.0	29.5	+2.5
		22F	19.5	20.0	19.0	20.5	20.5	21.5	+2.0
		23F	19.5	18.0	17.5	19.0	20.0	19.0	-0.5
		24F	19.5	18.0	17.5	18.0	19.5	19.5	0

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females**

Appendix D2 Liver Mass (g)

Group / Genotype	Treatment	Animal	Liver Mass (g)
1 (db/wt)	Control (chow only)	1	1.120
		2	1.031
		3	1.014
		4	0.769
		5	0.812
		6	0.681
2 (db/wt)	Sunflower Oil	7	0.836
		8	0.998
		9	0.977
		10	0.695
		11	1.017
		12	0.782
3 (db/wt)	Canola Oil	13	1.147
		14	1.109
		15	1.097
		16	1.081
		17	0.882
		18	0.832
4 (db/wt)	Sunflower Oil and Cholesterol	19	0.984
		20	1.062
		21	1.004
		22	0.896
		23	0.707
		24	0.679

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females**

Appendix D3 Liver Mass / Body Mass x 100 (%)

Group / Genotype	Treatment	Animal	Liver Mass / Body Mass x 100 (%)
1 (db/wt)	Control (chow only)	1	4.15
		2	3.89
		3	3.83
		4	3.75
		5	3.87
		6	3.58
2 (db/wt)	Sunflower Oil	7	3.41
		8	4.07
		9	3.69
		10	3.39
		11	3.98
		12	3.91
3 (db/wt)	Canola Oil	13	4.33
		14	4.03
		15	4.22
		16	4.50
		17	4.20
		18	4.27
4 (db/wt)	Sunflower Oil and Cholesterol	19	3.51
		20	3.60
		21	3.40
		22	4.17
		23	3.72
		24	3.57

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females**

Appendix D4 Glucose Concentration (mmol/l)

Group / Genotype	Treatment	Animal /Sex	Glucose conc. (mmol/l)
1 (db/wt)	Control (chow only)	1M	6.7
		2M	8.7
		3M	9.2
		4F	4.7
		5F	5.0
		6F	6.5
2 (db/wt)	Sunflower Oil	7M	9.1
		8M	6.8
		9M	7.5
		10F	6.2
		11F	7.8
		12F	8.2
3 (db/wt)	Canola Oil	13M	9.5
		14M	11.4
		15M	8.0
		16F	6.0
		17F	6.1
		18F	6.7
4 (db/wt)	Sunflower Oil and Cholesterol	19M	8.2
		20M	5.2
		21M	5.4
		22F	3.6
		23F	2.6
		24F	2.1

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females
Appendix D5 Triglycerides (mg TG/g. liver)**

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (db/wt)	Control (chow only)	1M	19.67	16.48	18.08
		2M	18.01	16.82	17.42
		3M	18.30	19.06	18.68
		4F	21.87	17.47	19.67
		5F	19.81	20.13	19.97
		6F	30.83	22.96	26.90
2 (db/wt)	Sunflower Oil	7M	20.15	18.22	19.19
		8M	19.32	18.39	18.86
		9M	18.08	19.97	19.03
		10F	29.23	24.61	26.92
		11F	32.30	27.45	29.88
		12F	25.05	21.33	23.19
3 (db/wt)	Canola Oil	13M	21.96	22.08	22.02
		14M	25.72	23.32	24.52
		15M	21.55	26.10	23.83
		16F	26.28	29.14	27.71
		17F	25.59	28.29	26.94
		18F	21.63	21.55	21.59
4 (db/wt)	Sunflower Oil and Cholesterol	19M	23.46	21.76	22.61
		20M	20.89	15.16	18.03
		21M	19.92	22.01	20.97
		22F	24.34	22.28	23.31
		23F	24.05	24.90	24.48
		24F	22.74	24.83	23.79

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females
Cholesterol (mg chol/g. liver)**

Appendix D6

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (db/wt)	Control (chow only)	1M	1.80	2.17	2.04
		2M	1.99	1.90	1.95
		3M	1.94	1.88	1.91
		4F	2.29	2.34	2.32
		5F	2.89	2.10	2.50
		6F	3.23	3.32	3.28
2 (db/wt)	Sunflower Oil	7M	5.21	4.72	4.95
		8M	5.31	5.30	5.31
		9M	2.37	2.42	2.40
		10F	5.30	5.26	5.28
		11F	5.01	4.91	4.96
		12F	4.07	3.76	3.92
3 (db/wt)	Canola Oil	13M	2.69	2.45	2.57
		14M	2.00	2.12	2.06
		15M	2.01	1.98	2.00
		16F	3.63	3.77	3.70
		17F	4.88	4.74	4.81
		18F	5.39	5.45	5.42
4 (db/wt)	Sunflower Oil and Cholesterol	19M	3.55	3.61	3.58
		20M	2.75	3.00	2.88
		21M	3.04	2.26	3.15
		22F	5.85	5.82	5.84
		23F	4.90	4.14	4.52
		24F	4.07	3.82	3.94

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females
Conjugated Dienes ($\mu\text{mol/g. liver}$)**

Appendix D7

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (db/wt)	Control (chow only)	1M	2.26	2.46	2.36
		2M	1.87	1.79	1.83
		3M	2.11	1.95	2.03
		4F	2.00	2.04	2.02
		5F	1.82	1.97	1.90
		6F	2.00	1.70	1.85
2 (db/wt)	Sunflower Oil	7M	2.50	2.55	2.53
		8M	2.56	2.38	2.32
		9M	2.32	2.32	2.32
		10F	3.05	3.30	3.18
		11F	2.62	2.84	2.78
		12F	2.81	2.83	2.82
3 (db/wt)	Canola Oil	13M	2.28	2.40	2.34
		14M	2.38	2.41	2.40
		15M	2.73	2.78	2.76
		16F	2.04	2.07	2.06
		17F	1.85	2.17	2.01
		18F	2.11	1.89	2.00
4 (db/wt)	Sunflower Oil and Cholesterol	19M	2.78	2.02	2.40
		20M	2.41	2.43	2.42
		21M	2.39	2.50	2.45
		22F	2.83	2.20	2.52
		23F	2.81	2.24	2.52
		24F	2.37	2.48	2.43

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females**

Appendix D8 Lipid Hydroperoxides (nmol LOOH/g. liver)

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (db/wt)	Control (chow only)	1M	303	357	330
		2M	277	271	274
		3M	394	372	383
		4F	461	449	455
		5F	399	315	357
		6F	961	955	958
2 (db/wt)	Sunflower Oil	7M	374	386	380
		8M	351	337	344
		9M	305	330	318
		10F	753	827	790
		11F	732	825	789
		12F	699	787	743
3 (db/wt)	Canola Oil	13M	400	452	426
		14M	502	454	483
		15M	388	370	379
		16F	548	498	523
		17F	551	579	565
		18F	623	625	624
4 (db/wt)	Sunflower Oil and Cholesterol	19M	354	357	356
		20M	349	381	365
		21M	349	405	376
		22F	683	726	709
		23F	749	783	766
		24F	372	357	408

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females**

Appendix D9 TTBARs (nmol/g. liver)

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (db/wt)	Control (chow only)	1M	3401	3291	3346
		2M	3273	3474	3374
		3M	3035	3108	3072
		4F	3383	3310	3347
		5F	4168	3583	3876
		6F	4498	3858	4178
2 (db/wt)	Sunflower Oil	7M	3492	3548	3520
		8M	3327	2486	2907
		9M	3346	2687	3017
		10F	2505	2395	2450
		11F	3784	3236	3510
		12F	3437	3831	3634
3 (db/wt)	Canola Oil	13M	4278	4004	4141
		14M	3583	3276	3430
		15M	3035	3602	3319
		16F	2669	2988	2829
		17F	3547	3565	3556
		18F	2834	2377	2606
4 (db/wt)	Sunflower Oil and Cholesterol	19M	2980	3199	3090
		20M	2157	2395	2276
		21M	2541	2852	2697
		22F	3656	3401	3529
		23F	4552	3218	3885
		24F	3876	3620	3748

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females**

Appendix D10 BCA Protein ($\mu\text{g}/\mu\text{g}$. liver)

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (db/wt)	Control (chow only)	1M	72.4	58.8	65.6
		2M	67.0	61.3	64.2
		3M	66.9	62.7	64.8
		4F	101.2	88.7	95.0
		5F	64.1	68.9	66.5
		6F	71.0	72.4	71.7
2 (db/wt)	Sunflower Oil	7M	64.4	67.1	65.8
		8M	74.8	65.9	70.4
		9M	79.6	73.6	76.6
		10F	79.2	72.4	75.8
		11F	73.5	74.0	73.8
		12F	72.8	69.2	71.0
3 (db/wt)	Canola Oil	13M	73.2	76.6	74.9
		14M	61.8	65.0	63.4
		15M	62.8	66.5	64.7
		16F	88.6	90.3	89.5
		17F	89.1	82.9	86.0
		18F	70.0	73.2	71.6
4 (db/wt)	Sunflower Oil and Cholesterol	19M	64.9	67.1	66.0
		20M	77.0	69.5	73.3
		21M	76.6	81.7	79.2
		22F	72.7	75.6	74.2
		23F	71.1	72.6	71.9
		24F	92.2	84.8	88.5

**Impact of Various Oils, Administered via the Diet (4 Weeks) –
Individual Values: Males and Females**

Appendix D11 Animal Cage Assignments

Cage Number	Animal No.s
A	1, 2, 3
B	7, 8, 9
C	13, 14, 15
D	19, 20, 21
E	4, 5, 6
F	10, 11, 12
G	16, 17, 18
H	22, 23, 24

Please note – the animals were allocated on a random basis

Appendix E1 **Study of Short Period of Alcohol Administration (4 Weeks) – Individual Values: Males and Females**
Weekly Body Mass (g)

Group / Genotype	Treatment	Animal / Sex	Age (weeks)							Body Mass Gain / Loss (g) 14-20 weeks
			14	15	16	17	18	19	20	
1 (wt/wt)	H ₂ O Control	1M	17.5	19.5	19.5	18.5	19.5	19.5	20.0	+2.5
		2F	17.0	18.0	18.5	18.5	19.0	18.5	19.0	+2.0
2 (wt/wt)	20% Alcohol	3M	19.5	20.5	21.5	20.5	21.5	21.0	21.5	+2.0
		4F	15.5	16.5	16.0	16.0	16.5	16.5	17.0	+1.5
3 (db/wt)	H ₂ O Control	5M	25.0	26.5	27.0	27.5	28.0	27.5	28.0	+3.0
		6M	28.0	28.5	28.0	29.0	28.5	29.0	29.5	+1.5
		7F	21.5	22.0	23.0	22.0	23.0	22.5	23.5	+2.0
		8F	21.5	20.5	21.5	21.5	21.0	21.5	22.0	+0.5
4 (db/wt)	20% Alcohol	9M	24.0	25.0	26.0	26.0	25.5	25.0	25.5	+1.5
		10M	25.0	25.5	25.5	25.5	24.5	24.5	25	0
		11F	22.0	21.5	21.5	20.5	22.5	22.0	21.5	-0.5
		12F	17.5	18.0	18.0	18.0	18.0	18.0	18.5	+1.0
5 (db/db)	H ₂ O Control	13M	39.0	40.0	41.5	41.0	41.5	42.0	42.5	+3.5
		14M	45.0	45.5	46.0	45.5	46.0	47.0	49.0	+4.0
		15F	28.0	28.5	28.0	26.5	26.0	26.5	27.0	-1.0
		16F	33.5	35.0	35.5	36.0	36.0	35.5	37.0	+3.5
6 (db/db)	20% Alcohol	17M	34.0	36.0	38.0	37.5	38.0	39.0	40.0	+6.0
		18F	35.0	35.5	36.0	35.5	35.5	36.0	36.5	+1.5
		19F	31.5	33.0	34.0	35.0	36.0	36.0	37.0	+5.5
		20F	36.5	38.0	38.5	38.5	39.5	40.0	40.5	+4.0

**Study of Short Period of Alcohol Administration (4 Weeks) –
Individual Values: Males and Females
Triglycerides (mg TG/g. liver)**

Appendix E2

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (wt/wt)	H ₂ O Control	1M	1.77	1.56	1.67
		2F	1.31	1.45	1.38
2 (wt/wt)	20% Alcohol	3M	1.96	1.79	1.88
		4F	1.99	1.61	1.80
3 (db/wt)	H ₂ O Control	5M	0.97	1.08	1.03
		6M	1.13	1.19	1.16
		7F	1.03	1.05	1.04
		8F	0.94	0.99	0.97
4 (db/wt)	20% Alcohol	9M	1.50	1.23	1.37
		10M	1.38	1.25	1.32
		11F	1.15	1.19	1.17
		12F	1.40	1.08	1.24
5 (db/db)	H ₂ O Control	13M	3.07	3.19	3.13
		14M	3.28	3.07	3.18
		15F	2.55	2.84	2.70
		16F	2.86	2.58	2.72
6 (db/db)	20% Alcohol	17M	7.04	6.83	6.94
		18F	6.52	6.89	6.71
		19F	5.88	5.82	5.85
		20F	5.80	6.72	6.26

**Study of Short Period of Alcohol Administration (4 Weeks) –
Individual Values: Males and Females
Cholesterol (mg chol/g. liver)**

Appendix E3

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (wt/wt)	H ₂ O Control	1M	3.65	4.47	4.06
		2F	3.09	2.94	3.02
2 (wt/wt)	20% Alcohol	3M	3.97	4.99	4.48
		4F	4.99	4.16	4.58
3 (db/wt)	H ₂ O Control	5M	3.89	2.94	3.42
		6M	3.26	2.78	3.02
		7F	1.87	2.05	1.96
		8F	2.00	2.13	2.07
4 (db/wt)	20% Alcohol	9M	4.50	4.86	4.68
		10M	4.61	5.63	5.12
		11F	3.39	4.65	4.02
		12F	3.99	4.30	4.15
5 (db/db)	H ₂ O Control	13M	6.52	4.63	5.58
		14M	5.41	5.39	5.40
		15F	4.21	4.10	4.16
		16F	3.04	3.83	3.44
6 (db/db)	20% Alcohol	17M	7.44	6.84	7.14
		18F	4.13	3.38	3.76
		19F	5.05	4.81	4.93
		20F	5.07	4.47	4.77

**Study of Short Period of Alcohol Administration (4 Weeks) –
Individual Values: Males and Females
Conjugated Dienes ($\mu\text{mol/g}$. liver)**

Appendix E4

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (wt/wt)	H ₂ O Control	1M	1.69	1.96	1.83
		2F	1.91	1.66	1.79
2 (wt/wt)	20% Alcohol	3M	2.15	2.09	2.12
		4F	1.81	1.86	1.84
3 (db/wt)	H ₂ O Control	5M	1.77	2.04	1.91
		6M	1.85	2.02	1.94
		7F	1.28	1.24	1.26
		8F	1.75	1.70	1.73
4 (db/wt)	20% Alcohol	9M	2.39	2.48	2.44
		10M	2.41	2.57	2.49
		11F	1.99	1.88	1.94
		12F	2.10	1.57	1.84
5 (db/db)	H ₂ O Control	13M	3.34	3.50	3.42
		14M	3.22	3.47	3.35
		15F	2.29	2.55	2.42
		16F	2.13	2.65	2.39
6 (db/db)	20% Alcohol	17M	5.36	4.85	5.11
		18F	2.21	3.14	2.68
		19F	2.89	3.86	3.38
		20F	2.72	3.87	3.30

**Study of Short Period of Alcohol Administration (4 Weeks) –
Individual Values: Males and Females
Lipid Hydroperoxides (nmol LOOH/g. liver)**

Appendix E5

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (wt/wt)	H ₂ O Control	1M	101	99	100
		2F	62	108	85
2 (wt/wt)	20% Alcohol	3M	277	306	292
		4F	299	280	290
3 (db/wt)	H ₂ O Control	5M	151	178	165
		6M	232	201	217
		7F	157	103	130
		8F	82	154	118
4 (db/wt)	20% Alcohol	9M	200	256	228
		10M	219	220	220
		11F	125	136	131
		12F	142	158	150
5 (db/db)	H ₂ O Control	13M	512	472	492
		14M	711	545	628
		15F	276	311	294
		16F	365	276	321
6 (db/db)	20% Alcohol	17M	1054	999	1027
		18F	656	714	685
		19F	709	480	595
		20F	380	235	308

**Study of Short Period of Alcohol Administration (4 Weeks) –
Individual Values: Males and Females
TTBARS (nmol/g. liver)**

Appendix E6

Group / Genotype	Treatment	Animal / Sex	Trial 1	Trial 2	Mean
1 (wt/wt)	H ₂ O Control	1M	1517	1481	1499
		2F	1481	1481	1481
2 (wt/wt)	20% Alcohol	3M	1572	1481	1527
		4F	1609	1645	1627
3 (db/wt)	H ₂ O Control	5M	1261	1188	1225
		6M	1207	1261	1234
		7F	1060	1097	1079
		8F	1134	1188	1161
4 (db/wt)	20% Alcohol	9M	1261	1335	1298
		10M	1298	1316	1307
		11F	1591	1517	1554
		12F	1335	1280	1308
5 (db/db)	H ₂ O Control	13M	1481	1517	1499
		14M	1426	1225	1326
		15F	1280	1207	1244
		16F	1188	1134	1161
6 (db/db)	20% Alcohol	17M	1901	1810	1856
		18F	1865	1792	1829
		19F	1847	1920	1884
		20F	1810	1847	1829

Appendix F1 **Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males**
Weekly Body Mass (g)

Group / Genotype	Treatment	Animal	Age (Weeks)						
			9	10	11	12	13	14	15
1 (db/wt)	H ₂ O Control	1	25.0	25.0	25.5	26.5	27.0	27.5	29.5
		2	26.0	27.5	28.5	29.5	30.5	30.5	32.0
		3	25.0	27.0	27.5	28.0	29.0	30.0	32.0
		4	26.5	28.0	28.5	29.5	30.5	31.5	33.0
		5	26.0	28.0	29.0	29.5	31.0	32.5	34.5
		6	26.0	26.5	27.5	28.0	28.5	28.5	29.5
		7	22.0	23.5	24.5	26.0	26.5	27.0	28.0
2 (db/wt)	20% Alcohol	8	28.5	29.5	29.0	29.5	30.0	30.0	31.0
		9	27.5	27.5	29.0	29.5	30.0	31.0	30.5
		10	25.5	26.5	27.0	27.5	28.5	28.5	29.5
		11	27.5	27.5	28.0	28.5	30.0	30.0	31.0
		12	26.0	26.5	26.5	27.5	27.0	27.5	28.0
		13	28.5	29.0	29.0	29.5	31.0	31.5	32.0
3 (db/db)	H ₂ O Control	14*	43.0	45.5	45.5	47.0	45.5	41.5	fd
		15	43.0	46.5	46.0	48.0	46.5	45.0	43.0
		16	47.0	50.5	50.5	53.0	54.5	54.0	54.0
		17	39.0	44.0	46.0	49.5	50.0	50.0	51.5
		18	47.5	49.0	50.5	53.0	53.5	55.0	55.5
		19	47.0	48.0	48.0	49.5	50.0	51.0	50.5
		20	44.0	44.5	46.5	48.0	47.5	48.5	49.5
4 (db/db)	20% Alcohol	21	41.5	44.5	45.5	48.0	48.0	51.0	53.0
		22	50.0	52.5	53.5	53.5	53.5	53.0	52.0
		23	42.0	43.0	44.0	44.0	43.0	42.0	41.0
		24	41.0	43.5	45.0	46.0	46.5	46.5	47.0
		25	47.0	50.5	52.0	53.0	54.0	55.5	57.0

* = Animal No. 14 was found dead aged 15 weeks

Appendix F1 *Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males*
(continued) *Weekly Body Mass (g)*

Group / Genotype	Treatment	Animal	Age (Weeks)					Body Weight Mass / Loss (g) 9-20 weeks
			16	17	18	19	20	
1 (db/wt)	H ₂ O Control	1	29.0	29.5	29.5	30.0	29.5	+4.5
		2	31.5	32.5	36.0	33.0	31.5	+5.5
		3	32.0	33.0	33.5	34.0	34.0	+9.0
		4	33.0	33.5	33.5	34.0	34.5	+8.0
		5	34.0	35.5	35.5	36.0	35.5	+9.5
		6	29.0	30.0	30.0	30.5	30.0	+4.0
		7	27.5	28.0	28.0	29.0	29.0	+7.0
2 (db/wt)	20% Alcohol	8	30.5	31.5	31.5	31.0	33.0	+4.5
		9	31.0	30.5	31.0	30.5	32.0	+4.5
		10	29.5	30.0	30.0	30.0	31.0	+5.5
		11	31.0	31.5	31.5	31.5	32.5	+5.0
		12	29.0	29.5	30.0	30.0	30.0	+4.0
		13	31.5	32.5	32.0	32.5	32.5	+4.0
3 (db/db)	H ₂ O Control	14*	-	-	-	-	-	-
		15	45.0	45.0	45.0	43.0	45.0	+2.0
		16	57.0	58.0	59.0	56.0	57.0	+10.0
		17	54.5	55.0	55.5	55.0	56.5	+17.5
		18	56.5	56.5	56.5	55.0	56.0	+8.5
		19	50.5	49.0	50.0	48.0	47.5	+0.5
		20	50.0	49.0	48.5	48.0	49.0	+5.0
4 (db/db)	20% Alcohol	21	54.0	55.0	56.5	56.5	55.5	+14.0
		22	50.5	51.0	52.0	51.0	50.0	0
		23	40.5	38.5	38.0	37.5	36.5	-5.5
		24	47.5	47.0	47.5	46.5	46.5	+5.5
		25	57.0	58.0	58.5	59.5	59.0	+12.0

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males

Appendix F2 Liver Mass (g)

Group / Genotype	Treatment	Animal	Liver Mass (g)
1 (db/wt)	H ₂ O Control	1	1.0213
		2	0.9649
		3	1.0617
		4	1.0932
		5	1.1143
		6	1.0912
		7	1.0716
2 (db/wt)	20% Alcohol	8	1.3854
		9	1.4263
		10	1.3767
		11	1.6781
		12	1.4904
		13	1.3683
3 (db/db)	H ₂ O Control	14*	-
		15	1.9390
		16	2.6287
		17	2.6939
		18	3.6215
		19	3.2134
		20	2.8890
4 (db/db)	20% Alcohol	21	2.8804
		22	2.9219
		23	2.4681
		24	2.4433
		25	3.4698

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F4 Glucose Concentration (mmol/l)

Group / Genotype	Treatment	Animal	Glucose conc. (mmol/l)
1 (db/wt)	H ₂ O Control	1	10.1
		2	13.2
		3	12.3
		4	15.1
		5	19.1
		6	9.9
		7	16.8
2 (db/wt)	20% Alcohol	8	8.9
		9	10.2
		10	9.1
		11	9.8
		12	6.0
		13	7.6
3 (db/db)	H ₂ O Control	14*	-
		15	30.9
		16	28.5
		17	32.9
		18	x
		19	32.6
		20	x
4 (db/db)	20% Alcohol	21	20.1
		22	32.8
		23	31.0
		24	x
		25	32.7

* = Animal No. 14 was found dead aged 15 weeks

x = Animal No.'s 18, 20 and 24 glucose concentration was to high to record

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F5 Blood/Alcohol Levels (mmol/l)

Group / Genotype	Treatment	Animal	Blood/Alcohol Levels (mmol/l)
1 (db/wt)	H ₂ O Control	1	0.40
		2	0.00
		3	1.20
		4	0.00
		5	0.70
		6	0.44
		7	0.99
2 (db/wt)	20% Alcohol	8	1.51
		9	1.56
		10	1.05
		11	0.42
		12	2.81
		13	0.64
3 (db/db)	H ₂ O Control	14*	-
		15	0.65
		16	1.32
		17	1.08
		18	0.37
		19	1.37
		20	1.94
4 (db/db)	20% Alcohol	21	2.56
		22	3.44
		23	15.33
		24	1.14
		25	1.19

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F6 Aspartic Transaminase (AST) Levels (U/l)

Group / Genotype	Treatment	Animal	AST levels (U/l)
1 (db/wt)	H ₂ O Control	1	27
		2	38
		3	20
		4	33
		5	13
		6	30
		7	67
2 (db/wt)	20% Alcohol	8	43
		9	55
		10	35
		11	25
		12	52
3 (db/db)	H ₂ O Control	13	40
		14*	-
		15	33
		16	92
		17	52
		18	63
		19	40
4 (db/db)	20% Alcohol	20	43
		21	57
		22	32
		23	148
		24	70
		25	82

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F7 Alanine Transaminase (ALT) Levels (U/l)

Group / Genotype	Treatment	Animal	ALT levels (U/l)
1 (db/wt)	H ₂ O Control	1	35
		2	5
		3	12
		4	15
		5	28
		6	12
		7	23
2 (db/wt)	20% Alcohol	8	25
		9	33
		10	27
		11	22
		12	17
3 (db/db)	H ₂ O Control	13	10
		14*	-
		15	38
		16	135
		17	33
		18	115
		19	43
4 (db/db)	20% Alcohol	20	28
		21	60
		22	58
		23	127
		24	37
		25	73

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F8 Triglycerides (mg TG/g. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	6.03	5.71	5.87
		2	4.43	4.54	4.49
		3	3.06	5.31	4.19
		4	5.64	4.16	4.90
		5	3.44	1.40	2.42
		6	1.29	1.07	1.18
		7	4.71	5.20	4.96
2 (db/wt)	20% Alcohol	8	5.53	7.07	6.30
		9	5.26	6.30	5.78
		10	7.13	5.04	6.09
		11	6.58	7.24	6.91
		12	7.62	8.34	7.98
		13	6.74	6.41	6.58
3 (db/db)	H ₂ O Control	14*	-	-	-
		15	4.49	3.99	4.24
		16	6.63	5.86	6.25
		17	6.74	5.81	6.28
		18	7.84	8.34	8.09
		19	7.29	8.68	7.99
		20	6.41	6.52	6.47
4 (db/db)	20% Alcohol	21	6.41	7.07	6.74
		22	10.54	11.86	11.20
		23	10.21	11.47	10.84
		24	10.54	9.49	10.02
		25	12.30	13.35	12.83

* = Animal No. 14 was found dead aged 15 weeks

Appendix F9 *Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males*
Cholesterol (mg chol/g. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	4.85	4.47	4.66
		2	5.08	2.06	3.57
		3	2.70	4.17	3.46
		4	4.14	3.23	3.69
		5	2.82	2.14	2.48
		6	1.81	1.71	1.76
		7	3.64	3.99	3.82
2 (db/wt)	20% Alcohol	8	3.84	3.48	3.66
		9	5.01	4.63	4.82
		10	4.93	4.37	4.62
		11	5.08	6.35	5.72
		12	6.25	6.35	6.30
		13	5.06	4.93	5.00
3 (db/db)	H ₂ O Control	14*	-	-	-
		15	5.26	5.95	5.61
		16	8.21	6.71	7.46
		17	5.46	4.85	5.16
		18	4.52	4.96	4.74
		19	7.01	6.56	6.79
		20	5.69	5.74	5.72
4 (db/db)	20% Alcohol	21	7.16	7.29	7.23
		22	7.34	8.16	7.75
		23	7.04	8.26	7.65
		24	8.82	8.16	8.49
		25	10.89	11.43	11.16

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F10 Conjugated Dienes ($\mu\text{mol/g. liver}$)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	2.06	2.20	2.13
		2	1.61	1.39	1.50
		3	1.54	1.63	1.59
		4	1.31	1.46	1.39
		5	2.09	1.71	1.90
		6	1.23	1.17	1.20
		7	1.39	1.63	1.51
2 (db/wt)	20% Alcohol	8	2.16	2.14	2.15
		9	1.83	1.88	1.86
		10	1.71	2.01	1.86
		11	1.61	1.85	1.73
		12	1.87	2.25	2.04
		13	2.26	2.45	2.36
3 (db/db)	H ₂ O Control	14*	-	-	-
		15	2.85	2.74	2.80
		16	2.61	2.60	2.61
		17	2.65	2.68	2.67
		18	2.75	2.48	2.62
		19	2.65	2.54	2.60
		20	2.60	2.76	2.68
4 (db/db)	20% Alcohol	21	3.11	3.38	3.25
		22	3.83	3.57	3.70
		23	3.43	3.38	3.41
		24	3.44	3.51	3.48
		25	3.55	3.25	3.40

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F11 Lipid Hydroperoxides (nmol LOOH/g. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	205	310	258
		2	293	298	296
		3	399	276	338
		4	382	407	395
		5	394	441	416
		6	331	335	333
		7	352	323	338
2 (db/wt)	20% Alcohol	8	614	875	745
		9	542	681	612
		10	706	803	755
		11	824	803	814
		12	512	1165	839
		13	542	609	576
3 (db/db)	H ₂ O Control	14*	-	-	-
		15	816	1047	932
		16	1149	1191	1170
		17	1057	951	1004
		18	989	1141	1065
		19	1068	1157	1113
		20	1250	1056	1153
4 (db/db)	20% Alcohol	21	1473	1326	1400
		22	1178	1506	1342
		23	1776	1350	1563
		24	1587	1679	1633
		25	1772	1216	1494

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F12 TTBARs (nmol/g. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	1810	1627	1719
		2	1956	1316	1636
		3	1700	1938	1919
		4	2084	1846	1965
		5	1883	1335	1609
		6	1901	2011	1956
		7	1627	1627	1627
2 (db/wt)	20% Alcohol	8	2870	2486	2678
		9	2066	1974	2020
		10	2596	2962	2779
		11	2925	2304	2615
		12	2541	2614	2578
		13	2834	2176	2505
3 (db/db)	H ₂ O Control	14*	-	-	-
		15	1682	1499	1591
		16	2212	1755	1984
		17	2322	1920	2121
		18	1810	1737	1774
		19	1773	1481	1627
		20	2120	2029	2075
4 (db/db)	20% Alcohol	21	3126	2632	2879
		22	2888	3071	2980
		23	3437	3218	3328
		24	3034	2980	3007
		25	2541	2687	2614

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F13 BCA Protein Assay ($\mu\text{g}/\mu\text{g}$. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Protein ($\mu\text{g}/\mu\text{g}$. liver)
1 (db/wt)	H ₂ O Control	1	58.7	59.5	59.1
		2	49.4	46.5	47.9
		3	50.8	53.0	51.9
		4	69.2	67.1	68.2
		5	45.4	30.9	38.2
		6	55.9	55.9	55.9
		7	66.9	65.5	66.2
2 (db/wt)	20% Alcohol	8	57.3	60.5	58.9
		9	64.8	64.4	64.6
		10	65.3	65.2	65.3
		11	63.8	57.7	60.8
		12	61.2	55.8	58.5
		13	53.1	59.5	56.3
3 (db/db)	H ₂ O Control	14*	-	-	-
		15	60.5	61.1	60.8
		16	79.1	80.2	79.6
		17	84.4	74.8	79.6
		18	52.8	50.3	51.5
		19	62.9	64.9	63.9
		20	80.5	81.7	81.1
		21	86.9	81.2	84.4
4 (db/db)	20% Alcohol	22	87.2	86.8	87.0
		23	86.1	83.7	84.9
		24	62.9	71.2	67.0
		25	57.7	62.9	60.3

* = Animal No. 14 was found dead aged 15 weeks

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F14 Fat Change and Inflammation Grading

Group / Genotype	Treatment	Animal	Fat Change (0-3)	Inflammatory Aggregates [†]
1 (db/wt)	H ₂ O Control	1 [^]	0	0
		2 [^]	0	0
		3 [^]	0	0
		4 [^]	0	0
		5 [^]	0	0
		6 [^]	0	0
		7 [^]	0	0
2 (db/wt)	20% Alcohol	8	0	0
		9	0	0
		10	0	0
		11	1	0
		12	0	0
		13	0	0
3 (db/db)	H ₂ O Control	14 ^{*^}	x	x
		15 [^]	2	0
		16 [^]	3	0
		17	3	2
		18	2	4
		19	2	0
		20	2	2
4 (db/db)	20% Alcohol	21	2	8
		22	3	6
		23	3	3
		24	3	9
		25	3	4

* = Animal No. 14 was found dead aged 15 weeks

+ = 20 x objective, 20 fields counted

[^] = Artefact was present in these sections due to a technical problem.

Assessment predominantly occurred in the periphery sections and is hopefully representative of the tissue as a whole.

x = The quantity of artefact was too severe for this animal to be fairly assessed.

Impact of Alcohol Administered over 8 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix F15 Animal Cage Assignments

Cage Number	Animal No.s
A	1, 2, 3, 4, 5
B	8, 9, 10
C	14, 15, 16, 17
D	21, 22, 23, 24, 25
E	6, 7
F	11, 12, 13
G	18, 19, 20

Please note – the animals were allocated on a random basis

Appendix G1 **Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males**
Weekly Body Mass (g)

Group / Genotype	Treatment	Animal	Age (Weeks)					
			9	10	11	12	13	14
1 (db/wt)	H ₂ O Control	1	26.0	26.0	27.0	28.0	29.5	29.5
		2	27.0	28.0	29.5	29.5	32.0	32.0
		3	27.0	28.5	28.5	29.5	30.5	32.0
		4	27.0	28.0	28.5	29.5	30.5	31.5
		5	28.5	30.0	30.5	31.5	32.5	33.5
		6	27.0	28.0	29.0	29.0	31.0	31.0
		7	26.5	28.5	28.5	29.0	30.5	30.5
2 (db/wt)	20% Alcohol	8	28.5	28.0	28.5	28.0	29.5	30.0
		9	28.0	27.0	28.5	27.5	27.5	28.5
		10*	27.0	26.0	26.5	26.0	27.5	28.0
		11*	27.5	28.5	29.0	28.5	28.0	28.0
		12	27.0	28.5	29.0	29.5	30.5	31.0
		13*	27.5	28.5	29.0	29.0	30.0	30.0
		14	27.5	28.5	29.0	29.0	30.0	30.0
3 (db/db)	H ₂ O Control	15	46.0	45.5	46.5	53.0	53.5	56.0
		16	45.5	45.0	46.5	52.5	52.5	55.5
		17	46.0	46.0	48.0	54.0	54.5	56.5
		18*	46.0	46.0	46.5	51.5	52.5	53.0
		19	44.0	44.0	45.5	50.5	52.0	54.0
		20	42.5	44.5	44.5	46.0	48.5	50.0
		21	45.5	46.5	46.0	47.0	49.0	49.5
4 (db/db)	20% Alcohol	22	41.5	46.0	47.5	50.0	52.5	48.5
		23*	41.0	44.0	46.0	47.0	47.0	fd
		24	42.5	46.5	48.5	50.0	50.0	47.0
		25	45.5	47.5	47.5	51.0	53.0	54.5
		26	44.5	47.0	47.0	50.5	51.5	52.0
		27	43.5	45.0	45.5	48.5	49.5	50.5
		28	45.5	48.0	47.5	51.5	53.0	53.5

* = Animal No. 18 was found dead aged 21 weeks

* = Animal No. 23 was found dead aged 14 weeks

* = Animal No's 10, 11 and 13 were found dead aged 29 weeks

Appendix G1 Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
(continued) Weekly Body Mass (g)

Group / Genotype	Treatment	Animal	Age (Weeks)					
			15	16	17	18	19	20
1 (db/wt)	H ₂ O Control	1	29.5	30.5	31.0	31.0	30.5	29.5
		2	31.5	32.5	32.5	33.0	33.5	32.0
		3	34.5	34.0	35.5	35.0	35.5	36.5
		4	33.0	32.5	33.0	33.5	33.5	33.5
		5	35.5	35.5	36.5	37.0	37.0	37.5
		6	32.0	31.5	32.0	32.5	32.5	32.5
		7	31.5	32.0	31.5	32.5	32.0	32.5
2 (db/wt)	20% Alcohol	8	30.0	31.0	31.0	31.0	29.0	30.0
		9	29.5	30.0	31.0	30.0	30.0	29.5
		10*	28.5	29.0	28.0	29.0	29.5	29.5
		11*	29.0	29.0	28.5	28.5	29.0	28.5
		12	31.0	31.5	30.5	31.5	31.5	30.5
		13*	31.0	30.5	30.5	31.0	30.5	30.5
		14	31.0	31.5	31.0	31.5	31.0	29.5
3 (db/db)	H ₂ O Control	15	58.0	59.0	58.5	60.5	61.0	60.5
		16	57.5	59.0	58.0	59.5	60.0	60.0
		17	59.0	60.5	60.0	62.0	62.5	62.0
		18*	52.5	55.0	53.5	54.5	56.0	52.5
		19	55.5	56.5	55.5	56.5	56.5	57.0
		20	50.5	52.0	51.5	52.5	53.5	52.5
		21	50.5	51.5	50.5	52.0	52.5	51.5
4 (db/db)	20% Alcohol	22	51.0	51.0	52.5	53.5	54.0	53.5
		23*	-	-	-	-	-	-
		24	48.0	48.0	47.0	47.0	45.0	45.5
		25	55.0	54.0	55.0	55.5	54.5	54.0
		26	53.0	52.5	52.5	53.5	52.5	54.0
		27	50.5	49.0	49.0	49.0	50.5	51.0
		28	53.5	53.5	53.0	52.5	52.0	53.0

* = Animal No. 18 was found dead aged 21 weeks

* = Animal No. 23 was found dead aged 14 weeks

* = Animal No's 10, 11 and 13 were found dead aged 29 weeks

Appendix G1 Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
(continued) Weekly Body Mass (g)

Group / Genotype	Treatment	Animal	Age (Weeks)					
			21	22	23	24	25	26
1 (db/wt)	H ₂ O Control	1	30.5	29.5	30.0	29.0	30.0	30.0
		2	32.0	31.5	32.5	32.0	32.0	32.5
		3	35.5	35.5	37.0	36.5	36.5	37.5
		4	34.0	33.5	35.0	34.5	34.5	35.0
		5	37.5	37.0	37.5	37.0	37.5	38.0
		6	32.5	32.0	33.0	32.0	32.0	32.5
		7	32.0	31.5	32.0	32.0	31.5	32.0
2 (db/wt)	20% Alcohol	8	29.0	30.0	31.5	32.0	33.0	33.5
		9	28.0	27.5	30.0	30.0	31.0	31.0
		10*	30.5	30.0	30.0	31.0	31.0	32.0
		11*	30.0	29.5	30.0	29.0	29.5	31.0
		12	31.0	30.0	31.5	31.0	31.0	31.5
		13*	31.0	29.5	30.5	29.5	30.0	31.0
		14	31.5	31.0	31.5	31.0	31.5	31.5
3 (db/db)	H ₂ O Control	15	58.0	59.0	59.5	56.5	59.0	60.5
		16	58.5	59.5	59.5	58.0	60.0	61.0
		17	61.0	61.0	62.5	60.5	62.0	64.5
		18*	fd	-	-	-	-	-
		19	54.5	55.0	55.0	51.0	53.5	55.5
		20	52.5	52.5	52.0	51.0	53.0	53.5
		21	51.5	50.5	49.5	50.0	51.5	52.5
4 (db/db)	20% Alcohol	22	53.5	53.5	53.5	54.0	54.0	54.5
		23*	-	-	-	-	-	-
		24	45.5	44.5	43.5	43.5	43.5	42.5
		25	52.5	52.0	51.0	50.0	52.0	53.0
		26	53.0	52.0	51.5	51.0	52.5	53.5
		27	51.5	51.5	52.5	50.0	53.0	54.5
		28	52.0	51.5	50.5	51.0	52.0	53.0

* = Animal No. 18 was found dead aged 21 weeks

* = Animal No. 23 was found dead aged 14 weeks

* = Animal No's 10, 11 and 13 were found dead aged 29 weeks

Appendix G1 Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
(continued) Weekly Body Mass (g)

Group / Genotype	Treatment	Animal	Age (Weeks)						Body Mass Gain / Loss (g) 9 – 32 Weeks
			27	28	29	30	31	32	
1 (db/wt)	H ₂ O Control	1	30.5	30.0	31.0	32.0	29.0	30.5	+ 4.5
		2	31.0	31.5	33.5	34.0	31.5	32.0	+ 5.0
		3	36.5	37.0	37.5	37.0	38.0	37.0	+ 10.0
		4	35.0	35.5	35.5	35.0	34.5	33.5	+ 6.5
		5	37.5	38.0	37.5	37.0	38.0	37.5	+ 9.0
		6	32.0	32.0	32.0	32.5	33.5	32.5	+ 5.5
		7	32.0	31.0	31.5	32.5	32.5	32.0	+ 5.5
2 (db/wt)	20% Alcohol	8	34.0	34.0	33.0	34.0	34.5	34.0	+ 5.5
		9	30.5	30.5	30.5	30.5	31.0	31.0	+ 3.0
		10*	31.0	31.0	-	-	-	-	-
		11*	30.0	30.5	-	-	-	-	-
		12	32.0	32.5	30.5	30.5	29.5	29.0	+ 2.0
		13*	31.0	30.5	-	-	-	-	-
3 (db/db)	H ₂ O Control	14	32.5	31.5	29.5	31.5	31.0	31.0	+ 3.5
		15	59.5	60.5	60.5	60.5	60.5	60.5	+ 14.5
		16	61.0	62.0	63.5	62.0	61.0	62.0	+ 16.5
		17	64.0	64.5	65.5	64.0	62.0	64.5	+ 18.5
		18*	-	-	-	-	-	-	-
		19	55.5	55.5	56.5	53.5	53.0	53.0	+ 9.0
		20	53.5	54.5	55.0	55.5	54.0	52.5	+ 10.0
4 (db/db)	20% Alcohol	21	52.5	54.5	53.0	54.5	54.0	52.0	+ 6.5
		22	55.0	56.5	56.0	56.0	55.5	55.5	+ 14.0
		23*	-	-	-	-	-	-	-
		24	42.0	43.0	42.0	41.0	40.0	39.0	- 3.5
		25	52.5	52.5	55.0	55.0	53.5	52.0	+ 6.5
		26	54.5	55.0	57.0	55.0	53.5	52.5	+ 8.0
		27	54.0	55.5	56.5	55.5	54.0	51.5	+ 8.0
28	53.0	54.0	54.5	53.5	51.5	50.5	+ 5.0		

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males

Appendix G2 Liver Mass (g)

Group / Genotype	Treatment	Animal	Liver Mass (g)
1 (db/wt)	H ₂ O Control	1	1.3364
		2	1.4096
		3	1.3206
		4	1.4007
		5	1.4060
		6	1.4173
		7	1.3154
2 (db/wt)	20% Alcohol	8	1.5816
		9	1.6246
		10*	-
		11*	-
		12	1.3716
		13*	-
		14	1.5659
3 (db/db)	H ₂ O Control	15	3.4396
		16	2.8176
		17	3.1934
		18*	-
		19	2.9237
		20	2.3980
		21	2.2932
4 (db/db)	20% Alcohol	22	3.1161
		23*	-
		24	2.8193
		25	3.6237
		26	3.0893
		27	2.4968
		28	2.8072

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G3 Liver Mass / Body Mass x 100 (%)

Group / Genotype	Treatment	Animal	Liver Mass / Body Mass x 100 (%)
1 (db/wt)	H ₂ O Control	1	4.38
		2	4.41
		3	3.57
		4	4.18
		5	3.75
		6	4.36
		7	4.22
2 (db/wt)	20% Alcohol	8	4.65
		9	5.24
		10*	-
		11*	-
		12	4.73
		13*	-
		14	5.05
3 (db/db)	H ₂ O Control	15	5.69
		16	4.54
		17	4.95
		18*	-
		19	5.52
		20	4.57
		21	4.41
4 (db/db)	20% Alcohol	22	5.61
		23*	-
		24	7.21
		25	6.97
		26	5.88
		27	4.85
		28	5.56

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males

Appendix G4 Glucose Concentration (mmol/l)

Group / Genotype	Treatment	Animal	Glucose conc. (mmol/l)
1 (db/wt)	H ₂ O Control	1	9.7
		2	9.6
		3	13.0
		4	18.9
		5	12.0
		6	16.7
		7	15.2
2 (db/wt)	20% Alcohol	8	19.1
		9	13.6
		10*	-
		11*	-
		12	7.5
		13*	-
		14	9.0
3 (db/db)	H ₂ O Control	15	30.9
		16	31.7
		17	32.1
		18*	-
		19	31.3
		20	32.2
		21	30.0
4 (db/db)	20% Alcohol	22	28.5
		23*	-
		24	20.1
		25	32.1
		26	31.8
		27	33.2
		28	29.2

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G5 Blood/Alcohol Levels (mmol/l)

Group / Genotype	Treatment	Animal	Blood/Alcohol Levels (mmol/l)
1 (db/wt)	H ₂ O Control	1	0.00
		2	1.08
		3	0.99
		4	0.72
		5	0.00
		6	0.51
		7	1.17
2 (db/wt)	20% Alcohol	8	1.72
		9	20.89
		10*	-
		11*	-
		12	19.32
		13*	-
		14	8.68
3 (db/db)	H ₂ O Control	15	0.63
		16	0.63
		17	0.03
		18*	-
		19	0.56
		20	0.74
		21	0.77
4 (db/db)	20% Alcohol	22	23.05
		23*	-
		24	0.96
		25	0.87
		26	0.91
		27	0.33
		28	0.85

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**Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Aspartic Transaminase (AST) Levels (U/l)**

Appendix G6

Group / Genotype	Treatment	Animal	AST Levels (U/l)
1 (db/wt)	H ₂ O Control	1	17
		2	29
		3	36
		4	14
		5	12
		6	31
		7	24
2 (db/wt)	20% Alcohol	8	26
		9	39
		10*	-
		11*	-
		12	36
		13*	-
		14	49
3 (db/db)	H ₂ O Control	15	51
		16	37
		17	34
		18*	-
		19	31
		20	46
		21	25
4 (db/db)	20% Alcohol	22	103
		23*	-
		24	20
		25	34
		26	44
		27	26
		28	34

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G7 Alanine Transaminase (ALT) Levels (U/l)

Group / Genotype	Treatment	Animal	ALT Levels (U/l)
1 (db/wt)	H ₂ O Control	1	19
		2	31
		3	10
		4	12
		5	9
		6	15
		7	10
2 (db/wt)	20% Alcohol	8	7
		9	26
		10*	-
		11*	-
		12	19
		13*	-
		14	15
3 (db/db)	H ₂ O Control	15	134
		16	105
		17	60
		18*	-
		19	29
		20	24
		21	39
4 (db/db)	20% Alcohol	22	113
		23*	-
		24	68
		25	60
		26	51
		27	54
		28	112

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**Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Triglycerides (mg TG/g. liver)**

Appendix G8

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	3.19	3.67	3.43
		2	2.45	4.04	3.25
		3	5.01	4.26	4.64
		4	3.33	3.92	3.63
		5	4.80	3.31	4.06
		6	3.39	4.57	3.98
		7	2.94	3.18	3.07
2 (db/wt)	20% Alcohol	8	5.01	4.83	4.92
		9	6.12	6.39	6.23
		10*	-	-	-
		11*	-	-	-
		12	3.77	6.48	5.13
		13*	-	-	-
		14	5.56	4.70	5.13
3 (db/db)	H ₂ O Control	15	3.29	4.82	4.06
		16	4.17	5.96	5.07
		17	5.34	7.05	6.20
		18*	-	-	-
		19	6.80	7.49	7.15
		20	4.89	7.08	5.99
		21	5.13	6.81	5.97
4 (db/db)	20% Alcohol	22	8.43	5.90	7.17
		23*	-	-	-
		24	6.66	7.78	7.22
		25	9.02	8.12	8.57
		26	7.54	7.54	7.54
		27	8.62	7.85	8.24
		28	6.09	8.41	7.25

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males

Appendix G9 Cholesterol (mg chol/g. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	3.08	4.80	3.94
		2	2.72	3.48	3.10
		3	2.03	4.80	3.42
		4	3.32	5.82	4.57
		5	3.92	4.64	4.28
		6	3.28	4.20	3.74
		7	3.96	4.20	4.08
2 (db/wt)	20% Alcohol	8	5.17	5.73	5.45
		9	5.09	5.21	5.15
		10*	-	-	-
		11*	-	-	-
		12	3.72	6.57	5.15
		13*	-	-	-
		14	7.62	5.49	6.56
3 (db/db)	H ₂ O Control	15	5.57	3.56	4.57
		16	7.62	5.13	6.38
		17	2.55	7.05	4.80
		18*	-	-	-
		19	10.07	6.17	8.12
		20	5.97	6.69	6.33
		21	6.49	6.81	6.65
4 (db/db)	20% Alcohol	22	10.11	12.68	11.40
		23*	-	-	-
		24	7.86	7.33	7.60
		25	6.29	9.02	7.66
		26	10.31	7.78	9.05
		27	9.42	7.82	8.62
		28	10.75	10.67	10.71

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G10 Conjugated Dienes ($\mu\text{mol/g}$. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	1.54	1.58	1.56
		2	1.65	1.47	1.56
		3	1.69	1.63	1.66
		4	1.70	1.73	1.72
		5	1.78	1.73	1.76
		6	1.90	1.87	1.89
		7	1.48	1.17	1.33
2 (db/wt)	20% Alcohol	8	2.70	2.43	2.57
		9	2.04	2.10	2.07
		10*	-	-	-
		11*	-	-	-
		12	2.26	2.20	2.23
		13*	-	-	-
		14	1.82	1.99	1.90
3 (db/db)	H ₂ O Control	15	2.52	2.39	2.46
		16	2.39	2.00	2.20
		17	2.23	2.20	2.21
		18*	-	-	-
		19	2.09	2.11	2.10
		20	1.97	2.11	2.04
		21	2.31	2.28	2.30
4 (db/db)	20% Alcohol	22	2.66	2.36	2.51
		23*	-	-	-
		24	2.40	2.46	2.43
		25	2.47	2.59	2.53
		26	2.45	2.45	2.45
		27	2.78	2.68	2.73
		28	2.86	2.81	2.84

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G11 Lipid Hydroperoxides (nmol LOOH/g. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	40	225	133
		2	244	84	164
		3	288	327	308
		4	230	199	215
		5	212	86	149
		6	212	235	224
		7	124	226	175
2 (db/wt)	20% Alcohol	8	478	549	514
		9	354	416	385
		10*	-	-	-
		11*	-	-	-
		12	531	239	385
		13*	-	-	-
		14	651	399	525
3 (db/db)	H ₂ O Control	15	571	761	666
		16	571	730	651
		17	744	951	848
		18*	-	-	-
		19	655	898	777
		20	934	823	879
		21	1053	770	912
4 (db/db)	20% Alcohol	22	792	920	856
		23*	-	-	-
		24	1141	987	1064
		25	1026	1371	1199
		26	969	881	925
		27	898	991	945
		28	938	1053	996

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males

Appendix G12 TTBARs (nmol/g. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	1589	1407	1498
		2	1535	1498	1517
		3	1754	1151	1453
		4	968	1352	1160
		5	1845	2119	1982
		6	1936	2028	1982
		7	1845	1608	1727
2 (db/wt)	20% Alcohol	8	2503	2923	2713
		9	3288	3343	3316
		10*	-	-	-
		11*	-	-	-
		12	2338	2667	2503
		13*	-	-	-
		14	2503	2959	2731
3 (db/db)	H ₂ O Control	15	2064	3690	2877
		16	3160	2393	2777
		17	3380	3051	3216
		18*	-	-	-
		19	2722	2905	2814
		20	2338	3252	2795
		21	2740	2996	2868
4 (db/db)	20% Alcohol	22	2905	3361	3133
		23*	-	-	-
		24	3964	3708	3836
		25	4403	2868	3636
		26	2722	3124	2923
		27	3142	4019	3581
		28	2594	3215	2905

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G13 BCA Protein Assay ($\mu\text{g}/\mu\text{g}$. liver)

Group / Genotype	Treatment	Animal	Trial 1	Trial 2	Mean
1 (db/wt)	H ₂ O Control	1	49.9	48.9	49.5
		2	51.6	69.9	60.8
		3	70.5	70.7	70.6
		4	67.7	60.0	63.8
		5	54.6	48.0	51.3
		6	49.6	53.0	51.3
		7	59.4	66.4	63.9
2 (db/wt)	20% Alcohol	8	61.6	63.7	62.7
		9	69.3	74.7	71.2
		10*	-	-	-
		11*	-	-	-
		12	63.2	70.3	66.7
		13*	-	-	-
		14	60.4	55.4	57.9
3 (db/db)	H ₂ O Control	15	66.3	74.5	70.5
		16	99.7	91.2	95.5
		17	96.8	93.5	95.1
		18*	-	-	-
		19	84.8	81.9	83.4
		20	76.1	79.5	77.8
		21	74.6	70.9	72.7
4 (db/db)	20% Alcohol	22	85.7	86.3	86.0
		23*	-	-	-
		24	93.1	91.1	92.1
		25	98.6	97.8	98.2
		26	88.1	87.9	88.0
		27	94.6	89.7	92.1
		28	78.9	70.1	74.5

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Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G14 Fat Change and Inflammation Grading

Group / Genotype	Treatment	Animal	Fat Change (0-3)	Inflammatory Aggregates ⁺
1 (db/wt)	H ₂ O Control	1	0	4
		2	0	0
		3	0	5
		4	0	3
		5	0	0
		6	0	8
		7	0	0
2 (db/wt)	20% Alcohol	8	0	0
		9	1	2
		10*	-	-
		11*	-	-
		12	0	3
		13*	-	-
		14	0	0
3 (db/db)	H ₂ O Control	15	2	4
		16	3	2
		17	2	5
		18*	-	-
		19	2	4
		20	1	5
		21	1	1
4 (db/db)	20% Alcohol	22	2	4
		23*	-	-
		24	2	0
		25	2	1
		26	2	0
		27	2	1
		28	2	3

* = Animal No. 18 was found dead aged 21 weeks

* = Animal No. 23 was found dead aged 14 weeks

* = Animal No's 10, 11 and 13 were found dead aged 29 weeks

+ = 20 x objective, 20 fields counted

Impact of Alcohol Administered over 20 Weeks in db/wt and db/db Mice – Individual Values: Males
Appendix G15 Animal Cage Assignments

Cage Number	Animal No.s
A	1, 2
B	8, 9
C	15, 16, 17, 18, 19
D	22, 23, 24
E	3, 4, 5, 6, 7
F	10, 11, 12, 13, 14
G	20, 21
H	25, 26, 27, 28

Please note – the animals were allocated on a random basis

Chapter 10; References

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