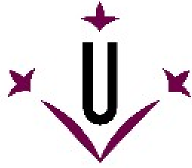

Safety assessment and efficacy investigations of genetically engineered corn accumulating high levels of carotenoids

Gemma Arjó Pont

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Safety assessment and efficacy investigations of genetically engineered corn accumulating high levels of carotenoids

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Doctoral dissertation

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Paul Christou professor del Departament de Producció Vegetal i Ciència Forestal i Carmen Piñol professora del Departament de Medicina, de la Universitat de Lleida, i directors de la tesi realitzada per la senyora Gemma Arjó Pont, “Safety assessment and efficacy investigations of genetically engineered corn accumulating high levels of carotenoids”,

INFORMEM:

Que el treball d’investigació es considera ja finalitzat i que compleix les condicions exigibles a la legislació vigent per optar al grau de Doctor. Per tant autoritzem la seva presentació perquè pugui ser jutjada pel Tribunal corresponent a la Universitat de Lleida.

Lleida, 12 de Juny de 2014

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L'important és no deixar de fer-se preguntes.

(Albert Einstein)

Als meus pares,

als meus germans,

i al Xavi

ABSTRACT, RESUM, RESUMEN

ABSTRACT

Malnutrition describes any nutritional imbalance, including undernutrition, overnutrition and the ‘dual burden’ of excess calorific intake combined with an insufficient intake of micronutrients. Malnutrition can be caused by a poor diet and/or by medical conditions that affect nutrient assimilation. In developing but also in industrialised countries, malnutrition often goes hand in hand with hunger, but increasingly results from the consumption of inexpensive, calorie-rich but nutrient-poor diets. Such diets reflect developments in agriculture and food processing technology that reduce the micronutrient content of foods, contributing to both malnutrition and obesity, and exacerbating many chronic diseases including cardiovascular disease, diabetes and cancer. Plants provide a good source of vitamins and minerals but these micronutrients are present at insufficient levels in staple cereal crops such as corn and rice. For example, cereal grains do not produce high levels of carotenoids, which confer health-promoting antioxidant activity and in some cases (provitamin-A carotenoids) act as essential nutrients. Biofortification by genetic engineering is a cost-effective strategy to improve the nutrient content of crops, but their development requires a mandatory risk assessment as an integral component of approval process prior to commercialization. The safety of a carotenoid-enriched engineered corn variety (High-carotenoid corn) produced by the Applied Plant Biotechnology group at Lleida University was therefore evaluated in a 90-day subchronic toxicity study in mice. This showed no evidence of toxicity compared to mice fed on control diet (based on near-isogenic unmodified M37W corn). Having established safety, diets based on the engineered corn were fed to a mouse cancer model associated with induced colitis, a mouse obesity and insulin resistance model developed by initial feeding on a high-fat diet, and finally a heterozygous phosphatase and tensin homolog knockout mouse line ($PTEN^{+/-}$) as a model of multiple-organ neoplasia and liver disease. These experiments showed that the carotenoid-enriched corn variety was chemopreventive and also induced promising health-promoting effects on risk parameters related to metabolic syndrome and insulin sensitivity.

RESUM

El terme malnutrició s'aplica a qualsevol condició de nutrició desequilibrada, ja sigui per una alimentació deficitària, per sobrealimentació o per una doble vessant d'ambdues: una ingesta calòrica excessiva combinada amb una ingesta insuficient de micronutrients. La malnutrició pot ser causada per una dieta pobra i/o per estats patològics que afectin la correcta assimilació dels nutrients. En països en vies de desenvolupament però també en països industrialitzats, la malnutrició sovint va de la mà amb la fam, i cada cop més, acompanyada d'un augment del consum de dietes riques en calories de baix cost i pobres en nutrients. Aquestes dietes són reflex del progrés en l'agricultura i en les tecnologies de processament d'aliments que contribueixen a reduir els continguts de micronutrients dels aliments, cosa que afavoreix tan a la malnutrició com a l'obesitat, i agreuja diverses malalties cròniques com les malalties cardiovasculars, diabetis i càncer. Les plantes són una font important de vitamines i minerals, però aquests micronutrients es troben en nivells insuficients en cultius bàsics de cereal com el panís o l'arròs. Per exemple, les llavors d'aquests cereals no produeixen nivells alts de carotens, els quals en alguns casos intervenen en la promoció de la salut pel seu efecte antioxidant, i en altres casos (com és el cas dels carotens provitamina A) actuen com a micronutrients essencials. La biofortificació a través de l'enginyeria genètica és una estratègia cost-efectiva per millorar el contingut nutricional del panís; no obstant això, la seva elaboració requereix d'una avaluació de riscs obligatòria com a component integral del procés d'aprovació prèvia a la comercialització. Així doncs, es va avaluar la seguretat d'un panís genèticament millorat per augmentar el seu contingut en carotens (*High-carotenoid corn*)**,** produït pel Grup de Biotecnologia Vegetal Aplicada de la Universitat de Lleida, mitjançant un estudi de toxicitat subcrònica de 90 dies en ratolins. No es van trobar evidències de toxicitat quan es va comparar amb ratolins alimentats amb una dieta control (basada en un panís no modificat anomenat M37W). Un cop establerta la seguretat del panís, es va administrar una dieta basada en el panís genèticament millorat a ratolins model de càncer de còlon associat a colitis, a ratolins model d'obesitat i resistència a la insulina induïdes a través d'una dieta amb alt contingut en greixos, i finalment, a una línia de ratolí heterozigot per l'homòleg de fosfatasa i tensina ($PTEN^{+/-}$) com a model de neoplàsia en múltiples òrgans i de malaltia hepàtica. Aquests experiments mostren que la varietat de panís enriquit en carotens presenta un efecte quimioprotector i també efectes prometedors en la promoció de la salut en paràmetres de risc relacionats amb la síndrome metabòlica i la sensibilitat a la insulina.

RESUMEN

El término malnutrición se aplica a cualquier condición de nutrición desequilibrada, ya sea por una alimentación deficitaria, por sobrealimentación o por la combinación de las dos: una ingesta calórica excesiva combinada con una ingesta insuficiente de micronutrientes. La malnutrición puede ser causada por una dieta pobre y/o por estados patológicos que afecten a la correcta asimilación de los nutrientes. En países en vías de desarrollo pero también en países industrializados, la malnutrición a menudo va de la mano del hambre, y cada vez más, acompañada de un aumento del consumo de dietas altas en calorías de bajo coste y pobres en nutrientes. Estas dietas son reflejo del progreso en la agricultura y en las tecnologías de procesado de alimentos que contribuyen a la reducción de los contenidos de micronutrientes de los alimentos, hecho que favorece tanto a la malnutrición como a la obesidad, y agrava diferentes enfermedades crónicas como las enfermedades cardiovasculares, diabetes y cáncer. Las plantas son una fuente importante de vitaminas y minerales, pero estos micronutrientes se encuentran en niveles insuficientes en cultivos básicos de cereal como el maíz o el arroz. Por ejemplo, las semillas de estos cereales no producen niveles altos de carotenos, los cuales en algunos casos intervienen en la promoción de la salud por su efecto antioxidante, y en otros casos (como es el caso de los carotenos provitamina A) actúan como micronutrientes esenciales. La biofortificación a través de la ingeniería genética es una estrategia costo-efectiva para mejorar el contenido nutricional del maíz; sin embargo, su elaboración requiere una evaluación de riesgos obligatoria como componente integral para un proceso de aprobación previa a la comercialización. Así pues, se evaluó la seguridad de un maíz genéticamente mejorado para aumentar su contenido de carotenos (*High-carotenoid corn*), producido por el grupo de Biotecnología Vegetal Aplicada de la Universidad de Lleida, mediante un estudio de toxicidad subcrónica de 90 días en ratones. No se encontraron evidencias de toxicidad cuando se comparó con ratones alimentados con una dieta control (basada en maíz no modificado llamado M37W). Una vez establecida la seguridad del maíz, se administró una dieta basada en maíz genéticamente mejorado a ratones modelos de cáncer de colon asociado a colitis, a ratones modelo de obesidad y resistencia a la insulina inducidas a través de una dieta con un alto contenido en grasa, y finalmente, a una línea de ratones heterocigotos por el homólogo de fosfatasa y tensina ($PTEN^{+/-}$) como modelo de neoplasia en múltiples órganos y de enfermedad hepática. Los experimentos muestran que la variedad de maíz enriquecido con carotenos presenta un efecto quimioprotector y también efectos prometedores en la promoción de la salud en parámetros de riesgo relacionados con el síndrome metabólico y la sensibilidad a la insulina.

TABLE OF CONTENTS

ABSTRACT I

RESUM III

RESUMEN V

INDEX OF FIGURES XXI

INDEX OF TABLES XV

LIST OF ABBREVIATIONS XVII

1. GENERAL INTRODUCTION 1

1.1. Forms of malnutrition: undernutrition and overnutrition 3

1.2. The impact of malnutrition on chronic diseases 4

1.2.1. Cancer and malnutrition 4

1.2.2. Type 2 diabetes and malnutrition 6

1.2.3. Non-alcoholic fatty liver disease and malnutrition 6

1.3. The biofortification of staple foods 7

1.3.1. Conventional breeding 7

1.3.2. Genetic engineering in plants 8

1.4. Genetically engineered plants 8

1.5. Safety assessment of genetically engineered crops 11

1.5.1. Safety assessment of new plant varieties 11

1.5.2. Regulatory issues concerning genetically engineered plants 12

1.5.3. Current comparative safety assessment methods for plant-derived foods 13

1.6. High-carotenoid corn 20

1.6.1. Carotenoids 20

1.6.2. Retinoids 21

1.6.3. Genetically engineered high-carotenoid corn 22

1.7. Animal models 25

2. AIMS AND OBJECTIVES 27

Aims and Objectives 29

3. EXPERIMENTAL CHAPTERS 27

3.1. CHAPTER 1. Mice fed on a diet enriched with genetically-engineered high-carotenoid corn exhibit no subchronic toxicity 33
3.1.1. INTRODUCTION 35
3.1.2. MATERIALS AND METHODS 37
3.1.2.1. Plant material 37
3.1.2.2. Compositional analysis of experimental and control diets 37
3.1.2.3. Animal feeding studies 38
3.1.2.4. Clinical observations 39
3.1.2.5. Statistical analysis 40
3.1.3. RESULTS 41
3.1.3.1. Compositional analysis 41
3.1.3.2. Ninety-day subchronic toxicity assessment 42
3.1.4. DISCUSSION 53
3.1.5. REFERENCES 55
3.2. CHAPTER 2. Chemoprevention of tumor development in the mouse AOM/DSS model of colitis-associated carcinogenesis by diets enriched with high-carotenoid corn .... .59
3.2.1. INTRODUCTION 61
3.2.2. MATERIALS AND METHODS 64
3.2.2.1 Animal feeding study, diets and chemicals 64
3.2.2.2. Colonoscopy 65
3.2.2.3. Blood analysis and histopathology 66
3.2.2.4. Total RNA extraction and quantitative real-time PCR 66
3.2.2.5. Immunohistochemistry 67
3.2.2.6. Western blot 67
3.2.2.7. Statistics 68
3.2.3. RESULTS 69
3.2.3.1. General observations 69
3.2.3.2. Incidence and multiplicity of colonic lesions after 6 to 12 weeks follow up by colonoscopy 72
3.2.3.3. Incidence and multiplicity of colonic lesions after 12 weeks 74
3.2.3.4. Histopathology of colonic lesions 76
3.2.3.5. Immunohistochemical analysis of COX-2 expression 77

3.2.3.6. Quantitative real time PCR analysis of inflammation markers 78
3.2.3.7. Western blot analysis of COX-2 and p44/42 MAPK 78
3.2.4. DISCUSSION 80
3.2.5. REFERENCES 83
CHAPTER 3. The effects of high-carotenoid corn on insulin sensitivity in a mouse model of obesity and insulin resistance induced by a high-fat diet 91
3.3.1. INTRODUCTION 93
3.3.2. MATERIALS AND METHODS 95
3.3.2.1. Diet 95
3.3.2.2. General nutrient compositional analysis 95
3.3.2.3. Animal feeding study.. 96
3.3.2.4. Subcutaneous glucose tolerance and insulin tolerance tests 96
3.3.2.5. Blood biochemistry 97
3.3.2.6. Antioxidant capacity... 97
3.3.2.7. Insulin measurements.. 98
3.3.2.8. Leptin measurements 98
3.3.2.9. DNA extraction 98
3.3.2.10. Tissue homogenization and western blot analysis 98
3.3.2.11. Analysis of oxidative damage by protein carbonyl detection 99
3.3.2.12. Carotenoid and retinoid extraction from liver and adipose tissues 100
3.3.2.13. Carotenoid and retinoid analysis by high-performance liquid chromatography 100
3.3.2.14. Statistical analysis... 100
3.3.3. RESULTS 101
3.3.3.1. Nutritional parameters and general observations.................................... 101
3.3.3.2. Biochemical characteristics 104
3.3.3.3. Glucose transport and GLUT4 translocation 108
3.3.3.4. AMPKα expression in visceral adipose tissue and skeletal muscle............ 109
3.3.3.5. Glycemic control: glycosylated hemoglobin and fructosamine................ 110
3.3.3.6. The role of visceral adipose tissue in insulin sensitivity............................ 111
3.3.3.7. Oxidative damage assays 114
3.3.3.8. HPLC analysis of the liver and adipose tissue retinoid content 117

3.3.4. DISCUSSION 119
3.3.5. REFERENCES 123
CHAPTER 4. Influence of a high-carotenoid corn diet on tumor development and risk parameters related to metabolic syndrome and insulin sensitivity in $PTEN^{+/-}$ mice 131
3.4.1. INTRODUCTION 133
3.4.2. MATERIALS AND METHODS 137
3.4.2.1. Diets 137
3.4.2.2. Animal feeding study 137
3.4.2.3. Blood analysis and histopathology 138
3.4.2.4. Immunohistochemistry 139
3.4.2.5. Oil Red O staining 139
3.4.2.6. Total RNA extraction and quantitative real-time PCR 139
3.4.2.7. Retinoid extraction 140
3.4.2.8. Retinoid identification and quantification by HPLC 140
3.4.2.9. Statistical analysis 140
3.4.3. RESULTS 141
3.4.3.1. Feeding experiments – general observations 141
3.4.3.2. Animal body weight and relative organ weights 141
3.4.3.3. Tumor incidence, multiplicity and histopathology in $PTEN^{+/-}$ mice 142
3.4.3.4. Histological evaluation of the liver 144
3.4.3.5. Biochemical analysis 145
3.4.3.6. Quantitative real-time PCR 149
3.4.3.7. Retinol and retinoic acid levels in liver tissue 149
3.4.4. DISCUSSION 150
3.4.5. REFERENCES 154
4. GENERAL DISCUSSION 163
5. CONCLUSIONS 169
6. REFERENCES 173

OUTPUTS 189

INDEX OF FIGURES

Figure 1. 1. Three generations of GE plants. Adapted from Sakakibara and Saito, 2006. 9

Figure 1. 2. Schematic representation of major allergenicity assessment areas, showing high risks (on the right) and lower risks (on the left) (Goodman et al., 2008)............... 16

Figure 1. 3. Pathways from β-carotene to retinoic acid in animals and humans. Symmetric (central) oxidative cleavage of β-carotene (BCO1) generates two molecules of retinaldehyde (retinal) which can be either reduced to retinol or oxidized into retinoic acid.. 22

Figure 1. 4. High-carotenoid corn and its conventional counterpart M37W................. 23

Figure 1. 5. Metabolic pathway leading to β-carotene, which is absent in the M37W corn variety because PSY is not expressed in the endosperm. 24

Figure 3.1. 1. Mean weekly feed consumption (mean ± CI 95%) of male and female mice.. 42

Figure 3.1. 2. Mean weekly body weights (mean ± Confidence Interval 95%) of male and female mice 43

Figure 3.1. 3. Histology (hematoxylin and eosin staining) from liver (a-f) and kidneys (g-l) x 20 (x 40 in the box) and heart (m-r) x 40 (x 63 in the box)..................................... 51

Figure 3.2. 1. Model for the regulation of COX-2 expression by IL-1. IL-1, a key cytokine associated with the inflammation of the intestinal mucosa, binds to its receptor and can activate the MAPK, NF-κB and protein kinase C (PKCζ) pathways. 62

Figure 3.2. 2. Experimental design and timeline of the colitis-associated colon carcinogenesis experiment... 64

Figure 3.2. 3. Endoscopic images, incidence, multiplicity and score of colonic tumors identified by endoscopic visualization.. 73

Figure 3.2. 4. (A) Macroscopic image of the colon dissected from i) Control WT, ii) DSS CAR, iii) AOM/DSS WT and iv) AOM/DSS CAR groups, and median and quartile distributions for colorectal tumor multiplicity (B) and mean colon tumor area (C) in tumor-bearing animals 75

Figure 3.2. 5. Histopathology of colonic lesions after hematoxylin and eosin staining in the AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) groups. (A) crypt dysplasia; (B) adenoma; (C) adenocarcinoma; (D) mucosal ulcer; (E) adenoma; (F) adenocarcinoma. 76

Figure 3.2. 6. Percentage of colonic lesion features found by investigative histology in AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) groups.. 76

Figure 3.2. 7. Immunohistochemical analysis of COX-2 expression in the normal colonic mucosa representing intensity scores of 1+ (A, D), 2+ (E), 3+ (B, F) and 4+ (C) in the AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) groups.. 77

Figure 3.2. 8. Mean COX-2 immunohistochemical score (± SEM) determined in AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) colonic tissues. 78

Figure 3.2. 9. Western blots showing the impact of high-carotenoid corn on the expression of (A) COX-2 and (B) p44/42 MAPK in normal (non-tumor) colonic mucosa 79

Figure 3.3. 1. . Blood biochemistry parameters in mice maintained on the NWT, NCAR, HWT and HCAR diets. (A) Subcutaneous glucose tolerance. (B) Glycemic response to insulin (C) Blood insulin levels and LDL/HDL cholesterol ratio.................................... 105

Figure 3.3. 2. Results from the ferric reducing ability of plasma (FRAP) and 3-ethylbenzothiazoline-6-sulphonate (ABTS) blood assays of mice fed on normocaloric and hypercaloric diets. 108

Figure 3.3. 3. Western blot analysis of GLUT4 expression in muscle (M) and visceral adipose tissue (VAT). 109

Figure 3.3. 4. Western blot analysis of AMPKα expression in skeletal muscle (Skl Msc), visceral adipose tissue (VAT) and liver. 110

Figure 3.3. 5. Analysis of the glycemic control indicators glycosylated hemoglobin and fructosamine.. 111

Figure 3.3. 6. Absolute amounts of DNA and protein per gram of visceral adipose tissue (VAT) in the four diet groups. 112

Figure 3.3. 7. Plasma leptin levels in the four diet groups. 112

Figure 3.3. 8. Western blot analysis of p44/42 MAPK expression in visceral adipose tissue.. 113

Figure 3.3. 9. Western blot analysis for protein reactive carbonyls (DNP) in skeletal muscle (Skl Msc) and liver. 115

Figure 3.3. 10. Western blot analysis of MDAL in skeletal muscle (Skl Msc) and visceral adipose tissue (VAT) to show the level of lipoxidation. The histogram shows the expression levels normalized against Gallyas staining and quantified by densitometry. 116

Figure 3.3. 11. Western blot analysis of ubiquitinylated proteins (Ubiq) in skeletal muscle (Skl Msc), visceral adipose tissue (VAT) and liver. 117

Figure 3.3. 12. HPLC analysis of retinol and retinoic acid levels in the liver and visceral adipose tissue in all four diet groups. 118

Figure 3.4. 1. The PI3K-AKT-mTOR pathway and the role of PTEN. 134

Figure 3.4. 2. Representative histopathology of colonic tissue sections stained with hematoxylin and eosin, and the percentage of animals with histopathological findings, i.e. follicular hyperplasia and adenoma. 143

Figure 3.4. 3. Representative histopathology of uterine endometrium sections stained with hematoxylin and eosin. 143

Figure 3.4. 4. Representative histopathology of liver sections stained with hematoxylin and eosin, with liver steatosis graded as described by Kleiner et al. (2005). 145

Figure 3.4. 5. Total cholesterol, lactate, fasting glucose and triglyceride levels in the blood of mice from the four different diet groups. 147

Figure 3.4. 6. Fructosamine, albumin and total protein levels. 148

Figure 3.4. 7. Levels of hepatic retinol and retinoic acid determined by HPLC. 149

INDEX OF TABLES

Table 1. 1. Sequence identity over an 80-amino-acid window between a recombinant protein and an allergen identified by BLAST/FASTA alignment, thus requiring serum affinity analysis (Goodman et al., 2008). 17

Table 3.1. 1. Wild-type M37W and high-carotenoid corn lines composition. 41

Table 3.1. 2. Biochemistry mean values ± SD for males (a. N=5; b. N=4). 44

Table 3.1. 3. Biochemistry mean values ± SD for females (a. N=5). 45

Table 3.1. 4. Hematology mean values ± SD for males (a. N=5; b. N=4). 46

Table 3.1. 5. Hematology mean values ± SD for females (a. N= 5). 47

Table 3.1. 6. Final body weight and absolute organ mean values ± SD for males. 48

Table 3.1. 7. Final body weight and absolute organ mean values ± SD for females. 49

Table 3.1. 8. Histopathologic analyses for male and female mice of reference (REF), WT and CAR groups. 50

Table 3.2. 1. Primary and secondary antibodies for western blot immunodetection. .. 68

Table 3.2. 2. Body weight (BW), food consumption (FC), liver weight and colon length. 70

Table 3.2. 3. Analysis of hematologic parameters: white blood cell count (WBC), neutrophils (Neutr), lymphocytes (Lym), monocytes (Mono), eosinophils (Eos), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HTC). 71

Table 3.2. 4. Tumor characteristics in dissected colons after 12 weeks: incidence and multiplicity of macroscopic colorectal lesions, tumor multiplicity, average tumor size

and total tumor area per tumor-bearing mouse presented as means ± SEM (n = 8 animals in the AOM/DSS WT group and n = 10 in the AOM/DSS CAR group). 75

Table 3.3. 1. Primary and secondary antibodies used for western blot immunodetection. 99

Table 3.3. 2. Composition of the NWT, NCAR, HWT and HCAR diets. 102

Table 3.3. 3. Final body weight, body weight gain and dietary intake (means ± SEM) of mice maintained on the NWT, NCAR, HWT and HCAR diets. 103

Table 3.3. 4. Blood biochemistry in animals fed on normocaloric and hypercaloric diets, showing total cholesterol (CHOL), HDL, LDL, non-esterified fatty acids (NEFA), triglycerides (TG), TG/HDL, LDL/HDL and total CHOL/HDL, all presented as means ± SEM.. 107

Table 3.4. 1. Final body weights and relative organ weights in the four experimental diet groups. 141

Table 3.4. 2. Macroscopic observations and histopathology of $PTEN^{+/-}$ mice in the WT and CAR diet groups. 142

ABBREVIATIONS

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di-[3-ethylbenzthiazolinesulfonate(6)] diammonium salt
AD	Adenoma
ADC	Adenocarcinoma
ALT	Alanine Aminotransferase
AMP	Adenosine monophosphate
AMPK	Adenosine-monophosphate-activated protein kinase
ANOVA	Analysis of Variance
AOM	Azoxymethane
ATP	Adenosine triphosphate
BCO1	β-carotene 15, 15′-oxygenase 1
BCO2	β-carotene 9′,10′-oxygenase 2
BHT	Butylated hydroxytoluene
Bt	*Bacillus thuringiensis*
BUN	Blood urea nitrogen
BW	Body weight
CA	California
CAR	High-carotenoid
CHOL	Cholesterol
COX-2	Cyclo-oxygenase-2
CRC	Colorectal cancer
crtI	Carotene desaturase
dhar	Dehydroascorbate reductase
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenylhydrazine
DSS	Dextran sulfate sodium
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay

EU	European Union
FAO	Food and Agriculture Organization
FC	Food consumption
FDA	Food and Drug Administration
FFA	Free fatty acids
FRAP	Ferric reducing ability of plasma
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCH1	Guanosine triphosphate (GTP) cyclohydrolase
GE	Genetically engineered
GLUT4	Glucose transporter type 4
Hb1Ac	Glycosilated hemoglobin
HDL	High-density lipoprotein
HGB	Hemoglobin
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HTC	Hematocrit
IBD	Inflammatory bowel disease
IHC	Scored immunohistochemistry
ILSI	International Life Sciences Institute
IR	Insulin resistance
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
LDL	Low-density lipoprotein
MA	Massachusetts
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MD	Maryland
MVC	Mean corpuscular volume
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis

NEFA	Nonesterified fatty acids
NF-κB	Nuclear factor kappaB
NRF-2	NF-E2-related factor 2
OECD	Organization for Economic Co-Operation and Development
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor
Ps Red	Ponceau Red
psy1	Phytoene synthase
PTEN	Phosphatase and tensin homolog
qPCR	Real-time polymerase chain reaction
QTL	Quantitative trait loci
RAR	Retinoic acid receptor
REF	Reference
RBC	Red blood cell count
RDI	Recommended daily intake
RNA	Ribonucleic acid
ROS	Reactive oxidative species
RXR	Retinoid X receptor
ScGTT	Subcuteaneous glucose tolerance test
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
SREBP	Sterol regulatory element-binding protein
T2D	Type-2 diabetes
TBP	TATA-binding protein
TE	Trolox equivalent
TG	Triglycerides
TNF-α	Tumor necrosis factor alpha
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
USA	United States of America
VAT	Visceral adipose tissue

WBC	White blood cell count
WHO	World Health Organization
WT	Wild-type

1. GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. Forms of malnutrition: undernutrition and overnutrition

Undernutrition is any condition resulting from the inadequate intake or assimilation of nutrients and it is responsible for more than 30% of all deaths worldwide in children under 5 years old (Relman, 2013). In developing countries, undernutrition reflects the common phenomenon of insufficient access to nutritious food, which is often caused by poverty. These factors act in concert to increase the prevalence of poor health and disability, impaired cognitive development and premature death (Berman et al., 2013; Pérez-Massot et al., 2013). There is an urgent need to find sustainable solutions that address these problems in developing countries, but strategies aiming to deal with undernutrition often fail because of poor infrastructure and organization. For example, attempting to address undernutrition by providing diverse sources of fresh food, supplements or fortified food products has not been successful because there is a lack of funding and because distribution networks are unstable (Gómez-Galera et al., 2010; Yuan et al., 2011). As a sustainable alternative, biofortification aims to modify staple crops, particularly local elite breeding varieties, so that they accumulate essential nutrients at source. This solution has no impact on the agricultural performance of the crop but provides added value in the form of single or multiple essential nutrients (Pérez-Massot et al., 2013; Zhu et al., 2013).

Undernutrition is a form of malnutrition, but the latter encompasses all deviations from an adequate or optimal nutritional status and therefore also includes excessive energy intake (overnutrition) and the intake of an unbalanced diet with an excess of some nutrients but a lack of others (Shetty, 2006). For example, it is quite possible for an individual with excess calorific intake to be malnourished due to a lack of vitamins (Kaidar-Person et al., 2008). Approximately 34% of adults in the developing world were classified as overweight or obese in 2008 (compared to 23% in 1980). In high-income countries the prevalence of obesity grew 1.7-fold over t he same period (Stevens et al., 2012). It seems paradoxical, but the rapid increase in obesity has not been

accompanied by a proportional reduction in undernutrition and these two nutritional states (known as the 'dual burden') persist alongside each other in many countries (FAO, 2011; Doak et al., 2005).

Obesity can be promoted by environmental, behavioral and genetic factors. Over the last few decades, the populations of many countries have become accustomed to inexpensive, calorie-rich but nutrient-poor diets resulting from developments in agriculture and food processing technology which reduce the micronutrient content of foods. This has a negative impact on nutritional health and increases susceptibility to obesity, with a startling impact on the many chronic diseases including cardiovascular disease, diabetes and cancer (Riaz et al., 2009; Swinburn et al., 2011).

1.2. The impact of malnutrition on chronic diseases

Because the prevalence of obesity is rising, metabolic syndrome and its concomitant diseases are becoming a severe health problem worldwide (Braun, 2011). Metabolic syndrome includes hypertension, abdominal obesity, hyperglycemia, dyslipidemia and it has been linked to insulin resistance and the resulting development of diabetes mellitus and nonalcoholic fatty liver disease (Jaggers et al., 2009; Braun, 2011).

1.2.1. Cancer and malnutrition

Malnutrition is a risk factor in cancer, but early studies failed to determine unequivocally whether increasing the intake of fruits and vegetables beyond the level needed to prevent malnutrition is necessarily beneficial (Bjelke, 1975; Armstrong and Doll, 1975). The World Cancer Research Fund and the American Institute for Cancer Research have reported 'convincing' evidence that consuming fruits and vegetables reduces the risk of various types of cancer (World Cancer Research Fund/American Institute for Cancer Research, 1997). However, more recent prospective investigations with more subjects did not show a clear association between higher consumption and lower cancer risk (Key, 2011). It is likely that dietary carcinogens have a greater impact

on malnourished individuals reflecting the lack of protective factors found in balanced diets, including the fiber found in fruits and vegetables (Lutz, 1999). Examples of dietary carcinogens include alcohol (excessive intake), heterocyclic aromatic amines, cadmium, polycyclic aromatic hydrocarbons and mycotoxins such as aflatoxin B_1, which have an adverse impact on mitochondrial metabolism, oxidative stress, protein synthesis, cell proliferation, hormonal and immune functions and gene regulation (Lutz, 1999). Moderate amounts of fruits and vegetables help to prevent nutrient deficiency and may reduce the risk of cancer, but further investigations are required to evaluate the risks of dietary carcinogens and overnutrition as risk factors in cancer (Key, 2011; Lutz, 1999).

On the one hand, dietary intake plays an important role in the progression of cancer because more than 85% of cancer patients develop clinical malnutrition. Poor nutritional status can inhibit the therapeutic response and increase the severity of side-effects, and these factors must be considered to improve prognosis and increase the chances of recovery (Davies, 2005). Cancer-associated malnutrition usually equates to undernutrition (Argilés, 2005), and its multifactorial etiology may reflect a generally lower food intake, metabolic changes in the patient, or the direct local effects of a tumor (Van Cutsem and Arends, 2005). Nutritional screening should therefore be used to identify malnourished patients or those at the highest nutritional risk (Kim et al., 2011).

On the other hand, obesity and diabetes have been associated with renal, pancreatic, breast, colorectal, endometrial and hepatic cancer (Calle et al., 2003). The American Cancer Society reported that nearly one in three cancer-related deaths in the United States was due to obesity, poor diet or inactivity (www.acscan.org). Metabolic syndrome (or its components) could play a decisive role, not only in the prognosis but also in the etiology and progression of certain cancer types (Braun, 2011).

1.2.2. Type 2 diabetes and malnutrition

Insulin resistance often manifests in obese individuals and it is a sign of pre-diabetes, which can develop into type 2 diabetes (T2D) subsequently if insulin sensitivity is not ameliorated (Kalupahana et al., 2012). Insulin resistance refers to the incapacity of insulin-sensitive tissues (e.g. fat, skeletal muscle and liver) to respond to insulin. However, pancreatic β cells compensate by secreting higher levels of insulin, which initially helps to maintain normal sugar levels. Over time, the progressive failure of β cells to secrete sufficient insulin induces hyperglycemia and eventually T2D (Guilherme et al., 2008). Oxidative stress is also increased in diabetic patients because persistent hyperglycemia and the oxidation of excess glucose can generate reactive oxidative species (ROS) that contribute to the development of diabetic complications (e.g. neuropathy, atherosclerosis, retinopathy and the destruction of β cells). Certain dietary micronutrients can counter these complications because of their antioxidant activity and their requirement as a cofactors in the metabolism of glucose, the insulin signaling cascade and the function of β cells (DeFronzo, 2009).

1.2.3. Non-alcoholic fatty liver disease and malnutrition

The associations among obesity, insulin resistance, T2D and steatosis have been recognized for a long time (Farrell and Larter, 2006). Non-alcoholic fatty liver disease (NAFLD) occurs when fat accumulates within liver cells (steatosis) in the absence of excess alcohol intake; the disease affects 20–30% of the general population (McCullough, 2004; Petta et al., 2009). NAFLD spans a range of symptoms, from steatosis alone to non-alcoholic steatohepatitis (NASH), which is the most severe form of NAFLD and includes hepatocellular carcinoma, cirrhosis and advanced liver disease (Ziouzenkova et al., 2007).

Malnutrition based on excess fat and carbohydrate intake often causes chronic increases in the levels of insulin, glucose and fatty acids in the blood. This induces resistance to insulin-stimulated glucose uptake by skeletal muscles as well as a suppression of triglyceride hydrolysis in adipose tissues, and thus causes glucose levels

in the blood and liver to rise. Non-insulin-dependent glucose uptake also increases, resulting in its conversion into free fatty acids and glycogen through insulin-mediated stimulation of *de novo* lipogenesis. The production of free cholesterol is also stimulated. The body therefore accumulates cholesterol esters and triacylglycerols in the liver (Ziouzenkova et al., 2007).

1.3. The biofortification of staple foods

Biofortification is a cost-effective strategy to address micronutrient deficiency and it is particularly advantageous in developing countries because it can increase the nutrient content of local elite staple crops at source. This is important because many staple crops (such as rice or corn) lack adequate amounts of essential micronutrients such as vitamins and minerals (Zorrilla-López et al., 2013). Biofortification can be achieved by conventional breeding and/or genetic engineering as discussed below (Zhu et al., 2007).

1.3.1. Conventional breeding

Conventional breeding methods involve the cross-breeding of sexually compatible plants of the same or different species and/or using *in vitro* culture techniques to generate plants with combinations of improved characteristics from parents with desirable attributes. When the desired trait is not present in sexually compatible plants that can be crossed with an elite breeding variety and selected by repetitive backcrossing, the elite lines can be mutated e.g. using chemicals or radiation, generating libraries of random mutations that can be selected for the trait of interest (Fitzmaurice et al., 1999; Ahloowalia and Maluszynski, 2001; Jander et al., 2003). Alternative approaches rely on natural variation within plant species, allowing the identification of quantitative trait loci (QTLs), i.e. genomic regions related to relevant phenotypic traits (Maloof, 2003). The major limitations of conventional breeding for nutritional improvement include the dependence on a compatible gene pool, the long

time required to breed a specific trait into an elite crop, and the layers of complexity introduced when attempting to select for different nutritional traits simultaneously (Bai et al., 2011).

1.3.2. Genetic engineering in plants

Genetic engineering allows the modulation of metabolic pathways directly in order to improve nutritional characteristics. The process begins with the identification of appropriate genes, followed by transformation, in which genetic constructs are introduced into the plant genome to confer novel or improved traits. *Agrobacterium tumefaciens* is a ubiquitous soil bacterium that is often used for genetic engineering in plants because it has a natural capacity to introduce a segment of DNA carried on a resident plasmid, and this segment of DNA integrates into the plant genome at the onset of pathogenesis (Comai et al., 1983). For plants that are recalcitrant to Agrobacterium-mediated transformation, electroporation or chemical transfection mediated by polyethylene glycol transformation are examples of direct DNA transfer, although these strategies work best with protoplasts and often require complex regeneration protocols (Zhang et al., 1988). Particle bombardment is a more versatile direct DNA transfer strategy based on the acceleration of DNA-coated metal particles that deliver genetic material into plant cells (Altpeter et al., 2005). The advantages of particle bombardment include the simple and straightforward technique, the ability to transfer DNA to any species of plant, and the ability to introduce multiple genes for simultaneous biofortification with different nutrients at the same genetic locus (Zhu et al., 2007).

1.4. Genetically engineered plants

The first commercial genetically engineered crops were deployed in 1996 (1.7 million hectares in six countries) and this has grown to 175.2 million hectares in 25 countries in 2013 (http://www.isaaa.org/). The four principal biotech crops are corn, soybean,

cotton and canola, and the biggest producers are the United States, Argentina, Brazil, Canada, India and China (Magaña-Gómez and de la Barca, 2009; Manjunath, 2010).

Three generations of biotech crops can be recognized according to the purpose of the introduced trait (Figure 1.1). The first-generation transgenic crops were generated to enhance productivity by avoiding biotic stress constraints caused by insects and weeds, without affecting other characteristics (e.g. appearance, flavor and nutritional composition). Second-generation transgenic crops were modified to enhance their nutritional traits, e.g. by increasing the quantity and/or quality of proteins, lipids, carbohydrates and essential micronutrients such as vitamins and minerals (Magaña-Gómez and de la Barca, 2009; Zhu et al., 2007). Third-generation transgenic crops are being developed as factories to produce pharmaceutical and industrial molecules (Sakakibara and Saito, 2006).

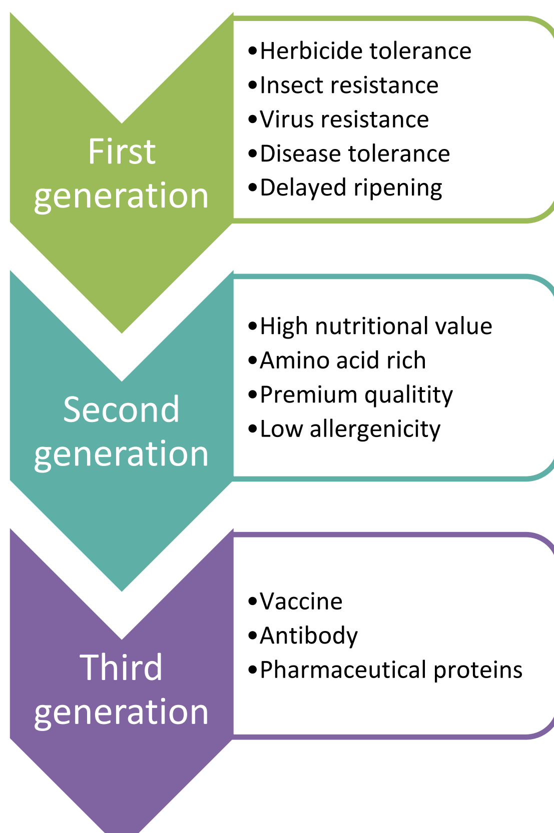

Figure 1. 1. Three generations of GE plants. Adapted from Sakakibara and Saito, 2006.

The early development and commercialization of transgenic crops occurred largely without incident, the products were labelled voluntarily to confirm the presence of ingredients from genetically engineered plants and there was no apparent hostility from consumers (Stewart et al., 2000).

However, this changed in 1999 with the publication of two controversial papers. The first claimed that potatoes genetically engineered to express the lectin *Galanthus nivalis* agglutinin were toxic towards rats, causing abnormalities in the immune system (Ewen and Pusztai, 1999), and the second claimed that pollen from corn plants engineered to express pest-specific *Bacillus thuringiensis* (Bt) toxins were harmful to monarch butterflies (Losey et al., 1999). These and other

similar papers were later discredited but the damage was already done (Enserink, 1999; Sears et al., 2001; Stanley-Horn et al., 2001; Gatehouse et al., 2002).

These initial publications resulted in a plethora of additional studies which were published in the general media and on the internet without peer review, and were seized upon by extreme environmental groups and Green political parties in Europe. These organizations mounted a campaign of misinformation to create alarm and distrust among the public, resulting in increasing hostility towards transgenic plants and the underlying technology (Stewart et al., 2000). It is often assumed by the public that this politically-motivated pressure precipitated the development of guidelines for the safety assessment of transgenic plants and products derived from them, but these guidelines have always been an integral part of the approval process. As in other technological areas, safety assessment guidelines evolve constantly in an attempt to keep pace with the most recent scientific developments (Magaña-Gómez and de la Barca, 2009).

More recently, a 2-year animal feeding trial was published in *Food and Chemical Toxicology* describing the potential toxicity towards Sprague-Dawley rats of a glyphosate herbicide-resistant corn variety and also independently the herbicide itself (Séralini et al., 2012). Once again, the study was riddled with errors and was comprehensively debunked, but it was nevertheless used as a justification to ban the import of genetically engineered crops into several countries and pressurized European Food Safety Authority (EFSA) to introduce an additional requirement for 2-year chronic toxicity assessments into the approval process (EFSA, 2013). Although the Séralini study was peer-reviewed, there were errors in experimental design, statistical analysis and interpretation (Arjó et al., 2013) which resulted in retraction of the paper by the journal one year later following intense pressure from the scientific community and regulatory agencies (Elsevier, 2013). These events remind us that the safety evaluation of genetically engineered crops is currently inseparable from politics and the media so there is a need to follow strict ethical guidelines and scientifically rigorous evaluation methods when dealing with such crops, to act with absolute transparency and to ensure the peer review system is closely monitored (Arjó et al., 2013).

It is remarkable that since the work described in this thesis began, EFSA has updated the safety assessment guidelines for transgenic crops four times (EFSA, 2010; EFSA, 2011a; EFSA, 2011b; EFSA, 2013). Even so, the EU has the most restrictive legislation in the world governing the “deliberate release” of transgenic plants. Only one additional crop (other than MON810 expressing Bt) has been approved in the last 14 years, and even this was intended for industrial use rather than human consumption (Amflora potato, event EH92-527-1), perhaps because the political consequences of positive approvals are too great (Masip et al., 2013). The current safety assessment procedure for new genetically engineered crops can cost up to €11 million and is described below (Masip et al., 2013; Kalaitzandonakes et al., 2007) . It is interesting to note that the EU applies different standards for the cultivation and import of transgenic crops (see Masip et al., 2013).

1.5. Safety assessment of genetically engineered crops

1.5.1. Safety assessment of new plant varieties

The creation of risk assessment guidelines has been discussed by scientific organizations such as the FAO (Food and Agriculture Organization, of the United Nations), WHO (World Health Organization), OECD (Organization for Economic Co-Operation and Development), EU (European Union) and ILSI (International Life Sciences Institute), and in other contexts, since foods derived from genetically engineered crops were first produced (Kuiper et al., 2001; König et al., 2004).

The first guidelines discussed the regulation of genetically engineered microorganisms and plants in the environment without any mention of food safety. The concept of substantial equivalence was introduced by the OECD in 1993, referring to appropriate comparators between genetically engineered plants and their conventional counterparts. When substantial equivalence exists in a novel product, it is considered as safe as its unmodified counterpart according to WHO and FAO (Kuiper et al., 2002). This inherently involves the comparison of genotypic, compositional and phenotypic

variations between the novel and parental varieties grown under the same conditions (Kok et al., 2008).

ILSI FAO/WHO guidelines therefore recommend that safety evaluation should be based on the concept of equivalence, according to parameters such as nutritional, phenotypic, molecular, toxicological and allergenic characteristics (FAO/WHO, 1996; Jonas et al., 1996). In 2003, the Codex Alimentarius published guidelines to develop risk assessment tests for genetically engineered organisms (FAO/WHO, 2003), and in 2004, an EU consortium comprising 60 members from 14 different European countries (Entransfood, http://www.entransfood.com/) published a paper listing strategies and methodologies to evaluate unintended effects and potential gene transfer consequences (Cellini et al., 2004; Kuiper, 2004; van den Eede et al., 2004). Hazard identification tests in this approach were based on substantial equivalence, analyzing the introduced gene and protein sequences (*in silico* analysis), testing to ascertain characteristics of novel sequences (mode and insertion site) and the phenotypic and compositional characteristics including key macronutrients, micronutrients, antinutrients and natural toxins (Kok et al., 2008). Currently such parameters are tested by DNA/mRNA microarray hybridization, event-specific polymerase chain reaction (PCR) detection methods and other specific techniques (Magaña-Gómez and de la Barca, 2009).

In 2006, OECD described procedures for the analysis of key plant components (nutrients and anti-nutrients) to screen for potential unintended effects in genetically engineered plants, including assays for toxicity, allergenicity, potential for gene transfer to non-target organisms and other unintended effects which are not usually defined a priori (OECD, 2006).

1.5.2. Regulatory issues concerning genetically engineered plants

In the EU, there are specific regulations (Regulation 1829/2003) describing the legal basis of the market approval process for genetically engineered foods/feeds, ingredients, additives or enzymes. The European Commission is advised by EFSA and member states, and for this purpose EFSA developed a “Guidance document for the

risk assessment of genetically modified plants and derived food and feed" based on comparative safety evaluations of genetically engineered plants and the corresponding unmodified parental lines (EFSA, 2006). This has recently been replaced with "Guidance for risk assessment of food and feed from genetically modified plants" which brings the rules concerning field trials, toxicological assessments and whole food/feed animal feeding studies up to date (EFSA, 2011a).

In the US, the requirement is that genetically engineered crops have equivalent compositional and nutritional characteristics to their non-engineered counterparts, except for any specifically introduced traits. The US Environmental Protection Agency (EPA) is responsible for the safety assessment of plants improved by pesticide proteins, and among other parameters the regulations require an assessment for allergenicity as mandated in the Federal Insecticide, Fungicide, and Rodenticide Act. The United States of America (USA) Food and Drug Administration (FDA) is responsible for the assessment of any remaining traits in genetically engineered plants (Kok et al., 2008).

The following section describes the tools currently used to evaluate the safety of food derived from genetically engineered crops. This information suggests strategies for the application of experimental procedures to evaluate the safety of such products.

1.5.3. Current comparative safety assessment methods for plant-derived foods

Molecular analysis. Unlike other authorities, the EU requires the analysis of genetically engineered plants according to the defined site of transgene insertion and the surrounding sequence to rule out mutational effects (Kok et al., 2008). Event-specific PCR detection methods are therefore used to identify and quantify genetically engineered plants (Kong-Sik Shin et al., 2013; Yang et al., 2013) .

Compositional characterization. Here the aim is to analyze key plant components (nutrients, anti-nutrients and toxins) phenotypic traits and compounds that are altered intentionally by genetic engineering. Additional data can be obtained by growing the genetically engineered plant (and its unmodified counterpart) under diverse field conditions and in different growing seasons (FAO/WHO, 1996; OECD, 1996; EFSA,

2011a). The ILSI Crop Composition Database shows compositional data from different crops grown in different countries (www.cropcomposition.org).

Toxicology. Internationally agreed protocols for the assessment of toxicology data are described in the OECD guidelines for the testing of chemicals (OECD, 1998; OECD, 2008; OECD, 2009) and in European Commission Directives on dangerous substances (EC, 2002). Strategies to assess toxicity should be chosen depending on the properties and function of the introduced protein, and the history of human or animal consumption. If both the newly synthesized protein and the plant have a history of safe consumption by humans and animals, then toxicity tests are not required at all (EFSA, 2011a).

Laboratory animals are recognized as good models for the prediction of toxicity in humans. EFSA recommends a 28-day oral toxicity study in which the introduce proteins are fed to rodents (OECD, 2008) plus additional tests as required by the initial results, e.g. immunotoxicity assessment (EFSA, 2011a).

EFSA also recommends a whole-food 90-day toxicity study in rodents if the composition of the transgenic crop is changed substantially or if there is any indication of potential unintended effects. A minimum of two test doses is recommended. It is important to be diligent in dose selection and to avoid nutritional imbalances and at least two dose levels are required for the genetically engineered food and comparator (at least the highest dose possible without producing a nutritional imbalance). Other animal species may be assessed to provide complementary data, e.g. broiler chicks or lambs (EFSA, 2008; EFSA, 2011a; EFSA, 2011b).

OECD test guidelines 407 (28-day oral toxicity study in rodents) and 408 (90-day oral toxicity study in rodents) are examples of repeated-dose sub-chronic tests. The aim in such studies is to determine adverse effects by repeated exposure to drugs, chemicals, food additives or food compounds for a period of one month or longer. The protocols are applied in young animals and are designed to reveal signs of toxicity in organs, tissues and cells. They provide information for a period sufficient to observe major toxic effects without age-associated changes in tissue morphology or function (EFSA, 2008).

EFSA recently published guidelines for whole-food/feed chronic toxicity and/or carcinogenicity studies in rodents, adapted from OECD TG 453 which was developed for chemicals (EFSA, 2013). These studies assess chronic effects over a long period (up to and including the entire life of the test animal) to identify any potential chronic toxicity or carcinogenicity effects of defined substances. Additional studies may assess reproductive and developmental toxicity (EFSA, 2008).

Direct immunotoxicity tests focus on the suppression or dysfunction of immune system components resulting in allergic or autoimmune reactions. OECD guidelines 407 and 408 as discussed above provide preliminary immunotoxicity data because they require hematological analysis (including differential cell counting, bone marrow analysis and serum immunoglobulin levels), weight measurements and histology of lymphoid organs and tissues. The immunotoxicity of food in the gut can be investigated by analyzing Peyer's patches and mesenteric lymph nodes, whereas systemic effects can be investigated by weighing and analyzing the thymus, spleen and distant lymph node. The accuracy of histopathology can be complemented by using techniques such as immunocytochemistry and flow cytometry (EFSA, 2008).

Nutritional evaluation. Two European initiatives (the EuroFIR program and the FAOSTAT database) describe macronutrients, micronutrients and bioactive components in food, and list consumption data for different countries. They are useful as reference databases for the assessment of food derived from genetically engineered crops (Kok et al., 2008). Nutritional assessment should demonstrate that such engineered food or feed products are no less nutritious than the equivalent unmodified crops. Studies in rodents, poultry and livestock have been published to assess the nutritional characteristics of several genetically engineered plants. The bioavailability and biological efficacy of nutrients in genetically engineered plants should also be considered, because they affect the feed conversion rate in animals (EFSA, 2011b).

Allergenicity. There are several guidelines describing food allergenicity assays (Metcalfe et al., 1996; FAO/WHO, 2001). The introduced protein should be considered in terms of the source organism, sequence/structural homology with known allergens (*in silico* analysis), digestibility, thermostability, IgE immunoaffinity tests and studies in

animal models (Goodman et al., 2008; Kok et al., 2008). These aspects are summarized in Figure 1.2.

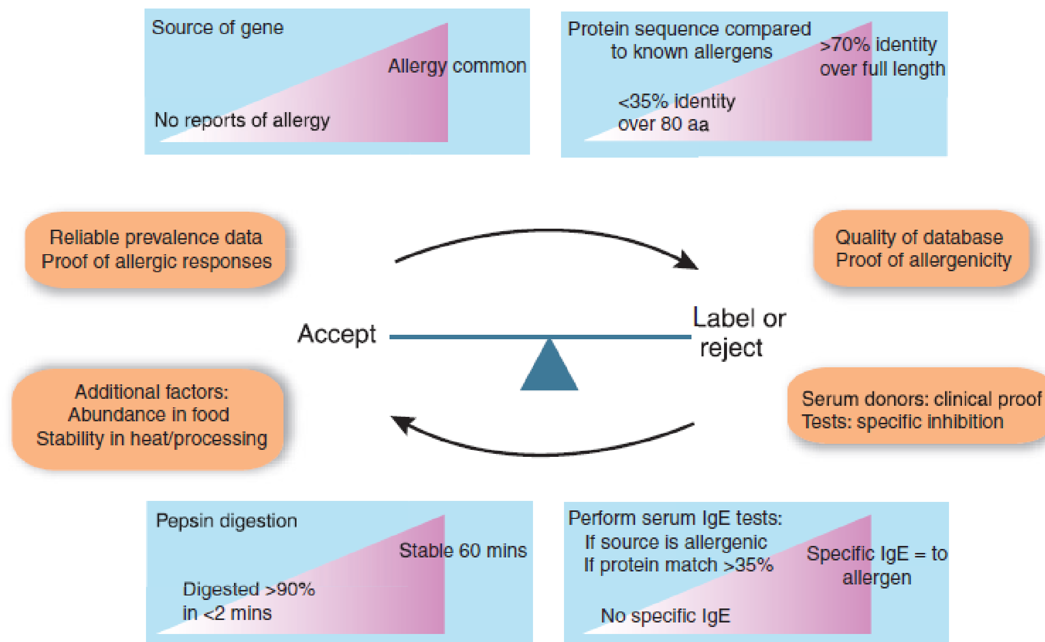

Figure 1. 2. Schematic representation of major allergenicity assessment areas, showing high risks (on the right) and lower risks (on the left) (Goodman et al., 2008).

The amount of recombinant protein present in foods derived from genetically engineered plants, and the ability of such proteins to withstand cooking, may influence their resistance to digestion and hence their likelihood of interacting with the intestinal mucosa. Most allergenic proteins in food are absorbed in the intestine, and patients sensitive to specific allergens may experience symptoms if they are exposed to proteins with similar structures, because they will cross-react with allergen-specific IgE (Herman et al., 2009).

The aim of allergenicity studies is therefore to minimize the likelihood that a novel food product is more allergenic than its conventional counterpart. One example of such an abandoned product was a genetically engineered soybean variety developed by Pioneer Hi-Bred International, which contained the Brazil nut 2S albumin. A serum IgE test was positive for individuals with Brazil nut allergies, and further development was discontinued (Herman et al., 2009).

If the source of the introduced transgenes is a known allergenic plant, or if the product is related to a respiratory allergen or a contact allergen such as latex, then an immunoafinity IgE test with serum from allergic patients should be performed, taking into account demographic and age factors, to rule out allergies to specific recombinant proteins in transgenic plants. Such studies require large amounts of human serum to generate statistically significant results, and sometimes it is difficult to find enough people allergic to specific foods to enable such tests (Goodman et al., 2008).

Bioinformatics analysis can be carried out by searching for amino acid sequence similarity between the recombinant protein and known allergens using algorithms such as FASTA or BLAST (Table 1.1). Following preliminary assessment, it is necessary to determine whether or not the protein has any potential to induce cross-reactivity with known allergens and thus induce allergic responses (Goodman et al., 2008).

Table 1. 1. Sequence identity over an 80-amino-acid window between a recombinant protein and an allergen identified by BLAST/FASTA alignment, thus requiring serum affinity analysis (Goodman et al., 2008).

Sequence identity percentage	Potential for cross-reaction	Need for IgE affinity test
> 70%	high	yes
50%-70%	moderate	yes
50%-35%	low	yes
<35%	inexistent	no

Two methods are used to evaluate structural similarity between allergens and recombinant proteins in genetically engineered plants. The method shown in Table1. 1 uses an 80-amino-acid window, based on the typical sequence length of a protein epitope recognized by antibodies. Other criteria include short peptides (6–8 residues)

based on theoretical B-cell and T-cell epitope sequences, but these are more susceptible to false positive matches (Cressman and Ladics, 2009; Herman et al., 2009).

The last and most definitive test to determine allergenicity is the serum affinity test. Serum IgE from allergic donors is tested by enzyme-linked immunosorbent assay (ELISA) and immunoblot using recombinant proteins from genetically modified plants to assess potential cross-reactivity. Ideally, the assay is carried out using a positive control comprising serum from patients known to be allergic to proteins from the transgene source or proteins that are similar to the transgene product, and a negative control comprising serum from donors allergic to non-related proteins (Goodman et al., 2008). However, these tests are complex and the results are often ambiguous. It is often unclear whether the tested protein is structurally identical to its naturally occurring counterpart, if the amount of protein used in the tests is the same as that found in the food, and whether the number of serum donors is statistically significant (Goodman et al., 2005; Goodman et al., 2008; Selgrade et al., 2009).

As mentioned above, if a protein is not digested in the alimentary canal it may be allergenic. Most proteins in food are digestible but certain protein allergens are stable when exposed to pepsin *in vitro*. Some non-allergenic proteins are also stable (Thomas et al., 2004; Goodman et al., 2008). Digestibility tests include assays with different digestion times in a liquid medium which simulates gastric (pepsin) and intestinal (pancreatin) fluids at the correct pH and temperature. It is important to consider the amount of the tested protein found in food because more abundant proteins are likely to survive digestion and are more likely to be allergenic. Currently, there are no scientifically objective criteria to assess such abundance data (Goodman et al., 2008) but EFSA proposes this as an additional criterion to be considered during the safety assessment (EFSA, 2010; EFSA, 2011a).

The majority of food allergens do not lose allergenicity when heated. Indeed, they can even become more allergenic due to covalent modifications caused by the Maillard reaction or proteases. In heat-stable allergens, the epitope recognized by IgE maintains its native structure when the protein is heated due to the presence of disulfide bonds and hydrophobic interactions. Heat stability is therefore an important part of safety assessment because processed foods are nearly always subjected to some form of

thermal treatment, e.g. oven roasting and frying or wet heating methods such as boiling and microwave cooking (Goodman et al., 2005).

FAO/WHO (FAO/WHO, 2001) recommend that novel foods are evaluated in two different species using two protein administration routes in each species. There are no standardized animal models to assess food allergies, even though many models have been used for this purpose (Singh et al., 2006; Wang et al., 2003; Singh et al., 2009). Many factors appear to influence the outcome of such tests, including genetic diversity that predisposes some individuals to allergy, genetic differences between humans and animal models, and the administration of sensitization doses (FAO/WHO, 2001). EFSA considers it useful to provide additional information although recognizes that *in vitro* cell models and animal models have not been validated (EFSA, 2011a).

Gene transfer. Horizontal gene transfer is the potential for genetic material to be transferred from one living cell or organism to another. Interspecies horizontal gene transfer has not been demonstrated between transgenic plants and microbes despite many efforts (Ramessar et al., 2007). Several studies have been carried out to evaluate this phenomenon, all of which emphasized the challenges of protecting the test tissue from contamination (Mazza et al., 2005; Nielsen et al., 2005).

EFSA initially recommended that the risk of homologous recombination with intestinal microflora should be investigated (EFSA, 2006). This has been dropped from the updated guidelines, although there is a recommendation to evaluate introduced DNA for the presence of replication origins and related sequences (EFSA, 2011a).

Post-market monitoring. Finally, although post-market monitoring should not replace pre-marketing safety assessments, such monitoring might complement and confirm the results of pre-market risk assessments (Kok et al., 2008).

1.6. High-carotenoid corn

1.6.1. Carotenoids

Carotenoids are C_{40} lipid-soluble pigments produced by plants, fungi, algae, bacteria and archaea. They play an important role in light perception during photosynthesis and also protect the photosynthetic apparatus from photo-oxidation (Naqvi et al., 2009). More than 750 carotenoids have been identified, although only 50 are typically found in the human diet, and only 10 can be detected in human blood, including α-carotene, β-carotene, lycopene, lutein, zeaxanthin and cryptoxanthin (Gerster, 1997). Humans need carotenoids as metabolic precursors, antioxidants and to prevent diseases such as cancer, blindness and immune system abnormalities. The only source of carotenoids for humans and many other animals is the diet because these molecules cannot be synthesized *de novo*. Fruits and vegetables are rich in carotenoids, but cereals which constitute the staple diet in most developing countries lack these metabolites because the metabolic pathway does not exist, or is truncated or inhibited in plant tissues (Zhu et al., 2008; Naqvi et al., 2009).

Lycopene, which gives red fruits and vegetables their color, protects cells against oxidative stress, and reduces the risk of cardiovascular diseases, cancer and degenerative disorders (Di Mascio et al., 1989). Xanthophylls such as lutein and zeaxanthin protect the retina against age-related macular degeneration and help to prevent the formation of cataracts (Snodderly, 1995; Agte and Tarwadi, 2010). The most abundant carotenoid in nature is β-carotene, and this is also the major source of vitamin A in the human diet. The absorption and metabolism of provitamin A carotemoids takes place in the intestine, and these molecules are predominantly stored in adipose tissue and in the liver as retinyl esters. If β-carotene is not metabolized, it is deposited in the epidermis. Vitamin A is necessary for night vision, the differentiation and proliferation of cells, embryonic development and to maintain correct immunological and reproductive functions (Niles, 2007; Fierce et al., 2008).

Ketocarotenoids are produced in algae, fungi and bacteria, but only rarely in plants. They contain at least one keto group in the linear chain or on one of the β-ionone

rings. Astaxanthin is a ketocarotenoid with potent antioxidant activity, and is often present in marine animals (such as salmon and prawn) and in birds (flamingos and quails). These animals cannot synthesize astaxanthin but they obtain it from their diet (Naqvi et al., 2009).

1.6.2. Retinoids

Carotenoids are absorbed in the gut and are incorporated into the liver and adipose tissue. The pro-vitamin A carotenoids (α-carotene, β-carotene, γ-carotene and β-cryptoxanthin) are converted to retinal (the reduced form of vitamin A) by the enzyme 15,15'-oxygenase (BCO1) (Figure 1.3). Retinal is then converted into retinol which can be converted into retinoic acid by oxidation (Bachmann et al., 2002; Tourniaire et al., 2009; Takaichi, 2011; Pérez-Massot et al., 2013).

Retinoids are metabolic regulators that activate specific nuclear receptors such as the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). RXRs act as partners to other nuclear receptors such as the vitamin D receptor, the thyroid hormone receptor, the liver X receptor, and the peroxisome proliferator-activated receptor (PPAR), and induce multiple biological effects. RXRs and RARs can also act as a heterodimerization partners for transcription factors and thus influence cell growth, differentiation, and apoptosis (Germain et al., 2006; Tourniaire et al., 2009).

The symmetric cleavage of β-carotene produces two retinaldehyde molecules which can subsequently be reduced to retinol or though the action of retinaldehyde dehydrogenases can produce two retinoic acid molecules (Ziouzenkova and Plutzky, 2008). However, the asymmetric cleavage of β-carotene by β-carotene 9',10'-oxygenase 2 (BCO2) leads the formation of β-apo-10'-carotenal and β-ionone, which can act as RAR and PPAR antagonists thus exerting anti-vitamin A activity and transcriptional repression (Ziouzenkova et al., 2007; Ziouzenkova and Plutzky, 2008; Eroglu et al., 2012; Shete and Quadro, 2013).

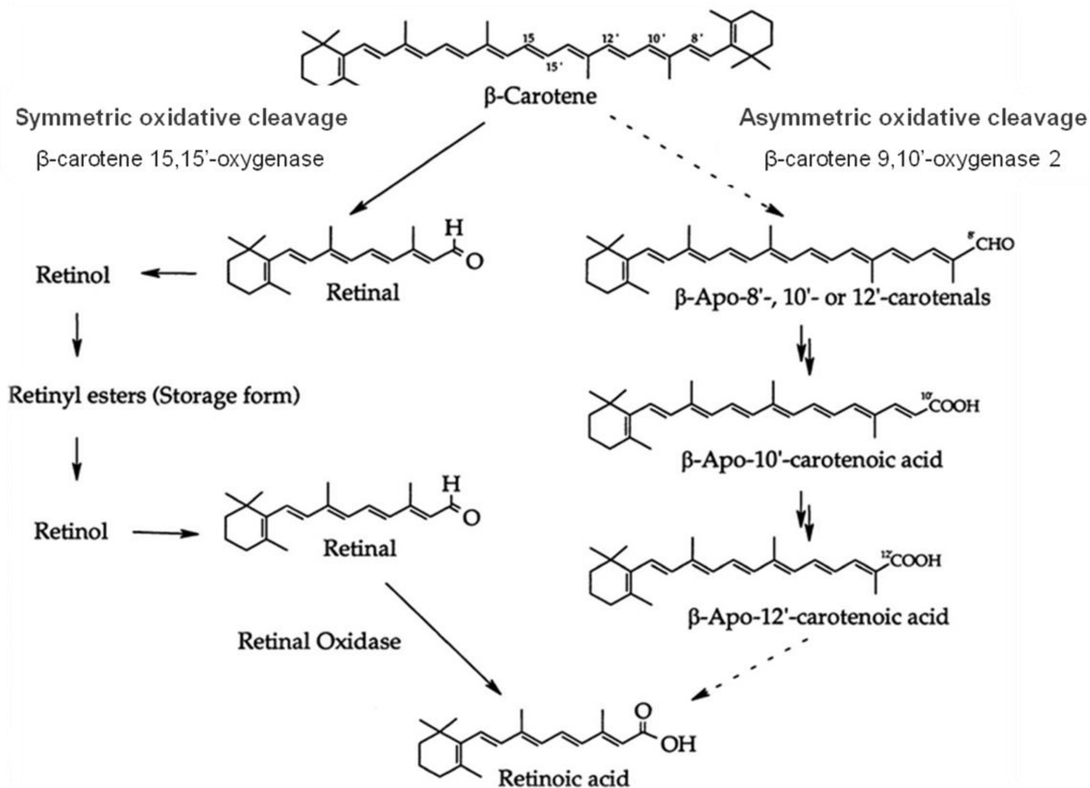

Figure 1. 3. Pathways from β-carotene to retinoic acid in animals and humans. Symmetric (central) oxidative cleavage of β-carotene (BCO1) generates two molecules of retinaldehyde (retinal) which can be either reduced to retinol or oxidized into retinoic acid. Moreover, apocarotenals can be generated from β-carotene by asymmetric cleavage catalyzed by β-carotene 9',10'-oxygenase 2 (BCO2) which leads to the formation of β-apo-10'-carotenal and β-ionone. β-apo-carotenal that can be oxidized to β-apo-carotenoic acids and then could be cleaved by BCO1 to yield retinal (however, the mechanisms of this conversion has not been completely known) which can be oxidized to retinoic acid (Shete and Quadro, 2013). Adapted from Bachmann et al., 2002.

1.6.3. Genetically engineered high-carotenoid corn

This study considers the evaluation of a genetically engineered corn variety (High-carotenoid) produced by the Applied Plant Biotechnology research group in the Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida (Naqvi et al., 2009). High-carotenoid corn is a carotenoid rich corn variety (Figure 1.4), engineered to express the *Zea mays* phytoene synthase gene (*psy1*), the *Pantoea ananatis* carotene desaturase gene (*crtI*), the rice dehydroascorbate reductase gene (*dhar)* and the *Escherichia coli folE* gene encoding guanosine triphosphate (GTP) cyclohydrolase (GCH1). Particle bombardment was used to transform 10–14-day-old

immature zygotic embryos[1] with five genetic constructs containing the four metabolic genes listed above and the selectable marker gene *bar*[2]. All four metabolic genes were expressed specifically in the endosperm using the wheat low-molecular-weight (LMW) glutenin promoter for *psy1* and the barley D-hordein promoter for the others. The selectable marker was expressed using a ubi1 promoter.

Figure 1. 4. High-carotenoid corn and its conventional counterpart M37W. Carotenoids are pigments so they are responsible for the orange color of the corn seeds.

Expressing the four metabolic genes listed above resulted in the modification of three distinct metabolic pathways, leading to β-carotene, ascorbate and folate. The pathway leading to β-carotene is shown in Figure 1.5. The genes were expressed in the South African white corn variety M37W, which lacks the corresponding pathways in the endosperm. This genetically engineered corn line produced 169-fold the normal amount of β-carotene, 6-fold the normal amount of ascorbate, and double the normal amount of folate (Naqvi et al., 2009).

[1] Immature zygotic embryos can give rise to undifferentiated cells in vitro with the capacity to differentiate into tissues and organs. They are isolated from corn seeds 10-14 days after pollination, and

[2] This is a herbicide-resistance gene from *Streptomyces hygroscopicus* which confers resistance to the herbicide bialaphos (active ingredient, phosphinothricin).

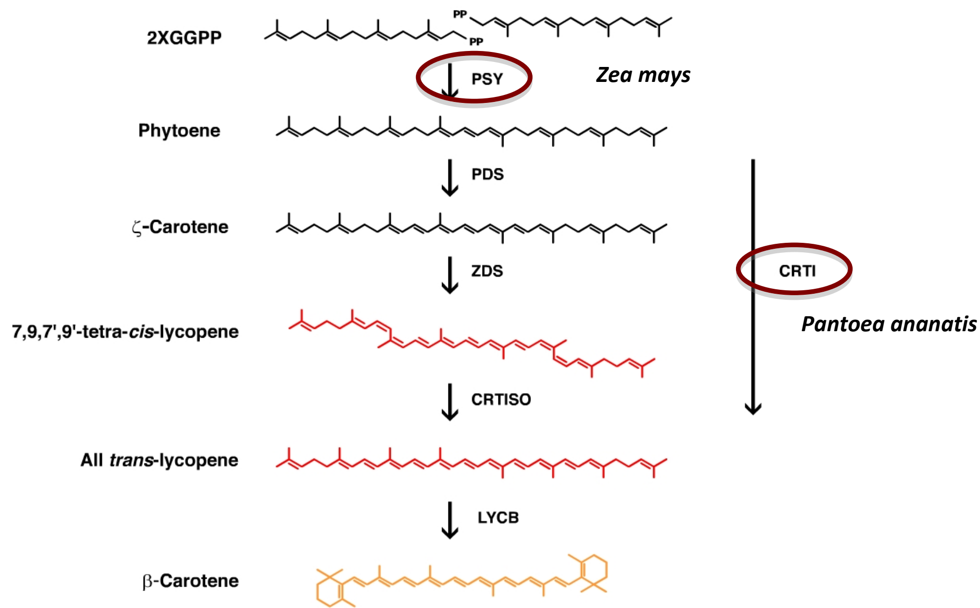

Figure 1. 5. Metabolic pathway leading to β-carotene, which is absent in the M37W corn variety because PSY is not expressed in the endosperm. ZmPsy1 and PaCrtI were introduced into M37W to create the high-carotenoid corn (Adapted from Naqvi et al., 2009).

Putative transformants were screened by genomic PCR using sets of three primers for each gene, resulting in overlapping PCR products that confirmed the transgenes were intact. This ruled out the presence of truncated or rearranged copies. Transgene expression was confirmed by mRNA blot analysis in immature kernels (Naqvi et al., 2009).

The seeds of the transgenic corn were orange in color, contrasting with the white color of the parental M37W variety reflecting the lack of carotenoids in corn varieties that are typically grown in sub-Saharan Africa for human consumption. Quantitative analysis of the carotenoid content was carried out by high-performance liquid chromatography (HPLC) showing that the total carotenoid content had increased 112-fold compared to the wild-type M37W. The high-carotenoid endosperm accumulated 60 µg of β-carotene, 23 µg of lycopene, 36 µg of zeaxanthin, 15 µg of lutein, 13 µg of α-cryptoxanthin, 7 µg of α-carotene, 5 µg of β-cryptoxanthin and 5 µg of γ-carotene per gram of dry seed weight (dsw). Furthermore, ascorbate levels increased to 110 µg/g dsw and folate levels to 1.94 µg/g dsw (Naqvi et al., 2009).

An intake of 100–200 g of high-carotenoid corn per day would provide the full recommended dietary intake (RDI) of β-carotene as a precursor of vitamin A (Naqvi et al., 2009).

1.7. Animal models

Animal models can contribute to the knowledge of cellular and molecular pathogenesis of human diseases and disorders. There is a broad range of available animals models: fly, zebrafish, mouse, rat, etc., (Lieschke and Currie, 2007). Focusing on mouse models, which is the animal of choice employed in the different experiments of this thesis, it has been used in research of several organs and for disorders and diseases: cardiovascular, urinary tract, kidney, viral, neural, cancer, obesity, metabolic, liver, etc. (Turner, 2013).

Mouse models useful for cancer research are: chemically-induced models, xenograft models and genetically engineered models. Chemically-induced mice are injected with carcinogenic compounds which initiate the tumor formation commonly by inducing structural DNA changes. They are useful for understanding the molecular mechanisms in sporadic cancers. In xenograft models, the tumors are formed by injecting human cancer cells into immune deficient mice, either subcutaneously in the flank of mice (ectopic) or directly into the target tissue (orthotopic) and are t used for drug development and the study of metastasis (Heindryckx et al., 2009; Taketo and Edelmann, 2009). Finally, genetically engineered mice give the opportunity to investigate carcinogenesis pathways in detail (Heindryckx et al., 2009).

Rodent models commonly used to better understand the mechanisms of human obesity and metabolic syndrome are induced with: high-fat diets, high-carbohydrate diets or a combination of the two, diets with high content of fructose or sucrose. Many more dietary components have been used to simulate the signs and the symptoms of metabolic syndrome (Panchal and Brown, 2010). Moreover, genetically engineered diabetic mice, chemically induced and genetic models (i.e. animals with autosomal recessive mutations in the leptin gene) are also employed (Panchal and Brown, 2010).

Whatever animal model is chosen to carry out experimental investigations, animal welfare, humane endpoints, and the application of the 3R principle (replacement: of animal with alternative methods if possible, reduction: in the number of animals used, and refinement: of methods to minimize animal suffering) should be considered and implemented in the design and the development of experiments (Russell and Burch, 1959; Workman et al., 2010)

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2. AIMS AND OBJECTIVES

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Aims

The overall aim of the work described in this thesis was to assess the safety of a carotenoid-enriched engineered corn line (high-carotenoid corn) using its near-isogenic unmodified counterpart M37W as a comparator in a subchronic toxicity study, and to explore the efficacy of the same engineered corn line in terms of its potential benefits in several mouse models of chronic disease.

Objectives

1. To investigate potential unintended effects of high-carotenoid corn in animal feeding experiments designed to evaluate subchronic toxicity.
2. To investigate the potential chemopreventive effects of high-carotenoid corn on an induced colitis-associated mouse cancer model.
3. To investigate the potential modulatory effects of high-carotenoid corn on insulin sensitivity and energy metabolism in a mouse model of obesity and insulin resistance generated by initial feeding on a high-fat diet.
4. To investigate the potential effects of high-carotenoid corn on heterozygous PTEN knockout mice (PTEN $^{+/-}$) as a model of multiple-organ neoplasia and liver disease.

3. EXPERIMENTAL CHAPTERS

CHAPTER 1

Mice fed on a diet enriched with genetically-engineered high-carotenoid corn exhibit no subchronic toxicity

CHAPTER 1

Mice fed on a diet enriched with genetically-engineered high-carotenoid corn exhibit no subchronic toxicity

3.1.1. INTRODUCTION

Up to 50% of the world's population suffers from multiple micronutrient deficiencies reflecting an over-dependence on foods that lack essential vitamins and minerals (Farre et al., 2010a, Farre et al., 2011a). As it was commented before, strategies that tackle micronutrient deficiency at source by creating nutrient-rich crops are known as biofortification approaches (Farre et al., 2011b). These should be targeted at staple crops such as rice and corn, which are the primary source of calories for more than 75% of the human population (Food and Agriculture Organization, 2009).

Genetic engineering is the most versatile approach for biofortification (Farre et al., 2011b). There have been many reports of genetically engineered staple crops accumulating high levels of vitamins (reviewed in Zhu et al., 2008; Farre et al., 2010b; Bai et al., 2011). Most of these enhanced varieties are still at the laboratory testing phase or in early field trials, but at least one is very near to broad release. This is Golden Rice which is engineered with two genes conferring the ability to synthesize β-carotene (pro-vitamin A) in the endosperm (Ye et al., 2000; Paine et al., 2005). The advantage of high-carotenoid corn is that it addresses multiple nutrient deficiencies simultaneously and as such it represents a "next generation" nutritional intervention.

There are two potential solutions to this challenge, the first to develop nutritionally enhanced staples that provide such high levels of a key target nutrient that a very small portion offers the full dietary reference intake. Mixed feed of different enhanced staples could then provide a full nutritional complement. This is unlikely to be practical because it would rely on the simultaneous availability of many varieties as well as user compliance to ensure the correct consumption ratio to achieve nutritional completeness while avoiding nutrient toxicity. The second solution is to develop staple crops simultaneously enhanced with different nutrients, providing a balanced and

nutritionally complete meal in a manageable portion. Such crops would be all but impossible to generate by conventional breeding even if sufficient genetic diversity were available, because many different traits would need to be targeted simultaneously (Naqvi et al., 2010). We have begun this process by developing a prototype high-carotenoid corn simultaneously engineered to accumulate high levels of β-carotene and other carotenoids (Zhu et al., 2008; Naqvi et al., 2009). These are biologically relevant levels, taking into account that 100-200g of high-carotenoid grain provides the full recommended daily intake (RDI) of β-carotene (provitamin A).

Genetic engineering provides the only practical approach to develop nutritionally complete staples but one limitation of genetically engineered crops is the onerous regulatory environment (Ramessar et al., 2010; Sabalza et al., 2011). Such crops must undergo extensive tests that are not required of conventional varieties even if they are genetically identical, because the trigger for testing is the process used to generate the crops not the actual product (in Europe and many developing countries). As it was stated before, the assessment of nutritionally-enhanced varieties created by genetic engineering must include (among other tests) compositional analysis, laboratory feeding trials in animals to test subchronic toxicity as well as tests for allergenicity and nutritional assessments (Kuiper et al., 2001; Konig et al., 2004).

The aim of 90-day subchronic toxicity evaluation is to determine any adverse effects caused by repeated exposure over a longer period. These assessments involve young animals and are designed to reveal signs of toxicity in organs, tissues and cells over a period long enough for major toxic effects to become apparent without any age-associated change in tissue morphology or function (van Haver et al., 2008). In the case of genetically engineered plants, the evaluations are based on substantial equivalence or comparative assessment with conventional varieties of the same crop.

As part of the development of our high-carotenoid corn, we therefore compared the proximates composition of high-carotenoid corn and non-transgenic corn diets and we carried out a 90-day subchronic feeding study, again with no indications of toxicity compared to mice fed on control diets.

3.1.2. MATERIALS AND METHODS

3.1.2.1. Plant material

High-carotenoid corn was created by the stable transformation of the South African inbred M37W with corn *psy1* (phytoene synthase), *Pantoea ananatis crt*I (carotene desaturase), rice *dhar* (dehydroascorbate reductase) and *Escherichia coli fol*E (GTP cyclohydrolase) under the control of various endosperm-specific promoters, together with the selectable marker gene *bar* (Naqvi et al., 2009). The corn used for the feeding experiments was from the T7 homozygous generation.

3.1.2.2. Compositional analysis of experimental and control diets

High-carotenoid and control corn plants were grown in the same greenhouse under exactly the same conditions over the same growing period. Carotenoid content was measured in a minimum of 10 individual plants by HPLC analysis and data were analyzed by Analysis of Variance (ANOVA).

Experimental diets containing high-carotenoid corn and control diets containing wild-type (WT) M37W corn were prepared from freeze-dried powdered kernels under hygienic conditions. Meals were prepared by mixing 2014 Teklad Global 14% Protein Rodent Maintenance Diet (Harlan Laboratories, Madison, USA) with the appropriate freeze-dried corn powder as a 60:40 ratio (w/w).

The composition of the corn was compared by quantifying basic chemical parameters such as moisture, fat, ash, crude fiber and protein, as well as the target micronutrient levels of carotenoids (AOAC International, 2000). Moisture was obtained as the loss of weight after drying in an oven at 100ºC to a constant weight. Fat content was estimated using Soxhlet extraction. Ash levels were estimated by gravimetric analysis after ignition in an electric furnace. Crude fiber was determined from the difference between the weight of the residue remaining after the sample was digested under specific conditions and the weight of the ash. Protein was estimated through total

nitrogen content by the Dumas combustion method. Protein content was calculated by applying a nitrogen-to-protein conversion factor of 6.25.

The β-carotene, ascorbate and folate levels were determined as described in Naqvi *et* al., 2009.

3.1.2.3. Animal feeding studies

The experimental design was adapted from OECD guidelines 408 and 409 for the 28 and 90-day studies respectively, following EFSA recommendations (European Food Safety Authority, 2006). The study complied with Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat) of Catalonia and EU Directives (EEC 63/2010), and was approved by the Ethics Committee on Animal Experiments of the University of Lleida.

Male and female albino BALB/c mice obtained from Harlan Laboratories Models S.L. (Sant Feliu de Codines, Spain) were acclimated in individual cages one week before the experiment. The mice had *ad libitum* access to a standard 2014 Teklad Global 14% Protein Rodent Maintenance Diet and water. The animal rooms were environmentally controlled and maintained at 20 ± 2ºC, relative humidity 50 ± 5%, with a 12-h photoperiod.

Following acclimation, 6-week-old animals were randomly assigned to three groups of 12 (six male, six female) excluding animals deviating significantly from the group mean body weight ($p < 0.05$). Fresh food was supplied 2–3 times per week as required, for 90 days. The reference group continued to be fed with the standard 2014 Teklad Global 14% Protein Rodent Maintenance Diet after acclimation. The control group was fed with a 40:60 mixture of powdered wild-type corn kernels and the reference diet. The experimental group was fed with a 40:60 mixture of powdered high-carotenoid corn kernels and the reference diet.

3.1.2.4. Clinical observations

Clinical observations were recorded before the experiment and daily while the experiment was in progress. No moribund or dead animals, abnormal behavior, abnormal appearance or abnormal droppings were observed. We also measured the feed intake and body weight of all mice on a weekly basis.

We carried out biochemical and hematological tests on blood samples taken from the submandibular vein while the animals were under isoflurane anesthesia preceding the terminal sacrifice. Whole blood was collected in a tube containing Ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and plasma was prepared by centrifugation at 1500 x g for 10 minutes prior to the analysis of alanine aminotransferase (ALT), total cholesterol, total protein, albumin and blood urea nitrogen (BUN) using a Hitachi Modular analyzer (Roche, Badalona, Spain). We also measured the white blood cell count (WBC), differential white blood cell count of neutrophils, lymphocytes, monocytes, eosinophils, basophiles, red blood cell count (RCB), hemoglobin (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) using a Sysmex XE-5000 analyzer (Roche, Badalona, Spain).

We also carried out autopsies on all animals, and determined the weight of adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus and uterus (paired organs were weighed together). The adrenals, aorta, cecum, cervix uteri, colon, duodenum, epididymides, eyes, heart, ileum, jejunum, kidneys, liver, lungs, esophagus, ovaries, pancreas, Peyer's patches, prostate, rectum, sciatic nerve, seminal vesicle, spinal cord, spleen, sternum (bone marrow), stomach, bone and muscle (from femur and quadriceps), testes, thymus, thyroid/parathyroid, trachea, urinary bladder, uterus, vagina, biliar vesicle and skin were then fixed, dehydrated, embedded in paraffin, sectioned (4–5 µm) and stained with hematoxylin-eosin. The adrenals, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pancreas, spleen, testes, thymus, thyroid/parathyroid and uterus of all animals were subject to histopathological analysis as these represent the major target organs for toxicity effects, and the

remaining samples were reserved for analysis in the event of abnormal histopathology of these initial specimens.

3.1.2.5. Statistical analysis

We compared animal weights, feed consumption, biochemical data, hematological data and organ weights between the high-carotenoid corn diet group and the reference diet group, and (separately) between the high-carotenoid corn diet group and the control corn diet group using PASW Statistics 18 (Version 18.0.0, 2009). The data were also analyzed separately in male and female groups. Differences were considered statistically significant at $p < 0.05$.

We carried out preliminary homogeneity of variances (Levene's Test) and normality of data distributions (Q-Q plots, histograms and Shapiro-Wilk tests) on all data (continuous values). If these tests were not statistically significant, we assumed Normal distribution, and we carried out ANOVA to compare the high-carotenoid corn diet group with both controls (*t*-Student test applying the Bonferroni method of correction for multiple comparisons). If one of the preliminary tests was significant, we carried out non-parametric tests using Kruskal-Wallis for k independent samples and if this yielded a significant result, we evaluated the contrasting data case-by-case using the Mann-Whitney test. For multiple comparisons we applied the Bonferroni method of adjustment to maintain a 5% type error rate per response variable in both bivariate tests.

3.1.3. RESULTS

3.1.3.1. Compositional analysis

There were minimal differences in proximate levels between the high-carotenoid corn and non-transgenic corn which did not have any impact on nutrition (supporting information Table 3.1.1).

Table 3.1. 1. Wild-type M37W and high-carotenoid corn lines composition. [a]Values are in µg per gram dry weight (DW).

Composition (%)	Wild-type M37W corn grain	High-carotenoid corn grain
Moisture	5.21	5.89
Fat	3.75	4.36
Ash (% DW)	2.09	2.07
Crude fiber	4.05	4.58
Protein	14.62	16.75
Starch	77.5	67.8
β-carotene[a]	0.35	59.32
Zeaxanthin[a]	0.32	35.76
Lycopene[a]	0	22.78
Lutein[a]	0.57	14.68
α-cryptoxanthin[a]	-	13.42
α-carotene[a]	0.12	7.26
β-cryptoxanthin[a]	-	5.28
γ-carotene[a]	0.09	4.79

3.1.3.2. Ninety-day subchronic toxicity assessment

The animals in all three groups exhibited normal clinical signs before and during the assessment. There were no signs of abnormal appearance or behavior, no unusual droppings and no aversion to any of the diets. No mice died during the experiment.

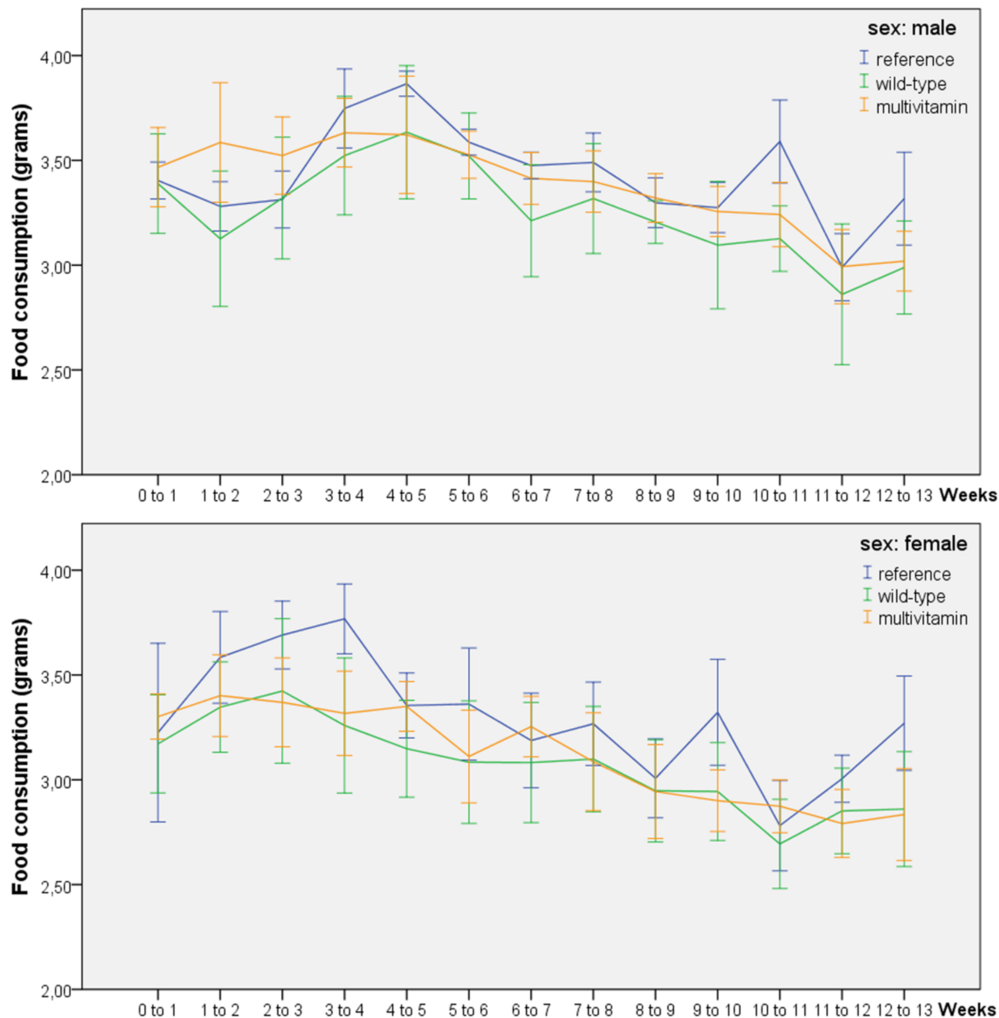

Figure 3.1. 1. Mean weekly feed consumption (mean ± CI 95%) of male and female mice. Animals were fed rodent diet (reference group), and rodent diets containing wild-type and high-carotenoid corn (multivitamin) for 13 weeks.

After 13 weeks, there was no statistically significant difference ($p < 0.05$) in either food consumption (Figure 3.1.1) or body weight (Figure 3.1.2) among the three diet groups when comparing the whole groups or individual sexes, although males fed on the high-carotenoid corn diet were on average marginally heavier (27.76 ± 0.75 g) than their counterparts in the other two groups (27.23 ± 1.01 g for the reference diet, 27.04 ±

1.13 g for the wild-type corn diet). The females in the reference, wild-type and high-carotenoid diet groups weighed 21.94 ± 1.44 g, 22.39 ± 1.42 g and 22.24 ± 0.99 g, respectively.

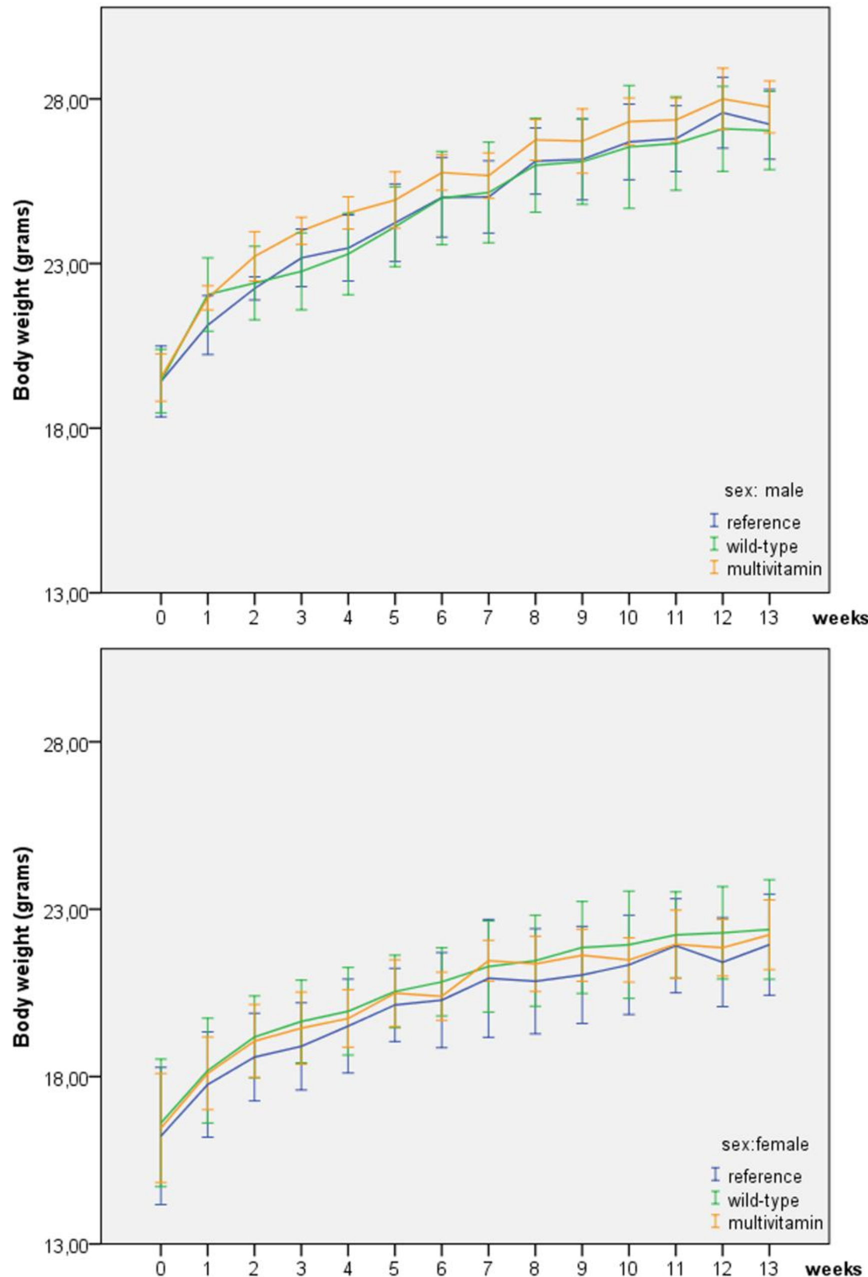

Figure 3.1. 2. Mean weekly body weights (mean ± Confidence Interval 95%) of male and female mice. Animals were fed rodent diet (reference group), and rodent diets containing wild-type and high-carotenoid corn (multivitamin) for 13 weeks.

There were no statistically significant differences ($p < 0.05$) between the high-carotenoid corn group and either of the controls in terms of biochemical markers

(Table 3.1.2 and Table 3.1.3) or hematological parameters (Table 3.1.4 and Table 3.1.5).

Table 3.1. 2. Biochemistry mean values ± SD for males (a. N=5; b. N=4). ALT: alanine aminotransferase; BUN: Blood Urea Nitrogen.

	Group		
	reference	WT	CAR
	Mean ± SD	Mean ± SD	Mean ± SD
ALT (U/l)	84 ± 27	79 ± 24[a]	132 ± 46[a]
BUN (ng/L)	0.224 ± 0.012	0.223 ± 0.035	0.215 ± 0.017[b]
Total cholesterol (mg/dl)	152 ± 24	148 ± 11	141 ± 10[b]
Total protein (g/dl)	4.9 ± 0.6	4.9 ± 0.2	4.8 ± 0.3[a]
Albumin (g/dl)	2.99 ± 0.32	3.07 ± 0.18	3.11 ± 0.23[a]

Blood samples were analyzed to determine the effect of each diet on known disease markers and to identify correlations between organ weights and histopathological findings. Because only a small amount of blood was available, we focused on a subset of the tests: ALT, alanine transaminase normally present in the liver and heart which is used to evaluate the health of those organs; BUN (blood urea nitrogen) which is a marker of kidney disease; total cholesterol, which is a cardiovascular disease risk biomarker; total proteins, which is a marker of immune system dysfunction as well as liver and kidney complications; and albumin, as an indicator of liver damage and nutritional status. One high-carotenoid group male blood sample was hemolized and other was not possible to determinable it because it was not enough amount.

Table 3.1. 3. Biochemistry mean values ± SD for females (a. N=5). ALT: alanine aminotransferase; BUN: Blood Urea Nitrogen.

	Group		
	reference	WT	CAR
	Mean ± SD	Mean ± SD	Mean ± SD
ALT (U/l)	136 ± 70	108 ± 40	135 ± 65
BUN (ng/L)	0.246 ± 0.053	0.246 ± 0.038	0.224 ± 0.045
Total cholesterol (mg/dl)	118 ± 18	115 ± 14	128 ± 19
Total protein (g/dl)	4.5 ± 0.3	4.4 ± 0.3	4.6 ± 0.3
Albumin (g/dl)	3.12 ± 0.13	2.99 ± 0.15[a]	3.11 ± 0.28

We assessed standard hematological values such as the white blood cell count (WBC) and the differential white blood cell count (neutrophils, lymphocytes, monocytes, eosinophils and basophils). We also assessed the red blood cell count (RCB), hemoglobin content (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) to identify potential preliminary immunotoxicity or anemia caused by liver disease, etc.

Table 3.1. 4. Hematology mean values ± SD for males (a. N=5; b. N=4). WBC: White Blood Cell Count; RCB: Red Blood Cell count; HGB: hemoglobin; HCT: hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

	Group		
	reference	WT	CAR
	Mean ± SD	Mean ± SD	Mean ± SD
WBC (10^3/uL)	5.80 ± 1.07[a]	5.94 ± 1.36	4.97 ± 1.45
Neutrophils (10^3/uL)	0.92 ± 0.22[a]	0.96 ± 0.26	0.75 ± 0.22[a]
Lymhpocytes (10^3/uL)	4.69 ± 0.90[a]	4.73 ± 1.11	4.09 ± 0.60[b]
Monocytes (10^3/uL)	0.13± 0.06	0.21± 0.10	0.13 ± 0.12
Eosinophils (10^3/uL)	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.03
Basophils (10^3/uL)	0.04 ± 0.08	0.01 ± 0.00	0.02 ± 0.03
RBC (10^6/uL)	9.88 ± 0.52[a]	10.14 ± 0.24	9.91 ± 0.35
HGB (g/dL)	15.12 ± 0.79[a]	15.57 ± 0.24	15.34 ± 0.56[a]
HCT %	43.54 ± 1.79[a]	44.93 ± 1.02	44.02 ± 1.29
MCV (fL)	44.45 ± 0.99	44.32 ± 0.22	44.42 ± 0.44
MCH (pg)	15.33 ± 0.28	15.35 ± 0.22	15.38 ± 0.19[a]
MCHC (g/dL)	34.48 ± 0.90	34.65 ± 0.50	34.70 ± 0.42[a]

Table 3.1. 5. Hematology mean values ± SD for females (a. N= 5). WBC: White Blood Cell Count; RCB: Red Blood Cell count; HGB: hemoglobin; HCT: hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

	Group		
	reference	WT	CAR
	Mean ± SD	Mean ± SD	Mean ± SD
WBC (10^3/uL)	5.34 ± 1.21	6.32 ± 2.65	5.52 ± 1.45
Neutrophils (10^3/uL)	0.67 ± 0.21	0.83 ± 0.20	0.85 ± 0.24
Lymhpocytes (10^3/uL)	4.09 ± 0.99	3.81 ± 1.00[a]	4.15 ± 1.19
Monocytes (10^3/uL)	0.53 ± 0.14	0.62 ± 0.28	0.48 ± 0.14
Eosinophils (10^3/uL)	0.02 ± 0.02	0.04 ± 0.03	0.03 ± 0.03
Basophils (10^3/uL)	0.03 ± 0.03	0.07 ± 0.14	0.01 ± 0.00
RBC (10^6/uL)	9.48 ± 0.26	9.41 ± 0.31	9.56 ± 0.32
HGB (g/dL)	15.07 ± 0.40	15.08 ± 0.42	15.33 ± 0.55
HCT %	42.25 ± 1.16	42.00 ± 1.24	42.50 ± 1.52
MCV (fL)	44.58 ±0.17	44.65 ± 0.23	44.45 ± 0.40
MCH (pg)	15.90 ±0.37	16.03 ± 0.27	16.03 ± 0.18
MCHC (g/dL)	35.67 ±0.70	35.93 ± 0.60	36.07 ± 0.42

We observed no significant differences ($p < 0.05$) between the high-carotenoid corn-fed group and control groups in terms of absolute organ weights, or organ weights relative to brain or body weights, changes in which often signify potential hepatocellular, myocardial, adrenal gland and renal tubular hypertrophy, neurotoxicity in the brain and toxicity-related alterations in reproductive or lymphoid organs. For these assays we measured the weights of the adrenals, brain, epididymis, heart, kidneys, liver, ovaries, spleen, testes thymus and uterus (Table 3.1.6 and Table 3.1.7)

Table 3.1. 6. Final body weight and absolute organ mean values ± SD for males.

	Group		
	reference	WT	CAR
	Mean ± SD	Mean ± SD	Mean ± SD
Final body weight (g)	27.23± 1.01	27,04 ± 1,13	27,76 ± 0,75
Adrenals (g)	0.0033 ± 0.0013	0,0033 ± 0,0016	0,0027 ± 0,0006
Brain (g)	0.3110 ± 0.0341	0,3352 ± 0,0476	0,3404 ± 0,0480
Epididymis (g)	0.1055 ± 0.0076	0,1093 ± 0,0122	0,1112 ± 0,0087
Heart (g)	0.1502 ± 0.0082	0,1479 ± 0,0077	0,1493 ± 0,0062
Kidneys (g)	0.4070 ± 0.0431	0,3975 ± 0,0322	0,3907 ± 0,0308
Liver (g)	1.3006 ± 0.1120	1,2889 ± 0,0514	1,2516 ± 0,1447
Spleen (g)	0.0707 ± 0.0132	0,0876 ± 0,0119	0,0822 ± 0,0039
Testes (g)	0.2169 ± 0.0225	0,2223 ± 0,0054	0,2153 ± 0,0123
Thymus (g)	0.0303 ± 0.0021	0,0299 ± 0,0050	0,0318 ± 0,0092

Table 3.1. 7. Final body weight and absolute organ mean values ± SD for females.

	Group		
	reference	WT	CAR
	Mean ± SD	Mean ± SD	Mean ± SD
Final body weight	21.94 ± 1.44	22,39 ± 1,42	22,24 ± 0,99
Adrenals (g)	0.0054 ± 0.0015	0,0061 ± 0,0014	0,0062 ± 0,0005
Brain (g)	0.3313 ± 0.0315	0,3299 ± 0,0137	0,3305 ± 0,0302
Heart (g)	0.1135 ± 0.0093	0,1167 ± 0,0096	0,1148 ± 0,0093
Kidneys (g)	0.2654 ± 0.0325	0,2629 ± 0,0296	0,2557 ± 0,0024
Liver (g)	1.0116 ± 0.0914	1,0126 ± 1,0639	1,0203 ± 0,0901
Ovaries (g)	0.0130 ± 0.0027	0,0106 ± 0,0027	0,0111 ± 0,0022
Spleen (g)	0.0833 ± 0.0064	0,0947 ± 0,0084	0,0832 ± 0,0110
Thymus (g)	0.0274 ± 0.0036	0,0272 ± 0,0041	0,0293 ± 0,0058
Uterus(g)	0.0708 ± 0.0384	0,0609 ± 0,0130	0,0778 ± 0,0054

We identified no histopathological anomalies specific to the high-carotenoid corn diet (Table 3.1.8). The few anomalies observed were distributed among all three animal groups and are commonly observed in rodents of this age and are considered spontaneous. Some of these histopathological events are shown with normal tissue structures for comparison in Figure 3.1.3.

Table 3.1. 8. Histopathologic analyses for male and female mice of reference (REF), WT and CAR groups.

		Males			Females		
		REF	WT	CAR	REF	WT	CAR
epididymides	oligospermia	1	0	0	-	-	-
heart	hypertrophy	4	4	3	1	3	3
	contraction band	1	3	1	2	0	0
kidneys	lymphoid aggregates	1	1	3	1	0	1
	extravased red blood cells in the interstitium	6	6	6	5	6	6
liver	sinusoid congestion and central vein dilatation	6	6	6	6	6	6
	steatosis	5	4	4	0	0	1
	inflammatory cells and necrotic debris	1	3	4	6	5	6
	iron deposits	1	1	0	3	2	2
	apoptotic cells	1	0	0	0	0	0
spleen	pigment, increased	5	6	6	6	6	6
thyroid/ parathyroid	cyst change	0	0	0	1	0	0

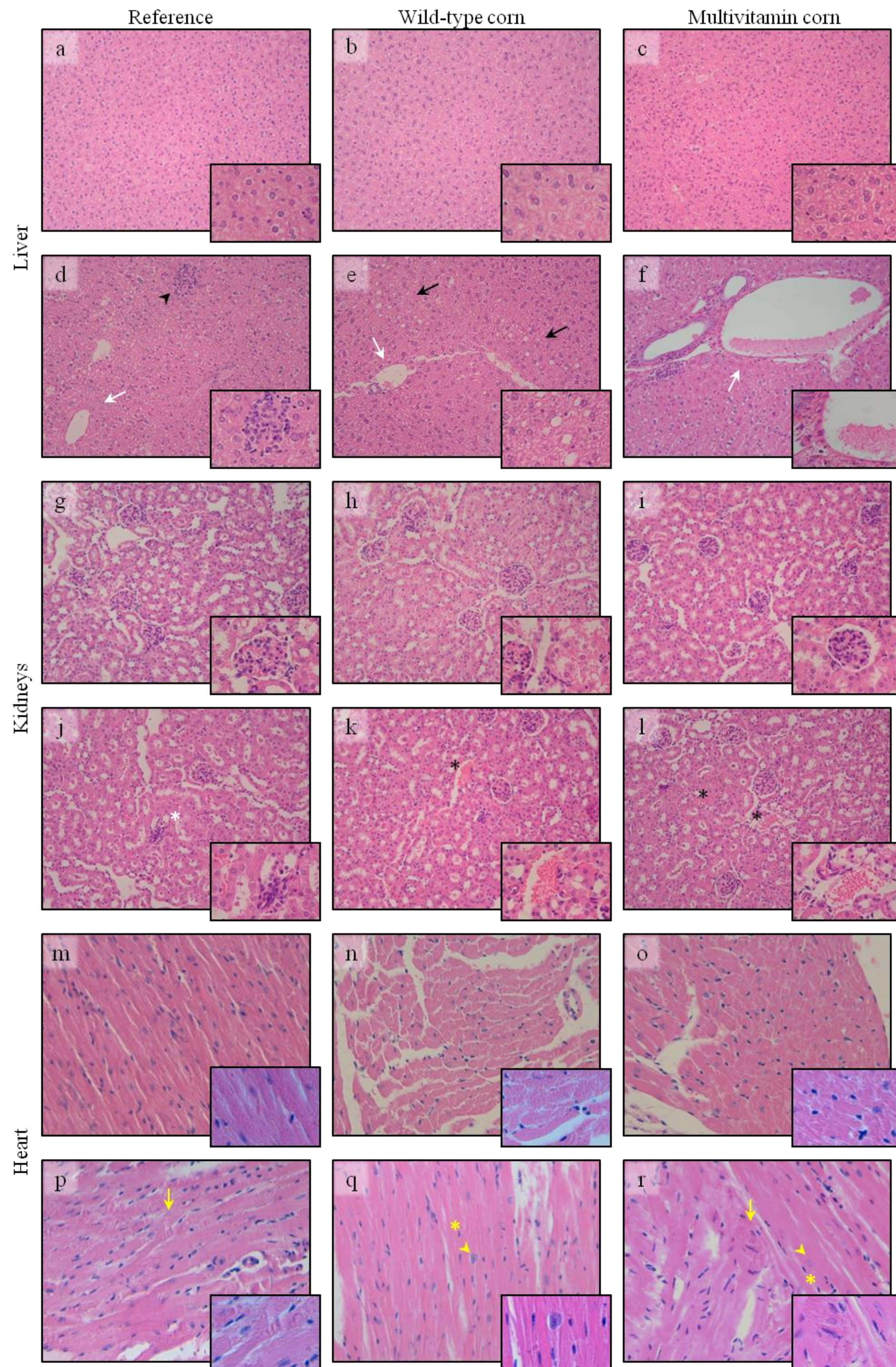

Figure 3.1. 3. Histology (hematoxylin and eosin staining) from liver (a-f) and kidneys (g-l) x 20 (x 40 in the box) and heart (m-r) x 40 (x 63 in the box); (a) (b) and (c) showing normal liver structure from reference,

wild-type and multivitamin (high-carotenoid) groups respectively; (d-f) showing lymphoid aggregates (black arrowhead), sinusoid congestion and/or central vein dilatation (white arrow) and steatosis (black arrow); (g) (h) and (i) showing normal kidney structure from reference, wild-type and multivitamin groups respectively; (j-l) showing lymphoid aggregates (white *) and red blood cells in the interestitium (black *); (m), (n) and (o) showing normal heart structure from reference, wild-type and multivitamin groups respectively; (p-r) showing contraction bands (yellow arrow) and hypertrophic cells (yellow arrowhead) with increased nuclear size in comparison with normal cell (yellow *).

3.1.4. DISCUSSION

We had previously reported the development of a prototype high-carotenoid corn variety based on the South African elite white corn inbred M37W. This corn is simultaneously engineered to accumulate high levels of carotenoids (Zhu et al., 2008; Naqvi et al., 2009).

All genetically engineered crops intended for consumption by humans must be rigorously tested to ensure safety, with the benchmark set by comparison to an equivalent unmodified variety which is 'Generally Regarded As Safe'. Genetically engineered crops are evaluated to determine acute and subchronic toxicity as well as allergenicity. Similar assessments are not required of non-transgenic varieties even if these are genetically identical to a transgenic crop but are produced by mutation or conventional breeding. They are not required even if the conventional crop is generated by crossing to wild species that are known to contain toxins or allergens. The double standards applied to genetically engineered and conventional crops have the unfortunate consequence that engineered crops such as Golden Rice, which could provide a real and immediate benefit to poor populations in developing countries riven by micronutrient deficiency diseases, are held up in a regulatory quagmire and lives continue to be lost unnecessarily (Potrykus, 2010).

The aim of subchronic toxicity evaluation is to determine any adverse effects caused by repeated exposure for a longer period. EFSA guidelines recommend that risk assessments should include at least a 90-day toxicity assessment on whole food/feed in rodents if the composition of the engineered crop is modified substantially or if there are indications for possible unintended effects (European Food Safety Authority, 2011a). However, these requirements have now become de facto standards for engineered crops even if there is no evidence of potential adverse effects, and are recommended in Europe to increase consumer confidence that food/feed from engineered crops is as safe as a conventional counterpart (European Food Safety Authority, 2011b).

Our compositional analysis clearly showed no nutritionally-relevant differences between the high-carotenoid corn and the control corn in terms of major constituents,

and indeed the only difference was the expected higher levels of carotenoids in the high-carotenoid corn. These nutritional differences represent the intended effects of the genetic modification and therefore do not constitute 'unintended effects' that the safety tests are ostensibly designed to identify. Having established the substantial equivalence of high-carotenoid and control corn, we carried out a 90-day subchronic toxicity evaluation to determine the effects of repeated exposure to the diet over a period long enough for toxicity to become apparent but too short for age-related effects to interfere with the results. The three groups were indistinguishable, showing similar food consumption rates, body weights and body weight gains. Food consumption and body weight are usually measured weekly in feeding trials as these parameters are health and wellbeing indicators, as well as highlighting unintended nutritional effects. The weight gain we observed was within the normal range for animals of this age. The mean consumption of high-carotenoid corn in this trial was ~57 g/kg body weight/day, which is approximately 210-fold more than the safety margin in humans (~0.27g/kg body weight/day; DEEM™, 2002).

There were no significant differences between the groups in terms of clinical observations, hematological parameters, biochemical markers and absolute or relative organ weights. Histopathology revealed a similar prevalence of anomalies among the three groups which are regarded as spontaneous, in agreement with data from previous feeding trials involving crops engineered for herbicide tolerance and pest resistance (Appenzeller et al., 2009; Hammond et al., 2004; Hammond et al., 2006; Healy et al., 2008).

In conclusion, the genetically engineered high-carotenoid corn did not show any unintended effects in animal feeding trials designed to evaluate subchronic toxicity, test which although are not mandated by law in most jurisdictions will facilitate public acceptance of high-carotenoid corn for human consumption. Diets prepared with high-carotenoid corn were palatable, nutritious and safe in animals, which should pave the way for human trials and the eventual deployment of high-carotenoid corn in developing country settings to help combat simultaneous multiple micronutrient deficiencies among populations that subsist on a predominantly cereal-based diet.

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CHAPTER 2

Chemoprevention of tumor development in the mouse AOM/DSS model of colitis-associated carcinogenesis by diets enriched with high-carotenoid corn

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3.2.1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the second most common in Europe (Bray et al., 2013). The disease has three etiologies: sporadic, hereditary and inflammatory (colitis-associated). Furthermore, inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is associated with a high risk of CRC (Wang and Dubois, 2010) .

Diets rich in fruit, vegetables and whole grains reduce the risk of CRC (Park et al., 2005; Haas et al., 2009; Pan et al., 2011; Aune et al., 2011). The ability of healthy diets to reduce the risk of cancer has been widely reported, particularly the role of dietary phytochemicals (Surh, 2003; Aggarwal and Shishodia, 2006; Pan et al., 2011). Although phytochemicals are non-essential nutrients, they can inhibit the signaling pathways that promote carcinogenesis (Surh, 2003; Aggarwal and Shishodia, 2006).

Carotenoids are a family of more than 750 natural, lipid-soluble pigments, 50 of which are found in the human diet (Ciccone et al., 2013). Vegetables and fruits typically contain α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene and zeaxanthin among others (Farré et al., 2011). Marine animals also accumulate carotenoids when they consume algae and plankton (Tanaka et al., 2012).

Carotenoids act as modulators of several inflammatory cytokines produced during colitis-associated carcinogenesis (Tanaka et al., 2012). Cyclo-oxygenase 2 (COX-2) a prostaglandin-endoperoxide synthase required for the formation of biological mediators of inflammatory responses, including the production of prostaglandins, prostacyclin and thromboxane, can be regulated by nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), as shown in Figure 3.2.1 (Sheng et al., 2001; Dong et al., 2003; Mari et al., 2007; Nagendraprablhu et al., 2011). The signaling

protein p44/42MAPK, also known as ERK1/2 and MAPK3/1, regulates cell proliferation, differentiation, motility and survival. The inhibition of MAPK pathways results in the downregulation and suppression of COX-2 expression (Roux and Blenis, 2004; Binion et al., 2008). COX-2 mediates inflammation, apoptosis, differentiation, cell migration and progression through the cell cycle, and can also be downregulated by carotenoids in CRC mice (Nagendraprablhu et al., 2011; Yasui et al., 2011).

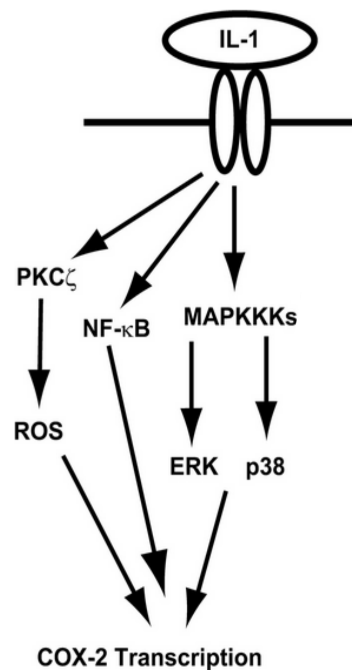


Figure 3.2. 1. Model for the regulation of COX-2 expression by IL-1. IL-1, a key cytokine associated with the inflammation of the intestinal mucosa, binds to its receptor and can activate the MAPK, NF-κB and protein kinase C (PKCζ) pathways. The transcription of *COX-2* depends on extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and p38 MAPK activation as well as the generation of reactive oxygen species (ROS), driving NF-κB-mediated activation of the *COX-2* promoter. Adapted from Mari et al. (2007).

Although the ability of carotenoids to prevent CRC in human trials is not clearly established (Park et al. 2009; Park et al. 2010; Williams et al. 2010), carotenoid dietary supplements have been shown to inhibit disease progression in rodent CRC models (Temple and Basu, 1987; Narisawa et al., 1996; Kim et al., 1998; Rijken et al., 1999;

Narisawa et al., 1999; Tanaka et al., 2000a; Suzuki et al., 2007; Tanaka et al., 2008; Nagendraprablhu et al., 2011; Yasui et al., 2011; Kawabata et al., 2012). The development of genetically-engineered high-carotenoid corn provides an opportunity to study the impact of the food matrix on carotenoid bioaccessibility and bioavailability compared to the provision of carotenoids as supplements. It also allows the analysis of the synergic effects caused by several carotenoids in the same feed. The beneficial effects can be determined by comparing animals fed on the high-carotenoid corn with those fed on a near-isogenic wild-type variety.

The azoxymethane/dextran sulfate sodium (AOM/DSS) mouse model of colitis-associated colorectal carcinogenesis closely resembles human inflammation-associated CRC pathogenesis and therefore provides a good model for chemoprotective efficacy experiments (Tanaka et al., 2008; Oyama et al., 2009; Butler et al., 2013). Unlike dimethylhydrazine (DMH) used in previous studies (Viñas-Salas et al., 1992; Viñas-Salas et al., 1998) the AOM/DSS model uses a single dose of AOM to induce colorectal tumors together with DSS as a promoter of colitis (Tanaka et al., 2003; De Robertis et al., 2011).

The aim of this study was to evaluate the potential chemoprotective effect of the mixture of carotenoids delivered by high-carotenoid corn in an inflammation-related AOM/DSS mouse model. The incidence and morphology of tumors was evaluated in each individual animal by high-resolution endoscopy during the feeding trial. The molecular basis of colitis-associated carcinogenesis was investigated by monitoring several inflammation-related markers, such as COX-2 and MAPK.

3.2.2. MATERIALS AND METHODS

3.2.2.1. Animal feeding study, diets and chemicals

The study complied with Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat) of Catalonia, as well as EU Directive EEC 63/2010. It was approved by the Ethics Committee on Animal Experiments of the University of Lleida and by the Ethics Commission on Animal Experimentation of the Generalitat de Catalunya (approved protocol number 7746).

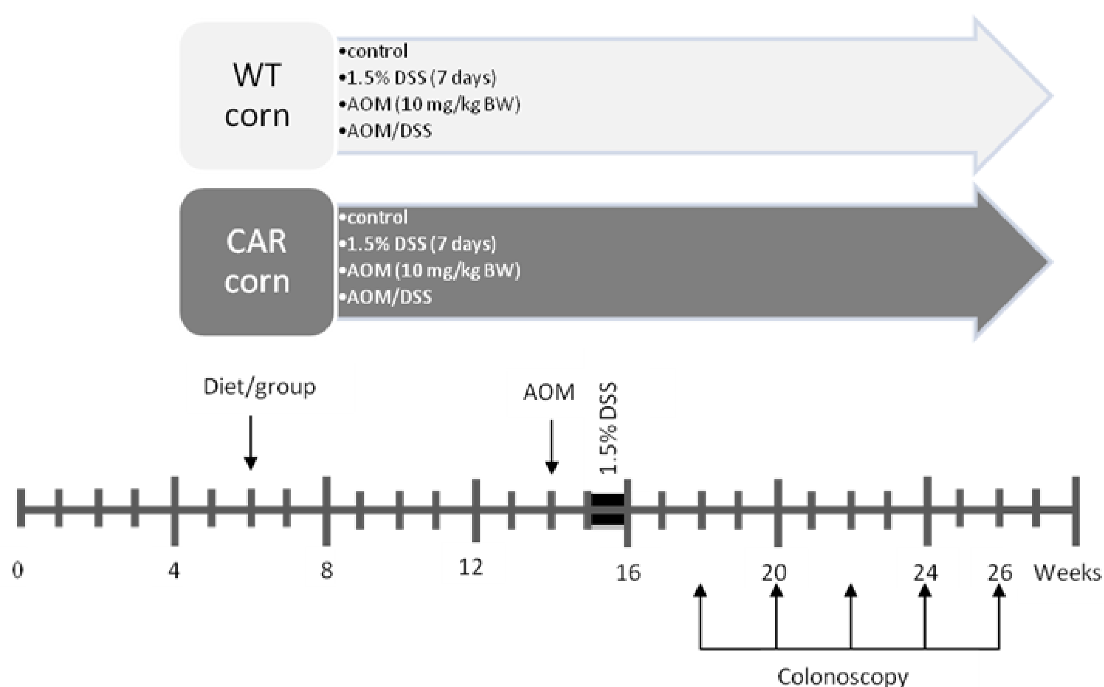


Figure 3.2. 2. Experimental design and timeline of the colitis-associated colon carcinogenesis experiment. The animals were fed on diets based on wild-type (WT) or high-carotenoid (CAR) corn commencing at 6 weeks old. After 8 weeks, the mice were injected with azoxymethane (AOM), and 1.5% dextran sodium sulfate (DSS) was introduced into the drinking water 1 week later. Endoscopic follow-up was carried out 6 weeks after the AOM injection at 2-week intervals for 2 months. The animals were assigned to each group (day 0) as follows: WT control n = 2, WT 1.5% DSS n = 2, WT AOM n = 2, WT AOM/DSS n = 14; CAR control n = 2, CAR 1.5% DSS n = 2, CAR AOM n = 2, CAR AOM/DSS n = 14.

Female ICR (CD-1®) mice obtained from Harlan Laboratories S.L. (Sant Feliu de Codines, Spain) were acclimated in individual cages 1 week before the experiment. The mice had *ad libitum* access to a standard 2014 Teklad Global 14% protein rodent

maintenance diet and water. The animal rooms were environmentally controlled and maintained at 20 ± 2°C and 50 ± 5% relative humidity, with a 12-h photoperiod.

Following acclimation, 40 6-week-old females were randomly assigned to eight groups (Figure 3.2.2) and housed in separate cages (five mice per cage) with access to food and water as above. Body weight and food consumption were measured weekly for 20 weeks. Experimental diets containing the wild-type or high-carotenoid corn (grown under the same conditions and harvested at the same time) were prepared from freeze-dried powdered kernels under hygienic conditions. Feed was prepared by mixing the purified AIN-76A diet (Research Diets, Inc.) with the appropriate freeze-dried corn powder in a 60:40 ratio (w/w).

The feeding trial was run for 8 weeks, and then the 14-week-old mice were injected intraperitoneally with one dose (10 mg/kg body weight) of AOM (Sigma-Aldrich, Taufkirchen, Germany) in phosphate-buffered saline (PBS). They were then provided with 1.5% DSS (50,000 molecular weight; MP Biomedicals, Illkirch, France) in drinking water for 7 days, one week after the AOM injection.

3.2.2.2. Colonoscopy

Six weeks after the AOM injection and at 2-week intervals thereafter, colonoscopy was carried out under anesthesia (2% isoflurane) using a Coloview rigid colonoscope (Karl Storz, Tuttlingen, Germany). Approximately 4 cm of the colon was visualized with air insulation to score the number, size and changing morphology of the lesions. The tumor size score was adapted from Becker et al. (2005) and graded 1 (ranging from a small detectable tumor to a tumor covering up to 12.5% of the colonic circumference) or 2 (tumor covering ≥ 25% of the colonic circumference).

3.2.2.3. Blood analysis and histopathology

Twelve weeks after the AOM injection (26-week-old mice) blood samples were obtained via cardiac puncture under 2% isoflurane anesthesia and the mice were sacrificed by exsanguination. Whole blood was collected in a tube containing EDTA, and plasma was prepared by centrifugation at 1500 x g (10 min) before analyzing hematological parameters. Complete necropsy was carried out and colons were excised and flushed with PBS so that the colon length (ceccum, distal, proximal) and liver weight could be measured. Colonic mucosa, colonic lesions and liver tissue were immediately frozen in liquid nitrogen for storage or were placed in 10% neutral-buffered formalin and embedded in paraffin for histopathology. Sections (4-5 µm) were stained with hematoxylin and eosin. Colonic tumors were analyzed for tumor incidence (number of animals presenting tumors), tumor multiplicity (number of tumors per tumor-bearing animal) and tumor size (mm^2).

3.2.2.4. Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from the colonic mucosa using the RNeasy Total RNA kit (Qiagen, Valencia, CA, USA) and cDNA was generated using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The cDNA was heated to 95°C for 10 min, and amplified by 40 PCR cycles of 95°C for 15 s and 60°C for 1 min. Quantitative real-time PCR was carried out on the ABI Prism 7900 Sequence Detection System (Applied Biosystems) using Promega GoTaq® qPCR Master Mix (California, USA). Relative mRNA expression levels were calculated using the ΔΔCt method and presented as ratios based on the housekeeping genes *GAPDH* encoding glyceraldehyde-3-phosphate dehydrogenase, used for the tumor necrosis factor alpha (*TNF-α*) control, or *TBP* encoding the TATA-binding protein, used as a control for all other genes. The primers were based on the following deposited sequences: *TNF-α*, Mm00443260_g1; *NF-κB1*, Mm00476361_m1; NF-E2-related factor 2 (*NRF-2*), Mm00477784_m1; *TBP*, Mm00446971_m1; *GAPDH*, Mm99999915_g1.

3.2.2.5. Immunohistochemistry

Paraffin-embedded sections of colon tissue (4-5 µm) were dried for 16 h at 56°C, cleared in xylene, rehydrated through a graded ethanol series (60%, 95% and 100%) and washed in PBS. The slides were microwave-heated for 2 min in 10 mmol/l citrate buffer (pH 6.5) or EDTA buffer (pH 8.0) for antigen retrieval. Endogenous peroxidase activity was blocked by immersing the sections in hydrogen peroxide. The primary antibody was anti-COX-2 (sc-1746, 1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After incubation, horseradish peroxidase activity was visualized using the EnVision Detection Kit (DAKO) with 3,3'-diaminobenzidine chromogen as the substrate. The sections were counterstained with hematoxylin. Immunoreactivity was scored by determining the percentage of COX-2 positive cells under an Olympus BX50 optical microscope as follows: 1+ = 1–24%, 2+ = 25–49%, 3+ = 50–74%, 4+ =75–100%.

3.2.2.6. Western blot

Colons were homogenized in a Polytron® device at 4°C using a buffer containing 180 mM KCl, 5 mM MOPS, 2 mM EDTA, 1 mM diethylene triamine penta-acetic acid, 1 µM butylated hydroxyltoluene, 10 µg/ml aprotinin, 1 mM NaF, 1 mM Na_3VO_4 and 1% v/v protease inhibitor mix 80–6501-23 (GE Healthcare, Piscataway, USA). Proteins were quantified using the Bradford method and mixed pools of each group (40 µg total protein per lane) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P Millipore, Bedford, MA, USA) and stained with Ponceau S as a loading control. Primary and secondary antibodies are listed in Table 3.2.1. Protein bands were visualized using chemiluminescent horseradish peroxidase (Millipore Corporation, Billerica, MA, USA) and band intensity was measured using the Bio-Rad Gel Doc/Chemi Doc Imaging System and Image Lab 4.0.1 software (Bio-Rad Laboratories, Munich, Germany).

Table 3.2. 1. Primary and secondary antibodies for western blot immunodetection.

Antigen	Supplier	Reference	Dilution
COX-2	Santa Cruz Biotechnology	sc-1746	1:200
p44/42 MAP Kinase	Cell Signaling	4695	1:1000
Anti-rabbit IgG	Thermo Scientific Pierce	31460	1:100000
Anti-goat IgG	Santa Cruz Biotechnology	SC2020	1:12000

3.2.2.7. Statistics

Values are presented as means ± standard errors of the mean (SEM). Data were compared by one-way ANOVA with Bonferroni correction or Student's *t*-test. Fisher's exact probability test was used to compare the incidence of lesions among groups.

3.2.3. RESULTS

3.2.3.1. General observations

Three animals from the WT AOM/DSS group and one from the CAR AOM/DSS group died or were sacrificed after exposure to AOM/DSS because they exhibited gastrointestinal bleeding and lethargy. One mouse in the WT AOM/DSS group was unable to recover from anesthesia after the first colonoscopy. The high-carotenoid corn diet showed no evidence of toxicity and there was no abnormal change in liver histology compared to animals fed on the wild-type corn diet. These results were consistent with our previously-reported 90-day subchronic toxicity feeding trial (Arjó et al. 2012). The mean initial and final body weights, liver weight and relative weight and colon length are shown in Table 3.2.2.

We first considered the impact of different treatments in each of the diet groups. The AOM/DSS WT group consumed significantly more food than the control WT group (p = 0.0004) and their mean liver weight was significantly lower (p = 0.0246). The mean liver weight of the AOM/DSS WT group was also significantly lower than the DSS WT group (p = 0.0267). In contrast, there were no significant differences between the treatment groups when mice were fed the high-carotenoid corn, except that the mean liver weight of the AOM/DSS CAR mice was significantly lower than the control CAR group (p = 0.0097). The lowest mean liver weights and relative liver weights were found in mice exposed to AOM/DSS or DSS alone. The latter group also had significantly shorter colons than the untreated control when both were fed on the wild-type corn diet (p = 0.0095).

The relevant comparison to determine the effects of high-carotenoid corn was between AOM/DSS-treated mice in the different diet groups (WT and CAR). There were no statistically significant differences between these groups in terms of initial and final body weight, colon length or liver weight, although the relative liver weight in the AOM/DSS CAR group was significantly lower than that of the AOM/DSS WT group (p = 0.0306) (Table 3.2.2). There were no significant differences in hematology parameters between diet groups under the same treatment regime (Table 3.2.3), only between the

DSS WT and DSS CAR groups in the percentage of lymphocytes, monocytes, hemoglobin and hematocrit.

Table 3.2. 2. Body weight (BW), food consumption (FC), liver weight and colon length. a Significant based on Student's t-test at $p < 0.05$ compared to AOM/DSS WT group. All values are presented as means ± SEM.

Group	Initial BW(g)	Final BW (g)	FC (g/mouse/day)	Liver weight (g)	Relative liver weight (g/100 g BW)	Length of large bowel (cm)
Control WT (n=2)	25.9 ± 1.4	34.4 ± 2.1	3.50 ± 0.07	1.49 ± 0.04	4.675 ± 0.005	14.65 ± 0.05
DSS WT (n=2)	24.2 ± 1.6	34.0 ± 3.1	3.87 ± 0.09	1.24 ± 0.09	3.650 ± 0.070	12.05 ± 0.25
AOM WT (n=2)	26.5 ± 1.6	30.7 ± 0.5	3.55 ± 0.15	1.43 ± 0.25	4.690 ± 0.910	13.05 ± 0.45
AOM/DSS WT (n=10)	25.9 ± 0.8	31.2 ± 0.6	3.91 ± 0.06	1.33 ± 0.03	4.264 ± 0.101	12.88 ± 0.41
Control CAR (n=2)	25.9 ± 0.5	36.0 ± 2.3	3.71 ± 0.08	1.62 ± 0.11	4.035 ± 0.265	13.95 ± 2.65
DSS CAR (n=2)	24.2 ± 0.2	34.2 ± 0.9	3.83 ± 0.071	1.24 ± 0.06	3.635 ± 0.095	13.65 ± 0.55
AOM CAR (n=2)	25.7 ± 0.3	30.5 ± 1.0	4.10 ± 0.21	1.32 ± 0.08	4.315 ± 0.115	12.20 ± 1.90
AOM/DSS CAR (n=13)	26.2 ± 0.5	32.7 ± 1.0	3.94 ± 0.11	1.29 ± 0.04	3.952 ± 0.089[a]	13.07 ± 0.38

Table 3.2. 3. Analysis of hematologic parameters: white blood cell count (WBC), neutrophils (Neutr), lymphocytes (Lym), monocytes (Mono), eosinophils (Eos), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HTC). All values are presented as means ± SEM. [a] RBC determination error in all samples.

	WBC (10^3/µl)	Neutr (%)	Lym (%)	Mono (%)	Eos (%)	RBC (10^6/µl)	HGB (g/dl)	HTC (%)
Control WT n=2	2.31 ± 0.86	19.3 ± 6.5	76.85 ± 7.75	3.85 ± 1.25	0	8.57 ± 0.32	13.25 ± 0.55	40.15 ± 1.05
DSS WT n=2	4.11 ± 1.31	11.55 ± 2.85	81.3 ± 3.0	11.5 ± 0.4	0	9.5 ± 0.5	14.8 ± 0.6	44.45 ± 2.95
AOM WT n=2	3.565 ± 1.42	14.1 ± 3.40	76.7 ± 11.2	8.85 ± 7.95	0	9.405 ± 0.38	14.9 ± 0.4	44.45 ± 1.55
AOM/ DSS WT n=10	2.815 ± 0.390	21.25 ± 4.46	72.14 ± 4.36	6.35 ± 1.90	0.06 ± 0.06	7.719 ± 0.55	12.61 ± 0.88	37.77 ± 2.42
Control CAR n=2	1.155 ± 0.285	15.8 ± 1.3	81.55 ± 2.95	1.55 ± 1.55	0.8 ± 0.2	9.53 ± 0.5	14.95 ± 0.55	45.85 ± 1.55
DSS CAR n=2	4.075 ± 0.945	3.985 ± 0.865	79.05 ± 4.25	17.95 ± 3.45	3.0 ± 0.8	- [a]	9.47 ± 0.18	15.1 ± 0.5
AOM CAR n=2	1.625 ± 0.105	20.75 ± 6.95	75.1 ± 4.2	3.5 ± 2.8	0.3 ± 0.3	8.815 ± 0.325	14.80 ± 0.60	43.9 ± 2.8
AOM/ DSS CAR n=13	2.8031 ± 0.6105	19.5846 ± 2.2839	73.1385 ± 2.6815	6.9615 ± 2.4912	0.2154 ± 0.1192	7.554 ± 0.4686	12.2923 ± 0.8486	37.4769 ± 2.4327

3.2.3.2. Incidence and multiplicity of colonic lesions after 6 to 12 weeks follow up by colonoscopy

High-resolution endoscopy allowed the progression of tumor growth to be followed in individual animals by visualizing 3-4 cm of colon, and was carried out every 2 weeks (Figure 3.2.3A). The incidence of tumors in the AOM/DSS CAR group was lower than in the AOM/DSS WT group at earlier stages although the difference was not statistically significant (Figure 3.2.3B), but the incidence was similar in both groups at 12 weeks ($p > 0.05$). The AOM/DSS CAR group also had a lower tumor multiplicity in tumor-bearing mice and a lower tumor score compared to the corresponding WT animals (e.g. the tumor multiplicity 6 weeks after the AOM injection was 2.50 ± 1.15 in the WT group *vs.* 0.87 ± 0.64 in the CAR group, and the tumor score was 10.00 ± 1.15 in the WT group *vs.* 4.33 ± 2.85 in the CAR group). Again, the difference in values was not statistically significant.

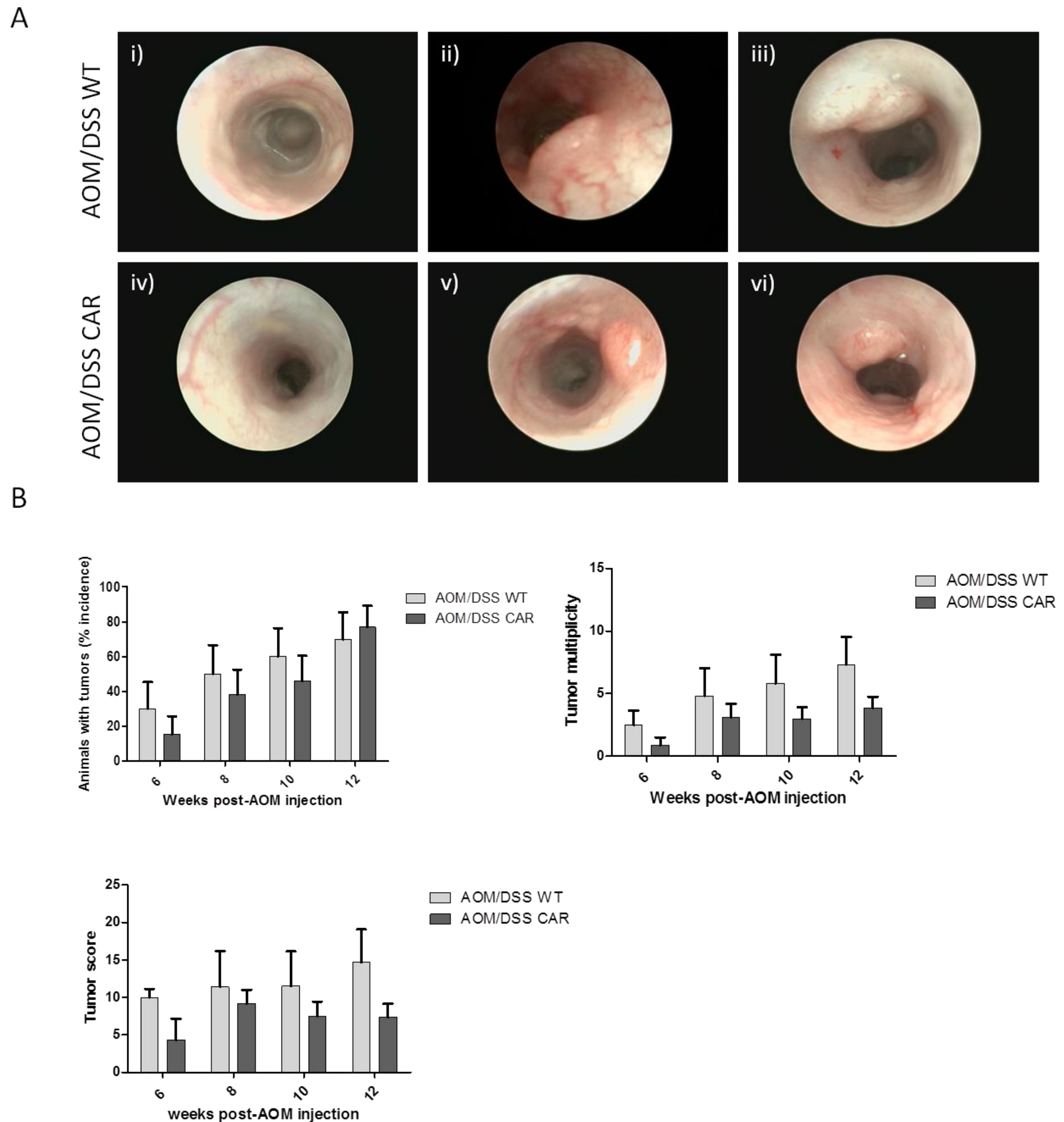


Figure 3.2. 3. Endoscopic images, incidence, multiplicity and score of colonic tumors identified by endoscopic visualization. (A) Representative colonoscopy images showing tumor progression: i) and iv) colitis at 6 weeks; ii) and v) small flat tumor at 8 weeks; iii) flat tumor at 12 weeks; vi) flat tumor and colitis at 10 weeks. (B) Quantitative analysis of tumor incidence in AOM/DSS WT (n = 10) and AOM/DSS CAR (n = 13) groups, and tumor multiplicity and score data in tumor-bearing mice (n = 8 in AOM/DSS WT and n = 10 in AOM/DSS CAR) obtained by endoscopic visualization. The tumor score per animal was based on the sum of size score of all tumors found by colonoscopy per animal and each tumor size was graded 1 (ranging from a from very small detectable tumor to a tumor covering up to 12.5% of the colonic circumference) or 2 (tumor covering ≥ 25% of the colonic circumference). All values are presented as means ± SEM.

3.2.3.3. Incidence and multiplicity of colonic lesions after 12 weeks

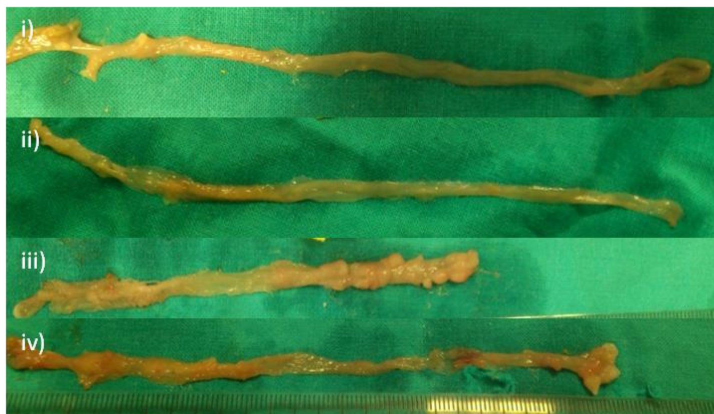
The colonoscopy and colonic lesion necropsy data were compared at the end of the experiment to determine the reliability of the colonoscopy method. Twelve weeks after the AOM injection, it was possible to visualize 71% of the total colorectal lesions in the AOM/DSS WT group and 54% in the AOM/DSS CAR group, compared to the numbers observed by necropsy.

The number of mice with macroscopic colonic lesions in dissected colons 12 weeks after the AOM injection was 8/10 (80%) in the AOM/DSS WT group and 10/13 (77%) in AOM/DSS CAR group, a difference that was not statistically significant (Table 3.2.4). One animal in the AOM WT group presented a tumor with an area of 0.375 mm^2 (data not shown). There was a higher multiplicity of colonic lesions in the AOM/DSS WT group compared to the AOM/DSS CAR group (8.71 ± 2.60 *vs.* 5.60 ± 2.001). The mean colon tumor area in the AOM/DSS WT group was 0.968 ± 0.294 mm^2 compared to 0.547 ± 0.1875 mm^2 in the corresponding CAR group, and the total tumor area was 10.73 ± 4.618 mm^2 in the WT group and 5.500 ± 3.905 mm^2 in the corresponding CAR group. All these differences represented a definite trend but were not statistically significant ($p > 0.05$).

Median values and quartiles were determined (Figure 3.2.4) showing that 50% of tumor-bearing mice in the AOM/DSS CAR group presented fewer than three tumors per animal, and 50% of tumor-bearing mice in the corresponding WT group presented more than eight tumors per animal. These data also revealed that 50% of the AOM/DSS WT mice had a mean colon tumor area greater than 0.090 mm^2 whereas for those in the AOM/DSS CAR group the area was less than 0.036 mm^2.

Table 3.2. 4. Tumor characteristics in dissected colons after 12 weeks: incidence and multiplicity of macroscopic colorectal lesions, tumor multiplicity, average tumor size and total tumor area per tumor-bearing mouse presented as means ± SEM (n = 8 animals in the AOM/DSS WT group and n = 10 in the AOM/DSS CAR group).

Group	% Animals with colon tumors (incidence)	Number of tumors/animal (multiplicity)	Average size of tumors (mm^2)	Total tumor area (mm^2)
AOM/DSS WT	80%	8.71 ± 2.60	0.968 ± 0.2941	10.73 ± 4.618
AOM/DSS CAR	77%	5.60 ± 2.00	0.547 ± 0.1875	5.500 ± 3.905



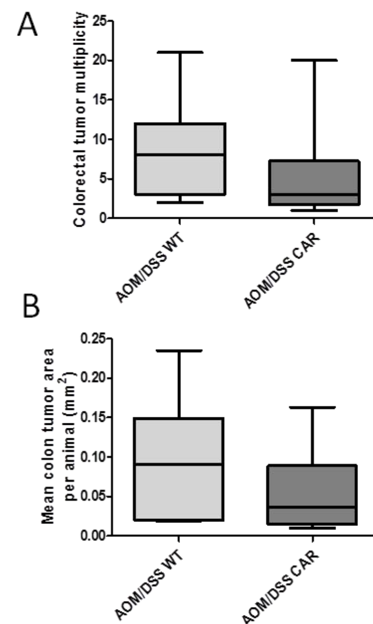


Figure 3.2. 4. (A) Macroscopic image of the colon dissected from i) Control WT, ii) DSS CAR, iii) AOM/DSS WT and iv) AOM/DSS CAR groups, and median and quartile distributions for colorectal tumor multiplicity (B) and mean colon tumor area (C) in tumor-bearing animals (n = 8 in AOM/DSS WT and n = 10 in AOM/DSS CAR).

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3.2.3.4. Histopathology of colonic lesions

The analysis of colonic lesions (Figure 3.2.5 and Figure 3.2.6) revealed that 18% of the lesions in the AOM/DSS WT group were inflammatory infiltrates containing lymphocytes responsible for the development of ulcers, 14% were instances of crypt dysplasia, 9% were adenomas and 59% were adenocarcinomas (ADCs). The AOM/DSS CAR group presented a higher proportion of mucosal ulcers (25%) but a lower proportion of ADCs (43%) compared to the AOM/DSS WT group. These data confirmed that there is statistically significant dependence between having neoplastic lesions and diet (p = 0.0336), and animals fed on high-carotenoid corn diet resulted in a reduction in neoplastic lesions.

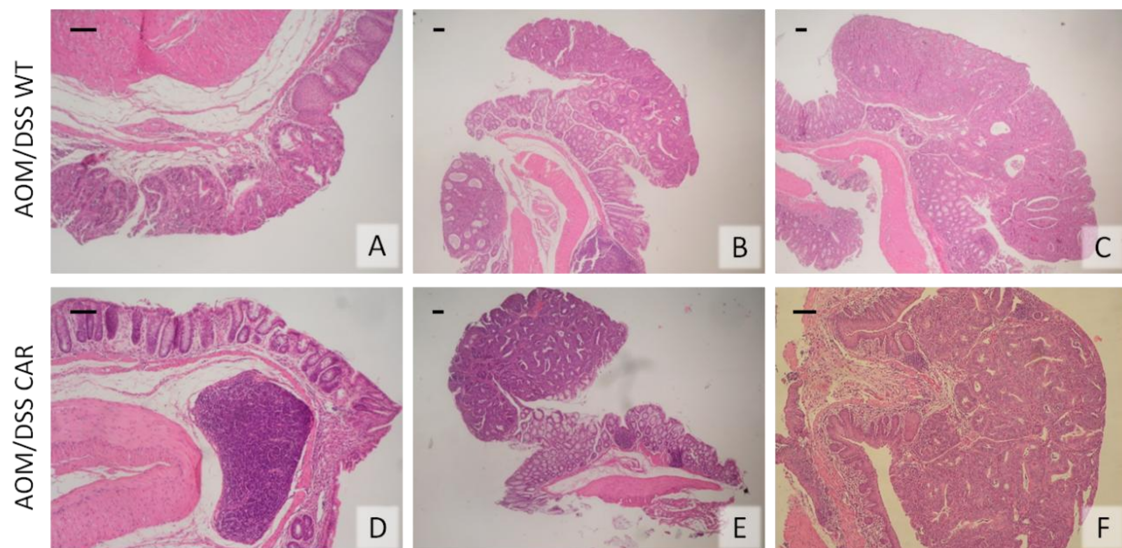

Figure 3.2. 5. Histopathology of colonic lesions after hematoxylin and eosin staining in the AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) groups. (A) crypt dysplasia; (B) adenoma; (C) adenocarcinoma; (D) mucosal ulcer; (E) adenoma; (F) adenocarcinoma. Scale bar = 100 µm.

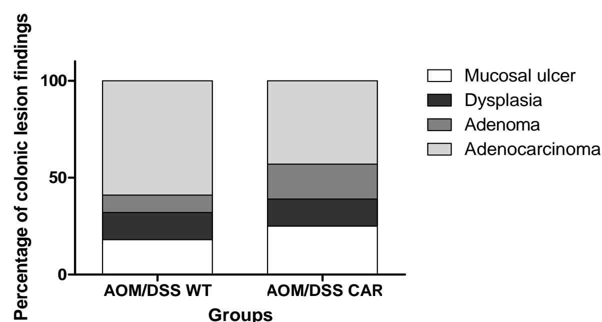

Figure 3.2. 6. Percentage of colonic lesion features found by investigative histology in AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) groups.

3.2.3.5. Immunohistochemical analysis of COX-2 expression

COX-2 is an important biological marker of inflammation and its levels are typically elevated in rodent colon tumor models (Sheng et al., 2001; Dong et al., 2003).

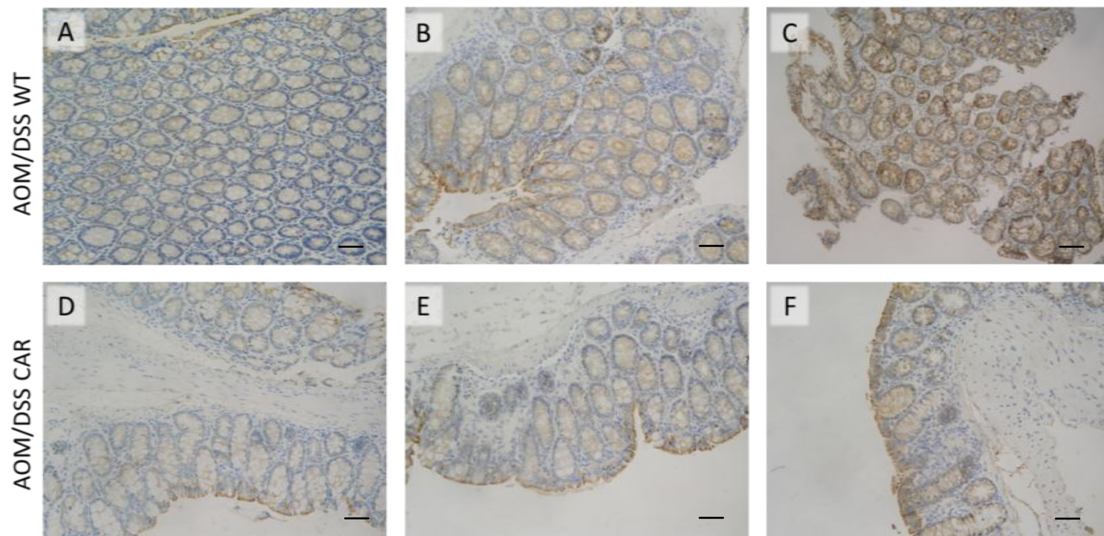

Figure 3.2. 7. Immunohistochemical analysis of COX-2 expression in the normal colonic mucosa representing intensity scores of 1+ (A, D), 2+ (E), 3+ (B, F) and 4+ (C) in the AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) groups. Scale bar = 100 µm.

The immunohistochemical detection of COX-2 showed that the consumption of high-carotenoid corn significantly reduced (p=0.042) the abundance of this marker in mice treated with AOM/DSS (Figure 3.2.7 and Figure 3.2.8). COX-2 was expressed primarily in colonic epithelial cells.

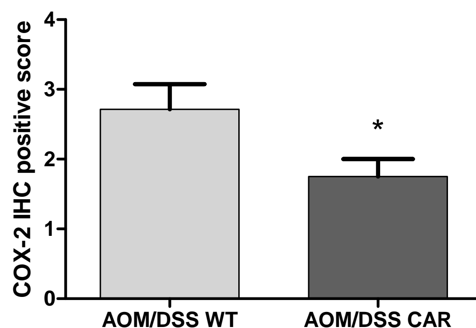

Figure 3.2. 8. Mean COX-2 immunohistochemical score (± SEM) determined in AOM/DSS WT (n = 8) and AOM/DSS CAR (n = 10) colonic tissues. *The differences between groups were significant according to Student *t*-test at $p < 0.05$.

3.2.3.6. Quantitative real time PCR analysis of inflammation markers

The expression of three additional inflammation markers (TNF-α, NF-κB and NRF-2) was investigated by quantitative real-time PCR. Tumor necrosis factor alpha (TNF-α) is a mediator of inflammation and nuclear factor-kappa B (NF-κB) is a transcription factor that plays an important role in inflammation, immunity, cell proliferation, apoptosis, and tumorigenesis (Kumar et al., 2004; Mukaida et al., 2011). Nuclear factor/erythroid 2-related factor 2 (NRF-2) is a novel target for the prevention of colorectal cancer and several chemoprotective compounds including carotenoids are associated with the activation of NRF-2 (Kawabata et al., 2012; Tuzcu et al., 2012; Yang et al., 2014). NF-κB and NRF-2 are both expressed in inflammatory and cancer cells in the AOM/DSS model (Suzuki et al., 2007b; Tanaka et al., 2010). However, there was no significant difference in the transcript levels of these markers when AOM/DSS-treated mice in the different diet groups were compared.

3.2.3.7. Western blot analysis of COX-2 and p44/42 MAPK

The abundance of COX-2 and MAPK was investigated by western blot, following the preparation of soluble extracts of the colonic mucosa from AOM/DSS-treated animals in both diet groups (Figure 3.2.9). Both markers were significantly depleted in mice fed

on the high-carotenoid corn diet, suggesting that the presence of carotenoids leads the suppression of COX-2 and p44/p42 MAPK expression in the colonic mucosa.

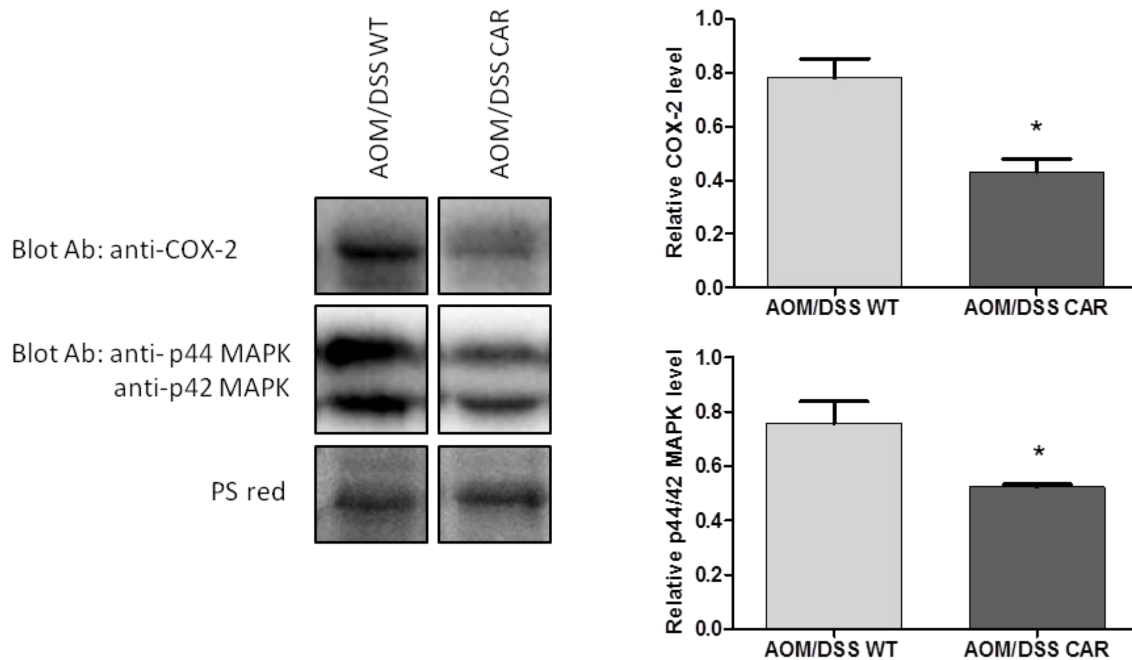

Figure 3.2. 9. Western blots showing the impact of high-carotenoid corn on the expression of (A) COX-2 and (B) p44/42 MAPK in normal (non-tumor) colonic mucosa. Right-side panels show the corresponding histograms of quantitative densitometric values of the blots, normalized against Ponceau S staining as a loading control. *The differences were significant according to Student's *t*-test ($p < 0.05$).

3.2.4. DISCUSSION

The comparison of mice fed on diets containing either high-carotenoid corn or a near isogenic wild-type comparator showed that the high-carotenoid corn significantly inhibited the progression of pre-neoplastic lesions in a mouse model of colitis-associated colorectal carcinogenesis induced by exposure to AOM/DSS. Although by the end of the feeding trial the incidence of tumors in mice was similar in both diet groups, there was two-fold increase in the percentage incidence of tumors in mice fed on the wild-type corn diet during the earlier stages of the trial (6 weeks after AOM injection) and these mice also showed a higher multiplicity of colonic lesions (1.42-fold) and a larger overall mean colon tumor area than mice fed on high-carotenoid corn, although the differences did not reach the threshold for statistical significance. The data show a definite trend indicative of chemoprotective activity, and the analysis of several inflammatory markers suggested that the chemoprotective mechanism could involve the suppression of COX-2, an important mediator of inflammatory signaling. This is based on immunohistochemical data showing the significantly greater abundance of COX-2 in the non-tumor colonic mucosa of mice fed on the wild-type corn. These data therefore provide evidence that supports the proposed inhibitory effect of carotenoids on colorectal carcinogenesis in animals (Temple and Basu, 1987; Narisawa et al., 1996; Kim et al., 1998; Narisawa et al., 1999; Rijken et al., 1999; Tanaka et al., 2000; Suzuki et al., 2007; Nagendraprablhu et al., 2011; Yasui et al., 2011; Kawabata et al., 2012).

The link between carotenoids, COX-2 expression and the prevention of colorectal cancer is unclear. The high-carotenoid corn contains 112-fold more total carotenoids than the wild-type comparator and in order of decreasing abundance these are β-carotene, zeaxanthin, lycopene, lutein, α-cryptoxanthin, α-carotene, β-cryptoxanthin and γ-carotene (Naqvi et al., 2009). Dietary carotenoids are absorbed primarily by the small intestine and are transported to the liver as chylomicrons, but they are also bioaccessible in the large intestine, where they could have a direct effect on the colonic mucosa (Goñi et al., 2006). It is not clear whether individual carotenoids have a specific beneficial impact or whether the chemoprotective effects result from the

synergic activity of several provitamin A and non-provitamin A carotenoids in the whole corn matrix.

The AOM/DSS mouse model is useful for the investigation of colitis-associated carcinogenesis because DSS induces chronic colitis and accelerates tumorigenesis (Tanaka et al., 2003; Clapper et al., 2007). Endoscopic colonoscopy in mice 6–12 weeks after the injection of AOM provided high-quality images allowing colon tumor development and progression to be evaluated at least in the distal 4 cm of colon. It is therefore a rapid and reproducible technique to evaluate colorectal tumor development in mice. Although the chemoprotective trend was not statistically significant, this is likely to reflect natural variability of disease progression in the mouse model and it is possible that larger studies with more animals would confirm the significance of the trends we observed in terms of tumor incidence, multiplicity and area. Biopsies taken over time would also provide more supportive evidence about the role of different biomarkers of inflammation. The incidence of animals developing macroscopic tumors 12 weeks after AOM injection was previously reported as 80–100% with a multiplicity of 10 (Becker et al., 2005). In our experiments, both the incidence and multiplicity were lower at the end of the study (80% incidence with a multiplicity of 8.7) potentially because a lower dose of DSS was used, i.e. 1.5% instead of the 3% used by Becker et al. (2005). This lower dose is often used to study the potential chemoprotective effects of food (Ju et al., 2009; Oyama et al., 2009; Miyoshi et al., 2011).

Although the molecular basis of the observed chemoprotective effect of carotenoids has not been unraveled, the anti-inflammatory effects attributed to carotenoids may reflect their antioxidant activity. Inflammatory markers such as iNOS, TNF-α, COX-2 and IL-1β are common modulators of tumor development and metastasis in chronic inflammatory diseases such as colitis-associated colorectal carcinogenesis (Tanwar et al., 2009; Nagendraprablhu et al., 2011; Yasui et al., 2011; Kawabata et al., 2012). COX-2 expression is upregulated during early colon carcinogenesis suggesting an important role in the progression of the disease, e.g. through the regulation of angiogenesis, apoptosis and tumor invasiveness (De Robertis et al., 2011). Western blotting and in situ immunohistochemistry showed that COX-2 levels in the normal

colonic mucosa were higher in AOM/DSS-treated mice fed on the wild-type corn than in those fed on the high-carotenoid corn. Previous studies have also suggested that carotenoids may be able to regulate the expression of COX-2 during the progression of colon cancer (Nagendraprablhu et al., 2011; Tanwar et al., 2009; Kawabata et al., 2012).

The inhibition NF-κB is known to suppress COX-2 expression and protect against colorectal cancer so this is a potential candidate that could be targeted by carotenoids (Tanwar et al., 2009; Nagendraprablhu et al., 2011; Yasui et al., 2011; Kawabata et al., 2012; Tuzcu et al., 2012). We found that there was no significant change in NF-κB mRNA expression levels when we compared the diet groups ($p = 0.42$) which indicates that the gene encoding NF-κB is unlikely to be a target, although a direct effect on the protein, or perhaps another component of the same pathway (such as the inhibitor IκB) cannot be ruled out. However, there was a significant reduction in the levels of p44/42 MAPK, an important modulator of cytokine production that controls the infiltration of monocytic cells, intestinal electrolyte and water secretion, and intestinal inflammation (Kyriakis and Avruch, 2001). Previous studies have shown that MAPKs induce COX-2 expression in intestinal epithelial cells and promote inflammatory diseases in the intestine (Hommes et al., 2002; Waetzig et al., 2002; Salh et al., 2003; Kim et al., 2005; Camacho-Barquero et al., 2007). Therefore, it is possible that carotenoids act upon this mediator, or upstream on other components of the receptor tyrosine kinase signaling pathway.

In conclusion, the data presented herein suggest that high-carotenoid corn inhibits the progression of colon cancer in an AOM/DSS mouse model and the mechanism involves the suppression of the inflammatory modulator COX-2, possibly by interfering indirectly in an upstream signaling pathway mediated by p44/42 MAPK.

3.2.5. REFERENCES

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CHAPTER 3

The effects of high-carotenoid corn on insulin sensitivity in a mouse model of obesity and insulin resistance induced by a high-fat diet

CHAPTER 3

The effects of high-carotenoid corn on insulin sensitivity in a mouse model of obesity and insulin resistance induced by a high-fat diet

3.3.1. INTRODUCTION

Overweight and obese individuals carry a higher risk of type-2 diabetes (T2D). This is a major concern given that 366 million people worldwide are now considered obese, 55.2 million of whom live in Europe (Pan et al., 2013; Breen et al., 2014).

Studies in both experimental animals and humans have shown that dietary fat composition has an impact on insulin sensitivity, i.e. saturated fats promote high plasma insulin levels whereas unsaturated fats help to improve insulin sensitivity (Riccardi et al., 2004). Recent studies have shown that neutrophils mediate the development of insulin resistance in animal models of obesity induced by high-fat diets, reflecting the production of the pro-inflammatory neutrophil elastase (Talukdar et al., 2012). This means that insulin resistance can be reversed by life-style changes including the adoption of a healthy diet before the onset of clinical T2D (Solomon et al., 2010; Macotela et al., 2011). As well as T2D, obesity also increases the risk of several other chronic diseases, including cardiovascular disease, hypercholesterolemia, asthma, hypertriglyceridemia, hypertension/stroke, and certain forms of cancer (Mokdad et al., 2003; Greenberg and Obin, 2006).

Adipose tissue grows by recruiting new fat cells from the adipose tissue precursor cell pool, which has a high capacity for adipocyte differentiation (Schling and Löffler, 2002; Jo et al., 2009). As individuals become obese, the adipose tissue undergoes molecular and cellular changes that affect systemic metabolism (Greenberg and Obin, 2006). Adipose tissue therefore plays an important role in the development of obesity-related insulin resistance because it secretes adipokines, cytokines and chemokines that influence glucose homeostasis, energy balance and fatty acid metabolism. Adipocytes accumulate triglycerides and thus reduce the circulation of free fatty acids, but when they reach their storage capacity they undergo senescence, apoptosis or necrosis, all

of which are linked to the infiltration of adipose tissue by immune cells and macrophages, causing inflammation that leads to vascular deterioration and hypoxia (Lafontan, 2014). The latter triggers the release of pro-inflammatory cytokines such as TNFα, interleukin-6 and C-reactive protein, and macrophage chemokines such as monocyte chemoattractant protein-1, which reduce the triglyceride storage capacity further and reinforce a feedback loop leading to greater dysfunction (Lafontan, 2014). Triglyceride synthesis declines and lipolysis is induced, increasing the levels of circulating free fatty acids and triglycerides which cause macrophages to infiltrate the liver and skeletal muscle, inhibiting the insulin signaling pathway (Schenk et al., 2008). The amount of adipose tissue can increase by hyperplasia (an increase of adipocyte number) or hypertrophy (an increase of adipocyte size) and the latter is associated with a worse lipid profile, greater glucose intolerance and higher levels of insulin (Roberts et al., 2009; Arner et al., 2010; Divoux et al., 2010).

Phytochemicals can help to improve insulin sensitivity (Rayalam et al., 2008) and carotenoids in particular are valuable because they possess antioxidant activity and can act as cell growth regulators, gap junction communicators, and modulators of gene expression, the immune response and drug metabolism (Rao and Rao, 2007). However, the impact of carotenoids on adipose tissue is unclear, because this is where carotenoids and related retinoids are stored, including retinal, retinoic acid and retinyl palmitate, all of which can act as regulators of adipocyte differentiation, lipid metabolism and adipokine secretion thus increasing insulin sensitivity (Kawada et al., 1996; Kumar et al., 1999; Bonet et al., 2003; Felipe et al., 2003; Ziouzenkova et al., 2007). Even apocarotenals, the metabolic breakdown products of carotenoids, can influence adipocyte biology (Ho et al., 2007).

The aim of the work described in this chapter was to investigate the potentially beneficial role of the mixture of carotenoids contained in the matrix of a genetically-engineered high-carotenoid corn variety, specifically its effects on insulin sensitivity and energy balance in a mouse model of obesity induced by a high-fat diet.

3.3.2. MATERIALS AND METHODS

3.3.2.1. Diet

Experimental diets containing wild-type M37W corn or genetically engineered high-carotenoid corn (grown under the same conditions and harvested at the same time) were prepared from freeze-dried powdered kernels under hygienic conditions. The diets were formulated to achieve isoproteic mixtures with a balanced macronutrient composition by complementing the corn component with corn starch, casein and corn oil to obtain the final normocaloric diets based on wild-type corn (NWT) and high-carotenoid corn (NCAR). The equivalent hypercaloric diets based on wild-type corn (HWT) and high-carotenoid corn (HCAR) were prepared by including pork lard as an additional source of fats.

3.3.2.2. General nutrient compositional analysis

Nutrient compositional analysis was carried out at the Chemistry Department (University of Lleida) as described in Chapter 3. Briefly, the moisture level was determined by measuring the loss of weight after drying in an oven at 100°C to a constant weight, the fat content was measured by Soxhlet extraction, and ash levels were estimated by gravimetric analysis after ignition in an electric furnace. Crude fiber levels were determined by measuring the difference between the weight of the residue remaining after the sample was digested under specific conditions and the weight of the ash. The amount of protein was estimated by determining the total nitrogen content using the Dumas combustion method and applying a nitrogen-to-protein conversion factor of 6.25 (AOAC International, 2000).

Corn starch was measured following 30 min alkaline hydrolysis (4 M KOH) at room temperature. The hydrolysate was neutralized by adding 2 M HCl and incubating with β-amiloglucosidase (Sigma) (pH 4.75, 60°C, 45 min) before measuring the glucose level using a commercial colorimetric enzyme assay (GOP-PAD, Spinreact 1001190)

3.3.2.3. Animal feeding study

The study complied with Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat) of Catalonia and EU Directive (EEC 63/2010), and was approved by the Ethics Committee on Animal Experiments of the University of Lleida.

Twenty-eight 5-week-old male ICR (CD-1®) mice obtained from Harlan Laboratories (Sant Feliu de Codines, Spain) were acclimated for 1 week before the experiment by providing them with *ad libitum* access to a 2014 Teklad Global 14% protein rodent maintenance diet and water. Following acclimation, 6-week-old mice were placed in individual cages and seven mice were randomly assigned to each of the four diet groups: NWT, NCAR, HWT and HCAR. The animal rooms were environmentally controlled and maintained at 20 ± 2°C and 50 ± 5% relative humidity, with a 12-h photoperiod. Body weight and food consumption were measured weekly over a duration of 4 months.

Before the end of the experiment, the animals were injected with glucose to carry out a subcutaneous glucose tolerance test (ScGTT) followed a week later by an insulin injection to carry out an insulin tolerance test (ITT). After one further week, the mice were fasted for 12 h and sacrificed by decapitation. Blood was collected into EDTA-coated capillary tubes, centrifuged at 1500 x g for 10 min and immediately frozen. Visceral and subcutaneous adipose tissue, skeletal muscle (*rectus femoris*) and liver tissue were collected and stored at –80°C.

3.3.2.4. Subcutaneous glucose tolerance and insulin tolerance tests

Mice that had been fasted for 12 h were injected subcutaneously with 2 g glucose per kg body weight and blood samples were taken for glucose measurements at 0, 20, 40, 60 and 120 min after the injection to determine glucose levels using a glucose meter. For ITT, mice fasted for 2 h were injected subcutaneously with human insulin (12343, Sigma, USA) at 0.5 U/kg body weight and were taken for glucose measurements at 0, 20, 40, 60 and 120 min after injection.

3.3.2.5. Blood biochemistry

Clinical chemistry kits (Spinreact, Girona, Spain) were used to measure total cholesterol, low-density and high-density lipoproteins (LDL/HDL), triglycerides, uric acid, HbA1c and fructosamine. The concentration of non-esterified fatty acids (NEFAs) was measured using a commercial colorimetric enzyme NEFA assay kit (Roche, Mannheim, Germany).

3.3.2.6. Antioxidant capacity

The antioxidant capacity of the plasma was measured using two different assays. The first was a ferric reducing antioxidant power (FRAP) assay based on the reagents 2,4,6-tripyridyl-S-triazine (T1253, Sigma-Aldrich, Steinheim, Germany), $FeCl_3$ and acetate buffer (Serrano et al., 2013). Briefly, 900 µl of the FRAP reagent was mixed with 90 µl distilled water and 30 µl plasma. The absorbance was recorded at 595 mm using a spectrophotometer (Multiskan Ascent 96/384 Plate Reader, Finland). The second used 3-ethylbenzothiazoline-6-sulphonate (ABTS) to determine antioxidant capacity in terms of free radical scavenging activity (Hervert-Hernández et al., 2011). Briefly, ABTS radical cation ($ABTS^{+\cdot}$) was produced by reacting 7 mmol/l ABTS stock solution with 2.45 mmol/l potassium persulphate in the dark at room temperature for 12-16 h. The $ABTS^{+\cdot}$ solution was diluted with 5 mM phosphate-buffered saline (PBS, pH 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. After adding 0.1 ml of sample to 3.9 ml of diluted $ABTS^{+\cdot}$ solution, the absorbance was measured every 20 s for 6 min using a Multiskan Ascent spectrophotometer. The inhibition of absorbance over time was plotted and the area below the curve (0–6 min) was calculated. A calibration curve was prepared using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as a standard.

3.3.2.7. Insulin measurements

Plasma insulin was measured using the MILLIPLEX magnetic bead-based immunoassay (Millipore, Billerica, USA).

3.3.2.8. Leptin measurements

Plasma leptin was measured by enzyme-linked immunosorbent assay (ELISA) using the Leptin Mouse ELISA Kit (Abcam, Cambridge, UK).

3.3.2.9. DNA extraction

Genomic DNA was extracted from visceral adipose tissue using the Genomic DNA Mini kit (Invitrogen, Barcelona, Spain) and quantified using a Nanodrop spectrophotometer.

3.3.2.10. Tissue homogenization and western blot analysis

Frozen liver, skeletal muscle and visceral/subcutaneous adipose tissues were homogenized at 4°C in a Polytron device containing the appropriate buffer: 180 mM KCl, 5 mM MOPS, 2 mM EDTA, 1 mM diethylenetriaminepentaacetic acid, 1 µM butylated hydroxyl toluene, 10 µg/mk aprotinin, 1 mM NaF, 1 mM Na_3VO_4 and 1% v/v 80–6501-23 protease inhibitor mix (GE Healthcare, USA). The total soluble protein concentration was determined by the Bradford method (after brief centrifugation to remove cellular debris) and pooled homogenates containing equal amounts of protein from all mice per group were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels (15-40 µg total protein per lane) and transferred to polyvinylidenedifluoride membranes (Immobilon-P Millipore, Bedford, MA, USA) using Bio-Rad apparatus. The primary and secondary antibodies are listed in Table 3.3.1. Protein bands were visualized with the Luminol ™ Western Chemiluminiscent HRP method (Millipore, Billerica, MA, USA) and the band intensity was measured using the Bio-Rad Gel Doc/Chemi Doc Imaging System and Image Lab

software v4.0.1 (Bio-Rad Laboratories). Anti-β-tubulin (Abcam, ab7291) and silver staining were used as protein loading controls.

Table 3.3. 1. Primary and secondary antibodies used for western blot immunodetection.

Antigen	Supplier	Reference	Dilution
AMPKα	Cell Signaling	2532	1:1000
p44/42 MAP kinase	Cell Signaling	4695	1:1000
GLUT4	Abcam	ab654	1:2500
Ubiquitin	Sigma	U5379	1:100
DNP	Sigma	D9565	1:2000
MDAL	Academy Bio-Medical Company	Md20a	1:1000
Anti-β-tubulin	Abcam	ab7291	1:1000
Anti-mouse IgG	GE-Healthcare	NA931	1:5000
Anti-rabbit IgG	Thermo Scientific Pierce	31460	1:100000
Anti-goat	Santa Cruz	SC2020	1:12000

3.3.2.11. Analysis of oxidative damage by protein carbonyl detection

Tissue damage by oxidation was evaluated by measuring protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNP) as previously described (Portero-Otín et al., 1999). Briefly, 15 µl of each homogenate was adjusted to 3.75 µg/µl total protein concentration and SDS was added to a final concentration of 6%. Samples were boiled for 3 min at 95°C and 20 µl of 10 mM DNP (in 10% trifluoroacetic acid) was added and incubated for 7 min at room temperature. The reaction was neutralized and samples were prepared for SDS-PAGE by adding 24 µl of a solution containing 2 M Tris base, 30% glycerol and 15% β-mercaptoethanol. Band intensities were measured in the range 30–250 kDa.

3.3.2.12. Carotenoid and retinoid extraction from liver and adipose tissues

Liver tissue (100 mg wet weight) was lyophilized in a Telstar cryodos-50 for 20 h and dried samples were ground to powder. Visceral adipose tissue (50 mg wet weight) was homogenized with a Polytron device using the buffer described above. The samples were mixed by vortexing with 200 µl 30% potassium hydroxide, 1 ml ethanol, 0.01% butylated hydroxytoluene in 100 µl ethanol and 20 µM β-apo-8'-carotenal in 100 µl ethanol (internal standard). After incubating at 37°C for 45 min, the samples were extracted three times with 3 ml ether:hexane (2:1 v:v) followed by centrifugation at 13,000 x g for 5 min to separate the phases. The extracts were evaporated to dryness under nitrogen, redissolved in 100 µl Phase A buffer and passed through an Eppendorf UltraFree 5-kDa filter (UFC3LTK00, Millipore, Bedford, MA, USA).

3.3.2.13. Carotenoid and retinoid analysis by high-performance liquid chromatography

The processed samples were separated by high-performance liquid chromatography (HPLC) using a YMC C30 column (250 x 4.6 mm, internal diameter 3 µm) at 35°C in a 110 series HPLC system (Agilent Waldbronn, Germany). Mobile phase A consisted of methanol/acetone (60:40) and mobile phase B consisted of acetone/deionized water (60:40). Carotenoids were separated at a flow rate of 0.5 ml/min using a gradient of 60–30% B (0–3 min), 30% B (3–22 min), 30–10% B (22–26 min), 10% B (26–41.5min), 10–60% B (41.5–45 min) and 60% B (45–90 min). Retinoids were separated using 100% mobile phase A (0–30 min). Carotenoids were detected at 450 nm and retinoids at 325 nm.

3.3.2.14. Statistical analysis

Student's *t*-test or either one-way or two-way ANOVA were used for statistical comparisons after data normalization, or in other cases a non-parametric test was used. Values of $p < 0.05$ were considered statistically significant.

3.3.3. RESULTS

3.3.3.1. Nutritional parameters and general observations

Five-week-old mice were fed for 16 weeks on normocaloric or hypercaloric diets supplemented with either wild-type M37W corn and or the genetically-engineered near isogenic high-carotenoid corn. The high-carotenoid corn was shown, as expected, to contain higher levels of several carotenoids: 60 µg/g β-carotene, 23 µg/g lycopene, 36 µg/g zeaxanthin, 15 µg/g lutein, 13 µg/g α-cryptoxanthin, 7 µg/g α-carotene, 5 µg/g β-cryptoxanthin and 5 µg/g γ-carotene (see Chapter 1 Table 3.1.1 for the full comparison).

Table 3.3. 2. Composition of the NWT, NCAR, HWT and HCAR diets.

	NWT (kg diet)	NCAR (kg diet)	HWT (kg diet)	HCAR (kg diet)
Energy (kcal/g)	3.7	3.7	5.2	5.2
Protein (g/kg diet)	249	249	249	249
Carbohydrate (g/kg diet)	476	476	285	285
Lipids (g/kg diet)	109	109	340	340
Dietary fiber (g/kg diet)	58	58	58	58
Casein (g/kg diet)	195.9	172.7	228.8	223.0
L-cysteine (g/kg diet)	3.6	3.5	3.5	3.5
Saccharose ((g/kg diet)	181.3	175.8	199.8	198.2
Lard (pork) (g/kg diet)	0.0	0.0	249.8	247.8
Corn oil (g/kg diet)	97.9	90.9	85.9	84.3
Cellulose (g/kg diet)	43.8	38.4	54.0	51.5
Calcium phosphate (g/kg)	3.5	3.4	3.4	3.4
Choline bitrate (g/kg)	3.1	3.0	3.0	3.0
Mineral mix AIN-93G (g/kg)	44.8	43.4	43.0	42.6
Vitamin mix AIN-93G (g/kg)	19.8	19.2	19.0	18.8
Freeze-dried corn (g/kg)	406.3	449.5	109.9	123.9

Table 3.3. 3. Final body weight, body weight gain and dietary intake (means ± SEM) of mice maintained on the NWT, NCAR, HWT and HCAR diets.

	NWT (n = 7)	NCAR (n = 7)	HWT (n = 5)	HCAR (n = 6)
Final body weight (g)	44.7 ± 1.5	45.0 ± 2.1	57.1 ± 2.4	56.0 ± 3.3
Body weight gain (%)	70.80 ± 5.41	69.17 ± 7.42	112.1 ± 8.04	111.2 ± 11.56
Weight gain/food intake (%)	3.627 ± 0.223	3.840 ± 0.348	6.714 ± 0.389	6.660 ± 0.595
Diet intake (g/day)	4.836 ± 0.118	4.523 ± 0.110	4.262 ± 0.112	4.215 ± 0.213
Energy (kcal/day)	17.70 ± 0.387	17.25 ± 0.4745	22.17 ± 0.579	21.80 ± 1.107
Protein (g/day)	1.206 ± 0.030	1.126 ± 0.027	1.060 ± 0.028	1.050 ± 0.053
Carbohydrates (g/day)	2.301 ± 0.056	2.153 ± 0.052	1.216 ± 0.0326	1.202 ± 0.0600
Fat (g/day)	0.527 ± 0.013	0.493 ± 0.012	1.45 ± 0.038	1.43 ± 0.073

Before the end of the trial, the mice were subjected to glucose tolerance and insulin tolerance tests. Blood samples were taken and analyzed for biochemical markers as well as insulin, leptin and GLUT4, which can be used to infer insulin resistance. The adipocyte cell number was investigated to determine whether the presence of carotenoids in the diet caused hypertrophy or hyperplasia in the adipose tissue. Additional protein markers for insulin sensitivity, energy homeostasis and oxidative damage were then analyzed by western blot. Finally, carotenoid and retinoid levels were tried to be measured in the liver and adipose to determine whether the high-carotenoid diet caused an increase in the levels of stored retinoids in these tissues, which would indicate that the carotenoids are bioavailable and their presence in these tissues may be responsible for differences between the animals in the four diet groups.

The corn component was complemented with additional corn starch, casein and corn oil to obtain the nutritionally-balanced and normocaloric diets, and pork lard was added to formulate the hypercaloric diets (Table 3.3.2). Two animals in the HWT group were excluded from the study because there was evidence of a cutaneous reaction, and the application of topical glucocorticoids had the potential to alter the results. One

animal in the HCAR group was also excluded due to spontaneous leg swelling (edema) which was also likely to interfere with the results, especially those based on the detection of inflammation markers. Neither of the conditions was directly associated with the diets.

The hypercaloric diet induced obesity as shown in Table 3.3.3. The final body weight, body weight gain and feed efficiency (weight gain in relation to food intake) were significantly higher in the animals reared on the hypercaloric diets compared to those on the normocaloric diets. Although the study was designed to compare the effects of the wild-type and high-carotenoid corn, comparisons between the hypercaloric and normocaloric diets nevertheless confirmed the ability of the hypercaloric diet to induce obesity efficiently. Mice reared on the HCAR diet gained less weight than those reared on the HWT diet although the mean difference of 1.1 g was not statistically significant. Nevertheless, these data hinted at the potential benefits of the high-carotenoid diet on insulin sensitivity, which were therefore investigated in more detail as described below.

3.3.3.2. Biochemical characteristics

The induction of obesity and the resulting insulin resistance cause the accumulation of fat and modify the fatty acid composition in several tissues. In the current study, this was tested by measuring glucose tolerance, the glycemic response to insulin and plasma insulin levels after 4 months on the experimental diets.

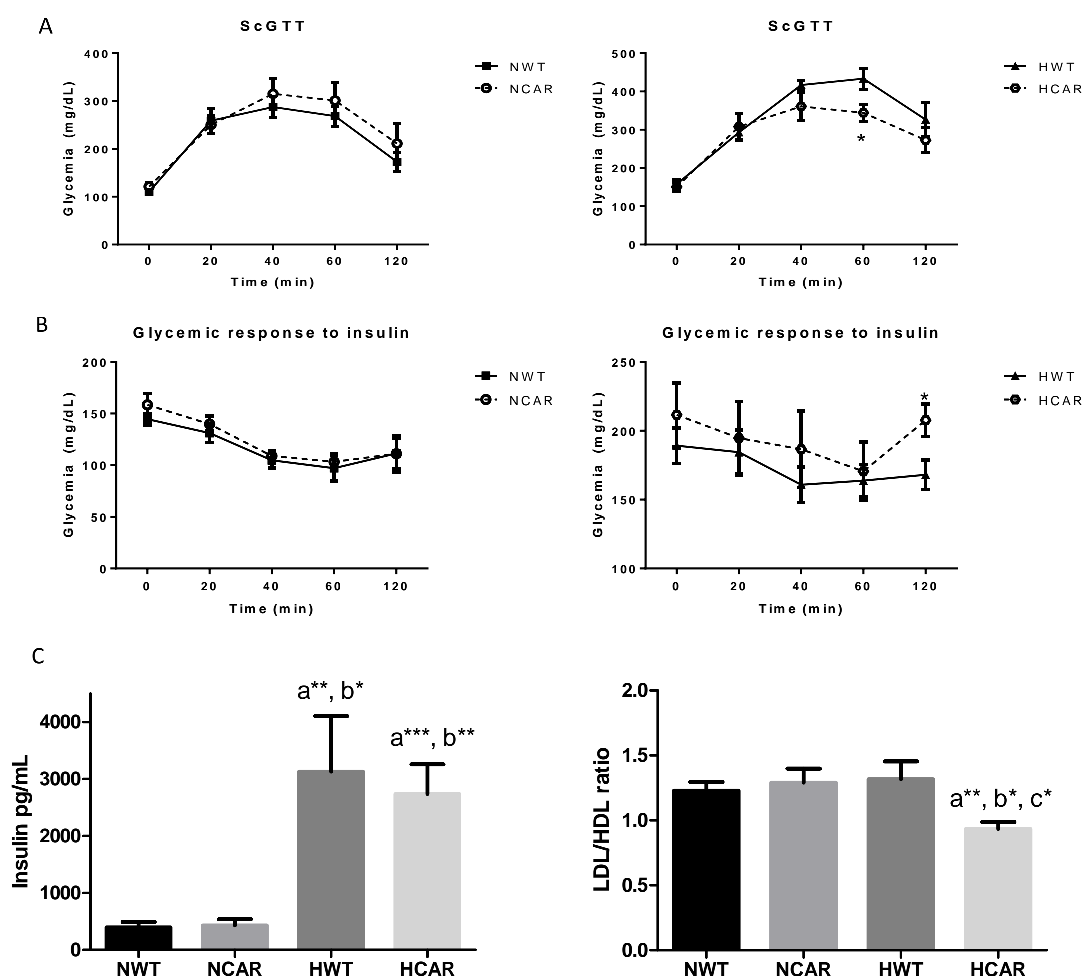

Figure 3.3. 1. . Blood biochemistry parameters in mice maintained on the NWT, NCAR, HWT and HCAR diets. (A) Subcutaneous glucose tolerance. (B) Glycemic response to insulin (C) Blood insulin levels and LDL/HDL cholesterol ratio. Differences were analyzed using Student's t-test: *p < 0.05, **p < 0.01; ***p<0.001. Statistically significant differences are shown with lower case letters: a: statistically significant difference versus NWT group; b: statistically significant difference versus NCAR group; c: statistically significant difference versus HWT group. Glucose, insulin and LDL/HDL cholesterol levels were determined in n = 7 (NWT), n = 7 (NCAR), n = 5 (HWT) and n = 6 (HCAR) animals. All values are presented as means ± SEM.

The glucose and insulin tolerance tests showed that the hypercaloric diet induced both glucose intolerance and insulin resistance. However the glucose homeostasis recovery period was shorter in the HCAR group than the HWT group, and the plasma glucose level 60 min after glucose injection was also significantly lower (p = 0.0318) in the mice fed on the HCAR diet (Figure 3.3.1A). The plasma glucose levels remained slightly lower in the HCAR diet group at the end of the test (120 min) and the area under the glucose curve was 42904 ± 1857 mg/dl x 2h in the HWT group but only 36853 ± 2418 mg/dl x

2h in the HCAR group ($p = 0.087$). The insulin tolerance test (Figure 3.3.1B) showed that mice in the HCAR had a lower hypoglycemic response to insulin than those in HWT group after the injection of 2 g/kg body weight of glucose. The HCAR group also showed a faster glucose recovery rate (tested 60 min after the insulin injection) and a higher glycemic response ($p < 0.05$) after 120 min, i.e. 168.0 ± 10.71 mg/dl in the HWT group compared to 207.7 ± 11.95 mg/dl in the HCAR group.

The basal plasma insulin concentrations were determined by immunoassay (Figure 3.3.1C). Animals fed on the hypercaloric diets showed evidence of hyperinsulinemia as expected, and this was more severe in the animals fed on the HWT diet (3130 ± 972.5 pg/ml) than in those fed on the HCAR diet (2734 ± 523.5 pg/ml) although the difference between the corn lines was not significant. There was also no significant difference when the two normocaloric diets were compared (NWT = 395.2 ± 94.82 pg/ml and NCAR = 430.9 ± 106.1 pg/ml).

Metabolic syndrome can result from fat accumulation in non-adipose tissue when its storage capacity is exhausted (Slawik and Vidal-Puig, 2007). Lipids accumulate in metabolically-active non-adipose tissues such as the liver and skeletal muscle, leading to lipotoxicity, insulin resistance, T2D and other chronic diseases (Slawik and Vidal-Puig, 2007). Differences in glucose and insulin sensitivity between the normocaloric and hypercaloric diet groups could therefore reflect differences in fat storage in adipose tissue and non-adipose tissues such as the liver and skeletal muscle. The levels of total cholesterol, HDL, LDL, NEFA and triglycerides were therefore tested, and the corresponding ratios were calculated as shown in Table 3.3.4. The most striking finding was the significantly lower ($p = 0.021$) LDL/HDL cholesterol ratio in the HCAR group compared to the HWT group (Figure 3.3.1).

Table 3.3. 4. Blood biochemistry in animals fed on normocaloric and hypercaloric diets, showing total cholesterol (CHOL), HDL, LDL, non-esterified fatty acids (NEFA), triglycerides (TG), TG/HDL, LDL/HDL and total CHOL/HDL, all presented as means ± SEM. Differences were analyzed using Student's *t*-test: $*p < 0.05$.

	NWT n=7	NCAR n=7	*t* test *p* value	HWT n=5	HCAR n=6	*t* test *p* value
CHOL (mg/dL)	187.2 ± 13.6	189.2 ± 15.6	0.92	228.4 ± 17.7	266.9 ± 23.3	0.23
HDL (mg/dL)	93.4 ± 5.6	93.3 ± 7.0	0.99	109.7 ± 9.0	127.2 ± 17.1	0.15
LDL (mg/dL)	115.7 ± 11.0	120.4 ± 13.0	0.79	147.6 ± 22.5	120.6 ± 13.1	0.31
NEFA (nmol/μL)	2.68 ± 0.05	2.68 ± 0.01	0.97	2.50 ± 0.07	2.47 ± 0.06	0.70
TG (mg/dL)	95.0 ± 32.9	122.0 ± 13.2	0.24	82.1 ± 17.6	81.0 ± 9.0	0.94
TG/ HDL (mg/dL)	1.04 ± 0.17	1.32 ± 0.19	0.29	0.74 ± 0.06	0.66 ± 0.11	0.55
LDL/ HDL (mg/dL)	1.23 ± 0.07	1.29 ± 0.29	0.11	1.32 ± 0.14	**0.93 ± 0.05***	**0.021**
CHOL/ HDL (mg/dL)	2.00 ± 0.09	2.03 ± 0.06	0.82	2.09 ± 0.09	2.08 ± 0.10	0.96

The levels of enzymatic and non-enzymatic plasma antioxidant activity were measured using the FRAP method (Figure 3.3.2). Although there was more antioxidant activity in the HCAR group (343.2 ± 19.84 μmol Trolox equivalent (TE)/g) than the HWT group (322.5 ± 15.72 μmolTE/g) the differences were not significant ($p > 0.05$), and were similar to the levels observed in the normocaloric diet groups (NWT = 346.9 ± 26.69 μmolTE/g, NCAR = 337.3 ± 23.94 μmolTE/g). There were also no significant differences among the diet groups in the plasma ABTS radical assay, although again the HCAR group showed the highest activity of 4273 ± 117.5 μmolTE/g.

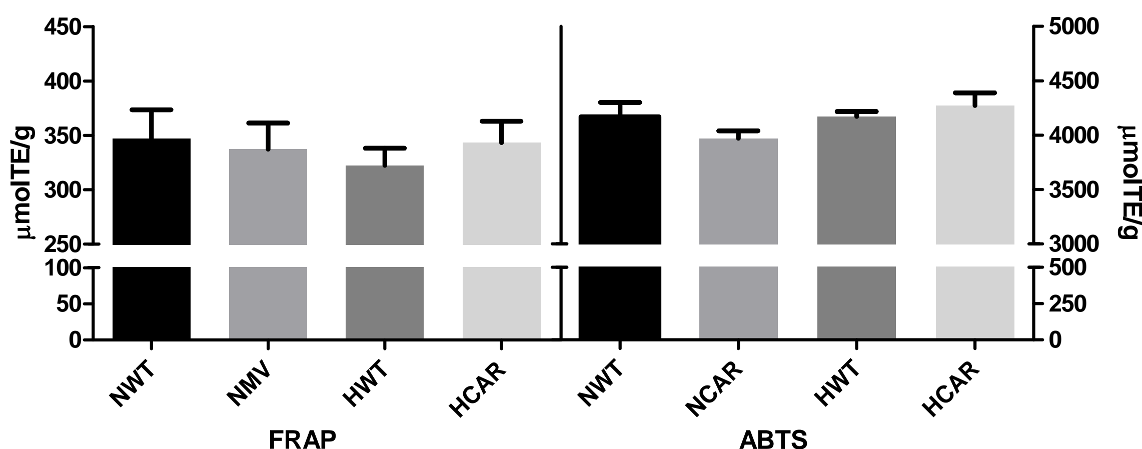

Figure 3.3. 2. Results from the ferric reducing ability of plasma (FRAP) and 3-ethylbenzothiazoline-6-sulphonate (ABTS) blood assays of mice fed on normocaloric and hypercaloric diets. FRAP and ABTS levels were determined in n = 7 (NWT), n = 7 (NCAR), n = 5 (HWT) and n = 6 (HCAR) animals. All values are presented as means ± SEM.

Uric acid is the final oxidation product of purine catabolism and it has been recognized as an effective antioxidant (Koenig and Meisinger, 2008). However, excess levels of uric acid in the blood are associated with a higher risk of cardiovascular disease, metabolic syndrome, obesity and insulin resistance among others, and it can act as a pro-oxidant (Hayden and Tyagi, 2004; Strazzullo and Puig, 2007). Blood uric acid levels were lower in the HCAR diet group (0.79 ± 0.12 µmol/l) than in the other three groups (NWT = 1.3 ± 0.30 µmol/l, NCAR = 1.14 ± 0.19 µmol/l, HWT = 1.03 ± 0.23 µmol/l in HWT) although the difference was not statistically significant.

3.3.3.3. Glucose transport and GLUT4 translocation

The uptake of glucose into skeletal muscle and visceral adipose tissue is mediated by insulin-responsive glucose transporter type 4 (GLUT4) which therefore plays a key role in glucose homeostasis (Olson, 2012). Western blots showed that animals fed on the high-carotenoid corn diet had higher levels of GLUT4 in skeletal muscle and visceral adipose tissue compared to animals fed on wild-type corn (Figure 3.3.3). In the HCAR group, with lower (albeit not significantly lower) blood insulin levels, the stronger expression of GLUT4 in the skeletal muscle may have contributed to the observed

insulin sensitivity and reduced glycemia, and in adipose tissue it may have ensured that the adipocytes retained the capacity to store energy.

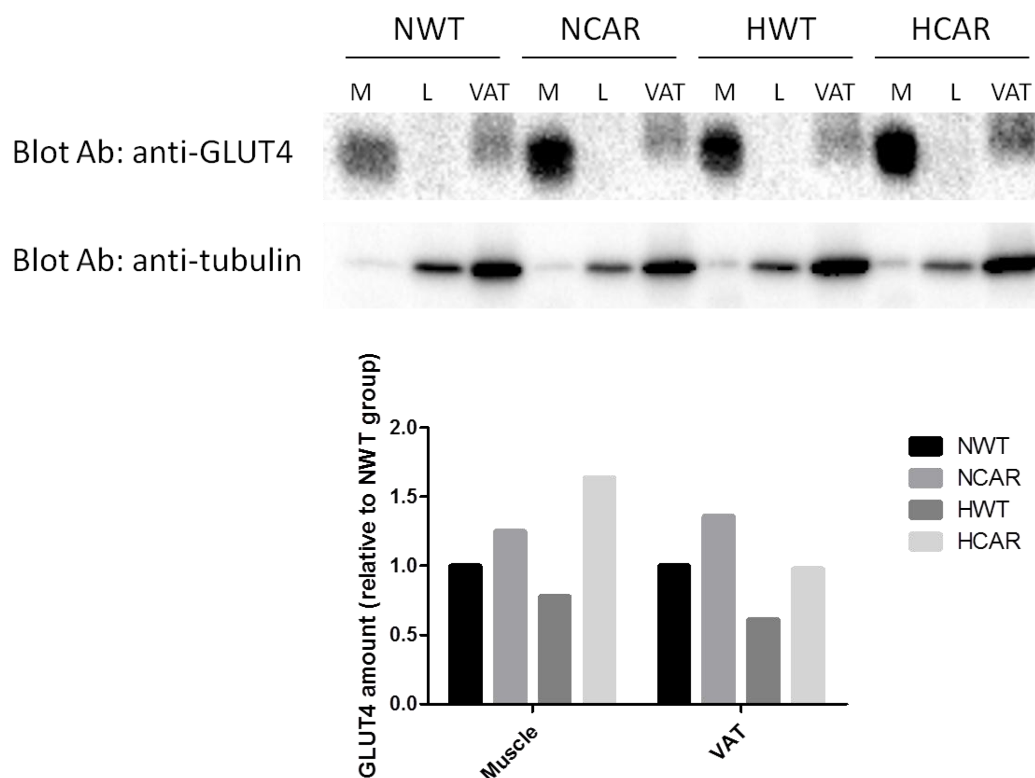

Figure 3.3. 3. Western blot analysis of GLUT4 expression in muscle (M) and visceral adipose tissue (VAT). The histogram shows the expression levels normalized against tubulin and presented as values relative to the expression level in the NWT diet group. Liver (L) homogenates were used as a negative control.

3.3.3.4. AMPKα expression in visceral adipose tissue and skeletal muscle

Adenosine-monophosphate-activated protein kinase (AMPKα) is a key mediator of glycemic homeostasis, fatty acid oxidation and the inhibition of lipogenesis in the liver (Viollet et al., 2006). AMPKα levels in the skeletal muscle, visceral adipose tissue and liver tissue from animals in the four different diet groups were analyzed by western blot. There were no significant differences among the diet groups in terms of AMPKα levels in skeletal muscle (Figure 3.3.4). However, there was a significant reduction in the level of AMPKα signaling in the visceral adipose tissue of the HCAR diet group

compared to the HWT and NCAR groups. This showed that the key pathway controlling glucose uptake and fatty acid oxidation was downregulated in response to the high-fat diet, suggesting that the adipose tissue was able to take up fatty acids. The significant increase in AMPKα levels in the livers of HCAR mice suggested that lipogenesis, cholesterol synthesis and triglyceride synthesis were inhibited and that hepatic AMPK activation may therefore play an important role in the prevention of fatty atherosclerosis, liver disease and dyslipidemia in T2D (Winder and Hardie, 1999; Li et al., 2011).

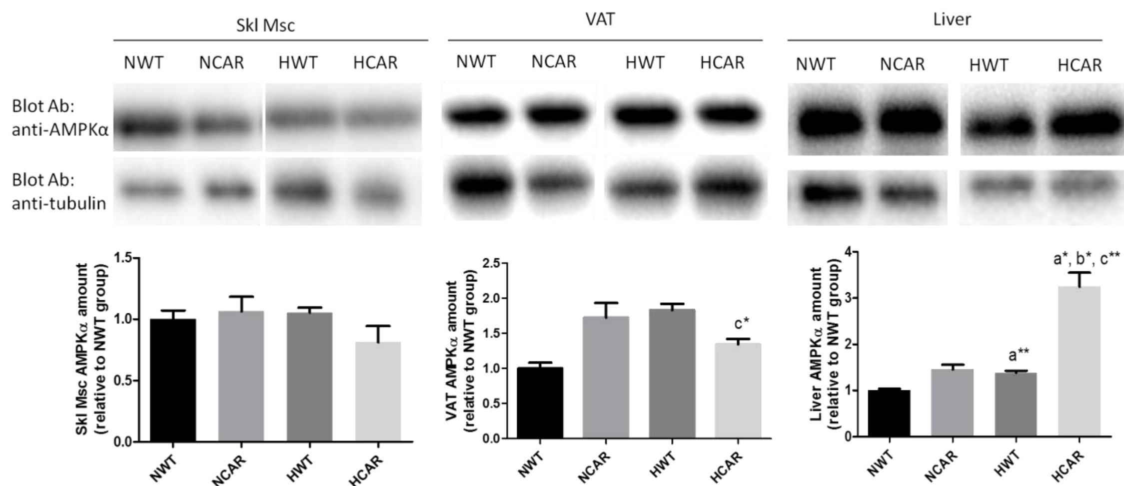

Figure 3.3. 4. Western blot analysis of AMPKα expression in skeletal muscle (Skl Msc), visceral adipose tissue (VAT) and liver. The histogram shows the expression levels normalized against tubulin and presented as values relative to the expression level in the NWT diet group. Differences were analyzed using Student's *t*-test: *p<0.05; **p<0.01; a: statistically significant difference versus NWT group, b: statistically significant difference versus NCAR group; c: statistically significant difference versus NWT group.

3.3.3.5. Glycemic control: glycosylated hemoglobin and fructosamine

Blood glucose levels can be estimated over time by measuring glycosylated hemoglobin (Hb1Ac) and this indicated lower average glucose levels in the HCAR group compared to the HWT group, although difference was not statistically significant (Figure 3.3.5). Hb1Ac levels can also be influenced by differences in the lifespan of red blood cells between the groups (Cohen et al., 2008) so fructosamine levels were also

tested to evaluate glycemic control because this shows the amount of plasma protein glycation, and plasma proteins are synthesized predominantly in the liver. There was a significant difference ($p < 0.05$) between the two carotenoid-enriched diets compared to the normocaloric diet based on wild-type corn, suggesting that the presence of carotenoids results in higher levels of plasma protein glycation regardless of whether the diet is normocaloric or hypercaloric.

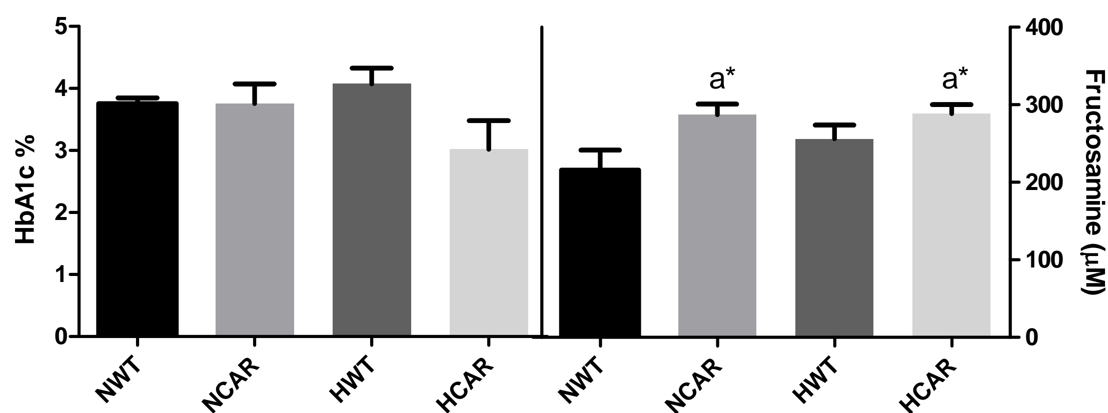

Figure 3.3. 5. Analysis of the glycemic control indicators glycosylated hemoglobin and fructosamine. Differences were analyzed using Student's *t*-test: *p<0.05; a: statistically significant difference versus NWT group

3.3.3.6. The role of visceral adipose tissue in insulin sensitivity

To determine the effect of high-carotenoid corn on adipocyte differentiation and whether the growth of adipose tissue was associated with a higher adipose cell number (hyperplasia) or an increase in size (hypertrophy), adipocyte genomic DNA was isolated and the relative amounts of DNA and total protein were quantified as previously described (Patel et al., 2003). The adipose tissue in the HCAR diet group contained nearly twice as much DNA (1.83-fold more) than the HWT group, which in turn contained about the same amount of DNA as the NWT and NCAR groups (Figure 3.3.6). The adipose tissue of the HCAR diet group also contained 1.52-fold more protein than the HWT group, which in turn was marginally lower than the NWT and NCAR groups. Although none of these differences were statistically significant, the data clearly indicated that the growth of adipose tissue in the HCAR diet group is mostly due to the higher number of adipocytes per gram of tissue, suggesting that

dietary carotenoids could promote hyperplasia over hypertrophy when combined with a hypercaloric diet.

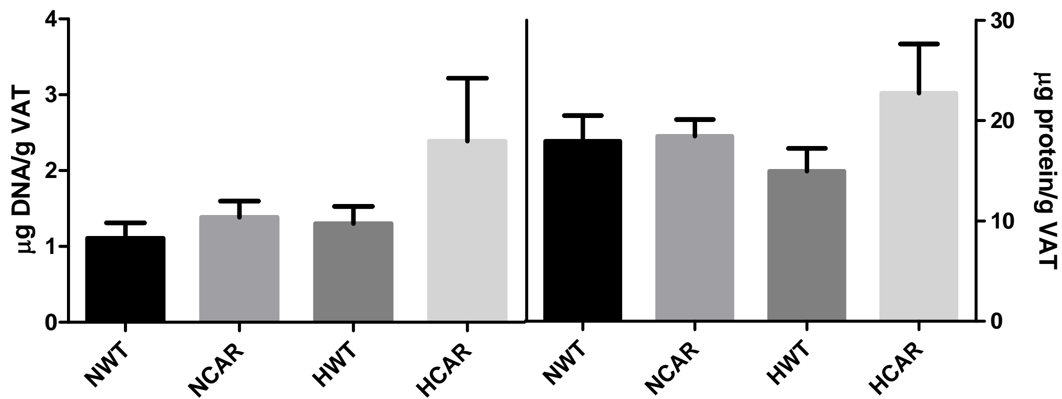

Figure 3.3. 6. Absolute amounts of DNA and protein per gram of visceral adipose tissue (VAT) in the four diet groups.

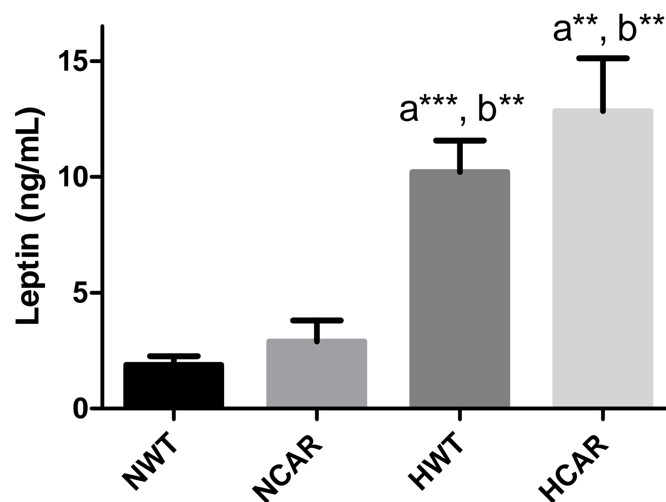

Figure 3.3. 7. Plasma leptin levels in the four diet groups. Differences were analyzed using Student's *t*-test: **p<0.01 and ***p<0.001; a: statistically significant difference versus NWT group; b: statistically significant difference versus NCAR group. Levels were determined in n = 7 (NWT), n = 7 (NCAR), n = 5 (HWT) and n = 6 (HCAR) animals. All values are presented as means ± SEM.

Leptin is an adipocyte-derived hormone that regulates energy expenditure, food intake and glucose metabolism (Friedman and Halaas, 1998). A leptin-specific ELISA was used to measure the levels of this hormone in the plasma of all four diet groups (Figure 3.3.7). The results showed that both hypercaloric diets induced the production of leptin resulting in significant differences between the HCAR/HWT and NCAR/NWT groups. There was also a slight increase in leptin levels in the mice fed on high-carotenoid corn (HCAR and NCAR) compared to their counterparts fed on wild-type corn, with the greatest difference observed between the HCAR group (12.84 ± 2.28 ng/ml) and the HWT group (10.21 ± 36 ng/ml). Although the carotenoid-dependent differences were not statistically significant, the lower leptin levels in animals fed on the wild-type corn diets may reflect the lower number of adipocytes (and the absence of adipocyte atrophy) based on the data presented in Figure 3.3.7.

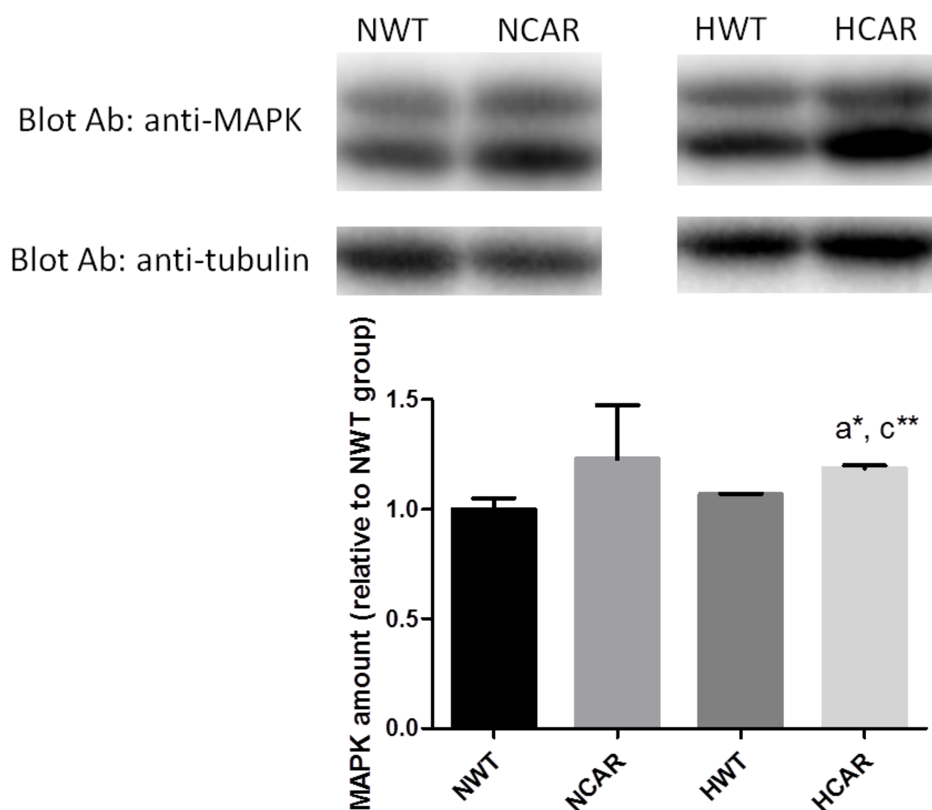

Figure 3.3. 8. Western blot analysis of p44/42 MAPK expression in visceral adipose tissue. The histogram shows the expression levels normalized against tubulin, presented as values relative to the expression level in the NWT diet group. Differences were analyzed using Student's *t*-test: **p*<0.05 and ***p*<0.01; a: statistically significant difference versus NWT group; c: statistically significant difference versus HWT group.

The expression of p44/42 MAPK was evaluated by western blot to determine whether the MAPK signaling pathway was affected by the carotenoid-rich diets, given that this pathway initiates a transcriptional program that leads to cellular proliferation or differentiation (Saltiel and Kahn, 2001). There was a statistically significant 1.1-fold increase (p = 0.0062) in the HCAR group compared to the HWT group, indicating that the pathway may be modulated by the presence of carotenoids in the diet (Figure 3.3.8).

3.3.3.7. Oxidative damage assays

Three assays were carried out to survey the degree of oxidative damage in the skeletal muscle, visceral adipose tissue and liver of mice from all four diet groups to determine if the antioxidant activity of carotenoids had a protective effect. The abundance of protein-reactive carbonyls was determined by western blot (based on the detection of carbonylated proteins) showing a significant increase in the skeletal muscle of mice fed on both hypercaloric diets compared to those fed on the corresponding normocaloric diets, but a significant (p = 0.0066) reduction in the livers of mice in the HCAR group compared to those in the HWT group (Figure 3.3.9).

A malondialdehyde-lysine (MDAL) assay was carried out because MDAL is an end product of lipid peroxidation (Gaweł et al., 2004). There were no significant differences in skeletal muscle MDAL levels among the different diet groups, but the level of MDAL was significantly higher in the visceral adipose tissue of mice fed on the HCAR diet compared to the HWT diet group, suggesting that animals in the HCAR diet group were exposed to increased lipid oxidative stress (Figure 3.3.10). There were no significant differences among the four diet groups in terms of the abundance of ubiquitinylated proteins in skeletal muscle or adipose tissue, but mice fed on the HCAR diet showed significantly higher levels of ubiquitinylated proteins in the liver (Figure 3.3.11).

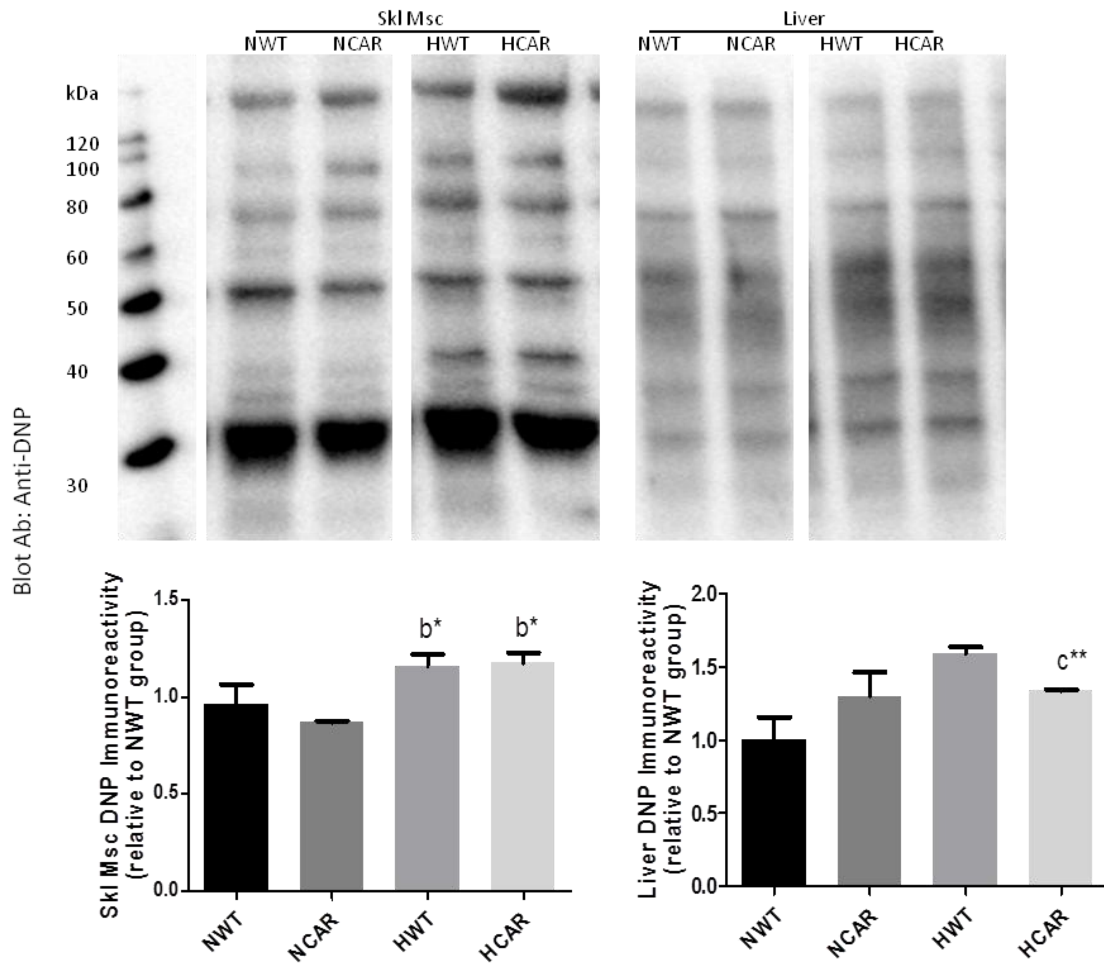

Figure 3.3. 9. Western blot analysis for protein reactive carbonyls (DNP) in skeletal muscle (Skl Msc) and liver. The histogram shows the expression levels normalized against Gallyas staining and quantified by densitometry. Differences were analyzed by Student's *t*-test: $*p < 0.05$ and $**p < 0.01$; b: statistically significant difference versus NCAR group, c: statistically significant difference versus HWT group.

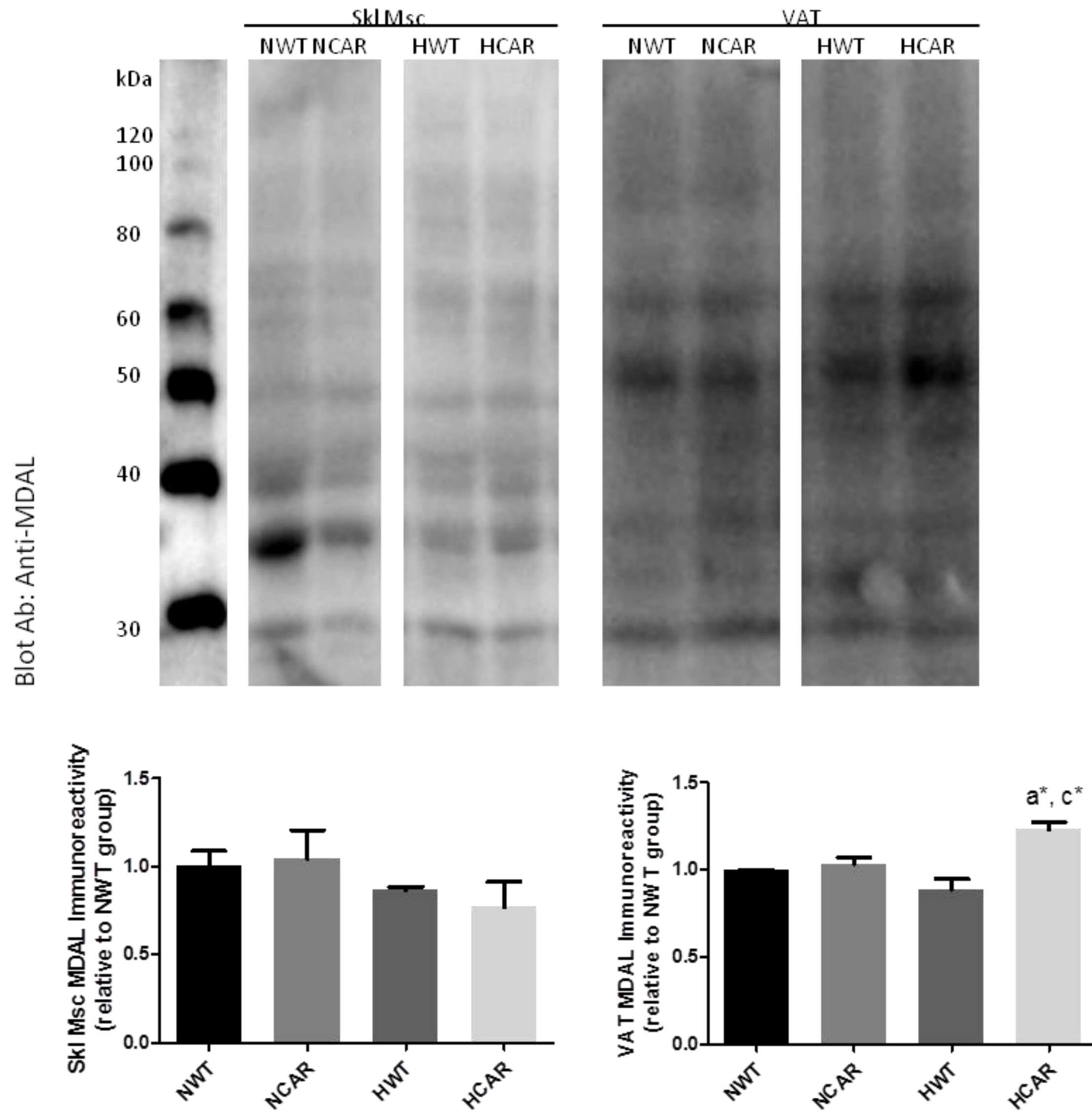

Figure 3.3. 10. Western blot analysis of MDAL in skeletal muscle (Skl Msc) and visceral adipose tissue (VAT) to show the level of lipoxidation. The histogram shows the expression levels normalized against Gallyas staining and quantified by densitometry. Differences were analyzed by Student's *t*-test: $*p < 0.05$; a: statistically significant difference versus NWT group; c: statistically significant difference versus HWT group.

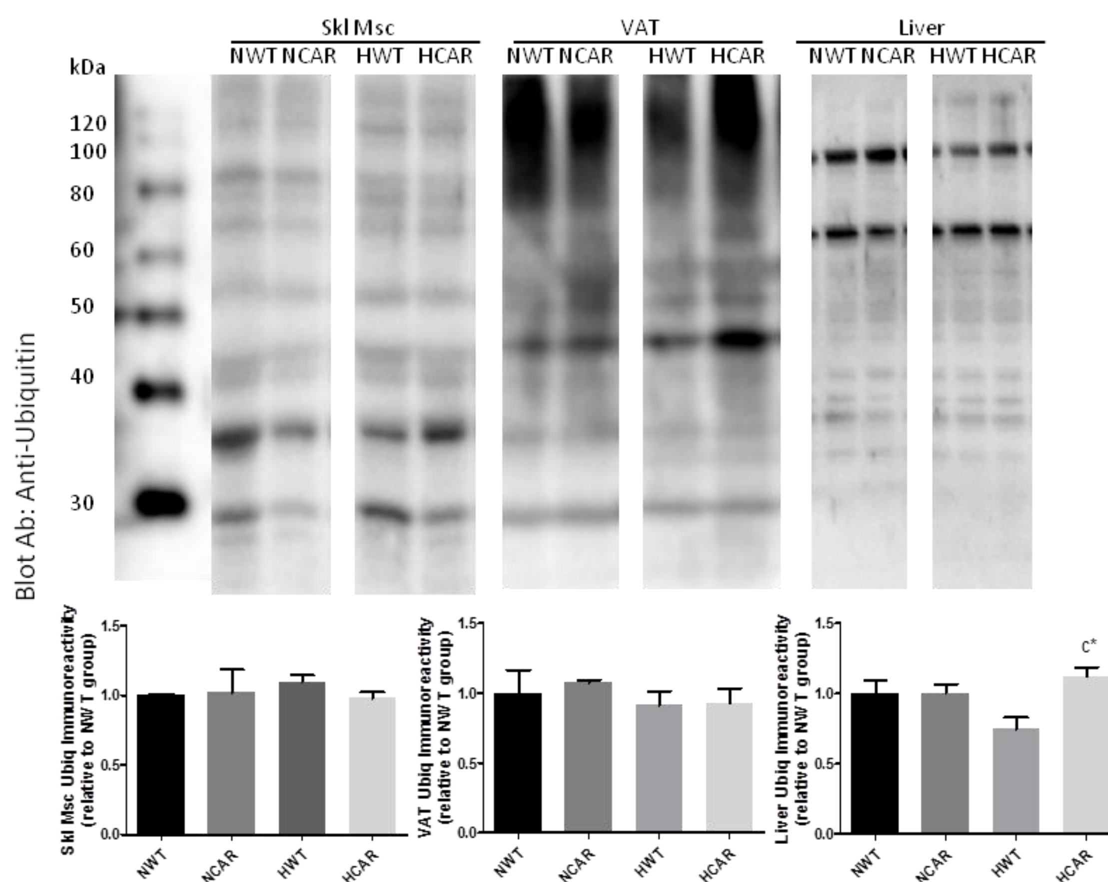

Figure 3.3. 11. Western blot analysis of ubiquitinylated proteins (Ubiq) in skeletal muscle (Skl Msc), visceral adipose tissue (VAT) and liver. The histogram shows the expression levels normalized against Gallyas staining and quantified by densitometry. Differences were analyzed by Student's *t*-test: $*p < 0.05$; c: statistically significant difference versus HWT group.

3.3.3.8. HPLC analysis of the liver and adipose tissue retinoid content

The retinoid content of the liver and visceral adipose tissue in the four diet groups was determined by HPLC (Figure 3.3.12). It was not possible to measure specific carotenoid levels directly because the retention times overlapped. The livers of animals reared on the hypercaloric diets stored lower levels of retinoids (retinol and retinoic acid) than those reared on the normocaloric diets, indicating that the hypercaloric diets interfere with the hepatic storage of pro-vitamin A carotenoids. The visceral adipose tissue generally stored 10-fold lower levels of retinoids than the liver, but the visceral

adipose tissue of the NCAR diet group stored more than twice the amount of retinoic acid as in any other diet group.

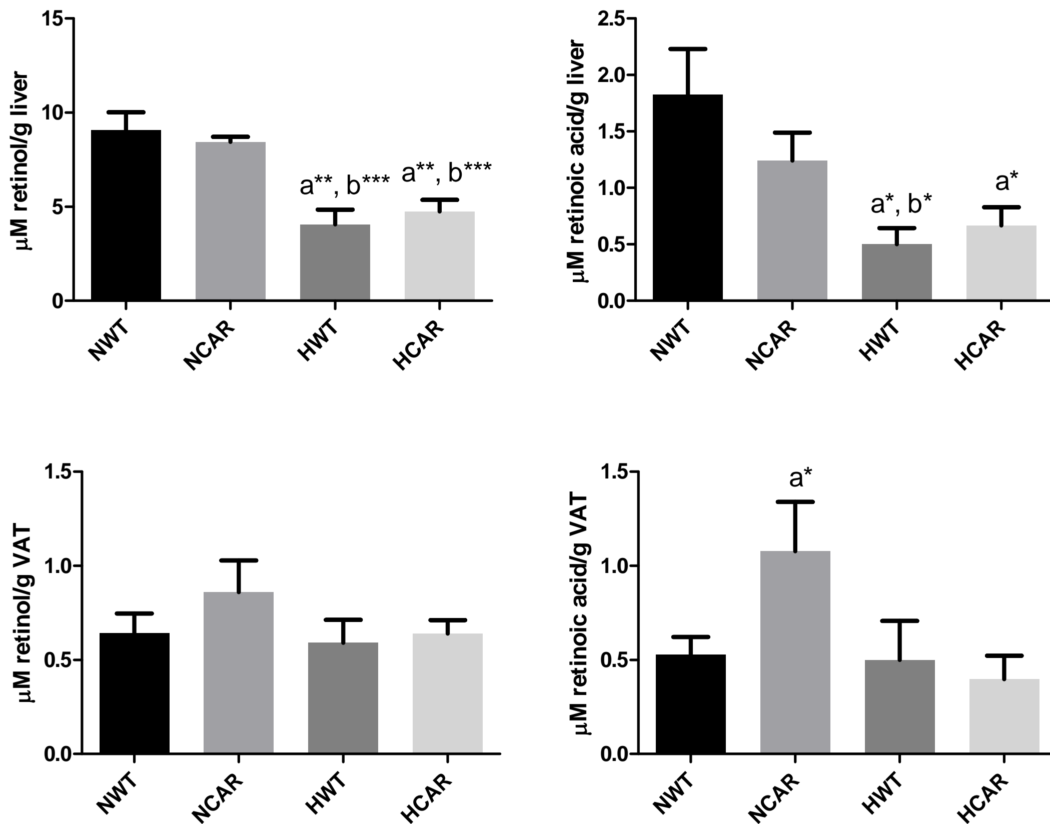

Figure 3.3. 12. HPLC analysis of retinol and retinoic acid levels in the liver and visceral adipose tissue in all four diet groups. Results are shown as µM/g wet tissue. Differences were analyzed by Student's *t*-test: *p < 0.05 and **p < 0.01; a: statistically significant difference versus NWT group; b: statistically significant difference versus NCAR group. Retinoic acid and retinol levels were determined in n = 7 (NWT), n = 7 (NCAR), n = 5 (HWT) and n = 6 (HCAR) animals. All values are presented as means ± SEM.

3.3.4. DISCUSSION

The comparative impact of high-carotenoid and wild-type corn on energy homeostasis was evaluated using a mouse model of obesity and insulin resistance induced by the consumption of a hypercaloric diet. Normocaloric control groups based on each type of corn were also tested, yielding four different diet groups: HCAR, HWT, NCAR and NWT.

Lipid-rich diets are known to modulate gene expression, hormone sensitivity and morphology in pre-existing adipocytes (Li et al., 2002). The hypercaloric diets administered to mice in our feeding trial contained threefold more lipids than the normocaloric diets resulting in a 1.25-fold increase in final body weight, a 1.6-fold increase in weight gain and a 1.8-fold increase in the weight gain/food intake ratio. There was a difference of just over 1 g in the mean final body weight of the HWT and HCAR diet groups, and although the difference was not significant it could nevertheless indicate that carotenoid-enriched corn can reduce weight gain in mice fed on high-fat diet, perhaps by affecting insulin sensitivity or perhaps through the modulation of adipose tissue responses.

Tests for glucose tolerance and the glycemic response to insulin showed that the hypercaloric diet induced insulin resistance, but not aggressively, because the basal levels of glucose in both groups were similar (see Figure 3.3.1). However, following the injection of glucose and insulin, the mice in the normocaloric diet groups normalized the basal glucose levels more quickly than those in the hypercaloric diet groups. Mice fed on the HCAR diet recovered more quickly than those on the HWT diet reflecting the stronger induction of GLUT4, and they demonstrated improved insulin sensitivity and insulin-mediated uptake of glucose by skeletal muscle and adipose tissue compared to HWT mice. However, all mice on the hypercaloric diets presented with insulin resistance (hyperglycemia and hyperinsulinemia) compared to the normocaloric diet groups). The enhanced insulin sensitivity and glucose tolerance in the HCAR group correlated with the observed modulation of the lipid profile, which was characterized by a 1.2-fold increase in the relative amount of HDL cholesterol and a significantly lower LDL/HDL ratio. HDL cholesterol helps to prevent atherosclerotic vascular disease

(Barter et al., 2007), and may also promote glucose homeostasis and insulin sensitivity through its anti-inflammatory activity, by increasing glucose uptake and by stimulating pancreatic β-cell insulin secretion (Brunham et al., 2007; Fryirs et al., 2010; Drew et al., 2012).

The increase in adipose tissue mass in obese mice can be driven by increasing the number of adipocytes (hyperplasia) or the size of existing cells (hypertrophy). Comparison of total DNA and protein levels across groups showed a 1.8-fold increase in the amount of DNA and a 1.5-fold increase in the amount of protein in the adipose tissue of the HCAR group, strongly suggesting hyperplasia as the underlying mechanism even though the differences were not statistically significant. Moreover, the activation of MAPK kinase can phosphorylate transcription factors that promote cellular proliferation or differentiation (Saltiel and Kahn, 2001). The data from this study suggest that MAPKs were significantly upregulated in HCAR mice and could therefore induce cellular differentiation and proliferation in visceral adipose tissue. Adipose tissue growth is therefore likely to involve hyperplasia (adipogenesis) in which new adipocytes are recruited from precursor cells via the proliferation and differentiation of preadipocytes (Hausman et al., 2001). The induction of adipogenesis reflects the need to store excess calories and the formation of new cells is a safe way to adapt to this increased energy intake (Rosen and Spiegelman, 2014).

The analysis of oxidative stress in skeletal muscle showed that high-carotenoid corn had no effect in this tissue, but MDAL levels were significantly higher in the HCAR group compared to the HWT group, suggesting a higher rate of lipid oxidation. Oxidative stress caused by exposure to hydrogen peroxide can directly modulate adipose cell differentiation (Schneider and Chan, 2013). The HCAR mice also showed a significant reduction in the level of oxidative damage in the liver (DNP assay) and significantly higher levels of ubiquitin-conjugated proteins compared to HWT mice, the latter indicating that any increase in oxidation damage was counteracted by endogenous repair processes. Levels of oxidation were similar between the normocaloric and hypercaloric groups indicating that the high-fat diet did not induce severe oxidative stress. However, further experiments are needed to determine

whether high-carotenoid corn has a specific impact on the regulation of oxidative stress.

A higher calorific intake reduces the adenosine monophosphate: adenosine triphosphate (AMP:ATP) ratio (Kahn et al., 2005) by inhibiting AMPK, reflecting a feed-forward effect of lipid overload (Liu et al., 2006; Martin et al., 2006; Wu et al., 2007). The significant increase of AMPK levels in hepatocytes could protect against hyperlipidemia and hepatic steatosis in this mouse model of diet-induced obesity and insulin resistance as previously reported (Li et al., 2011). There was a significant reduction in the level of AMPKα in the visceral adipose tissue of the HCAR diet group, showing that the key pathway controlling glucose and lipid uptake was downregulated in response to the high-fat diet, and suggesting that adipocytes remain functional and can incorporate fats when AMPKα levels are low, thus stimulating adipogenesis and increasing the activity of MAPK. The larger number of fatty cells means that adipose tissue avoids energy excess in the adipose tissue and allows the uptake of glucose (shown by GLUT4 results). The increased MAPK levels in HCAR mice suggests that these animals were able to respond to insulin signaling and regulate fundamental cellular functions including survival and cell differentiation (Eisenmann et al., 2003). Further investigations should be carried out to confirm the reduced AMPK signaling in HCAR mice i.e. by analyzing downstream targets such as PPAR-γ and SREBP (Li et al., 2011; Yoon, 2011).

The levels of retinoids in liver and adipose tissue were similar to those published in earlier studies (Kane et al., 2008; Kim et al., 2008). The elevation of retinoid levels in the normocaloric diet groups may reflect the lower intake of high-carotenoid corn in the hypercaloric groups, where the corn represented only 12% of the diet compared to 45% in the normocaloric group.

In conclusion, the high-carotenoid corn improved systemic glucose homeostasis and the LDL/HDL cholesterol ratio in obese mice by increasing the sensitivity of skeletal muscle and adipose tissue to circulating insulin, probably by inducing the expression of GLUT4. The increase in adipose tissue mass was explained by hyperplasia rather than hypertrophy (suggested by the increased DNA and protein levels) and adipogenesis

was stimulated by an increased level of MAPK signaling. It appears that carotenoids (and their conversion to retinal) could offer an alternative treatment for insulin resistance. A mouse model of obesity and insulin resistance induced by a high-fat diet was fed on high-carotenoid corn for 4 months with no changes in oxidative damage. However, further experiments are required to determine the precise impact of high-carotenoid corn in this model.

3.3.5. REFERENCES

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CHAPTER 4

Influence of a high-carotenoid corn diet on tumor development and risk parameters related to metabolic syndrome and insulin sensitivity in PTEN $^{+/-}$ mice

CHAPTER 4

Influence of a high-carotenoid corn diet on tumor development and risk parameters related to metabolic syndrome and insulin sensitivity in PTEN+/- mice

3.4.1. INTRODUCTION

PTEN (lipid and protein phosphatase and tensin homolog) is a tumor suppressor protein encoded by a gene on human chromosome 10. It plays an important role in the PI3K-AKT-mTOR signaling pathway, named after the components phosphatidylinositol-3-kinase, Akt and mammalian target of rapamycin (Figure 3.4.1). The PTEN protein is a phosphoinositide phosphatase that antagonizes PI3K by dephosphorylating phosphatidylinositol trisphosphate $PI(3,4,5)P_3$ to produce phosphatidylinositol bisphosphate $PI(4,5)P_2$. PTEN opposes the activity of PI3K signaling upstream of the AKT-mTORC1 components of the pathway, which are implicated in the promotion of cancer and metabolic dysfunction (Maehama and Dixon, 1998; Myers et al., 1997).

The phosphatase activity of PTEN regulates migration, apoptosis, cell cycle progression, transcription and translation (Stambolic et al., 1998; Yin and Shen, 2008). The mutation or loss of PTEN causes hyperactive PI3K signaling and this is thought to be the basis of several cancers (Carracedo et al., 2008). Germ-line mutations in the *PTEN* gene cause Cowden syndrome, a rare autosomal dominant disorder characterized by hamartomas of the skin (Liaw et al., 1997). Homozygous deletion or somatic mutations of *PTEN* have been described in several sporadic (nonhereditary) cancers such as glioblastoma (20–75%), endometrial cancer (34–50%), small-cell lung cancer (40%), prostatic cancer (10-49%), thyroid cancer, leukemia, lymphoma, breast cancer, colon cancer and anaplastic meningioma (Suzuki et al., 1998; Hollander et al., 2011). Loss of heterozygosity or mutation at the *PTEN* locus increases in cases of advanced glioma and late-stage breast and prostate tumors (Suzuki et al., 1998).

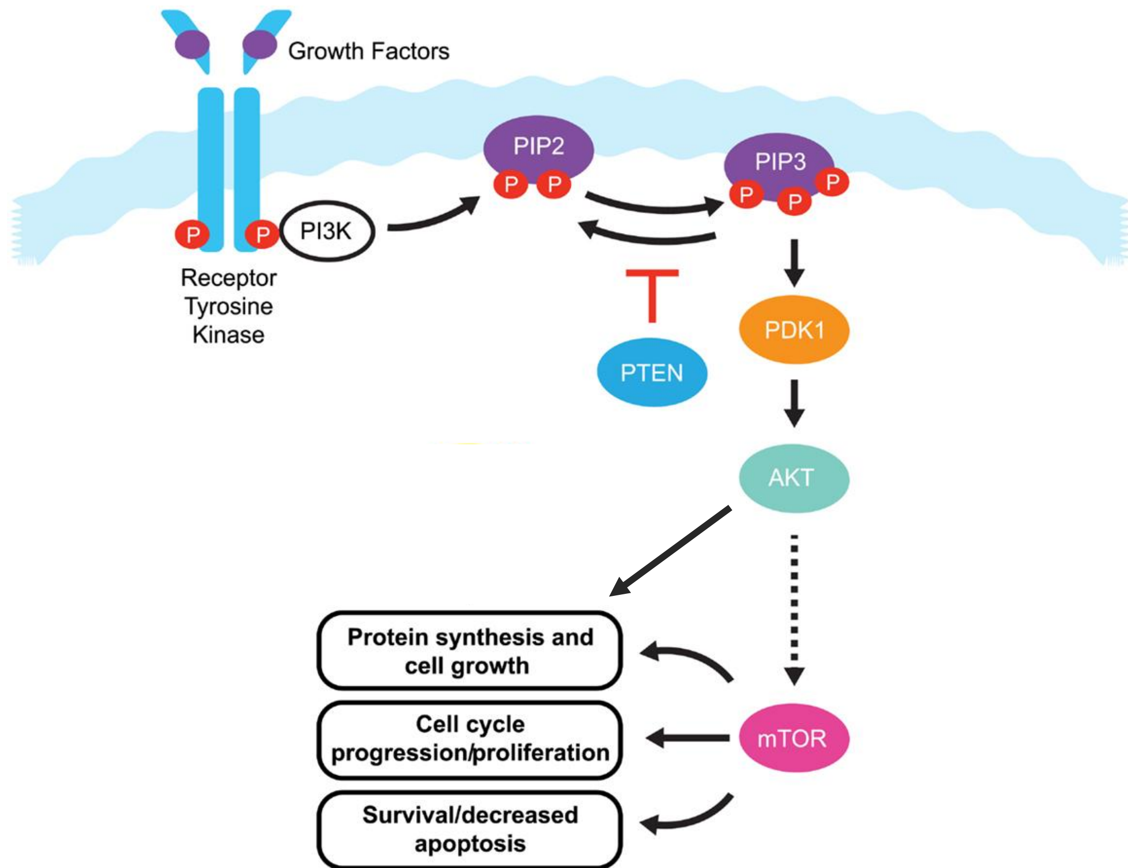

Figure 3.4. 1. The PI3K-AKT-mTOR pathway and the role of PTEN. PI3K can be activated by the binding of growth factors to a receptor tyrosine kinase, whereupon P13K is recruited to the receptor complex. Activated PI3K converts PIP2 to PIP3, which subsequently phosphorylates Akt via phosphoinositide-dependent kinase 1 (PDK1). Phosphorylated Akt acts on a large number of substrates, but one of its most important targets is mTOR, which controls cell growth, proliferation and survival. PTEN negatively regulates the pathway by removing the 3-phosphate from PIP3, converting it back to PIP2. Loss of PTEN leads to Akt hyperactivity, which in turn is linked to uncontrolled cell proliferation, inhibition of apoptosis and enhanced tumor angiogenesis. Adapted from Phin et al. (2013).

Endometrial cancer is the most common malignancy of the female genital tract in Europe and North America (Murali et al., 2014). The PI3K/AKT pathway is activated by growth factors stimulated by the endometrial stromal cells in response to estrogen, boosting the levels of PIP3 and thus promoting the phosphorylation of AKT and in turn the activation of proteins that regulate survival, growth and cell proliferation (Joshi et al., 2012). The antagonistic activity of PTEN is an important gatekeeper role and its loss of function therefore results in the hyperactivation of the pathway, leading then to the development of endometrial hyperplasia and carcinoma (Joshi et al., 2012). $PTEN^{+/-}$ mice are heterozygous for a null *PTEN* allele and spontaneously develop complex

hyperplasia at a higher rate than wild-type mice, with 20% progressing to endometrial cancer (Joshi et al., 2012). Loss of *PTEN* expression has also been reported in 20–40% of colorectal cancers (Naguib et al., 2011) and the frequency of loss and mutation increases as the disease progresses from normal colonic mucosa to adenoma, primary colorectal cancer and ultimately metastasis (Jang et al., 2010; Hocking et al., 2014).

PTEN is not only involved in the PI3K signaling pathway as a tumor suppressor. Abnormal PTEN activity is also linked to the development of hepatic diseases related to obesity, including diabetes, hepatitis B and C, alcohol abuse, insulin resistance and hepatocellular carcinoma (Peyrou et al., 2010).

In the liver, insulin activates PI3K signaling to promote lipid accumulation (He et al., 2010). Because PTEN antagonizes the PI3K/AKT pathway, hepatic PTEN deficiency causes the liver to accumulate high levels of triglycerides even though there is systemic hypoglycemia in mice lacking PTEN activity in the liver, thus promoting insulin sensitivity (Stiles et al., 2004; Knobbe et al., 2008; He et al., 2010). Loss of PTEN activity also causes hepatic steatosis in mice, which is aggravated with increasing age (Horie et al., 2004; Stiles et al., 2004). A similar link has been found in humans (Vinciguerra et al., 2008). Fatty liver disease is attributed to the activation of genes involved in fatty acid and triglyceride synthesis (Horie et al., 2004). Upregulation of the transcription factor sterol regulatory element binding protein 1c (SREBP-1c) increases the uptake of free fatty acids into liver cells, and the upregulation of peroxisome proliferator-activated receptor γ (PPARγ) promotes the generation of reactive oxygen species (ROS) and inflammatory cell infiltration (Ishii et al., 2009). The loss of PTEN activity not only affects liver homeostasis and insulin metabolism but also increases susceptibility to hepatic adenomas and hepatocellular carcinomas (Knobbe et al., 2008). The restoration of physiological PTEN activity in the liver could therefore help to prevent or reduce the incidence of non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, alcoholic liver disease, viral hepatitis, hepatic adenoma and hepatocellular carcinoma (Peyrou et al., 2010).

The potential chemoprotective effect of carotenoids has been reported in the context of several liver disorders (Shimizu et al., 2011; Sugiura et al., 2006; Gradelet et al.,

1998; Chamberlain et al., 2009; Chung et al., 2012). Carotenoids also reduce the risk of several cancers, including colorectal cancer as discussed in Chapter 4 (Tanaka et al., 2012). Carotenoids appear to inhibit estrogenic signaling in estrogen-dependent cancer cells, and to inhibit breast and endometrial cancer cell proliferation (Amir et al., 1999; Nahum et al., 2001; Hirsch et al., 2007; Veprik et al., 2012). The aim of the work described in this chapter was to analyze the dietary effect of a high-carotenoid corn diet in $PTEN^{+/-}$ mice during a 36-week feeding trial. This model is genetically engineered mice to delete a single *PTEN* allele, because the loss of both alleles is embryonic lethal (Hollander et al., 2011). The experiments compared high-carotenoid corn and wild-type corn diets, focusing on the impact on colon, endometrium and liver tissues.

3.4.2. MATERIALS AND METHODS

3.4.2.1. Diets

Experimental diets containing wild-type M37W corn or high-carotenoid corn (grown under the same conditions and harvested at the same time) were prepared from freeze-dried powdered kernels under hygienic conditions. Compositional analysis was carried out as described in Chapter 3. The feed was prepared by mixing the purified AIN-76A diet (Research Diets, Inc., New Brunswick, USA) with the appropriate freeze-dried corn powder in a 60:40 (w/w) ratio.

3.4.2.2. Animal feeding study

The study complied with Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat of Catalonia) and EU Directive EEC 63/2010, and was approved by the Ethics Committee on Animal Experiments of the University of Lleida and the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya.

Female C57BL/6 PTEN$^{+/-}$ mice (B6.129-Ptentm1Rps) were obtained from the National Cancer Institute mouse repository (NCI, Frederick, MD, USA). Mice were acclimated in individual cages 1 week before the experiment, with *ad libitum* access to a standard 2014 Teklad Global 14% Protein Rodent Maintenance Diet (www.harlan.com) and water. The animal rooms were environmentally controlled (20 ± 2°C, relative humidity 50 ± 5%, 12-h photoperiod).

Mice were genotyped by earmarking and DNA was isolated from tail tissue in proteinase K lysis buffer. PCR was carried out with GoTaq Polymerase (Promega, Madison, USA) using a common forward primer 5'-TTG CAC AGT ATC CTT TTG AAG-3' and the PTEN$^{+/+}$ reverse primer 5'-GTC TCT GGT CCT TAC TTC-3' or the PTEN$^{+/-}$ reverse primer 5'-ACG AGA CTA GTG AGA CGT GC-3'.

Following acclimation, 48 6-week-old female $PTEN^{+/-}$ mice and 10 6-week-old female $PTEN^{+/+}$ mice were randomly assigned to the two diets, resulting in four groups: $PTEN^{+/+}$ WT, $PTEN^{+/+}$ CAR, $PTEN^{+/-}$ WT and $PTEN^{+/-}$ CAR. The mice were housed in cages (4–5 mice per cage) with *ad libitum* access to food and water as above. Body weight was measured weekly during the first month, every two weeks during the second month and monthly thereafter until the animals were 42 weeks old. Fresh food was supplied twice each week.

3.4.2.3. Blood analysis and histopathology

Fasted animal blood was collected from the submandibular vein under 4% isoflurane anesthesia 8 and 20 weeks after the start of the experiment. Blood was collected into an EDTA-coated tube and the plasma was separated by centrifugation at 1500 x g for 10 min and stored at –80°C. At the end of the experiment (36 weeks) blood samples were taken by cardiac puncture under 4% isoflurane anesthesia. Whole blood was collected and separated as above. The glucose, total cholesterol, triglycerides, total protein, albumin, lactate and fructosamine concentrations were measured using commercial clinical chemistry kits (Spinreact, Girona, Spain).

When the mice had been sacrificed, the organs were removed to determine the colon length (cecum, distal and proximal) and liver and spleen weights. Colons were excised, flushed with PBS, fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (4-5 µm) were stained with hematoxylin and eosin for histopathology. Uterus and liver tissue samples were also sectioned and stained as above for histopathology and the remaining samples were frozen in liquid nitrogen and stored at –80°C. Normal colon mucosa and colon tumors were analyzed for colon length (cm), tumor incidence (number of animals with tumors), tumor multiplicity (number of tumors per tumor-bearing animal) and tumor size (mm^2). The incidence of lymphoma was determined macroscopically. For lipid staining, frozen sections (10-20 µm) were stained with Oil Red O and counterstained with hematoxylin (see Section 3.4.5). Hepatic histopathology was carried out as described by Kleiner et al. (2005) and abnormalities were graded as none (0–5%), mild (5–33%), moderate (33–66%) or severe (> 66%).

3.4.2.4. Immunohistochemistry

Uterine tissue was fixed embedded and sectioned as above, and the sections were dried for 16 h at 56°C, cleared in xylene, dehydrated through a graded ethanol series and washed in phosphate-buffered saline (PBS). The sections were heated in a pressure cooker for 2 min in 10 mmol/l citrate buffer (pH 6.5) or EDTA buffer (pH 8.0) to achieve antigen retrieval. Endogenous peroxidase activity was blocked by immersing the sections in hydrogen peroxide. The primary antibody was anti-PTEN (D4.3) XP (Cell Signaling Technology, Beverly, MA, USA) used at 1:200 dilution. After incubation, horseradish peroxidase activity was visualized using the EnVision Detection Kit (DAKO, Carpinteria, USA) with 3,3'-diaminobenzidine chromogen as the substrate. All sections were counterstained with hematoxylin.

3.4.2.5. Oil Red O staining

Oil Red O staining (Sigma-Aldrich, Munich, Germany) was used to measure lipid accumulation. Tissue was fixed in 10% formaldehyde, washed in PBS and stained with 0.21% (w/v) Oil Red O solution (60% isopropanol, 40% water). The triacylglyceride content was determined after drying the cells and extracting the Oil Red O with 100% isopropanol followed by photometric measurement at 495 nm.

3.4.2.6. Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from the uterine endometrium using the RNeasy Total RNA kit (Qiagen, Valencia, CA, USA) and cDNA was generated using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA). The cDNA was amplified by heating to 95°C for 10 min, followed by 40 PCR cycles of 95°C for 15 s and 60°C for 1 min using the ABI Prism 7900 Sequence Detection System (Applied Biosystems) and Promega GoTaq® qPCR Master Mix (Madison, USA). Relative mRNA expression levels were calculated using the ΔΔCt method and are presented as ratios to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primers were based on

the following GenBank sequences: TNF-α, Mm00443260_g1; Nfκb1, Mm00476361_m1; Nrf-2, Mm00477784_m1; GAPDH, Mm99999915_g1.

3.4.2.7. Retinoid extraction

Liver tissue (~100 mg wet weight) was lyophilized in a Telstar cryodos-50 for 20 h and the dried samples were ground to powder and pooled from all mice per group before vortexting with 200 µl 30% potassium hydroxide, 1 ml ethanol, 0.01% butylated hydroxytoluene in 100 µl of ethanol and 100 µl 20 µM apo-carotenal in ethanol (internal standard) and incubating at 37°C for 45 min. The samples were extracted three times with 3 ml ether:hexane (2:1, v:v), centrifuged at 13,000 x g for 5 min to separate the phases and evaporated to dryness under nitrogen. The powder was redissolved in 100 µl phase A buffer and passed through an Eppendorf UltraFree 5-kDa filter (UFC3LTK00, Millipore, Bedford, MA, USA).

3.4.2.8. Retinoid identification and quantification by HPLC

The processed samples were separated using a YMC C30 column (250 x 4.6 mm, internal diameter 3 µm) at 35°C in a 110 series HPLC system (Agilent Waldbronn, Germany) at a flow rate of 0.5 ml/min in 100% mobile phase A (methanol/acetone, 60:40 v/v) 0–30 min. Retinol and retinoic acid were detected at 325 mm.

3.4.2.9. Statistical analysis

Values are presented as means ± standard errors of the mean (SEM). Data were compared using Student's *t*-test or one-way ANOVA, with $p < 0.05$ considered as significant. Fisher's Exact Probability test was used to compare the incidence of histopathological lesions between groups.

3.4.3. RESULTS

3.4.3.1. Feeding experiments – general observations

Two animals originally assigned to the PTEN$^{+/-}$ CAR group were reassigned to the PTEN$^{+/+}$ CAR group following the discovery of a genotyping error. Two animals from the PTEN$^{+/-}$ CAR group died following blood extraction after failing to recover from anesthesia. Two animals each from the PTEN$^{+/-}$ WT and CAR groups were sacrificed when lymphoma tumors reached the ethical limit. The high-carotenoid corn diet did not produce any observable unexpected effects.

3.4.3.2. Animal body weight and relative organ weights

There were no significant differences in final body weights or relative spleen weights between the diet groups, but the relative liver weights were significantly lower in the PTEN$^{+/-}$ CAR group compared to the PTEN$^{+/-}$ WT group (Table 3.4.1).

Table 3.4. 1. Final body weights and relative organ weights in the four experimental diet groups. Values are means ± SEM and differences are significant* at $p < 0.05$.

	PTEN$^{+/+}$ WT (n=5)	PTEN$^{+/+}$ CAR (n=7)	*p* value	PTEN$^{+/-}$ WT (n=22)	PTEN$^{+/-}$ CAR (n=18)	*p* value
Final body weight (g)	23.25 ± 0.80	22.74 ± 0.68	0.6328	28.00 ±0.52	28.36 ± 0.52	0.6304
Relative liver weight (%)	3.743 ± 0.087	3.752 ± 0.116	0.9593	4.939 ± 0.174	**4.098 ± 0.322***	**0.0207**
Relative spleen weight (%)	0.3277 ± 0.05752	0.2880 ± 0.03010	0.5215	0.7809 ± 0.1157	0.5591 ± 0.1395	0.1202

3.4.3.3. Tumor incidence, multiplicity and histopathology in PTEN+/- mice

PTEN$^{+/-}$ mice fed on the wild-type corn (PTEN$^{+/-}$ WT) showed a higher incidence of 100% lymphoid tumors than those fed on the high-carotenoid corn (PTEN$^{+/-}$ CAR), but the tumor multiplicity was slightly lower in the PTEN$^{+/-}$ CAR group although the difference between diets was not statistically significant (Table 3.4.2). Similarly, the PTEN$^{+/-}$ WT group showed a higher incidence of colorectal tumors (52%) than the PTEN$^{+/-}$ CAR group (38%) but the differences were not significant. The multiplicity of colorectal tumors and the tumor area were similar in both groups (Figure 3.4.2). Finally, the PTEN$^{+/-}$ WT group showed a higher incidence of endometrial lesions (cystic hyperplasia, complex hyperplasia and carcinoma) than the PTEN$^{+/-}$ CAR group. However, there was no statistically significant relationship between the wild-type corn diet and the incidence of malignant endometrial lesions such as complex hyperplasia and carcinoma (Figure 3.4.3). The incidence of endometrial carcinoma was 9.5% in the PTEN$^{+/-}$ WT group and 0% in the PTEN$^{+/-}$ CAR group. The *PTEN* genotype of the endometrium (normal vs heterozygous) was confirmed by immunohistochemistry.

Table 3.4. 2. Macroscopic observations and histopathology of PTEN$^{+/-}$ mice in the WT and CAR diet groups. The incidence of lymphomas, colorectal lesions and endometrial lesions is represented by the percentage of tumor-bearing animals. The multiplicity of lymphomas and colorectal tumors is represented by the mean number of tumors in tumor-bearing animals. The mean area of colorectal tumors was evaluated by necropsy. Normal endometrial appearance and mild cystic changes are not included in table data. Data show means ± SEM.

Group	Lymphomas	Colorectal lesions		Endometrial lesions		
	Multiplicity/Incidence	Multiplicity/Incidence	Area (mm)	Cystic hyperplasia	Complex hyperplasia	Carcinoma
PTEN$^{+/-}$ WT n=22	3.273 ± 0.3430 (100%)	1.67 ± 0.28 (52.2%)	0.5125 ± 0.167	52.4%	19%	9.5%
PTEN$^{+/-}$ CAR n=18	2.667 ± 0.2801 (100%)	1.14 ± 0.14 (38.8%)	0.6214 ± 0.3489	50%	16.7%	-

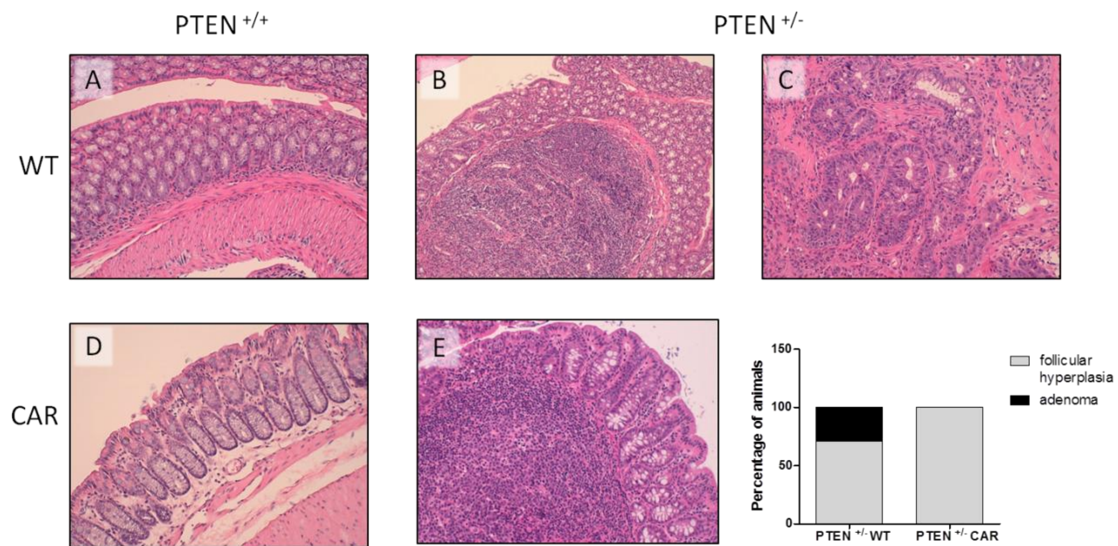

Figure 3.4. 2. Representative histopathology of colonic tissue sections stained with hematoxylin and eosin, and the percentage of animals with histopathological findings, i.e. follicular hyperplasia and adenoma. (A) and (D) represent normal colonic mucosa, (B) and (E) represent follicular hyperplasia, and (C) represents adenoma. The incidence of animals presenting these colonic findings (follicular hyperplasia and adenomas) analyzed by histopathology was n = 7 in the PTEN$^{+/-}$ WT group and n = 5 in the PTEN$^{+/-}$ CAR group. Magnification = 20x.

The vast majority of lesions in the colonic mucosa were classed as follicular hyperplasia (71% in the PTEN$^{+/-}$ WT group and 100% in the PTEN$^{+/-}$ CAR group) whereas 29% of the PTEN$^{+/-}$ WT group progressed to adenoma, which is considered a pre-malignant neoplasm (Figure 3.4.2).

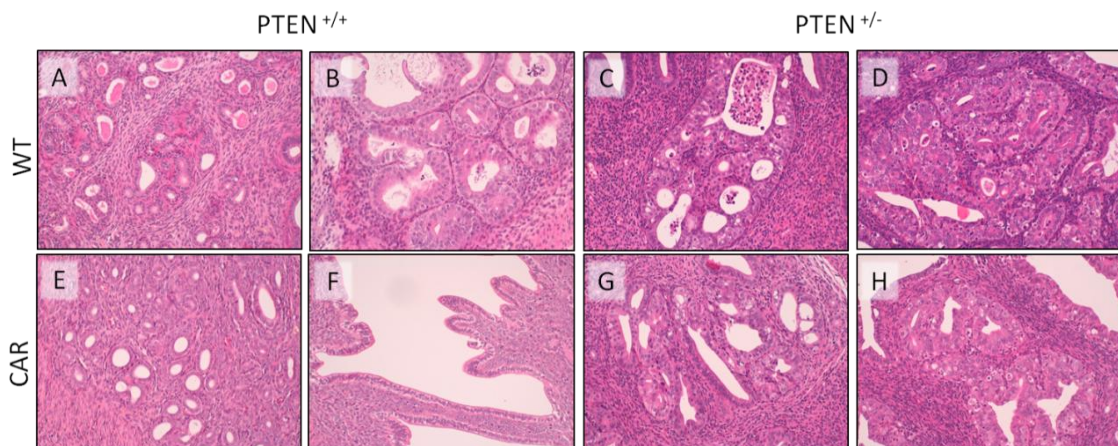

Figure 3.4. 3. Representative histopathology of uterine endometrium sections stained with hematoxylin and eosin. (A) and (E) represent normal endometrium, (B) and (G) represent cystic hyperplasia, (C) and (H) represent complex hyperplasia, (D) represents carcinoma and (F) represents mild cystic changes. Magnification = 20x.

Furthermore, 19% of the PTEN$^{+/-}$ WT group presented lesions classed as complex hyperplasia and 52% presented lesions classed as cystic hyperplasia. These data suggested that mice in the PTEN$^{+/-}$ WT group tended more towards the advanced stages of the disease compared to the PTEN$^{+/-}$ CAR group, in which none of the animals developed endometrial carcinomas, only 17% presented lesions classed as complex hyperplasia and 50% presented lesions classed as cystic hyperplasia (Figure 3.4.3).

3.4.3.4. Histological evaluation of the liver

Histological evaluation of the liver (Figure 3.4.4) showed that 20% of the PTEN$^{+/+}$ WT group and 17% of the PTEN$^{+/+}$ CAR group presented with mild steatosis (5–33%), whereas 18% of the PTEN$^{+/-}$ WT group presented with moderate steatosis (33–66%) and an additional 14% with mild steatosis. In the PTEN$^{+/-}$ CAR group, there were no animals with moderate steatosis, 28% with mild steatosis and 72% with no steatosis (0–5%). There was a significant correlation (p = 0.0486) between the high-carotenoid corn diet and the absence of steatosis, and between the wild-type corn diet and the presence of mild to moderate steatosis. Oil Red O staining was also used to support the analysis of hepatic lipid content.

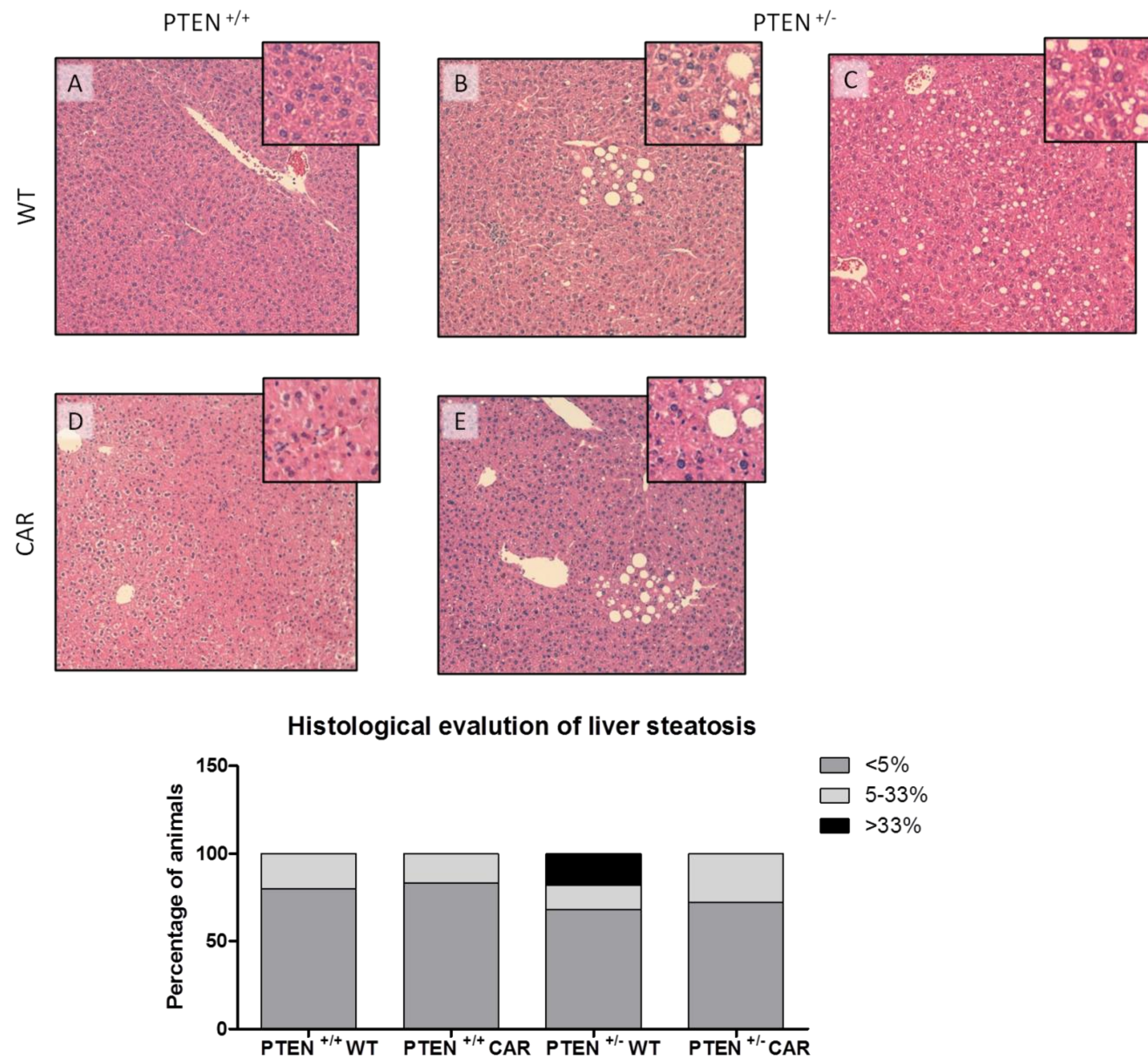

Figure 3.4. 4. Representative histopathology of liver sections stained with hematoxylin and eosin, with liver steatosis graded as described by Kleiner et al. (2005): none = 0–5%; mild = 5–33%, moderate = 33–66%. (A) and (D) represent no steatosis, (B) and (E) represent mild steatosis, and (C) represents moderate steatosis. Lower magnification (x10) and higher (x40). Histogram shows the percentage of animals in each liver steatosis grade. The incidence of mild and moderate steatosis was 7/22 (32%) animals in the PTEN$^{+/-}$ WT group and 5/18 (28%) animals in the PTEN$^{+/-}$ CAR group.

3.4.3.5. Biochemical analysis

The significant correlation between the different diets and the prevalence of steatosis in PTEN$^{+/-}$ mice was investigated by studying the lipid profile and other biochemical parameters in blood drawn from mice in each of the diet groups. The accumulation of fatty acids in the liver could reflect the increased uptake of fatty acid by liver cells or a higher rate of *de novo* lipogenesis (Stahl et al., 2001). Furthermore, fasting glucose and

fructosamine levels were monitored due to the improved glucose tolerance observed in mice with a liver-specific $PTEN^{+/-}$ genotype (Stiles et al., 2004, Horie et al., 2004).

The fructosamine and glucose levels were significantly higher ($p < 0.05$) in the $PTEN^{+/-}$ CAR group compared to the $PTEN^{+/-}$ WT group (Figure 3.4.5 and Figure 3.4.6). The fructosamine levels in the $PTEN^{+/-}$ WT group were also significantly lower than in the $PTEN^{+/+}$ WT group ($p = 0.0327$).

Total plasma cholesterol was lower in both $PTEN^{+/-}$ and $PTEN^{+/+}$ mice fed on the high-carotenoid corn diet compared with their counterparts fed on wild-type corn. There were no significant differences in plasma triglycerides suggesting that the loss of one *PTEN* allele does not change the systemic levels of triglycerides because of hepatic fatty acid secretion and synthesis.

Total protein and albumin levels were measured to confirm that the changes in fructosamine levels (which indicate the levels of glycated proteins) did not reflect a general loss of liver function. No significant differences were observed between the diet groups (Figure 3.4.6) confirming that the observed differences in fructosamine levels were due to underlying changes in the abundance of glucose.

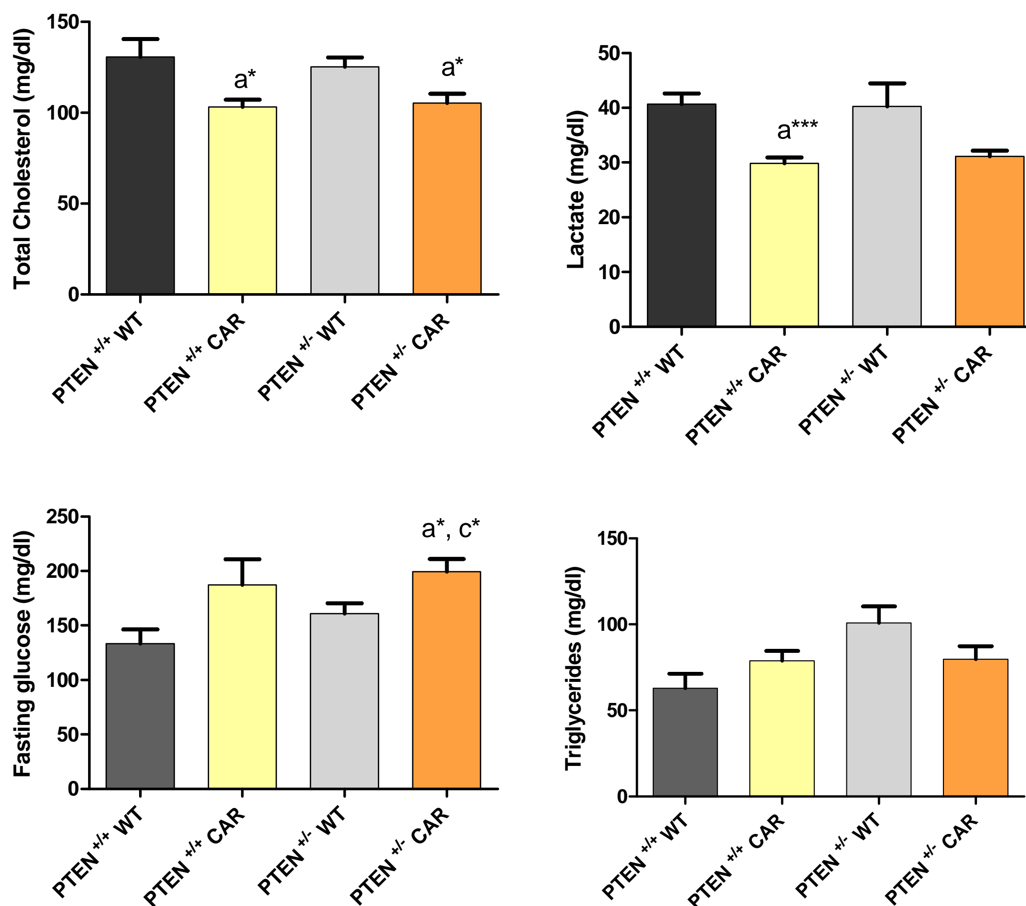

Figure 3.4. 5. Total cholesterol, lactate, fasting glucose and triglyceride levels in the blood of mice from the four different diet groups. Differences are significant according to Student's *t*-test at $*p < 0.05$ $***p < 0.001$; a - statistically significant difference versus $PTEN^{+/+}$ WT group; c - statistically significant difference versus $PTEN^{+/-}$ WT group. Total cholesterol levels were analyzed in n = 5 $PTEN^{+/+}$ WT, n = 7 $PTEN^{+/+}$ CAR, n = 20 $PTEN^{+/-}$ WT and n = 13 in $PTEN^{+/-}$ CAR mice. Glucose was analyzed in overnight fasted mice (n = 4 $PTEN^{+/+}$ WT, n = 7 $PTEN^{+/+}$ CAR, n = 13 $PTEN^{+/-}$ WT and n = 16 $PTEN^{+/-}$ CAR) and lactate and triglycerides were analyzed also in fasted animals (n = 4 $PTEN^{+/+}$ WT, n = 7 $PTEN^{+/+}$ CAR, n = 13 $PTEN^{+/-}$ WT and n = 12 $PTEN^{+/-}$ CAR). The remaining samples were not analyzed because there was insufficient blood. All values are presented as means ± SEM.

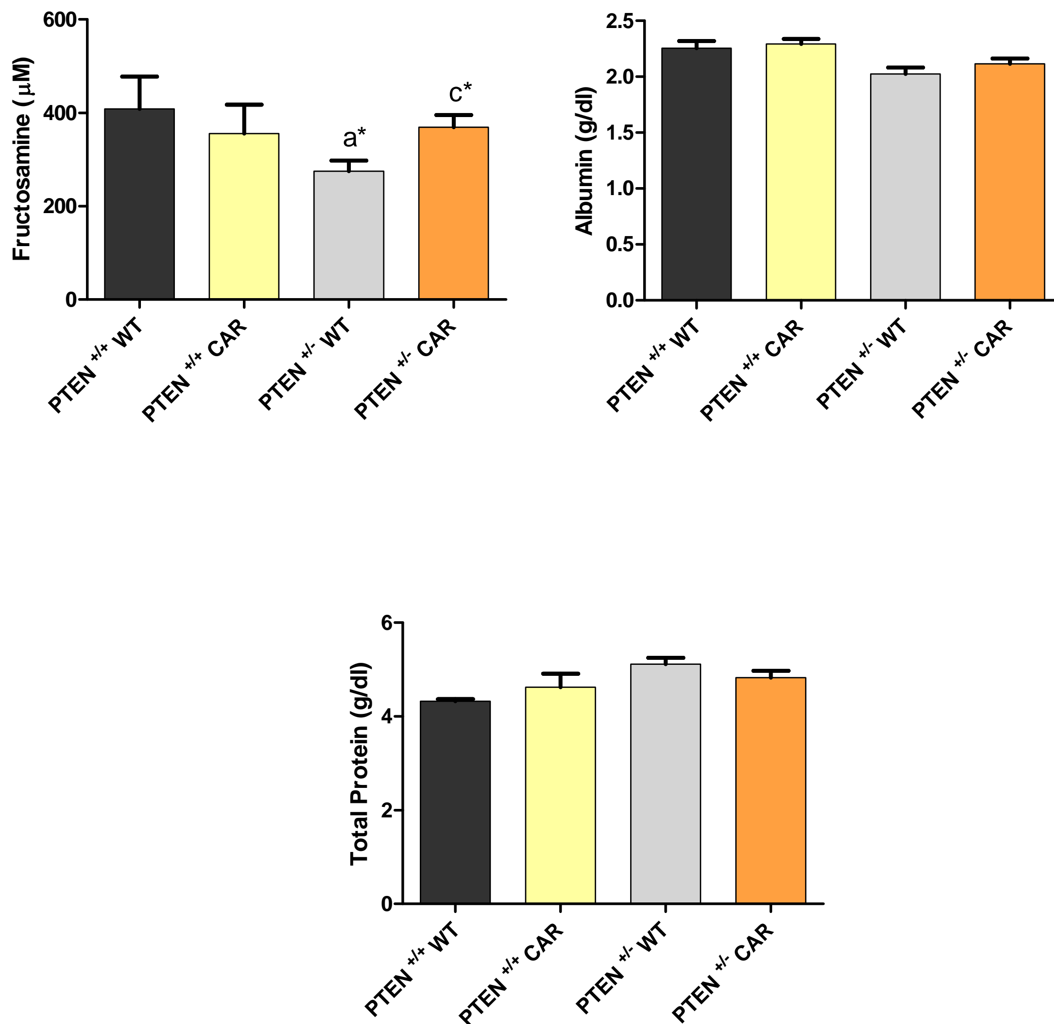

Figure 3.4. 6. Fructosamine, albumin and total protein levels. Differences are significant according to Student's *t*-test at $*p < 0.05$; a - statistically significant difference versus PTEN$^{+/+}$ WT group; c - statistically significant difference versus PTEN$^{+/-}$ WT group. Fructosamine, albumin and total protein levels were analyzed in n = 5 PTEN$^{+/+}$ WT, n = 7 PTEN$^{+/+}$ CAR, n = 20 PTEN$^{+/-}$ WT and n = 18 PTEN$^{+/-}$ CAR mice. The remaining samples were not analyzed because there was insufficient blood. All values are presented as means ± SEM.

3.4.3.6. Quantitative real-time PCR

The expression levels of TNFα, NF-κB and Nrf-2 (key markers of inflammation) showed no significant differences between the PTEN$^{+/-}$ WT and PTEN$^{+/-}$ CAR groups. There was also a large background variation in expression levels between animals in the same group.

3.4.3.7. Retinol and retinoic acid levels in liver tissue

PTEN$^{+/-}$ mice fed on the high-carotenoid corn diet showed a significant increase in the levels of hepatic retinol (p = 0.0010) and retinoic acid (p = 0.0229) compared to the PTEN$^{+/-}$ WT group (Figure 3.4.7). PTEN $^{+/+}$ mice fed on the high-carotenoid corn diet also showed a significant increase in the levels of hepatic retinol (p = 0.0277) compared to the PTEN $^{+/+}$ WT group, but there was no significant difference in liver retinoic acid levels between the PTEN $^{+/+}$ CAR and WT groups. The levels of retinol and retinoic acid in the PTEN $^{+/-}$ WT group were very low and differed significantly from all three other groups.

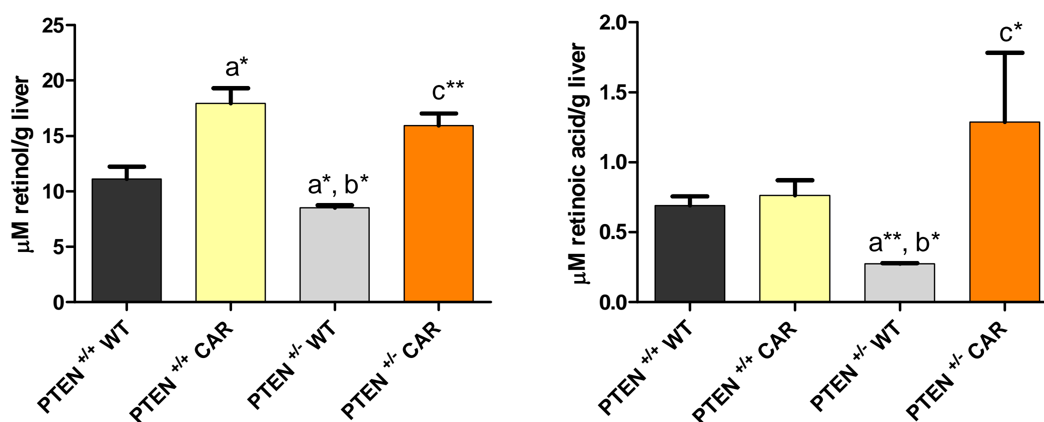

Figure 3.4. 7. Levels of hepatic retinol and retinoic acid determined by HPLC. Differences are significant according to Student's t-test at $*p < 0.05$ and $**p < 0.01$; a - statistically significant differences versus PTEN$^{+/+}$ WT group; b - statistically significant differences versus PTEN$^{+/+}$ CAR group; c - statistically significant differences versus PTEN$^{+/-}$ WT group.

3.4.4. DISCUSSION

The feeding trial described in this chapter compared the effects of a diet supplemented with genetically-engineered high-carotenoid corn and a diet supplemented with a near-isogenic wild-type variety on PTEN$^{+/-}$ and PTEN$^{+/+}$ mice over 36 weeks. The heterozygous PTEN$^{+/-}$ knockout model has been used to study diverse organ neoplasias (Suzuki et al., 1998; Podsypanina et al., 1999) although in this study, the analysis was restricted to the liver, colon and endometrial tissue (Viñas-Salas et al., 1992; Viñas-Salas et al., 1998; Llobet et al., 2009; Mirantes et al., 2013). Previous studies have indicated that carotenoids may protect against endometrial carcinogenesis by inhibiting estrogen signaling (Hirsch et al., 2007) or by inhibiting cell cycle progression (Nahum et al., 2001). PTEN also plays a role in the development of hepatic disorders such as insulin resistance and steatosis, so PTEN$^{+/-}$ and PTEN$^{+/+}$ mice reared on the two diets were also investigated in terms of markers of liver disease.

There was a statistically significant relationship between the wild-type corn diet and the progression of colorectal cancer, with the most severe form (colorectal adenoma) occurring only in the PTEN$^{+/-}$ WT group. The most severe endometrial pathology (endometrial complex hyperplasia and carcinoma) was also 1.65-fold more prevalent in the PTEN$^{+/-}$ WT group, although this difference was not statistically significant. A 100% incidence of endometrial hyperplasia (both cystic and complex types) was not observed in all PTEN$^{+/-}$ females, in contrast to previous studies (Podsypanina et al., 1999; Stambolic et al., 2000). However, the incidence of endometrial hyperplasia and carcinoma was higher in PTEN$^{+/-}$ mice fed on wild-type corn (81%) than those fed on the high-carotenoid variety (67%).

Several studies have demonstrated that carotenoids inhibit colorectal and endometrial cancer, based on in vivo data (Tao et al., 2005; Pelucchi et al., 2008) and experiments with endometrial cancer cells (Nahum et al., 2001; Hirsch et al., 2007). Carotenoids can inhibit estradiol signaling in mammals and genistein signaling in plants, suggesting a general inhibitory role in the estrogen pathway. However, carotenoids are not among the substances which have been tested in PTEN$^{+/-}$ mice to determine their effects on endometrial hyperplasia (Begum et al., 2006; Wu et al., 2008; Yu et al., 2010). The

results presented in this chapter suggest that the high-carotenoid corn variety inhibits the development and progression of colon and endometrial tumors.

In addition to its impact on colorectal and endometrial carcinogenesis, the high-carotenoid corn variety also has a beneficial impact on hepatic disorders. The relative liver weights of the PTEN$^{+/-}$ mice were higher than those in the corresponding PTEN$^{+/+}$ diet groups confirming that PTEN deficiency causes hepatomegaly as previously reported (Butler et al., 2002; Horie et al., 2004). However, the liver weights of PTEN$^{+/-}$ mice fed on the high-carotenoid corn diet were closer to those of the PTEN$^{+/+}$ mice and significantly lower than the liver weights of PTEN$^{+/-}$ mice fed on wild-type corn. PTEN deficiency also induces steatosis, steatohepatitis and hepatocellular carcinoma (Horie et al., 2004; Stiles et al., 2004; Vinciguerra et al., 2008). Accordingly, the feeding trial showed that liver steatosis was more severe in PTEN$^{+/-}$ mice fed on wild-type corn (18% of animals showed moderate steatosis) than in those fed on the high-carotenoid corn, where all the animals showed either mild steatosis or the complete absence of steatosis. This suggests that the high-carotenoid corn reduces hepatic triglyceride deposition, which could be caused by elevated circulating free fatty acids and their incorporation into the liver, as well as factors such as the inhibition of lipid β-oxidation and enhanced *de novo* lipogenesis (Musso et al., 2009). Blood glucose and fructosamine levels were higher in the PTEN$^{+/-}$ CAR group than the PTEN$^{+/-}$ WT group, and were similar to the fasting glucose and fructosamine levels in PTEN$^{+/+}$ mice. This suggested that only PTEN$^{+/-}$ mice fed on the wild-type corn diet showed increased glucose tolerance. Fructosamine levels were lower in PTEN$^{+/-}$ mice fed on the wild-type corn diet compared with PTEN$^{+/+}$ mice on the same diet. The observed increase in insulin sensitivity confirm the results of several previous studies (Wong et al., 2007; Pal et al., 2012).

Biochemical analysis also indicated that the high-carotenoid corn reduced total plasma cholesterol levels as revealed by other carotenoid feeding experiments (Palozza et al., 2011; Choi et al., 2013; Harari et al., 2013). Hepatic steatosis involves an inflammatory response and oxidative stress, hyperinsulinemia, hyperleptinemia and abnormal cholesterol loading (Takahashi, 2012). However, the total cholesterol levels in PTEN$^{+/-}$

and PTEN$^{+/+}$ mice were similar suggesting that the steatosis was mild and not accompanied by severe inflammation, supporting the histopathological data.

Further experiments, including the measurement of insulin, would confirm the enhanced glucose tolerance and insulin hypersensitivity of PTEN$^{+/-}$ mice feeding on wild-type corn and would demonstrate any differences with the same mice on feeding on the high-carotenoid variety. The PI3K-PKB-Akt pathway is activated by insulin signaling, so PTEN deficiency is accompanied by the loss of inhibition of the PI3k signaling pathway which is indispensable for the transduction of insulin signals in insulin-responsive adipose, muscle and hepatic tissues (Guenzl et al., 2013) Furthermore, the analysis of nonesterified fatty acids, leptin, adiponectin and liver triglycerides (Stiles et al., 2004) would show whether the PTEN$^{+/-}$ genotype would be a useful replacement for the liver-specific PTEN-deficient mice as a model for non-alcoholic steatohepatitis or hepatic steatosis.

The improved glucose tolerance of mice in the PTEN $^{+/-}$ WT group could reduce blood glucose levels and promote glucose uptake by the liver. In addition, enhanced insulin signaling in the liver may help to redistribute body fat from adipose tissue to the liver as previously described (Stiles et al., 2004). The liver could then use the glucose for lipogenesis and triglyceride storage, leading to hepatic steatosis and steatohepatitis as the liver is sacrificed to maintain glucose control (Stiles et al., 2004; Qiu et al., 2008). However, high-carotenoid corn helps to reverse the PTEN-deficiency effects because animals in the PTEN$^{+/-}$ CAR group exhibited glucose levels similar to PTEN$^{+/+}$ mice, developed less severe steatosis and had lower relative liver weights than animals in the PTEN$^{+/-}$ WT group. High-carotenoid corn provides an important source of vitamin A through the conversion of three provitamin A carotenoids (β-carotene, α-carotene and β-cryptoxanthin) into active forms of vitamin A such as retinol, retinal and retinoic acid (Harrison, 2005). The levels of liver retinol and retinoic acid were higher in PTEN$^{+/-}$ mice fed on the high-carotenoid corn compared to those fed on wild-type corn. The levels of retinoids in all groups were similar to those previously reported (Kane et al., 2008; Kim et al., 2008) and also similar to the levels reported in Chapter 3 5, although retinol was slightly more abundant in the PTEN$^{+/+}$ CAR mice compared to the normocaloric CAR mice discussed in that chapter.

Retinoids not only play an important role in liver metabolism but also in glucose and lipid metabolism (Chen, 2013). The asymmetric cleavage of β-carotene can produce apocarotenals such as β-apo-14'-apocarotenal, which has the ability to inhibit the activities of the peroxisome proliferator-activated receptors PPARγ and PPARα, and also the retinoid X receptors (RXRs), which are important regulators of transcription (Ziouzenkova et al., 2007). All three of these regulators are induced in PTEN-deficient mice and contribute to hepatic steatosis and to the activation of genes involved in fatty acid oxidation, which could cause oxidative stress and inflammation in the liver (Desvergne and Wahli, 1999; Browning and Horton, 2004). The PPARγ gene is activated by SREBP-1c, which is also strongly expressed in PTEN-deficient mice because insulin-mediated activation of the PI3/Akt pathway acts on the same target, and it may be inhibited by AMPK (Browning and Horton, 2004; Horie et al., 2004; Piguet et al., 2010). Interestingly, the accumulation of lutein and zeaxanthin in the liver can lead to the activation of AMPK thus preventing hepatic steatosis in mice (Lin et al., 2014). In this last study, adding wolfberry to a high-fat diet showed increased the hepatic levels of lutein and zeaxanthin and the subsequent activation of AMPKα, which in turn activated lipid β-oxidation and secretion (i.e. PPARα) and other signaling pathways resulting in the prevention of hepatic steatosis (Lin et al., 2014). The diet-induced obesity and insulin resistance mouse model described in Chapter 3 showed a significant increase in hepatic AMPK levels when fed on high-carotenoid corn, so this variety appears not only to reduce the progression of endometrial and colorectal cancer, but also to reduce the risk of hepatic steatosis in $PTEN^{+/-}$ mice, improve glucose homeostasis and regulate insulin sensitivity. Further experiments are needed to determine the molecular mechanisms underlying this phenomenon and the potential role of carotenoids and their metabolic products (retinoids and apocarotenals) in the regulation of PPAR, RXR, AMPK and SREBP-1c signaling.

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4. GENERAL DISCUSSION

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The work described in this thesis links two major global challenges: undernutrition and overnutrition, both of which can be described as types of malnutrition. Although it may seem paradoxical to consider these opposite conditions under one umbrella, they are united by a common basis – the inability to achieve a balanced and healthy diet that provides adequate nutrition (Shetty, 2006). One of the consequences of malnutrition is a higher risk of chronic disease. For example, overnutrition leads to obesity, increasing the risk of cancer, insulin resistance, diabetes, nonalcoholic fatty liver disease and metabolic disease, which affect many people and cause millions of deaths globally every year.

The consequences of malnutrition are major concerns not only in developing countries but also in industrialized countries, where undernourishment and obesity are both increasing in prevalence. High-carotenoid corn is a biofortified staple that can help to address undernourishment in developing countries (Berman et al., 2013). However, the high content of carotenoids embedded in the corn matrix may also provide additional health-promoting effects that counteract the diseases listed above. This hypothesis can be tested by using the high-carotenoid corn and its near isogenic wild-type comparator (which was transformed to generate the enriched variety) in head-to-head feeding trials using the same animal models of chronic disease. This approach can also be used to compare the effects of carotenoids added as supplements to those integrated in the corn matrix, which might affect both their bioaccessibility (the amount released from the food in the gut, i.e. the amount with the potential to be absorbed) and their bioavailability (the amount which actually is taken up and either stored or metabolized). The bioavailability of a nutrient depends on its bioaccessibility and it is likely that the latter is affected by how the nutrient is presented – i.e. as a supplement or as a complex with other components of the plant matrix, such as dietary fibers (Palafox-Carlos et al., 2011).

Carotenoids are lipophilic molecules, so bioavailability depends on their efficient release from the food matrix by digestive enzymes and subsequent solubilization by

bile acids. This results in the formation of micelles and solubilized free carotenoids (Palafox-Carlos et al., 2011). For example, soluble dietary fibers (the indigestible components of plant cell walls) may attenuate the absorption of dietary lipids in the gut and could therefore also inhibit the absorption of carotenoids (Rock et al., 1998). There is little data concerning the bioavailability of dietary fats and carotenoids contained in the food matrix (Palafox-Carlos et al., 2011). In most animal studies, carotenoid supplements have been added to standard diets, e.g. carotenoids isolated from plants or algae or synthetic commercial carotenoid powders (Kim et al., 1998; Narisawa et al., 1999; Tanaka et al., 2008; Kawabata et al., 2012). In other studies, animals disease models have been fed on standard diets supplemented with carotenoid-rich foods such as tomato to investigate the impact of carotenoids and other antioxidants contained in the food matrix (Pannellini et al., 2010; Tanaka et al., 2012). The carotenoid-rich corn can be analyzed in the same comparative manner using the wild-type corn variety as a comparator, but because it is a novel corn variety the first priority is to evaluate its safety as an ingredient.

To monitor for unintended effects, a whole-food 90-day subchronic toxicity study in mice was carried out using one dose of feed, a 40:60 mixture of powdered high-carotenoid corn with a standard diet. No other doses were tested because only a limited quantity of transgenic maize seeds was available at the time, although different doses are recommended by EFSA (EFSA, 2011a). This was the first animal feeding trial involving high-carotenoid transgenic corn and it provided information about palatability as well as safety. The corn was palatable, thereby avoiding the need to feed the mice by oral gavage, and the results confirmed that the high-carotenoid corn was as safe as its unmodified counterpart.

This initial toxicity assessment was the first step towards the eventual deregulation of high-carotenoid corn as a new genetically-engineered crop aiming to address hunger and malnutrition in developing countries. Laboratory animals are needed to evaluate novel foods for any unintended negative effects (safety assessment) but can also be used to investigate potential beneficial effects, such as nutritional enhancement and protection against chronic diseases (Argilés, 2005; Key, 2011; Kalupahana et al., 2012).

The effect of a high-carotenoid corn was tested on the mouse AOM/DSS model of colitis-associated cancer, using rigid colonoscopy during the trial and gross necropsy, histopathology and molecular analysis at its conclusion. The data showed that high-carotenoid corn has a chemoprotective effect and delays the progression of the disease from colitis to the development of adenocarcinomas, although the differences in values in some of the comparisons failed to reach the threshold for statistical significance because there was a degree of background variability in the model itself. Similar conclusions can be drawn about the ability of high-carotenoid corn to prevent the formation of colon adenomas in the $PTEN^{+/-}$ mouse model. Both models therefore confirmed a trend towards chemoprotective activity, and it is likely that larger-scale studies with more animals would confirm a statistically significant difference. The high-carotenoid corn also delayed the development and progression of endometrial cancer.

The high-carotenoid corn was also fed to mice maintained on a high-fat diet to induce obesity and insulin resistance, thus testing its ability to prevent or ameliorate the symptoms of this disease. The high-carotenoid corn improved systemic glucose homeostasis and promoted higher insulin sensitivity than wild-type corn. In the $PTEN^{+/-}$ model of fatty liver disease and insulin hypersensitivity, the high-carotenoid corn also reduced the incidence and severity of hepatic steatosis. As was the case for the AOM/DSS model, some of the observed differences were not statistically significant, but there was a distinct trend showing that high-carotenoid corn has health-promoting effects and reduces risk parameters related to metabolic syndrome, such as total cholesterol, the LDL/HDL ratio and insulin resistance.

The work described in this thesis evaluated the effects of feeding a novel, carotenoid-enriched corn (compared to a wild-type variety lacking these carotenoids) to mouse models of diseases that affect millions of people. Although further investigations are required with larger numbers of animals to define the beneficial effects more clearly, the data strongly suggest that high-carotenoid corn is beneficial in a number of pathological scenarios and should be tested in humans not only to address malnutrition in developing countries but also for the development of ‘functional foods’ with health-promoting effects. The high-carotenoid corn could also be used as the basis for further studies that compare the benefits of carotenoid supplements with

carotenoids produced as part of the food matrix, and the potential synergic effects of multiple carotenoids present in the same food.

5. CONCLUSIONS

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1. High-carotenoid corn did not exhibit any unintended effects towards experimental animals and it was shown to be as safe as its conventional counterpart.

2. High-carotenoid corn delayed the progression of pre-neoplasms to malignant neoplasms in an induced AOM/DSS colitis-associated carcinogenesis murine model .

3. In animals fed with high-carotenoid corn inhibition of MAPK signaling appeared to mediate COX-2 down-regulation in non-lesional colonic mucosa in the AOM/DSS model.

4. High-carotenoid corn diets improved systemic glucose homeostasis and insulin sensitivity through increased GLUT4 expression in adipose tissue and skeletal muscle in a high-fat diet induced obesity and insulin resistance mouse model.

5. Hypercaloric feed diets induced glucose intolerance and insulin resistance in mice. Glucose tolerance assessments revealed a faster recovery of blood glucose basal levels in animals fed on a fat diet enriched with high-carotenoid corn.

6. High-carotenoid enriched fat diets modulated the lipid profile in mice by increasing HDL cholesterol and lowering LDL/HDL cholesterol ratios.

7. An increase in adipose tissue cell number was observed through total DNA and protein quantification which suggests that it increase by hyperplasia rather than by hypertrophy in animals fed on a fat diet enriched on high-carotenoid corn.

8. The increased adipogenesis can be related to the increase of MAPK through an increase of insulin signaling in animals fed on a fat diet enriched on high-carotenoid corn.

9. Increases in adipose tissue cell numbers were attributed to increased content of retinoids (retinol and retinoic acid) in animals fed on a fat diet enriched with high-carotenoid corn.

10. High-carotenoid corn exhibited a protective effect towards the development of colon and endometrial carcinogenesis in PTEN $^{+/-}$ mice.

11. High-carotenoid corn reduced hepatic steatosis and improved liver metabolism by decreasing insulin hypersensitivity in PTEN $^{+/-}$ mice.

12. Pro-vitamin A carotenoids in high-carotenoid corn increased hepatic vitamin A reserves in PTEN $^{+/-}$ mice.

6. REFERENCES

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OUTPUTS

SCIENTIFIC PUBLICATIONS

Authors: Yuan D, Bassie L, Sabalza M, Miralpeix B, Dashevskaya S, Farre G, Rivera S, Banakar R, Bai C, Sanahuja G, Arjo G, Avilla E, Zorrilla-Lopez U, Ugidos-Damboriena N, Lopez A, Almacellas D, Zhu C, Capell T, Hahne G, Twyman R and Christou P.

Title: The potential impact of plant biotechnology on the Millennium Development Goals.

Journal: 904276 - Plant Cell Reports

Number or authors: 21

Volume: 30 **Number:** 3 **Pages, Initial:** 249 **final:** 265 **Year:** 2011 **Place of publication:** UNITED STATES **ISSN:** 0721-7714

Key: Review **article code:** 016242 **Order:** 001

Authors: Arjó G, Capell T, Matias-Guiu X, Zhu C, Christou P, Piñol C

Title: Mice fed on a diet enriched with genetically engineered multivitamin corn show no sub-acute toxic effects and no sub-chronic toxicity

Journal: 913023 - Plant Biotechnology Journal

Number or authors: 6

Volume: 10 **Number:** 9 **Pages, Initial:** 1026 **final:** 1034 **Year:** 2012 **Place of publication:** ENGLAND **ISSN:** 1467-7644

Key: **Paper **article code:** 018551 **Order:** 003

Authors: Zhu C, Sanahuja G, Yuan D, Farré G, Arjó G, Berman J, Zorrilla-López U, Banakar R, Bai C, Pérez-Massot E, Bassie L, Capell T, Christou P.

Title: Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies

Journal: 913023 - Plant Biotechnology Journal

Number or authors: 13

Volume: 11 **Number:** 2 **Pages, Initial:** 129 **final:** 141 **Year:** 2013 **Place of publication:** ENGLAND **ISSN:** 1467-7644

Key: Review **article code:** 018552 **Order:** 004

Authors:Pérez-Massot E, Banakar R, Gómez-Galera S, Zorrilla-López U, Sanahuja G, Arjó G, Miralpeix B, Vamvaka E, Farré G, Rivera SM, Dashevskaya S, Berman J, Sabalza M, Yuan D, Bai C, Bassie L, Twyman RM, Capell T, Christou P, Zhu C.

Title: The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints

Journal: 914086 - Genes And Nutrition

Number or authors: 20

Volume: --- **Number:** 8 **Pages, Initial:** 29 **final:** 41 **Year:** 2013 **Place of publication:** UNITED STATES **ISSN:** 1555-8932

Key: Review **article code:** 018553 **Order:** 005

Authors: Berman J, Zhu C, Perez-Massot E, Arjo G, Zorrilla-Lopez U, Masip G, Banakar R, Sanahuja G, Farre G, Miralpeix B, Bai C, Vamvaka E, Sabalza M, Twyman RT, Bassie L, Capell T, Christou P.

Title: Can the world afford to ignore biotechnology solutions that address food insecurity?

Journal: 900548 - Plant Molecular Biology

Number or authors: 17

Volume: 83 **Number:** 1-2 **Pages, Initial:** 5 **final:** 19 **Year:** 2013 **Place of publication:** NETHERLANDS **ISSN:** 0167-4412

Key: Review **article code:** 019804 **Order:** 006

Authors: Arjó G; Portero M; Piñol C; Viñas J; Matias-Guiu X; Capell T; Bartholomaeus A; Parrott W; Christou P.

Title: Plurality of opinion, scientific discourse and pseudoscience: an in depth analysis of the Séralini et al. study claiming that Roundup™ Ready corn or the herbicide Roundup™ cause cancer in rats.

Journal: 908103 - Transgenic Research

Number or authors: 9

Volume: --- **Number:** --- **Pages, Initial:** --- **final:** --- **Year:** 2013 **Place of publication:** NETHERLANDS **ISSN:** 0962-8819

Key: Review **article code:** 019784 **Order:** 007

Authors: UXUE ZORRILLA-LÓPEZ, GEMMA MASIP, GEMMA ARJÓ, CHAO BAI, RAVIRAJ BANAKAR, LUDOVIC BASSIE, JUDIT BERMAN, GEMMA FARRÉ, BRUNA MIRALPEIX, EDUARD PÉREZ-MASSOT,MAITE SABALZA, GEORGINA SANAHUJA, EVANGELIA VAMVAKA, RICHARD M. TWYMAN, PAUL CHRISTOU, CHANGFU ZHU and TERESA CAPELL

Title: Engineering metabolic pathways in plants by multigene transformation

Journal: 908667 - International Journal of Developmental Biology

Number or authors: 17

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Key: Review **article code:** 868 **Order:** 008