An Investigation into the Effect of Dietary Lipids and Lovastatin on the Multi-Step Process of Colon Carcinogenesis and Associated Cellular and Molecular Events

by

Kathleen Chambers Clouston

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Food and Nutritional Sciences

University of Manitoba

Winnipeg

Copyright © 2007 by Kathleen Chambers Clouston

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

An Investigation into the Effect of Dietary Lipids and Lovastatin on the Multi-Step Process of Colon Carcinogenesis and Associated Cellular and Molecular Events

BY

Kathleen Chambers Clouston

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

DOCTOR OF PHILOSOPHY

Kathleen Chambers Clouston © 2007

Permission has been granted to the University of Manitoba Libraries to lend a copy of this thesis/practicum, to Library and Archives Canada (LAC) to lend a copy of this thesis/practicum, and to LAC's agent (UMI/ProQuest) to microfilm, sell copies and to publish an abstract of this thesis/practicum.

This reproduction or copy of this thesis has been made available by authority of the copyright owner solely for the purpose of private study and research, and may only be reproduced and copied as permitted by copyright laws or with express written authorization from the copyright owner.

ABSTRACT

An Investigation into the Effect of Dietary Lipids and Lovastatin on the Multi-Step Process of Colon Carcinogenesis and Associated Cellular and Molecular Events

Colorectal Cancer (CRC) is the second leading cause of cancer deaths in Canada[1]. Diets high in saturated and unsaturated lipid promote CRC[2] via different mechanisms[3], including differential regulation of cholesterol biosynthesis. The rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCAR), produces an essential intermediate for $p21^{K\text{-}ras}$ membrane association. $p21^{K\text{-}ras}$ plays a critical role in cell signal transduction regulating cell growth and differentiation. $p21^{K\text{-}ras}$ mutations have been found in over 50% of colonic tumors. Lovastatin (LOV) decreases CRC mortality rates and is known to inhibit HMGCAR[4]. Inhibition of HMGCAR function disrupts $p21^{K\text{-}ras}$ membrane association and function leading to disruption of downstream protein regulating growth signals.

Our objectives were to determine: (1) whether LOV would retard CRC development, (2) if the different stages of CRC growth and their responsiveness to LOV is effected by dietary lipid, and (3) the influence of dietary lipids and LOV on the expression of HMGCAR, $p21^{K\text{-}ras}$, LDLr, as well as specific signal transduction and cell cycle related molecules involved in cellular growth (ERK-1/2, CD1) and apoptosis (caspase-3).

Male F344 rats were injected with azoxymethane (15mg/kg body weight/week for two weeks) and fed a low fat corn oil (LFC) diet for 12 weeks. They were divided into two more groups receiving a high fat corn oil (HFC) diet or beef tallow (HFB) diet for an additional nine weeks, after which a subset of rats from the HFC and HFB groups were treated with LOV (20 mg/kg body weight/day) for three weeks. Colonic aberrant crypt

foci (ACF), microadenomas (MA), and tumors were assessed before and after LOV treatment. Colonic tumors and mucosa were procured for biochemical assessment.

In light of the ACF, MA, and tumor findings along with the tumor expression data for the molecular targets mentioned above, we have substantial evidence demonstrating critical phenotypic differences between tumors in the HFC and HFB environments. The type of dietary lipid and LOV modulate $p21^{K\text{-}ras}$ expression and function, cell cycle regulatory and apoptotic proteins in normal and neoplastic colonic tissue. Our findings are the first to uncover critical interactions between dietary lipids and LOV in gene expression in CRC.

1. Colorectal Cancer Association of Canada. 2007. Colorectal Cancer Statistics.
2. Potter, J.D., Slattery, M.L., Bostick, R.M. and Gapstur, S.M. 1993. Epidemiol. Rev. 15: 499-545.
3. Bird, R.P. and Good C.K. 2000. Toxicology Letters: 112-113:395-402.
4. Demierre, M-F., Higgins, P.D., Gruber, S.B., Hawk, E. and Lippman, S.M. 2005. Nat. Rev. Cancer. 5(12): 930-942.

Acknowledgements

Without the guidance and support of many people in my life, this accomplishment would not have been realized. I am extremely grateful to Dr. Ranjana Bird who gave me such a wonderful opportunity to participate in her ongoing and cherished research endeavours. Her dedication, intelligence, and support throughout the many phases of the research are very much appreciated. I am also thankful for the financial assistance form Dr. Bird in facilitating participation in a number of conferences. I am also thankful for the assistance I received from Dr. Bird's students and laboratory technicians including Carolyn Good, Darcy Salo, Natsha Sablic, Ann Kiunga, Mark Pasetka, Brendan McCarthy, and Nita Modi. I would like to thank Dr. Crow for his assistance with developing the SAS statistical programs used for analyzing our data. I would also like to acknowledge the assistance provided from the animal care staff.

Thank you to my committee members, Drs. Michael Eskin, Raj Bhullar, Ron Marquardt Gopinadhan Paliyath, for their time and support as well as for reading the thesis and providing constructive suggestions for improvement.

I greatly appreciate the financial support provided by the National Science Engineering Research Council of Canada as well as the student financial assistance from both the University of Manitoba Student Fellowship and the Manitoba Health and Research Council.

I would like to take this opportunity to thank my family, for which I am extremely grateful, Darren, Michael, Alace and Ted Chambers, and Chandi Chambers.

Table of Contents

	Page Number
Prefatory Pages	
Title Page	i
Abstract	ii
Acknowledgements	iv
Table of Contents	v
List of Tables	xiv
List of Figures	xviii
Copyright Permission Forms	xix
List of Abbreviations	xxv
Chapter 1: Introduction	
The Multi-Step Process of Colon Carcinogenesis	1
The Effect of Dietary Lipids and Lovastatin on the Multi-Step Process of Colon Carcinogenesis	4
The Effect of Dietary Lipids and Lovastatin on $p21^{K\text{-}ras}$, 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase (HMGCAR) and Low-Density Lipoprotein Receptor (LDLr) in Colon Carcinogenesis	5
The Effect of Dietary Lipids and Lovastatin on Cellular Signalling Molecules Related to the Multi-Step Process of Colon Carcinogenesis	10

The Effect of Dietary Lipids and Lovastatin on Apoptosis in Colon Carcinogenesis 13

Research Objectives and Hypothesis 13

Chapter 2: Literature Review

Anatomy of the Large Intestine 15

Colorectal Cancer 15

Epidemiology and Etiology of Colorectal Cancer 18

Pathology of Colorectal Cancer 20

The Multi-Step Process of Colorectal Carcinogenesis 22

Aberrant Crypt Foci (ACF) 24

Animal Models and the ACF System 28

Dietary Lipids and ACF Development 30

Molecular Mechanisms Involved in Signal Transduction and Cell Growth in Colorectal Cancer 33

The $p21^{K\text{-}ras}$ Proto-Oncogene in Colorectal Cancer 35

Subcellular Localization of $p21^{K\text{-}ras}$ 37

$p21^{K\text{-}ras}$ Signalling 40

Extracellular Signal Regulated Kinases: ERK-1 and ERK-2 42

Cyclin D1 and the Cell Cycle 45

Caspase-3 and Apoptosis 46

The Effect of Dietary Lipids on Cell Signalling Molecules Involved in Colorectal Cancer 46

Cholesterol Biosynthesis and Homeostasis 52

Involvement of HMGCAR and LDLr in Regulating Plasma Cholesterol 56

3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGCAR) Regulation 58

Low Density Lipoprotein and its Receptor 61

The Relationship between Cholesterol Metabolism and $p21^{K\text{-}ras}$ in Colorectal Cancer 64

The Effect of Dietary Lipids on HMGCAR, LDLr, and $p21^{K\text{-}ras}$ in Cancer 67

Chemopreventive / Chemotherapeutic Agents in Colorectal Cancer 69

Lovastatin and Cancer 70

Chapter 3: Materials and Methods

Colon Carcinogen 78

Experimental Animals 78

Experimental Diets 78

Experimental Design 80

Lovastatin (LOV) 84

Quantification of ACF 85

Assessment of Tumors 86

Serum Lipids 87

Total Cholesterol 87

Triacylglycerol 88

Low Density Lipoprotein 89

$p21^{K\text{-}ras}$ Activity 89

Analysis of Tissue Protein Concentration 91

Tissue Preparation for Western Blot Analysis 91

Preparation and Separation of Cytosolic and Membrane Mucosal Tissue Fractions 91

Preparation of Whole Cell (Colonic Mucosa, Tumor and Liver) Homogenate 92

SDS-PAGE, Western Blotting, Immunodetection and Quantification 93

Immunoblot Analysis of $p21^{K\text{-}ras}$ 101

Immunoblot Analysis of HMGCAR and LDLr 102

Analysis of Tissue mRNA Expression by RT-PCR 103

Statistical Analysis 108

Chapter 4: Results

The Effect of Type and Amount of Dietary Lipid and Lovastatin Treatment on the Multi-Step Process of Colon Carcinogenesis 109

PART A: An Investigation of the Effects of Dietary Lipids and Lovastatin on the Growth and Development of Aberrant Crypt Foci, Microadenomas, and Tumors in the Multi-Step Process of Colon Carcinogenesis 109

Experimental Approach 109

Effect of High Fat Diets and Lovastatin on Body Weight and Blood Lipids of F344 Rats 109

The Effect of Dietary Lipid and Lovastatin on ACF, Microadenoma and Tumor Development in Colon Carcinogenesis 119

The HFC Diet Exerts Rapid Growth Promoting Effects on ACF 119

The Effect of Lovastatin on ACF Development is Dependent on the Dietary Lipid Environment 122

Lovastatin Lowers Tumor Incidence in the HFC Group 128

Lovastatin Treatment Retards Tumor Growth Without affecting Total Tumor Number in the HFB Diet Group 128

Tumor Size is affected by Dietary Lipid Type 131

Combined with an HFC Diet, Lovastatin Treatment Inhibits the Appearance of Small Tumors 131

The Effect of Lovastatin on Tumor Distribution Depends on Dietary Lipid Type 135

PART B: An Investigation of the Effects of Dietary Lipids and Lovastatin on Critical Molecules Involved in Cholesterol Biosynthesis and Associated Events in the Multi-Step Process of Colon Carcinogenesis 137

Experimental Approach 137

The Level and Type of Dietary Lipid affects Cytosolic $p21^{K\text{-}ras}$ Protein Levels in Colonic Mucosa 137

Lovastatin Treatment Modulates $p21^{K\text{-}ras}$ Protein Expression in the Cytosolic and Membrane-Associated Fraction of Colonic Mucosa 140

Hepatic Cytosolic and Membrane-Associated p21$^{K\text{-}ras}$ Protein Expression Depends on the Level and Type of Dietary Lipid 140

The Effect of Lovastatin on Hepatic Cytosolic and Membrane-Associated p21$^{K\text{-}ras}$ Protein Expression Depends on Dietary Lipid Type 141

The Lipid Composition of the Diet Modulates the Effect of Lovastatin on p21$^{K\text{-}ras}$ Protein Expression in Normal Mucosa 142

p21$^{K\text{-}ras}$ Protein Levels in Colonic Tumors are Significantly Higher Compared to Colonic Mucosa and Depend on Dietary Lipid Type 142

p21$^{K\text{-}ras}$ Activity in Normal and Tumorigenic Colonic Tissue 144

The Level and Type of Dietary Lipid affect p21$^{K\text{-}ras}$ Activity in Colonic Mucosa and Tumors 144

The Effect of Lovastatin Treatment on p21$^{K\text{-}ras}$ Activity in Colonic Mucosa and Tumors Depends on Dietary Lipid Type 147

HMGCAR Gene Expression is Significantly Higher in Colonic Tumors Compared to Mucosa and is affected by the Level and Type of Dietary Lipid 147

The Effect of Lovastatin Treatment on HMGCAR Gene Expression in Colonic Tumors and Mucosa Depends on Dietary Lipid Type 149

The Amount and Type of Dietary Lipid affects HMGCAR Protein Expression in Colonic Mucosa 150

Lovastatin Significantly Decreases HMGCAR Protein Expression in Colonic Mucosa 150

HMGCAR Protein is Significantly Higher in Colonic Tumors Compared to Colonic Mucosa 150

The Effect of Lovastatin on HMGCAR Protein Expression in Colonic Tumors Depends on Dietary Lipid Type 152

Hepatic HMGCAR Protein Expression is Significantly Higher in the HFC Compared to the HFB Dietary Lipid Group and is affected by Lovastatin Treatment 152

LDL Receptor (LDLr) Gene Expression in Normal and Tumorigenic Colonic Tissue 153

The Effect of Lovastatin Treatment on LDLr Gene Expression in Colonic Mucosa and Tumors Depends on Dietary Lipid Type 153

The Level and Type of Dietary Lipid affects LDLr Protein Expression in Colonic Mucosa and Tumors 153

The Effect of Lovastatin on LDLr Protein Expression in Colonic Mucosa Depends on Dietary Lipid Type 157

Lovastatin Treatment Results in Decreased LDLr Protein Expression in Colonic Tumors 158

LDL Receptor (LDLr) Protein Expression in Rat Hepatic Tissue 158

Lovastatin Treatment Results in Increased LDLr Protein Expression in Hepatic Tissue 159

PART C: An Investigation of the Effects of Dietary Lipids and Lovastatin on the Gene and Protein Expression of Critical Cell Signalling and Apoptotic Molecules in the Multi-Step Process of Colon Carcinogenesis 159

Experimental Approach 159

ERK-1 Gene Expression in Colonic Mucosa and Tumors is affected by a Low Fat Diet 159

The Amount of Dietary Lipid and Lovastatin Significantly affect ERK-1 Gene Expression in Colonic Mucosa and Tumors 161

ERK-1 Protein Expression in Colonic Mucosa and Tumors is affected by Dietary Lipid 161

The Effect of Lovastatin Treatment on ERK-1 Protein Expression in Colonic Tumors Depends on Dietary Lipid Type 163

Dietary Lipid Type and Level affect ERK-2 Protein Expression in Colonic Mucosa 164

ERK-2 Protein Expression is Significantly Higher in Colonic Tumors Compared to Mucosa 164

The Amount of Dietary Lipid affects CD1 Gene Expression in Colonic Mucosa 166

Differences in CD1 Gene Expression Between Colonic Mucosa and Tumors Depend on the Type of Dietary Lipid 166

Lovastatin Treatment Reduces CD1 Gene Expression in Colonic Tumors 168

The Type of Dietary Lipid affects CD1 Protein Expression in Colonic Mucosa and Tumors 168

The Effect of Lovastatin Treatment on CD1 Protein Expression in Colonic Mucosa and Tumors Depends on Dietary Lipid Type 171

Dietary Lipid affects Caspase-3 Protein Expression in Colonic Mucosa and Tumors 171

Caspase-3 Protein Expression is Significantly Higher in Colonic Tumors Compared to Mucosa and affected By Dietary Lipid Type 174

The Effect of Lovastatin Treatment on Caspase-3 Protein Expression in Colonic Mucosa and Tumors Depends on Dietary Lipid Type 174

Chapter 5: Discussion

The Effect of Dietary Lipids and Lovastatin on the Multi-Step Process of Colon Carcinogenesis 175

Part I: ACF, Microadenoma and Tumor Findings 177

Part II: p21^{K-ras}, HMGCAR, and LDLr Expression in Colonic Tissues 180

Part III: ERK-1, ERK-2, Cyclin D1, and Caspase-3 Expression in Colonic Tissues 190

Summary and Conclusions 195

References 200

Appendices

Appendix A: Composition of AIN-76A Vitamin And Mineral Mix 233

Appendix B: Fatty Acid Composition of Experimental Lipids 234

Appendix C: Fatty Acid Composition of Experimental Diets 235

Appendix D: Composition of Experimental Diets 236

Appendix E: RIPA Buffer Recipe 237

Appendix F: Buffers Used For Tissue Cell Lysate Preparation (Cytosolic, Membranous and whole cell lysate) 238

Appendix G: Western Blotting/Immunoblotting Buffers and Acrylamide Gel Recipes 239

Appendix H: RT-PCR Buffer Recipes 242

Appendix I: Cyclin D1 and Caspase-3 Protein Expression in Hepatic Tissue 244

Appendix J: Representative Western Blot Images for p21^{K-ras}, HMGCAR, LDLr, ERK-1, ERK-2, CD1, and Caspase-3 246

Appendix K: Genestein Findings 250

List of Tables

Page Number

Table 1.	Names and Chemical Structures of Common Saturated and Unsaturated Fatty Acids.	31
Table 2.	Composition of Experimental Diets.	79
Table 3.	Fatty Acid Composition of Experimental Lipids.	81
Table 4.	Fatty Acid Composition of Experimental Diets.	82
Table 5.	$p21^{K\text{-}ras}$ Protein Immunoblotting Conditions for Colonic Mucosa, Tumor and Liver Tissue.	94
Table 6.	HMGCAR and LDLr Protein Immunoblotting Conditions for Colonic Mucosa, Tumor and Liver Tissue.	96
Table 7.	ERK-1, ERK-2, Cyclin D1, and Caspase-3 Protein Immunoblotting Conditions for Colonic Mucosa, Tumor and Liver Tissue.	98
Table 8.	Primer Sequences and Conditions for RT-PCR.	107
Table 9.	Body Weights of Male F344 Rats after 24 Weeks of Intervention with LFC, HFC, HFB, HFCα and HFBα Diets.	111
Table 10.	Day Time Levels of Plasma Total Cholesterol and LDL at 21 and 24 Weeks after Treatment with LFC, HFC, HFB Diets.	112
Table 11.	Day Time Levels of Plasma Total Cholesterol, Triacylglycerol, and LDL at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin.	115
Table 12.	Night Time Levels of Plasma Total Cholesterol, Triacylglycerol, and LDL at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin.	116

Table 13. Combined Day and Night Time Levels of Plasma Total Cholesterol and LDL At 24 Weeks after Treatment With LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 118

Table 14. Enumeration of ACF Growth Characteristics in Male F344 Rats after at 21Weeks Representing Nine Weeks of Intervention with LFC, HFC, and HFB Diets. 120

Table 15. Number of ACF with Different Growth Features in Male F344 Rats as Affected by LFC, HFC and HFB Diets: 21 Weeks. 121

Table 16. Enumeration of ACF Growth Characteristics in Male F344 Rats at 24 Weeks of the Study Representing 12 Weeks of Intervention with LFC, HFC, HFB Diets and Three Weeks of Lovastatin Treatment. 123

Table 17. Number of ACF With Different Growth Features in Male F344 Rats as Affected by LFC, HFC, HFB, HFCα, and HFBα Diets at 12 Weeks. 125

Table 18. Tumor Parameters in Male F344 Rats Fed LFC, HFC, and HFB Diets and Lovastatin Treatment of a Subset of the HFC an HFB Diet Groups. 129

Table 19. Classification of Tumor Size in Male F344 Rats AT 24 Weeks Representing 12 Weeks of Dietary Intervention with LFC, HFC, and HFB Diets and Three Weeks of Lovastatin Treatment of a Subset of the HFC and HFB Groups. 132

Table 20. Average Tumor Size of Tumors Along the Length of The Colon in Male F344 Rats as affected by LFC, HFC, HFB Diets and Lovastatin Treatment. 133

Table 21. Percentage Distribution of Tumors Along the Length of the Colon in Male F344 Rats as Affected by LFC, HFC, HFB Diets and Lovastatin Treatment. 136

Table 22. Cytosolic and Membrane-Associated p21$^{K\text{-}ras}$ Protein Expression in Rat Colonic Mucosa and Liver at 24 Weeks After Treatment with LFC, HFC, HFB Diets And Three Weeks Of Lovastatin. 138

Table 23. p21$^{K\text{-}ras}$ Protein Expression in Rat Colonic Mucosa and Tumors at 24 Weeks after Treatment With LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 143

Table 24. p21$^{K\text{-}ras}$ Activity in Rat Colonic Mucosa and Tumors at 24 Weeks after Treatment with a High Fat Diet and Three Weeks of Lovastatin. 145

Table 25. p21$^{K\text{-}ras}$ Activity in Rat Colonic Mucosa and Tumors at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 146

Table 26. HMGCAR Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 148

Table 27. HMGCAR Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 151

Table 28. LDL Receptor Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 154

Table 29. LDL Receptor Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 155

Table 30. ERK-1 Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 160

Table 31. ERK-1 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 162

Table 32. ERK-2 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 165

Table 33. Cyclin D1 Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 167

Table 34. Cyclin D1 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 169

Table 35. Caspase-3 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin. 172

Table 36. Phenotypic Differences in Colonic Mucosa and Tumors from HFC and HFB Experimental Treatments. 198

Table 37. Phenotypic Differences in Colonic Mucosa and Tumors from HFCα and HFBα Dietary Treatments. 199

List of Figures

		Page Number
Figure 1.	The Four Sections of the Human Colon (Large Bowel)	16
Figure 2.	A Molecular Model for the Evolution of CRC through the Adenoma-Carcinoma Sequence	23
Figure 3.	Aberrant Crypt Foci (ACF)	25
Figure 4.	The Ras Signalling Cascade	34
Figure 5.	The Structure of Cholesterol	53
Figure 6.	The Cholesterol Biosynthetic Pathway as affected by Statins	54
Figure 7.	Experimental Protocol	83

Copyright Permission Forms

Figure 1

Hello Kathleen,

Thanks for contacting the CCAC. As per your request to use the diagrams from the Understanding reference guide, the booklet was actually produced by AstraZeneca and we do not have versions to send in electronic format. We do have another version attached which has been used on our website and you are welcome to use for your thesis.

kind regards,

Heidi Watts
Program Director
Colorectal Cancer Association of Canada
60 St. Clair Avenue East
Suite 204
Toronto, ON
M4T 1N5
tel: 416.920.4333
fax: 416.920.3004
Toll-free support line 1.877.50COLON
www.colorectal-cancer.ca

Figure 2

ELSEVIER LIMITED LICENSE
TERMS AND CONDITIONS

Nov 30, 2007

This is a License Agreement between Kathleen M Chambers Clouston ("You") and Elsevier Limited ("Elsevier Limited"). The license consists of your order details, the terms and conditions provided by Elsevier Limited, and the payment terms and conditions.

License Number	1812560113983
License date	Oct 19, 2007
Licensed content publisher	Elsevier Limited
Licensed content publication	Cell
Licensed content title	A genetic model for colorectal tumorigenesis
Licensed content author	Eric R. Fearon and Bert Vogelstein
Licensed content date	1 June 1990
Volume number	61
Issue number	5
Pages	9
Type of Use	Thesis / Dissertation
Portion	Figures/table/illustration/abstracts
Quantity	1
Format	Print
You are an author of the Elsevier article	No
Are you translating?	No
Purchase order number	
Expected publication date	Jan 2008
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD
Total	0.00 USD

Terms and Conditions

Figure 4

ELSEVIER LIMITED LICENSE
TERMS AND CONDITIONS

Nov 30, 2007

This is a License Agreement between Kathleen M Chambers Clouston ("You") and Elsevier Limited ("Elsevier Limited"). The license consists of your order details, the terms and conditions provided by Elsevier Limited, and the payment terms and conditions.

License Number	1812590167830
License date	Oct 19, 2007
Licensed content publisher	Elsevier Limited
Licensed content publication	The Lancet Oncology
Licensed content title	The MAPK signalling pathways and colorectal cancer
Licensed content author	Jing Yuan Fang and Bruce C Richardson
Licensed content date	May 2005
Volume number	6
Issue number	5
Pages	6
Type of Use	Thesis / Dissertation
Portion	Full article
Format	Print
You are an author of the Elsevier article	No
Are you translating?	No
Purchase order number	
Expected publication date	Jan 2008
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD
Total	0.00 USD

Terms and Conditions

ELSEVIER LIMITED LICENSE
TERMS AND CONDITIONS

Nov 30, 2007

This is a License Agreement between Kathleen M Chambers Clouston ("You") and Elsevier Limited ("Elsevier Limited"). The license consists of your order details, the terms and conditions provided by Elsevier Limited, and the payment terms and conditions.

License Number	1812590047653
License date	Oct 19, 2007
Licensed content publisher	Elsevier Limited
Licensed content publication	The Lancet Oncology
Licensed content title	The MAPK signalling pathways and colorectal cancer
Licensed content author	Jing Yuan Fang and Bruce C Richardson
Licensed content date	May 2005
Volume number	6
Issue number	5
Pages	6
Type of Use	Thesis / Dissertation
Portion	Figures/table/illustration/abstracts
Quantity	1
Format	Print
You are an author of the Elsevier article	No
Are you translating?	No
Purchase order number	
Expected publication date	Jan 2008
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD
Total	0.00 USD

Figure 5

Kathleen Chambers Clouston,

On 10/13/2007 we received your request. Your request **ID is 169097**. This confirmation does not constitute permission to use the material requested but we recommend that you keep this email for your records.

ISBN: 0534559867
Author: Gropper, Smith, Groff
Title: Advanced Nutrition and Human Metabolism
Material Requested: pages 133 and 145, Figure 6.21 & Figure 6.7

Your request to use Thomson Learning materials is now being processed. You can expect a response once the information is complete and we have researched the request. Response time increases during peak permissions seasons (August - October, and January), so plan accordingly and submit all requests well before they're needed. Use the **request ID 169097** to reference this request. Please email any questions to thomsonrights@thomson.com. If this is a republishing request, please fax a copy of the pages you want to use to 800-730-2215. Put the request **ID 169097** on each page you fax.

Thank you,

Thomson Learning Global Rights Group
thomsonrights@thomson.com

All requests to use Thomson Learning materials must be submitted online at http://www.thomsonrights.com

Dear Ms. Kathleen Chambers Clouston,

Your request (**ID number 160907**) has been granted.

Please note that if your thesis is published further with a publisher for sale, you must request additional permission.

Thank you,

Jane Park, Permissions Granting Associate
Cengage Learning

10 Davis Drive, Belmont CA 94002
Phone: 650 413-7787 or 800 730-2214 Fax: 800 730-2215
jane.park@thomson.com

Figure 6

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Nov 30, 2007

This is a License Agreement between Kathleen M Chambers Clouston ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

License Number	1812570943043
License date	Oct 19, 2007
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Cancer
Licensed content title	Statins and cancer prevention
Licensed content author	Marie-France Demierre , Peter D. R. Higgins , Stephen B. Gruber , Ernest Hawk and Scott M. Lippman
Volume number	
Issue number	
Pages	
Year of publication	2005
Portion used	Full paper
Requestor type	Student
Type of Use	Thesis / Dissertation
PO Number	
Total	$0.00

List of Abbreviations

AA	Arachidonic Acid
ACAT	Acyl CoA:Cholesterol Acyl Transferase
ACF	Aberrant Crypt Foci
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
CD1	Cyclin D1
Cdk	Cyclin dependent kinases
CEA	Carcinoembryonic Antigen
CRC	Colorectal Cancer
CVD	Cardiovascular Disease
DAG	Diacylglycerol
DMH	Dimethylhydrazine
EGF	Epidermal Growth Factor
EPA	Eicosapentaenoic acid (C20:5n3)
ER	Endoplasmic Reticulum
ERK-1	Extracellular Signal-Regulated Kinases –1 ($p44^{MAPK}$)
ERK-2	Extracellular Signal-Regulated Kinases –2($p42^{MAPK}$)
FA	Fatty Acid
FPP	Farnesyl Pyrophosphate
FPTase	Farnesyl Pyrophosphate Transferase
GAP	GTPase Activating Proteins

Grb-2	Growth Factor Receptor Binding Protein 2
GNEF	Guanine Nucleotide Exchange Factors
HMGCAR	3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase
IGF-1R	Insulin-Like Growth Factor –1 Receptor
IR	Insulin Receptor
LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein Receptor
MA	Microadenoma
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase (also MEK1 and MEK2)
PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reactions
PUFA	Polyunsaturated Fatty Acid
RCT	Randomized Control Trial
RT	Reverse Transcription
SOS	Son of Sevenless Protein
SRE	Sterol Response Elements
SREBP	Sterol Response Element Binding Proteins
TAG	Triacylglycerol
TBST	Tris Buffered Saline with 0.1% Tween-20
VEGF	Vascular Endothelial Growth Factor

Chapter 1: Introduction

The Multi-Step Process of Colon Carcinogenesis

Understanding the molecular basis underlying the ability of specific dietary lipids, along with preventive agents such as lovastatin (LOV), in modulating aberrant crypt foci (ACF) and tumor growth in the colon is essential to formulating interventions that will inhibit both the appearance and progression of colon cancer. ACF represent preneoplastic lesions and are the earliest representatives of the multi-stage process of colon carcinogenesis. Hence, they are useful endpoints in assessment of the dietary lipid environment as well as chemotherapeutic/chemopreventive compounds. Using the ACF system, we are able to quantitatively assess this process and identify specific modulators of ACF growth and development as well as microadenoma and tumor development and growth.

One of the goals of our research was to investigate if LOV affects the growth of preneoplastic lesions and tumors and if this effect of LOV on colonic tissues is modulated by dietary lipids varying in the levels of unsaturated and saturated fatty acids. It was hypothesized that a high saturated fat diet (beef tallow), would augment the cholesterol biosynthetic pathway, hence preneoplastic lesions and/or tumors would be less sensitive to LOV. On the other hand, a highly unsaturated fat diet would exert a synergistic effect with LOV in retarding the growth of these lesions. This hypothesis was based on the assumption that the cholesterol biosynthetic pathway is regulated by the level of saturated or unsaturated fatty acids in a manner similar to other tissues (Goldstein and Brown, 1990; Ness and Chambers, 2000) and plays a critical role in growth and development of preneoplastic lesions and tumors.

Our investigation focused on investigating the role of specific dietary lipids and LOV in the late stages of colon cancer development, signified by advanced colonic lesions. Therefore, experimental treatment before, during or soon after carcinogen injection was avoided to prevent altered tumor outcome due to modulation of the early events of colon carcinogenesis. This approach allows investigation of the process of colon carcinogenesis most similar to those of high-risk humans.

F344 rats were injected with AOM, a colon specific carcinogen, according to the protocol outlined in Chapter 3. This protocol allowed us to analyze preneoplastic (ACF and MA) and neoplastic (colonic tumors) tissue. Two tumor-promoting diets, HFC and HFB, were used, as well as a LFC diet to allow investigation of the effect of total amount of dietary lipid on colon carcinogenesis. We intervened in the CRC process using the HMGCAR inhibitor LOV (Figure 7; Schematic of Experimental Protocol). LOV has been demonstrated to have clinical relevance in colorectal cancer treatment in humans (Poynter et al, 2005; Demierre et al, 2005) and has multi-functional effects on tumor cell growth, some of which are HMGCAR-dependent. The colons of the experimental animals contained a large number of ACF with varying crypt multiplicities. Therefore, many developmental states were represented as ACF crypt multiplicity indicates its development state. Using this approach, we were able to study the response of preneoplastic lesions at different developmental stages to the growth modulating effects of specific dietary lipids (highly saturated versus unsaturated), high and low fat diets, as well as the cholesterol biosynthetic pathway inhibitor, LOV. Corn oil and beef tallow were used due to their contrasting fatty acid composition and differential effects in promoting colon tumor development. ACF growth features, microadenoma (MA) as well

as tumor incidence were assessed before and after dietary intervention. Normal and neoplastic tissue samples were harvested and processed for biochemical analysis.

In the second part of the study, the proposed research determined the influence of different dietary lipids and LOV on the expression of HMGCAR (the rate-limiting enzyme of cholesterol biosynthesis), $p21^{K\text{-}ras}$, and LDLr expression, as well as expression of specific signal transduction and cell cycle related factors important in cellular growth (ERK-1, ERK-2, CD1) and apoptosis (caspase-3).

Dietary lipid and LOV have been implicated in the regulation of cell cycle and proliferative proteins. Therefore, the effects of the above experimental protocol on the expression of ERK-1 and ERK-2 as well as CD1 were investigated. Whether these effects are related to $p21^{K\text{-}ras}$ function in normal and neoplastic colonic tissue remains to be confirmed. Due to findings that LOV induces cellular apoptosis (Agarwal et al, 1999; Agarwal et al, 2002a; Swamy et al, 2002), a preliminary investigation into caspase-3 protein expression was conducted. As the primary target organ of LOV metabolism is the liver (Moghadasian, 1999), in some cases, we used this organ as a comparison tissue when investigating such molecules as $p21^{K\text{-}ras}$, HMGCAR, LDLr, ERK-1/2, and CD1. In most cases, the expression of target molecules was investigated at both the transcriptional level as well as the translational level. It appears that the more useful and consistent parameter is the protein expression data, and if possible, the protein activity level. It is widely accepted that a plethora of processes affects the eventual conversion of mRNA transcripts to protein. Even though mRNA transcript levels may not reflect treatment effects and remain relatively constant even between colonic mucosa and tumor tissue, altered post-transcriptional regulation of proteins in these tissues does occur (Kiunga et

al, 2004). In addition, protein activity can be increased without parallel increases in protein quantity (Spence et al, 1985).

It is well established that altered expression of specific genes at the levels of transcription and translation, as well as post-translational processing, underlies the adenoma-adenocarcinoma sequence in the multi-stage process of colon carcinogenesis. Identification of differences in gene expression between healthy and abnormal colonic tissue is of interest in understanding and formulating disease treatment approaches. It is also imperative to investigate and delineate the effects of different dietary lipids on both normal and abnormal growth in the context of gene expression. Dietary lipids have been shown to affect regulation of gene expression as well as protein function and activity (Clarke and Jump, 1994; Sellmayer et al, 1997; El-Sohemy and Archer, 1999). However, research investigating the complex interactions between therapeutic treatments and different lipids is sparse. Research investigating the effects of saturated fat on gene expression in colonic mucosa and tumors is also limited. Based on epidemiological and experimental studies, diets high in saturated fat are most closely associated with colon tumor promoting effects. However, there is a gap in our knowledge of the effects of saturated fat on gene and protein expression as well as activity of the target genes implicated in colon carcinogenesis.

The Effects of Dietary Lipid and Lovastatin on the Multi-Step Process of Colon Carcinogenesis

The growth regulatory effect of dietary lipids on ACF and tumor development in the rat is dependent on its characteristic fatty acid composition. Corn oil and beef tallow promote colon tumor development in carcinogen treated rats (Bird and Good, 2000). In animal models, both a high fat corn oil diet rich in linoleic acid (HFC) and a high fat beef

tallow diet (a poor source of linoleic acid), have been proven to promote colon tumorigenesis. If the fatty acid (FA) composition of the diet is indeed important in the CRC process, it is reasonable to propose that these diets affect CRC via different mechanisms. Under similar conditions, fish oil inhibits tumor development (Good, 1999). High corn oil diets have been shown to stimulate cell proliferation in the proximal and distal colon compared to fish oil, whereas the hyperproliferative effects of a high fat beef tallow diet on colon cells was found only in the distal colon (Kim et al, 1998). Compared to rats fed a highly unsaturated fat diet, those fed a diet high in saturated fat excrete more fecal lipids. Fecal lipid excretion is significantly correlated to colon cell proliferation in that distal colon cell proliferation increases as fecal lipids increase. The hypothesis underlying the research was that the two tumor promoting diets, with markedly different fatty acid composition (a highly polyunsaturated (PUFA) and a highly saturated fat diet), would affect the development of colon carcinogenesis via different mechanisms based on differential regulation of the rate-limiting enzyme of cholesterol biosynthesis. Based on this assumption, we predicted that the environment created by these two diets would produce preneoplastic and neoplastic lesions with differing phenotypes and that their growth and development would be regulated differently by the HMGCAR inhibitor, LOV.

The Effect of Dietary Lipids and Lovastatin on $p21^{K\text{-}ras}$, 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGCAR) and Low Density Lipoprotein Receptor (LDLr) in Colon Carcinogenesis

$p21^{K\text{-}ras}$ plays a critical role in the early stages of colon carcinogenesis. $p21^{K\text{-}ras}$ is mutated in approximately 50% of all colonic tumors, as well as demonstrating over-expression of native ras in colonic tumors. It is a key regulatory mitigator of cellular

growth signals linked to the cellular cycle (through CD1) and signalling processes (ERK-1 and ERK-2) involved in colon tumorigenesis. Proper function of $p21^{K\text{-}ras}$ is essential for cellular growth and its abnormal function, related to mutation or over-expression, is involved in colon tumor growth. Therapeutic interventions aimed at interrupting the continuous growth signal established by mutated or over-expressed $p21^{K\text{-}ras}$ may ultimately decrease or abolish tumor appearance and growth.

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCAR) is the rate-limiting enzyme of cholesterol biosynthesis and its product, mevalonate, is required for production of farnesyl, which is used in post-translational modification of $p21^{K\text{-}ras}$ to facilitate attachment to the plasma membrane necessary for its function. Farnesylation of $p21^{K\text{-}ras}$ and plasma membrane attachment is part of the requirement for its activation. Overall, the amount of farnesyl available is one factor affecting the amount of membrane-associated $p21^{K\text{-}ras}$ and its activity. LOV inhibits HMGCAR and is used therapeutically as a competitive inhibitor of HMGCAR resulting in decreased farnesyl production. Whether LOV treatment alters transcription of the HMGCAR gene and protein expression in colonic tissue is unknown. HMGCAR expression and activity is intimately associated with LDLr expression and function. Evidence from six human colonic adenocarcinomas cell lines indicated that all tumors grow well in lipoprotein-deficient serum, implying that LDL in culture medium was not crucial for cell growth (Fabricant and Broitman, 1990). In addition, five of the six colon tumor cell lines were unable to overcome a LOV block in cholesterol metabolism indicating that these cells were deficient in LDL receptors. The most aggressive colonic tumors have been shown to down-regulate LDLr (Caruso et al, 1998, 1999, 2002). However, no research has been

conducted on the affect of dietary saturated and unsaturated fat on these molecules in the colorectal cancer disease process. Therefore, investigation of the effect of dietary lipids and LOV treatment on $p21^{K\text{-}ras}$, HMGCAR and LDLr expression is in keeping with the research hypothesis that LOV will modulate HMGCAR which in turn will influence $p21^{K\text{-}ras}$ and LDLr.

Cellular growth is dependent on cholesterol (Siperstein, 1984) which is obtained either by cellular synthesis via regulation of the rate-limiting enzyme of cholesterol biosynthesis, HMGCAR, or from cellular uptake via the LDLr, or both. HMGCAR and LDLr are regulated according to the cholesterol requirements of the cell (Goldstein and Brown, 1990). Cancer cells have an increased cholesterol requirement that may be obtained by increased HMGCAR activity (endogenous synthesis) and/or increased LDLr (exogenous uptake) (Caruso et al, 1999). Research findings have documented the fact that, compared to normal tissue, various human tumors have an increased HMGCAR and LDLr expression at both the mRNA and protein level. These findings support the idea that HMGCAR and LDLr play vital roles in cellular growth, especially in selected tumors. In one study of HMGCAR activity in human brain tumors, findings supported the contention of phenotypic differences among tumors in regards to expression of HMGCAR (Maltese, 1983). The findings of Caruso et al (1999 and 2002) support this contention relative to human colorectal carcinogenesis. In human colorectal adenomas, HMGCAR activity was significantly higher in tumors not expressing LDLr compared to those expressing LDLr (Caruso et al, 1999). Serum cholesterol levels did not differ between the groups expressing or not expressing LDLr. Our research is based on the assumption that these differences depend on environmental factors, including dietary

lipids, and that these environmental factors affect gene expression and phenotype in both normal and tumorigenic colonic tissue. Hence, the lipid environment may also play an important role in determining HMGCAR and LDLr expression as well as amenability to clinical intervention with LOV. Reliance, by the tumor, on increased HMGCAR activity exposes a vulnerability to possible interference with an HMGCAR inhibitor such as LOV. *In vivo* studies have demonstrated decreased tumor growth using HMGCAR inhibitors (Maltese et al, 1985; Sumi et al, 1992). Whether the effects of LOV are dependent on the type of dietary fat is currently unknown. The ability of a tumor cell to down-regulate LDLr may provide it with a growth advantage in that the suppressing effect of exogenous cholesterol on HMGCAR is negated, allowing the cell to up-regulate HMGCAR resulting in increased production of isoprenoid products required for ras farnesylation. $p21^{K\text{-}ras}$ mutation and over-expression is well documented in colon carcinogenesis (Bos, 1989).

Due to the multitude of regulatory effects utilized by a cell, including post-translational modification, as well as the many regulatory steps involved, it is not surprising that gene expression does not always corroborate protein expression (Spady and Cuthbert, 1992). HMGCAR activity correlates well with immunoreactive protein levels (Nakanishi et al, 1988; Chambers and Ness, 1998). Cholesterol regulates HMGCAR gene expression in rats primarily at the translation level (Chambers and Ness, 1998).

HMGCAR expression is regulated partly by the LDLr. Increased expression of LDLr by diets high in PUFAs leads to increased cellular uptake of cholesterol resulting in inhibition of HMGCAR expression. PUFAs and statins both inhibit HMGCAR activity (Proksch et al, 1992; Watanabe et al, 1999). Factors resulting in decreased LDLr

expression, such as saturated fatty acids, result in increased HMGCAR expression and blood LDL cholesterol levels. Therefore, an intimate relationship between HMGCAR and LDLr exists in normal tissues. However, this association appears to be disengaged during tumorigenic growth. At the level of gene expression, HMGCAR and LDLr demonstrate coordinated regulation. However, the activities of these two proteins are independently regulated (Spady and Cuthbert, 1992).

Research supports the existence of phenotypic differences related to HMGCAR and LDLr in colorectal tumors. Absence of the LDLr predicts shorter time to metastasis and survival and has been demonstrated to occur in over 60% of human colorectal tumors (Notarnicola et al, 2004). Tumors not expressing the LDLr have significantly higher HMGCAR activity than those tumors expressing LDLr (Caruso et al, 1999). The cholesterol requirement of tumors not expressing the LDLr may be met by up-regulating endogenous synthesis via HMGCAR. Therefore, our research attempts to elucidate the therapeutic advantage of LOV (an HMGCAR inhibitor) treatment, on colorectal tumors expressing high HMGCAR activity dependence for growth.

Due to the effect of PUFA on the HMGCAR enzyme in the pathway of cholesterol biosynthesis and the HMGCAR-dependent cellular processes, we also predicted that LOV, an HMGCAR inhibitor, would have a synergistic effect with the PUFA diet resulting in a retardation or regression of growth of preneoplastic and neoplastic lesions. Based on the assumption that saturated fat acts via a mechanism different from PUFAs, we predicted that LOV would be a less effective inhibitor of colon carcinogenesis in this dietary lipid environment. The rational behind our hypothesis is based on epidemiological and animal studies supporting the dogma that the fatty acid composition

of dietary lipids influences colorectal cancer (CRC) risk and development. Looking at the vast amount of accumulated evidence on cardiovascular disease (CVD) and carcinogenesis, it is well documented that UFA diets, including corn oil, lowers HMGCAR expression and activity (Goldstein and Brown, 1990; El-Sohemy and Archer, 1997 and 1999). This results in decreased endogenous cholesterol biosynthesis as well as increased LDLr expression and activity and removal of cholesterol from the blood producing decreased blood LDL cholesterol levels. On the other hand, saturated fat diets, including beef tallow, augments HMGCAR expression and activity. This results in increased endogenous cholesterol biosynthesis as well as inhibition of LDLr expression and activity and leads to increased blood LDL cholesterol levels (Goldstein and Brown, 1990). Therefore, one can speculate that UFA diets would produce similar decreases in HMGCAR expression and activity in preneoplastic and neoplastic lesions and that these effects would be substantially lower compared to the effects on HMGCAR found with an HFB diet. Combined with a PUFA diet, it is reasonable to expect that LOV will act synergistically in lowering HMGCAR expression and activity. However, this effect is not expected when LOV treatment is combined with an HFB diet.

The Effect of Dietary Lipids and Lovastatin on Cellular Signalling Molecules Related to the Multi-Step Process of Colon Carcinogenesis

The extracellular signal-regulated kinases, ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK) are downstream targets of the $p21^{K\text{-}ras}$ signal and as such play a critical role in cell growth and differentiation in normal and neoplastic colonic tissue. Both kinases are activated via phosphorylation as a result of activation of the ras-raf-MAPK kinase pathway and translocate to the nucleus where they activate transcription factors regulating transcription of specific genes (Eggstein et al, 1999). Human colonic tumors of various

types and stages have shown different ERK-2 expression levels and generally a lower activity level compared to colonic mucosa. Activity and protein expression of ERK-2 have been shown to be significantly correlated (Eggstein et al, 1999). Increased expression of MAP kinases have been reported in both mammary (Maemura et al, 1999), human renal carcinoma (Oka et al, 1995), chicken crypt cells (Mamajiwalla and Burgess, 1995) and colorectal cancer (Ostrowski et al, 1998). A positive correlation has been observed between MAP kinase activity and tumor size in human hepatocellular carcinoma (Ito et al, 1998).

The MAP kinase cascade is a critical signalling pathway involved in facilitating transmission of the $p21^{K\text{-}ras}$ signal to the nuclear transcription machinery of the cell leading to growth control. Increased ERK-1 and ERK-2 protein levels in colonic tumors have been reported (Licato and Brenner, 1998; Ostrowski et al, 1998; Eggstein et al, 1999; Sebolt-Leopold et al, 1999). A two-fold increase in ERK-1 and ERK-2 in colonic tumors versus mucosa in rats fed a high corn oil diet has also been observed (Davidson et al, 1999). The effects of saturated lipid or LOV on ERK-1 and ERK-2 expression in colonic tissues are unknown. Comparing ERK-1 and ERK-2 protein expression between normal colonic mucosa and tumors for each dietary lipid and LOV treatment group may provide insight into changes essential to tumor formation and progression as well as establish whether phenotypic differences exist among tumors from each group. Along with assessment of $p21^{K\text{-}ras}$, HMGCAR, LDLr, ERK-1, ERK-2, and CD-1 expression, possible relationships among these factors in specific tumor phenotypes may be uncovered.

Growth factor signalling is linked to cell cycle progression by CD1. CD1 gene induction is induced as a secondary response gene following mitogenic stimulation and cyclin dependent kinase (cdk) activity depends on forming a complex with cyclins such as CD1. CD1 is a downstream target of the $p21^{K\text{-}ras}$ signalling pathway. $p21^{K\text{-}ras}$ plays a critical role in signal transmission resulting in cell cycle progression and is required for induction of CD1 as well as for down-regulation of $p27^{KIP1}$. Regulation of CD1 is a critical target of the $p21^{K\text{-}ras}$ signalling cascade based on findings that constitutive expression of CD1 negates the requirement for $p21^{K\text{-}ras}$ in cell proliferation (Aktas et al, 1997). Oncogenic $p21^{K\text{-}ras}$ can induce expression of CD1 (Winston et al, 1996). CD1 protein expression is dependent on $p21^{K\text{-}ras}$ signalling with $p21^{K\text{-}ras}$ modulating CD1 gene expression at the level of mRNA accumulation (Aktas et al, 1997). The dependence of CD1 mRNA accumulation on $p21^{K\text{-}ras}$ function is supported by studies demonstrating that the regulatory elements of the CD1 promoter are induced in response to oncogenic $p21^{K\text{-}ras}$ proteins or activation of the ERK mitogen activated protein kinase (Albanese et al, 1995; Lavoie et al, 1996; Aktas et al, 1997). In addition, cdk activity is regulated by inhibitory compounds including $p27^{KIP1}$. Ras signalling regulates expression of $p27^{KIP1}$ and its interaction with different cdk-cyclin complexes (Aktas et al, 1997). $p27^{KIP1}$ is expressed at high levels in cells treated with LOV (Hengst and Reed, 1996). Comparative studies investigating the differential effects of saturated and n6 dietary lipids on CD1 gene and protein expression in normal and tumorigenic colonic tissue in animal models are limited. It is essential to investigate this protein in the context of both its upstream and downstream targets. There is an absence of data available on the possible interactions of dietary lipid with LOV treatment on CD1 expression in colonic tissues.

The Effect of Dietary Lipids and Lovastatin on Apoptosis in Colon Carcinogenesis

Inhibition of isoprenylation of ras superfamily proteins by HMGCAR inhibitors, such as LOV, has been demonstrated to have cellular effects including increased apoptosis (Demierre et al, 2005). The modulatory effect of LOV on caspase-3 activity and expression in relationship to apoptosis in colon cancer cell lines has been investigated (Swamy et al, 2002). Caspase-3 protein expression was only moderately altered by LOV treatment at low doses (5 or 10 micromoles). However, at higher doses (20-25 micromoles), a significant increase in protein expression and activity was found. These findings support the role of HMGCAR as an important regulator of apoptosis and sheds light on further roles of this chemopreventive agent in therapeutic strategies for colon cancer prevention. Investigation into the effects of dietary lipid and LOV on caspase-3 expression and its role in normal and neoplastic colonic tissue *in vivo* have not been conducted.

Research Objectives and Hypothesis

The thesis research attempted to develop a more thorough understanding of the CRC disease process at both the cellular and molecular levels based on the following concept that: (1) cancer development is a multi-step, multi-factorial, and complex process involving continuous clonal selection and propagation of initiated cells (Farber and Rubin, 1991). The successful transition of a preneoplastic lesion to a malignant state involves sequential acquisition of novel genotypic and phenotypic features compatible with enhanced growth autonomy and survival, (2) preneoplastic lesions at various "preneoplastic stages" may respond differently to specific growth regulatory

environments including LOV and dietary lipid, and (3) preneoplastic lesions are biologically heterogeneous.

One of the goals of our research was to investigate if LOV affects the growth of preneoplastic lesions and tumors and if this effect of LOV on the colonic tissues is modulated by dietary lipids varying in the levels of unsaturated and saturated fatty acids. It was hypothesized that a high saturated fat diet (beef tallow), would augment the cholesterol biosynthetic pathway, hence preneoplastic lesions and/or tumors would be less sensitive to LOV. On the other hand, a highly unsaturated fat diet would exert a synergistic effect with LOV in retarding the growth of these lesions. This hypothesis was based on the assumption that the cholesterol biosynthetic pathway is regulated by the level of saturated or unsaturated fatty acids in a manner similar to other tissues (Goldstein and Brown, 1990; Ness and Chambers, 2000) and plays a critical role in growth and development of preneoplastic lesions and tumors. Different types and amounts of dietary lipids, as well as LOV, have been shown to affect $p21^{K\text{-}ras}$ post-translational modification via affects on farnesyl pyrophosphate production, modulated through HMGCAR and LDLr, leading to modulation of $p21^{K\text{-}ras}$ farnesylation, membrane localization and activity and differential expression of $p21^{K\text{-}ras}$ function.

In the second part of the study, the proposed research determined the influence of different dietary lipids and LOV on the expression of the rate-limiting enzyme of cholesterol biosynthesis (HMGCAR), $p21^{K\text{-}ras}$, and LDLr expression, as well as expression of specific signal transduction and cell cycle related factors important in downstream signalling responsible for cellular growth (ERK-1, ERK-2, CD1) and apoptosis (caspase-3).

Chapter 2: Literature Review

Anatomy of the Large Intestine

As part of the gastrointestinal system, the large intestine includes the colon and rectum. The proximal end (right side) of the colon leads off of the small intestine and is approximately 90 cm long. The distal section (left side) of the colon is attached to the rectum. The rectum, which is the final 15 cm of the large intestine, leads to the anus. The four sections of the colon include the ascending, transverse, descending and sigmoid sections (Figure 1). The role of the colon is to absorb water from digested food and convert the liquid contents from the small intestine into semi-solid stool. The colon also breaks down substances for reabsorption and reuse by the body. It is inhabited by bacteria that break down the waste material. The rectum functions as a reservoir for stool formed in the colon. Stool is held in the rectum until it passes through the anus in a bowel movement (Colorectal Cancer Association of Canada, 2004).

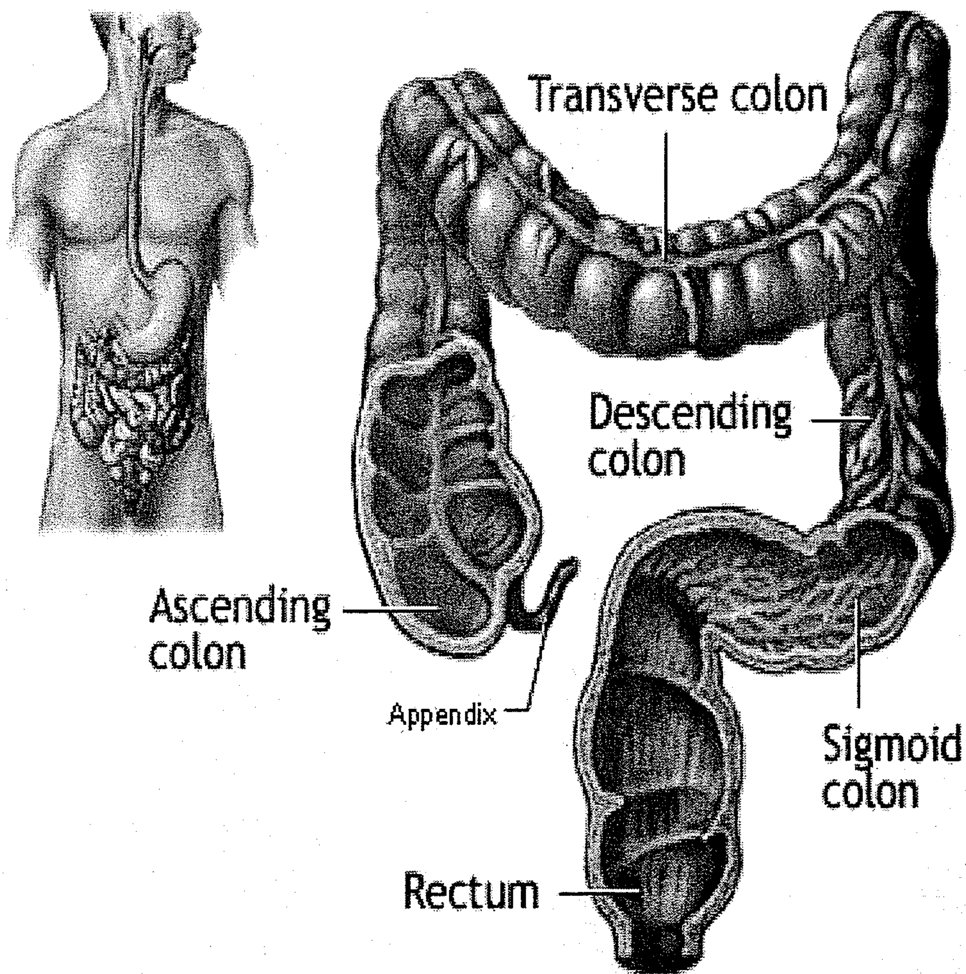
The primary functional cell of the intestinal mucosa is the enterocyte which is produced from a stem cell deep in the colonic crypt, matures, and migrates to the top of the crypt, undergoes apoptosis and is shed into the bowel lumen (Bertagnolli et al, 1997). This process of cell renewal, maturation and death, is tightly regulated and takes approximately 406 days to complete (Lipkin et al, 1993).

Colorectal Cancer

Factors involved in predisposing an individual to colorectal cancer (CRC) development include both genetic predisposition (harbouring a mutation in highly penetrant cancer-predisposition genes leading to increased chance of developing CRC) as well as more

Figure 1. The Four Sections of the Human Colon

(Copyright permission from the Colorectal Cancer Association of Canada, 2004)



subtle genetic alterations that may affect the natural history of tumors, their prognosis, and their response to different treatments. Knowledge of the genetic basis of inherited forms of colon cancer has positively impacted directly on the management of patients as well as symptom-free individuals. Understanding who has an increased risk for developing colorectal cancer (CRC), including the lifestyle and dietary factors involved in promoting and inhibiting cancer growth and development is critical to prevention and treatment of the disease. Understanding and awareness, as well as the stigma associated with early detection procedures, of the disease is low partially accounting for the fact that CRC is one of the most deadly. However, CRC is one of the most preventable cancers. Today, 50-55 out of 100 individuals who are diagnosed with CRC will be alive and healthy in 10 years. For CRC detected early, the cure rate is higher, therefore early detection is the key to survival (Canadian Cancer Society, 2004).

Prevention strategies aimed at identifying the agents promoting and inhibiting neoplastic growth are highly valuable. As colon carcinogenesis is a multifactorial process, multiple factors culminate to increase the likelihood of any step in the series of events that lead to malignancy. Hence, a focus of the thesis research was to investigate those interactions between dietary lipid environment, gene expression and LOV treatment in determining the course of normal and neoplastic colonic cell growth. Superior strategies for prevention and treatment of CRC may result from detailed understanding of the role of cholesterol biosynthesis in normal and neoplastic growth in the context of environments differing in amount and type of fatty acids as well as the effects of inhibition of cholesterol biosynthesis (use of LOV). The possible involvement of lipid-gene interactions must also be investigated.

Epidemiology and Etiology of Colorectal Cancer

CRC is the third leading cause of cancer death worldwide (Dollinger et al, 2002). It is the fourth most common cancer for both men and women and the second leading cause of cancer deaths in Canada. In 2006, approximately 20,000 new cases of CRC were diagnosed, 90% of which were men and women over the age of 50. In 2007, an estimated 20,800 Canadians will be diagnosed with CRC and 8,700 will die of the disease. Approximately 400 Canadians will be diagnosed with CRC every week and 167 Canadians will die of CRC weekly. An almost equal number of women and men are diagnosed annually. According to recent statistics, one in 14 men is expected to develop CRC during their lifetime and one in 28 will die of it. One in 16 women is expected to develop CRC during their lifetime and one in 31 will die of it. In Manitoba, approximately 700 people per year are diagnosed with the disease (Colorectal Cancer Association of Canada, 2007). The average person has a one in six chance of developing CRC over their lifetime. Although the incidence and death rates for CRC have been declining steadily, the number of people affected has continued to increase. Extensive research into the molecular basis and signalling pathways leading to colon carcinogenesis has established effective modes of treatment and prevention of CRC. Some gaps in the research remain especially in the differential effects of saturated and unsaturated fatty acids on expression of genes involved in the molecular process of CRC.

Colon carcinoma is about twice as common as rectal carcinoma (Cooper, 1992). Rectal tumors account for approximately 27%, while 50% of tumors occur proximal to the splenic flexure. Cancer of the proximal colon is more frequent in young females, countries with low risk of colon cancer and in some hereditary neoplastic disorders of the

large bowel (Moller-Jensen, 1983). Cancer of the distal colon is more frequent in older males, in high risk Western countries, and is epidemiologically related to most environmental factors known (Ponz de Leon et al, 1990). Therefore, cancer prevention initiatives employing dietary strategies is worthwhile and an important goal of human nutritional research.

CRC incidence rates show significant geographical variation, suggesting environmental factors play a major role in CRC. Geographical variation in CRC incidence has been linked to diet as it is more common in industrialized countries including the United States, Canada, New Zealand and the United Kingdom (Neal and Hoskin, 1997). Immigrants moving from their country of origin with low risk of colorectal cancer develop the same risk of CRC as individuals in the new country of residence which has higher risk of CRC. An example is Japanese individuals immigrating from Japan to Hawaii (Cooper, 1992).

Epidemiological studies have strengthened the contention that diet plays an important role in the etiology and prevention of certain types of cancer, including colon cancer. Dietary components and are thought to play a major role in the etiology of CRC (Greenberg, 1996). Multiple factors have been linked to increasing risk of colon cancer (Giovannucci et al, 1994; Giovannucci et al, 1995; Colorectal Cancer Association of Canada, 2004). Consumption of a high fat diet, especially saturated animal fat, increases risk for colon cancer (Potter et al, 1993; Giovannucci and Golden, 1997; Yen et al, 2006). A direct relationship has been found between dietary fat intake and development of CRC (Broitman et al, 1993). Case-control studies have demonstrated an association between high fat consumption and increased CRC risk (Potter, 1992). Animal studies suggest that

diets high in saturated and n6 polyunsaturated lipids promote the growth of preneoplastic colonic lesions and increase tumor incidence (Bird et al, 1996). Epidemiological and animal model studies have proven a strong association with development of CRC and a high intake of dietary fat (Rao et al, 2001). In a large U.S study of approximately 90,000 women, colon cancer was nearly twice as frequent among women whose diet contained approximately 44% of calories from fat than among women whose diet contained 30% of calories as fat (Cooper, 1992). Animal model research has lead to the discovery that both amount and type of dietary fatty acids are important in modulating the disease process (Sakaguchi et al, 1986; Willet et al, 1990; Reddy et al, 1991; Rao et al, 2001). High fat diets consisting primarily of n6 PUFA enhance colon tumor development (Deschner et al, 1990; Reddy et al, 1991), whereas n3 PUFA-containing diets reduce colon tumor incidence (Minoura et al, 1988; Deschner et al, 1990; Reddy et al, 1991). Multiple mechanisms have been suggested to explain these effects. Dietary lipids influence plasma membrane FA composition that may modulate the activation of membrane bound receptors and enzymes, affecting intracellular signalling pathways. Specific fatty acids (FAs) posses different biological activities and seem to contribute independently to colon tumor development. This fact must be taken into account when investigating and considering their role, whether promotional or inhibitory, in colon carcinogenesis.

Pathology of Colorectal Caner

Approximately half of the population over 40 years are believed to have polyps protruding from the mucosa of the colon or rectum. Most polyps are benign. However, one type of polyp called an adenomatous polyp may be a precursor to cancer (Gordon and Nivatvongs, 1999). About 90% of CRC are thought to arise from these polyps and a

person with colorectal adenoma has a three-fold risk of developing CRC. The most common of the colonic polyps are the adenomatous polyps which account for between 50 to 66% of all polyps found in the colon and rectum. It is important to identify and remove these polyps as soon as possible as the risk of cells becoming cancerous increases with size and number of polyps. Increased susceptibility to colon cancer is inherited by 10-20% of the population (Cooper, 1992). Approximately 75% of all new CRC cases occur in individuals with no known risk factors for the disease, other than being 50 or older (Colorectal Cancer Association of Canada, 2004).

Rectal and rectosigmoid cancers account for approximately 33% of large intestinal tumors. Approximately 25% are located in the sigmoid colon, 10% in the caecum, and 32% evenly distributed along the colon (Neal and Hoskin, 1997). Most CRC are adenocarcinomas which are tumors that develop in the glands of the inner lining of the mucosa (Dollinger et al, 2002) and are usually well differentiated. Glandular adenocarcinomas represent approximately 85%, mucinous tumors 15%, and signet ring approximately 2% (Neal and Hoskin, 1997). Tumor growth is both longitudinal and circumferential along the mucosa, sometimes causing obstruction of the bowel lumen, and infiltrates the layers penetrating into the muscular wall of the bowel and serosa. This causes infiltration of the surrounding abdominal and pelvic viscera. Submucosal spread in the lamina propria may cause skip lesions away from the primary tumor. Regional (mesenteric) lymph node involvement may occur and risk of lymph node metastases increases with the depth of bowel invasion. Tumor metastases may occur to the liver via the portal circulation, and from there to the lungs, bone, brain and skin.

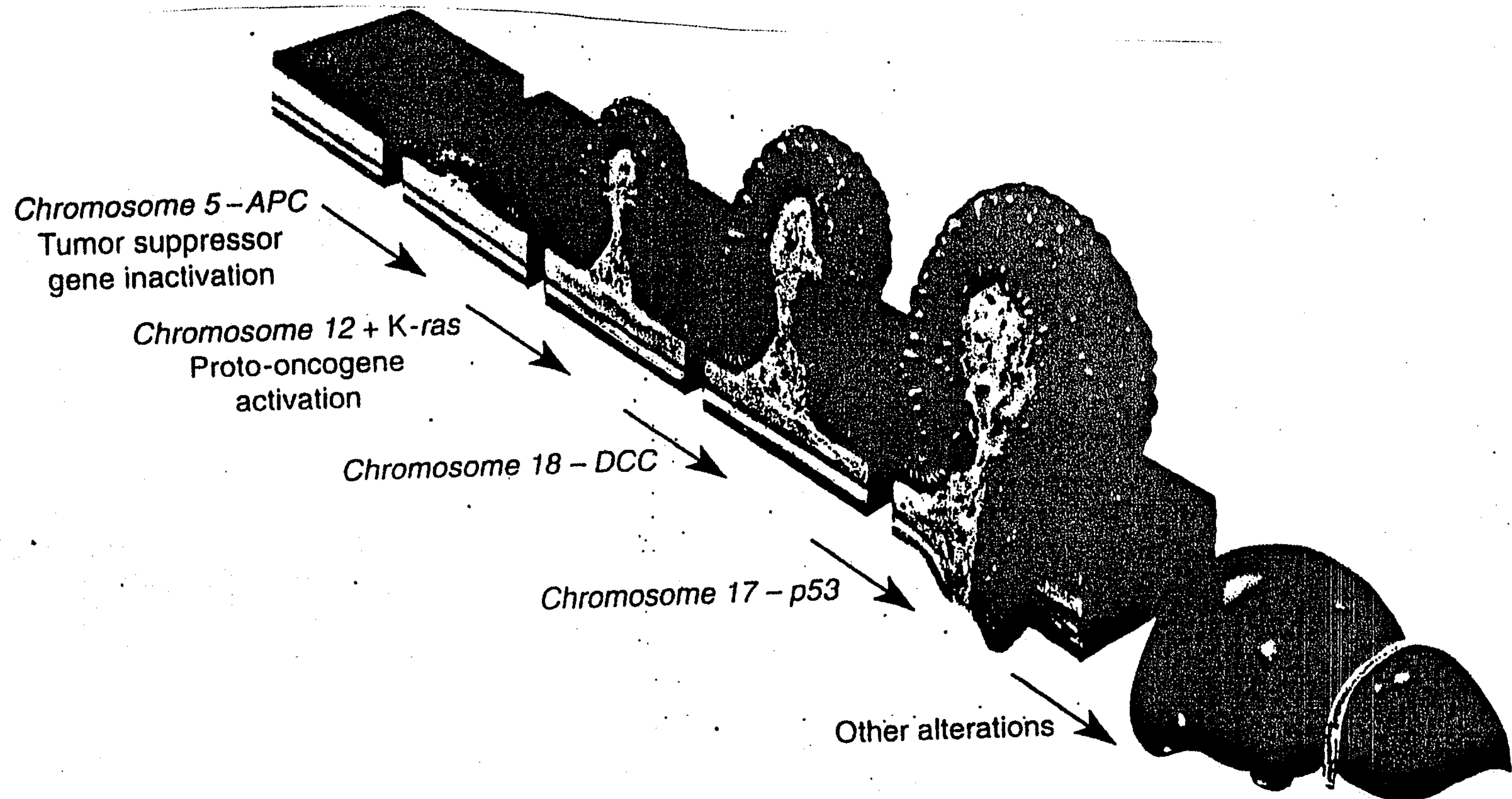
The Multi-Step Process of Colorectal Carcinogenesis

Colon and rectal carcinomas are well characterized with respect to the roles of both oncogenes and tumor-suppressor genes in tumorigenesis. Normally functioning proto-oncogenes control many signalling pathways that regulate cell renewal in adult tissues, differentiation, and programmed cell death (apoptosis) (Jothy et al, 1996; Haber and Fearon, 1998). Mutations in proto-oncogenes (point mutation, chromosomal translocation, gene amplification) occur in cancer cells and may lead to deregulated cell growth. These mutations are sometimes referred to as gain of function mutations as they lead to increased or novel function. Genetic factors involved in CRC include mutation of the K-ras gene, which is found in 50% of all people with colorectal adenomas and cancers (BArbacid, 1987; Goodsell, 1999; Dollinger et al, 2002). In most cases, the tumor suppressor gene p53 (chromosome17) is inactivated. Once a polyp has one of these chromosomal changes, other "hits" to the cell may cause cancer to develop.

Carcinogenesis in the gastrointestinal epithelium is the result of successive accumulation of multiple genetic mutations, producing a transformed phenotype and progression of the enterocyte to invasive cancer. Each mutation confers a selective growth advantage to a particular cell, allowing a clone of cells to overgrow the others, ultimately leading to carcinoma derived from that clone (Fearon and Vogelstein, 1990; Farber and Rubin, 1991; Harris, 1991; Fearon and Jones, 1992; Kumar et al, 1993; Bird, 1995).

The genetic alterations involved, including mutational activation of oncogenes coupled with mutation or loss of tumor suppressor genes, in CRC was described by Vogelstein and colleagues (1988) who defined their relationship to the adenoma-carcinoma sequence (Figure 2). The multi-step process of colon carcinogenesis is accepted as the basis for

Figure 2. **A Molecular Model for the Evolution of Colorectal Cancer through the Adenoma-Carcinoma Sequence** (Copyright permission provided from Elsevier Limited; License Number 1812560113983)



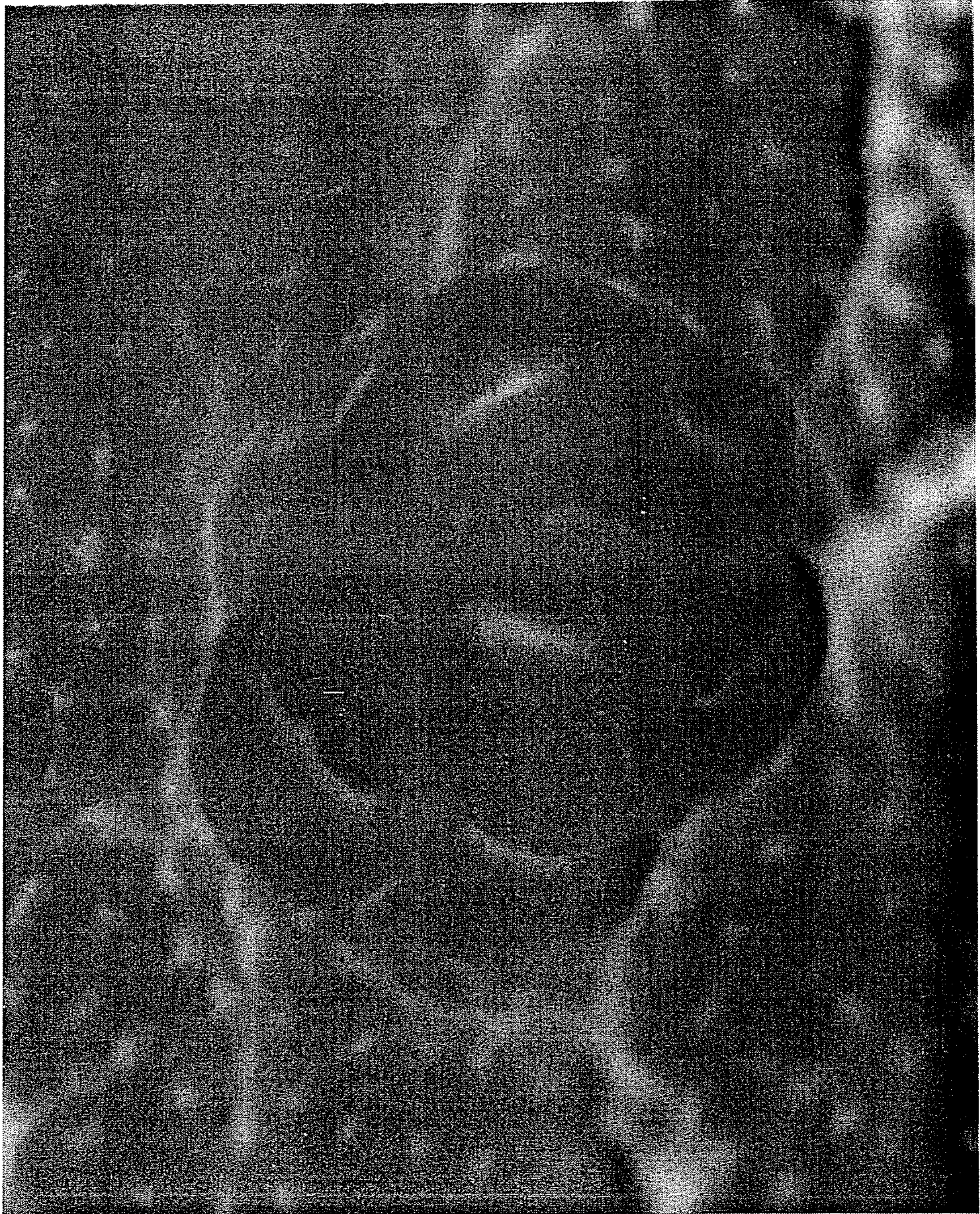
malignant transformation and provides a genetic perspective of tumor initiation, promotion and progression.

Colorectal carcinogenesis begins in the epithelial lining (mucosa) of the colon and rectum due to genetic mutation leading to progressive loss of the normal balance and controls of proliferation, maturation and apoptosis of colonic enterocytes. Loss of proliferative control results in the inability of the enterocyte to repress DNA synthesis during migration from the base to the surface of the crypt (Anti et al, 1993). Normally confined to the lower three-quarters of the colonic crypt, the proliferative zone expands throughout the entire length of the crypt. Prior to the formation of the neoplastic polyp, there is an increased proliferation of preneoplastic cells, called aberrant crypt foci (ACF; Figure 3), within the colonic epithelium of rats (Bird, 1995; Bird and Good, 2000) and humans (Pretlow et al, 1992). Through clonal selection, one cell from this hyperproliferative population gives rise to a small benign neoplasm referred to as a small adenoma or polyp. Further rounds of clonal selection result in the formation of adenomas of increasing size and growth potential. Malignant carcinomas arise from the benign adenomas.

Aberrant Crypt Foci (ACF)

Bird (1987) was the first to identify ACF by microscopic examination of methylene blue stained carcinogen treated rodent colons. The ACF system is used to enumerate preadenomatous stages in the entire colon by a simple method (Bird, 1995) and are purported to be preneoplastic lesions (Bird and Good, 2000). Enumeration of ACF is used as a biomarker in the identification of individuals with increased susceptibility to CRC (Roncucci et al, 1991; Pretlow et al, 1993) as well as serving as valuable end points in assessing the risk of colon cancer incidence and as biomarkers in the identification of

Figure 3. Aberrant Crypt Foci, ACF



cancer modulators and chemopreventive agents (Bird et al, 1985; Bird, 1987; McLellan and Bird, 1988a and 1988b; Tudek et al, 1989; Bruce, 1990; Pretlow et al, 1990; Bird, 1991; Caderni et al, 1991; Lam and Zhang, 1991; McLellan and Bird, 1991a and 1991b; McLellan et al, 1991; Pretlow et al, 1991; Takahashi et al, 1991; Archer et al, 1992; Bird and Pretlow, 1992; Kendell et al, 1992; Pretlow et al, 1992a and 1992b; Rao et al, 1992; Roncucci, 1992; Shivapurkar et al, 1992; Singh et al, 1992; Stopera and Bird, 1992; Stopera et al, 1992a and 1992b; Wargovich et al, 1992; Bruce et al, 1993; Magnuson and Bird, 1993a and 1993b; Pretlow et al, 1993; Rao et al, 1993a and 1993b; Shirtliff et al, 1993; Stopera and Bird, 1993; Takahashi et al, 1993; Vivona et al, 1993; Kawamori et al, 1994; Lafave et al, 1994; Magnuson and Bird, 1994; Pereira et al, 1994; Smith et al, 1994; Bird, 1995; Lasko and Bird, 1995; Bird and Good, 2000; Wargovich et al, 2000; Kiunga et al, 2004; Kiunga, 2006; Reddy, 2004; Reddy et al, 2005; Raju and Bird, 2007). In animal models, genetic mutation and abnormal cell proliferation (ACF) are initiated using AOM injection (Chapter 3). Tumor promoting (high fat diets) and inhibiting (low fat diet, and LOV) agents are used to analyze the different types of proliferating tumor cell populations as well as to determine whether interactions among dietary lipid treatment and gene expression and/or mutations exist. Some mutations, as well as environmental agents (different fatty acids and/or LOV treatment) may have deleterious effects on certain cells leading to inhibition of growth. Eventually one change will result in more rapid cell growth and that cell will gain a selective growth advantage (clonal selection) (Fearon and Vogelstein, 1990). These cells have increased proliferative potential and take over the tumor cell population (Cooper, 1992). The clonal selection process is repeated many times during tumor progression, resulting in the formation of

more and more rapidly growing tumor cells. Genetic changes in ACF have been detected, including gene mutations and amplification. Elevated transcripts of K-ras mRNA have been demonstrated (Stopera et al, 1992a and 1992b; Sugimura et al, 1992; Stopera and Bird, 1993). The presence of p53 mutated proteins in select ACF and rat colon tumors suggests that these changes may occur very early on in selected ACF. It has been demonstrated that both human and rat ACF harbour K-ras mutations and APC mutations (Pretlow et al, 1993; Smith et al, 1994). One study (Jen et al, 1994) found K-ras mutations in 19 out of 20 ACF examined. Evidence suggests that the earliest detectable genetic changes occurring during colon carcinogenesis are also present in ACF, hence supporting the belief that ACF are preneoplastic lesions of colon cancer.

Interest in the ACF system has intensified after the observation that these lesions are present in human colons and are associated with high risk of developing colon cancer (Roncucci, 1992; Pretlow et al, 1993). Investigation of the multi-step process of colon carcinogenesis is aided by enumerating the number and growth features of ACF. Research has demonstrated that crypt multiplicity, a growth feature of ACF, can be used to predict the disease outcome as well as determine the preneoplastic state of lesions, allowing for more detailed analysis of the multi-step process of colon carcinogenesis. Biological and genetic differences exist among ACF exhibiting different crypt multiplicity. In addition, ACF with different crypt multiplicity are regulated differently and transition of microadenomas to adenomas and carcinomas can be affected by dietary and environmental manipulation. Once ACF are formed, only selected ACF proceed through the multi-step process leading to formation of microadenomas (MA). Only a select number of MA will develop into adenomas, some of which subsequently develop

into adenocarcinomas (Bird, 1995). In this light, the ACF system can be used to explore the events critical to and causally related to the genesis of CRC. Known promoters and inhibitors of colon carcinogenesis enhance, or inhibit, the number and growth features of ACF (Bruce, 1990; Lam and Zhang, 1991; McLellan and Bird, 1991; Pereira and Khoury, 1991; Archer et al, 1992; Bruce et al, 1993; Pereira et al, 1994). It is believed that the tumor modulating effects of various agents are achieved by different mechanisms. It seems likely that precursor lesions adapt to a changing environment.

Animal Models and the ACF System

Studies using animal models allow a systematic evaluation of the growth, morphologic, cellular and molecular features of ACF. In order to determine differences in growth regulation in primal and advanced preneoplastic lesions, it is important to allow preneoplastic lesions to develop for several weeks post carcinogen (Azoxymethane; AOM) injection, before any dietary or chemotherapeutic agent intervention. ACF are quantified before and after intervention and, microadenoma and tumor outcome are assessed. Enumeration of the population of ACF with various growth features (crypt multiplicities) allows speculation on the transition of lesions from one stage to the next. An intervention duration of four weeks sufficiently allows advanced ACF to progress to tumors, and for primal ACF to progress into intermediate or advanced ACF. If significant alterations in the tumor outcome occur, it is likely the test agent was affecting mainly the number and growth features of advanced ACF to form tumors (Bird and Good, 2000).

The use of animal models in CRC provides a bridge between descriptive epidemiological studies and mechanistic studies carried out in biologically homogeneous populations of

cells. Inducing colon carcinogenesis in rodents using Azoxymethane (AOM) is simple and provides a reproducible and effective model in which to study the multi-step process of colon carcinogenesis as well as allowing investigation into the interaction between tumor development and exogenous factors. AOM is one of the two most commonly used carcinogenic chemicals inducing colonic neoplasms in rodents (Fiala, 1977; Bird, 1987; Greene et al, 1987; Potter, 1999). Alkylation of AOM occurs in the liver and the activated carcinogen reaches the colon by the blood, producing the first "hit" in the genetic material of the stem cell (Potter, 1999). Carcinogen administration is either intraperitoneally (i.p.) or subcutaneously (s.c.) and usually at a concentration of 15mg/kg body weight in order to induce similar tumor incidence in the distal colon as seen in humans (Greene, 1987). The standard injection protocol is a total of two to three s.c. injections of AOM (15mg/kg body weight/week) given to male, weanling rats. Aberrant crypts appear within two weeks after carcinogen injection (Bird and Good, 2000).

The AOM induced colon carcinogenesis rodent model is a useful investigative tool as it mimics the adenoma-carcinoma sequence observed in humans, including the early event of ras mutation. Identification of the first K-ras point mutation in ACF was identified by Stopera et al (1992a). The mutation was in K-ras on codon 12, leading to a G to A transition and substitution of the amino acid aspartic acid (asp) for glycine (gly) and was detected in 32% of ACF examined (Stopera et al, 1992b). This mutation was also found in adenocarcinoma tissue but was not present in normal colonic epithelium. In one study, the frequency of K-ras mutations were followed during the ACF-adenoma-carcinoma sequence in F344 rats and 2 out of 27 ACF were found to have K-ras mutations; one ACF with a crypt multiplicity of five and the other with crypt multiplicity of 10. In addition,

one adenoma out of 23 and 10 adenocarcinomas out of 27 had K-ras mutations (Vivona et al, 1993). These findings may suggest that K-ras mutations are not always an early event in the development of some ACFs and occur at higher frequency in colonic tumors.

Dietary Lipids and ACF Development

Fatty acid (FA) chain length in food and body tissues ranges from 4 to about 24 carbon atoms. They may be saturated, monounsaturated, or polyunsaturated (Table 1). Both the number and location of the fatty acid double bonds are of interest to researchers and demonstrate different effects on human physiology and disease progression. FAs having a chain length of 14 or more carbon atoms are most important nutritionally and functionally. Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2n6) together account for more than 90% of the average US diet. Much of the current research being conducted in CRC prevention focuses on the n3 fatty acids, usually comparing them with n6 PUFAs (Field et al, 1987; El-Sohemy and Archer, 1999; Chen and Istfan, 2001; Roynette et al, 2004). This has left a gap in the research surrounding the effects of saturated fat, especially at the molecular level, on the disease processes contributing to CRC.

In the ACF model, a high fat diet promotes ACF growth at an accelerated rate allowing them to attain an autonomous state of growth within a few weeks of feeding (Bird et al, 1996). This is in contrast to a low fat diet in which ACF require a considerably longer duration of time to achieve this growth state (Reddy, 1992; Bird et al, 1996; Good et al, 1998; Lasko et al, 1999). Animals fed a low fat diet usually have fewer ACF and tumors compared to those fed a high fat diet. In animal models, a high fat beef tallow (HFB) diet provides a more stable growth environment for ACF with advanced features than LFB

Table 1. Names and Chemical Structures of Common Saturated and Unsaturated Fatty Acids

Notation	Common Name	Formula
Saturated Fatty Acids		
14:0	Myristic acid	CH_3-$(CH_2)_{12}$-COOH
16:0	Palmitic acid	CH_3-$(CH_2)_{14}$-COOH
18:0	Stearic acid	CH_3-$(CH_2)_{16}$-COOH
Unsaturated Fatty Acids		
18:2n6	Linoleic acid	CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH
18:3n3	Linolenic acid	CH_3-$(CH_2$-CH=CH$)_3$-$(CH_2)_7$-COOH
20:4n6 (AA)	Arachidonic acid	CH_3-$(CH_2)_3$-$(CH_2$-CH=CH$)_4$-$(CH_2)_3$-COOH
20:5n3 (EPA)	Eicosapentaenoic acid	CH_3-$(CH_2$-CH=CH$)_5$-$(CH_2)_3$-COOH
22:6n3 (DHA)	Docosahexaenoic acid	CH_3-$(CH_2$-CH=CH$)_6$-$(CH_2)_2$-COOH

diet (Bird, 1995). An accelerated transition of ACF towards advanced stages at earlier time points was reflected in the early appearance of tumors and larger tumors in the HBT diet group than in the LBT group. Once preneoplastic lesions attain a certain growth state, they exhibit growth autonomy and become increasingly more resistant to growth modulatory effects (Bird et al, 1996). Due to the demonstrable differences in dietary lipid effects on ACF and eventual tumor incidence, it is important that research take into consideration the possibility of interactions between dietary lipids and any chemopreventive agent being tested.

Some dietary fatty acids (saturated fatty acids and n-6 unsaturated fatty acids) and total amounts (high fat diets) are considered promoting agents for CRC whereas others (n-3 fatty acids and low fat diets) are considered inhibitory for CRC. Bird demonstrated that fatty acids, differing in their degree of saturation, exerted colonic tumor modulating effects by different mechanisms (Bird, 1998; Bird and Good, 2000). Most importantly, it has been shown that the effect of an HFB diet differs from the effect of a high corn (HFC) oil diet, even though both diets enhance colon carcinogenesis. These lipids affect different stages of carcinogenesis leading to the same tumor outcome. Findings also suggest that the nutrient requirements of ACF may differ depending on their stage of development (primal versus advanced). The HFB diet exerts a growth enhancing effect on the primal or intermediate ACF. The HFC diet does not influence the conversion of ACF from primal to intermediate or advanced stages. The HFC diet accelerates a higher proportion of advanced ACF to develop into tumors (Robblee and Bird, 1994; Bird, and Good, 2000).

In studies using animal models, differences in experimental design, carcinogen treatment, diet composition, length of dietary intervention, and timing of experimental diet introduction (prior to, during, or immediately following carcinogen administration), make comparison of results challenging. Dietary nutrients may interfere with carcinogen metabolism and effectiveness, resulting in decreased tumor incidence and number of initiated cells. This effect could be mistakenly interpreted as inhibitory due to the influence of dietary intervention (Bird and Good, 2000).

Molecular Mechanisms Involved in Signal Transduction and Cell Growth in Colorectal Cancer

Cells communicate with one another through an elaborate mechanism of signal transduction involving extracellular protein messengers that bind to cell surface receptors and transmit the message from the cell surface to the nucleus resulting in a change in gene expression in response to the particular message. Alteration of the proteins involved in signal transduction pathway(s), including mutations and/or over-expression of the genes responsible for their expression, may lead to inappropriate messages delivered to cells and a disruption of normal growth regulatory signals. In this light, a cell may receive a message to continue proliferating which leads to neoplastic growth and cancer formation. Oncogenes are genetically altered proto-oncogenes of signal transduction proteins that lead to cell transformation (Goodsell, 1999). The ras signal transduction pathway is critical to control of cell growth and proliferation (Bos, 1989; Campbell et al, 1998). Ras is situated at the center of a complex cascade (Figure 4) of molecular interactions and has been recognized as a major etiological factor in the initiation and development of several human tumors, including colon cancer (Spandidos et al, 2002).

Figure 4. The Ras Signalling Cascade
(Copyright permission provided from Elsevier Limited; License Number 1812590167830)

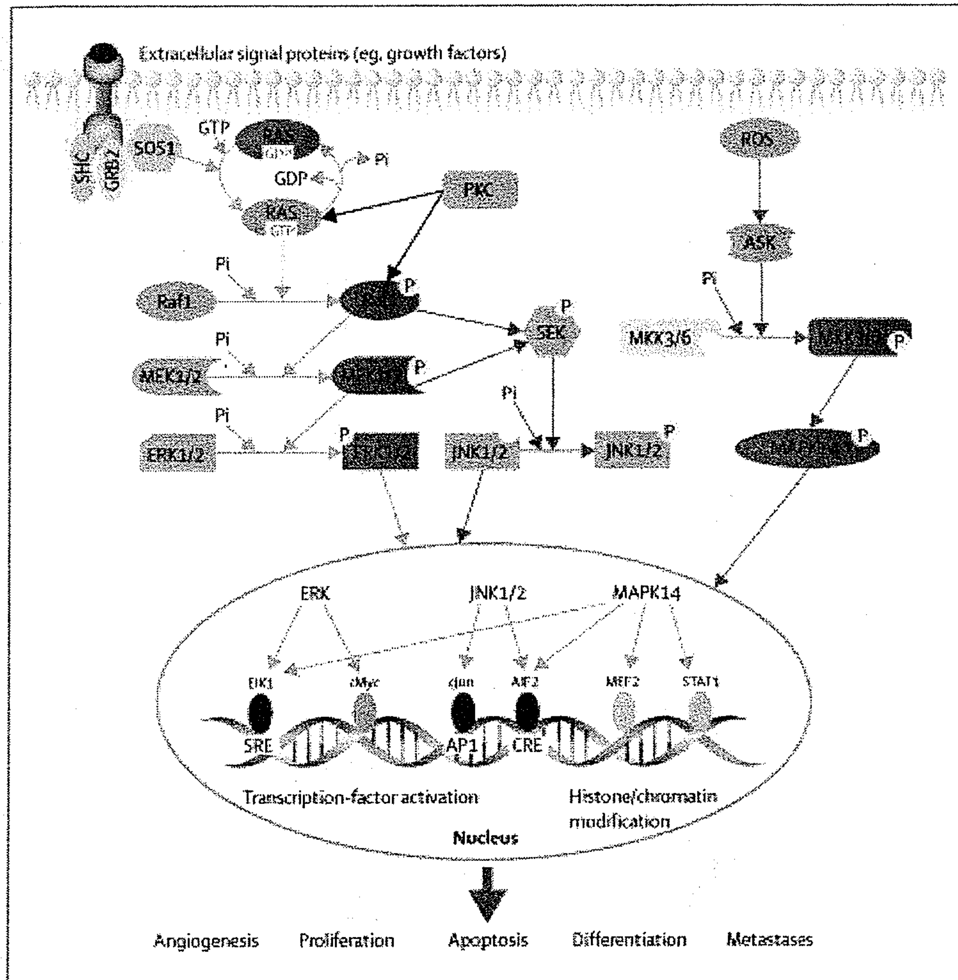


Figure 4: Inter-related signal transduction cascades activated by various stimuli (such as growth factors, stress, and inflammation). Downstream effectors of the Ras pathway include activation of cMyc and cJun to form AP1 (activator protein 1) for gene activation. AIF2=apoptosis inducing factor. ASK=apoptosis signal-regulating kinase. CRE=cyclic AMP/calcium response element. ELK=mixed lineage kinase. MKK=MAPK kinase. MEF2=myocyte enhancer factor 2. PKC=protein kinase C. ROS=reactive oxygen species. SOS=son of sevenless. STAT1=signal transducer and activator of transcription factor 1.

A majority of the proteins involved in this signalling cascade are regulated (activated) via phosphorylation.

The p21$^{K\text{-}ras}$ Proto-Oncogene in Colorectal Cancer

Three types of ras genes (K-ras, N-ras and H-ras) all encode 21-kDa proteins (p21s). In most tissues, all the ras genes are expressed, however, the levels of expression of each type can vary widely. Ras proteins transmit signals from cell surface receptors along several different pathways, ultimately effecting mitogenic functions such as growth and differentiation, DNA synthesis, apoptosis, lipid metabolism, and cytoskeletal organization (Fang and Richardson, 2005). Ras proteins bind guanine nucleotides and have intrinsic GTPase activity (McLeod and Murray, 1999). The K-Ras2 gene is found on chromosome 12p12 and produces a 2 kb transcript encoding the p21$^{K\text{-}ras}$ protein. p21$^{K\text{-}ras}$ proteins must undergo a series of post-translational modifications that facilitate their association with the plasma membrane from which they relay growth and differentiation signals to the cell interior (Howe and Guillem, 1997) as well as functioning in cell cycle progression (McLeod and Murray, 1999). The earliest step in p21$^{K\text{-}ras}$ maturation involves the transfer of the farnesyl group from farnesyl pyrophosphate (FPP), a cholesterol biosynthetic pathway intermediate, to p21$^{K\text{-}ras}$ via the enzyme farnesyltransferase (FPTase) (Hancock et al, 1989). The attachment site on the p21$^{K\text{-}ras}$ protein for farnesyl is called the CAAX box and consists of a sequence of four amino acids: a cysteine followed by two aliphatic amino acids, followed by any one of several different amino acids. This process is referred to as farnesylation and is a target for drugs and strategies aimed at blocking/inhibiting ras activity. Once attached, farnesyl acts as a "hook" allowing ras to attach to the inner plasma membrane (Travis, 1993).

The human K-ras gene is one of those critical components of cell-signalling pathways frequently activated by point mutation in CRC. The majority of ras mutations are located on codon 12 (Fearon and Vogelstein, 1990), with additional mutations on codons 13 and 61. Point mutations in the K-ras gene occur in approximately 50% of CRC and 58% of adenomas greater that 1 cm in size. Only 9% of adenomas less than 1 cm have been found to have K-ras mutations. K-ras gene mutations are considered to be relatively early events in colon tumor development, however not the earliest due to findings that small adenomas infrequently manifest these mutations (Vogelstein et al, 1988). Point mutations in ras proto-oncogenes convert them into oncogenes and result in continuous stimulation of cell proliferation (Kiaris and Spandidos, 1995; Fung et al, 1997). Mutations are usually limited to only one of the ras family members and the frequency has been shown to be tissue- and tumor type-dependent (Bos, 1989). Mutations in these codons confer a proliferative advantage in the cells bearing these mutations and therefore, they are selected within the cell population (Spandidos et al, 2002). K-ras gene mutation may be useful as a prognostic indicator of CRC (Pajkos et al, 1999). The presence of ras mutations is associated with disease invasiveness (Web et al, 1998), independent of pathological stage, but is not of prognostic significance within a given stage of disease. One study found that ras mutations were associated with tumor progression and poor survival in individuals with descending colon tumors, but not in those with ascending tumors (Elnatan et al, 1996). Positive staining for p21 may be associated with poor prognosis and/or an independent prognostic factor in colon tumorigenesis (McLeod and Murray, 1999).

Some genetic evidence exists supporting the anti-oncogenic properties of ras genes in human tumors. Studies carried out in human carcinomas suggest ras genes may act as tumor suppressor genes and that increased expression of the wild-type alleles may function in favour of reducing the transforming potential rather than promoting the malignancy (Spandidos et al, 2002). The wild-type ras allele has also been proposed to inhibit cell proliferation by promoting differentiation (Pfeifer, 2001). Bissonnette et al (2000) has demonstrated a sub-population of colonic adenomas over-expressing wild-type $p21^{K\text{-}ras}$ that did not have increased expression of CD1 which is typical of colonic tumors. Conversely, over-expression of wild-type $p21^{K\text{-}ras}$ without somatic mutational activation can induce formation of malignant tumors. *In vitro* studies have demonstrated the existence of wild-type $p21^{K\text{-}ras}$ that has been activated by epigenetic events (Bissonnette et al, 2000). A subset (21%) of AOM-induced tumors in F344 rats has shown constitutively activated wild-type $p21^{K\text{-}ras}$. Wild-type $p21^{K\text{-}ras}$ was constitutively activated in both adenomas and adenocarcinomas which led to Bissonnette's speculation that constitutive activation of wild-type $p21^{K\text{-}ras}$ occurs early in colonic malignant transformation.

Subcellular Localization of $p21^{K\text{-}ras}$

Synthesis of the ras protein occurs in the cytoplasm on free ribosomes to produce pro-$p21^{K\text{-}ras}$ with a half-life of at least 24 hours. Post-translational modification of Pro-$p21^{K\text{-}ras}$ increases its hydrophobicity and results in its association with the inner face of the plasma membrane. Activated ras genes lose their transforming activity when mutations are introduced that render the protein cytosolic. C-terminus sequences are essential for membrane association and conserved Cys186 is required to initiate post-

translational modifications of pro-p21$^{K\text{-}ras}$ (Lowy and Willumsen, 1993). Cys186 is first modified by the isoprenoid farnesyl pyrophosphate. The three amino acids C-terminal to Cys186 are proteolytically cleaved and the carboxy-terminal group of Cys186 is methylated, producing a more hydrophobic protein with higher affinity for membranes. In the final modification, cysteine residues upstream of the farnesylated cysteine become reversibly palmitylated which further increases membrane affinity and biological activity (Hancock et al, 1990). Residues 30-40 of ras are highly conserved and specify the area for the interacting surface for effector molecules (Lowy and Willumsen, 1993). The catalytic domain of ras is considered to be the conserved 164 N-terminal amino acids (Gideon et al, 1992). It is possible to interfere with the farnesylation of ras by limiting the intracellular availability of farnesyl. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCAR) prevent the synthesis of mevalonate, a precursor in farnesyl synthesis, which leads to accumulation of non-processed, cytosolic p21$^{K\text{-}ras}$ (Rine and Kim, 1990).

The biological activity of ras is also determined by the proportion of bound nucleotides GTP and GDP. The normal functioning ras protein has GTPase activity that functions to limit the lifetime of the GTP bound ras form. The concentration of bound GTP-ras form in the cell is regulated by catalyzing the binding or hydrolysis of GTP, including GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GNEF) (Lowy and Willumsen, 1993; Goodsell, 1999). Compared with normal ras, a higher proportion of mutationally activated ras exists in the cell as the GTP complexed form.

Mutationally activated ras is associated with amino acid alterations related to guanine nucleotide association. Based on the mutation location, decreased GTPase activity and/or

increased nucleotide exchange rate results in abnormally high proportions of GTP bound ras. Substitution of the normal Glycine 12 (Seeburg et al, 1984) or Glutamine 61 (Der et al, 1986) by a variety of amino acids results in reduced intrinsic GTPase activity of ras, renders its GTPase resistant to acceleration by GAP and makes the protein transforming (Lowy and Willumsen, 1993). When mutated, there is a reduction in the ability of $p21^{K\text{-}ras}$ protein to interact with ras-GTPase activating proteins (ras-GAP), allowing it to remain in the GTP-bound active state and leading to alterations in signal transduction (Goodsell, 1999). The half-life rate of hydrolysis in for normal GTP bound ras has been measured to be 1-5 hours, with that of activated ras being 3-9 times longer (John et al, 1988).

In normal tissues, $p21^{K\text{-}ras}$ activation is regulated by guanine nucleotide binding. In its "off" state, $p21^{K\text{-}ras}$ is bound to GDP. Guanine nucleotide exchange factors (GNEFs), such as Son of Sevenless (SOS), initiate a message to $p21^{K\text{-}ras}$ by expelling GDP, allowing GTP to bind in its place (Goodsell, 1999). GTP binding causes a conformational change in the ras protein triggering a cascade of mitogen-activated protein (MAP) kinases leading to phosphorylation of the target (such as a transcription factor) and delivery of the message to its final destination. In normal cells, this signal is self-limiting and the amount of active ras within the cell is tightly linked to the amount of bound receptor at the cell surface. This is attained by ras hydrolyzation of GTP to GDP via intrinsic ras GTPase activity and a number of GTP-ase activating proteins (GAPs) which speed up the process. GAPs bind to the effector domain of the ras protein (Bos, 1989). In the case of cells containing oncogenic ras, the protein is insensitive to the

regulation of guanine nucleotide binding by GAPs and GNEFs and is constantly activated, resulting in an uncontrolled continuous stimulation of cell proliferation.

GAP is a cytoplasmic factor accelerating the intrinsic GTPase activity of normal ras by up to five orders of magnitude (Gideon et al, 1992). The relative biological activity of mutant ras proteins correlates better with their resistance to down-regulation by GAP than with their relative intrinsic GTPase activity. GAP binds preferentially to GTP bound ras and all normal ras proteins appear to be sensitive to GTPase acceleration by GAP (Lowy and Willumsen, 1993). Both GAPs and GNEF can regulate activation of wild-type $p21^{K\text{-}ras}$, whereas they do not influence mutated $p21^{K\text{-}ras}$ due to its constitutively activated state (Maruta et al, 1994). GAP expression is inversely proportional to levels of wild-type $p21^{K\text{-}ras}$ activation (Bissonnette et al, 2000). The alteration in GAP expression is suspected to be partly responsible for the activation of wild-type $p21^{K\text{-}ras}$ in a subset of colonic adenocarcinomas. Whether dietary lipid composition affects GAP expression and activity is unknown.

$p21^{K\text{-}ras}$ Signalling

In healthy tissue, $p21^{K\text{-}ras}$ protein is involved in regulating the normal cellular functions of proliferation, differentiation, and apoptosis (Chen et al, 1998). One critical function of $p21^{K\text{-}ras}$ is in growth signal transduction involving activation by growth factor receptors binding their ligand and subsequent activation of $p21^{K\text{-}ras}$ activity. Non-receptor protein tyrosine kinases are also involved in transduction of extracellular signals in this pathway (Lowy and Willumsen, 1993). Growth factor stimulation of GTP bound $p21^{K\text{-}ras}$ and its increased activity suggests $p21^{K\text{-}ras}$ functions early in the cell cycle. In addition, a requirement for $p21^{K\text{-}ras}$ in G_0 to G_1 transition in the cell cycle has been demonstrated as

well as being necessary for the transition from G_2 to mitosis (Durkin and Whitfield, 1987; Dobrowolski et al, 1994).

Activation of several serine/threonine kinases implicated in mitogenesis have been shown to depend on $p21^{K\text{-}ras}$, including mitogen-activated protein (MAP) kinases (also known as extracellular regulated kinases; ERK-1 and ERK-2) and the kinase that activates the MAP kinase (MAP kinase kinase) (Lowy and Willumsen, 1993). Oncogenic $p21^{K\text{-}ras}$ induces the activity of these kinases (Wood et al, 1992) as well as the Rac/Rho pathways (Khosravi-Far et al, 1995). Activation of the ERK pathway results in up-regulation of transcription factors promoting tumorigenic transformation (Oldham et al, 1996).

Ras mediated signals inhibiting apoptotic cell death act through the PI3-kinase and AKT pathway (Fang and Richardson, 2005). AKT has also been shown to have an anti-apoptotic effect through direct inactivation of caspases-9 and -3 by direct phosphorylation (Zhou et al, 2000). A ras-mediated model in which ras functions as a sensor of caspase activity to determine whether or not a cell should survive has been reported (Yang and Widman, 2001). When caspases are mildly activated, the partial cleavage of Ras-GAP protects cells from apoptosis. When caspase activity reaches levels allowing completion of Ras-GAP cleavage, the resulting Ras-GAP fragments turn into potent pro-apoptotic molecules (Spandidos et al, 2002).

In a unique study, Bissonnette et al (2000) characterized two fundamentally different types of colonic tumors based on ras mutation or activation of wild-type $p21^{K\text{-}ras}$, expression of the ERK cascade, and CD1 in colonic tumors of F344 rats. His research demonstrated significantly higher $p21^{K\text{-}ras}$ activation levels in colonic tumors harbouring $p21^{K\text{-}ras}$ mutations as well as a subset of tumors (18 out of 70) with wild-type $p21^{K\text{-}ras}$

with significantly higher $p21^{K\text{-}ras}$ activation levels. His research also demonstrated increased expression of MAPK in both types of tumors (mutated and activated wild-type $p21^{K\text{-}ras}$). Tumors with mutated $p21^{K\text{-}ras}$ had significantly increased CD1 expression, whereas those tumors with activated wild-type $p21^{K\text{-}ras}$ did not. Tumors with activated wild-type $p21^{K\text{-}ras}$ demonstrated decreased Ras-GAP.

Researchers have speculated that alterations in growth factors and/or their receptors, which regulate GAP and GNEF activity, may be involved in activation of wild-type $p21^{K\text{-}ras}$. Over-expression of growth factor receptors has been implicated in $p21^{K\text{-}ras}$ activation (Bissonnette et al, 2000). In addition, increased expression of IGF-1R in colonic tumors has been found (Kiunga, 2006). Therefore, both decreased GAP expression combined with increased expression of growth factor receptors may be responsible for increased $p21^{K\text{-}ras}$ activation.

Evidence is mounting regarding the theory that different dietary lipids regulate the expression of specific genes (Singh et al, 1997; El-Sohemy and Archer, 1999; Hofmanova et al, 2005). However, the underlying mechanisms are not fully understood. Dietary lipids may alter gene expression by modulating plasma membrane composition, membrane receptor activity, and cell signalling intermediates. One promising line of investigation is that of down-stream affects on $p21^{K\text{-}ras}$, ERK-1 and ERK-2, and CD1 that induce nuclear transcription factors, including the proto-oncogenes c-fos, c-myc, and c-jun (Sellmayer et al, 1997).

Extracellular Signal Regulated Kinases: ERK-1 and ERK-2

The "classical" MAP kinase cascade consisting of the Ras/Raf-1/MEK/MAP kinase pathway in which MAP kinase activation provides the link between cytoplasmic and

nuclear signalling events is considered to be pivitol in differentiation, carcinogenesis and tumor progression. ERK-1 (p44) and ERK-2 (p42) belong to a group of protein-serine/threonine kinases that are activated in response to various stimuli including growth factors and differentiating agents (Milanini et al, 1998; Maemura et al, 1999; Chen and Davis, 2003). Growth factors bind cell surface receptors that have intracellular tyrosine kinase activity, causing activation of a complex intracellular signal transduction cascade (Figure 4) (Dudley and Saltiel, 2000; Cassano et al, 2002). The magnitude of ERK signalling affects the cellular response (Vial and Marshall, 2003). Constitutive activation of these pathways leads to cellular transformation and possible neoplasm and cancer. CRC is associated with MAP kinase pathway dysregulation involving altered expression of EGFR (Good, 1999), IGF-1R (Kiunga, 2006), and $p21^{K\text{-}ras}$ mutation (24-61%) in tumors (Woodburn, 1999; Porebska et al, 2000).

Activation of ERK-1 and ERK-2 leads to activation of genes involved in the regulation of cellular proliferation, differentiation, and malignant transformation (Wasylyk et al, 1998). Activation of MAP kinase has been reported in several human malignancies. Hepatocellular carcinoma demonstrated increased MAPK activity with a positive correlation observed between MAPK activity and tumor size (Ito et al, 1998). Inhibition of MAPK activation has been shown to inhibit colonic tumor growth *in vivo* (Sebolt-Leopold et al, 1999; Wang et al, 2004). Compared to normal tissue, increased MAPK activity has been observed in human colorectal cancer (Shao et al, 2004; Caron et al, 2005). Due to the finding that oncogenic $p21^{K\text{-}ras}$ is established in approximately 66% of colon tumors, the activation and involvement of ERK-1 and ERK-2 in colon carcinogenesis has been investigated (Shao et al, 2004; Caron et al, 2005). Carcinogen-

induced changes in p21^{K-ras} expression and membrane localization are associated with the *in vivo* activation of the ERK pathway (Davidson et al, 1999; Bissonnette et al, 2000). Ras activation can lead to sequential activation of ERK (Collett et al, 2001). Sakakura (1999) demonstrated that MAPK activation in human colon tumors occurred at low frequency (18%) in those tumors harbouring a p21^{K-ras} mutation, whereas in tumors without a p21^{K-ras} mutation, MAPK activation was high (75%). These findings suggested that MAPK activation correlated with the presence of p21^{K-ras} mutation. However, other types of mutation may also be involved in MAPK activation in human colorectal tumors. Bissonnette et al (2000) found that colonic tumors with either activated wild-type p21^{k-ras} or mutated p21^{K-ras} have increased activation of ERK-1 and ERK-2. The effect of different types of dietary lipids on these signalling molecules has not been investigated.

ERK-2 (p42) activity has been shown to be down-regulated in human colorectal neoplasms. During the mucosa-adenoma-carcinoma sequence, the activity of p42 MAPK was found to decrease stepwise, suggesting inactivation of p42 MAPK may be an early event in CRC (Eggstein et al, 1999). MAPK cascade involvement has been demonstrated in signalling that induces differentiation (Troppmaier et al, 1992; Porras, et al, 1994) and apoptosis (Watanabe et al, 1996; Drosopoulos et al, 2005). Eggstein (1999) speculated that reduced p42 activity in colonic carcinoma could be a cause of de-differentiation or the inability of the cells to undergo apoptosis.

Compared to normal appearing colonic mucosa, MAPK expression is increased in colonic tumors with both mutated p21^{K-ras} as well as those tumors with activated wild-type p21^{K-ras}. However, only colonic tumors with mutated p21^{K-ras} had significantly increased CD1 expression (Bissonnette, 2000). This distinction is an important

consideration in characterizing colonic tumor phenotype as different types and /or amounts of dietary lipids may effect activation of wild-type p21$^{K\text{-}ras}$. Bissonnette (2000) has already demonstrated that activation of wild-type p21$^{K\text{-}ras}$ stimulates MAPK but not CD1 expression.

Cyclin D1 and the Cell Cycle

CD1 is a cell cycle regulated gene shown to play a role in normal progression of cells through the cell cycle (Haber and Fearon, 1998). One link between cell cycle progression and growth factor signalling is provided by CD1, whose gene is induced as a secondary response gene following mitogenic stimulation. Regulation of cyclin D1 (CD1) is a critical target of the p21$^{K\text{-}ras}$ signalling cascade. CD1 is rapidly degraded, therefore, its expression is dependent on continued growth factor stimulation until cells pass the G_1 restriction point. Ras proteins play a key role in integrating mitogenic signals with cell cycle progression through G_1 (Aktas et al, 1997). Studies have indicated the ability of oncogenic ras to induce expression of CD1 (Albanese et al, 1995; Liu et al, 1995; Arber et al, 1996, Winston et al, 1996). Regulatory elements of the CD1 promoter are induced in response to oncogenic ras proteins or activation of the ERK mitogen activated protein kinase (Albanese et al, 1995; Lavoie et al, 1996). Ras modulates expression of CD1 at the level of mRNA accumulation (Aktas et al, 1997). CD1 is amplified in some cancers, including colon cancer (Bissonette et al, 2000). Evidence exists supporting the fact that colonic malignant transformation in humans and animals involves alterations in both p21$^{K\text{-}ras}$ and CD1 (Arber et al, 1996; Bissonnette et al, 2000), both of which are important cell signal transduction elements. *In vitro* and *in vivo* studies have found increased CD1 expression due to mutations in p21$^{K\text{-}ras}$ and have been shown to be elevated in a subset of

AOM-induced tumors (Otori et al, 1999). Progression through the cell cycle is also controlled by cyclin-dependent protein kinases (cdks). Ras signalling is required for both induction of CD1 and for down-regulation of the Cdk inhibitor $p27^{KIP1}$ (Sherr and Roberts, 1995). $p27^{KIP1}$ is expressed in high levels in quiescent cells and in cells arrested in G_1 by lovastatin (Polyak et al, 1994; Hengst and Reed, 1996).

Caspase-3 and Apoptosis

Apoptosis is a process involved in the removal of genetically altered cells (Radtke and Clevers, 2005). Neoplastic growth in colon cancer has been related to defects in the apoptotic mechanism (Evertsson et al, 1999). Activation of caspases is a critical step in apoptotic cell death and caspase-3 is known to be a key enzyme during apoptosis (Kim et al, 2000; Jonges et al, 2001). The frequency of apoptosis increases from mucosa to adenoma and carcinoma (Hawkins et al, 1997). Biochemical detection of caspase-3 activity has been used to measure apoptosis in colorectal tumor cells as well as having prognostic value in the disease. Frequency of apoptosis in tumor tissue may have an impact on the outcome of the disease. High caspase-3 activity significantly correlates with higher recurrence and is preferentially found in tumors of the right side of the colon (Jonges et al, 2001).

The Effect of Dietary Lipids on Cell Signalling Molecules Involved in Colorectal Cancer

As dietary lipids have proven to be a major factor involved in CRC development, the mechanisms by which it alters incidence of the disease have become of major interest to investigators. Many types of dietary lipid have been implicated in regulating cholesterol biosynthesis, a fact that provides the necessary link between dietary lipid, $p21^{K\text{-}ras}$ farnesylation and function. Interest exists in elucidating what effects different dietary

lipids have on p21$^{K\text{-}ras}$ function in colon cancer. There is only limited data investigating the effects of dietary lipids on p21$^{K\text{-}ras}$ function at the molecular level in colon cancer *in vivo*. Research that does exist usually involves comparisons between n3 and n6 PUFAs, thereby leaving a substantial gap in the literature on the effects of saturated fat, as well as the amount of dietary lipid on these processes. In one study comparing the saturated fatty acids myristic, palmitic and stearic acids to the PUFA arachidonic acid in the rat fibroblast cell line Rat 6 transfected with an activated human c-H-ras oncogene, a significant increase in the number of transformed foci were found (Hsiao et al, 1990). Targeting p21$^{K\text{-}ras}$ for investigation may occur at the level of mRNA expression, protein expression, and activity of the protein. Many modulatory factors are involved at each level. For example, once p21$^{K\text{-}ras}$ protein is synthesized, it exists in two forms in the cell, membrane-associated and cytosolic. Normally, five to eight fold more p21$^{K\text{-}ras}$ resides at the membrane compared with the cytosol (Collett et al, 2001). An indication of p21$^{K\text{-}ras}$ activation state is the ratio between membrane-associated to cytosolic p21$^{K\text{-}ras}$. Ras membrane localization is a key regulatory point and is affected by dietary lipids in several ways, including, (1) HMGCAR activity and farnesylation, and (2) independent effects on membrane association (affecting GTP binding and/or membrane structure).

One study investigating the differences in n6 versus n3 FA in CRC found adenocarcinoma incidence to be significantly lower with fish oil (56%) compared to corn oil (70%). Total p21$^{K\text{-}ras}$ expression and membrane:cytosol ratio was demonstrated to be four to six fold higher in colonic tumors than in mucosa in AOM injected rodents (Davidson et al, 1999). Expression of p21$^{K\text{-}ras}$ in the membrane fraction was demonstrated to be 13% higher in animals fed corn oil diet than in those fed fish oil diet.

Dietary n-3 fatty acids were shown to decrease carcinogen-induced colon adenocarcinoma formation and tumorigenesis by decreasing membrane localization of $p21^{K\text{-}ras}$ (Davidson et al, 1999). Another study (Collett et al, 2001) reported that n3 FA (DHA) decreased membrane-associated $p21^{K\text{-}ras}$ compared to n6 FAs. However, the effect was unrelated to $p21^{K\text{-}ras}$ farnesylation as there was no change in HMGCAR protein expression. Data from this study also suggested that 18:2n6 increased membrane-associated $p21^{K\text{-}ras}$ compared to the control group. Because this study was conducted on mouse colon cells over-expressing H-ras, obvious limitations exist in interpreting the findings.

Dietary lipids have been shown to significantly influence $p21^{K\text{-}ras}$ subcellular localization, with fish oil reducing membrane-associated $p21^{K\text{-}ras}$ levels (Singh et al, 1997; Davidson et al, 1999). Membrane localization is important as GTP bound $p21^{K\text{-}ras}$ must be anchored to the plasma membrane in order to function (Hancock et al, 1989; James and Olson, 1989). In addition, docking of $p21^{K\text{-}ras}$ in the cell membrane may regulate its proteolytic degradation (Oldham et al, 1996; Haklai et al, 1998). Findings by Singh et al (1997) have demonstrated a dramatic decrease in colonic expression and membrane-association of $p21^{K\text{-}ras}$ after feeding a high fat fish oil diet (50% of the HFC diet) compared with a high corn oil diet. Singh et al (1997) also demonstrated that AOM induced increasingly higher levels of $p21^{K\text{-}ras}$ expression with advancing stages of colon tumor development. Their findings indicate that the high fat corn oil diet enhanced expression of $p21^{K\text{-}ras}$, whereas the high fish oil diet inhibited $p21^{K\text{-}ras}$ expression. In addition, there was an increased incidence and multiplicity of colonic tumors from the high n6 PUFA diet, whereas the high n3 PUFA diet had decreased incidence and

multiplicity of colon tumors. Feeding a high n3 PUFA diet resulted in an increased accumulation of $p21^{K\text{-}ras}$ in the cytoplasm with a concomitant decrease in membrane-associated $p21^{K\text{-}ras}$. This study suggests that diets high in n6 PUFA promote colon carcinogenesis by increasing $p21^{K\text{-}ras}$ expression, whereas those high in n3 PUFA exert their tumor inhibitory effects by interfering with post-translational modification and membrane-association of $p21^{K\text{-}ras}$.

Several possible explanations for the observed decrease in membrane-association of $p21^{K\text{-}ras}$ as a result of fish oil feeding have been suggested in the literature. The first possibility involves alteration in the post-translational palmitoylation of $p21^{K\text{-}ras}$, which is essential for its association with the intracellular portion of the plasma membrane and its ability to transform cells (Hancock et al, 1989; James and Olson, 1989). Arachidonic acid (AA) and eicosapentaenoic acid (EPA) can covalently bind to G proteins via a thioester linkage, suggesting the possibility for esterification of $p21^{K\text{-}ras}$ with these fatty acids instead of palmitate resulting in modulatory effects on plasma membrane anchorage and transforming activity (Davidson et al, 1999). The second possibility for decreased $p21^{K\text{-}ras}$ membrane localization in fish oil fed animals is reduced availability of farnesyl for esterification due to decreased activity of HMGCAR. Decreased levels and activity of HMGCAR in the mammary gland of fish oil fed rats compared with those fed safflower oil have been found (El-Sohemy and Archer, 1997). A third effect involves altered expression of farnesyl protein transferase (FPTase), the enzyme catalyzing post-translational farnesylation of $p21^{K\text{-}ras}$ precursors facilitating plasma membrane anchorage. Singh et al (1998) found that high amounts of corn oil enhanced FPTase expression,

whereas high amounts of fish oil reduced FPTase expression in both colonic mucosa and colonic tumors.

Limited data is available investigating the effects of dietary lipids in ERK in CRC. Phosphorylated p42 and p44 mitogen activated protein kinase (ERK-1 and ERK-2) expression has been found to be two-fold higher in colonic tumors compared with normal mucosa in AOM injected rodents (Davidson et al, 1999), establishing a link between $p21^{K\text{-}ras}$ and the cell signalling cascade involved in cellular growth. Research supports the fact that $p21^{K\text{-}ras}$ activation leads to the sequential stimulation of ERK (Collett et al, 2001). K-ras mutation frequency between corn oil and fish oil diet groups has been reported to be similar with a trend towards higher incidence of mutation in codon 12 or 13 in tumors from corn oil fed animals (85%) compared with fish oil fed animals (58%) (Davidson et al, 1999).

Currently, there are many gaps in our understanding of the effects of specific dietary lipids on cell cycle control and gene expression. Some evidence exists supporting the role of dietary lipids in cancer development (Hildkivi-Clarke et al, 2004). Limited data exists describing the effect of specific dietary fatty acids on CD1 expression and its involvement in colon carcinogenesis. CD1 expression in normal appearing mucosa has been shown to be increased by feeding both high fat corn oil and beef tallow diets. Increased CD1 expression has been found in colonic mucosa of AOM injected compared to non-injected rats (Fujise et al, 2006). In addition, increased CD1 expression has been demonstrated to be significantly higher in rats fed corn oil and beef tallow diets with AOM versus non-AOM treatment. Research investigating the effects of n-6 and n-3 PUFAs on CD1 expression demonstrate that n-6 PUFAs increase CD1 in T47D breast

cancer cells (Razananahefa et al, 2000). CD1 expression is decreased by n-3 fatty acids in both HT-29 colon cancer cells (Chen and Istfan, 2001) and NIH 3T3 cells (Palakurthi et al, 2000) as well as in mammary tissue (Wang et al, 2003). In addition, decreased expression of CD1 was demonstrated in CaCo-2 colon cancer cells treated with DHA (Narayanan, et al, 2004). No data exists on the effects of saturated fat on CD1 expression in the colon.

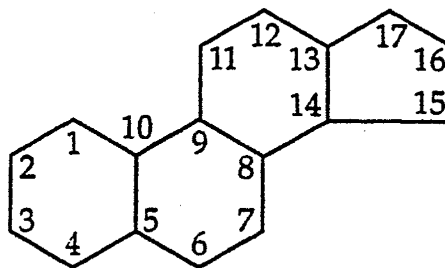
Investigation into the effects of dietary lipids on caspase expression in colon cancer is extremely limited. Understanding of the effect of type and amount of dietary lipid, as well as drugs shown to inhibit CRC, on induction of apoptotic pathways involving caspase-3 expression may be of therapeutic benefit in treatment of the disease. The proliferative and apoptotic response of human colon adenocarcinomas HT-29 cells has been shown to be modulated by pre-treatment with AA or DHA. Hofmanova (2005) demonstrated that pre-treatment of HT-29 human adenocarcinomas cells with these lipids significantly reduced the number of cells in the S-phase of the cell cycle and was accompanied by an increase in the number of cells in G_2/M (for AA) and G_0/G_1 (for DHA). Pre-treatment with AA or DHA induced processes promoting the sensitivity of cells to endogenous apoptotic regulators including activation of caspase-8, -9, and –3 as well as cleavage of poly (ADP-ribose) polymerase (PARP) protein. These treatments initiated a cascade of processes which prepared a permissive environment for a more effective action of apoptotic inducers and suggested that the composition of dietary lipid be included in strategies for colon cancer prevention and therapy. Treatment of CaCo-2 colon caner cells with DHA has been shown to up-regulate caspases 5, 8, 9 and 10 (Narayanan et al, 2001; Narayanan et al, 2003). Diosgenin, a food saponin, has been

shown to induce a dose-dependent apoptotic response in HCT-116 human colon carcinoma cells involving cleavage of PARP. Diosgenin reduced the expression of HMGCAR mRNA and protein as well as $p21^{K\text{-}ras}$ and beta-catenin. It also to reduced expression of HMGCAR and $p21^{K\text{-}ras}$ in HCT-116 human colon carcinoma cells and produced a dose-dependent apoptotic response involving cleavage of PARP (Raju and Bird, 2007).

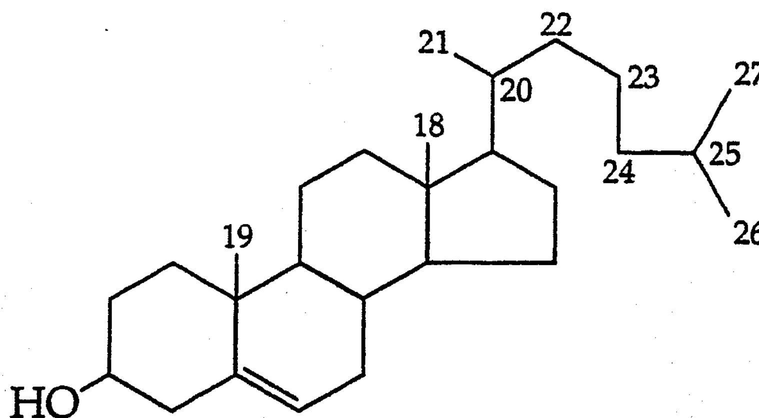
Cholesterol Biosynthesis and Homeostasis

Cholesterol biosynthesis from acetyl CoA occurs in nearly all body tissues. Cholesterol is characterized by its four ring steroid nucleus and is classified as a monohydroxy alcohol. The structure of cholesterol and a schematic representation of its biosynthesis can be found in Figures 5 and 6. Cholesterol is found only in animal tissues in free form or esterified with a FA. It is an essential component of cellular membranes and critical to normal cell function (Rao, 1995). Additionally, cholesterol biosynthetic pathway precursors serve vital physiological purposes (Gropper et al, 2005). One such example, related to the dissertation topic, is the production of farnesyl from mevalonate catalyzed by HMGCAR (EC 1.1.1.34) which is used to anchor $p21^{K\text{-}ras}$ to the plasma membrane. The rate-limiting enzyme and primary control site of the cholesterol biosynthetic pathway is the hepatic HMGCAR. HMGCAR is the most elaborately regulated enzyme of this pathway. For the sake of brevity and in light of the dissertation topic, the reaction catalysed by HMGCAR in the pathway of cholesterol biosynthesis will be highlighted. For additional information, the reader is referred to a more detailed explanation of cholesterol biosynthesis (Gropper et al, 2005).

Figure 5. **The Structure of Cholesterol**
(Copyright permission provided from Thompson Learning Global Rights Group; License Number 169097)

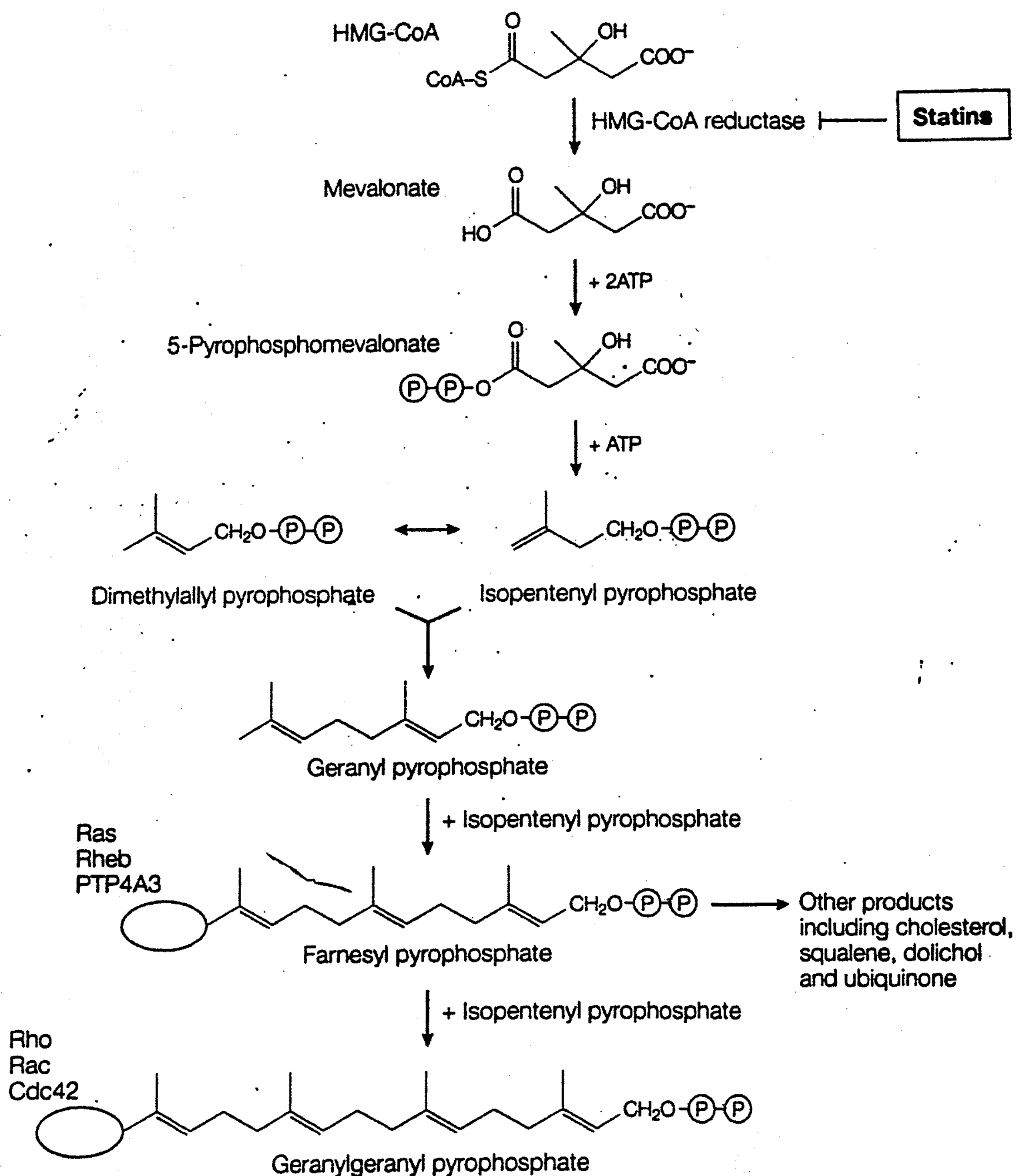


Steroid nucleus



Cholesterol

Figure 6. **The Cholesterol Biosynthetic Pathway as affected by Statins** (Copyright permission provided from Nature Publishing Group; License Number 1812570943043)



The liver accounts for approximately 20% of endogenous cholesterol synthesis. Among the extrahepatic tissues accounting for the remaining 80%, the enterocytes have a predominant role (Nguyen et al, 1994). An intricate balance is maintained between biosynthesis, utilization, and transport of cholesterol. Cholesterol metabolism must be regulated as humans have the ability to synthesize it as well as ingest it from animal food sources. The liver and extrahepatic tissues have two ways of obtaining cholesterol, either synthesizing it from acetyl CoA by the de novo pathway or obtaining it from exogenous dietary sources, and in the case of peripheral tissues from the liver, via the bloodstream by LDL receptor-mediated endocytosis. Cellular cholesterol metabolism is regulated primarily through the coordinate expression of the low density lipoprotein receptor (LDLr) and HMGCAR (Lindgren et al, 1985). As total body cholesterol increases, negative feedback regulation of HMGCAR results in decreased cholesterol synthesis. This suppression by dietary cholesterol is unique to the liver and feedback regulation by dietary cholesterol in colonic enterocytes is essentially unresponsive to dietary cholesterol (Ness and Chambers, 2000). Cholesterol biosynthesis and transport is highly regulated and cellular supply is maintained by (1) regulating HMGCAR activity, (2) regulating LDLr synthesis, and (3) regulating the rate of esterification and removal of free cholesterol. HMGCAR regulation is accomplished by both long-term and short-term regulation. Short-term regulation of HMGCAR catalytic activity is via competitive inhibition, allosteric regulation, and covalent modification involving reversible phosphorylation. Long-term regulation of HMGCAR enzyme concentration is accomplished via modulation of synthesis and degradation rates (Gropper et al, 2005).

High intracellular concentrations of cholesterol suppress LDLr synthesis, whereas low cholesterol concentrations stimulate it.

LDLr function is involved in cholesterol homeostasis and is modulated by the type of dietary lipid (Hayes et al, 1997). When dietary cholesterol intake is kept constant, long-chain saturated fatty acids suppress hepatic LDLr activity leading to elevated LDL cholesterol levels, whereas polyunsaturated fatty acids increase LDLr activity leading to decreased LDL cholesterol levels (Mustad et al, 1997; Dietschy, 1998). Only triacylglycerides (TAG) rich in lauric (12:0) and myristic (14:0) fatty acids are especially hypercholesterolemic, whereas palmitic acid (16:0) and stearic acid (18:0) rich TAGs are usually neutral (Hayes, 1995). Studies investigating the effects of PUFA on LDLr have shown that hepatic LDLr mRNA is significantly increased as the PUFA content of the diet increases (Ramjiganesh et al, 2002). As a result of LDLr up-regulation, more cholesterol from plasma is taken up by cells leading to decreased plasma LDL concentrations. Fatty acid-induced changes in hepatic LDLr activity have been reported to be accompanied by parallel changes in hepatic LDLr protein and mRNA levels suggesting that dietary fatty acids regulate the hepatic LDLr pathway largely at the mRNA level (Horton et al, 1993). Whether this is true in colonic enterocytes has yet to be investigated.

Involvement of HMGCAR and LDLr in Regulating Plasma Cholesterol

Plasma cholesterol levels in humans (and rats) are determined by the net balance between input of cholesterol into plasma via hepatic synthesis (regulated by HMGCAR) and removal rate from plasma which is governed partly by the number of LDLr present (Rudling, 1992). The liver plays a critical role in cholesterol metabolism. The regulation

of HMGCAR is extremely high and rapid due to regulation at the transcriptional, translational and protein level (Goldstein and Brown, 1990). In both *in vitro* and *in vivo* experiments, the LDLr seems to be regulated at the levels of transcription and post-transcription (Ostlund et al, 1991; Owen et al, 1991). LDLr and HMGCAR activities show coordinate regulation in cultured cells (Goldstein and Brown, 1990). It has been demonstrated that hepatic down regulation of LDLr and HMGCAR mRNA levels are small even during extreme dietary fat loads. The effects of feeding cholesterol only (0.4% cholesterol), a standard experimental design producing decreased HMGCAR activity and LDLr, leads to decreased levels of LDLr and HMGCAR mRNA but only a small increase in plasma and hepatic cholesterol levels. Higher concentrations of dietary cholesterol double hepatic cholesterol concentration but LDLr and HMGCAR mRNA levels do not decrease further and total plasma cholesterol levels are unchanged (Rudling, 1992). These results suggests post-transcriptional regulation including translational effects and protein cycling and/or altered degradation rate which have been subsequently verified by further research (Nakanishi et al, 1988).

Hepatic mRNA levels for the LDLr and HMGCAR are regulated in parallel *in vivo* under normal physiological conditions as well as during administration of LOV. LOV is a powerful inducer of hepatic HMGCAR transcription (Lopez et al, 1997). Treatment with competitive inhibitors of HMGCAR, such as LOV, result in a decreased rate of hepatic cholesterol biosynthesis as well as induction of LDL receptors and HMGCAR (Gropper et al, 2005). Treatment with a combination of LOV and colestipol lead to increased hepatic transcript levels of HMGCAR and LDLr producing only slight decreases in plasma and hepatic cholesterol levels but resulted in significant elevations in HMGCAR

(545%) and LDLr (169%) protein expression. When LDLr protein concentration was compared to LDLr mRNA levels in the LOV plus colestipol group, hepatic mRNA level for the LDLr mirrored LDLr protein levels. In this group, LDLr was only slightly reduced. The strong positive correlation between hepatic LDLr and HMGCAR mRNA levels indicates that these genes are regulated in parallel at the mRNA level *in vivo* (Rudling, 1992). Hepatic enzyme activity of HMGCAR and the LDLr binding activity in humans indicate that the regulatory pattern for HMGCAR and LDLr are similar to that found at the mRNA level in the mouse (Reihner et al, 1990).

3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGCAR) Regulation

Feedback regulation of cholesterol biosynthesis occurs predominantly in the liver of rats and humans (Ness, 1993; Chambers and Ness, 1998). Hepatic HMGCAR activity undergoes diurnal variation due to changes in the level of immunoreactive protein mediated by changes in insulin and glucagons levels (Lakshamanan et al, 1975; Ness et al, 1994; Ness and Chambers, 2000). The overall activity of the HMGCAR enzyme is regulated by multiple factors and occurs at a multitude of levels. Feedback regulation of hepatic HMGCAR gene expression plays an important role in overall cholesterol homeostasis. Molecular mechanisms proposed to contribute to HMGCAR regulation of gene expression include changes in transcription rate (Liscum et al, 1983; Lopez et al, 1997), translation (Ness et al, 1994; Chambers and Ness, 1998) as well as protein turnover rate (Brown and Goldstein, 1997; Ness and Chambers, 2000) and activity (Ness et al, 1991; Lopez et al, 1997). This combination of transcriptional and post-transcriptional controls can regulate the amount of HMGCAR protein over several hundred fold range in animal cells (Nakanishi et al, 1988). Although there are several

possible methods of post-translational regulation of hepatic HMGCAR, a large reduction in HMGCAR translation rate accounts for most of the observed reductions in enzyme activity and protein that occur in response to dietary cholesterol and endogenously synthesized cholesterol. Comparisons of mRNA levels with the corresponding levels of HMGCAR immunoreactive protein and/or enzyme activity offers the most beneficial understanding of the mechanism(s) of regulation of this complex enzyme.

Significant species differences exist with respect to the portion of feedback regulation of hepatic HMGCAR attributable to transcriptional or translational control (Chambers and Ness, 1998). Rats display primarily translational regulation (Ness and Chambers, 2000). Studies have found that non-sterols regulate HMGCAR gene expression at the level of translation (Goldstein and Brown, 1990). The possible mechanism suggested for this type of regulation is production of mRNAs of different length 5' untranslated regions (between 68 to 670 nucleotides long) with or without AUG codons upstream of the initiator methionine (Reynolds et al, 1985). LOV has been shown to produce HMGCAR mRNAs with the shorter 5' untranslated regions associated with the actively translating polysome fraction (Gayen and Peffley, 1995). The decreased translational efficiency of HMGCAR mRNA in livers from rats fed cholesterol supplemented diets was found to be due to a shift from association with actively translating polysomes to inactive monosomes with the monosomes containing over ten times more HMGCAR mRNA. The distribution of beta-actin mRNA was unaffected (Chambers and Ness, 1997). Humans display a combination of transcriptional and translational regulation with differences among individuals in their ability to "buffer" cholesterol synthesis and maintain cholesterol homeostasis (Ness and Chambers, 2000). Rats are more resistant to dietary cholesterol

possibly due to much higher levels of hepatic cholesterol biosynthesis and HMGCAR activity (Spady et al, 1993), providing an increased capacity for feedback regulation. In humans, differences have been detected in HMGCAR activity as measured by mevalonate levels (Naoumova et al, 1996). Those individuals expressing higher levels of HMGCAR are more able to tolerate cholesterol in their diets. This theory of cholesterol buffering capacity has been described by Ness and Chambers (2000).

In chow fed rats, the half-life of HMGCAR is approximately 2.5 hours (Keller et al, 1996). Modulation of HMGCAR half-life can markedly and rapidly change the level of protein (Ness and Chambers, 2000). Modulation of enzyme half-life has been demonstrated with treatment of LOV in which enzyme half-life increased to approximately 12 hours (Sinensky and Logel, 1983). Both sterols and non-sterols are required to regulate the degradation rate of HMGCAR (Chun et al, 1990; Goldstein and Brown, 1990; Ness and Chambers, 2000). Studies have determined that the eight-membrane spanning domain of HMGCAR is required for sterol-mediated regulation of degradation (Moriyama et al, 1998), whereas farnesol is the non-sterol regulator of HMGCAR degradation (Meigs et al, 1996). Mevalonate also accelerates degradation of HMGCAR (Nakanishi et al, 1988). Regulation of HMGCAR enzyme by controlled degradation is dependent upon its association with the ER membrane (Gil et al, 1985). HMGCAR protein has a molecular weight of 97,000 (97 kDa) which is divided into two regions: (1) the NH_2 terminal third of the protein is extremely hydrophobic and is firmly embedded in the ER membrane, and (2) the COOH terminal two-thirds of the protein which is hydrophilic and contains all of the catalytic activity (Nakanishi et al, 1988). Rapid degradation of HMGCAR is dependent on the presence of its hydrophobic NH_2

terminal region (Jingami et al, 1987). Farnesylated proteins could influence this degradation by regulating the rate at which HMGCAR is incorporated into vesicles that transport it from the ER to a degradative site. Transport of lipid vesicles from the ER requires a membrane bound GTP-binding protein (Melancon et al, 1987) which is likely to be a member of the $p21^{K\text{-}ras}$ family and thus be farnesylated. Goldstein and Brown (1990) suggest that sterols may function in part by diverting small amounts of mevalonate into non-sterol products that include farnesylated GTP-binding proteins of the $p21^{K\text{-}ras}$ family and that the seven membrane-spanning regions of HMGCAR are a possible candidate for interaction with such a GTP-binding protein.

The finding that cells will not eliminate their HMGCAR activity unless mevalonate is available (Nakanishi et al, 1988), and that growth inhibition can be observed in a single cell cycle when treated with an HMGCAR inhibitor (Fairbanks et al, 1984), suggests that the non-sterol isoprenoid allowing entrance into the S phase is the same as that which suppresses HMGCAR (Goldstein and Brown, 1990). The reason for the complex mevalonate mediated post-transcriptional control of HMGCAR links it to cell cycle control (Nakanishi et al, 1988). HMGCAR activity rises just before the S phase of the cell cycle even in the presence of sterols (Quesney-Huneeus et al, 1979). When mevalonate is unavailable, unfarnesylated $p21^{K\text{-}ras}$ proteins accumulate (Repko and Maltese, 1989) and may affect HMGCAR translation or degradation.

Low Density Lipoprotein and its Receptor

Low density lipoprotein (LDL) is the primary carrier of cholesterol in the blood, binding approximately 60% of total serum cholesterol. Its function is to transport cholesterol to tissues where it can be used for membrane synthesis or converted into other metabolites

required for other functions. LDL interacts with LDL receptors on cells via its apoB-100 and is removed from circulation complexed to receptors that cluster in coated pits (Brown and Goldstein, 1986). The LDL receptor is a transmembrane glycoprotein encoded by a gene on chromosome 19. A detailed outline of lipoprotein metabolism is given by Gropper et al (2005). Inside the cell, free cholesterol has regulatory functions including: (1) lowering the concentration of LDLr mRNA resulting in decreased synthesis of LDLr which prevents further entry of LDL into the cell; (2) modulating the activity of HMGCAR; and (3) modulating activity of the enzyme acyl CoA: cholesterol acyl transferase (ACAT). The number of receptors synthesized by cells varies according to its cholesterol requirements. Dietary lipid intake is an environmental factor effecting hepatic LDLr expression. Feedback regulation of LDLr along with that of cholesterol biosynthesis ensures production of sufficient mevalonate for the end-products of the mevalonate pathway necessary to cholesterol homeostasis as well as cellular growth.

Reducing serum cholesterol through treatment with HMGCAR inhibitors, such as LOV, can cause an increase in hepatic LDLr gene transcription (Ness and Chambers, 2000; Gropper et al, 2005). LOV increases hepatic LDLr protein synthesis and degradation, therefore increasing cycling and uptake of LDL (Ness and Chambers, 2000). When LOV is given, a transient depletion of hepatic cholesterol occurs and then is followed by a marked increase in hepatic HMGCAR protein levels and LDLr function in an attempt to increase endogenous cholesterol production and uptake from serum, respectively (Ness et al, 1994; Goldstein et al, 1995). Induction of HMGCAR alone does not restore hepatic cholesterol levels as long as inhibitors of HMGCAR are present. Therefore, overall *in vivo* hepatic cholesterol biosynthesis is reduced and in order to meet its cholesterol

requirement, increased LDL uptake from the serum occurs (Bilheimer et al, 1989) and is mediated by increased expression of hepatic LDLr (Ma et al, 1986) which reduces serum LDL cholesterol concentrations (Bradford et al, 1991). The central role of LDLr in modulating the effect of HMGCAR inhibitors on serum cholesterol levels has been demonstrated in studies of individuals with the LDLr negative form of familial hypercholesterolemia, in which treatment produces no reduction in serum cholesterol levels (Uauy et al, 1988). In a study investigating the importance of LDLr in the cholesterol lowering effect of HMGCAR inhibitors, the effect of LOV treatment (5 days) on LDLr mRNA and protein levels in rats was investigated and found that LDLr mRNA was increased while protein levels were unaffected. However, LOV increased the rate of hepatic LDLr protein degradation by decreasing LDLr half-life (Ness et al, 1996b). The HMGCAR inhibitors that produced the largest increase in LDLr mRNA had the greatest effect on LDLr degradation. Therefore, it was suggested that HMGCAR inhibitors increase rates of both synthesis and degradation of hepatic LDLr protein in order to maintain constant steady state levels. LOV was also found to increase the half-life of HMGCAR to approximately eight hours, whereas cholesterol feeding had no affect on HMGCAR half-life (remained at 3 hours). Therefore, the effects on LDLr turnover are distinct from those on HMGCAR degradation, suggesting that different regulatory systems are involved (Ness et al, 1996b). One study found feeding LOV to rabbits for a period of 8 days caused a two-fold increase in hepatic LDLr mRNA and protein levels (Ma et al, 1986). Similar effects of LOV have been found in humans (Angelin, 1995).

The Relationship between Cholesterol Metabolism and $p21^{K\text{-}ras}$ in Colorectal Cancer

Understanding of cholesterol metabolism in normal and neoplastic colonic enterocytes is relatively limited. Low serum cholesterol as a biomarker of CRC was considered in epidemiological studies focusing on obese men with lower than normal serum cholesterol levels found to be at increased risk of colon cancer. Genetic studies have provided insight on a possible association involving alterations on chromosome 5 which occur during the progression of CRC. This chromosome carries two genes vital to the biosynthesis and regulation of systemic and cellular cholesterol metabolism, namely, HMGCAR and 3-hydroxy-3-methylglutaryl coenzyme A synthase (Broitman et al, 1993). Research findings support the involvement of the cholesterol biosynthetic pathway in colon carcinogenesis (Rao, 1995; Rao et al, 1995; Notarnicola et al, 2004). However, its exact role is not fully understood, partly due to its multiple roles and underlying effects. Stimulated HMGCAR activity is only partially reversible by LDL but completely reversible by mevalonolactone addition, supporting the theory of multivalent control by both sterol and non-sterol products (Brown et al, 1987; Nakanishi et al, 1988). To support the high turn over rate, intestinal epithelial cells must synthesize enough cholesterol for cellular membrane synthesis and proper function (Stange et al, 1981). Compared to normal cells, cancer cells have an increased requirement for cholesterol (Rao, 1995) which is met by increased HMGCAR activity and/or LDLr expression (Caruso et al, 2002). Higher HMGCAR activity and LDLr levels have been detected in human colonic tumors compared to normal mucosa (Caruso et al, 2002; Notarnicola et al, 2004). Up-regulation of HMGCAR has also been detected in left-sided tumors (Demierre et al, 2005). Inhibiting cholesterol biosynthesis inhibits cell growth,

suggesting a linkage between cholesterol and DNA synthetic pathways (Rao, 1995). The normal feedback regulation of HMGCAR is lost in tumors (Siperstein and Fagan, 1964; Azrolan and Coleman, 1989). Due to the increased cholesterol requirement of tumor cells (Rao, 1995), HMGCAR gene expression in tumor cells differs from that in normal cells and tissues (Ness and Chambers, 2000). Existing evidence supports the involvement of HMGCAR and LDLr in malignant transformation in various tissues (Rudling et al, 1990). In one study, the level of LDLr was shown to be up-regulated in tumors (Notarnicola et al, 2004) but was unchanged in another (Caruso et al, 2002). In contrast and unlike normal fibroblasts, colonic cancer cells, *in vitro*, have been shown to possess a diminished or nonexistent ability to use LDL to support cellular growth. Diminished LDLr activity is a significant alteration in a metabolic pathway with such fundamental ties to cellular growth and activation (via mevalonate effects on isoprenylation of G-proteins). This phenotype seems to be selected for in the development of human colonic tumors, providing a growth advantage to the tumor. LDL has been shown to inhibit the proliferative capacity of certain human colonic adenocarcinomas that have been shown to posses a high rate of cholesterol synthesis relative to fibroblasts (Broitman et al, 1993). Chronic lymphocytic leukemia (CLL) cells express lower LDLr activity and higher HMGCAR activity than normal mononuclear blood cells (Vitols et al, 1997). LDLr and HMGCAR mRNA levels are also reduced in kidney tumors compared to normal mucosa (Rudling and Collins, 1996). The LDLr mRNA levels correlated significantly to the mRNA levels for HMGCAR in tumors and normal kidney tissue. LDLr mRNA expression was found to be significantly increased in human colon carcinoma tissues compared to tissue from the tumor free margin. The

extent of LDLr mRNA expression positively correlated to the percentage rise of plasma cholesterol levels three months and twelve months after curative surgery suggesting that the tumor tissue itself contributes to decreased plasma cholesterol levels in patients with CRC and supports the hypothesis that low cholesterol levels in cancer patients are a consequence, not a cause, of the malignancy (Niendorf et al, 1995).

A study of the regulation of HMGCAR and LDL binding in human colon cancer cells (Caco-2 carcinoma cell line) demonstrated that HMGCAR and LDL binding is subject to the availability of non-sterol products of mevalonic acid and of exogenous cholesterol. The availability of endogenously synthesized mevalonate products influenced the expression of LDL activity in these Caco-2 cells (Reimann et al, 1992). Inhibition of HMGCAR by LOV, and hence endogenous cholesterol biosynthesis, increased both LDL binding and HMGCAR activity significantly. Increased HMGCAR activity was probably due to enhanced protein synthesis (Brown et al, 1987) as well as reduced enzyme degradation (Nakanishi et al, 1988) reflecting an attempt, by cells, to compensate for reduced levels of sterols and non-sterol products of HMGCAR necessary for cell cycling (Siperstein, 1984). A study investigating the effects of LDL and LOV on metastatic behaviour (adhesion and migration) of human colon adenocarcinomas cell lines (Mehta et al, 1998; Web et al, 1998) found LOV to reduce transendothelial tumor migration. In addition, LDL significantly affected tumor migration but not adhesion of primary and metastatic colonic adenocarcinomas.

Evidence that the farnesyl moiety from the cholesterol biosynthetic pathway is necessary for the activation of $p21^{K\text{-}ras}$ oncoprotein provides a probable basis for understanding the linkage through signal transduction pathways. Farnesylation of $p21^{K\text{-}ras}$ is one factor

regulating membrane localization during malignant transformation in the colon (Reddy, 2005). Therefore, $p21^{K\text{-}ras}$ farnesylation underscores the importance of the cholesterol biosynthetic pathway in cell growth and carcinogenesis. The activation of $p21^{K\text{-}ras}$ by gene mutation in CRC is aided by continuous farnesylation due to stimulation of the cholesterol biosynthetic pathway in tumors (Rao, 1995). It is important to investigate the phenotypic differences in ACF and colonic tumors to elucidate dependence of their growth on this pathway. In addition, due to the multifactorial effect of environment on growth, dietary lipid composition may also play a role in mediating the effects of the pathway on growth. This area of research remains highly unexplored. Depending on their phenotype, some tumors may be more reliant on cholesterol biosynthesis than others. The cholesterol biosynthetic pathway and $p21^{K\text{-}ras}$ are targets for chemoprevention in CRC. One study also showed that statin treatment produced significant anti-proliferative and pro-apoptotic effects (Notarnicola et al, 2004). The possibility of dietary fatty acid-gene and dietary fatty acid-drug treatment interactions has never been investigated at the level of ACF and colon tumor development *in vivo*.

The Effect of Dietary Lipids on HMGCAR, LDLr, and $p21^{K\text{-}ras}$ in Cancer

HMGCAR activity is modulated differently by specific types of dietary lipids (Goldfarb and Pitot, 1971). Different types and amounts of dietary FAs modulate post-translational $p21^{K\text{-}ras}$ farnesylation and and membrane localization, by regulating HMGCAR activity leading to alterations in $p21^{K\text{-}ras}$ function and cell growth (Reddy, 1991; El-Sohemy and Archer, 1997 and 1999). With the demonstrated critical role of $p21^{K\text{-}ras}$ activation in the multi-step process of colon carcinogenesis, which has also been shown to be regulated by different types and amounts of FA, there has been interest in delineating the modulatory

role of these FAs on p21$^{K\text{-}ras}$ expression via effects on HMGCAR and LDLr during the exclusive stages of ACF and tumor development. The modulatory role of saturated fat, as well as low fat diets, on p21$^{K\text{-}ras}$ expression during the multi-step process of colon carcinogenesis has received minimal attention.

Post transcriptional regulation of HMGCAR has been found in tumor cell lines (Brown and Goldstein, 1997). Research conducted by El-Sohemy and Archer (1999) has shown that changes in HMGCAR mediate the differential effects of n3 and n6 PUFA on experimental mammary tumorigenesis whereby a significant diet-gene interaction was observed. In addition, the LDLr has been shown to mediate the effects of PUFA on HMGCAR in the mammary gland but not the liver (El-Sohemy and Archer, 1999). These results suggest that the composition of dietary PUFA profoundly influences the effects of deleting the LDLr on HMGCAR and serum lipids suggesting that diet may influence the phenotype of other knockout or transgenic animals (El-Sohemy and Archer, 1999). Research investigating the role of the cholesterol biosynthetic pathway in human breast cancer cells and tumors grown in nude mice demonstrated elevated HMGCAR activity in malignant cells demonstrating the growth promoting effect of intermediates of the cholesterol biosynthetic pathway in these cells and tumors (Duncan et al, 2005). Dietary factors shown to have anti-cancer effects in experimental breast cancer models via inhibition of HMGCAR include cholesterol, plant isoprenoids, n3 PUFA, and genestein.

Additional evidence suggests PUFA block HMGCAR activity (Field et al, 1987; Proksch et al, 1992; Hromadova et al, 1994; El-Sohemy and Archer, 1997 and 1999). Hence, there exists a possible interaction between statins and dietary fatty acids on HMGCAR

(Das, 2001). PUFA in combination with LOV may be very effective in inhibiting colon carcinogenesis based on suppression of HMGCAR, inhibition of farnesylation of oncogenic $p21^{K\text{-}ras}$ and tumor progression. Carcinogen-induced $p21^{K\text{-}ras}$ activation in the colon was shown to be suppressed by n3 PUFA prior to development of neoplasia (Chapkin et al, 1997).

Chemopreventive / Chemotherapeutic Agents in Colorectal Cancer

In its broadest concept, cancer prevention involves regression, inhibition, or elimination of precancerous lesions, especially advanced preneoplastic lesions, with the net effect of reducing cancer incidence. The multifactorial nature of CRC complicates attempts to delineate exact causes of colon cancer and prevention strategies are thus increasingly complex. Cancer preventive agents are usually classified based on their ability to affect preneoplastic lesions of different stages (Bird and Good, 2000). Whether known modulators of colon cancer affect all or selected preneoplastic lesions remains elusive. A need exists to quantify the cellular and molecular events which permit the transition of preneoplastic lesions from an early stage to a more advanced stage so that they can be targeted for inhibition of disease progression. Strategies for prevention include attempting to decrease risk by limiting exposure to lifestyle factors known to be positively correlated with risk, as well as more recent chemopreventive strategies aimed at inhibiting or reversing the process of colon carcinogenesis using pharmacological agents including cholesterol biosynthesis inhibitors (statins) (Narisawa et al, 1994; Narisawa et al, 1996). Certain types of clinically tested medications, including lovastatin (LOV), have been implicated in reducing CRC incidence and progression. Therapeutic intervention in the carcinogenic process related to $p21^{K\text{-}ras}$ mutation and misfunction are

aimed at blocking p21$^{K\text{-}ras}$ function in the cell. Due to its structural components, there are several aspects of p21$^{K\text{-}ras}$ that may be targeted. One such target involves the short isoprenoid group (farnesyl pyrophosphate). Any factor blocking this attachment inhibits p21$^{K\text{-}ras}$ involvement in the delivering its growth signal. Due to the fact that farnesyl pyrophosphate is an intermediate of the cholesterol biosynthetic pathway, it has been suggested that drugs, such as LOV, which inhibit this pathway may also affect p21$^{K\text{-}ras}$ function and tumor development. Recently, researchers from Belgium and Italy have reported that the efficacy of Vectibix (panitumumab) for treating metastatic colorectal cancer is limited to patients with tumors lacking K-ras mutations (Amodol et al, 2007). Therefore, it is important to determine possible phenotypic differences in colonic tumors that may affect their ability to be modulated by chemoprevetnive drugs, including LOV, as well as dietary fatty acid modulation of drug effectiveness in these tumors.

Lovastatin and Cancer

Statins, including LOV, are reversible competitive inhibitors of the microsomal enzyme HMGCAR (Lennernas and Fager, 1997). HMGCAR is bound approximately 1,000 times more efficiently by statins with open-ring structures than by its natural substrate, HMG CoA (Chan et al, 2003). The chromatographic pattern of LOV metabolites produced by human and rat liver microsomes have been found to be similar (Greenspan et al, 1988). LOV is a cholesterol-lowering agent isolated from a strain of *Aspergillus terreus*. It is ingested orally and its inactive lactone form is hydrolysed to the corresponding β-hydroxyacid form, which is a specific inhibitor of HMGCAR (CPS, 1998). The serum concentration of statins therapeutically relevant for lipid lowering is in the low (1-10 μmol/L) micromolar range (Thibault et al, 1996). LOV reduces cholesterol production

by the liver and induces some changes in cholesterol transport and disposition in the blood and tissues. As a consequence of extensive hepatic extraction of LOV, the availability of the drug to the general circulation is low and variable and estimated to be less than 5% of an oral dose (American Hospital Formulary Service, 2004). LOV and its β-hydroxyacid metabolite are highly bound (95%) to human plasma proteins. The major active metabolites in human plasma are the β-hydroxy derivative, its 6'-hydroxy, 6'-hydroxymethyl, and 6'exomethylene derivatives. Peak plasma concentrations of both active and total inhibitors are attained within two to four hours of dose administration. The recommended therapeutic dose range for humans is between 20-80 mg/day. When LOV is given under fasting conditions, plasma concentrations of active and total inhibitors are, on average, approximately two-thirds those found when administered immediately after a standard test meal (CPS, 1998).

Inhibition of HMGCAR by statins decreases intracellular cholesterol biosynthesis (Figure 6), resulting in transcriptionally up-regulated production of microsomal HMGCAR and cell surface LDLr. The liver is the target organ for statins. However, cholesterol biosynthesis in extrahepatic tissues is necessary for normal cell function (Lennernas and Fager, 1997).

In addition to decreasing cholesterol concentration, statins inhibit other mevalonate pathway reactions and their products including isoprenoid (farnesyl and geranylgeranyl isoprenoids) synthesis (Hawke and Viner, 2005). Cell culture and animal model studies have demonstrated the effect of stains in preventing CRC by suppression of farnesylation, which is critical to its activation, and $p21^{K\text{-}ras}$ inhibition (Kim et al, 2004; Demierre et al, 2005; Hawke and Viner, 2005). Research reported by various researchers support the

differential effects of dietary n3 and n6 FA on colonic ACF development and tumor growth as well as mammary tumor growth, in rodents (Singh et al, 1997; Reddy, 2004; El-Sohemy and Archer, 1997 and 1999). Research has demonstrated that many of the effects of statins in cancer development are due to inhibition of synthesis of prenylation precursor isoprenoid compounds (farnesyl and geranylgeranyl) and prevention of prenylation of ras and rho proteins (Wong et al, 2002) resulting in interruption of critical molecular signalling pathways in carcinogenesis. Farnesyl pyrophosphate (FPP) prenylates $p21^{K\text{-}ras}$ proteins, whereas geranylgeranyl pyrophosphate (GGPP) prenylates rho proteins (Demierre et al, 2005). Drug induced inhibition of mevalonate biosynthesis by HMGCAR inhibitors such as LOV, have been shown to prevent $p21^{K\text{-}ras}$ farnesylation and block cell growth *in vitro* and *in vivo* (Cox and Der, 1992; Demierre et al, 2005). Oncogenic $p21^{K\text{-}ras}$ proteins that are non-farnesylated and unprocessed are unable to localize properly into the plasma membrane and are both non-transforming as well as displaying a dominant inhibitory phenotype that has antagonistic effects on the activity of membrane bound oncogenic $p21^{K\text{-}ras}$ (Stacey et al, 1991). Farnesylation of $p21^{K\text{-}ras}$ is crucial in regulating the inhibitory effects of statins on carcinogenesis (Demierre et al, 2005). In normal cells, HMGCAR is subject to complex feedback regulation at the transcriptional, translational, and post-translational levels by both sterol and non-sterol products of cholesterol biosynthesis pathway intermediates (Demierre et al, 2005). Tumor cells are resistant to sterol-mediated feedback and are more sensitive than normal cells to isoprenoid-mediated suppression (Edwards and Ericsson, 1999; Wong et al, 2002; Houten et al, 2003; Mo and Elson, 2004). Compared with normal cells, tumor cells have an increased HMGCAR expression and an increased requirement for mevalonate-derived

isoprenoids (Hentosh et al, 2001), making certain populations of tumor cells more vulnerable to statin treatment. LOV inhibits tumor cell proliferation by suppressing HMGCAR and the cholesterol biosynthetic pathway (Alberts et al, 1980; Vincent et al, 1991; Sumi et al, 1992; Gebhardi and Niendorf, 1995). *In vivo* studies in mouse mammary tumor models showed that LOV and simvastatin lead to reductions of tumor incidence as well as inhibiting metastasis (Alonso et al, 1998; Farina et al, 2002). When compared to control, LOV (50ppm) has been shown to have significantly reduced, (by 49%), the mean number of colonic ACF in F344 rats (Kim et al, 2004). Statins have been shown to reduce colon tumor incidence by 30-67% in various animal model studies (Narisawa et al, 1994; Narisawa et al, 1996; Agarwal et al, 1999). LOV has been shown to reduce the initiation and mean number of ACF compared to normal cells (Agarwal et al, 1999; Kim et al, 2004). In one study, this effect was prevented by the addition of GGPP. In contrast, Singh et al (1997) found dietary n3 to increase the membrane-associated to cytosolic $p21^{K\text{-}ras}$ ratio in colonic tumors and mucosa, resulting in a reduction of ACF as well as tumor incidence. Additional research on the effects of dietary lipids on mammary cancer development demonstrated reductions in HMGCAR and tumor incidence in animals with increased intake of n3 versus n6 FAs (El-Sohemy and Archer, 1997 and 1999). To our knowledge, no studies have been conducted evaluating the possible modulatory effect of dietary lipid type on LOV effects on $p21^{K\text{-}ras}$ activity and processing and the downstream cell signalling events affected in these models. LOV may have several effects on ACF depending on the stage and dietary fatty acid milieu of the diet. LOV may result in decreased farnesylation and active $p21^{K\text{-}ras}$ for a sub-population of ACF, whereas it could increase apoptosis in another, different

population. Therefore, it is important to delineate the separate effects and cellular and molecular pathways involved including those of cholesterol biosynthesis and homeostasis (HMGCAR and LDLr), $p21^{K\text{-}ras}$ expression and activity, as well as factors involved in cell signalling (ERK-1 and ERK-2) and apoptosis (caspase-3).

Data from observational, preclinical and randomized controlled studies of statin use in cardiovascular disease (CVD) treatment underlie the deep interest in these agents for use in cancer prevention and treatment (Strandberg et al, 2004; Singal et al, 2005), including CRC (Ehrenstein et al, 2005). RCT safety results indicate a benefit of statin treatment in cancer prevention associated with reductions in CRC, prostate cancer, breast cancer, and melanoma (Oliver, 1991). Statins are one of the most promising classes of agents currently available for testing in cancer prevention. The overall safety of statins for use in humans has already been established in CVD treatment and prevention (CPS, 1998). Statins have a highly favourable safety profile for long-term use in cancer prevention (Demierre et al, 2005). Maximal statin concentration in serum, with standard anti-cholesterol dosing, is 10-200 nanomolar (Wong et al, 2002; Chan et al, 2003).

Research has shown that cholesterol biosynthesis inhibiting drugs, such as LOV, can reduce CRC incidence (Taylor et al, 2000; Demierre et al, 2005). However, the molecular basis behind their chemopreventive action, and the effects of various dietary lipid environments, remains elusive. Two large population based studies have shown statin-associated reductions in overall, CRC and prostate cancer incidence. Detailed reviews of the preclinical, epidemiological and clinical studies of statins in cancer prevention are available (Demierre et al, 2005; Hawke and Viner, 2005). Significant statin-associated reductions (14-28%) in overall cancer incidence has been demonstrated

by observational studies in humans from four major prospective prescription or medical database studies of statins (Blaiset al, 2000; Kaye et al, 2002; Graaf et al, 2004; Kaye and Jick, 2004; Friis et al, 2005). In some of these studies, a reduction (non-significant) in colorectal cancer incidence was reported (Blais et al, 2000; Graaf et al, 2004). Additional observational studies of the effect of statins on human cancer have been conducted (Beck et al, 2003; Cauley et al, 2003; and Boudreau et al, 2004; Platz et al, 2005; Singal et al, 2005). A significant reduction (50%) in CRC was found in a large population-based, case-control study called the Molecular Epidemiology of Colorectal Cancer (MECC) study (Poynter et al, 2005). In this study, statin use for at least five years was associated with a 47% reduction in the risk of developing CRC. Additional data from secondary analysis of a statin RCT emphasize the strong inverse association between CRC and long-term statin use. Although short-term statin use was not associated with colorectal adenoma risk (Wei, 2005), the effect of statins in these studies on the development of preneoplastic lesions development was not assessed. Despite these positive findings of a reduced risk of a variety of cancers, including CRC, with statin use, interpretation of data from observational findings has limitations. No RCT of statins has had cancer as a primary endpoint and the analysis of cancer as a secondary endpoint is hampered by their limited ability to assess cancer outcomes due to relatively small sample sizes and short follow-up periods, resulting in very small numbers of overall or specific cancer cases (Demierre et al, 2005).

Complete understanding of the cellular and molecular mechanisms underlying the effect of statins remains elusive as evidence suggests they are involved at many levels of cell regulation and interrelated pathways. The effect of statins in cancer, including CRC, can

be viewed as HMGCAR dependent and independent (Demierre et al, 2005). Not all effects of LOV are clearly related to inhibition of HMGCAR. Additional targets of statins have been identified, including, targeting the protein-degradation machinery of proteasomes which may account for the effects of statins on the cyclin-dependent kinase inhibitors p21 and p27 (Demierre et al, 2005), and inducing apoptosis (Agarwal et al, 2000) via modulation of several signalling pathways including the RAF-mitogen activated protein kinase kinase 1-extracellular regulated kinase (ERK) pathway (Wu et al, 2004). Up-regulation of p21 and p27 via HMGCAR-dependent and independent mechanisms has been found (Rao et al, 1999; Ukomadu and Dutta, 2003) and the effects are attainable in humans at standard LOV doses (Demierre et al, 2005). LOV treatment has been shown to increase apoptosis induction and that the modulatory effect on this process was related to caspase-3 activity and expression in leukaemic HL-60 cells (Wang et al, 2000). Lovastatin combined with celecoxib was demonstrated to induce apoptosis in human HT-29 colon cancer cells via induction of caspase-3 (Swamy et al, 2002). Similar results have been found using a combination of lovastatin and sulindac (Feleszko et al, 2002). Supportive research using rodent models demonstrated low-dose atorvastatin plus low-dose celecoxib suppressed development of invasive and non-invasive colonic adenocarcinomas by 95% (Reddy et al, 2005). Activation of caspases is supported by various additional research (Marcelli et al, 1998; Cafforio et al, 2005). LOV has also been found to inhibit cellular proliferation through induction of G1/S arrest and/or G2/M arrest (Park et al, 2001). The anti-proliferative and apoptotic effects of statins seem to be independent. Statins seem to induce apoptosis and inhibit proliferation to a greater degree in malignant than non-malignant cells (Wong et al, 2002; Wu et al,

2004), possibly due to the increased expression of HMGCAR and increased requirement of mevalonate-derived isoprenoids in tumor versus normal cells (Hentosh et al, 2001). Data suggests statins inhibit growth and induce apoptosis in CRC cell lines independent of the mutational status of $p21^{K\text{-}ras}$ (Ukomadu and Dutta, 2003). It has been shown that LOV induces apoptosis by inhibiting geranylgeranylation of the rho protein family (Agarwal et al, 2002a). In a recent study, the effect of LOV on apoptotic cell death triggered by radiation treatment was found to be dose dependent, with antiapoptotic effects occurring at low concentration (those achieved in humans), and proapoptotic effects at high concentration (Nubel et al, 2006). *In vitro* studies have shown that LOV is able to reverse radiation resistance of human tumor cells caused by oncogenic $p21^{K\text{-}ras}$ (Miller and Samid, 1995). The effect of LOV on radiosensitivity of human tumor cells harbouring wild-type $p21^{K\text{-}ras}$ is cell type specific (Fritz, 2003).

Chapter 3: Materials and Methods

Unless otherwise stated, chemicals and reagents used were purchased from Sigma Chemical Company, Mississauga, ON, Canada.

Colon Carcinogen

Azoxymethane (AOM; Sigma Chemical, Mississauga, ON, Canada) was used as the colon specific carcinogen and was dissolved in 0.9% saline. Animals were injected subcutaneously (s.c.) once weekly for three weeks at a concentration of 15 mg/kg body weight.

Experimental Animals

Male Fisher 344 rats (6 weeks old; weanling) were purchased from the Central Animal Care Facility (University of Manitoba) and acclimatized for one week prior to the experiments. Animals were housed in single wire meshed stainless steel cages (three rats per cage) using a 12:12h light/dark cycle. Temperature and humidity were controlled at 22°C and 50%, respectively. Animals had free access to food and water and were cared for according to the guidelines of the Canadian Council for Animal Care. Animals were killed at the fourth hour of the light cycle by CO_2 asphyxiation. Body weights were assessed twice weekly and recorded.

Experimental Diets

Experimental diets were formulated based on the AIN-76A diet (American Institute of Nutrition, 1977) as described in Table 2 and Appendix A to D. The low fat corn oil diet contained 5% fat by weight added as corn oil (LFC; Mazola Brand, Canada Safeway) and was considered the control group for initial comparisons before LOV treatment. The

Table 2: Composition of Experimental Diets[1, 2, 3]

Diet Ingredient	LFC	HFC	HFB
Casein	20.0	23.0	23.0
Corn Starch	52.0	33.8	33.8
Dextrose	13.0	8.5	8.5
Corn Oil	5.0	5.0	5.0
Cellufil	5.0	5.9	5.9
DL-Methionine	0.3	0.3	0.3
Choline Bitartrate	0.2	0.2	0.2
AIN-76 mineral mix	3.5	4.1	4.1
AIN-76 vitamin mix	1.0	1.2	1.2
Additional Dietary lipid [4]	-	18.0	18.0

1. All values represent the percentage composition unless otherwise stated and are isocaloric.
2. Dietary groups are as follows: low fat corn oil (LFC), high fat corn oil (HFC), and high fat beef tallow (HFB).
3. LOV treated groups were orally gavaged 20 mg/kg body weight per day for the last three weeks of the study.
4. Additional dietary lipid consisted of corn oil for the HFC group and beef tallow for the HFB group.

high fat diets contained an additional 18% fat by weight added as corn oil (HFC; Mazola Brand, Canada Safeway) or beef tallow (HFB; Maple Leaf Foods Inc., Winnipeg, MB, Canada) at the expense of an isocaloric amount of carbohydrate. The fatty acid composition of the experimental lipids and diets is given in Tables 3 and 4. The fatty acid composition of the experimental diets were modified in two distinct ways: (1) according to the absolute amount of total fat, and (2) the level of unsaturation. For example, the LFC diet had a lower absolute amount of total fat compared to both HFC and HFB diets. However, both the LFC and HFC diets have the same level of unsaturation on a percentage basis. The fatty acid composition of the experimental diets are shown in Table 4. Accordingly, the HFC diet has a higher level of unsaturation than the HFB diet. In the present study, we examined the effects of the following five dietary treatments: a low fat corn oil diet (LFC), a high fat corn oil diet (HFC), a high fat beef tallow diet (HFB), and an HFC and HFB dietary group treated with LOV (HFCα and HFBα, respectively). LOV was gavaged daily for three weeks (20 mg/kg body weight/day).

Experimental Design

A summary of the study protocol is detailed in Figure 7. Male weanling F344 rats were injected once per week for three weeks with azoxymethane (AOM; Sigma Chemical, Mississauga, ON, Canada) in fresh saline (15 mg/kg/week sc). Animals were given free access to lab chow over the duration of the injection period. One week after the last injection, all animals were placed on the low fat corn oil (LFC) diet. After 12 weeks on the LFC diet, one group of animals continued to receive the LFC diet (n=35) while the

Table 3: Fatty Acid Composition of Experimental Lipids[1,2]

Fatty Acid	Beef Tallow	Corn Oil
14:0	3.10	ND
16:0	23.00	10.10
16:1	2.20	ND
18:0	19.40	1.70
18:1n9	38.60	26.50
18:2n6	3.40	59.20
18:3n3	1.00	0.80
20:5n3	ND	ND
22:5n3	ND	ND
22:6n3	ND	ND

1. Expressed as percent composition; ND, non-detectable.

2. Fatty acid composition assessed by gas chromatography.

Table 4: Fatty Acid Composition of Experimental Diets[1,2]

Fatty Acid	Beef Tallow	Corn Oil
14:0	2.40	ND
16:0	20.60	10.60
16:1	1.80	0.10
18:0	16.00	2.20
18:1n9	34.90	25.50
18:2n6	14.30	57.80
18:3n3	2.60	2.40
20:5n3	ND	ND
22:5n3	ND	ND
22:6n3	ND	ND

1. Expressed as percent composition; ND, non-detectable.
2. Fatty acid composition assessed by gas chromatography.

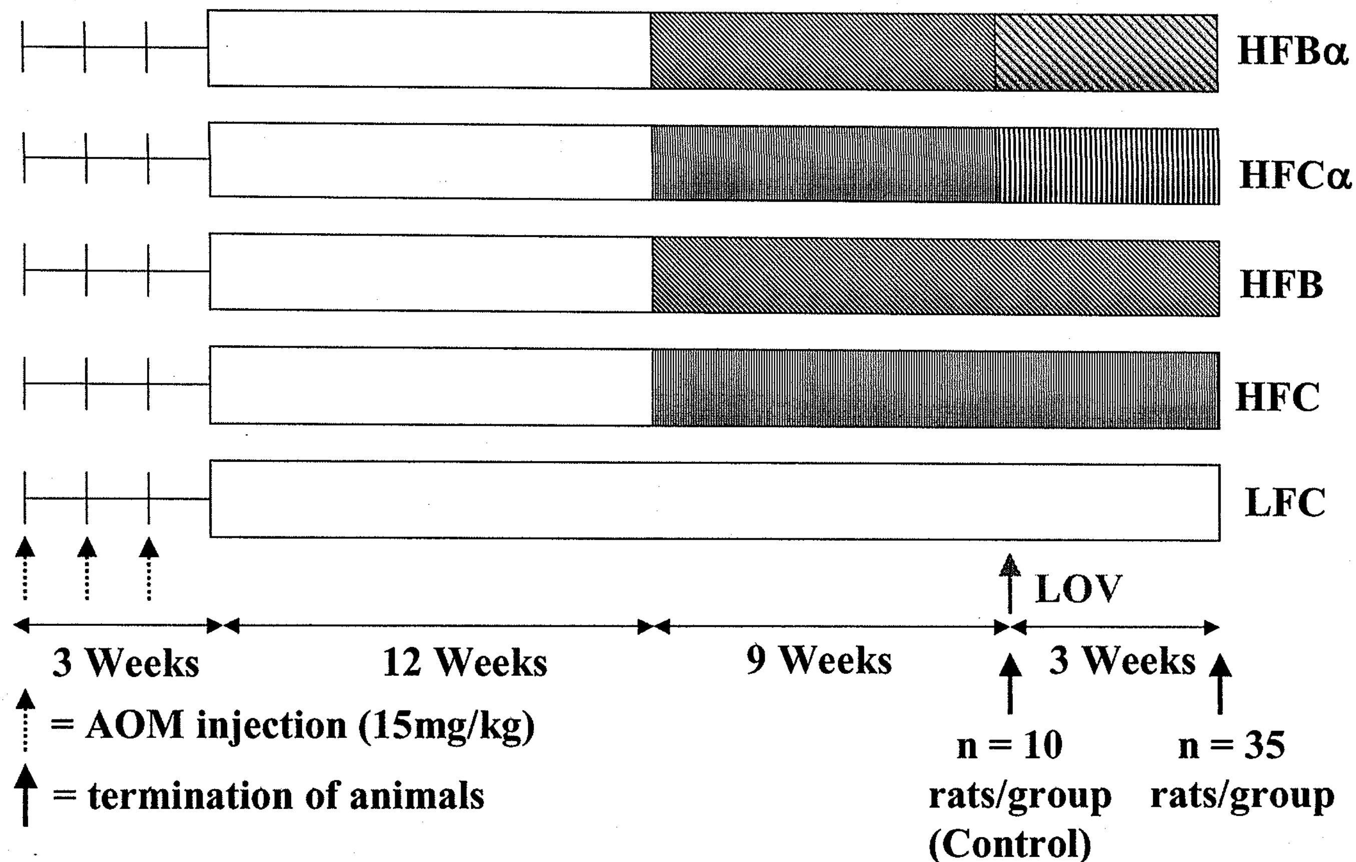


Figure 7. Experimental Design. Animals were fed standard laboratory chow during AOM injections, then placed on the LFC diet 1 week after last injection. Animals were allowed free access to LFC diet for 12 weeks. Animals were separated into 3 diet groups and fed an LFC (n-35), HFC (n=70), or HFB (n=70) diet. After 9 weeks of feeding, 10 animals per group were terminated. At this time, a subset of animals from the HFC and HFB groups (n=35/group) were treated with LOV (20 mg/kg body weight/day by gavage) for 3 weeks. At the end of the 3 weeks, 30-35 animals/group were terminated.

remaining animals were randomly allocated into the following two dietary groups: high fat corn oil (HFC; n=70) and high fat beef tallow (HFB; n=70). After nine weeks of feeding the experimental diets, 10 animals per group were killed by CO_2 asphyxiation and their colons assessed for ACF, microadenomas (MA) and tumors. These were considered the baseline/control groups for each dietary fat group, representing baseline values of ACF and MA before LOV treatment. At this time, a subset of animals from the HFC and HFB diet groups (n = 35/group) were randomly allocated to receive the LOV treatment (20mg/kg/day by gavage; APO-LOVASTATIN, Apotex Inc.). It should be emphasized that 12 weeks of dietary intervention is sufficient for selected advanced preneoplastic microscopic stages to proceed to the MA and/or tumor stage, whereas 24 weeks is required for selected initiated cells to complete sequential clonal expansion and appear as MA and/or tumors. An observed change in the population of ACF with various growth features allows us to speculate on the transition of lesions from one stage to the next. The total number of advanced preneoplastic lesions and/or the rate of growth will influence the eventual tumor outcome. All animals were killed after three additional weeks of dietary and/or LOV treatment (12 weeks after initial dietary randomization). Tumor incidence and MA enumeration (12 weeks) was assessed in 32-42 animals per group. Between eight to ten animals per group were designated for ACF analysis.

Lovastatin (LOV)

LOV was purchased from the Health Sciences Centre Pharmacy, Winnipeg, MB, Canada. To ensure accurate dose delivery to each animal and protection from ultraviolet light degradation, 40 mg LOV tablets were ground in a mortar and pestle daily, individually weighed out to provide a dose of 20 mg/kg body weight, and allocated to microcentrifuge

tubes. As LOV is insoluble in water and sparingly soluble in ethanol, the LOV crystalline powder was dissolved in 975 μl water plus 25 μl ethanol. Each dose was delivered via gavage between 1500 to 1600 hours daily for three weeks. Animals in each of the LOV treated groups were weighed twice weekly and an average group weight was calculated to establish and ensure an appropriate dosing of LOV.

Quantification of ACF

All rats were killed via CO_2 asphyxiation. The number of animals killed per group each day was similar among each dietary group. Colons for enumeration of ACF were removed immediately after termination, flushed with cold phosphate-buffered saline (PBS), cut along the longitudinal axis (from cecum to anus) on a cold plate (4°C) and palpated for tumors and microadenomas (MA). After screening for tumors and MA, colons were laid flat between filter papers and fixed in cold 70% ethanol for at least 24 hours. Each filter paper containing a colon was coded in order to be scored blindly. Following the protocol established by Bird (1987), fixed colons were scored by staining in a 0.2% solution of methylene blue in phosphate buffer saline (PBS) for 5-10 minutes, placed mucosal side up on a microscope slide and assessed by light microscopy (10X magnification) for the number and growth features of ACF defined as crypt multiplicity (number of crypts per focus). Criteria used to identify ACF include: (1) increased size, (2) thicker epithelial lining, and (3) increased pericryptal zone, and (4) elongated luminal opening, relative to normal crypts (Bird, 1995). Topographic enumeration of ACF in the whole mount of colon was used to represent preneoplastic lesions of microscopic dimensions. The number, distribution and multiplicity of ACF were determined along the entire length of the colon. Distribution of ACF was determined by counting and

recording the number of ACF in every 2 cm section of colon starting at the rectal (distal) end and working up to the ceacal (proximal) end. Crypt multiplicity was determined by counting and recording the number of crypts per focus. ACF with different growth features were categorized based on their crypt multiplicity as small (1-3 crypts/focus), medium (4-6 crypts/focus), or large (≥7 crypts /focus).

Assessment of Tumors

Colons destined for enumeration of ACF were removed immediately after termination, flushed with cold phosphate-buffered saline, placed on a cold plate (4^0C), cut along the longitudinal axis and palpated for tumors. The location and size (width and length) of MA and tumors were recorded for each colon. Size was the criterion used to distinguish MA from tumors; a lesion containing several crypts that protruded from the colonic surface and was smaller than $1mm^2$ was defined as an MA. Tumors were dissected out with 0.5 cm surrounding mucosa and fixed in 4% paraformaldehyde or 70% ethanol (for immunohistochemical analysis). Tumors designated for RT-PCR analysis were treated with RNAguard, dissected out without attached normal mucosa and snap-frozen in liquid nitrogen for RNA isolation. Tumors designated for Western blot analysis were dissected out without attached normal mucosa and snap-frozen in liquid nitrogen. The tumor assessment parameters used were those described previously by Bird (1996). Tumor parameters were assessed as follows: tumor incidence (percentage of total animals with tumors); tumor multiplicity (average number of tumors/tumor-bearing rat); average tumor size (mm^2) per tumor-bearing rat; average tumor size/group (average size of all tumors in a group); and tumor burden (average of the total tumor area in each tumor-bearing rat).

Tumor size (mm^2) per tumor-bearing rat was further examined by allocating tumors to one of three size categories: small (<12mm^2); medium (12-25mm^2); and large (>25mm^2).

Serum Lipids

Following a 12 hour fast, each animal was terminated and approximately 8-10 mL of blood was collected via cardiac puncture into 10 mL vacutainer tubes (containing 0.10 mL of 15% EDTA solution as anticoagulant) and immediately chilled on ice. Plasma separation was done via centrifugation at 14,000 x g for 10 minutes. Plasma total cholesterol, triacylglycerol, and low density lipoprotein (LDL) were determined via microplate assay using the Spectra Max 3000 analyzer (Molecular Devices, Sunnyvale, CA, USA). Assay Kits for total cholesterol, triacylglycerides and LDL cholesterol were purchased from Bio-Pacific Diagnostics Inc. and carried out according to manufacturers instructions. Measurements were carried out in triplicate for total cholesterol and triacylglyceride analysis and in duplicate for LDL analysis.

Total Cholesterol Cat # DCL 225-26

Four standards were prepared (0, 1.25, 2.50, and 5.00 mmol CHOL/L) in fresh saline (0.9% NaCl). Samples were prepared by mixing 15 μL plasma with 45 μL saline. Ten microlitres of each sample was added to each well along with 200 μL working reagent (1:1 ratio of cholesterol colour reagent to cholesterol phenol reagent; Cat. # DCL 210-75) and then incubated at 37°C for 5 minutes. Briefly, cholesterol esters are hydrolysed by cholesterol esterase. The free cholesterol is oxidized by cholesterol oxidase to cholesten-3-one with simultaneous production of hydrogen peroxide (H_2O_2). The H_2O_2 produced couples with 4-aminoantipyrine and phenol, in the presence of peroxidase, to yield a chromogen with maximum absorbance at 505 nm. The intensity of the colour produced

is directly proportional to the concentration of total cholesterol in the sample. Absorbance was read at 505 nm. Total serum cholesterol can be expressed as mmol/L or mg/dl. Total serum cholesterol in mmol/L is equal to absorbance of unknown sample divided by the absorbance of the 5 mmol/L (193 mg/dl) standard and then multiplied by the concentration of the standard (either 5 mmol/L or 193 mg/dl).

Triacylglycerol

Four standards were prepared (0, 0.5, 1.0, and 2.0 mmol TAG /L (177mgTAG/dL)) in fresh saline (0.9% NaCl). Samples were prepared by mixing 15 μL plasma with 45 μL saline. Ten microlitres of each sample was added to each well along with 100 μL of working reagent (20 mL triglyceride buffer per vial of triglyceride colour reagent; Cat. # DCL 210-75) and incubated at room temperature for 10 minutes. After incubation, 140 μL deionized water was added to each well and absorbance read at 515 nm. Briefly, serum TAG are hydrolysed to glycerol and free fatty acids by lipase. In the presence of ATP and glycerol kinase, the glycerol is converted to glycerol-1-phosphate. The glycerol-1-phosphate is oxidized by glycerol phosphate oxidase to yield H_2O_2. The condensation of H_2O_2 with 3,5-dichloro-2-hydroxy-benzenesulfonic acid (DHBS) and 4-aminoantipyrine in the presence of peroxidase produces a red colored quinoneimine dye which absorbs at 515 nm. The increase in absorbance at 515 nm due to the formation of the quinoneimine dye is directly proportional to the concentration of TAG in the sample. TAG concentration is expressed as mmol/L or mg/dl. TAG are calculated as the absorbance of the unknown sample divided by the absorbance of the 2 mmol TAG/L (177 mg TAG/dl) standard, and then multiplied by the concentration of the standard (either 2 mmol TAG/L or 177 mg TAG/dl).

Low Density Lipoprotein

Four standards were prepared (0, 0.7, 1.4, and 2.8 mmol/L Direct LDL calibrator; Cat # 249-10 for LDL analysis kit; SE 249 for LDL calibrator) in fresh saline (0.9% NaCl). To each well, 2 microlitres of each sample was added along with 200 μL and 66.7 μL of Direct LDL Buffer (Reagent 1) and Direct LDL Colour Reagent (Reagent 2), respectively. Samples were incubated at 37°C for 10 minutes. Absorbance was read at 546 nm. Briefly, the direct LDL assay is comprised of a detergent system that solubilizes only the non LDL lipoprotein particles, allowing reaction of the remaining particles with cholesterol enzymes resulting in a non-colour forming product. A second detergent system then solubilizes the LDL particles and allows a colour formation as a result of reaction with N,N-bis (4-sulfobutyl)-m-toluidine, disodium (DSBmT). This colour formation at 550 nm is proportional to the concentration of LDL cholesterol in the sample. LDL concentration can be expressed as either mmol/L or mg/dl and is calculated by dividing the absorbance of the unknown sample by the absorbance of the known standard (2.8 mmol/L) and multiplying by the concentration of the standard (2.8 mmol/L).

$p21^{K\text{-}Ras}$ Activity

A non-radioactive assay kit for Ras activation was purchased from Upstate Biotechnology (Cat #17-218). This assay depends on the affinity of GTP-Ras for the Raf-1 amino terminal domain which comprises the Ras-binding region and which is used as an affinity ligand to selectively precipitate active GTP bound Ras. Immobilized Raf-1 Ras-binding domain (RBD) was incubated with whole tumor tissue lysate/homogenate. Active Ras was bound to the bead containing the Raf-1 binding domain. Bound material

was assayed via Western blot analysis. It was probed with a pan-isoform-specific anti-Ras antibody (clone RAS10, Cat #05-516). The quantity of activated Ras is directly proportional to the intensity of the Ras signal, hence this assay provides a relative measure of the Ras activation state.

Immediately after animal termination, fresh whole tissue homogenate of normal appearing colonic mucosa and colonic tumors was prepared using 1X Mg^{2+} Lysis/Wash buffer (MLB) according to manufacturer's directions and diluted to 1 μg/μL total protein. Ten microlitres of Raf-1 RBD, agarose conjugate was added to 1 mg cell lysate per assay (one affinity reaction per lane of SDS-PAGE). Reaction mixture was gently rocked and incubated at 4°C for 30 minutes. Agarose beads were collected by pulsing (5 seconds in the microcentrifuge at 14, 000 x g) and the supernatant drained. The agarose beads were washed three times with MLB using 1.0 mL, 350 μL, and 350 μL, respectively and then resuspended in 20 μL 2X Laemmli sample buffer. Samples were heated for 5 minutes and the beads collected by microcentrifuge pulse. SDS-PAGE was performed on the supernatant and the proteins transferred to nitrocellulose which was then blocked in freshly prepared PBS containing 5% non-fat dry milk and 0.05% Tween-20 (PBS-MLK-T) for 20 minutes at 25°C with constant agitation. The nitrocellulose was incubated with 1 μg/mL of Ras, clone RAS10, diluted in freshly prepared PBS-MLK-T overnight with agitation at 4°C. The nitrocellulose was washed twice with water and incubated in the secondary reagent (goat anti-mouse HRP conjugated IgG, 1:5000 dilution) in PBS-MLK-T for 1.5 hours at room temperature with agitation. The nitrocellulose was washed twice with water, once in PBS-0.05% Tween 20, and four times with water for 5

minutes each. The detection method used was the same as in the Western blot analysis procedure described below.

Analysis of Tissue Protein Concentration

The supernatant of each sample homogenate (representing whole, cytosolic, or membranous fractions) was measured for protein concentration using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) as the standard. This microassay is based on the Bradford method (1976) using a Coomassie protein assay reagent (Pierce, Rockford, IL, USA). Standard and experimental samples were analyzed in triplicate using a Spectra Max 3000 (Molecular Devices, Sunnyvale, CA, USA).

Tissue Preparation for Western Blot Analysis

The recipes for the buffer systems and gels used for Western blot analysis are outlined in detail in Appendix E to G. Linearity of detection was validated using a range of sample protein amounts. To ensure equal amounts of protein were loaded on the gels and transferred, β-actin was probed. Densitometric analysis using Scion Image for windows software was conducted on visible immunoreactive protein bands and expressed as pixels. Representative Western blots for the experimental molecules investigated are in Appendix J.

Preparation and Separation of Cytosolic and Membrane Mucosal Tissue Fractions

For determination of hepatic and colonic mucosa cytosolic and membrane bound K-ras, the tissues were washed in cold PBS and re-suspended in PBS-PMSF [10 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, and 0.25 mM PMSF] containing 0.5 μg/ml leupeptin, 2.0 ug/ml aprotinin, 10 μg/ml trypsin inhibitor, and 1 mM SOV protease

inhibitors. Samples were homogenized on ice, and all debris and nuclei were removed by centrifugation at 800 X g for 10 minutes at 4°C. The supernatants were further centrifuged at 40,000 X g for 30 minutes at 4°C. The resulting supernatant (cytosolic fraction) was saved, and the 40,000 X g pellets (membrane fraction) was solubilized in lysis buffer [10 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, 10 mM sodium deoxycholate, 1 mM PMSF, and 1% Triton X-100] containing 0.5 ug/ml leupeptin, 2.0 ug/ml aprotinin, 10 ug/ml trypsin inhibitor, and 1 mM SOV protease inhibitors, and centrifuged at 40,000 X g for 30 minutes at 4°C. The clear supernatants representing cytosolic and solubilized membrane fractions were measured for their protein concentration using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA) with BSA as the standard as described above. It should be noted that due to limited availability, it was not possible to separate tumor tissue into these fractions and instead tumor whole homogenate was used for analysis.

Preparation of Whole Cell (Colonic Mucosa, Tumor and Liver) Homogenate

Fresh, thawed colonic mucosal scrapings, tumors and liver tissues were placed in 2 mL of ice cold PBS-PMSF [10 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, and 0.2 mM PMSF]. Just prior to homogenization, the following protease inhibitors were added to each sample, 1 ug/mL leupeptin, aprotinin, and trypsin inhibitor, as well as 1.0 mM PMSF and SOV (Na_3VO_4). Each sample was homogenized on ice with a polytron and all debris and nuclei were removed by centrifugation at 15,000X g for 20 minutes at 4°C. The supernatant was drawn, separated into small aliquots (50-100 µL each) and stored at minus 80°C until further analysis. The supernatants of each sample representing whole

homogenate was measured for protein concentration using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA) with BSA as the standard.

SDS-PAGE, Western Blotting, Immunodetection and Quantification

SDS-PAGE and Western transfers were carried out by the methods of Laemmli (Laemmli, 1976). The specific protocols for each protein analyzed by Western blot technique are outlined in Tables 5 through 7. All antibodies were diluted with fresh PBS. To ensure that the detection of immunoblotted proteins by ECL was linear, a trial linear range was performed for each specific protein (antibody) analyzed. This protocol included loading increasing amounts of protein (between 10-120 μg depending on the protein being analyzed and the type of tissue) to determine the optimum protein concentration for the analysis. The amount of protein loaded for each antibody was determined based on the median of the linear range. A carry over sample was loaded onto each gel to control for gel-to-gel variation, allowing for comparison of expression from one gel to another for the same antibody. The antibodies used included $p21^{K\text{-}ras}$, HMGCAR, LDLr, ERK1, ERK2, cyclin D1, and caspase-3. All antibodies were incubated overnight at 4°C in a humid chamber. All proteins were solubilized in appropriate (either 2X or 6X SDS) sample buffer (Appendix G) and heated for three minutes at 95°C and subjected to SDS-PAGE via separation on a discontinuous denaturing gel system using 5% stacking gel and the appropriate percentage resolving gel (Appendix G). A pre-stained molecular weight protein ladder (Gibco) was used as a standard to assess the molecular weight of the protein bands. Appropriate positive controls were utilized for each blot. The amount of protein from colonic mucosa, tumor, or liver whole homogenate lysate used for immunoblotting

Table 5. K-Ras Protein Immunoblotting Conditions for Colonic Mucosa, Tumor and Liver Tissue[1]

Protein	Tissue[2]	Molecular Weight (kDa)	Amount of Protein Loaded (μg)	Gel %	Primary Antibody Dilution[3]	Secondary Antibody Dilution[4]	Positive Control[5]
p21$^{K\text{-}ras}$	Mucosa (m)	21	60	15	1 : 100	1 : 1,000	KNRK
	Mucosa (c)	21	60	15	1 : 100	1 : 1,000	KNRK
	Mucosa (w)	21	30	15	1 : 100	1 : 1,000	KNRK
	Tumors	21	30	15	1 : 100	1 : 1,000	KNRK
	Liver (m)	21	30	15	1 : 100	1 : 1,000	KNRK
	Liver (c)	21	30	15	1 : 100	1 : 1,000	KNRK
	Liver (w)	21	30	15	1 : 100	1 : 1,000	KNRK

1. All gels were transferred for 90 minutes. A Benchmark Prestained Protein Ladder with a 10-200 kDa range of molecular weight markers (GIBCO, BRL; cat#10748-010) was loaded on each gel (10 µg/lane/gel). After ECL (Chemiglow) treatment, each gel was exposed for 15 minutes.

2. Colonic mucosa and liver tissues were separated into membranous and cytosolic fractions in addition to whole cell homogenate. Membranous and cytosolic fractions and whole cell homogenate are designated by (m), (c), and (w), respectively.

3. Primary Antibody used was $p21^{K\text{-}ras}$ (F234) from Santa Cruz Biotechnology (Cat # sc-30).

4. Anti-mouse HRP from Santa Cruz Biotechnology.

5. KNRK whole cell lysate from Santa Cruz Biotechnology was loaded on each gel (20 µg/lane/gel).

Table 6. HMGCAR and LDLr Protein Immunoblotting Conditions for Colonic Mucosa, Tumor and Liver Tissue[1]

Protein	Tissue	Molecular Weight (kDa)[2]	Amount of Protein Loaded (μg)	Gel %	Primary Antibody Dilution[3]	Secondary Antibody Dilution[4]	Positive Control[5]
HMGCAR	Mucosa	97	20	12	1 : 100	1 : 1,000	Liver homogenate
	Tumor	97	20	12	1 : 100	1 : 1,000	
	Liver	55	20	12	1 : 100	1 : 1,000	
LDLr	Mucosa	160	60	10	1: 1,500	1:100,000	Liver homogenate
	Tumor	160	60	10	1: 1,500	1:100,000	
	Liver	160	10	10	1: 1,500	1:100,000	

1. All gels were transferred for 90 minutes. A Benchmark Prestained Protein Ladder with a 10-200 kDa range of molecular weight markers (GIBCO, BRL; cat#10748-010) was loaded on each gel (10 μg/lane/gel). After ECL (Chemiglow) treatment, each gel was exposed for 2-4 and 10 minutes for HMGCAR and LDLr respectively.

2. Depending on the tissue, HMGCAR may separate as the different isoforms of protein at 97, 60, or 30kDa molecular weights (El-Sohemy and Archer, 1999; Ness et al, 1986).

3. The LDLr primary antibody was a generous gift from Dr. G. Ness.

4. The secondary antibody for HMGCAR and LDLr were anti-rabbit HRP from Santa Cruz Biotechnology.

5. Liver homogenate was used as the positive control and was loaded at 20μg/lane/gel.

Table 7. ERK-1, ERK-2, Cyclin D1, and Caspase-3 Protein Immunoblotting Conditions for Colonic Mucosa, Tumor and Liver Tissue[1]

Protein	Tissue	Molecular Weight (kDa)	Amount of Protein Loaded (μg)	Gel %	Primary Antibody Dilution	Secondary Antibody Dilution	Positive Control[2]
ERK-1/2	Mucosa	44/42	15	12	1 : 2,000	1 : 5,000	NIH 3T3
	Tumors	44/42	15	12	1 : 2,000	1 : 5,000	NIH 3T3
	Liver	44/42	15	12	1 : 2,000	1 : 5,000	NIH 3T3
Cyclin D1	Mucosa	34	30	12	1 : 2,000	1 : 2,000	KNRK
	Tumors	34	30	12	1 : 2,000	1 : 2,000	KNRK
	Liver	34	30	12	1 : 2,000	1 : 2,000	KNRK
Caspase-3	Mucosa	33	60	10	1 : 100	1 : 5,000	HuT78 cells
	Tumors	33	60	10	1 : 100	1 : 5,000	HuT78 cells
	Liver	33	10	10	1 : 100	1 : 5,000	HuT78 cells

1. All gels were transferred for 90 minutes. A Benchmark Prestained Protein Ladder with a 10-200 kDa range of molecular weight markers (GIBCO, BRL; cat#10748-010) was loaded on each gel (10 µg/lane/gel). After ECL (Chemiglow) treatment, each gel was exposed for 30 seconds, one, and three minutes respectively for ERK 1 & 2, CD1, and Caspase-3. For additional information, see Materials and Methods section.

2. NIH 3T3 cell lysate (Upstate Biotechnology Lake Placid, N.Y.; cat# 06-182) and HuT78 cells were used as positive controls for CD1 and Caspase-3, respectively.

depended on the antibody being analyzed. As described previously, determination of the optimum amount of protein to utilize for each blot was determined by running a linear range test. To allow comparisons of protein expression among tissues, the same amount of protein was used for mucosa, tumors and liver homogenate.

Electrophoretically separated proteins were electrotransferred onto Hybond ECL nitrocellulose membrane (Amersham Life Sciences Co., Arlington Heights, IL, USA) for one hour at room temperature. After transblotting the electrophoretically resolved proteins, the blots were blocked with 5% non-fat dry milk (Carnation non-fat dry milk) dissolved in TBST for an hour at room temperature. Blots were then incubated with the primary antibody under investigation (Tables 5 to 7) and diluted in TBST containing 0.5% non-fat dry milk overnight at 4°C. Blots were washed extensively in TBST and re-incubated with the appropriate peroxidase-linked secondary antibody (Tables 5 to 7) diluted in TBST containing 0.5% non-fat dry milk. The blots were then thoroughly washed in excess TBST. Immunoreactive protein was assessed using the enhanced chemiluminescence detection system (ECL; Amersham Life Sciences, Arlington Heights, IL, USA). Each blot was incubated with ECL reagents for one minute and analyzed using the Fluorchem analyzer. Exposure time was dependent on the protein under investigation and ranged from 10 seconds to 5 minutes. Hepatic LDLr protein migrated as a 160 kDa band, whereas HMGCAR was detected at a size of approximately 97 kDa. The molecular weights of HMGCAR in tumors (55 kDa), mucosa (97 kDa) and liver (97 and 200 kDa). The relative amount of immunoreactive protein was quantified by densitometric analysis using the Fluorchem analyzer Software. Coomassie blue was used to stain total proteins to ensure equal loading. In order to ensure equal sample loading, a

common "carry over" sample was loaded onto each gel. In addition, analysis of β-actin protein was performed on randomly selected gels.

Immunoblot Analysis of p21$^{K\text{-}ras}$

Colonic mucosa cytosolic and membrane fractions corresponding to 60 μg of protein were solubilized in an appropriate amount of 6X SDS sample buffer. Whole cell homogenate from colonic mucosa, tumors and liver were performed using 30 μg protein solubilized in 2X SDS sample buffer. Samples were heated for three minutes at 95°C and separated on a discontinuous denaturing gel system using 5% stacking gel and then further resolved by extended electrophoresis on a 15% reduced polyacrylamide minipro gel, along with pre-stained SDS-PAGE molecular weight markers (GIBCO BRL), and a positive control (KNRK whole cell lysate, Santa Cruz Biotechnology for K-ras). To ensure equal loading and even transfer of proteins, each gel was stained with coomassie blue after transfer completion. Electrophoresis was run at 170 volts for 90 minutes using the molecular weight standard to track the progress of protein separation. Electrophoretically resolved proteins were electrotransferred onto 0.45 micron nitrocellulose hybond-C membrane (Hybond ECL membrane, Amersham Life Sciences Co., Arlington Heights, IL, USA) in a Trans-blot Electrophoretic BIO-RAD miniprotean cell. The transfer was performed in a 20% Tris-methanol buffer at 100 volts 60 minutes at 4°C. The nitrocellulose hybond-C membrane was prepared before use by soaking it in distilled water for 1 hour and then in transfer buffer for 10 minutes prior to transfer. After transblotting the electrophoretically resolved proteins, the blots were blocked with 5% non-fat dry skim milk powder (SMP) dissolved in TBST (Tris buffered saline with 0.1% Tween-20) for 1 hour at room temperature on a rocker. The membrane was washed

for 40 minutes with a minimum of three changes of TBST (50 ml). Blots were then incubated and probed with primary antibody (anti-K-ras mouse monoclonal IgG_{2a} antibody; K-Ras (F234); cat #sc-30, Santa Cruz Biotechnology) diluted (1:100) in TBST containing 0.5% non-fat dry milk. This antibody is specific for the K-ras p21 translational product of the K-ras gene and is non cross-reactive with p21 translational products of the H- or N- ras genes. Blots were washed in TBST for one hour with five changes of TBST. Incubation with a peroxidase-linked secondary antibody (anti-mouse horseradish peroxidase (HRP) conjugated IgG; Santa Cruz Biotechnology) diluted (1:1000) in TBST containing 0.5% non-fat dry milk was performed for one hour at room temperature. The blots were then thoroughly washed in excess TBST as previously described. Immunoreactivity was detected using the ECL and Fluorchem analyzer and densitometric analysis was performed as described previously.

Immunoblot Analysis of HMGCAR and LDLr

Polyclonal antisera to a peptide corresponding to a portion of the C-terminal region of the rat LDL receptor was generated in rabbits according to the method by Ness (Ness and Zhao, 1994) and was generously provided to our lab by Dr. Ness. The molecular weight of the mature LDLr has been determined to be about 160 kDa using a monoclonal antibody (Takahashi et al, 1992). The antisera recognized a single band at approximately 160 kDa which agrees with the size of the purified LDL receptor (Ness et al, 1996). Thus the antisera appears specific for the LDLr. The LDLr migrated as a 160 kDa band, whereas HMGCAR was detected at a size of approximately 97 kDa. Relative amounts of immunoreactive protein were quantified as described previously. Under the conditions used (excess primary antibody, secondary antibody, amount of LDLr protein in sample,

and exposure time) good linearity was obtained with respect to protein concentrations. In some studies investigating the effects of LOV on protein expression, the predominant form of HMGCAR was found to be the 100 kDa form instead of the 200 kDa possibly due to cleavage of intermolecular disulfide linkages. In addition, the molecular weight of HMGCAR in colonic tumors has been found to be most predominant at 55 kDa (Sample and Ness, 1986).

Analysis of Tissue mRNA Expression by RT-PCR

All reagents and enzymes were obtained from Gibco BRL (Burlington, ON, Canada) unless otherwise specified. Recipes and additional details regarding buffers, TBE, gels and ladders used are found in appendix H. Extraction of total RNA was based on the method developed by Chomczynski and Sacchi (1987). Colonic mucosa, tumor and hepatic tissues were placed in 500 μl denaturation solution (4M guanidine thiocyanante, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). All samples were homogenized for 30 seconds with a tissue grinder pestle (Kontes #749515-000) in 1.5 ml conical microcentrifuge tubes. One-tenth volume of 2M sodium acetate (pH 5.2) and 1 volume water-saturated ultra pure phenol with 0.1% hydroxyquinoline (w/w) were added to each sample and vortexed. One-fifth volume of chloroform-isoamyl alcohol (49:1 v/v) was added to each sample and then vortexed and incubated on ice for 40 minutes. The resulting suspension was centrifuged at 16,000 x g for one hour at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 2 volumes of absolute ethanol, and placed at minus 80°C overnight in order to allow the RNA to precipitate out of solution. Centrifugation at 16,000 x g for one hour at 4°C allowed recovery of the RNA. The resultant RNA pellets were washed by adding 400 μl 80% ethanol and

centrifuged at 16,000 x g for ten minutes at 4°C. The ethanol was removed and the pellets were air-dried at room temperature for 15 minutes. The pellets were resuspended in 33 μl of autoclaved ultra pure deionized water. DNAse 1 (37U) and RNA guard (38.9U; Pharmacia, Montreal, Quebec, Canada) were added to a solution consisting of 40 mM Tris (pH 7.5) and 6 mM $MgCl_2$ to a total volume of 50 μl, then added to the suspended RNA, and incubated for one hour at 37°C. The DNAse treatment was inactivated by adding 100 mM EDTA. Each sample was then phenol extracted and ethanol precipitated as described previously. The RNA pellet was re-suspended in 30 μl autoclaved ultrapure deionized water. The concentration and integrity of each sample was determined by spectrophotometry at 260 nm and 280 nm absorbance, respectively. cDNA was synthesized by reverse transcription (RT) from 1 μg total RNA in a 20 μl reaction volume according to the method detailed by Gibco BRL. One microgram of RNA, 8 μl of water and 1 μl of oligo DT (500 μg/ml) were combined and heated to 65°C for 10 minutes and the mixture placed immediately on ice for 5 minutes. After cooling, 1 μl (39 U) of RNA Guard (Pharmacia), 2 μl of 100 mM DTT, 2 μl of 5 mM dNTP and 5X first strand buffer were added and briefly vortexed. RT was carried out by the addition of 1μl (200U/μl) of M-MLV (Moloney murine leukemia virus) at 42°C for 2 hours.

All polymerase chain reactions (PCR) were carried out using a PTC-100 Thermocycler (MJ Research Inc.). After cDNA synthesis, 0.5-5.0 μl of 10X diluted cDNA was amplified in a 50 μl PCR reaction. The PCR reaction mixture contained 1.87 U Taq DNA polymerase, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 20 mM Tris (pH 8.4), 50 mM KCl, and 0.5 μM 5' and 3' primers (Gibco BRL, Life Technologies Inc.) HMG CoA

reductase, LDL receptor, ERK-1, ERK-2, Cyclin D1, and Beta-actin expression was analyzed using the appropriate primers and conditions (Table 8). Beta-actin was used as the house-keeping gene. All reaction mixtures were covered with mineral oil (Sigma) and preheated for 2 minutes at 94°C. The primer sequences for Beta-actin are as follows (Good, 1999): 5' GTG GGG CGC CCC AGG CAC CA 3' (forward) and 5' CTC CTT AAT GTC ACG CAC GAT TTC 3' (reverse). RT-PCR was completed in 23 cycles by denaturation at 94°C for one minute, annealing at 52°C for 2 minutes, and extension at 72°C for three minutes. The primer sequences for HMG CoA reductase are as follows (Genebank Accession No. Ax13722): 5' ATG CTC CTT GAA CAC CTA GCA TCT 3' (forward) and 5' AGG TTC CAA TGG CAA CAA CAG AAG 3' (reverse). RT-PCR was completed in 35 cycles by denaturation at 95°C for one minute, annealing at 60°C for one minute, and extension at 72°C for one minute. The primer sequences for the LDL receptor are as follows (Genebank Accession No. Ax13722): 5' GCC TTA ACT TAG GCA GGT CAT 3' (forward) and 5' ACG CTA CCG GGA TTG TTT AAG 3' (reverse). RT-PCR was completed in 35 cycles by denaturation at 95°C for one minute, annealing at 60°C for one minute, and extension at 72°C for one minute. The primer sequences for the ERK-1 are as follows (Genebank): 5'GCA TCA AAC CTA CTG TCA GCG CAC G 3' (forward) and 5' 3' (reverse). RT-PCR was completed in 27 cycles by denaturation at 94°C for one minute, annealing at 60 °C for one minute, and extension at 72°C for one minute. The primer sequences for the Cyclin D1 (CD1) are as follows (Genebank; D14014): 5' TCA AGT GTG ACC CGG ACT GC 3' (forward) and 5' ACT TCC CCT TCC TCC TCG GT 3' (reverse). RT-PCR was completed in 40 cycles by denaturation at 95°C for one minute, annealing at 60°C for one minute, and extension at 72°C for one

minute (Genebank; CD1; Accession number D14014). All samples were subjected to a final elongation period of ten minutes at 72°C. When the PCR reactions were complete, 5μl of 10X PCR loading buffer was added and all samples were stored at -20°C until further analysis. For each primer, sequential amounts of cDNA were used to ensure sample amounts were within the linear range and to avoid the saturation limit of the Taq DNA polymerase (Appendix I).

PCR products were separated on a 2% agarose gel containing 3 ml 10X TBE and 4.5μl ethidium bromide in TBE buffer using the Gibco BRL Horizon 11-14 gel electrophoresis apparatus at 150 volts for 25 minutes. Equal volumes of each sample were loaded (15μl) and all primers for one sample were loaded on a single gel to reduce variation within samples. The resulting gels were photographed under ultraviolet illumination with Polaroid film. The resulting photographs were scanned using a Hewlett Packard ScanJet4c scanner and Corel Photopaint version 5.0 PC software. The area of the product bands detected on film (in pixels) was calculated using Scion Image Software (Version 2.0). The area of the band corresponding to the particular primer (HMG-CAR, LDLr, ERK-1, or CD1 primers) was expressed as a ratio relative to the area of the band corresponding to beta-actin for each sample. Beta-actin is considered the "housekeeping gene" that is present in equal amounts in all cells (Wong et al, 1994) and has been used previously to quantify PCR products from rat colonic neoplasms (Yoshimi et al, 1994; Bird et al, 1997). The very limited amount of sample available for RT-PCR analysis necessitated that each gel contain all genes under investigation for each tissue sample. Therefore, representative comparison gels were not able to be conducted. The images were stored for future reference.

Table 8. Primer Sequences and Conditions for RT-PCR

Primer	Sequence (forward/reverse)	Product Size (bp)	Conditions (Temp °C /min)			Cycles
HMGCAR[1]	ATGCTCCTTGAACACCTAGCATCT		95	60	72	
	AGGTTCCAATGGCAACAACAGAAG	700	(1)	(1)	(1)	3
LDLr[1]	GCCTTAACTTAGGCAGGTCAT		95	60	72	
	ACGCTACCGGGATTGTTTAAG	399	(1)	(1)	(1)	35
ERK-1[1]	GCATCAAACCTACTGTCAGCGCACG		94	60	72	
	TGTACTGAGGCCCCGGAGGATCT	178	(1)	(1)	(1)	27
Cyclin D1[2]	TCAAGTGTGACCCGGACTGC		95	60	72	
	ACTTCCCCTTCCTCCTCGGT	424	(1)	(1)	(1)	40
Beta-Actin[3]	GTGGGGCGCCCCAGGCACCA		94	52	72	
	CTCCTTAATGTCACGCACGATTTC	541	(1)	(2)	(3)	23

1. Genebank (LDLr; Accession number Ax13722).

2. Genebank (CD1; Accession number D14014).

3. Good, 1999.

Statistical Analysis

All statistical analyses were carried out using the Statistical Analysis System (SAS) for microcomputers (Version 6.06, SAS Institute Inc., Cary NC, USA) as described for each experiment. Statistical significance was taken at p [0.05 or less.

Differences among the dietary groups in gene expression were determined by analysis of variance (ANOVA) combined with the Duncan's multiple range test at $p \leq 0.05$. Differences between normal colonic mucosa and tumors were analysed using a student's t-test at $p \leq 0.05$.

Chapter 4: Results

THE EFFECT OF TYPE AND AMOUNT OF DIETARY LIPID AND LOVASTATIN TREATMENT ON THE MULTI-STEP PROCESS OF COLON CARCINOGENESIS

For purposes of clarity, the experimental findings have been sub-divided into three main sections based on the effect of dietary lipids and LOV on (1) the development of ACF, MA and tumors, (2) the cholesterol biosynthetic pathway and associated molecular target molecules and, (3) associated cellular signalling and apoptotic molecules.

PART A: An Investigation of the Effects of Dietary Lipids and Lovastatin on the Growth and Development of Aberrant Crypt Foci, Microadenomas, and Tumors in the Multi-Step Process of Colon Carcinogenesis

Experimental Approach

Male F344 rats were injected with azoxymethane (15mg/kg body weight/week for three weeks) and fed a low fat corn oil (LFC) diet for 12 weeks. They were then divided into two additional groups receiving a high fat corn oil (HFC) diet or beef tallow (HFB) diet for an additional nine weeks, after which a subset of rats from the HFC and HFB groups were orally gavaged with LOV (20 mg/kg body weight/day) for three weeks.

Body weights of each animal were measured weekly as well as at final termination. Average body weight per group was then calculated. Blood samples were collected at weeks 21 and 24 terminations and followed the procedures outlined in the Chapter 3.

Effect of High Fat Diets and Lovastatin on Body Weight and Blood Lipids of F344 Rats

At the conclusion of the study period, the body weights of the male F344 rats in both the HFC and HFB tallow groups were significantly higher ($p < 0.05$) than the body weights of the LFC group. The order from highest mean body weight to lowest, was HFC > HFB > HFCα > HFBα > LFC (417.6g, 413.0g, 404.4g, 399.5g, 390.2g; Table 9).

In rats, LOV treatment has been demonstrated to be ineffective at lowering blood cholesterol levels (Endo et al, 1979; Fears et al, 1980). In these animals, LOV treatment results in elevation of HMGCAR enzyme level due to increased mRNA levels and stability of the enzyme (Singer et al, 1984). Although most hepatocytes of control rats do not synthesize detectable amounts of HMGCAR, they can be induced to do so by LOV treatment. At the diurnal high point, four hours after darkness, rat liver expresses upwards of 5-fold more HMGCAR activity. These increases in hepatic HMGCAR partially explain the reason that long-term LOV treatment does not lower serum cholesterol levels in rats (Singer et al, 1984). As part of the study, serum lipids were measured at selected endpoints and are discussed below. In the rat, the intestine clears plasma-derived cholesterol through the receptor-dependent and independent uptake of low density lipoproteins (Spady et al, 1983). *In vivo* studies in the rat show that LDL uptake in isolated enterocytes is constant irrespective of dietary manipulations that varied local cholesterol synthesis (Stange and Dietschy, 1983; Reimann et al, 1992). In rats, statin treatment has been shown to produce little, if any, cholesterol-lowering effects (Yamauchi et al, 1991; Moghadasian, 1999).

After nine weeks of treatment with LFC, HFC, and HFB diets, serum total cholesterol, triacylglycerol and LDL levels were assessed (Table 10). Total cholesterol concentration was significantly higher ($p<0.04$) in the LFC group compared to the HFB group. The total cholesterol concentration for the dietary groups, in decreasing order was

Table 9: **Body Weights of Male F344 Rats after 24 Weeks of Intervention with LFC, HFC, HFB, HFCα and HFBα Diets[1, 2, 3, 4].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Body Weight[5, 6]	390.2 ± 5.4	417.6 ± 8.2*	404.4 ± 6.0	413.0 ± 5.6*	399.5 ± 4.7

1. Values are means ± SE.
2. Statistical significance is as follows: *, different from LFC ($p<0.05$).
3. Number of animals per group was between 32 and 42.
4. Animals were terminated 24 weeks after the last AOM injection.
5. Body weight was recorded in grams.
6. The designation "α" denotes the LOV treated group. LOV was gavaged at 20mg/kg body weight/day for three weeks.

Table 10: Day Time Levels of Plasma Total Cholesterol, Triacylglycerol, and LDL at 21 and 24 Weeks after Treatment with LFC, HFC, HFB Diets[1, 2, 3].

	Diet Group		
	LFC	**HFC**	**HFB**
Total Cholesterol (mmol/L) [π, λ]			
21 Weeks	3.31 ± 0.17[a, *]	2.97 ± 0.12[ab]	2.60 ± 0.16[b, *]
24 Weeks	3.96 ± 0.08[a]	2.97 ± 0.11[c]	3.46 ± 0.13[b]
Triacylglyceride (mmol/L) [π, λ]			
21 Weeks	1.86 ± 0.07[a]	0.79 ± 0.02[b]	0.88 ± 0.07[b]
24 Weeks	1.35 ± 0.04[a]	1.09 ± 0.08[b]	1.12 ± 0.13[ab]
LDL (mmol/L)			
21 Weeks	1.73 ± 0.12[a]	1.86 ± 0.05[a]	1.49 ± 0.12[a]
24 Weeks	1.76 ± 0.13[a]	1.63 ± 0.09[a]	1.57 ± 0.06[a]

1. Values are means ± SE.

2. Values in a row with different superscripts are significantly different at $p<0.04$ and $p<0.05$ for total cholesterol and triacylglyceride, respectively at 21 weeks and $p<0.0001$ and $p<0.02$ for total cholesterol and LDL, respectively at 24 weeks. * represents significant differences between weeks 21 and 24 of the study. π represents significant main effects low versus high fat diet treatments ($p< 0.02$, $p<0.058$, $p<0.0003$ for total cholesterol, LDL and triacylglycerol, respectively); λ represents significant main effect of dietary saturated versus unsaturated fat ($p<0.05$ and $p<0.0001$ for total cholesterol and triacylglycerol, respectively). Note that due to significant differences between day and night time triacylglycerol levels, comparison between the two study time points was not possible.

3. Animals were terminated during the day at 21 and 24 weeks of the study, representing 9 and 12 weeks of dietary intervention with the LFC, HFC, or HFB diets. n=8-12 animals/group.

LFC > HFC > HFB. Serum LDL concentration was significantly higher in the HFC group compared to the HFB group. The LDL concentration for the dietary groups, in decreasing order was HFC > LFC > HFB. Serum triacylglyceride concentration was significantly higher ($p<0.0001$) in the LFC group compared to either the HFC or HFB dietary groups. For both total cholesterol and triacylglyceride, significant main effects of low versus high dietary fat treatment as well as saturated versus unsaturated dietary fat treatment were detected. These effects did not quite reach the level of statistical significance for LDL cholesterol. The LFC diet resulted in the highest concentration of total cholesterol, whereas the HFB diet demonstrated the lowest concentration at 21 weeks. The unsaturated fat diets seemed to result in a higher total cholesterol concentration compared to the saturated fat diet. The low fat diet produced the highest triacylglyceride level, whereas rats in the high fat diet groups both had significantly lower triacylglyceride concentrations.

Diurnal variation in blood lipid and metabolic enzyme activity produced significant differences between day and night time triacylglyceride blood levels. Statistical analysis of total cholesterol, triacylglycerides and LDL cholesterol was performed to ensure no differences existed between day-time and night-time samples before dietary groups were compared between 21 (day time samples available only) and 24 weeks (day and/or night samples available).

After 12 weeks of treatment with the LFC, HFC, and HFB diets, as well as three weeks LOV treatment of a subset of the HFC and HFB dietary groups, serum total cholesterol, triacylglycerol and LDL levels were assessed (Tables 10, 11, and 12). Total cholesterol concentrations significantly increased between weeks 21 and 24 of the study

Table 11: Day Time Levels of Plasma Total Cholesterol, Triacylglycerol, and LDL at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1,2,3].

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Total Cholesterol (mmol/L)$^{\Psi, \pi, \lambda}$	3.96 ± 0.08^{a}	2.97 ± 0.11^{c}	2.49 ± 0.17^{d}	3.46 ± 0.13^{b}	2.99 ± 0.13^{c}
Triacylglycerol (mmol/L)$^{\lambda}$	1.35 ± 0.04^{a}	1.09 ± 0.08^{b}	1.14 ± 0.07^{ab}	1.12 ± 0.13^{ab}	1.06 ± 0.08^{b}
LDL (mmol/L)$^{\kappa}$	1.76 ± 0.13^{a}	1.63 ± 0.09^{ab}	1.51 ± 0.09^{ab}	1.57 ± 0.06^{a}	1.45 ± 0.07^{b}

1. Values are means ± SE.

2. Values in a row with different superscripts are significantly different at $p<0.05$; Ψ represents significant main effects of LOV ($p< 0.002$) and fat treatments ($p< 0.0001$); π represents significant effect of low versus high fat diet treatments for total cholesterol ($p< 0.0025$) and triacylglycerol ($p< 0.05$); λ represents significant main effect of LOV by dietary fat interaction ($p< 0.0001$); κ represents significant main effect of dietary fat ($p < 0.015$).

3. Animals were terminated during the day and 12 weeks after dietary intervention with the LFC, HFC, or HFB diets with a subset of animals from the HFC and HFB diet groups receiving three weeks of LOV treatment (20 mg/kg body weight/day). α designates the presence of LOV treatment. n=8-12 animals/group.

Table 12: **Night Time Levels of Plasma Total Cholesterol, Triacylglycerol, and LDL at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Total Cholesterol (mmol/L)$^{\Psi, *, \pi, \kappa}$	3.40 ± 0.07^{a}	2.95 ± 0.13^{b}	2.95 ± 0.10^{b}	3.43 ± 0.07^{a}	2.89 ± 0.09^{b}
Triacylglycerol (mmol/L)$^{\Psi, \pi, \kappa}$	0.67 ± 0.02^{a}	0.50 ± 0.01^{c}	0.41 ± 0.02^{d}	0.49 ± 0.02^{c}	0.59 ± 0.01^{b}
LDL (mmol/L)$^{\lambda, \kappa}$	1.99 ± 0.08^{a}	1.51 ± 0.14^{c}	1.66 ± 0.04^{bc}	1.74 ± 0.05^{abc}	1.53 ± 0.07^{c}

1. Values are means ± SE.

2. Values in a row with different superscripts are significantly different at $p<0.05$; Ψ represents significant main effects of LOV ($p< 0.001$ and $p<0.059$ for TAG and Total cholesterol, respectively); * represents significant main effect of dietary fat treatments ($p< 0.02$); π represents significant effect of low versus high fat diet treatments for total cholesterol ($p< 0.007$) and triacylglycerol ($p< 0.0005$); λ represents significant main effect of LOV by dietary fat interaction ($p< 0.03$); κ represents significant main effect of dietary saturated versus unsaturated fat ($p < 0.003$, $p < 0.015$ $p < 0.03$ for triacylglycerol, total cholesterol and LDL, respectively).

3. Animals were terminated at night and 12 weeks after dietary intervention with the LFC, HFC, or HFB diets with a subset of animals from the HFC and HFB diet groups receiving three weeks of LOV treatment (20 mg/kg body weight/day). α designates the presence LOV treatment. n=8-12 animals/group.

in the LFC and HFB groups, whereas for the HFC group, it remained at the same level. LDL cholesterol concentration remained similar between 21 and 24 with no significant effects of dietary fat treatment on LDL cholesterol concentrations.

Blood samples from day and night-time sampling were available at the 24 week study period. Hence, data is provided for both sampling times (Tables 11 and 12).

No significant differences were found between day and night time samples for total cholesterol (except LFC group) and LDL cholesterol. Hence, day and night samples were combined for all groups and are shown in Table 13 (except LFC for total cholesterol).

At 24 weeks, total cholesterol concentration significantly increased in the LFC and HFB treated groups, whereas levels remained the same for the HFC group. In decreasing order, total cholesterol concentration was HFB > LFC > HFC = HFCα > HFBα. LOV treatment significantly ($p<0.03$) reduced total cholesterol levels in the HFBα group but had no effect in the HFCα compared with the HFC group. There was a significant effect of dietary fat ($p<0.04$) for total cholesterol, whereby the HFB group had higher levels compared to the HFC group. Saturated fat resulted in higher total cholesterol levels compared to unsaturated fat. A significant effect ($p<0.0001$) of low fat versus high fat treatment was found with the LFC group demonstrating higher total cholesterol levels compared to the HFC group. At 24 weeks, LDL concentration was significantly ($p<0.004$) higher in the LFC group compared to all other groups. In decreasing order, LDL concentration was LFC > HFB > HFC = HFCα > HFBα.

Table 13: **Combined Day and Night Time Levels of Plasma Total Cholesterol and LDL at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Total Cholesterol (mmol/L)$^{\Psi, *, \pi, \kappa}$	3.40 ± 0.07^{a}	2.92 ± 0.11^{bc}	2.73 ± 0.10^{bc}	3.45 ± 0.08^{a}	2.94 ± 0.08^{bc}
LDL (mmol/L)$^{\Psi, *}$	1.87 ± 0.08^{a}	1.57 ± 0.08^{b}	1.57 ± 0.06^{b}	1.64 ± 0.04^{b}	1.49 ± 0.05^{b}

1. Values are means ± SE.

2. Values in a row with different superscripts are significantly different at $p<0.0001$ and $p<0.001$ for total cholesterol and LDL respectively; Ψ represents significant main effects of LOV ($p< 0.03$ and $p<0.005$ for total cholesterol, and LDL, respectively); * represents significant main effect of dietary fat treatments ($p< 0.04$ and $p<0.004$ for total cholesterol and LDL, respectively); π represents significant effect of low versus high fat diet treatments for total cholesterol ($p< 0.0001$); κ represents significant main effect of dietary saturated versus unsaturated fat ($p < 0.005$ for total cholesterol).

3. Animals were terminated at night and 12 weeks after dietary intervention with the LFC, HFC, or HFB diets with a subset of animals from the HFC and HFB diet groups receiving three weeks of LOV treatment (20 mg/kg body weight/day). α designates the presence of LOV treatment. n=17-24 animals/group for total cholesterol and 13-14 animals per group for LDL cholesterol.

The Effect of Dietary Lipid and Lovastatin on ACF, Microadenoma and Tumor Development in Colon Carcinogenesis

The HFC Diet Exerts Rapid Growth Promoting Effects on ACF

Modulation of ACF by dietary lipid type was analyzed at 21 weeks, after nine weeks of dietary intervention, and prior to LOV treatment. Following nine weeks of dietary intervention, the HFC group had a significantly higher ($p<0.05$) average number of total ACF/colon compared to both the HFB and LFC groups which were not statistically different from each other (Table 14). The HFC group also had a significantly higher ($p<0.05$) number of MAs compared to the HFB group. Therefore, we can conclude that the ACF in the HFC group are more rapid growing compared to the ACF in the HFB group which are more slow growing and in which MA appearance was significantly lower (2.3 ± 0.7 versus 0.5 ± 0.3, for HFC and HFB, respectively). In each of the crypt multiplicity categories (1-3, 4-6, and ≥ 10), the HFC group demonstrated significantly higher numbers compared to the LFC group (Table 15). The HFB group demonstrated a consistent trend towards higher numbers within each crypt multiplicity category but was not significantly different compared to the LFC group. The HFC group had significantly ($p<0.05$) higher numbers of ACF with small crypt multiplicity (ACF-2, ACF-3, ACF-4, and ACF-5) compared to LFC. The HFC group had significantly higher numbers of ACF with three crypts compared to HFB, with the HFB group demonstrating a trend of lower numbers of ACF with smaller crypt numbers. Compared to the LFC group, the HFC group demonstrated significantly higher ($p<0.05$) numbers of ACF with a crypt multiplicity of ≥ 10. The HFB group had significantly lower ($p<0.05$) numbers of singular ACF (ACF-1) compared to the LFC and HFC groups. The crypt multiplicity of the HFB group seemed to be intermediate between the LFC and HFC groups.

Table 14: **Enumeration of ACF Growth Characteristics in Male F344 Rats after 21 Weeks Representing Nine Weeks of Intervention with LFC, HFC, and HFB Diets[1, 2, 3, 4, 6].**

Group	Total ACF	ACF[5] 1-3	4-6	7-9	≥10	MA
LFC	216.2 ± 23.5[c]	167.5 ± 19.8[b]	43.0 ± 4.3[b]	5.2 ± 1.3[a]	0.5 ± 0.2[b]	0.7 ± 0.4[ab]
HFC	362.0 ± 26.8[a]	264.7 ± 21.4[a]	86.3 ± 7.0[a]	8.3 ± 0.7[a]	2.7 ± 0.5[a]	2.3 ± 0.7[a]
HFB	250.2 ± 26.2[bc]	179.8 ± 18.6[b]	60.7 ± 8.1[ab]	8.5 ± 2.3[a]	1.2 ± 0.7[ab]	0.5 ± 0.3[b]

1. Values are means ± SE.
2. Values in a column with different superscripts are significantly different ($p<0.05$).
3. Animals were terminated nine weeks after dietary treatment with the LFC, HFC, or HFB diets; n=10 rats/group.
4. Abbreviations are as follows: ACF, aberrant crypt foci, MA, microadenomas representing microscopic lesions < 1mm^2, see List of Abbreviations for additional abbreviations.
5. ACF are categorized by number of crypts per focus: 1-3, 4-6, 7-9, ≥10.
6. Number of tumors per group was as follows: out of 8 rats in the LFC group, four rats had a total of four tumors (66-90mm^2), 3 of which were proximal; out of 9 rats in the HFC group, 5 rats had a total of 7 tumors (14-64mm^2), 2 of which were proximal; out of 8 rats in the HFB group, four rats had a total of five tumors (6-66mm^2), one of which was proximal.

Table 15: **Number of ACF with Different Growth Features in Male F344 Rats as affected by LFC, HFC and HFB Diets: 21 Weeks[1, 2, 3].**

No. of Crypts per Focus	Diet Group		
	LFC	HFC	HFB
ACF-1	42.0 ± 4.5^{a}	56.3 ± 10.8^{a}	35.0 ± 3.7^{b}
ACF-2	70.2 ± 7.8^{b}	122.0 ± 9.9^{a}	86.0 ± 11.2^{ab}
ACF-3	55.3 ± 8.7^{b}	86.3 ± 3.7^{a}	58.8 ± 6.0^{b}
ACF-4	27.3 ± 3.0^{c}	50.5 ± 5.6^{ab}	35.7 ± 4.9^{bc}
ACF-5	11.8 ± 1.2^{b}	27.0 ± 4.2^{a}	17.3 ± 2.7^{ab}
ACF-6	3.8 ± 1.0^{a}	8.8 ± 1.3^{a}	7.7 ± 1.7^{a}
ACF-7	2.3 ± 0.9^{a}	5.3 ± 0.8^{a}	3.7 ± 1.2^{a}
ACF-8	1.8 ± 0.4^{a}	2.3 ± 0.4^{a}	3.8 ± 1.1^{a}
ACF-9	1.0 ± 0.4^{a}	0.7 ± 0.2^{a}	1.0 ± 0.4^{a}
ACF ≥10	0.5 ± 0.2^{b}	2.7 ± 0.5^{a}	1.2 ± 0.7^{ab}

1. Values are means ± SE.
2. Values in a row with different superscripts are significantly different ($p<0.05$).
3. ACF are categorized by number of crypts per focus: 1 through 9 and ≥10.

The Effect of Lovastatin on ACF Development is Dependent on the Dietary Lipid Environment

At 24 weeks, all animals were terminated. This time period represents 12 weeks of LFC, HFC and HFB dietary intervention in addition to two subsets of animals, one within both the HFB and HFC groups treated with LOV for three weeks (20mg/kg body weight/day; Figure 7). The colons of animals were examined for preneoplastic and neoplastic changes and compared with the baseline (nine weeks of treatment with LFC, HFC, and HFB) findings (Table 16). Compared to baseline (21 weeks), there were marked increases in ACF number in both the HFC and HFB groups in the ACF categories 1-3, 4-6 and 7-9. These differences were significant for the HFB group in the ACF categories 1-3 and >10 (Table 17).

At 24 weeks, the HFC group had the highest average number of total ACF/colon, followed by HFB, LFC, HFCα, and HFBα. The HFBα group had a significantly lower ($p<0.05$) number of total ACF/colon compared to the HFB group. Consistent with these findings, the HFBα group had significantly lower ($p<0.05$) numbers of ACF in the crypt multiplicity groupings of 1-3 (mostly due to reduced numbers of ACF with two crypts), whereas all other dietary groups were similar.

When compared to baseline, the average total ACF/colon was significantly higher ($p<0.05$) for the LFC and HFB groups but not for the HFC group. Compared to baseline, the LFC group had significantly higher ($p<0.05$) average ACF/colon in the crypt multiplicity categories 1-3, 4-6, and ≥10. Compared to baseline, the HFB group had significantly higher ($p<0.05$) average ACF/colon in the crypt multiplicity categories 1-3

Table 16: Enumeration of ACF Growth Characteristics in Male F344 Rats at 24 Weeks Representing 12 Weeks of Intervention with LFC, HFC, HFB Diets and Three Weeks of Lovastatin Treatment[1-6].

Group	Total ACF	ACF 1-3	ACF 4-6	ACF 7-9	ACF ≥10	MA
LFC	420.0 ± 76.5[ab, Ψ] (216.2 ± 23.5)	314.5 ± 54.5[ab, Ψ]	90.2 ± 19.0[a, Ψ]	11.7 ± 3.2[ab]	3.7 ± 1.5[a, Ψ]	2.7 ± 0.6[ab, Ψ] (0.7 ± 0.4)
HFC	478.9 ± 67.9[a] (362.0 ± 26.8)	357.6 ± 50.7[a]	103.1 ± 15.9[a]	14.3 ± 2.4[ab]	3.9 ± 0.7[a]	1.7 ± 0.6[ab] (2.33 ± 0.7)
HFB	458.9 ± 61.3[a, Ψ] (250.2 ± 26.2)	338.0 ± 44.4[a,Ψ]	100.6 ± 19.9[a]	13.1 ± 2.6[ab]	2.6 ± 0.8[a, Ψ]	3.38 ± 0.3[a, Ψ] (0.50 ± 0.34)
HFCα	406.1 ± 55.5[ab]	289.6 ± 35.9[ab]	103.6 ± 14.5[a]	12.6 ± 2.4[ab]	4.5 ± 1.1[a]	0.8 ± 1.5[b]
HFBα	283.5 ± 28.2[b]	206.5 ± 18.7[b]	66.5 ± 9.5[a]	8.4 ± 1.4[b]	2.1 ± 0.9[a]	0.9 ± 0.4[b]

1. Values are means ± SE.

2. Values in a column with different superscripts are significantly different at $p<0.05$; Ψ represents significant differences between nine and twelve weeks within a group ($p<0.05$).

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment (20 mg/kg/day) for a subset of HFC and HFB animals, HFCα and HFBα, respectively; n=10 rats/group.

4. Abbreviations are as follows: ACF, aberrant crypt foci; MA, microadenomas representing microscopic lesions < $1mm^2$; see List of Abbreviations for additional abbreviations.

5. Bracketed values represent control data from nine weeks of dietary intervention.

6. ACF are categorized by number of crypts per focus: 1-3, 4-6, 7-9, and ≥10.

Table 17: Number of ACF with Different Growth Features in Male F344 Rats as affected by LFC, HFC, HFB, HFCα, and HFBα Diets at 12 Weeks[1, 2, 3, 4].

No. of Crypts per Focus	Diet Group				
	LFC	HFC	HFCα	HFB	HFBα
ACF-1	$81.5 \pm 12.6^{ab\Psi}$	$87.1 \pm 13.2^{a\Psi}$	66.3 ± 7.0^{ab}	86.8 ± 10.7^{ab}	54.4 ± 6.1^{b}
ACF-2	$132.6 \pm 22.6^{ab\Psi}$	159.4 ± 23.9^{a}	128.5 ± 17.6^{ab}	$148.5 \pm 19.6^{a\Psi}$	89.4 ± 8.7^{b}
ACF-3	81.3 ± 16.3^{ab}	111.0 ± 14.6^{a}	94.9 ± 13.6^{ab}	$102.8 \pm 14.7^{ab\Psi}$	62.8 ± 5.9^{b}
ACF-4	47.7 ± 10.8^{a}	62.1 ± 9.0^{a}	61.9 ± 11.6^{a}	61.8 ± 8.2^{a}	38.0 ± 5.8^{a}
ACF-5	24.1 ± 4.7^{a}	26.3 ± 5.8^{a}	28.1 ± 6.1^{a}	29.0 ± 5.2^{a}	18.5 ± 2.9^{a}
ACF-6	$12.0 \pm 2.5^{a\Psi}$	14.7 ± 2.7^{a}	10.6 ± 2.9^{a}	13.0 ± 2.5^{a}	10.0 ± 1.2^{a}
ACF-7	$7.6 \pm 1.9^{a\Psi}$	7.9 ± 1.2^{a}	7.3 ± 1.7^{a}	6.4 ± 1.2^{a}	4.6 ± 1.0^{a}
ACF-8	2.6 ± 1.0^{a}	3.4 ± 1.0^{a}	3.6 ± 0.9^{a}	3.8 ± 0.9^{a}	2.6 ± 0.5^{a}
ACF-9	0.7 ± 0.5^{b}	$3.0 \pm 0.9^{a\Psi}$	2.3 ± 0.5^{ab}	2.5 ± 0.8^{ab}	1.1 ± 0.4^{ab}
ACF ≥10	$3.1 \pm 1.4^{a\Psi}$	3.9 ± 0.7^{a}	2.8 ± 0.8^{a}	$4.5 \pm 1.1^{a\Psi}$	2.1 ± 0.9^{a}

1. Values are means ± SE; n=10/group.

2. Values in a row with different superscripts are significantly different ($p<0.05$); Ψ represents significant differences between nine and twelve weeks within a group ($p<0.05$).

3. ACF are categorized by number of crypts per focus: 1 through 9 and ≥10.

4. Abbreviations are as follows: HFCα, HFC diet group treated with LOV (20 mg/kg/day); HFBα, HFB diet group treated with LOV (20 mg/kg/day); see List of Abbreviations for additional abbreviations.

and ≥10. It is important to note that for the HFC group, there was no difference in the average number of total ACF per colon at 24 weeks compared to baseline (21 weeks).

LOV treatment caused a significant reduction ($p<0.05$) in average total ACF/colon in the HFBα group compared to the HFB group. In the HFBα group, compared to the HFB baseline, no changes seemed to occur in ACF with different crypt multiplicities. That is, total ACF in the HFBα group at 24 weeks was similar to total ACF in the HFB group at 21 weeks, therefore appearing as though growth and progression were inhibited. LOV treatment combined with an HFB diet caused a significant decrease in ACF crypt multiplicity in the category 1-3. Most interesting is that LOV treatment had a negligible effect on ACF numbers in the HFCα group compared to the HFC group. No differences were found in any of the crypt multiplicity categories when the HFC and HFCα groups were compared.

At 24 weeks, the highest average number of MAs, or total MA per group, were found in the HFB (3.38) group followed by the LFC (2.7), HFC (1.7), HFBα (0.9), and HFCα (0.8) groups (Table 16). There was a significantly higher ($p<0.05$) number of MA in both the LFC and HFB groups at 24 weeks compared to 21 weeks (2.7 versus 0.7 and 3.38 versus 0.50 for LFC and HFB, respectively), representing a steady and slower growth rate. The HFC group demonstrated an insignificant decrease in MA between weeks 21 and 24 of the study (1.7 versus 2.33). In the HFBα group, LOV treatment caused a significant ($p<0.05$) decrease in MA formation (3.38 versus 0.9 for HFB and HFBα, respectively). However, there was no difference in the number of MAs between the HFC and HFCα groups (1.7 versus 0.8 for HFC and HFCα, respectively), although the HFCα group demonstrated a trend towards lower MA numbers compared to the HFC group.

Lovastatin Lowers Tumor Incidence in the HFC Group

At 21 weeks, four animals in the LFC group (n=8) had four tumors with a size range of 66-90 mm^2, three of which were in the proximal region. This shows the LFC tumors are of a more aggressive phenotype. In the HFC group (n=9), 5 rats contained 7 tumors with a size range of 14-64mm^2, two of which were proximally located. Four rats in the HFB group (n=8) had 5 tumors with a size range of 6-66mm^2, one of which was proximal.

AT 24 weeks (Table 18), the total number of tumors among the treatment groups was in the order: HFC (47) > HFBα (40) > HFB (39) > HFCα (22) = LFC (22). LOV treatment significantly reduced ($p<0.05$) the total number of tumors in the HFCα group. However, the total number of tumors remained relatively unchanged in the LOV treated HFBα group compared to the non-treated HFB group. The total number of tumors was also significantly higher ($p<0.05$) in the HFC group compared to the LFC group. This demonstrates that both the amount and type of dietary lipid as well as LOV treatment, influences the development of colorectal tumors and suggests possible differences in overall tumor phenotype.

Lovastatin Treatment Retards Tumor Growth without affecting Total Tumor Number in the HFB Diet Group

One of the most interesting and provoking findings of the study involves tumor incidence (Table 18). In order of highest to lowest, tumor incidence for the experimental groups was HFBα (70.3 %) > HFC (66.7 %) > HFB (60.5 %) > LFC (59.4 %) > HFCα (41.2 %). Although there were no differences in tumor incidence between the HFC and HFB groups, LOV treatment led to a significant ($p<0.05$) reduction in tumor incidence in the HFCα group, whereas the same treatment in the HFBα group seemed to support a

Table 18: **Tumor Parameters in Male F344 Rats Fed LFC, HFC, and HFB Diets and Lovastatin Treatment of a Subset of the HFC and HFB Diet Groups[1-8].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Total No. of Rats	38	42	34	38	37
No. of Rats with Tumors	19	27	14	23	26
Total Tumors per Group	22^{b}	47^{a}	22^{b}	39^{ab}	40^{ab}
Tumor Incidence (%)	50.0 ± 0.09^{ab}	64.0 ± 0.07^{a}	41.2 ± 0.09^{b}	60.5 ± 0.08^{ab}	70.3 ± 0.08^{a}
Average Tumor Size (mm^2) per Group	20.9 ± 0.03^{a}	21.2 ± 0.04^{a}	26.3 ± 0.02^{a}	21.9 ± 0.03^{a}	17.6 ± 0.04^{b}
Average Tumor Size (mm^2) per Tumor Bearing Rat	17.28 ± 5.83^{a}	16.83 ± 3.34^{a}	11.91 ± 4.96^{a}	13.05 ± 2.76^{a}	11.99 ± 2.59^{a}
Tumor Multiplicity	1.16^{a}	1.74^{b}	1.57^{b}	1.70^{b}	1.54^{ab}
Tumor Burden	19.38 ± 6.23^{a}	25.0 ± 4.33^{a}	16.68 ± 5.97^{a}	22.5 ± 5.06^{a}	18.68 ± 4.19^{a}
Total MA per Group	2.67 ± 0.61^{ab}	1.71 ± 0.59^{ab}	0.75 ± 1.51^{b}	$3.38 \pm 0.30^{a\Psi}$	0.88 ± 0.40^{b}

1. Values are means ± SE.

2. Values in each row not sharing a common superscript letter are significantly different ($p<0.05$).

3. "α" denotes LOV treated subgroups, respectively; LOV dosage was 20 mg/kg body weight per day for three weeks.

4. Tumor incidence: number of animals with tumors/ total number of animals.

5. Average tumor size/group: total tumor area/number of tumors in the group.

6. Tumor burden: average of total tumor area/ tumor bearing rat.

7. Tumor multiplicity: average number of tumors/tumor bearing rat.

8. See List of Abbreviations for additional abbreviations.

trend towards increased tumor incidence. There were no significant differences in tumor incidence among the LFC, HFC and HFB groups. HFBα tumor incidence was significantly higher ($p<0.05$) than HFCα tumor incidence.

Tumor Size is affected by Dietary Lipid Type

In light of the tumor incidence data, the tumor size and location findings become even more interesting (Table 18). The average size of tumors per treatment group was in the order HFCα (26.3mm^2) > HFB (21.9mm^2) > HFC (21.2mm^2)> LFC (20.9mm^2) > HFBα (17.6mm^2). Although there were no significant differences in average tumor size among the LFC, HFC, HFCα, and HFB dietary groups, LOV treatment of the HFBα dietary group resulted in a significant reduction ($p<0.05$) in tumor size compared to the HFB and HFCα groups. LOV treatment in the HFCα dietary group resulted in a trend (insignificant) towards increased tumor size. Average tumor size was significantly larger ($p<0.05$) in the HFCα compared to the HFBα group.

Combined with an HFC Diet, Lovastatin Treatment Inhibits the Appearance of Small Tumors

Characterization of tumors based on size (small, medium, and large) was completed (Tables 19 and 20). In decreasing order, the number of small tumors was HFC (26) > HFBα (23) > HFB (19) > LFC (13) > HFCα (12). The HFC and HFBα groups had the highest number of small tumors (26 and 23, respectively), with the HFCα group having the lowest number of small tumors. There was a two-fold reduction in number of small tumors in the HFCα group compared to the HFC group. The number of medium sized tumors was in the order HFC > HFBα > HFB > HFCα > LFC. The

Table 19: Classification of Tumor Size in Male F344 Rats at 24 Weeks Representing 12 Weeks of Dietary Intervention with LFC, HFC, and HFB Diets and Three Weeks of Lovastatin Treatment of a Subset of the HFC and HFB Groups[1, 2, 3].

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Tumor Size[4]					
Small	59.1 (13)[a]	56.5 (26)[a]	54.5 (12)[a]	48.7 (19)[a]	57.5 (23)[a]
Medium	13.6 (3)[a]	21.7 (10)[a]	18.2 (4)[a]	17.9 (7)[a]	22.5 (9)[a]
Large	27.3 (6)[a]	23.9 (11)[a]	27.3 (6)[a]	33.3 (13)[a]	20.0 (8)[a]
Total Tumors	22[b]	47[a]	22[b]	39[ab]	40[ab]

1. Values are expressed as a percentage of tumors; the actual numbers of tumors are in parentheses.

2. Rows with different superscripts are statistically significant ($p<0.05$).

3. Animals were terminated after 12 weeks of dietary intervention. A subset of the HFC and HFB dietary groups, were treated with LOV (20 mg/kg body weight /day) for three weeks and are designated as "α". See List of Abbreviations for additional abbreviations.

4. Small tumors size, < 12 mm^2, medium tumor size, 12-25mm^2, large tumor size, > 25 mm^2.

5. Values represent the total number of tumors/group (n=32-42 rats/group); tumors represent macroscopic lesions > 1mm^2.

Table 20: **Average Tumor Size along the Length of the Colon in Male F344 Rats as affected by LFC, HFC, HFB Diets and Lovastatin Treatment[1,2].**

Group	Colon Segment[3,4]				
	A	**B**	**C**	**D**	**E**
LFC	14.3 ± 6.0 (4)	34.3 ± 13.2 (7)	15.1 ± 4.2 (8)	67.3 ± 46.6 (3)	NA (0)
HFC	19.7 ± 11.8 (6)	20.5 ± 4.4 (20)	20.6 ± 4.6 (16)	14.0 ± 8.1 (3)	15.0 ± 0.0 (1)
HFCα	11.0 ± 4.5 (3)	54.6 ± 25.2 (5)	23.9 ± 11.3 (9)	19.0 ± 16.0 (2)	6.7 ± 0.7 (3)
HFB	24.5 ± 12.2 (4)	21.6 ± 6.6 (12)	24.4 ± 4.7 (19)	10.3 ± 5.2 (3)	4.0 ± 0.0 (1)
HFBα	12.5 ± 4.37 (7)	14.0 ± 4.7 (15)	20.5 ± 4.3 (16)	25.0 ± 0.0 (1)	NA (0)

1. Values represent the average tumor size ± standard error (mm^2). Values with different superscripts are significantly different at $p<0.05$.

2. Abbreviations are as follows: HFCα, HFC diet with LOV treatment (20 mg/kg body weight/day); HFBα, HFB diet with LOV treatment (20 mg/kg body weight/day); NA, not applicable due to lack of tumors in that region. See List of Abbreviations for additional abbreviations.

3. Colonic mucosal segments A to E represents approximately 17 cm of unfixed colon. Each segment is a four cm segment with A representing the most distal segment and E the most proximal segment harbouring colonic tumors; location from the rectal end in cm: 0-4 (A), 4-8 (B), 8-12 (C), 12-16 (D), >16 (E).

4. Values in parenthesis represent the number of tumors in each region for the particular treatment group.

number of large sized tumors was in the order HFB > HFC > HFBα > HFCα = LFC. These differences were not significantly different.

The Effect of Lovastatin on Tumor Distribution Depends on Dietary Lipid Type

Tumor distribution along the length of the colon is shown in Table 21. For all dietary groups, the average tumor size along the length of the colon is shown in Table 20. Compared to the HFC group, the HFCα group demonstrated a four-fold decrease in number of tumors in the distal region between 4-8 cm (Region B) along the colon. The HFCα group also had the highest number of tumors in the most proximal segment (Region E). Therefore, in the HFC group, the effect of LOV in decreasing tumor growth was most pronounced in the distal region. Conversely, LOV treatment in the HFBα group resulted in higher numbers of tumors in the most distal segment of the colon between the 0-4 cm (Region A). Compared to HFB, the HFBα group had lower numbers of tumors in the proximal segment of the colon (Region E). Therefore, in the HFB group, the effect of LOV in decreasing tumor growth was most pronounced in the proximal region. Our findings support the contention that tumors developing in the distal and proximal regions of the colon are biologically and phenotypically different from one another. This is supported by one study in which even though no difference in tumor incidence was found between rats fed corn oil and beef tallow, malignant tumors of the colon, causing death, occurred earlier in rats fed corn oil as compared to those fed beef tallow (Wilson et al, 1977). In addition, rats fed beef tallow have been shown to harbour fewer ACF in the distal colonic mucosal region compared to those fed soybean oil as well as having increased mucosal apoptosis (Khil and Gallaher, 2004).

Table 21: Percentage Distribution of Tumors along the Length of the Colon in Male F344 Rats as Affected by LFC, HFC, HFB Diets and Lovastatin Treatment[1, 2].

		Colon Segment[3]				
Group	**Total Tumors**	**A**	**B**	**C**	**D**	**E**
LFC	22	23.0 (4)	32.0 (7)	32.0 (8)	14.0 (3)	0.0 (0)
HFC	47	13.0 (6)	48.0 (22)	30.0 (14)	6.5 (3)	2.0 (1)
HFCα	22	13.6 (3)	27.0 (6)	36.0 (8)	9.0 (2)	13.6 (3)
HFB	39	13.0 (5)	38.5 (15)	36.0 (14)	10.0 (4)	2.5 (1)
HFBα	40	20.0 (8)	42.5 (17)	32.5 (13)	2.5 (1)	0.0 (0)

1. Values are expressed as percentages. Values in parentheses represent the actual number of tumors present.

2. Abbreviations are as follows: HFCα, HFC diet with LOV treatment (20 mg/kg body weight/day); HFBα, HFB diet with LOV treatment (20 mg/kg body weight/day). See List of Abbreviations for additional abbreviations.

3. Colonic mucosal segments A to E represent approximately 17 cm of unfixed colon. Each segment is a four cm segment with A representing the most distal segment and E the most proximal segment harbouring colonic tumors; location from the rectal end in cm: 0-4 (A), 4-8 (B), 8-12 (C), 12-16 (D), >16 (E).

Total tumor burden, represented by the total tumor area per tumor bearing rat was highest in the HFC group and decreased in the order HFC > HFB > LFC > HFBα > HFCα. However, these differences did not reach statistical significance.

PART B: An Investigation of the Effects of Dietary Lipids and Lovastatin on Critical Molecules Involved in Cholesterol Biosynthesis and Associated Events in the Multi-Step Process of Colon Carcinogenesis

Experimental Approach

Normal colonic mucosa and tumor tissue were harvested from male F344 rats. Preparation of whole cell, cytosolic and membrane homogenate fractions of colonic mucosa and tumors, as well as liver tissue was conducted. Harvested tissues were also processed for Western blot and RT-PCR analysis as described in Chapter 3.

In light of the fact that the tissue volume from harvested colonic tumors was insufficient to allow separation into cytosolic and membranous fractions, only the normal colonic and hepatic tissues were separated in this way (Table 22). Assessing these fractions provides further insight into the cellular distribution of $p21^{K\text{-}ras}$. In this way, we can take into consideration total $p21^{K\text{-}ras}$ protein, cellular distribution and activity in an attempt to gain a clearer understanding of what is occurring at the cellular level.

The Level and Type of Dietary Lipid affects Cytosolic $p21^{K\text{-}ras}$ Protein Levels in Colonic Mucosa

In the normal appearing colonic mucosa, from highest to lowest, $p21^{K\text{-}ras}$ protein expression in the cytosolic fraction of the colonic mucosa was as follows: HFC > HFCα > LFC > HFB > HFBα (Table 22). Cytosolic $p21^{K\text{-}ras}$ expression was significantly higher ($p<0.0001$) compared to membrane expression in all dietary and

Table 22: Cytosolic and Membrane-Associated $p21^{K\text{-}ras}$ Protein Expression in Rat Colonic Mucosa and Liver at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1,2,3].

	Diet Group				
	LFC	HFC	HFCα	HFB	HFBα
Protein Expression[4]					
Colonic Mucosa					
Cytosol[*,5,6,8]	14.1 ± 1.1[bc]	21.2 ± 1.3[a]	15.5 ± 1.3[b]	12.1 ± 1.1[c]	8.8 ± 0.7[d]
Membrane[5,7,8]	3.5 ± 0.2[b]	2.9 ± 0.2[b]	6.5 ± 0.3[a]	3.0 ± 0.1[b]	3.3 ± 0.3[b]
Memb:Cyt Ratio	0.25	0.14	0.42	0.25	0.38
Liver					
Cytosol[*,6,7,9]	7.5 ± 0.8[b]	10.4 ± 0.5[a]	5.4 ± 0.2[b]	11.3 ± 1.3[a]	10.2 ± 0.9[a]
Membrane[6,7]	3.3 ± 0.1[a]	2.2 ± 0.1[b]	2.1 ± 0.3[b]	3.9 ± 0.4[a]	2.3 ± 0.1[b]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values represent K-Ras protein expression in the cytosolic and membrane fractions of the colonic mucosa and liver tissues. The amount of protein loaded for colonic mucosa cytosol and membrane fractions was 60 μg. The amount of protein loaded for liver cytosol and membrane fractions was 30 μg. See Materials and Methods for additional information. Memb:Cyt ratio represents the ratio between membrane-associated and cytosolic k-ras protein.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=6-8 rats/group). α designates the presence of LOV treatment.

4. Values in a row with different superscripts are significantly different at $p<0.0001$; * represents significant differences between the cytosolic and membrane K-ras protein expression in both colonic mucosa and liver for all treatment groups ($p<0.0001$).

5. Significant main effect of dietary fat treatment ($p<0.0001$) for colonic cytosolic and membrane fractions.

6. Significant main effect of saturated versus unsaturated fat for colonic mucosa cytosolic fraction ($p<0.0002$) and liver cytosolic ($p<0.03$) and membrane ($p<0.0003$) fractions, respectively.

7. Significant main effect of LOV x dietary fat interaction for colonic mucosa membrane fraction ($p<0.0001$) and liver cytosolic ($p<0.04$) and membrane ($p<0.0003$) fractions, respectively.

8. Significant main effect of LOV for colonic cytosolic ($p<0.0003$), and membrane ($p<0.0001$) fractions.

9. Significant main effect of low versus high dietary fat treatments ($p<0.0003$).

treatment groups. There was a significant ($p<0.0001$) main effect of dietary lipid treatment found in that the HFC group had higher cytosolic expression of $p21^{K\text{-}ras}$ protein than the HFB group. A significant ($p<0.0002$) main effect of saturated versus unsaturated fatty acids on cytosolic $p21^{K\text{-}ras}$ protein expression was detected with the LFC and HFC groups showing higher levels compared to the HFB group.

Lovastatin Treatment Modulates $p21^{K\text{-}ras}$ Protein Expression in the Cytosolic and Membrane-Associated Fraction of Colonic Mucosa

A significant main effect of LOV treatment ($p<0.0003$) on cytosolic $p21^{K\text{-}ras}$ protein expression was found in colonic mucosa. LOV treatment produced significant decreases in cytosolic $p21^{K\text{-}ras}$ protein expression in both the HFCα and HFBα compared with the HFC and HFB groups, respectively ($p<0.0001$). In addition, cytosolic $p21^{K\text{-}ras}$ protein levels were significantly lower in the HFBα compared to the HFCα group.

No differences in levels of membrane-associated $p21^{K\text{-}ras}$ were found among the LFC, HFC and HFB diet treatment groups. Therefore, there was no lipid effect on membrane-associated $p21^{K\text{-}ras}$ protein expression (Table 22).

LOV treatment significantly increased ($p<0.05$) membrane-associated $p21^{K\text{-}ras}$ protein in the HFCα compared to the HFC group. The level of $p21^{K\text{-}ras}$ was highest only in the HFCα compared to those noted in the other groups. The interactive effect between LOV and dietary lipid was significant ($p<0.0001$).

Hepatic Cytosolic and Membrane-Associated $p21^{K\text{-}ras}$ Protein Expression Depends on the Level and Type of Dietary Lipid

The major regulatory organ of cholesterol biosynthesis and lipid metabolism and transport is the liver. Hence, we investigated hepatic expression of the target molecules of interest in an attempt to use it as a comparative tissue. Our findings support the

contention that colonic tissue is responsive to the effects of LOV as colonic mucosa from both HFC and HFB groups responded differently to LOV treatment. From highest to lowest, $p21^{K\text{-}ras}$ protein expression in the cytosolic fraction of hepatic tissue was as follows: HFB > HFC > HFBα > LFC > HFCα (Table 22). Cytosolic $p21^{K\text{-}ras}$ expression was significantly higher ($p<0.0001$) compared to $p21^{K\text{-}ras}$ associated with the membrane in all dietary and treatment groups. There was a significant main effect of saturated versus unsaturated dietary fat on hepatic cytosolic and membrane-associated $p21^{K\text{-}ras}$ concentrations ($p<0.03$ and $p<0.0003$, respectively) in that the saturated fat diet resulted in higher cytosolic $p21^{K\text{-}ras}$ protein compared to the UFA diet. There was a significant ($p<0.0003$) main effect of low versus high dietary fat treatment in that a high fat diet resulted in significantly higher ($p<0.03$) cytosolic $p21^{K\text{-}ras}$ concentrations compared to a low fat diet.

The level of PUFA in the diet significantly ($p< 0.0003$) affected hepatic membrane-associated $p21^{K\text{-}ras}$ levels. The LFC diet resulted in a higher level of membrane-associated $p21^{K\text{-}ras}$ compared to the HFC diet.

The Effect of Lovastatin on Hepatic Cytosolic and Membrane-Associated $p21^{K\text{-}ras}$ Protein Expression Depends on Dietary Lipid Type

The liver is the primary target tissue of LOV. LOV treatment resulted in significant reduction in cytosolic $p21^{K\text{-}ras}$ in the HFC group but not the HFB group. There was a significant interactive effect between LOV and dietary lipids ($p<0.04$).

In contrast to the cytosolic fraction, LOV treatment produced a significant reduction in hepatic membrane-associated $p21^{K\text{-}ras}$ protein in the HFBα group but not the HFCα

group. There was a significant interactive effect between LOV and dietary lipids ($p<0.0003$).

The Lipid Composition of the Diet Modulates the Effect of Lovastatin on $p21^{K\text{-}ras}$ Protein Expression in Normal Mucosa

The HFC diet induced a higher level of $p21^{K\text{-}ras}$ in colonic mucosa. From highest to lowest, expression of $p21^{K\text{-}ras}$ protein in colonic mucosa (whole homogenate) was as follows: HFC > HFCα > LFC > HFB > HFBα (Table 23). $p21^{K\text{-}ras}$ protein expression was significantly higher in the HFC group compared to the LFC and HFB group ($p<0.05$). A significant main effect of dietary unsaturated versus saturated fat was found in the colonic mucosa ($p<0.0001$).

LOV treatment had no effect on $p21^{K\text{-}ras}$ protein expression within the HFC or HFB groups (Table 23). However, the HFCα group had significantly higher ($p<0.05$) $p21^{K\text{-}ras}$ expression compared to the HFBα group. The effect of LOV on $p21^{K\text{-}ras}$ protein expression was found to be significantly dependent on the dietary lipid environment ($p<0.002$).

$p21^{K\text{-}ras}$ Protein Levels in Colonic Tumors are Significantly Higher Compared to Colonic Mucosa and Depend on Dietary Lipid Type

In colonic tumor whole homogenate, from highest to lowest, the expression of $p21^{K\text{-}ras}$ protein was as follows: HFCα > HFB > LFC > HFC > HFBα (Table 23). In all dietary treatment groups, except HFC, $p21^{K\text{-}ras}$ protein expression was significantly higher in colon tumors compared to mucosa ($p<0.0001$). $p21^{K\text{-}ras}$ expression was similar for the LFC, HFC and HFB dietary groups.

LOV treatment produced opposite effects on $p21^{K\text{-}ras}$ protein expression depending on the dietary lipid environment (Table 23). LOV treatment of

Table 23: p21$^{K\text{-}ras}$ Protein Expression in Rat Colonic Mucosa and Tumors at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].

	Diet Group				
	LFC	HFC	HFCα	HFB	HFBα
Protein Expression[4]					
Colonic Mucosa[*, 5, 6, 7]	17.3 ± 1.8^{b}	21.4 ± 0.4^{a}	19.2 ± 0.9^{ab}	12.5 ± 0.6^{c}	12.2 ± 0.4^{c}
Colonic Tumors[5, 7]	35.1 ± 5.6^{ab}	27.5 ± 0.4^{bc}	45.2 ± 4.0^{a}	35.8 ± 2.8^{ab}	20.9 ± 1.4^{c}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Protein expression is for colonic mucosa and tumor whole homogenate. The amount of protein loaded for colonic mucosa and tumors was 30 µg. See Methods and Materials for additional information.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=6-8 rats/group). α designates the presence of LOV treatment.

4. Values in a row with different superscripts are significantly different at $p<0.05$; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the LFC, HFCα, HFB, and HFBα dietary fat groups, respectively.

5. Significant main effect of dietary fat treatment for colonic mucosa ($p<0.0001$) and tumors ($p<0.04$).

6. Significant main effect of saturated versus unsaturated fat ($p<0.0001$).

7. Significant main effect of LOV x dietary fat interaction for colonic mucosa ($p<0.002$) and tumors ($p<0.0002$).

the HFCα group resulted in significantly higher ($p<0.05$) $p21^{K\text{-}ras}$ protein expression compared to the HFC group. The same treatment in the HFBα dietary group produced a significant reduction ($p<0.05$) in $p21^{K\text{-}ras}$ protein expression compared to the HFB group. In addition, $p21^{K\text{-}ras}$ protein expression was significantly lower ($p<0.05$) in the HFBα group compared to the HFCα group. There was a significant ($p<0.0002$) interactive effect between LOV and dietary lipid. Hence, the effect of LOV on $p21^{K\text{-}ras}$ protein expression in colonic tumors depends on the lipid environment.

$p21^{K\text{-}ras}$ Activity in Normal and Tumorigenic Colonic Tissue

In an attempt to increase the statistical power of the analysis and due to the low sample size from each treatment group in assessing $p21^{K\text{-}ras}$ activity, we initially combined all non-LOV treated tumors into one group and all non-LOV treated mucosa samples into a separate group (Table 24). $p21^{K\text{-}ras}$ activity was significantly higher in colonic tumors than mucosa ($p< 0.0001$) indicating that increased $p21^{K\text{-}ras}$ is specific to tissue type. In addition, LOV treatment resulted in significant reductions in $p21^{K\text{-}ras}$ activity in both colonic mucosa and tumors ($p<0.0001$), indicating that both tissue types responded to LOV treatment. Therefore, it seems that LOV treatment could be used to target $p21^{K\text{-}ras}$ activity in colonic tumors.

The Level and Type of Dietary Lipid affect $p21^{K\text{-}ras}$ Activity in Colonic Mucosa and Tumors

From highest to lowest, $p21^{K\text{-}ras}$ activity in normal appearing colonic mucosa was as follows: HFB > HFC > LFC > HFCα > HFBα (Table 25). $p21^{K\text{-}ras}$ activity was significantly higher in the HFB group compared to the HFC group. The level of significance of main effect of saturated versus unsaturated fatty acids was $p<0.0001$.

Table 24: **$p21^{K\text{-}ras}$ Activity in Rat Colonic Mucosa and Tumors at 24 Weeks after Treatment with a High Fat Diet and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group	
	HFC/HFB	**HFCα/HFBα**
K-Ras Activity		
Colonic Mucosa*	16.35 ± 3.36[b]	0.16 ± 0.02[c]
Colonic Tumors	29.15 ± 5.40[a]	2.26 ± 0.40[c]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.0001$; * represents significant differences between colonic mucosa and tumor $p21^{K\text{-}ras}$ activity ($p<0.0001$).

3. Animals were terminated 12 weeks after dietary intervention with a high fat diet and three weeks of LOV treatment for a subset of animals (n=6-9 rats/group). α designates the presence of LOV treatment (20mg/kg body weight/day).

Table 25: $p21^{K\text{-}ras}$ Activity in Rat Colonic Mucosa and Tumors at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
K-Ras Activity[4, 5, 6]					
Colonic Mucosa*	5.50 ± 0.50^{b}	8.70 ± 1.50^{b}	0.10 ± 0.01^{c}	26.40 ± 2.30^{a}	0.20 ± 0.03^{c}
Colonic Tumors	34.50 ± 4.10^{a}	18.70 ± 4.40^{b}	1.40 ± 0.10^{c}	39.60 ± 4.00^{a}	2.90 ± 0.50^{c}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.05$; * represents significant differences between colonic mucosa and tumor $p21^{K\text{-}ras}$ activity ($p<0.0001$) for the LFC, HFC, HFB, and HFBα dietary lipid groups, respectively.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=3-5 rats/group). α designates the presence of LOV treatment.

4. Significant main effect of LOV x dietary fat interaction for colonic mucosa ($p<0.0001$) and tumors ($p<0.008$), respectively.

5. Significant main effect of saturated versus unsaturated fat for colonic mucosa ($p<0.0001$) and tumors ($p<0.005$), respectively.

6. Significant main effect of low versus high fat dietary treatments for colonic mucosa ($p<0.0001$).

High fat diets significantly increased the level of active $p21^{K\text{-}ras}$ in the colonic mucosa ($p<0.0001$) compared to a low fat diet.

From highest to lowest, $p21^{K\text{-}ras}$ activity in colonic tumors was as follows: HFB > LFC > HFC > HFCα > HFBα. $p21^{K\text{-}ras}$ activity in colonic tumors was significantly higher ($p<0.0001$) than in the mucosa for all dietary groups except HFCα. Colonic tumors in the HFB group had significantly higher ($p<0.05$) $p21^{K\text{-}ras}$ activity than the HFC group but not the LFC group. There was a significant main effect ($p<0.005$) of dietary saturated versus unsaturated fatty acid treatment.

The Effect of Lovastatin Treatment on $p21^{K\text{-}ras}$ Activity in Colonic Mucosa and Tumors Depends on Dietary Lipid Type

LOV treatment resulted in a significant ($p<0.05$) reduction in $p21^{K\text{-}ras}$ activity in colonic mucosa as noted for both the HFCα and HFBα diet treatment groups (Table 25). However, the effect of LOV on $p21^{K\text{-}ras}$ activity was significantly dependent on the lipid environment as there was a significant interactive effect between LOV and dietary fat ($p<0.0001$). LOV treatment resulted in a significant reduction ($p<0.05$) in colonic tumor $p21^{K\text{-}ras}$ activity in both the HFCα and HFBα groups. However, the effect of LOV on $p21^{K\text{-}ras}$ activity in tumors was dependent on the lipid environment as a significant ($p<0.008$) interactive effect between LOV and dietary fat was found for colonic tumors.

HMGCAR Gene Expression is Significantly Higher in Colonic Tumors Compared to Mucosa and is affected by the Level and Type of Dietary Lipid

In colonic mucosa, HMGCAR gene expression (mRNA) levels were similar among the HFC, HFCα, HFB and HFBα dietary treatment groups (Table 26). The LFC group had significantly higher ($p<0.0002$) HMGCAR gene expression compared to all other treatment groups.

Table 26: HMGCAR Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].

	Diet Group				
	LFC	HFC	HFCα	HFB	HFBα
Gene Expression[4]					
Colonic Mucosa[*, 6, 7]	0.78 ± 0.05[a]	0.21 ± 0.05[b]	0.38 ± 0.07[b]	0.37 ± 0.08[b]	0.28 ± 0.06[b]
Colonic Tumors[5, 6, 7, 8]	0.68 ± 0.05[c]	1.16 ± 0.10[a]	0.92 ± 0.05[bc]	0.71 ± 0.06[c]	1.04 ± 0.09[ab]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.0002$ and $p<0.001$ for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the HFC, HFCα, HFB and HFBα dietary fat groups, respectively.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=6-8 rats/group). α designates the presence of LOV treatment.
4. HMGCAR gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.
5. Significant main effect of dietary fat treatment for colonic tumors ($p<0.05$).
6. Significant main effect of low versus high fat dietary treatments for colonic mucosa ($p<0.0001$) and tumors ($p<0.02$), respectively.
7. Significant main effect of LOV x dietary fat interaction for colonic mucosa ($p<0.05$) and tumors ($p< 0.002$), respectively.
8. Significant main effect of saturated versus unsaturated fat for colonic tumors ($p<0.004$).

Colonic tumors in all treatment groups, except the LFC group, had significantly ($p<0.001$) higher HMGCAR gene expression compared to normal appearing colonic mucosa (Table 26).

HMGCAR gene expression was significantly lower ($p<0.02$) in the LFC tumors compared to the HFC tumors (Table 26). The HFC group had significantly higher HMGCAR gene expression compared to the HFB group ($p<0.001$). A significant main effect of dietary lipid treatment was found ($p<0.05$). Saturated versus unsaturated dietary fat treatments significantly affected HMGCAR gene expression ($p<0.004$).

The Effect of Lovastatin Treatment on HMGCAR Gene Expression in Colonic Mucosa and Tumors Depends on Dietary Lipid Type

In colonic mucosa, even though there was no significant effect of LOV treatment on HMGCAR gene expression in either of the HFC or HFB groups, there was a significant interactive effect of dietary lipid on the effect of LOV ($p<0.05$) (Table 26). LOV treatment resulted in a trend towards increased HMGCAR gene expression in the HFCα group, whereas it reduced HMGCAR gene expression in the HFBα group.

Colonic tumors present in the HFCα group had significantly reduced HMGCAR gene expression compared to the HFC group ($p<0.001$) (Table 26). An opposite effect was noted in the tumors from the HFBα compared to the HFB group ($p<0.001$). The main interactive effect of dietary lipid on LOV effects was significant ($p<0.002$). The level of HMGCAR gene expression was similar in both the HFCα and HFBα groups. These findings are supported by the HMGCAR protein findings described below.

The Amount and Type of Dietary Lipid affects HMGCAR Protein Expression in Colonic Mucosa

The level of HMGCAR protein expression in normal appearing rat colonic mucosa, in decreasing order, was LFC > HFB > HFC > HFBα > HFCα (Table 27). The level of dietary lipid significantly influenced HMGCAR protein expression in the colonic mucosa. A significant ($p<0.02$) effect of level (low versus high) of dietary PUFA on HMGCAR protein expression was found. LFC group had the highest expression of HMGCAR which was significantly higher than the HFC group. In addition, there was a significant ($p<0.01$) main effect of dietary lipid type on HMGCAR protein expression. The HFB group had significantly higher HMGCAR protein expression compared to the HFC group ($p<0.0001$).

Lovastatin Significantly Decreases HMGCAR Protein Expression in Colonic Mucosa

LOV treatment produced a significant ($p<0.0002$) reduction in HMGCAR protein expression in both the HFCα and HFBα groups (Table 27). The level of statistical significance for the main effect of the LOV treatment was $p<0.001$.

HMGCAR Protein is Significantly Higher in Colonic Tumors Compared to Colonic Mucosa

In all dietary treatment groups, colonic tumors had significantly higher HMGCAR protein expression than the normal appearing colonic mucosa ($p<0.0001$). This finding supports the contention that colonic tumors require higher levels of HMGCAR and its products to support growth. It also suggests an interruption in the regulatory control mechanisms of cholesterol biosynthesis in colonic tumors. We wonder whether tumors

Table 27: **HMGCAR Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Protein Expression[4]					
Colonic Mucosa[*, 5, 6, 7]	19.5 ± 0.5^{a}	16.7 ± 0.06^{b}	14.6 ± 0.7^{c}	18.6 ± 0.6^{a}	16.0 ± 0.7^{bc}
Colonic Tumors[8]	28.0 ± 1.1^{b}	33.8 ± 2.7^{a}	21.5 ± 1.1^{c}	27.1 ± 1.3^{b}	33.8 ± 2.4^{a}
Liver[5, 8]	40.5 ± 3.7^{c}	63.7 ± 2.8^{b}	87.1 ± 10.3^{a}	45.0 ± 2.1^{c}	93.5 ± 7.5^{a}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.0001$, $p<0.004$, and $p<0.0001$ for colonic mucosa, tumors and liver, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for all treatment groups.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=6-8 rats/group). α designates the presence of LOV treatment.
4. HMGCAR protein expression is for colonic mucosa, tumor, and liver whole homogenate. The amount of protein loaded was 20μg. The 97 KDa band was assessed. See Materials and Methods for additional information.
5. Significant main effect of LOV treatment for colonic mucosa ($p<0.001$) and liver ($p<0.0001$).
6. Significant main effect of dietary fat treatment ($p<0.01$).
7. Significant main effect of low versus high fat dietary treatments ($p<0.02$).
8. Significant main effect of LOV x dietary fat interaction for colonic tumors ($p<0.0001$) and liver ($p< 0.05$), respectively.

differ in their ability to up-regulate the expression of this protein and, depending on how successful they are, how their growth is affected.

The Effect of Lovastatin on HMGCAR Protein Expression in Colonic Tumors Depends on Dietary Lipid Type

The level of HMGCAR protein expression in rat colonic tumors, in decreasing order, was HFC = HFBα > LFC > HFB > HFCα (Table 27). The HFC group had significantly ($p<0.004$) higher HMGCAR protein expression compared to the HFB and LFC groups. HFB and LFC groups had similar expression. A significant interactive effect between LOV and dietary lipid was found ($p<0.0001$). LOV treatment resulted in a significant ($p<0.0004$) reduction in HMGCAR expression in the HFC group, whereas LOV treatment in the HFB group had the opposite effect, producing a significant ($p<0.0004$) increase in HMGCAR protein expression.

Hepatic HMGCAR Protein Expression is Significantly Higher in the HFC Compared to the HFB Dietary Lipid Group and is affected by Lovastatin Treatment

The level of HMGCAR protein expression in rat hepatic tissue, in decreasing order, was HFBα = HFCα > HFC > HFB >LFC (Table 27). Hepatic HMGCAR protein expression was significantly ($p<0.0001$) higher in the HFC group compared to the HFB group. Both the HFB and LFC groups had similar levels of HMGCAR expression.

LOV treatment produced significant ($p<0.0001$) increases in hepatic HMGCAR protein expression in both the HFCα and HFBα groups which had similar levels of HMGCAR expression (Table 27). There was a significant ($p<0.05$) interactive effect of between LOV and dietary lipid treatment in which the increase in HMGCAR protein expression in the HFBα group was higher compared to that of the HFC group.

LDL Receptor (LDLr) Gene Expression in Normal and Tumorigenic Colonic Tissue

The Effect of Lovastatin Treatment on LDLr Gene Expression in Colonic Mucosa and Tumors Depends on Dietary Lipid Type

LDLr gene expression in the normal appearing rat colonic mucosa, in decreasing order was HFBα > HFB > HFCα > HFC > LFC (Table 28). There was a significant ($p<0.04$) main effect of low versus high fat dietary treatment in which the LFC group had lower LDLr mRNA levels compared to the high fat diet groups. The LFC group had significantly lower LDLr mRNA levels compared to the HFB group. LOV treatment did not affect LDLr gene expression in colonic mucosa.

LDLr gene expression was significantly ($p<0.0001$) higher in colonic tumors compared to mucosa for the LFC and HFB groups only (Table 28). LDLr gene expression in colonic tumors, in decreasing order was HFBα > HFB > LFC > HFC > HFCα. There were no differences between the HFC and HFCα or the HFB and HFBα groups. However, there was a significant ($p<0.04$) main effect of LOV by dietary lipid interaction. In the HFBα group, LOV treatment resulted in significantly higher LDLr mRNA levels compared to LOV treatment of the HFCα group.

The Level and Type of Dietary Lipid affects LDLr Protein Expression in Colonic Mucosa and Tumors

LDLr protein expression in the normal appearing rat colonic mucosa, in decreasing order was HFC > HFBα > HFB > LFC > HFCα (Table 29). There was a significant ($p<0.0001$) main effect of low versus high dietary fat treatment with the LFC group showing significantly lower LDLr protein expression compared to the high fat groups.

Table 28: LDL Receptor Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].

	Diet Group				
	LFC	HFC	HFCα	HFB	HFBα
Gene Expression [4]					
Colonic Mucosa [*, 5]	0.35 ± 0.05[c]	0.47 ± 0.05[bc]	0.54 ± 0.05[ab]	0.55 ± 0.09[ab]	0.65 ± 0.05[a]
Colonic Tumors [6, 7]	0.67 ± 0.06[ab]	0.59 ± 0.13[ab]	0.46 ± 0.07[b]	0.91 ± 0.26[a]	0.92 ± 0.16[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.02$ and $p<0.05$ for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the LFC and HFB dietary fat groups, respectively.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=6-8 rats/group). α designates the presence of LOV treatment.
4. LDLr gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.
5. Significant main effect of low versus high fat dietary treatment for colonic mucosa ($p<0.04$).
6. Significant main effect of dietary fat treatment for colonic tumors ($p<0.009$).
7. Significant main effect of LOV by dietary fat interaction ($p<0.04$).

Table 29: **LDL Receptor Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Protein Expression [4]					
Colonic Mucosa[*, 5, 6, 7]	3.4 ± 0.2[c]	5.3 ± 0.1[a]	3.0 ± 0.1[d]	4.0 ± 0.2[bc]	4.1 ± 0.2[b]
Colonic Tumors [6, 8, 9]	1.7 ± 0.2[c]	3.0 ± 0.2[a]	2.4 ± 0.2[b]	2.3 ± 0.1[b]	1.4 ± 0.2[c]
Liver [6, 8, 9]	1.3 ± 0.1[d]	1.5 ± 0.1[cd]	2.0 ± 0.1[b]	1.6 ± 0.1[c]	2.4 ± 0.1[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.0002$, $p<0.001$, and $p<0.0001$ for colonic mucosa, tumors, and liver, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for all treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=6-8 rats/group). α designates the presence of LOV treatment.

4. LDLr protein expression is for colonic mucosa, tumor, and liver whole homogenate. The amount of protein loaded for colonic mucosa and tumors was 60μg. The amount of protein loaded for liver was 10μg. The 160 KDa band was assessed. See Materials and Methods for additional information.

5. Significant main effect of LOV x dietary fat interaction for colonic mucosa ($p<0.0001$).

6. Significant main effect of low versus high fat dietary treatments for colonic mucosa ($p<0.0001$), colonic tumors ($p<0.0003$), and liver ($p<0.02$).

7. Significant main effect of saturated versus unsaturated dietary fat for colonic mucosa ($p<0.004$).

8. Significant main effect of LOV treatment for colonic tumors ($p<0.0002$) and liver ($p<0.0001$), respectively.

9. Significant main effect of dietary fat treatment for colonic tumors ($p<0.0001$) and liver ($p<0.02$).

There was a significant ($p<0.004$) main effect of saturated (HFB) versus unsaturated dietary fat on LDLr protein expression in colonic mucosa. LDLr protein expression was significantly ($p<0.0002$) higher in the HFC group compared to the HFB group. This finding was expected as diets high in saturated fat result in decreased hepatic LDLr expression, whereas diets high in unsaturated fat result in increased hepatic LDLr expression (Ness et al, 1985a and 1995b).

Compared to the colonic mucosa, LDLr protein expression in colonic tumors was significantly ($p<0.0001$) lower in all treatment groups. In decreasing order, the level of LDLr protein expression in colonic tumors was HFC > HFCα > HFB > LFC > HFBα. The established trend of a highly unsaturated fat diet producing increased LDLr expression was followed by colonic tumors in the HFC group (Table 29) As expected, a highly saturated fat diet lowered LDLr as HFB tumors exhibited decreased LDLr protein expression. There was a significant main effect of dietary fat ($p<0.0001$) on LDLr protein expression as the HFC group had significantly higher LDLr levels compared to the HFB and LFC groups. In addition, the HFB group had significantly higher LDLr levels compared to the LFC group ($p<0.001$). A significant ($p<0.0003$) main effect of low versus high dietary fat on LDLr protein expression in colonic tumors was found. Colonic tumors from the LFC group had significantly ($p<0.001$) lower LDLr protein expression compared to the HFC and HFB groups.

The Effect of Lovastatin on LDLr Protein Expression in Colonic Mucosa Depends on Dietary Lipid Type

In colonic mucosa, LOV treatment in the HFCα group produced a significant reduction in LDLr protein expression compared to the HFC group (Table 29). This effect was not observed in the HFBα group compared to the HFB group. The interactive effect between

LOV and dietary lipid was significant ($p<0.0001$). The HFCα group had significantly lower LDLr protein expression than the HFBα group.

Lovastatin Treatment Results in Decreased LDLr Protein Expression in Colonic Tumors

As LOV treatment inhibits HMGCAR and endogenous cholesterol synthesis, one would expect that LDLr expression in tumors would increase to compensate for the blocked HMGCAR activity and facilitate the necessary requirement for cholesterol to support tumor growth. In contrast, LOV treatment significantly ($p<0.0002$) reduced LDLr protein expression in colonic tumors (Table 29). Tumors in the HFBα group had significantly ($p<0.001$) lower LDLr protein expression compared to the HFCα group.

LDL Receptor (LDLr) Protein Expression in Rat Hepatic Tissue

Hepatic LDLr protein expression, in decreasing order, was HFBα > HFCα > HFB > HFC > LFC (Table 29). There was a significant main effect of low versus high fat dietary treatment ($p<0.02$). A high fat diet resulted in higher hepatic LDLr protein expression compared to a low fat diet. There was no significant difference in hepatic LDLr protein expression between the HFC and HFB dietary treatment groups. Cholesterol synthesis in the rat liver can be regulated over a wide range with no change in receptor-dependent LDL transport (Spady and Cuthbert, 1992). LOV increases transcription of LDLr gene (Brown and Goldstein, 1986). Immunoblotting studies have demonstrated that hepatic LDLr protein levels are not increased in rats treated with LOV, pravastain, fluvastatin, cerivastatin or atorvastatin (Ness et al, 1996).

Lovastatin Treatment Results in Increased LDLr Protein Expression in Hepatic Tissue

LOV treatment resulted in a significant ($p<0.0001$) increase in hepatic LDLr protein expression in both the HFCα and HFBα groups compared to the respective non-treated groups. There was a significant ($p<0.0001$) main effect of LOV treatment. The HFBα group had significantly ($p<0.0001$) higher hepatic LDLr protein expression compared to the HFCα group.

PART C: An Investigation of the Effects of Dietary Lipids and Lovastatin on the Gene and Protein Expression of Critical Cell Signalling and Apoptotic Molecules in the Multi-Step Process of Colon Carcinogenesis

Experimental Approach

Normal colonic mucosa and tumor tissue were harvested from male F344. Preparation of whole cell lysate from colonic mucosa and tumors, as well as hepatic tissue was conducted. Harvested tissues were processed for Western blot and RT-PCR analysis as described in Chapter 3.

Assessing the level of expression of the ERK-1, ERK-2, cyclin D1, and caspase-3 proteins provides further insight into the molecular changes occurring in cellular signalling and apoptotic molecules in normal and neoplastic colonic tissue. We can also gain further insight into modulation of these molecules by dietary lipid and/or LOV treatment as well as possible modulation of LOV treatment effects by dietary lipids.

ERK-1 Gene Expression in Colonic Mucosa and Tumors is affected by a Low Fat Diet

A low fat diet increased ERK-1 gene expression in colonic mucosa. In decreasing order, the level of ERK-1 mRNA in colonic mucosa was LFC > HFB > HFBα > HFC > HFCα (Table 30). There was a significant main effect of low versus high dietary lipid treatment

Table 30: **ERK-1 Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Gene Expression[4]					
Colonic Mucosa[*, 5]	1.89 ± 0.24[a]	1.13 ± 0.19[b]	1.01 ± 0.07[b]	1.36 ± 0.23[b]	1.15 ± 0.12[b]
Colonic Tumors[5, 6]	0.77 ± 0.10[b]	1.47 ± 0.21[a]	0.76 ± 0.27[b]	1.34 ± 0.11[a]	0.88 ± 0.07[b]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.02$ and $p<0.001$ for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.05$) for the LFC dietary fat group.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=5-6 rats/group). α designates the presence of LOV treatment.
4. ERK-1 gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.
5. Significant main effect of low versus high fat dietary treatment for colonic mucosa ($p<0.008$) and tumors ($p<0.0004$).
6. Significant main effect of LOV treatment for colonic tumors ($p<0.0002$).

($p<0.008$). The LFC group had significantly ($p<0.02$) higher ERK-1 mRNA expression compared to HFC and HFB groups. The type of high fat diet had no effect on ERK-1 gene expression in colonic mucosa. In contrast to mucosa, a low fat diet significantly decreased ERK-1 gene expression in colonic tumors compared to mucosa. In decreasing order, the level of ERK-1 mRNA in colonic tumors was HFC > HFB > HFBα > LFC > HFCα. In the LFC group only, colonic mucosa had significantly ($p<0.05$) higher mRNA expression compared to tumors.

The Amount of Dietary Lipid and Lovastatin Significantly affect ERK-1 Gene Expression in Colonic Mucosa and Tumors

LOV treatment had no effect on ERK-1 mRNA expression in colonic mucosa in either of the HFCα or HFBα groups (Table 30).

In colonic tumors, the high fat diet treatments resulted in significantly higher ($p<0.001$) ERK-1 mRNA levels than the low fat treatment (Table 30). The level of significance of the main effect of low versus high fat dietary treatment was $p<0.0004$. LOV treatment resulted in significantly ($p<0.001$) decreased ERK-1 gene expression in colonic tumors in both the HFCα and HFBα groups (Table 30). LOV treatment itself exerted a significant effect on ERK-1 gene expression $p<0.0002$). It was interesting that ERK-1 levels in the LOV groups were similar to that of the LFC group.

ERK-1 Protein Expression in Colonic Mucosa and Tumors is affected by Dietary Lipid

In decreasing order, the level of ERK-1 protein expression in colonic mucosa was HFCα > HFBα > LFC > HFB >HFC (Table 31). The LFC group had significantly ($p<0.0001$) higher ERK-1 protein levels compared to the HFC and HFB groups. There was a significant ($p<0.03$) main effect of saturated versus unsaturated fat treatment on

Table 31: **ERK-1 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
	LFC	**HFC**	**HFCα**	**HFB**	**HFBα**
Protein Expression [4]					
Colonic Mucosa[*, 7, 9]	2.95 ± 0.24[a]	1.94 ± 0.06[b]	3.17 ± 0.24[a]	2.25 ± 0.12[b]	3.00 ± 0.18[a]
Colonic Tumors[5, 6, 7, 8]	6.70 ± 0.30[a]	4.30 ± 0.20[c]	6.09 ± 0.40[a]	6.13 ± 0.30[a]	5.04 ± 0.40[b]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.0001$ and $p<0.0003$ for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for all treatment groups.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=5-7 rats/group). α designates the presence of LOV treatment.
4. ERK-1 protein expression is for colonic mucosa and tumor whole homogenate. ERK-1 was detected as a 44 kDa band. The amount of protein loaded for colonic mucosa and tumors was 15μg. See Materials and Methods for additional information.
5. Significant main effect of LOV x dietary fat interaction for colonic tumors ($p<0.05$).
6. Significant main effect of low versus high fat dietary treatments for colonic tumors ($p<0.002$).
7. Significant main effect of saturated versus unsaturated dietary fat for colonic mucosa ($p<0.03$) and tumors ($p<0.005$).
8. Significant main effect of LOV treatment for colonic tumors ($p<0.0001$).
9. Significant main effect of dietary fat treatment for colonic mucosa ($p<0.0001$).

ERK-1 protein levels in the colonic mucosa. No difference in ERK-1 protein expression between the HFC and HFB groups was found.

The type and amount of dietary lipid were important variables in affecting ERK-1 protein expression in colonic tumors. In decreasing order, the level of ERK-1 protein expression in colonic tumors was LFC > HFB > HFCα > HFBα > HFC (Table 31). Colonic tumors had significantly ($p<0.0001$) higher ERK-1 protein expression compared to colonic mucosa for all dietary treatment groups. Compared to a diet low in polyunsaturated fat (LFC group), tumors in the high polyunsaturated fat (HFC group) had significantly ($p<0.0003$) lower ERK-1 protein expression. The significance level for the main treatment effect of low versus high dietary fat treatment in colonic tumors was $p<0.002$. The HFB diet resulted in significantly higher ERK-1 protein expression compared to the HFC diet. The main treatment effect of saturated versus unsaturated dietary fat treatment was $p<0.005$.

The Effect of Lovastatin Treatment on ERK-1 Protein Expression in Colonic Tumors Depends on Dietary Lipid Type

LOV treatment resulted in a significant ($p<0.0001$) increase in colon mucosa ERK-1 protein expression in both the HFCα and HFBα groups compared to their non-treated groups (Table 31). Both the HFCα and HFBα groups had similar levels of ERK-1 protein expression.

Depending on the type of dietary lipid, LOV treatment had opposing effects on ERK-1 protein expression in colonic tumors. LOV treatment resulted in a significant ($p<0.0001$) increase in ERK-1 protein expression in the HFCα group, whereas in the HFBα group, LOV treatment resulted in a significant ($p<0.0001$) reduction in ERK-1 protein expression (Table 31). HFCα tumors had significantly higher ERK-1 expression than

HFBα tumors. The interactive effect between LOV and dietary lipid in colonic tumors was significant ($p<0.05$).

Dietary Lipid Type and Level affect ERK-2 Protein Expression in Colonic Mucosa

In decreasing order, the level of ERK-2 protein expression in colonic mucosa was HFBα > LFC > HFCα > HFB > HFC (Table 32). The LFC group had significantly ($p<0.003$) higher ERK-2 protein expression compared to the HFC group. For colonic mucosa, there was a significant ($p<0.002$) main effect of low versus high dietary fat treatment in ERK-2 protein expression. No difference in ERK-2 protein expression was found between the HFC and HFB groups.

LOV treatment had no effect on ERK-2 protein expression in the colonic mucosa of the HFCα group compared to the HFC group (Table 32). However, LOV treatment in the HFBα group resulted in a significant ($p<0.003$) increase in ERK-2 protein expression compared to the HFB group.

ERK-2 Protein Expression is Significantly Higher in Colonic Tumors Compared to Mucosa

In decreasing order, the level of ERK-2 protein expression in colonic tumors was HFB > HFBα > HFCα > HFC >LFC (Table 32). With the exception of the LFC group, ERK-2 protein expression was significantly ($p<0.0001$) higher in colonic tumors compared to mucosa.

Table 32: **ERK-2 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
	LFC	HFC	HFCα	HFB	HFBα
Protein Expression [4]					
Colonic Mucosa*, [5]	1.94 ± 0.16^{ab}	1.44 ± 0.08^{c}	1.59 ± 0.04^{bc}	1.56 ± 0.11^{bc}	2.13 ± 0.17^{a}
Colonic Tumors	2.51 ± 0.20^{a}	2.56 ± 0.20^{a}	2.91 ± 0.20^{a}	3.02 ± 0.40^{a}	3.00 ± 0.30^{a}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.003$ for colonic mucosa; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the HFC, HFCα, HFB, and HFBα dietary fat treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=5-7 rats/group). α designates the presence of LOV treatment.

4. ERK-2 protein expression is for colonic mucosa and tumor whole homogenate. The amount of protein loaded for colonic mucosa and tumors was 15μg. ERK-2 was detected as a 42 kDa band. See Materials and Methods for additional information.

5. Significant main effect of low versus high fat dietary treatments for colonic mucosa ($p<0.002$).

ERK-2 protein expression in all treatment groups was similar. Therefore, the type and amount of dietary fat as well as LOV treatment had no effect on ERK-2 protein expression in colonic tumors. It seems that ERK-2 is important for tumors but that it is less prone to modulation by exogenous factors and is playing an important role as a protective factor in maintaining tumor phenotype.

The Amount of Dietary Lipid affects CD1 Gene Expression in Colonic Mucosa

In decreasing order, CD1 mRNA expression in colonic mucosa was LFC > HFC > HFB > HFBα > HFC (Table 33). The LFC group had significantly ($p<0.02$) higher CD1 expression compared to all other treatment groups. There was a significant ($p<0.02$) main effect of low versus high dietary fat on CD1 gene expression in colonic mucosa. LOV treatment had no effect on CD1 gene expression in colonic mucosa.

Differences in CD1 Gene Expression between Colonic Mucosa and Tumors Depend on the Type of Dietary Lipid

In decreasing order, CD1 mRNA expression in colonic tumors was HFB > HFC > HFBα > LFC > HFCα. With the exception of the LFC and HFCα groups, CD1 gene expression was significantly ($p<0.05$) higher in colonic tumors compared to colonic mucosa in all dietary treatment groups.

In colonic tumors, there were no differences in CD1 gene expression among the LFC, HFC and HFB groups (Table 33). In addition, a significant ($p<0.03$) main effect of low versus high dietary fat treatment on CD1 gene expression in colonic tumors was found. CD1 expression was lower in the low fat group compared to the high fat groups.

Table 33: Cyclin D1 Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].

	Diet Group				
Gene Expression[4]	**LFC**	**HFC**	**HFCα**	**HFB**	**HFBα**
Colonic Mucosa[*, 5]	0.52 ± 0.05^{a}	0.39 ± 0.04^{b}	0.30 ± 0.03^{b}	0.37 ± 0.01^{b}	0.34 ± 0.06^{b}
Colonic Tumors[5, 6]	0.57 ± 0.03^{ab}	0.80 ± 0.08^{ab}	0.48 ± 0.13^{b}	0.91 ± 0.23^{a}	0.68 ± 0.09^{ab}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.02$ and $p<0.05$ for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.05$) for the HFC, HFB, and HFBα dietary fat treatment groups.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=5-6 rats/group). α designates the presence of LOV treatment.
4. Cyclin D1 gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.
5. Significant main effect of low versus high fat dietary treatment for colonic mucosa ($p<0.02$) and tumors ($p<0.03$).
6. Significant main effect of LOV treatment for colonic tumors ($p<0.02$).

Lovastatin Treatment Reduces CD1 Gene Expression in Colonic Tumors

Although CD1 gene expression in colonic tumors was similar in the HFC compared to HFCα and HFB compared to HFBα groups, a significant main effect of LOV treatment was found for colonic tumors at $p<0.02$ (Table 33). LOV treatment resulted in a reduced CD1 gene expression.

The Type of Dietary Lipid affects CD1 Protein Expression in Colonic Mucosa and Tumors

In decreasing order, the level of CD1 protein expression in colonic mucosa was HFBα > HFCα > HFB > LFC > HFC (Table 34). There was a significant ($p<0.0001$) main effect of dietary lipid treatment on CD1 protein expression in colonic mucosa. In addition, there was a significant ($p<0.0006$) main effect of saturated versus unsaturated dietary fat on CD1 protein expression in colonic mucosa whereby CD1 expression was significantly ($p<0.001$) higher in colonic mucosa from the HFB compared to the HFC group. No difference in CD1 expression between LFC and HFC groups was noted.

In all dietary groups, colonic tumors had significantly ($p<0.0001$) higher CD1 protein expression compared to colonic mucosa. In decreasing order, the level of CD1 protein expression in colonic tumors was HFC > HFB > HFCα > LFC > HFBα. In colonic tumors, CD1 protein expression in the HFC group was significantly ($p<0.0001$) higher compared to the HFB group (Table 34). There was a significant ($p<0.0001$) main effect of saturated versus unsaturated dietary fat treatment on CD1 protein expression in colonic tumors. CD1 expression in the HFC group was significantly higher than that of the LFC group ($p<0.002$).

Table 34: **Cyclin D1 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
Protein Expression [4]	**LFC**	**HFC**	**HFCα**	**HFB**	**HFBα**
Colonic Mucosa[*, 6, 8]	2.78 ± 0.17^{bc}	2.63 ± 0.09^{c}	3.15 ± 0.04^{ab}	3.02 ± 0.06^{ab}	3.31 ± 0.15^{a}
Colonic Tumors[5, 6, 8]	4.63 ± 0.11^{bc}	5.80 ± 0.31^{a}	4.66 ± 0.09^{bc}	4.97 ± 0.18^{b}	4.14 ± 0.09^{c}
Liver[5, 6, 7, 8]	3.12 ± 0.07^{bc}	2.75 ± 0.16^{c}	2.79 ± 0.10^{c}	3.23 ± 0.11^{b}	3.92 ± 0.23^{a}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.001$, $p<0.0001$, and $p<0.0001$ for colonic mucosa, colonic tumors, and liver respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for all treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=5-8 rats/group for colonic mucosa and 5-6 rats/group for colonic tumors). α designates the presence of LOV treatment.

4. Cyclin D1 protein expression is for colonic mucosa, tumor, and liver whole homogenate. CD1 was detected as a 34 kDa band. The amount of protein loaded for colonic mucosa, tumors, and liver was 30μg. See Materials and Methods for additional information.

5. Significant main effect of LOV x dietary fat interaction for colonic tumors ($p<0.0001$) and liver ($p<0.0001$).

6. Significant main effect of saturated versus unsaturated dietary fat for colonic mucosa ($p<0.0006$), tumors ($p<0.0001$), and liver ($p<0.0001$).

7. Significant main effect of LOV for liver ($p<0.01$).

8. Significant main effect of dietary fat treatment for colonic mucosa ($p<0.0001$), tumors ($p<0.0002$), and liver ($p<0.007$).

The Effect of Lovastatin Treatment on CD1 Protein Expression in Colonic Mucosa and Tumors Depends on Dietary Lipid Type

CD1 expression in colonic mucosa was significantly higher in the HFCα group compared to that of the HFC group (Table 34). However, CD1 protein expression in the HFBα group was similar to the HFB group.

In colonic tumors, LOV treatment in both the HFCα and HFBα groups resulted in significantly ($p<0.0001$) lower CD1 protein expression in colonic tumors compared to their respective HFC and HFB groups (Table 34). The reduction in CD1 expression was more pronounced in the HFCα group than in the HFBα group. A significant interactive effect between LOV and dietary fat was found ($p<0.0001$). Both the HFCα and HFBα groups had similar CD1 protein expression levels.

Dietary Lipid affects Caspase-3 Protein Expression in Colonic Mucosa and Tumors

In decreasing order, caspase-3 protein expression in colonic mucosa was HFC > HFB > HFCα > LFC > HFBα (Table 35). A significant ($p<0.01$) main effect of dietary fat on caspase-3 expression was found. The LFC group had significantly ($p<0.007$) lower caspase-3 protein expression compared to the HFC group.

No difference in caspase-3 expression was found between the HFC and HFB groups. However, a significant ($p<0.007$) main effect of saturated versus unsaturated fatty acid treatment was found whereby caspase-3 expression was significantly higher in the saturated fat group compared to the unsaturated fat groups.

Caspase-3 protein expression in colonic tumors was unaffected by various amounts or types of dietary lipid. There were no differences in caspase-3 protein expression among the LFC, HFC and HFB groups (Table 35).

Table 35: **Caspase-3 Protein Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Lovastatin[1, 2, 3].**

	Diet Group				
Protein Expression [4]	**LFC**	**HFC**	**HFCα**	**HFB**	**HFBα**
Colonic Mucosa[*, 5, 6, 7]	2.75 ± 0.25^{bc}	3.43 ± 0.03^{a}	2.82 ± 0.10^{bc}	3.12 ± 0.16^{ab}	2.39 ± 0.23^{c}
Colonic Tumors[5, 7]	4.78 ± 0.61^{bc}	6.09 ± 0.68^{bc}	9.25 ± 0.87^{a}	4.12 ± 0.17^{c}	6.82 ± 0.80^{b}
Liver[8]	3.75 ± 0.53^{ab}	3.28 ± 0.31^{bc}	4.52 ± 0.35^{a}	2.31 ± 0.25^{c}	3.84 ± 0.37^{ab}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.007$, $p<0.0001$, and $p<0.007$ for colonic mucosa, colonic tumors, and liver respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the LFC, HFC, HFCα, and HFBα dietary fat treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and three weeks of LOV treatment for a subset of the HFC and HFB animals (n=5-8 rats/group for colonic mucosa, 5-6 rats/group for colonic tumors and 6-7 rats/group for liver, respectively). α designates the presence of LOV treatment.

4. Caspase-3 protein expression is for colonic mucosa, tumor, and liver whole homogenate. Caspase-3 was detected as a 33 kDa band. The amount of protein loaded for colonic mucosa and tumors was 60μg. The amount of protein loaded for liver was 10μg. See Materials and Methods for additional information.

5. Significant main effect of LOV x dietary fat interaction for colonic mucosa ($p<0.005$) and tumors ($p<0.01$).

6. Significant main effect of saturated versus unsaturated dietary fat for colonic mucosa ($p<0.007$).

7. Significant main effect of dietary fat treatment for colonic mucosa ($p<0.01$) and tumors ($p<0.0009$).

8. Significant main effect of low versus high dietary fat treatment for liver ($p< 0.02$).

Caspase-3 Protein Expression is Significantly Higher in Colonic Tumors Compared to Mucosa and is affected by Dietary Lipid Type

In decreasing order, caspase-3 protein expression in colonic tumors was HFCα > HFBα > HFC > LFC > HFB. With the exception of the HFB group, colonic tumors had significantly higher caspase-3 protein expression compared to the corresponding groups of the colonic mucosa.

The Effect of Lovastatin Treatment on Caspase-3 Protein Expression in Colonic Mucosa and Tumors Depends on Dietary Lipid Type

LOV treatment in the colonic mucosa resulted in a significant ($p<0.007$) reduction in caspase-3 protein expression in both the HFCα and HFBα groups (Table 35). Dietary lipid type significantly modulated of the effect of LOV on caspase-3 protein expression ($p<0.005$).

In colonic tumors, caspase-3 protein expression was significantly ($p<0.0001$) increased by LOV treatment in the HFCα and HFBα groups (Table 35). The HFCα group had significantly ($p<0.0001$) higher caspase-3 protein expression compared to the HFBα group. Dietary lipid type significantly modulated of the effect of LOV on caspase-3 protein expression ($p<0.01$).

Chapter 5: Discussion

The Effect of Dietary Lipids and Lovastatin on the Multi-Step Process of Colon Carcinogenesis

Considering that the typical North American diet is high in saturated fat as well as the overwhelming evidence in support of the association between a diet high in saturated fat and increased risk of CRC, there is a surprising gap in the research related to the molecular basis of the promotional effects of saturated fat on CRC. One of the goals of our research was to investigate if LOV affects the growth of preneoplastic lesions and tumors and if this effect of LOV on the colonic tissues is modulated by dietary lipids varying in the levels of unsaturated and saturated fatty acids. It was hypothesized that a high saturated fat diet (beef tallow), would augment the cholesterol biosynthetic pathway, hence preneoplastic lesions and/or tumors would be less sensitive to LOV. On the other hand, a highly unsaturated fat diet would exert a synergistic effect with LOV in retarding the growth of these lesions. This hypothesis was based on the assumption that, in the colon, the cholesterol biosynthetic pathway is regulated by the level of saturated or unsaturated fatty acids in a manner similar to other tissues (Goldstein and Brown, 1990; Ness et al, 1994, 1996, 1998; Ness and Chambers, 2000) and plays a critical role in growth and development of preneoplastic lesions and tumors. LOV and the family of statin drugs are being explored for their anticancer efficacies. It is now known that these drugs exert multiple physiological effects and impact several pathways including the activity of HMGCAR. Nevertheless, we were interested in exploring if HMGCAR and the cholesterol biosynthetic pathway are dysregulated in experimentally induced colonic neoplasms. The experimental model we used emulated a high risk model. F344 rats were injected with AOM and 12 weeks after the last injection they were allocated into

groups receiving a highly unsaturated (corn oil) or saturated (beef tallow) diet for nine weeks. Therefore, the initial 12 week duration after AOM injections allowed the preneoplastic lesions to develop, at which time they were exposed to the high fat diets for nine additional weeks. At this time, ten animals per group were terminated and ACF and tumors were enumerated. Then, each high fat group was further subdivided into two groups. Two groups continued to receive the designated high fat diets (HFC or HFB), however one sub-group in each high fat diet received LOV by oral gavage daily for three weeks.

This approach allowed us to assess the effect of lipid type on the different stages of colon carcinogenesis and also to assess the sensitivity of these lesions to short term exposure to LOV. After the final three weeks, all animals were terminated and ACF and tumors were enumerated. This approach and model allowed us to determine the effect of lipid type and LOV on the multiple stages of colon carcinogenesis. Analyses of colonic tissues (mucosa and tumors) for specific genes and proteins provided a mechanistic insight into how these lesions were responding. In addition to quantifying preneoplastic lesions and tumors, we analysed tissues for molecules that are associated with the cholesterol biosynthetic pathway (HMGCAR and LDLr) as well as $p21^{K\text{-}ras}$ protein whose function is dependent on the cholesterol biosynthetic pathway through post-translational modification. In addition, we also analysed genes and corresponding proteins which are indicators of cell growth or death (ERK-1, ERK-2, and CD1 and caspase-3).

In spite of experimental and epidemiological findings supporting the tumor promoting effect of diets high in saturated fat, a large portion of the studies investigating the link between colon cancer and the modulatory effects of dietary lipids are limited to PUFA.

Few studies have investigated the effects of saturated fatty acids on gene and protein expression of the cellular targets established as playing an integral role in the step-wise development of colon cancer.

PART I: ACF, Microadenoma and Tumor Findings

The experimental protocol of the present thesis research allowed for observation of the change in population of ACF with various growth features and on the transition of lesions from one stage to the next. The duration of intervention with the test diets was 12 weeks and three weeks for LOV. This duration was sufficient to allow advanced ACF to progress into MA and tumors as well as for primal ACF to progress into intermediate or advanced lesions. After nine weeks of dietary intervention, ACF appearance and development was more rapid in the HFC group. The HFC diet stimulated more rapid growth and clonal expansion of primal lesions compared to the HFB diet. Development of primal lesions occurred at a slower rate in the HFB and LFC groups as evidenced by lower ACF numbers.

One of our objectives was to determine the effect of LOV on the growth and development of late stage preneoplastic lesions (ACF) and tumors under different dietary lipid environments. We accomplished this objective by treating each high fat dietary group (HFC and HFB) with LOV (20mg/kg/d). The mode of LOV treatment was in an orally gavaged bolus form (once per day, as in humans), which differs from other experimental protocols in which LOV is provided to animals within the diet, at continuous levels throughout the day, and soon after carcinogen injection. We preferred to gavage the animals with a freshly prepared solution of LOV based on body weight. LOV is sensitive to degradation by ultraviolet light. In the present study, LOV was

prepared in dim light and administered to the animals within 30 minutes of preparation. LOV treatment affected different stages of colon carcinogenesis and was dependent on the dietary lipid environment. LOV treatment seemed to be ineffective at reducing total ACF numbers in the HFCα group, whereas in the HFBα group, LOV significantly reduced total ACF, especially in ACF with crypt multiplicity of 1-3 representing the earlier stage of growth. This finding is further supported by the MA data in which LOV treatment significantly reduced MA formation in the HFBα group but not the HFCα group. This finding suggests that ACF developing in a highly unsaturated lipid environment are less sensitive to LOV than those developing under a saturated lipid environment. This concept was further strengthened by findings from the analyses of colonic tissues for the various molecular markers discussed in the following sections. In contrast to the findings in the ACF population, tumor incidence was significantly lower in the HFCα compared to the HFC group (41% and 67%, respectively), whereas it was similar for both HFB and HFBα (61% and 70%, respectively). This difference was mainly due to reduction in the number of small tumors in the HFCα group. Therefore, it would appear that the stages representing pre-adenomatous (advanced) lesions were more sensitive to LOV in the HFCα group. Any lesions that were present as adenomas or adenocarcinomas did not respond to LOV. The other possibility is that there were a sub-group of lesions in the HFCα group that were sensitive to LOV (LOV sensitive phenotype) and thus were unable to progress to the tumor stage.

In the HFBα group, LOV resulted in an overall growth retarding effect that was noted as reduction in total ACF number, especially ACF in the 1-3 crypt multiplicity category, and accompanied by a reduced tumor size but not incidence. The significantly lower tumor

size in the HFBα group suggests that LOV treatment was having a more generalized growth inhibitory effect on tumors. These findings support the contention that growth and development, as well as the biology of preneoplastic lesions, are affected by the dietary lipid environment.

It is worth noting that LOV treatment produced different responses on tumor growth, especially in tumor number, which seemed to be modulated via different mechanisms depending on the type of growth promoting high fat diet. We propose that tumors appearing in two different high fat diets are biologically different in responding to environmental stressors. In a parallel study (Kiunga, 2006) investigating the effect of tumor development under the two tumor promoting dietary lipid environments (HFC and HFB), it was noted that receptors implicated in cell survival and growth, including insulin receptor (IR) and insulin-like growth factor receptor (IGF-1R), were differentially expressed. All tumors, regardless of the lipid type, expressed higher levels of IR and IGF-1R compared to mucosa. However, HFC tumors exhibited significantly higher IR levels compared to HFB tumors. We do not know if the IR and IGF-1R are involved in resisting LOV effects. Mevalonate-dependent mechanisms involved in growth control include N-linked glycosylation of the IGF-1 receptor (Dricu et al, 1997). LOV treatment of malignant melanoma cell line SK-MEL-2 demonstrated 50% inhibition of ras prenylation as well as a 95% inhibition of N-linked glycosylation of IGF-1 receptors (Carlberg et al, 1996). Inhibition of HMGCAR activity depresses the expression of functional IGF-1R in colon cancer cells (Carlberg et al, 1996). It would be interesting to know if this sensitivity to LOV treatment is specific to fatty acid composition or level of unsaturation. The issue of two tumor promoting diets with markedly different effects has

not received enough attention as results suggest it should. One future direction would be to investigate the role of n-3 fatty acids under similar conditions.

Distribution of tumor number and size along the length of the colon was used to ascertain information on the sensitivity of specific colonic regions to dietary lipids and LOV treatment. It is noteworthy that differences existed among different regions of the colon with respect to the number of tumors. This observation supports the notion that colonic regions respond differently to dietary lipid manipulation and chemopreventive substances. Although tumor distribution in both the HFC and HFB groups was similar, LOV treatment of each group produced opposing effects on the distribution of tumors along the length of the colon. In the HFCα group, there were more proximal tumors and less distal tumors compared to the HFC group. In the HFBα group, there were less proximal tumors and more distal tumors compared to the HFB group. These findings provide evidence in support of the concept that fundamental differences exist among colonic tumors depending on the physiology of the tissue and the surrounding environment which in turn affect cellular pathways involved in supporting their growth.

PART II: $p21^{K\text{-}ras}$, HMGCAR, and LDLr Expression in Colonic Tissues

In the second part of the study, the research determined the influence of different dietary lipids and LOV on the expression of the rate-limiting enzyme of cholesterol biosynthesis (HMGCAR), $p21^{K\text{-}ras}$, and LDLr expression, as well as expression of specific signal transduction and cell cycle related factors important in cellular growth (ERK-1, ERK-2, CD1) and apoptosis (caspase-3).

$p21^{K\text{-}ras}$ is a critical molecular protein involved in transmitting growth signals and promoting survival of colonic mucosa and tumors. Oncogenic $p21^{K\text{-}ras}$ protein has been

implicated in the etiology of colon cancer (Stopera and Bird, 1992; Singh et al, 1997). In addition, over-expression of wild-type p21$^{K\text{-}ras}$ and its activation has been implicated in CRC (Bissonnette et al, 2000). Ras gene mutations have been found in approximately 50% of colorectal cancers, but less than 10% are found in adenomas less than 1 cm in diameter (Bos et al, 1987; Forrester et al, 1987).

Previous research findings support the concept of dietary lipid mediated effects on p21$^{K\text{-}ras}$ expression, membrane-association and signalling in colonic tissue (Singh et al, 1997; Davidson et al, 1999). Additional research suggests the link between the effects of dietary lipids on p21$^{K\text{-}ras}$ function may be via alteration of expression and activity of HMGCAR (El-Sohemy and Archer, 1997 and 1999). Our research findings demonstrate that both the level and type of dietary lipid, including saturated and unsaturated fatty acids, as well as the chemopreventive drug LOV, modulate the expression, activity and membrane localization of p21$^{K\text{-}ras}$ in the multi-step process of colon carcinogenesis. Based on our findings, we speculate that the HFC and HFB diets select tumors with distinctly different p21$^{K\text{-}ras}$ phenotypes (mutated versus activated wild-type p21$^{K\text{-}ras}$ for the HFC and HFB tumors, respectively). The significance of this finding lies in the fact that activated p21$^{K\text{-}ras}$ oncogene is a relatively early step in the process of colon carcinogenesis (Fearon and Vogelstein, 1990).

Our findings also provide compelling evidence that differences in dietary lipid promote the growth of colonic tumors with critical differences in the genetic expression of regulatory proteins as well as reliance on the ERK signalling pathway.

Our results demonstrate that p21$^{K\text{-}ras}$ protein expression is higher in colonic tumors compared to mucosa, supporting the findings of Davidson et al (1999) and provide new

insight into the differential effects of different types of dietary lipids on $p21^{K\text{-}ras}$ expression in colonic mucosa and tumor tissues. We found expression of $p21^{K\text{-}ras}$ protein in normal colonic mucosa to be significantly higher in the HFC group compared to the HFB group. In tumors, $p21^{K\text{-}ras}$ protein expression was similar in both the HFB and HFC groups. However, HFB tumors had higher $p21^{K\text{-}ras}$ activity, suggesting that HFB tumors are more reliant on $p21^{K\text{-}ras}$ and its downstream signalling cascade for growth. The effect of LOV treatment on $p21^{K\text{-}ras}$ expression in both the mucosa and tumors was dependent on dietary lipid type. Tumors from the HFCα group had significantly higher $p21^{K\text{-}ras}$ expression compared to the HFC group, whereas tumors from the HFBα group had significantly lower $p21^{K\text{-}ras}$ expression compared to the HFB group. However, further investigation of $p21^{K\text{-}ras}$ activity revealed that it did not parallel $p21^{K\text{-}ras}$ protein expression in these tissues. In general, colonic tumors had significantly higher $p21^{K\text{-}ras}$ activity compared to colonic mucosa. Tumors from both LOV treated groups had similar $p21^{K\text{-}ras}$ activity with HFBα tumors showing a trend towards higher activity than HFCα tumors. It is possible that HFCα tumors had a higher proportion of $p21^{K\text{-}ras}$ in cytosolic form, whereas HFBα tumors had a higher proportion as membrane-associated form. This would explain the low $p21^{K\text{-}ras}$ activity of the tumors in the HFCα group compared to that of the HFBα group as increased cytosolic $p21^{K\text{-}ras}$ has been shown to antagonize membrane-associated $p21^{K\text{-}ras}$ activity (Stacey et al, 1991). In addition, $p21^{K\text{-}ras}$ activity in tumors from the HFC group was significantly lower than in tumors from the HFB group. To our knowledge, this is the first report of differential effects of dietary lipids on $p21^{K\text{-}ras}$ activity in normal and neoplastic colonic tissue *in vivo*. These findings suggest the possibility of enhanced membrane-associated $p21^{K\text{-}ras}$ in the HFB tumors and

increased cytosolic $p21^{K\text{-}ras}$ in the HFC tumors. Due to the small size and limited tissue availability, we were unable to separate tumor tissue into membrane and cytosolic fractions. Singh et al (1997) reported a reduced colon tumor incidence in AOM injected rats fed diets high in fish oil compared to corn oil. This study demonstrated that the $p21^{K\text{-}ras}$ expression was enhanced by the high fat corn oil diet, whereas it was inhibited by the high fat fish oil diet. In addition, their findings demonstrated that colonic tumors from the high fat fish oil group had reduced membrane-associated $p21^{K\text{-}ras}$ and increased cytosolic $p21^{K\text{-}ras}$ compared to tumors from the high fat corn oil group. Our findings support those of Singh et al (1997) in that colonic mucosa from the HFC diet had enhanced total $p21^{K\text{-}ras}$ expression compared to the LFC group. LOV treatment led to significant reductions in $p21^{K\text{-}ras}$ activity in both colonic mucosa and tumors, irrespective of dietary lipid treatment group. We speculate that the low $p21^{K\text{-}ras}$ activity level of tumors in the HFCα group may be due to the antagonistic action of cytosolic $p21^{K\text{-}ras}$ on membrane-associated $p21^{K\text{-}ras}$ proposed by Stacey et al (1991). The question remains as to how the HFC α and HFBα tumors were surviving if $p21^{K\text{-}ras}$ activity is so essential to growth. Because both tumor types were able to survive, there must be other pathways involved in their survival and growth. These tumors may be so advanced that they were able to up-regulate other pathways to overcome the lack of $p21^{K\text{-}ras}$ activity. It is important to consider that the HFCα tumors were fewer in number. Therefore, the tumors which did not appear in this group were unable to tolerate the adverse effects of LOV treatment and/or were unable to up-regulate the other molecular machinery and related pathways to overcome the adverse effect. Some genetic evidence exists supporting the anti-oncogenic properties of ras genes in human tumors. It is important to

keep in mind that chronically activated $p21^{K\text{-}ras}$ may occur either through mutation (Vogelstein et al, 1988) or over-expression of wild-type $p21^{k\text{-}ras}$ (Bissonnette et al, 2000), both of which have been described as relatively early events in CRC. It has also been demonstrated, using human carcinomas that ras genes may act as tumor suppressor genes and that increased expression of the wild-type alleles may function in favor of reducing the transforming potential rather than promoting the malignancy (Spandidos et al, 2002). The wild-type ras allele has also been proposed to inhibit cell proliferation by promoting differentiation (Pfeifer, 2001; Spandidos et al, 2002).

Factors that may function as a link between $p21^{K\text{-}ras}$ protein expression and $p21^{K\text{-}ras}$ activity are: (1) HMGCAR expression and activity and, (2) differential effects of dietary lipid related to palmitoylation and regulation of GTPases as well as GNEFs.

Cholesterol can be obtained for cellular growth via two mechanisms: (1) synthesis through HMGCAR and/or (2) LDLr uptake of LDL (Caruso et al, 1999). The cell's need for cholesterol dictates the activity of both HMGCAR and LDLr (Goldstein and Brown, 1990). Cancer cells have a higher demand for cholesterol which is met by increasing synthesis (Clayman et al, 1986) or LDL uptake (Vitols et al, 1992) or both (Rudling et al, 1990). Research has demonstrated that certain human tumors have increased expression of HMGCAR and LDLr at the protein and mRNA levels (Maltese, 1983; Engstrom and Schofield, 1987; Caruso et al, 1993; Niendorf et al, 1995). Data on the effect of dietary lipids on the expression of the gene and protein products is limited. We found that tumors from the high fat diets had increased levels of HMGCAR mRNA compared to normal mucosa (except for the LFC group) and that HMGCAR mRNA expression in tumors with or without LOV treatment was dependent on fat type. Tumors from the

HFCα group had a significant reduction in HMGCAR mRNA compared to the HFC group, whereas the HFBα group had a significant increase in HMGCAR mRNA levels compared to the HFB group. This pattern of HMGCAR mRNA expression is also reflected in the HMGCAR protein expression.

In the colonic mucosa, the higher HMGCAR protein expression found in the HFB group is expected as a highly saturated fatty acid diet decreases hepatic LDLr levels and cellular cholesterol uptake (Ness et al, 1996). Therefore, in an attempt to ensure adequate cholesterol levels, cellular biosynthesis would increase and would be reflected in increased HMGCAR levels. In colonic mucosa, the increase in LDLr protein expression and reduced HMGCAR protein expression in the HFC diet was expected and is supported by previous research findings (Goldstein and Brown, 1984; Goldstein and Brown, 1990; Ness, 1994; Ness et al, 1996; Ness and Chambers, 2000).

Changes at the mRNA transcript level are not always corroborated by changes at the protein level. For example, significant reductions in HMGCAR protein expression occurred in colonic mucosa from both HFCα and HFBα. Hence, this suggests other factors affecting HMGCAR status such as mRNA and enzyme stability as well as transcription and translation rate. One possible explanation is that regardless of treatment with LOV, tumors have a higher requirement for HMGCAR than corresponding normal mucosa. Our findings in relation to HMGCAR protein expression in tumors compared to mucosa are consistent with Caruso et al (2002) and Notarnicola et al (2004) in that colonic tumors had significantly higher HMGCAR protein expression compared to mucosa. Tumors in the HFC group had higher HMGCAR protein expression compared to HFB tumors. Our findings of a differential effect of dietary lipid type on HMGCAR

protein expression in both colonic mucosa and tumors are in direct contrast to those of Collett et al (2001) who reported no effect of linoleic acid or DHA on HMGCAR protein expression in young adult mouse colon cells over-expressing H-ras.

Phenotypic differences in colonic adenocarcinomas in relation to HMGCAR activity has been described (Maltese et al, 1983) in which three of four colonic adenocarcinomas expressed high HMGCAR activity and one expressed low activity. Notarnicola's research findings support differences in HMGCAR expression based on tumor distribution within the colon and has demonstrated increased HMGCAR activity in left-sided (distal) human colon tumors (Notarnicola, 2004). Our findings show that in the HFBα group, a higher proportion of tumors were distally located and that these tumors had the highest HMGCAR protein expression. These tumors seem to rely more heavily on cholesterol biosynthesis to support their growth, a suggestion that is supported by investigations of human colonic adenocarcinomas cell lines (Broitman et al, 1993 and Cerda et al, 1995). Increased HMGCAR leads to increased cholesterol biosynthesis, and also increases the production of mevalonate and its metabolites, including farnesyl pyrophosphate. Oncogenic $p21^{K\text{-}ras}$ has been detected in approximately 50% of human colonic tumors and requires farnesylation for activation. It has been hypothesized that increased HMGCAR activity in colonic neoplastic tissue not expressing LDLr may play a critical role in malignancy by increasing mevalonate and farnesyl pyrophosphate synthesis (Caruso et al, 1999). It may also provide an opportunity for chemoprevention using statins to target HMGCAR. Indeed, in humans, the use of statins for at least five years (versus non-use of statins) was associated with a 47% relative reduction in risk of colon cancer (Poynter et al, 2005). In animal models, the tumor preventive effects of

HMGCAR inhibitors have also been demonstrated *in vivo* (Maltese et al, 1985; Sumi et al, 1992). Our findings demonstrate that the effect of LOV treatment on colonic tumor mRNA and protein expression depends on the dietary lipid composition. LOV treatment combined with an HFC diet (HFCα) results in a reduction of HMGCAR mRNA, whereas the same treatment in the HFB group (HFBα) produces an increase in HMGCAR mRNA. LOV treatment had no effect on HMGCAR mRNA in normal mucosa. The effect of LOV treatment on colon tumor HMGCAR protein expression corresponded with the trend of mRNA expression in this tissue. HMGCAR protein expression was significantly lower in the HFCα group compared to the HFC group and significantly higher in the HFBα compared to the HFB group. HFBα tumors had lower LDLr and higher HMGCAR, whereas the HFCα tumors had higher LDLr and lower HMGCAR expression. However, in the non-LOV treated groups, HFB tumors had lower LDLr and HMGCAR, whereas the HFC tumors had higher LDLr and HMGCAR expression. These findings support our contention that the HFC tumors are less dependent on the cholesterol biosynthetic pathway to support their growth and development and that the HFC dietary environment selected tumors with the ability to survive HMGCAR inhibition. It should also be emphasized that increases in the level of a specific protein may not be reflected in a corresponding increase in its activity. Nevertheless, these findings do attest to the fact that the tissues were responding to the experimental treatments. It may be that the tumors rely on alternate pathways to support their growth. One possible pathway may involve PKC activation of $p21^{K\text{-}ras}$ and/or PKC activation of ERK-1. Another factor that must be considered is the possibility that the rapidly appearing HFC tumors have mutated $p21^{K\text{-}ras}$ and require less protein farnesylation and hence are less reliant on HMGCAR, whereas

HFB tumors rely on increased farnesylation of wild-type activated $p21^{K\text{-}ras}$ and hence, increased levels of HMGCAR to support growth. We investigated hepatic HMGCAR expression as a baseline tissue measure. Our findings support those of Ness et al (1994), in that LOV treatment significantly increased hepatic HMGCAR protein expression in both the HFCα and HFBα groups. This finding supports the proposal that LOV may inhibit the activity of HMGCAR and does not inhibit the synthesis or may affect the turnover rate of this protein.

To our knowledge, the effect of dietary lipid composition and LOV on LDLr expression in normal and neoplastic colonocytes, *in vivo*, has not been investigated. Hepatic LDLr activity, mRNA and protein expression has been shown to decrease in response to SFA and increase in response to PUFA. Due to the association between HMGCAR and LDLr in establishing and maintaining cholesterol metabolism and status, LDLr was included as one of the parameters investigated in the study. Our findings demonstrate that in normal appearing colonic mucosa, LDLr mRNA expression is similar in both the HFC and HFB groups. However, feeding a low fat diet produced a significant reduction in LDLr mRNA compared to the high fat diet which is consistent with feedback regulation of cholesterol biosynthesis. Our findings demonstrate a trend towards increased LDLr mRNA expression in tumors compared to mucosa. The effect of LOV treatment on LDLr mRNA expression was dependent on the type of dietary lipid. HFCα tumors had significantly lower LDLr mRNA than HFBα tumors. This finding is consistent with other research supporting the notion that more aggressive tumors lack the LDLr (Caruso et al, 1999). Due to the multi-level regulation of the LDLr and tissue differences in expression among tissues, it was important to investigate the expression of LDLr protein

in normal as well as neoplastic colonic tissue. The effect of a high saturated and PUFA diet on LDLr protein expression in normal appearing colonic tissue followed the same trend as that described for hepatocytes. The HFB diet resulted in significantly lower LDLr protein compared to the HFC diet. The effect of LOV treatment on LDLr protein expression in the colon was dependent on the dietary lipid environment. LDLr expression was significantly lower in the HFCα compared to both the HFC and HFBα groups.

Colonic tumors seemed to be less dependent on the LDLr for growth as tumors in all experimental treatment groups had significantly lower LDLr protein expression compared to mucosa. Notarnicola et al (2004) demonstrated that HMGCAR activity and LDLr levels were higher in colonic tumors compared to normal appearing mucosa. We found significant reductions in colonic tumor LDLr protein expression for all treatment groups. Caruso et al (1999) reported that in a population of human colonic tumors, 63% did not express the LDLr and that absence of the LDLr predicted shorter survival (Caruso et al, 1998). Tumors not expressing the LDLr had significantly higher HMGCAR activity than those expressing the LDLr. The tumors with low LDLr may be classified as more aggressive. The present findings support the conjecture that experimentally induced tumors are less dependent on pre-formed cholesterol uptake through LDLr than normal cells and rely more heavily on HMGCAR in the metabolic pathway responsible for the synthesis of cholesterol. The finding that colonic tumors in both LOV treated groups had substantially lower LDLr expression is unique and the mechanism underlying such an effect requires further investigation.

In light of the effect of LOV on HMGCAR and LDLr protein expression in colonic tumors, it is apparent that phenotypic differences exist. It is difficult to explain exactly how these tumors are surviving and whether HMGCAR and LDLr are playing a role. When looking at the LDLr protein expression data in colonic tumors, it seems that LOV treatment resulted in decreased LDLr protein and a corresponding increase in HMGCAR in the HFBα group. This may represent an attempt to provide cholesterol and/or related compounds, such as farnesyl pyrophosphate, to maintain tumor growth.

PART III: ERK-1, ERK-2, Cyclin D1, and Caspase-3 Expression in Colonic Tissues

Extracellular signal-regulated kinases (ERKs), otherwise known as mitogen activated protein kinases (MAPKs), are serine-threonine kinases involved in major cell-proliferation signalling pathways from the cell surface to the nucleus (Dong et al, 2002). The ERK pathway, involving Ras/Raf1/MEK/ERK, is one of three subfamilies of MAPK. This signalling cascade leads to altered gene expression and promotes cell proliferation. Several key growth factors and proto-oncogenes transduce the signals that promote growth and differentiation through this cascade (Fang and Richardson, 2005). Hence, therapeutic agents able to disrupt this growth-stimulatory signalling cascade may be useful in treatment of CRC (Watanabe, 2000; Fang and Richardson, 2005). Our findings support this concept in that normal colonic mucosa demonstrated significantly lower ERK-1 expression compared to tumors from all treatment groups. Compared to normal tissue, increased MAPK activity has been observed in human gastric (Bang et al, 1998) and colorectal (Ostrowski et al, 1998; Aliaga et al, 1999; Shao et al, 2004; Caron et al, 2005) cancer. The link between MAPK pathway signalling and cell adhesion, angiogenesis, invasion and metastasis in CRC is also well established (Fang and

Richardson, 2005). Evidence is emerging in support of a role for MAPK in regulating apoptosis related to growth factors and their receptors in CRC. Insulin-like growth factor-1 has been shown to have anti-apoptotic function mediated by the ERK/MAPK signal cascade (Remacle-Bonnet, 2000) and has been demonstrated to be significantly higher in HFC tumors compared to HFB tumors (Kiunga, 2006). In addition, the EGF receptor has been implicated in playing a role in apoptosis in human CRC cells induced via the ERK pathway (Im, 2004).

In the current thesis research, ERK-1 and ERK-2 protein expression was assessed based on the fact that $p21^{K\text{-}ras}$ activation can lead to sequential activation of the ERK signalling cascade. Our findings support those previously reported (Davidson et al, 1999) in that colonic tumors up-regulated ERK-1 as well as ERK-2 expression to support their growth. Colonic tumors from the HFB group had significantly higher ERK-1 protein expression compared with tumors from the HFC group. Sakakura (1999) demonstrated that MAPK activation in human colon tumors occurred at low frequency (18%) in those tumors harbouring a $p21^{K\text{-}ras}$ mutation, whereas in tumors without a $p21^{K\text{-}ras}$ mutation, MAPK activation was high (75%). Hence, our theory that HFB tumors phenotypically harbor over-expressed levels of wild-type $p21^{K\text{-}ras}$, whereas HFC tumors phenotyically harbor mutated $p21^{K\text{-}ras}$ is reasonable. In addition, tumors from the LFC group had significantly higher ERK-1 expression and $p21^{K\text{-}ras}$ activity compared to the HFC group. Davidson (1999) suggested that the carcinogen-induced changes in $p21^{K\text{-}ras}$ expression and membrane localization were associated with activation of the ERK pathway. Our findings support this contention in colonic tumors as well as broaden the concept to include the fact that saturated and PUFA have differential effects on $p21^{K\text{-}ras}$ activity

leading to differential downstream effects on ERK-1 expression. In the HFC group, colonic tumors had significantly lower $p21^{K\text{-}ras}$ activity which corresponded with decreased ERK-1 protein expression, whereas tumors in the HFB group had significantly higher $p21^{K\text{-}ras}$ activity corresponding with increased ERK-1 protein expression. The fact that the HFB group had higher ERK-1 expression than the HFC group leads us to believe that the tumors use different combinations of pathways to survive and grow. The effect of LOV on tumor expression of ERK-1 was dependent on the dietary lipid environment. Colonic tumors from the HFCα group had up-regulated ERK-1 and demonstrated significantly higher expression of ERK-1 compared to the HFBα and HFC group. Studies using NSAIDs (sulindac) in CRC have shown that inhibition of ERK1/2 phosphorylation leads to increased apoptosis (Soh, 2000; Rice, 2003). The fact that tumors from the HFCα group demonstrated significantly higher ERK-1 expression leads us to believe that this may be one mechanism by which they are escaping apoptosis and have the ability to continue to grow in an inhibitory environment. In addition, HFBα tumors demonstrated significantly less ERK-1 expression which may "sensitize" them to increased likelihood of apoptosis.

Based on our findings, as well as the findings of other researchers, we propose that tumors from the HFCα group harbour a higher frequency of $p21^{K\text{-}ras}$ mutation and are more aggressive in that they are able to employ molecular mechanisms which allow them to escape growth inhibition and continue to grow at a rapid rate. In phase III clinical trials of a farnesyl protein transferase inhibitor (Zarnestra; R115777) in patients with CRC, it did not improve overall survival compared with supportive-care alone in refractory advanced CRC (Rao, 2004).

Cyclin D1 (CD1) plays an important role in normal progression of cells through the cell cycle (Haber and Fearon, 1998) and is amplified in colon cancer (Bissonette et al, 2000). Evidence exists supporting the fact that colonic malignant transformation in humans and animals involves alterations in both $p21^{K\text{-}ras}$ and CD1 (Arber et al, 1996; Bissonnette et al, 2000), both of which are important cell signal transduction elements. *In vitro* and *in vivo* studies have demonstrated increased CD1 expression due to mutations in $p21^{K\text{-}ras}$ and to be increased in a subset of AOM-induced tumors (Otori et al, 1999). That CD1 protein expression in HFC tumors is significantly higher compared to HFB tumors supports the theory that HFC tumors may harbour a higher frequency of $p21^{K\text{-}ras}$ mutations. Regulatory elements of the CD1 promoter are induced in response to oncogenic ras proteins or activation of the ERK mitogen activated protein kinase (Albanese et al, 1995; Lavoie et al, 1996). Ras activity plays a key role in integrating mitogenic signals with cell cycle progression and is required for induction of CD1 (Aktas et al, 1997).

Currently, there are many gaps in our understanding of the effects of specific dietary lipids on cell cycle control and gene expression. Our results support previous findings of increased CD1 expression in colonic mucosa of AOM injected compared to non-injected rats (Fujise et al, 2006). In addition, CD1 expression has been shown to be significantly higher in rats fed corn oil and beef tallow diets with and without AOM treatment. CD1 expression in normal appearing mucosa has been shown to be increased by feeding both high fat corn oil and beef tallow diets (Fujise et al, 2006). Our findings contradict these as we found that an HFC diet resulted in decreased CD1 protein expression, whereas an HFB diet led to increased CD1 expression in normal colonic mucosa. Limited data exist

describing the effect of specific dietary lipid on CD1 expression and involvement in colon carcinogenesis. The present study found significantly higher CD1 protein expression in colonic tumors from the HFC group compared to the HFB group. CD1 gene (and protein) expression was consistently elevated in tumors regardless of treatment. CD1 expression in both LOV treated groups were reduced to similar levels. Bissonnette (2000) has speculated that the differential expression of CD1 in tumors with mutated versus activated wild-type $p21^{K\text{-}ras}$ expression may involve alternate signalling pathways (including GAP and GNEF regulation) that activate the gene product of wild-type but not mutant $p21^{K\text{-}ras}$.

Neoplastic growth in colon cancer has been related to defects in the apoptotic mechanism (Duke et al, 1996; Evertsson et al, 1999). Activation of caspases is a critical step in apoptotic cell death and caspase-3 is known to be a key enzyme during apoptosis (Jonges, 2001). We investigated caspase-3 expression in normal and neoplastic colonic tissue via Western blot analysis in all the experimental groups in a preliminary attempt to establish whether caspase-3 expression is affected by dietary lipid environment as well as to determine if this protein plays a role in the apoptotic effect of LOV in normal and neoplasic colonic tissue.

It is intriguing that high caspase-3 activity has been found to be significantly correlated with a higher risk of recurrence and is preferentially found in tumors of the right (proximal) side of the colon (Jonges et al, 2001). In fact, our findings demonstrate that tumors from the HFCα group, which we suspect are more aggressive tumors due to their significantly larger size and reduced incidence, had a proximal (right-sided) distribution as well as a significantly higher level of caspase-3 expression compared to all other

treatment groups. Our findings corroborate those of Jonges et al (2001) in that colonic tumors had a significantly higher caspase-3 expression compared to normal colonic mucosa.

Human colonic adenocarcinomas HT-29 cells pretreated with AA and DHA both showed increased ability to induce cell death, including increases in caspase-3 activity (Hofmanova et al, 2005). These findings demonstrated that PUFA induce processes that promote the sensitivity of colon cancer cells to endogenous apoptotic regulators. The researchers concluded that although dietary PUFAs alone were incapable of inducing apoptosis, they were able to, through changes in membrane properties, oxidative metabolism and additional mechanisms, start a cascade of processes which prepare permissive environment for a more effective action of apoptotic inducers (Hofmanova, J. et al, 2005) and led them to conclude that the composition of dietary lipid should be included in strategies for colon cancer prevention and therapy. Our results strongly support this contention as caspase-3 expression was found to be higher in colonic tumors from the HFC group compared to the HFB group. In addition, the effect of LOV on tumor caspase-3 expression was modulated by the dietary lipid environment.

Summary and Conclusions

The primary objective of this research was to investigate the amenability of preneoplastic colonic lesions and tumors to orally administered LOV and explore the cellular and molecular changes in the molecules involved in regulation of cholesterol homeostasis and cell growth in normal colonic mucosa and tumors. The secondary objective was to determine if preneoplastic lesions and/or colonic tumors which, were allowed to develop in rats consuming dietary lipids varying in fatty acid composition, would respond

differently to LOV. The rats, bearing established preneoplastic lesions after consuming a low fat corn oil diet for 12 weeks, were allowed to consume specific (LFC, HFC, or HFB) diets for an additional 9 weeks. LOV intervention was carried out daily for three weeks only. This short duration of LOV treatment was used to assess the sensitivity of the lesions to LOV.

The diets used were high in fat and varied markedly from each other with respect to the level of n-6 linoleic acid. One diet contained beef tallow (HFB) and the other contained corn oil (HFC). It is noteworthy that the tumor incidence was significantly lower in the LOV treated HFCα group compared to HFC group. This effect was not noted in the HFBα group compared to the HFB group. It was also noted that, generally, LOV was less effective in reducing the number and growth of ACF in both groups. All large tumors, which probably existed at the time of LOV intervention, did not respond to LOV. The reduction in the tumor incidence in the HFCα group was mainly due to reduction in the number of small tumors. These findings demonstrate that dietary lipid was altering the responses of developing preneoplastic lesions and/or tumors to LOV. The ability of dietary lipid and LOV to interact and change the phenotype of tumors was noted among the experimental measurements carried out in the studies. The findings are summarized in Tables 36 and 37. It is clear that dietary lipids profoundly affected the tumor phenotype and the effect of LOV was influenced by the dietary lipid environment. These findings demonstrate that tumors are a clonally expanding tissue mass, deviating markedly from the surrounding tissue. These findings provide an impetus to continue research in order to improve our understanding of the influence of the tumor macro-environment in selecting lesions with different phenotypes. Our findings also

demonstrate that the preneoplastic stages, even if morphologically they may appear to be similar, are biologically different and represent both rapid and slow growing lesions. In addition, the research demonstrates the importance of the *in vivo* model. Using this model we were able to demonstrate that the different stages of colon cancer development respond differently to LOV and that this drug may be more effective in cancer preventive strategies than as therapeutic agents.

Future studies should be conducted to determine if indeed the responsiveness of tumors to LOV depends on whether or not they have $p21^{K\text{-}ras}$ mutations as well as to determine the pathways (survival and pro-apoptotic) that are activated in response to LOV. It is also important to investigate whether the ability of tumors to metabolize and detoxify LOV varies depending on their macro-environment and stage of growth and development.

Table 36: Phenotypic Differences in Colonic Mucosa and Tumors from HFC and HFB Experimental Treatments

MUCOSA Parameter		HFC		HFB
p21$^{K\text{-}ras}$ Protein	↑	(21.4 ^{a})	↓	(12.5 ^{c}) ✚
p21$^{K\text{-}ras}$ Activity	↓	(8.7 ^{b})	↑	(26.4 ^{a}) ✚
HMGCAR Protein	↓	(16.7 ^{b})	↑	(18.6 ^{a})
LDLr Protein	↑	(5.3 ^{a})	↓	(4.0 ^{bc}) ✚
ERK-1 Protein (equal)		(1.94 ^{b})	↔	(2.2 ^{b}) ✚
ERK-2 Protein (equal)		(1.4 ^{c})	↔	(1.5 ^{bc})
CD1 Protein	↓	(2.6 ^{c})	↑	(3.0 ^{ab}) ✚
Caspase-3 Protein (equal)		(3.4 ^{a})	↔	(3.1 ^{a}) ✚

TUMORS: tumor incidence and size were the same between HFC & HFB

Parameter		HFC		HFB
p21$^{K\text{-}ras}$ Protein (equal)		(27.5 ^{bc})	↔	(35.8 ^{ab}) ❋
p21$^{K\text{-}ras}$ Activity	↓	(18.7 ^{b})	↑	(39.6 ^{a}) ✱, ✚
HMGCAR Protein	↑	(33.8 ^{a})	↓	(27.1 ^{b}) ✱
LDLr Protein	↑	(3.0 ^{a})	↓	(2.3 ^{b}) ◆
ERK-1 Protein	↓	(4.3 ^{c})	↑	(6.1 ^{a}) ✱, ✚
ERK-2 Protein (equal)		(2.5 ^{a})	↔	(3.0 ^{a}) ✱
CD1 Protein	↑	(5.8 ^{a})	↓	(4.97 ^{b}) ✱, ✚
Caspase-3 Protein (equal)		(6.1 ^{bc})	↔	(4.1 ^{c})

❋ designates higher in tumors compared to mucosa except for the HFC group; ✱ designates higher in tumors compared to mucosa; ◆ designates lower in tumors compared to mucosa; ✚ designates a significant effect of saturated versus unsaturated fat.

Table 37: Phenotypic Differences in Colonic Mucosa and Tumors from HFCα and HFBα Dietary Treatments

MUCOSA

Parameter	**HFCα**		**HFBα**
$p21^{K\text{-}ras}$ Protein	↑ (19.2 ^{ab})		↓ (12.2 ^{c}) ✧
$p21^{K\text{-}ras}$ Activity (equal)	(0.1 ^{c})	↔	(0.2 ^{c}) ✧
HMGCAR Protein (equal)	(14.6 ^{c})	↔	(16.0 ^{bc})
LDLr Protein	↓ (3.0 ^{d})		↑ (4.1 ^{b}) ✧
ERK-1 Protein (equal)	(3.2 ^{a})	↔	(3.0 ^{a})
ERK-2 Protein	↓ (1.6 ^{bc})		↑ (2.1 ^{a})
CD1 Protein (equal)	(3.2 ^{ab})	↔	(3.3 ^{a})
Caspase-3 Protein (equal)	(2.8 ^{bc})	↔	(2.4 ^{c}) ✧

TUMORS: HFCα (41%) had significantly lower tumor incidence compared to HFBα (70%); HFCα tumors were significantly larger (26 mm^2) than the HFBα tumors (17.6 mm^2)

Parameter	**HFCα**		**HFBα**
$p21^{K\text{-}ras}$ Protein	↑ (45.2 ^{a})		↓ (20.9 ^{c}) ✱, ✧
$p21^{K\text{-}ras}$ Activity (equal)	(1.4 ^{c})	↔	(2.9 ^{c}) ✧
HMGCAR Protein	↓ (21.5 ^{c})		↑ (33.8 ^{a}) ✱, ✧
LDLr Protein	↑ (2.4 ^{b})		↓ (1.4 ^{c}) ◆
ERK-1 Protein	↑ (6.1 ^{a})		↓ (5.0 ^{b}) ✱, ✧
ERK-2 Protein (equal)	(2.9 ^{a})	↔	(3.0 ^{a}) ✱
CD1 Protein (equal)	(4.7 ^{bc})	↔	(4.14 ^{c}) ✱, ✧
Caspase-3 Protein	↑ (9.25 ^{a})		↓ (6.82 ^{b}) ✱, ✧

✱ designates higher in tumors compared to mucosa; ◆ designates lower in tumors compared to mucosa; ✧ designates a significant lipid x lovastatin interaction.

REFERENCES

A

Agarwal, B, Bhendwal, S., Halmos, B., Moss, S.F., Ramey, W., and Holt, P. 2000. Lovastatin augments apoptosis induced by chemotherapeutic agents in colon cancer cells. Clin. Cancer Research. 6 (3): 1198-1199.

Agarwal, B, Rao, C., Bhendwal, S., Ramey, W., Shirin, H., Reddy, B., and Holt, P. 1999. Lovastatin augments sulindac-induced apoptosis in colon cancer cells and potentiates chemopreventive effects of sulindac. Gastroenterology. 117: 838-847.

Agarwal, B. Halmos, B., Feoktistov, A.S., Protiva, P., Ramey, W.G., Chen, M., Pothoulakis, C., Lamont, J.T., Holt, P.R. 2002a. Mechainsm of lovastatin-induced apoptosis in intestinal epithelial cells. Carcinogenesis. 23 (3): 521-528.

Aktas, H, Cai, H, and Cooper, G.M. 1997. Ras links growth factor signalling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor $p27^{KIP1}$. Molecular and Cellular Biology. 17(7): 3850-3857.

Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. and Pestell, R.G. 1995. Transforming $p21^{ras}$ mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J. Biol. Chem. 270: 23589-23597.

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, R., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. 1980. Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductse and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. USA, 77 (7): 3957-3961.

Aliaga, J.C., Deschenes, C., Beaulieu, J.F., Calvo, E.L., and Rivard, N. 1999. Requirement of the MAP kinase cascade for cell cycle progression and differentiation of human intestinal cells. Am. J. Physiol. 277(3 Pt 1): G631-G641.

Alonso, D.F. et al. 1998. Reduction of mouse mammary tumor formation and metastasis by lovastatin, an inhibitor of the mevalonate pathway of cholesterol biosynthesis. Breast Cancer Res. Treat. 50: 83-93.

American Hospital Formulary Service; Drug Information. 2004. American Society of Health-System Pharmacists.

American Institute of Nutrition: Report of the *ad hoc* committee on standards for nutrition studies. 1977. J. Nutr. 110: 1726.

Amodol RG, Wolf M, Freeman D, et al. 2007. European Journal of Cancer Supplements. 5: 8.

Angelin, B. 1995. Eur. J. Clin. Invest. 25: 215-224.

Anti, M., Marra, G., Armelao, F., et al. Rectal epithelial cell proliferation patterns as predictors of adenomatous polyp recurrence. 1993. Gut 34: 525-530.

Arber, N, Sutter, T., Miyake, M., Kahn, S.M., Venkatraj, V.S., Sobrino, A., Warburton, D., Holt, P.R. and Weinstein, I.B. 1996. Increased expression of cyclin D1 and the RB tumor suppressor gene in c-K-ras transformed rat enterocytes. Oncogene. 12: 1903-1908.

Archer, M.C, Bruce, W.R., Chan, C.C., Corpet, D.F., Medline, A, Roncucci, I. Stamp, D., and Zhand, X.M. 1992. Aberrant crypt foci and microadenoma as marker for colon cancer. Environ. Health Persect. 98: 195-197.

Azrolan, N.I. and Coleman, P.S. 1989. A discoordinant increase in the cellular amount of 3-hydroxy-3-methylglutary coenzyme A reductase results in the loss of rate-limiting control over cholesterogenesis in a tumor cell-free system. Biochem. J. 258: 421-425.

B

Bang, Y.J., Kwon, J.H., Kang, S.H., Kim, J.W., Yang, Y.C. 1998. Increased MAPK activity and MKP-1 overexpression in human gastric adenocarcinomas. Biochem. Biophys. Res. Coomun. 250: 43-47.

Barbacid, M. 1987. Ras genes. Annu. Rev. Biochem. 56: 799-827.

Beck, P., Wysowski, D.K, Downey, W, and Butler-Jones, D. 2003. Statin use and the risk of breast cancer. J. Clin. Epidemiol. 56: 280-285.

Bertagnolli, M., McDougall, C., and Newmark, H. Colon cancer prevention: intervening in a multistage process. Proc. Soc. Exp. Biol. Med. 216: 266-274, 1997

Bilheimer, D.W., Grundy, S.M., Brown, M.S., and Goldstein, J.L. 1989. Proc. Natl. Acad. Sci. USA. 80: 4124-4128.

Bissonette M., Khare, S., von Lintig, F.C., Wali, R.K., Nguyen, L., Zhang, Y., Hart, J., Skarosi, S., Varki, N., Boss, G.R., and Brasitus, T.A. 2000. Mutational and nonmutational activation of $p21^{ras}$ in rat colonic azoxymethane-induced tumors: Effects on mitogen-activated protein kinase, cyclooxygenase-2, and cyclin D1. Cancer Research. 604602-4609.

Bird R.P. 1987. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Letters. 37: 147-151.

Bird, R.P. 1991. Effect of cholic acid on the number and growth of aberrant crypt foci: putative preneoplastic lesions. Proc. Am. Assoc. Cancer Research. 32: 76 (abstract).

Bird R.P. 1995. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Letters. 93: 55-71.

Bird, R.P. 1998. Aberrant crypt foci to study cancer preventive agents in the colon. In: Hanaucek, M., Walaszek, Z., (Eds.), Tumor Marker Protocols. Human Press Inc., New Jersey, USA, pp. 465-474.

Bird, R.P. and Good C.K. 2000. The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. Toxicology Letters. 112-113:395-402.

Bird, R.P., Mercer, N., and Draper, H. 1985. Animal models for the study of nutrition and human disease: colon cancer, atherosclerosis and osteoporosiss. Advances in Nutritional Research. Volume 7. H.H. Draper (Ed.). Plenum Press, New York, USA. Pp.155-186.

Bird, R.P. and Pretlow, T.P. 1992. Letter to the editor. Cancer Research. 52: 4291-4292.

Bird, R.P, Salo, D., Lasko, C., and Good, C. 1997. A novel methodological approach to study the level of specific protein and gene expression in aberrant crypt foci putative preneoplastic colonic lesions by Western blotting and RT-PCR. Cancer Letters. 116: 15-19.

Bird, R.P., Yao, K., Lasko, C. and Good, C.K. 1996. Inability of –low or –high fat diet to modualte late stages of colon carcinogenesis in Sprague-Dawley rats. Cancer Research. 56: 2896-2899.

Blais, L., Desagne, A. and LeLorier, J. 2000. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and the risk of cancer: a nested case-control study. Arch. Intern. Med. 160: 2363-2368.

Bos J.L., Fearon, E.R., Hamilton, S.R., et al. 1987. Prevalence of ras gene mutations in human colorectal cancers. Nature. 327: 293-297.

Bos, J.L. 1989. Ras oncogenes in human cancer: a review. Cancer Research. 49: 4682-4689.

Boudreau, D.M et al. 2004. The association between 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor use and breast carcinoma risk among postmenopausal women: a case-control study. Cancer. 100: 2308-2316.

Bradford, M. 1976. A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principles of dye binding. Anal. Biochem. 84: 639-641.

Bradford, R.H., Shear, C.L, Chremos, A.N., Dujovne, C., Downton, M., Franklin, F.A., Gould, A.L., Hesney, M., Higgins, J., Hurley, D.P., Langendorfer, A. Nash, D.T., Pool, J.L. and Schnaper, H. 1991. Arch. Intern. Med. 151: 43-49.

Broitman, S.A., Cerda, S., Wilkinson, J. 1993. Cholesterol metabolism and colon cancer. Prog. Food Nutr. Sci. 17(1): 1-40.

Brown, M.S. and Goldstein, J.L. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell. 89: 331-340.

Brown, M.S., Faust, J.R. and Goldstein, J.L. 1987. Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-256-B), a competitive inhibitor of the reductase. J. Biol. Chem. 253: 1121-1128.

Bruce, W.R. 1990. Aberrant crypt foci in the detection of colon carcinogenesis. In: Mutagens and Carcinogens in the diet. Pp. 129-137. Editors: M.W. Pariza, H.U. Aeshbacher, J.S. Felton and S. Sato. Wiley, New York.

Bruce, W.R., Archer M.C., Corpet, D.F., Medline, A., Minkin, S., Stamp, D., Yin, Y., and Zhang, X.M. 1993. Diet, aberrant crypt foci and colorectal cancer. Mutat. Res. 290(1):111-118.

C

Caderni, G., Bianchini, F, Mancina, A., Spagnesi, M.T. andDolara, P. 1991. Effect of dietary carbohydrates on the growth of dysplastic crypt foci in the colon of rats treated with 1,2 dimethylhydrazine. Cancer Research. 51: 3721-3725.

Cafforio, P., Dammacco, F. Gernone, A. and Silvestris, F. 2005. Statins activate the mitochondrial pathway of apoptosis in human lymphoblasts and myeloma cells. Carcinogenesis. 26: 883-891.

Campbell, S.L., Khosravi-Far, R., Rossman, K.L., Clark, G.J., and Der, C.J. 1998. Increasing complexity of Ras signalling. Oncogene. 17: 1395-1413.

Canadian Cancer Society. 2004. Colorectal Cancer: What you need to know.

Carlberg, M., Dricu, A., Blegen, H. Wang, M., Hjertman, M., Zickert, P., Hoog, A., Larsson, O. 1996. Mevalonic acid is limiting for N-linked glycosylation and translocation of the insulin-like growth factor to the cell surface. Evidence for a new link between 3-hydroxy-3-methylglutaryl coenzyme A reductase and cell growth. J. Biol. Chem. 271 (29): 17453-17462.

Caron, R.W.,Yacoub, A., Mitchell, C. Zhu, X., Hong, Y., Sasazuki, T., Shirasawa, S., Hagan, M.P., Grant, S., Dent, P. 2005. Radiation-stimulated ERK1/2 and JNK1/2 signalling can promote cell cycle progression in human colon caner cells. Cell Cycle. 4(3): 456-464.

Caruso, M.G., Notarnicola, M., Cavallini, A., Di Leo, A. 2002. 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and low density lipoprotein receptor expression in diffuse-type and intestinal-type human gastric cancer. J. Gastroenterol. 37(7): 504-508.

Caruso, M.G., Notarnicola, M., Santillo, M., Cavallini, A., Di Leo, A. 1999. Enhanced 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Human Colorectal Cancer not Expressing Low Density Lipoprotein Receptor. Anticancer Research. 19: 451-454.

Caruso, M.G., Osella, A.R., Notarnicola, M., Berloco, P., Leo, S., Bonfiglio, C. Di Leo, A. 1998. Prognostic value of low density lipoprotein receptor expression in colorectal carcinoma. Oncol. Report. 5 (4): 927-930.

Caruso, M.G., Notarnicola, M., Cavallini, A. et al. 1993. Demonstration of LDL receptor in human colonic carcinoma and surrounding mucosa by immunoenzymeatic assay. Ital. J. Gastroenterology. 25: 361-367.

Cassano, A., Bagala, C., Battelli, C., Schinzari, G., Quirino, M., Ratto, C., Landriscina, M., Barone, C. 2002. Expression of vascular endotheilial growth factor, mitogen-activated protein kinase and p53 in human colorectal cancer. Anticancer Res. 22(4): 2179-2184.

Cauley, J.A. et al. 2003. Lipid-lowering drug use and breast cancer in older women: a prospective study. J. Womens Health (Larchmt). 12: 749-756.

Cerda, S.R., Wilkinson, J. IV, Broitman, S.A. 1995. Regulation of cholesterol synthesis in four colonic adenocarcinomas cell lines. Lipids. 30: 1083-1092.

Chambers C. M. and Ness G. C. 1997. Translation regulation of hepatic HMG-CoA reductase by dietary cholesterol. Biochemical and Biophysical Research Communications, 232: 278-281.

Chambers, C. M. and Ness G. C. 1998. Dietary cholesterol regulates hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in rats primarily at the level of translation. Archives of Biochemistry and Biophysics, 354: 317-322.

Chan, K.K., Oza, A.M. and Siu, L.L. 2003. The statins as anti-cancer agents. Clin. Cancer Res. 9: 10-19.

Chen, D.B. and Davis, J.S. 2003. Epidermal growth factor induces c-fos and c-jun mRNA via Raf-1/MEK1/ERK-dependent and independent pathways in bovine luteal cells. Mol. Cell Endocrinol. 200: 141-154.

Chen, Z.Y. and Istfan, N.W. 2001. Docosahexaenoic acid, a major constituent of fish oil diets, prevents activation of cyclin-dependent kinases and s-phase entry by serum stimulation in HT-29 cells. Prostaglandins Leukotreines and Essential Fatty Acids. 64: 67-73.

Chen, C.Y., Liou, J., Forman, L.W., and Faller, D.V. 1998. Differential regulation of discrete apoptotic pathways by Ras. J. Biol. Chem. 273: 16700-16709.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-161.

Chun, K.T., Bar-Nun, S. and Simoni, R.D. 1990. The regulated degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase requires a short-lived protein and occurs in the endoplasmic reticulum. J. Biol. Chem. 265: 22004-22010.

Chapkin, R.S., Jinag, Y.H., Davidson, L.A. and Lupton, J.R. 1997. Modulation of intracellulare second messengers by dietary fat during colonic tumor development. Adv. Exp. Med. Biol. 422: 85-96.

Clarke, S. and Jump, D. 1994. Dietary polyunsaturated fatty acid regulation of gene transcription. Ann. Rev. Nutr. 14: 83:98.

Clayman R.V., Bilhartz L.E., Buja L.M., Spady D.K., and Dietschy J.M. 1986. Renal cell carcinoma in the Wistar-Lewis rat: a model for studying the mechanisms of cholesterol acquisition by a tumor in vivo. Cancer Research. 46(6): 2958-2963.

Collett, E.D., Davidson, L.A., Fan, Y-Y., Lupton, J.R. and Chapkin, R.S. 2001. N6 and n3 polyunsaturated fatty acids differentially modulate oncogenic Ras activation in colonocytes. Am.J. Physiol. Cell Physiol. 280: C1066-C1075.

Colorectal Cancer Association of Canada. 2004. Nicholson, N., Shah, A., Charbonneau, F., Gallinger, S., Couture, F., and Tremblay, F. Understanding Colorectal Cancer.

Colorectal Cancer Association of Canada. 2007. Colorectal Cancer Statistics. www.ccac-accc.ca/statistics.html.

Cooper, G.M. 1992. Elements of Human Cancer. Jones and Bartlett Publishers, Inc.

Cox, A.D. and Der, C.J. 1992. The ras/cholesterol connection: implications for ras oncogenicity. Crit. Rev. Oncog. 3: 365.

CPS, 1998. Compendium of Pharmaceuticals and Specialties. 33rd edition.

D

Das, U.N. 2001. Essential fatty acids as possible mediators of the actions of statins. Prostaglandins, Leukotrienes and Essential Fatty Acids. 65(1): 37-40.

Davidson, L.A, Lupton, J.R., Jiang, Y-H. and Chapkin, R.S. 1999. Carcinogenesis. 20:785-791.

Demierre, M-F., Higgins, P.D., Gruber, S.B., Hawk, E. and Lippman, S.M. 2005. Statins and cancer prevention. Nat. Rev. Cancer. 5(12): 930-942. (www.nature.com/reviews/cancer).

Der, C.J., Finkel, T., Cooper, G.M. 1986. Cell. 44: 167-176.

Dergham, S.T., Dugan, M.C., Kucway, R. et al. 1997. Prevalence and clinical significane of combined k-ras mutation and p53 aberration in pancreatic adenocarcinomas. Int. J. Pancreatol. 21: 127-143.

Deschner E.E., Lytle, J., Wong, G., Ruperto, J. and Newmark, H.L. 1990. The effect of dietary omega-3 fatty acids (fish oil) on azoxymethanol-induced focal areas of dysplasia and colon tumor incidence. Cancer. 66: 2350-2356.

Dietschy, J.M., 1998. Dietary fatty acids and the regulation of plasma low density lipoprotein cholesterol concentrations. J. Nutr. 128(2 Suppl): 444S-448S.

Dobrowolski, S., Harter, M. and Stacey, D.W. 1994. Cellular ras activity is required for passage through multiple points of the G_0/G_1 phase in BALB/c 3T3 cells. Mol. Cell Biol. 14: 5441-5449.

Dollinger, M., Rosenbaum, E.H., Tempero, M., Mulvihill, S.J. 2002. Everyone's Guide to Cancer Therapy: How Cancer is Diagnosed, Treated, and Managed Day to Day. Andrews McMeel Publishing. Fourth Edition.

Dong, C., Davis, R.J., and Flavell, R.A. 2002. Map kinases in the immune response. Annu. Rev. Immunol. 20: 55-72.

Dricu, A., Wang, M., Hjertman,M., Malec, M., Blegen, H., Wejde, J., Carlberg, M., Larsson, O. 1997. Glycobiology. 7 (5): 625-623.

Drosopoulos, K.G., Roberts, M.L., Cermak, L., Sasazuki, T., Shirasawa, S., Andera, L. and Pintzas, A. 2005. Transformation by oncogenic ras sensitizes human colonic cells to TRAIL-induced apoptosis by up-regulating death receptor4 and death receptor 5 through a MEK-dependent pathway. J. Biol. Chem. 280(24): 22856-22867.

Dudley, D.T. and Saltiel, A.R. 2000. A pharmacological approach to the MAP kinase cascade. In: Gutkind, J.S., ed. Signalling Networks and Cell Cycle Control: The Molecular Basis of Cancer and Other Diseases. Totowa, N.J.: Humana Press Inc. pp. 467-481.

Duke, R.C., Ojcius, D.M., and Ding, E.Y.J. 1996. Cell suicide in health and disease. Sci Am. 275: 48-55.

Duncan, R.E., El-Sohemy,A., Archer, M.C. 2005. Dietary factors and the regulation of 3-hydroxy-3-methylglutaryl Coenzyme A reductase: implications for breast cancer development. Mol. Nutr. Food Res. 49(2): 93-100.

Durkin, J.P. and Whitfield, J.F. 1987. Mol. Cell Biol. 7: 444-449.

E

Edwards, P.A. and Ericsson, J. 1999. Sterols and isoprenoids: signalling molecules derived from the cholesterol biosynthetic pathway. Annu. Rev. Biochem. 68: 157-185.

Eggstein, S., Franke, M., Kutschka, I., Manthey, G., von Specht, B.U., Ruf, G. and Farthmann, E.H. 1999. Expression and activity of mitogen activated protein kinases in human colorectal carcinoma. Gut. 44: 834-838.

Ehrenstein, M.R., Jury, E.C. and Mauri, C. 2005. Statins for atherosclerosis-as good as it gets? N. Engl.J. Med. 352: 73-75.

Elnatan, J., Goh, H-S., and Smith, D.R. 1996. C-KI-RAS activation and the biological behaviour of proximal and distal colonic adenocarcinomas. Eur. J. Cancer. 32A: 491-497.

El-Sohemy, A and Archer, M.C. 1997. Regulation of mevalonate synthesis in rat mammary glands by dietary n-3 and n-6 polunsaturated fatty acids. Cancer Research. 57: 3685-3687.

El-Sohemy, A. and Archer, M.C. 1999. Regulation of mevalonate synthesis in low density lipoprotein receptor knockout mice fed n3 or n6 polyunsaturated fatty acids. Lipids. 34(10): 1037-1073.

Endo, A., Tsujita, Y., Kuroda, M. and Tanzawa, K. 1979. Biochim. Biophys. Acta. 575: 266-276.

Engstrom, W. and Schofield, P.M. 1987. Expression of the 3-hydroxy-3-methylglutaryl Coenzyme A reductase and LDL receptor and LDL receptor genes in human embryonic tumors and in normal foetal tissues. Anticancer Research. 7(3 Pt B): 337-342.

Evertsson, S., Bartik, Z., Zang, H., Jansson, A. and Sun, X.F. 1999. Apoptosis in relation to proliferating cell nuclear antigen and Duke's stage in colorectal adenocarcinomas. Int. J. Oncol. 15: 53-58.

F

Fabricant, M. and Broitman, S.A. 1990. Evidence for deficiency of low density lipoprotein receptor on human colonic carcinoma cell lines. Cancer Research. 50 (3): 632-636.

Fang, J.Y. and Richardson, B.C. 2005. The MAPK signalling pathways in colorectal cancer. The Lancet Oncology. 6(5): 322-327.

Farina, H.G., Bublik, D.R., Alonso, D.F. and Gomex, D.E. 2002. Lovastatin alters cytoskeleton organization and inhibits experimental metastasis of mammary carcinoma cells. Clin. Exp. Metastasis. 19: 551-559.

Farber, E. and Rubin, H. 1991. Cellular adaptation in the origin and development of cancer. Cancer Research. 51: 2751-2761.

Fearon, E.R. and Jones, P.A. 1992. Progressing toward a molecular description of colorectal cancer development. FASEB J. 6: 2783-2790.

Fearon, E.R. and Vogelstein B., 1990. A genetic model for colorectal tumorigenesis. Cell. 1990; 61:759-767.

Fears, R., Richards, D.H. and Ferres, H. 1980. Atherosclerosis. 35: 439-449.

Feleszko, W. et al. 2002. Synergistic interaction between highly specific cyclooxygenase-2 inhibitor, MF-tricyclic and lovastatin in murine colorectal cancer cell lines. Oncol. Rep. 9: 879-885.

Fiala, E. 1977. Investigation into the metabolism and mode of action of the colon carcinogens 1,2 dimethylhydrazine and azoxymethane. Cancer. 40: 2436-2445.

Field, F.J., Albright, E.J. and Mathur, S.N. 1987. Effect of dietary n-3 fatty acids on HMG-CoA reductase and ACAT activities in liver and intestine of the rabbit. J. Lipid Research. 28: 50-58.

Forrester, E., Almoguers, C. Hang, K., et al. 1987. Detection og high incidence of K-ras oncogenes during human colon tumorigenesis. Nature. 327: 298-303.

Friis, S. et al. 2005. Cancer risk among statin users: a population based cohort study. Int. J. Cancer. 114: 643-647.

Fritz, G., Brachetti, C., Kaina, B. 2003. Lovastatin causes sensitization of HeLa cells to ionizing radiation-induced apoptosis by the abrogation of G_2 blockage. Int. J. Radiot. Biol. 79:601-610.

Fujise, T., Iwakiri, R., Kakimoto, T., Shiraishi, R., Sakata, Y., Wu, B., Tsunada, S., Ootani, A., and Fujimoto, K. 2006. Long-term feeding of various fat diets modulates azoxymethane-induced colon carcinogenesis through Wnt/Beta-Catenin signalling in rats. Am. J. Physiol. Gastrointest. Liver Physiol. 292: G1150-G1156.

Fung, C., Bragg, T., Newland, R. et al. 1997. K-ras mutation and loss of heterozygosity of chromosome 17p and survival of colorectal cancer. Aust. N. Z.J. Surg. 67: 239-244.

G

Gayen, A.K. and Peffley, D.M. 1995. The length of 5' untranslated leader sequences influences distribution of 3-hydroxy-3-methyl glutaryl coenzyme A reductase mRNA in polysomes: effects of lovastatin, oxysterols, and mevalonate. Arch. Biochem. Biophys. 322: 475-485.

Gebhardi, A. and Niendorf, A. 1995. Effects of prevastatin, a hydroxymethylglutaryl coenzyme A reductase inhibitor, on two human tumor cell lines. J. Can. Research Clin. Oncol. 121: 343-349.

Gideon, P., John, J., Frech, M., Lautwein, A., Clark, R. et al. 1992. Mol. Cell Biol. 12: 2050-2056.

Gil, G., Faust, J.R., Chin, D.J., Goldstein, J.L. and Brown, M.S. 1985. Cell. 41: 249-258.

Giovannucci, E. and Golden, B. 1997. The role of fat, fatty acids, and total energy intake in the etiology of human colon cancer. Am. J. Clin. Nutr. 66(Suppl): 1564S-1571S.

Giovannucci, E., Rimm, E., Ashiero, A. et al. 1995. Alcohol, low methionine, low folate diets, and risk of colon cancer in men. J. Natl. Cancer Inst. 87:265-273.

Giovannucci, E., Rimm, E., Stampfer, M. et al. 1994. Intake of fat, meat, and fiber in relation to risk of colon cancer in men. Cancer Research. 54: 2390-2397.

Goldfarb, S and Pitot, H.C. 1971. The regulation of beta-hydroxy-beta-methylglutaryl coenzyme A reductase in Morris hepatomas 5123C, 7800, and 9618A. Cancer Research. 31: 1879-1882.

Goldstein, J.L. and Brown, M.S. 1984. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. J. Lipid Res. 25: 1450-1461.

Goldstein, J.L. and Brown, M.S. 1990. Regulation of the mevalonate pathway. Nature (Lond.). 343: 425-430.

Good, C.K. Ph.D. Thesis. 1999. Regulation of the cellular, molecular and morphological determinants of colonic precancerous stages by dietary lipids. Department of Food and Nutritional Sciences, The University of Manitoba, Winnipeg, Manitoba, Canada.

Good, C.K., Lasko, C., Adam, J., and Bird R.P. 1998. Diverse effect of fish oil on the growth of aberrant crypt foci and tumor multiplicity in F344 rats. Nutr. Cancer. 3: 204-211.

Goodsell, D.S. 1999. The molecular Perspective: The *ras* Oncogene. The Oncologist. 4: 263-264.

Gordons and Nivatvongs. 1999. Principles and Practice of Surgery for the Colon, Rectum and Anus. Digestion Colon Rectume. 42(7): 921-929.

Graaf, M.R., Beiderbeck, A.B., Egberts, A.C., Richel, D.J. and Guchelaar, H.J. 2004. The risk of cancer in users of statins. J. Clin. Oncol. 22: 2388-2394.

Greenberg, E. 1996. Preventing colon cancer. Recent Res. Cancer Res. 142: 9-18.

Greene, F., Lamb, L., and Barwick, M. 1987. Colorectal cancer in animal models: a review. J. Surg. Res. 43: 476-487.

Greenspan, M.D., Yudkovitz, J.B., Alberts, A.W., Argenbright, L.S., Arison, B.H., Smith, J.L. 1988. Metabolism of lovastatin by rat and human microsomes in vitro. American Society for Pharmacology and Experimental Therapeutics. 16(5): 678-682.

Gropper, S.S., Smith, J.L., and Groff, J.L. 2005. Advanced Nutrition and Human Metabolism. Thomson Wadsworth. Fourth Edition.

Goldstein, J.L. and Brown, M.S. 1990. Regulation of the mevalonate pathway. Nature, 343: 425-430.

Goodsell, D.A. 1999. The molecular perspective: the ras oncogene. The Oncologist. 4: 263-264.

H

Haber D. A. and Fearon E. R. 1998. The Promise of Cancer Genetics. Lancet. 351 (suppl II): 1-8.

Haklai, R., Weisz, M.G., Elas, G., Paz, A., Marciano, D., Egozi, Y., Ben-Baruch, G., and Kloog, Y. 1998. Dislodgement and accelerated degredation of ras. Biochemistry. 37: 1306-1314.

Hancock, J.F, Magee, A.I., Childs, J.E., Marshall, C.C., 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell. 57: 1167-1177.

Harris, C.C. 1991. Chemical and physical carcinogenesis: advances and perspectives for the 1990's. Cancer Re. 51 (Suppl): 5023-5044.

Hawkins, N., Lees, J., Hargrave, R., O'Connor, T., Meagher, A., and Ward, R. 1997. Pathological and genetic correlates of apoptosis in the progression of colorectal neoplasia. Tumour Biol. 18: 146-156.

Hayes, K.C., Khosla, P., Hajri, T, and Pronczuk, A. 1997. Saturated fatty acids and LDL receptor modulation in humans and monkeys. Prostalandins Leukot. Essent. Fatty Acids. 57 (4-5); 411-418.

Hayes, K.C 1995. Saturated fats and blood lipids: new slant on an old story. Can. J. Cardiol. 11(SupplG): 39G-46G.

Hawke, E and Viner, J.L., 2005. Statins and cancer-beyond the "one drug, one disease" model. N. Engl. J. Med. 352: 2238-2239.

Hengst, L. and Reed, S.I. 1996. Translational control of $p27^{KIP1}$ accumulation during the cell cycle. Science. 271: 805-816.

Hentosh, P., Yuh, S.H., Elson, C.E. and Peffley, D.M. 2001. Sterol-independent regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in tumor cells. Mol. Carcinogen. 32: 154-166.

Hill, M., Moroson, B., and Bussey, H. 1974. Aetiology of adenoma-carcinoma sequence in large bowel. Lancet. 1: 245-247.

Hildkivi-Clarke, L., Wang, C., Kalil, M., Riggins, R., Pestell, R.G. 2004. Nutritional Modulation of the Cell Cycle and Breast Cancer. Endocrine-Related Cancer. 11: 603-622.

Hofmanova, J., Vaculova, A., and Kozubik, A. 2005. Polyunsaturated fatty acids sensitize human colon adenocarcinomas HT-29 cells to death receptor-mediated apoptosis. Cancer Letters. 218 (1): 33-41.

Hohl, R.J. and Lewis, K. 1995. Differential effects of monoterpenes and lovastatin on ras processing. The Journal of Biological Chemistry. 270(29): 17508-17512.

Horton, J.D., Cuthbert, J.A. and Spady, D.K. 1993. Dietary fatty acids regulate hepatic low density lipoprotein (LDL) transport by altering LDL receptor and mRNA levels. J. Clin. Invest. 92(2): 743-749.

Houten, S.M., Frenkel, J and Waterham, H.R. 2003. Isoprenoid biosynthesis in hereditary periodic fever syndromes and inflammation. Cell. Mol. Life Sci. 60: 1118-1134.

Howe, J., and Guillem, J. 1997. The genetics of colorectal cancer. Surg. Clin. North Amer. 77: 175-195.

Hromadova, M., Sebokova, E. and Klimes, I. 1994. HMG-CoA reductase activity in the liver of rats with hereditary hypertriglyceridemia: effect of dietary fish oil. Endocr. Regul. 28: 211-214.

Hsiao, W.L., Pai, H.L., Matsui, M.S., Weinstein, I.B. 1990. Effects of specific fatty acids on cell transformation induced by an activated c-H-ras oncogene. Oncogene. 5(3): 417-421.

I

Im, E., and Martinez, J.D. 2004. Ursodeoxycholic acid (UDCA) can inhibit deoxycholic acid (DCA)-induced apoptosis via modulation of EGFR/Raf-1/ERK signalling in human colon cancer cells. J. Nutr. 134: 483-486.

Ito, Y., Sasaki, Y., Horimoto, M., Wada, S. Tanaka, Y. Kasahara, A. Ueki, T. Hirano, T. Yamamoto, H., Fugimoto, J., Okamoto, E. Hayashi, N. and Hori, M. 1998. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology. 27: 951-958.

J

James, G. and Olson, E.N. 1989. Fatty acylated proteins as components of intracellular signalling pathways. Biochemistry. 29: 2623-2634.

Janes, P.W., Daly, R.J., deFazio, A, et al. 1994. Activation of the ras signalling pathway in human breast cancer cells overexpressing erbB-2. Oncogene. 9: 3601-3608.

Jen, J., Powell, S., Papadopoulos, N. et al. 1994. Molecualr determinants of dyspalsia in colorectal lesions. Cancer Research. 54: 5523-5526.

Jingami, H, Brown, M.S., Goldstein, J.L., Anderson, R.G.W. and Luskey, K.L. 1987. J. Cell Biol. 104: 1693-1704.

John, J., Frech, M., Wittinghofer, A., 1988. J. Biol. Chem. 263: 11792-11799.

Jonges, L.E, Nagelkerke, J.F., Ensink, N.G., van der Velde, E.A., Tollenaar, R.A.E.M., Fleuren, G.J., van de Velde, C.J.H., Morreau, H., Kuppen, P.J.K. 2001. Caspase 3 Activity as a prognostic factor in colorectal carcinoma. 81: 681-688.

Jothy, S., Flanders, T. and Nowacki, P. 1996. New developments in the molecular pathology of humancolon cancer: relevance to pathogenesis and diagnosis. Adv. Anat. Pathol. 3: 343-350.

K

Kaye, J.A. and Jick, H. 2004. Statin use and cancer risk in the General Practice Research Database. Br. J. Cancer. 90: 635-637.

Kaye, J.A., Meier, C.R., Walker, A.M. and Jick, H. 2002. Statin use, hyperlipidaemia, and the risk of breast cancer. Br. J. Cancer. 86: 1436-1439.

Kawamori, T., Tanaka, T., Kojima, T. Suzui, M. Ohinishi, M., and Mori, H. 1994. Suppression of azoxymethane-induced rat colon aberrant crypt foci by dietary protocatchic acid. Jpn. J. Cancer Research. 85: 686-691.

Keller, R.K., Zhao, Z., Chambers, C.M., Ness, G.C. 1996. Farnesol is not the nonsterol regulator mediating degredation of HMGCAR in rat liver. Arch. Biochem. Biophys. 328: 324-330.

Kendell, C.W., Janezic, S.A., Friday, D., and Rao, V. 1992. Dietary cholesterol enhances preneoplastic aberrant crypt formation and alters cell proliferation in the murine colon treated with azoxymethane. Nutr. Cancer. 17: 107-114.

Khil, J. and Gallaher, D.D. 2004. Beef tallow increases apoptosis and decreases aberrant crypt foci formation relative to soybean oil in rat colon. Nutr. Cancer. 50 (1): 55-62.

Kiaris, H. and Spandidos, D.A. 1995. Mutations of ras genes in human tumours (review). Int. J. Oncol. 7: 413-421.

Kim, D.Y., Chung, K.H., Lee, J.H. 1998. Stimulatory effects of high fat diets on colon cell proliferation depend on the type of dietary fat and site of the colon. Nutr. Cancer. 30 (2): 118-123.

Kim, K.P., Whitehead, C., Piazza, G., and Wargovich, M.J. 2004. Combinatorial Chemoprevention: Efficacy of Lovastatin and Exisulind on the formation and Progression of Aberrant Crypt Foci. Anticancer Research. 24: 1805-1812.

Kim, W.H., Yeo, M., Kim, M.S., Chun, S.B., Shin, E.C., Park, J.H., Park, I.S. 2000. Role of Caspase 3 in apoptosis of colon cancer cells induced by nonsteroidal anti-inflammatory drugs. International Journal of Colorectal Disease. 15(2): 105-111.

Kiunga, G.A. 2006. Ph.D. Thesis. Department of food and Nutritional Sciences. University of Manitoba, Winnipeg, Manitoba, Canada.

Kiunga, G.A., Raju, J., Sabljic, N., Bajaj, G, Good, C.K., Bird, R.P. 2004. Elevated insulin receptor protein expression in experimentally induced colonic tumors. Cancer Letters. 211 (2): 145-153.

Kumar, V., Cotran, R., and Robbins, S. 1993. Basic Pathology. W.B. Saunders, Philaldelphia.

L

Lafave, L.M., Kumarathasan, P. and Bird,R. P. 1994. Effect of dietary fat on colonic protein kinase C and induction of aberrant crypt foci. Lipids. 29: 693-700.

Laemmli, U.K. 1976 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 22: 680-685.

Lakshmanan, M. R., Dugan, R. E., Nepokroeff, C. M., Ness, G. C., and Porter, J. W. 1975. Regulation of rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase activity and cholesterol levels of serum and liver in various dietary and hormonal states. Archives of Biochemistry and Biophysics, 168: 89-95.

Lam, L.K. and Zhang, J. 1991. Reduction of aberrant crypt formation in the colon of CF1 mice by potential chemopreventive agents. Carcinogenesis. 12: 2311-2315.

Lasko, C, M. and Bird, R.P. 1995. Modulation of aberrant crypt foci by dietary fat and caloric restriction: the effects of delayed intervention. Cancer Epidemiol. Biomarkers Prev. 4: 49-55.

Lasko, C., Good, C., Adam, J. and Bird, R.P. 1999. Energy restriction modulates the development of advanced preneoplastic lesions depending on the level of fat in the diet. Nutr. Cancer. 33: 69-75.

Lavoie, J.N., L'Allemain, G., Brunet, A., Muller, R. and Pouyssegur, J. 1996. Cyclin D1 expression is regulated positively by the $p42/p44_{MAPK}$ and negatively by the $p38/HOG_{MAPK}$ pathway. J. Biol. Chem. 271: 20608-20616.

Lennernas, H. and Fager, G. 1997. Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. Clin. Pharmacokinet. 32(5): 403-425.

Lewis, T.S., Shapiro, P.S. and Ahn, N.G. 1998. Signal transduction through MAP kinase cascades. Adv. Cancer Res. 74: 49-139.

Licato, L. and Brenner, D. 1998. Analysis of signalling protein kinases in human colon or colorectal carcinomas. Dig. Dis. Sci. 43: 1454-1464.

Lindgren, V., Luskey, K.L., Russel, D.W. and Francke, U. 1985. Human genes involved in cholesterol metabolism: chromosomal mapping of the loci for the LDL receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase with cDNA probes. Proc. Natl. Acad. Sci. USA. 82(24): 8567-8571.

Lipkin, M., Bell, B., and Sherlock, P. Cell proliferation kinetics in the gastrointestinal tract of men. I. Cell renewal in colon and rectum. 1993. J. Clin. Invest. 42:767-776.

Liscum, L. L., Luskey, K. L., Chin, D. J., Ho, Y. K., Goldstein J. L., and Brown, M. S. 1983. Regualtion of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. The Journal of Biological Chemistry, 258: 8450-8455.

Liu, J-J., Chao, J-R., Jiang, M-C., Ng, S-Y., Yen, J-Y. and Yang-yen, H-F. 1995. Ras transformation results in an elevated level of cyclin D1 and acceleration of G_1 progression in NIH 3T3 cells. Mol. Cell Biol. 15: 3654-3663.

Lopez, D., Chambers C. M., and Ness, G.C. 1997. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors unmask cryptic regulatory mechanisms. Archives of Biochemistry and Biophysics, 343: 118-122.

Lowy, D.R. and Willumsen, B.M. 1993. Function and regulation of ras. Annu. Rev. Biochem. 62: 851-91.

M

Ma, P.T.S., Gil, G., Sudhof, T.C., Bilheimer, D.W. Goldstein, J.L. and Brown M.S. 1986. Proc. Natl. Acad. Sci. USA. 83: 8370-8374.

Maemura, M., Iino, Y, Koibuchi, Y., Yokoe, T. and Morishita, Y. 1999. 57(suppl): 37-44.

Magnuson, B.A. and Bird, R.P. 1993. Reduction of aberrant crypt foci in rat olon with azoxymethane or methylnitrosouria by feeding cholic acid. Carcinogenesis. 68: 15-23.

Magnuson, B.A. and Bird, R.P. 1994. Resistance of aberrant crypt foci to apoptosis induced by azoxymethane in rats chronically fed cholic acid. Carcinogenesis. 15: 1459-1462.

Magnuson, B.A., Carr, I. and Bird, R.P. 1993. Ability of aberrant crypt foci characteristics to predict colonic tumor incidence in rats fed cholic acid. Caner Research. 53: 4499-4504.

Maltese, W.A. 1983. 3-Hydroxy-3-methylglutaryl coenzyme A reductase in human brain tumors. Neurology. 33: 1294-1299.

Maltese, W.A., Defendini, R., Green, R.A., Sheridan, K.M., Donley, D.K. 1985. Suppression of murine neuroblastoma growth in vivo by mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. J. Clin. Invest. 76: 1748-1754.

Mamajiwalla, S.N. and Burgess, D.R. 1995. Differential regulation of the activity of the 42 kDa mitogen activated protein kinase ($p42^{MAPK}$) during enterocyte differentiation in vivo. Oncogene. 11: 377-386.

Marcelli, M. et al. 1998. Cspase-7 is activated during lovastatin-induced apoptosis of the prostate cancer cell line LNCaP. Cancer Research. 58: 76-83.

Maruta, H., and Burgess, A.W. 1994. Regualtion of the Ras signalling network. BioEssays. 16: 489-496.

McLellan, E.A and Bird, R.P. 1988. Aberrant crypts: potential preneoplastic lesions in the murine colon. Cancer Research. 48: 6187-6192.

McLellan, E.A and Bird, R.P. 1988. Specificity study to evaluate induction of aberrant crypts in murine colons.. Cancer Research. 48: 6183-6186.

McLellan, E.A and Bird, R.P. 1991. Effect of disulfiram on 1,2-dimethylhydrazine and azoxymethane-induced aberrant crypt foci. Carcinogenesis. 12:969-972.

McLellan, E.A., Medline, A. and Bird, R.P. 1991. Sequential anaylsis of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. Canaer Research. 51: 5270-5274.

McLellan, E.A., Medline, A. and Bird, R.P. 1991. Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. Carcinogenesis. 12: 2093-2098.

McLeod, H.L. and Murray G.I. 1999. Tumor markers of prognosis in colorectal cancer. British Journal of Cancer. 79: 191-203.

Mehta, N., Hordines, J, Sykes, D., Doerr, R. and Cohen, S. 1998. Low density lipoproteins and lovastatin modulate the organ-specifc tranendothelial migration of primary and metastatic human colon adenocarcinomas cell lines in vitro. Clin. Exp. Metastasis. 16: 587-594.

Meigs, T.E., Roseman, D.S., Simoni, R.D. 1996. Regualtion of 3-hydroxy-3-methylglutaryl coenzyme A reductase degradation by the nonsterol mevalonate metabolite farnesol in vivo. J. Biol. Chem. 271: 7916-7922.

Melancon, P. et al. 1987. Cell. 51: 1053-1062.

Milanini, J., Vinals, F, Pouyssegur, J. et al. 1998. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. J. Biol. Chem. 273: 18165-18172.

Miller, A.C. and Samid, D. 1995. Tumor resistance to oxidative stress: association with ras oncogene expression and reversal by lovastatin, an inhibitor of p21ras isoprenylation. Int. J. Cancer. 60:240-254.

Minoura, T., Takata, T. Sakaguchi, M. Takada, H., Yamamura, M. and Yamamoto, M. 1988. Effect of dietary eicosapentaenoic acids on azoxymethane-induced colon carcinogenesis in rat. Cancer Research. 48: 4790-4794.

Mo, H. and Elson, C.E. 2004. Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. Exp. Biol. Med. 229: 567-585.

Moghadasian, M.H. 1999. Clinical pharmacology of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Life Sciences. 65 (13): 1329-1337.

Moller-Jensen, O. 1983. Different age and sex relationship for cancer subsites of the large bowel. Br. J. Cancer. 50:825-829.

Mustad, V.A., Etherton, T.D., Cooper, A.D., Mastro, A.M., Pearson, T.A., Jonnalagadda, S.S. and Kris-Etherton, P.M. 1997. Reducing saturated fat intake is associated with increased levels of LDL receptors on mononuclear cells in healthy men and women. J. Lipid Research. 38(3): 459-468.

N

Nakanishi, M., Goldstein, J.L., and Brown, M.S. 1988. Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degredation of enxyme. The Journal of Biological Chemistry. 263(18): 8929-8937.

Naoumova, R.P., Marais, A.D., Mountney, J., Firth, J.C., Rendell, N.B., Taylor, G.W. and Thompson, G.R. 1996. Atherosclerosis.119: 203-213.

Narayanan, B.A., Narayanan, N.K., and Reddy, B.S. 2001. Docosahexaenoic acid (DHA) regulated genes and transcription factors inducing apoptosis in human colon cancer cells. Int.J. Oncol. 19: 1255-1262.

Narayanan, B.A., Narayanan, N.K., Simi, B., and Reddy, B.S. 2003. Modulation of inducible nitric oxide synthase and related pro-inflammatory genes by the omega-3 fatty acid docosahexaenoic acid in human colon cancer cells. Cancer Research. 63: 972-979.

Narayanan, B.A., Narayanan, N.K., Pittman, B., and Reddy, B.S. 2004. Effects of a combination of docosahexaenoic acid and 1,4-phenylenebis (methylene) selenocyanate on cyclooxygenase-2, inducible nitric oxide synthase and beta-catenin pathways in colon cancer cells. Carcinogenesis.

Narisawa, T., Fukaura, Y., Terada, K., Umezawa, A., Tanida, N., Yazawa, K., Ishikawa, C. 1994. Prevention of 1,2-dimethylhydrazine-induced colon tumorigenesis by HMG-CoA reductase inhibitors, pravastatin and simvastatin in ICR mice. Carcinogenesis. 15(9): 2045-2048.

Narisawa, T. et al. 1996. Chemoprevention by pravastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, ofN-methyl-N-nitrosourea-induced colon carcinogenesis in F344 rats. Jpn. J. Cancer Res. 87: 798-804.

Neal, A.J. and Hoskin, P.J. 1997. Clinical Oncology: Basic Principles and Practice. Second Edition. Oxford University Press, Inc.

Ness, G.C. 1983. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Molecular and Cellular Biochemistry, 53/54: 299-306.

Ness, G.C. 1994. Developmental regulation of the expression of genes encoding proteins involved in cholesterol homeostasis. American Journal of Medical Genetics, 50: 355-357.

Ness, G.C. and Chambers, C. M. 1996a. The diurnal variation of hepatic HMG-CoA reducatse activity is due to changes in the level of immunoreactive protein. Archives of Biochemistry and Biophysics, 327: 41-44.

Ness, G. C. and Chambers, C. M. 2000. Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzymeA reductase: the concept of cholesterol buffering capacity. Society for Experimental Biology and Medicine. 224: 8-19.

Ness, G.C., Eales S., Lopez, D., and Zhao, Z. 1994. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression by sterols and non-sterols in rat liver. Archives of Biochemistry and Biophysics, 308: 420-425.

Ness, G. C., Eales, S. J., Pendleton L. C., and Smith M. 1985a. Activation of rat liver microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase by NADPH. Effects of dietary treatments. The Journal of Biological Chemistry, 260: 12391-12393.

Ness, G. C., Keller, R. K., and Pendleton, L. C. 1991. Feedback regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by dietary cholesterol is not due to altered mRNA levels. The Journal of Biological Chemistry, 266: 14854-14857.

Ness, G. C., Sample, C. E., Smith, M., Pendleton L. C., and Eichler, D. C. 1986. Charactersitics of rat liver microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductse. Biochem J., 233: 167-172.

Ness, G. C. and Zhao, Z. 1994. Thyroid hormone rapidly induces hepatic LDL receptor mRNA levels in hypophysectomized rats. Archives of Biochemistry and Biophysics, 315: 199-202.

Ness, G. C., Zhao, Z., and Lopez, D. 1996b. Inhibitors of cholesterol biosynthesis increase hepatic low density lipoprotein receptor protein degredation. Archives of Biochemistry and Biophysics, 325: 242-248.

Ness, G. C., Zhao, Z., and Wiggins, L. 1994. Insulin and glucagons modulate hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by affecting immunoreactive protein levels. The Journal of Biological Chemistry, 269: 29168-29172.

Nguyen, L.B., Shefer, S., Salen, G., Ness, G.C., Batta, A., Chowdhary, I.R., Paroulek, E., Hauser, S. 1994. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in the rat ileum: Effects of bile acids and lovastatin. Metabolism. 43(11): 1446-1450.

Niendorf, A., Nagele, H., Gerding, D., Meyer-Pannwitt, U., Gebhardt, A. 1995. Increased LDL receptor mRNA expression in colon cancer is correlated with a rise in plasma cholesterol levels after curative surgery. Int. J. Cancer. 61(4): 461-464.

Notarnicola, M., Messa, C., Pricci, M., Guerra, V., Altomare, D.F., Montemurro, S., Caruso, M.G. 2004. Up-regulaiton of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in left-sided colon cancer. Anticancer Res. 24(6): 3837-3842.

Nubel, T., Damrot, J., Roos, W.P., Kaina, B., Fritz, G. 2006. Lovastatin protects endothelial cells from killing by ionizing radiation without impairing induction and repair of DNA double-strand breaks. Clinical Cancer Research. 12: 933-939.

O

Oka, H., Chatani, Y., Hoshino, R., Ogawa, O., Kakehi, Y., Terachi, T., Okada, Y. Kawaichi, M., Kohno, M., Yoshida, O. 1995. Constitutive activation of MAP kinases in human renal cell carcinoma. Cancer Research. 55: 4182-4187.

Oliver, M.F. 1991. Might treatment of hypercholesterolaemia increase non-cardiac mortality? Lancet. 337: 1529-1531.

Oldham, S.M., Clark, G.J., Gangarosa, L.M., Coffey, R.J. and Der, C.J. 1996. Activation of the Raf-1/Map kinase cascade in not sufficient for ras transformation of RIE-1 epithelial cells. Proc. Natl. Acad. Sci. USA. 93: 6924-6928.

Ostlund, R.E. Jr., Iang, J.W., Heath-Monning, E., Semenkovich, C.F. and Daughaday, W.H. 1991. IGF I increases LDL receptor expression in fibroblasts by a post-transcriptional mechanism involving IGF I-receptors. Arterioscler. Thromb. 11: 1414.

Ostrowski, J., Trzeciak, L., Kolodziejski, J., Bomsztyk, K. 1998. Increased constitutive activity of MAPK and renatirable 85 kDa kinase in human-colorectal cancer. Br. J. Cancer.78: 1301-1306.

Otori, K., Sugiyama, K., Fukushima, S. and Esumi, H. 1999. Expression of the cyclin D1 gene in rat colorectal aberrant crypt foci and tumors induced by azoxymethane. Cancer Letters. 140: 99-104.

Owen, C.H., Sorci-Thomas, M. and Rudel, L.L. 1991. Dietary fat and cholesterol-induced modifications of LRL receptor function and mRNA abundance in non-human primates. Arterioscler. Thromb. 11: 1447.

P

Pajkos, G. Kiss, I., Sandor, J et al. 1999. Prognostic value of the presence of the mutation of the codons 12,13, and 61 in k-ras oncogene in colorectal cancer. Orv. Hetil. 140: 1673-1679.

Palakurthi, S.S., Fluckiger, R., Aktas, H., Changolkar, A.K, Shahsafaei, A., Harneit, S., Kilic, E., and Halperin, J.A. 2000. Inhibition of translation inititation mediates the anticancer effect of the n-3 polyunsaturated fatty acid eicosapenteanoic acid. Cancer Research. 60: 2919-2925.

Park, C., Lee, I., Kang, W.K. 2001. Lovastatin induced E2F-1 modulation and its effect on prostate cancer cell death. Carcinogenesis. 22: 1727-1731.

Pereira, M.A., Barnes, L.H., Rassman, V.L., Kelloff, G.V., and Steele, V.E. 1994. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. Carcinogenesis. 15: 1049-1054.

Pereira, M.A. and Khoury, M.D. 1991. Prevention by chemoprevetive agents of azoxymethane-induce foci of aberrant crypts in rat colon. Cancer Letters. 61: 27-33.

Pfeifer, G.P. 2001. A new verdict for an old convict. Natl Genet. 29: 3-4.
Platz, E.A. et al. 2005. Cancer risk among statin users: apopulaiton-based cohort study. Int. J. Cancer. 114: 643-647.

Platz et al, 2005. Cholesterol-lowering drugs including statins and the risk of prostate cancer in a large prospective cohort study. American Association for Cancer Research 96th Annual Meeting. April 16-20. Anaheim, Calif.

Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J.M., Roberts, J.M. and Koff, A. 1994. $p27^{KIP1}$, a cyclin-cdk inhibitor, links transformating growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. 8: 9-22.

Ponz de Leon, M., Sacchetti et al. 1990. Evidence for the existence of different types of large bowel tumor: suggestions from the clinical data of a population-based registry. J. Surg. Oncol. 44: 35-43.

Potter, J.D. 1999. Colorectal cancer: molecules and populations. J Natl. Cancer Inst. 91: 916-932.

Potter, J.D., Slattery, M.L., Bostick, R.M. and Gapstur, S.M. 1993. Colon cancer: a review of the epidemiology. Epidemiol. Rev. 15: 499-545.

Porebska, I., Harlozinska, A., Bojarowski, T. 2000. Expression of the tyrosine kinase activity growth factor receptors (EGFR, ERB B2, ERB B3) in colorectal adenocarcinomas and adenomas. Tumor Biol. 21: 105-115.

Porras, A. Muszynski, K., Rapp, U.R. et al. 1994. Dissociation between activation of Raf-1 kinase and the 42-kDa mitogen activated protein kinase/90kDa S6 kinase (MAPK/RSK) cascade in the insulin/ras pathway of adipocytic differentiation of 3T3 L1 cells. J. Biol. Chem. 269: 12741- 12748.

Poynter, J.N., Gruber, S.B., Higgins, P.D., Almog, R. Bonner, J.D., Rennert, H.S., Low, M., Greenson, J.K., Rennert, G. 2005. Statins and the risk of colorectal cancer. N. Engl. J. Med. 352: 2184-2192.

Pretlow, T.P., Barrow, B.B., Ashton, W.S., O'Riordan, M.A., Pretlow, T.G., and Jurcisek, J.A. 1991. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. Cancer Research. 51: 1564-1567.

Pretlow, T.P., O'Riordan, M.A., Kolman, M.F., and Jurcisek, J.A.. 1990. Colonic aberrant crypts in azoxymethane treated F344 rats have decreased hexosaminidase activity. Am.J. Pathol. 136: 13-16.

Pretlow, T.P., O'Riordan, M.A, Pretlow, T.G. and Stellato, T.A. 1992. Aberrant crypts in human colonic mucosa: putative preneoplastic lesions. J. Cell Biochem. (Suppl). 16G: 55-62.

Pretlow, T.P., O'Riordan, M.A., Somich, G.A., Amini, S.B., and Pretlow, T.G. 1992. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. Carcinogenesis. 13:1509-1512.

Pretlow, T.P., Rrasitus, T.A., Fulton, N.C., Chayer, C. and Kaplan, F.I. 1993. K-ras mutations in putative preneoplastic lesions in human colon. J. Natl. Cnacer Inst. 85(24): 2004-2007.

Proksch, E., Feingold, K.R. and Elias, P.M. 1992. Epidermal HMG CoA reductase activity in essential fatty acid deficiency: barrier requirements rather than eicosanoid generation regulate cholesterol biosynthesis. J. Invest. Dermatol. 99: 216-220.

Q

Quesney-Huneeus, V., Wiley, M.H. and Siperstein, M.D. 1979. Proc. Natl. Acad. Sci. USA. 76: 5056-5060.

R

Radtke, F. and Clevers, H. 2005. Self-renewal and cancer of the gut: two sides of a coin. Science. 307: 1904-1909.

Raju, J. and Bird, R.P. 2007. Diosgenin, a natuarally occurring furostanol suppresses 3-hydroxy-3-methylglutaryl CoA reductase expression and induces apoptosis in HCT-116 human colon carcinoma cells. Cancer Letters.

Ramjiganesh, T., Roy, S., Freake, H.C., McIntyre, J.C. and Fernandez, M.L. 2002. Corn fiber oil lowers plasma cholesterol by altering hepatic cholesterol metabolism and up-regulating LDL receptors in guinea pigs. J. Nutr. 132(3): 335-340.

Rao, A.V., Janezic, S.A., Friday, D., and Kendall, C.W. 1992. Dietary cholesterol enhances the induction and development of colonic preneoplastic lesions in C57BL/6J and BALB/CJ mice treaed with azoxymethane. Cancer Letters. 63: 249-257.

Rao, C.V., Desai, D., Simi, B., Kulkarni, N., Amin, S., and Reddy, B.S. 1993a. Inhibitory effect of caffeic acid esters on azoxymethane-induced biochemical changes and aberrant crypt foci formation in rat colon. Cancer Res., 53(18):4182-4188.

Rao, C.V., Hirose, Y., Indranie, C. and Reddy, B.S. 2001. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. Cancer Research. 61(5):1927-1933.

Rao, C.V., Rivenson, A., Simi, B., Zang, E., Kelloff, G., Steele, V. and Reddy B.S. 1995. Chemoprevention of colon carcinogenesis by sulindac, a non-steroidal anti-inflammatory agent. Cancer Research. 55: 1464-1472.

Rao, C.V., Simi, B., and Reddy, B.S. 1993b. Inhibition by dietary curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon. Carcinogenesis. 14(11): 2219-2225.

Rao, K.N. 1995. The significance of the cholesterol biosynthetic pathway in cell growth and carcinogenesis. Anticancer Res. 15(2): 309-314.

Rao, S., Cunningham, D., de Gramont, A. 2004. Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. J. Clin. Oncol. 22: 3950-3957.

Rao, S., et al. 1999. Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethylglutaryl CoA reductase. Proc. Natl. Acad. Sci. USA. 96: 7797-7802.

Razanamahefa, L., Prouff, S., and Bardon, S. 2000. Stimulatory effect of arachidonic acid on T-47D human breast cancer cell growth is associated with enhancement of CD1 mRNA expression. Nutrition and Cancer. 38: 274-280.

Reddy, B.S. 2004. Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. Environ. Mol. Mutagen. 44 (1): 26-35.

Reddy, B.S. 1992. Dietary fat and colon cancer: animal model studies. Lipids. 27: 807-813.

Reddy, B.S. et al. 2005. Synergistic effects of the combination of low doses of aspirin or celecoxib with Lipitor against colon carcinogenesis: a promising chemoprevention strategy. Proc. Amer. Assoc. Cancer Research. 46: LB-4.

Reddy, B.S., Burill, C. and Rigotty. 1991. Effect of diets high in w3 and w6 fatty acids on initiation and post-initiation stages of colon carcinogenesis. Cancer Research. 51: 487-491.

Reihner, E, Angelin, B., Rudling, M., Ewerth, S., Bjorkhem, I. and Einarsson, K. 1990. Regulation of hepatic cholesterol metabolism in humans: stimulatory effects of cholestyramine on HMG-CoA reductase activity and low density lipoprotein receptor expression in gallstone patients. J. Lipid Research. 31: 2219-2226.

Reimann, F.M., Herold, G., Grosshans, I., Rogler, G., Fellermann, K. and Strange, E.F. 1992. Regulation of cholesterol metabolism and low-density lipoprotein binding in human Caco-2 cells. Digestion. 51: 10-17.

Remacle-Bonnet, M.M., Garrouste, F.L., et al. 2000. Insulin-like growth factor-1 protects colon cancer cells from death factor-induced apoptosis by potentiating tumor necrosis factor alpha-induced mitogen-activated protein kinase and nuclear factor kappaB signalling pathways. Cancer Research. 60: 2007-2017.

Reynolds, G.A., Goldstein, J.L. and Brown, M.S. 1985. Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase determined by multiple transcription initiation sites and intron splicing sites in the 5' untranslated region. J. Biol. Chem. 260: 10369-10377.

Rice, P.L., Washington, M., Schleman, S., et al. 2003. Sulindac sulfide inhibits epidermal growth factor-induced phosphorylation of extracellular-regulated kinase 1/2 and Bad in human colon cancer cells. Cancer Research. 63: 616-620.

Rine, J. and Kim, S-H. 1990. New Biol. 2: 219-226.

Robblee, N. and Bird, R.P. 1994. Effects of a high corn oil diet on preneoplastic murine colons: prostanoid production and lipid composition. Lipids. 29: 67-71.

Roncucci, I. 1992. Early events in human colorectal carcinogenesis. Aberrant crypts and microadenoma. Ital. J. Gastroenterol. 24 (9): 498-501.

Roncucci, L., Stamp, D., Medline, A., Cullen, J.B., and Bruce, W.R. 1991. Identification and quantification of aberrant crypt foci and microadenomas in the human colon. Hum Pathol. 22: 287-294.

Roynette, C.E., Calder, P.C., Dupertuis, Y.M. and Pichard C. 2004. n3 polyunsaturated fatty acids and colon cancer prevention. Clin. Nutr. 23(2): 139-151.

Rudling, M.J., Angelin, B.O., Peterson, C.O., and Collins, V.P. 1990. LDL receptor activity in human intracranial tumors and its relation to the cholesterol requirement. Cancer Research. 50: 483-487.

Rudling, M. 1992. Hepatic mRNA levels for the LDL receptor and HMG-CoA reductase show coordinate regulation *in vivo*. Journal of Lipid Research, 33: 493-501.

Rudling, M and Collins, V.P. 1996. Low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA levels are co-ordinately reduced in human renal cell carcinoma. Biochim. Biophys. Acta. 1299(1): 75-79.

S

Sakaguchi, M., Minoura, T., Hiramatsu, Y. Takada, H., Yamamura, M., Hioki, K. and Yamamoto, M. 1986. Effects of dietary saturated and unsaturated fatty acids on fecal bile acids and colon carcinogenesis induced by azoxymethane in rats. Cancer Research. 46: 61-65.

Sakakura, C., Hagiwara, A., Shirahama, T., Nakanishi, M., Yasuoka, R., Fujita, Y., Inazawa, J., Abe, T., Kohno, M., Yamagishi, H. 1999. Infrequent activation of mitogen-activated protein kinase in human colon cancers. Hepatogatroenterology. 46(29): 2831-2834.

Sample, C. E. and Ness, G. C. 1986. Regulation of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase by insulin. Biochemical and Biophysical Communications, 137: 201-207.

Sebolt-Leopold, J., Dudley, D., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R., Tecle, H., Barrett, S., Bridges, A., Przybranowski, S., Leopold, W., Saltiel, A. 1999. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat. Med. 5: 810-816.

Seeburg, P.H., Colby, W.W., Capon, D.J., Goeddel, D.V., Levinson, A.D. 1984. Nature. 312: 71-75.

Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S., Leopold, W.R., and Saltiel. 1999. Blockade of the MAP kinase pathway suppresses growth of colonic tumors in vivo. Nat. Med. 5: 810-815.

Sellmayer, A., Danesch, U. and Weber, P. 1997. Modulaiton of the expression of early genes by polyunsaturated fatty acids. Prost. Leuko. Essential fatty acids. 57: 353-357.

Shao, J., Evers, B.M. and Sheng, H. 2004. Prostaglandin E2 synergistically enhances receptor tyrosine kinas-dependent signalling system in colon cancer cells. J. Biol. Chem. 279(14): 14287-14293.

Sherr, C.J. and Roberts, J.M. 1995. Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev. 9: 1149-1163.

Shirtliff, N., Carr, I., and Bird, R.P. 1993. Variable effect of cholic acid on the induction and growth of aberrant crypt foci and colonic tumors in Sprgue Dawley rats. Proc. Am. Assoc. Cancer Research. 34: 792.

Shivapurkar, N. Tang, Z.C. and Alabaster, O. 1992. The effect of high-risk and low-risk diets on aberrant crypt and colonic tumor formation in Fischer-F344 rats. Carcinogenesis. 13: 887-890.

Sinensky, M. and Logel, J. 1983. Inhibition of degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by mevinolin. J. Biol. Chem. 258: 8547-8549.

Singal, R., Khurana, V., Caldito, G. and Fort, C. 2005. Statins and prostate cancer risk: a case control study. Am. J. Epidemiol. 162: 318-325.

Singer, I. I., Kawka, D. W., Kazazis, D. M., Alberts, A. W., Chen, J. S., Huff, J. W., and Ness G. C. 1984. Hydroxymethylglutaryl coenzyme A reductase-containing hepatocytes are distributed periportally in normal and mevinolin-treated rat livers. Proceedings National Academy of Sciences, USA, 81: 5556-5560.

Singh, J., Hamid, R. and Reddy, B.S. 1997. Dietary fat and colon cancer: modulating effect of types and amount of dietary fat on ras-p21 function during promotion and progression stages of colon cancer. Cancer Research. 57: 253-258.

Singh, J., Hamid, R. and Reddy, B.S. 1998. Dietary fish oil inhibits the expression of farnesyl protein transferase and colon tumor development in rodents. Carcinogenesis. 19: 985-989.

Singh, J. Kelloff, G. and Reddy, B.S. 1992. Effect of chemopreventive agents on intermediate biomarkers during different stages of azoxymethane induced colon carcinogenesis. Cancer Epidemiol. Biomarkers Prev. 1: 405-411.

Siperstein, M.D. 1984. Role of cholesterogenesis and isoprenoid synthesis in DNA-replication and cell growth. J. Lipid Research. 25: 1463-1468.

Siperstein, M.D. and Fagan, V. 1964. Deletion of the cholesterol-negative feedback system in liver tumors. Cancer Research. 24: 1108-1115.

Smith, A.J., Stern, H.S., Penner, M., Hay, K., Mitri, A., Sapat, B.V., and Gallinger, S. 1994. Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. Cancer research. 54: 5527-5530.

Soh, J.W., Mao, Y., Kim, M.G., et al. 2000. Cyclin GMP mediates apoptosis induced by sulindac derivatives via activation of c-Jun NH2-terminal kinase 1. Clin. Cancer Res. 6: 4136-4141.

Spady, D. K. and Cuthbert, J. A. 1992. Regulation of hepatic sterol metabolism in the rat. Parallel regulation of activity and mRNA for 7α-hydroxylase but not 3-hydroxy-3-methylglutaryl coenzyme A reductase or low density lipoprotein receptor. The Journal of Biological Chemistry, 267: 5584-5591.

Spady, D. K., Woollett, L.A. and Dietschy, J.M. 1993. Annu. Rev. Nutr. 13: 355-381.

Spandidos, et al. 2002. Ras genes, tumor suppression and apoptosis. International Journal of Oncology. 21: 237-241.

Spence, J.T., Koudelka, A.P and Tseng-Crank, J.C.L. 1985. Biochem. J. 227: 939-947.

Stacey, D.W., Feig, L. A., and Gibbs, J.B. 1991. Dominant inhibitory ras mutants selectively inhibit the activity of either cellular or oncogenic ras. Mol. Cell Biol. 11: 4053-4064.

Stange, E.F. and Dietschy, J.M. 1983. Cholesterol synthesis and low density lipoprotein uptake are regulated independently in rat small intestine epithelium. Proc. Natl. Acad. Sci. USA. 80: 5739-5743.

Stange. E.F., Preclik, G., Schneider, A. Seiffer, E., Ditschuneit, H. 1981. Intestinal cholesterol synthesis and its relation to growth in cultured mucosa; in Robinson J.W.L., Dowling, R.H., Riecken, E.O. (eds): Mechanisms of Intestinal Adaptation. Lancaster, MTP Press. pp 91-100.

Stopera, S.A., and Bird, R.P. 1992. Expression of ras oncogene mRNA and protein in aberrant crypt foci. Carcinogenesis. 13:1863-1868.

Stopera, S.A., Davie, J.A., and Bird, R.P. 1992. Colonic aberrant crypt foci are associated with increases expression of c-fos: the possible role of modified c-fos expression in preneoplastic lesions in colon cancer. Carcinogenesis. 13: 573-578.

Stopera, S.A., Murphy, L.C., and Bird, R.P. 1992. Evidence for a ras gene mutation in azoxymethane-induced colonic aberrant crypts in Sprague-Dawley rats: earliest recognizable precursor lesions of colon cancer. Carcinogenesis. 13: 2081-2085.

Stopera, S.A. and Bird, R.P. 1993. Immunohistochemical demonstration of mutant p53 tumor suppressor gene product in aberrant crypt foci. Cytobios. 73: 73-88.

Strandberg, T.E. et al. 2004. Mortality and incidence of cancer during 10-year follow-up of the Scandinavian Simvastatin Survival Study (4S). Lancet. 364: 771-777.

Sugimura, T., Terada, M., Yokota, J., Hirohashi, S. and Wakabayashi, K. 1992. Multiple genetic alterations in human carcinogenesis. Environ. Health Perspect. 98: 5-12.

Sumi, B., Beauchamp, R.D., Townsend, C.M., Uchida, T., Murakami, M., Rajaraman, S., Ishizuka, J., Thompson, J.C. 1992. Inhibition of pancreatic adenocarcinomas cell growth by lovastatin. Gastroenterology. 103: 982-989.

Swamy, M.V., Cooma, I., Reddy, B.S., Rao, C.V. 2002. Lamin B, caspase 3, and apoptosis induction by a combination of HMG-CoA reductase inhibitor and COX-2 inhibitors: a novel approach in developming effective chemopreventive regimens. Int. J. Oncol. 20(4): 753-759.

T

Takahashi, S., Kawarabayasi, Y., Nakai, T., Sakai, J. and Yamamoto, T. 1992. Proc. Natl. Acad. Sci. USA. 89: 9252-9256.

Takahashi, M. Minamoto, T., Yamashita, N., Yazawa, K, Suimura, T. and Fsumi, H. 1993. Reduction in formation and growth of 1,2-dimethylhydrazine-induced aberrant crypt foci in rat colon by docosaheaenoic acid. Cancer Research. 53(12): 2786-2789.

Takahashi, M., Ogawa, K., Ohshima, H., Fsumi, H., Tto, N, and Sugimura T. 1991. Induction of aberrant crypt foci in the large intestine of F344 rats by oral administration of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Jpn. J. Cancer Research. 82(2): 135-137.

Thibault, A., Samid, D., Tompkins, A.C. et al. 1996. Phase ! study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin. Cancer Res. 2: 483-491.

Travis, J. 1993. Novel anticancer agents move closer to reality. Science. 260: 1877-1878.

Troppmaier, J., Bruder, J.T., App, H et al. 1992. Ras controls coupling of growth factor receptors and protein kinase C in the membrane to Raf-1 and B-Raf protein serine kinases in the cytosol. Oncogene. 7: 1867-1873.

Tudek, B, Bird, R.P., and Bruce, W.R. 1989. Foci of aberrant crypts in the colons of mice and rats exposed to carcinogens associated with foods. Cancer Research. 49:1236-1240.

U

Uauy, R., Vega, G.L., Grundy, S.M. and Bilheimer, D.W. 1988. J. Pediatr. 113: 387-392.

Ukomadu, C and Dutta, A. 2003. p21-dependent inhibition of colon cancer cell growth by mevastatin in independent of inhibition of G1 cyclin-dependent kinases. J. Biol. Chem. 278: 43586-43594.

V

Vial, E. and Marshall, C.J. 2003. Elevated ERK-MAP kinase activity protects the fos family member fra-1 against proteasomal degradation in colon carcinoma cells. J. Cell Sci. 116(Pt24): 4957-4963.

Vincent, T.S., Wulfert, E. and Merler, E. 1991. Inhibition of growth factor signalling pathways by lovastatin. Biochem Biophys. Res. Commun. 180: 1284-1289.

Vitols S., Peterson C., Larsson O., Holm P., and Aberg B. 1992. Elevated uptake of LDL by human lung cancer tissue in vivo. Cancer Research. 52(22):6244-6247.

Vitols, S., Angelin, B. and Juliusson, G. 1997. Simvastatin impairs mitogen-induced proliferation of malignant B-lymphocytes from humans-in vitro and in vivo studies. Lipids. 32(3): 255-262.

Vivona, A.A., Shpitz, B., Medline, A., Bruce, W.R., Hay, K., Ward, M.A., Stern, H.S. and Gallinger, S. 1993. K-ras mutations in aberrant crypt foci, adenomas and adenocarcinomas during azoxymethane-induced colon carcinogenesis. Carcinogenesis. 14(9):1777-1781.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M.M. and Bos, J.L. 1988. Genetic alterations during colorectal tumor development. New England Journal of Medicine. 319:525-532.

W

Wang, C. Pattabiraman, N., Zhou, J.N., Fu, M., Sakamaki, T. Albanese, C., Li, Z., Wu, K., Hulit, J., et al. 2003. Cyclin D1 expression of peroxisome proliferator-activated receptor gamma expression and transactivation. Mol. Cell Biol. 23: 6159-6173.

Wang, I.K., Lin-Shiau, S.Y., and Lin, J.K. 2000. Induction of apoptosis by lovastatin through activation of caspase-3 and DnaseII in leukaemic HL-60 cells. Pharmacology and Toxicology. 86(2); 83-91.

Wang, Z., Li, Y., Liu, E.T. and Yu, Q. 2004. Susceptibility to cell death induced by blockade of MAPK pathway in human colorectal cancer cells carrying ras mutations is dependent on p53 status. Biochem. Biophys. Res. Commun. 322(2): 609-613.

Wargovich, M.J., Jimenez, A., McKee, K., Steele, V.E., Velasco, M., Woods, J., Price, R., Gray, K., and Kelloff, G.J. 2000. Efficacy of potential chemopreventive agents on rat colon aberrant crypt formation and progression. Carcinogenesis. 21 (6): 1149-1151.

Wargovich, M.J., Harris, C., Chen, C-D., Palmer, C., Steele, V.E., and Kelloff, G.J. 1992. Growth kinetics and chemoprevention of aberrant crypts in the rat colon. J. Cell Biochem. (Suppl). 16G:51-54.

Wasylyk, B., Hagman, J., Gutierrez-Hartmann, A. 1998. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signalling pathway. Trends Biochem. Sci. 23: 213-216.

Watanabe, M., Ishiwata, T., Nishigai, K. et al. 2000. Overexpression of keratinocyte growth factor in cancer cells and enterochromaffin cells in human colorectal cancer. Pathol. Int. 50: 363-372.

Watanabe, M., Umekawa, H., Takahashi, T. and Furuichi, Y. 1999. Effects of dietary alpha- or gamma-linolenic acid on levels and fatty acid compositions of serum and hepatic lipids, and activity and mRNA abundance of 3-hydroxy-3-methylglutaryl coenzyme A reductase in rats. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 122: 213-220.

Watanabe, M., Masuda, Y., Nakajo, S et al. 1996. The cooperative interaction of two different signalling pathways in response to bufalin induces apoptosis in human leukemia U937 cells. J. Biol. Chem. 271: 14067-14072.

Web, C.P., Van Aelst, L., Wigler, M.H., et al. 1998. Signalling pathways in ras-mediated tumorigenisity and metastasis. Proc. Natl. Acad. Sci. USA. 95: 8773-8778.

Wei, J.T., et al. 2005. Reported use of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors was not associated with reduced recurrence of colorectal adenomas. Cancer Epidemiol. Biomarkers Prev. 14: 1026-1027.

Willet, W.C., Stampfer M.J., Colditz, G.A. Rosner, B.A. and Speizer, F.E. 1990. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. N. Engl. J. Med. 323: 1664-1672.

Wilson, R.B., Hutcheson, D.P., Wideman, L. 1977. Dimethylhydrazine-induced colon tumors in rats fed diets containing beef fat or corn oil with and without wheat bran. Am. J. Clin. Nutr. 30 (2): 176-181.

Winston, J.T., Coats, S.R., Wang, Y-Z. and Pledger, W.J. 1996. Regulation of the cell cycle machinery by oncogenic ras. Oncogene. 12: Liu, J-J., Chao, J-R., Jiang, M-C., Ng, S-Y., Yen, J-Y. and Yang-yen, H-F. 1995: 127-134.

Wong, H., Anderson, W., Cheng, T. and Riabowol, K. 1994. Monitoring mRNA expression by polymerase chain reaction: the primer dropping method. Anal. Biochem. 223: 251-258.

Wong, W.W.L., Dimitroulakos, J., Minden, M.D. and Penn, L.Z. 2002. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia. 16: 508-519.

Woodburn, J.R. 1999. The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol. Ther. 82: 241-250.

Wu, J., Wong, W.W.L., Khosravi, F., Minden, M.D. and Penn, L.Z. 2004. Blocking the Raf-MEK-ERK pathway sensitizes acute myelogenous leukemia cells to lovastatin-induced apoptosis. Cancer Research. 64: 6461-6468.

Y

Yamauchi, S., Linscheer, W.G. and Beach, D.H. 1991. Am. J. Physiology. 260G625-G630.

Yang, J.Y. and Widman, C. 2001. Antiapoptotic signalling generated by caspase-induced cleavage of RasGAP. Mol. Cell Biol. 21:5346-5358.

Yen, C.S., Wang, J.W., Cheng, T.L, Juan, C.H., Wu, C.H., and Lin, S.R. 2006. Fatty acid metabolism pathway play an important role in carcinogenesis of human colorectal cancers by Microarray_Bioinformatics analysis. 233 (2): 297-308.

Yoshimi, N., Wang, A., Makita, H., Suzuki, M., Mori, H., Okano, Y., Banno, Y. and Nozawa, Y. 1994. Reduced expression of phospholipase C-delta, a sigal transducing enzyme in rat colon neoplasms induced by methylazoxymethanol acetate. Mol. Carcinogenesis. 11: 192-196.

Z

Zhou, H., Li, X.M., Meinkoth, J., Pittman, R.N. 2000. Akt regulates cell survival and apoptosis at a postmitochondrial level. J. Cell Biol. 151: 483-494.

APPENDICES

APPENDIX A: Composition Of AIN-76A Vitamin And Mineral Mix

Vitamin Mix, AIN-76A	g/kg
Thiamin HCl	0.60
Riboflavin	0.60
Pyridoxin HCl	0.70
Niacin	3.00
Calcium pantothenate	1.60
Folic acid	0.20
Biotin	0.02
Vitamin B12	1.00
Dry Vitamin A palmitate	0.80
Dru Vitamin E acetate	10.0
Vitamin D3 trituration	0.25
Menadione sodium bisulfate complex	0.15
Sucrose, fine powder	981.08

Mineral Mix, AIN-76A	g/kg at 3.5% of diet
Calcium	5.1550
Phosphorus	3.9840
Potassium	3.6020
Sodium	1.0190
Chloride	1.5710
Sulphur	0.3370
Magnesium	0.5070
Iodine	0.0002
Iron	0.0351
Copper	0.0056
Manganese	0.0585
Zinc	0.0414

APPENDIX B

Fatty Acid Composition of Experimental Lipds[1,2]

Fatty Acid	Beef Tallow	Corn Oil
14:0	3.10	ND
16:0	23.00	10.10
16:1	2.20	ND
18:0	19.40	1.70
18:1n9	38.60	26.50
18:2n6	3.40	59.20
18:3n3	1.00	0.80
20:5n3	ND	ND
22:5n3	ND	ND
22:6n3	ND	ND

1. Expressed as percent composition; ND, non-detectable.
2. Fatty acid composition assessed by gas chromatography.

APPENDIX C

Fatty Acid Composition of Experimental Diets[1,2]

Fatty Acid	Beef Tallow	Corn Oil
14:0	2.40	ND
16:0	20.60	10.60
16:1	1.80	0.10
18:0	16.00	2.20
18:1n9	34.90	25.50
18:2n6	14.30	57.80
18:3n3	2.60	2.40
20:5n3	ND	ND
22:5n3	ND	ND
22:6n3	ND	ND

1. Expressed as percent composition; ND, non-detectable.
2. Fatty acid composition assessed by gas chromatography.

APPENDIX D

Composition of Experimental Diets[1,2,3]

Diet Ingredient	LFC	HFC	HFB
Casein	20.0	23.0	23.0
Corn Starch	52.0	33.8	33.8
Dextrose	13.0	8.5	8.5
Corn Oil	5.0	5.0	5.0
Cellufil	5.0	5.9	5.9
DL-Methionine	0.3	0.3	0.3
Choline Bitartrate	0.2	0.2	0.2
AIN-76 mineral mix	3.5	4.1	4.1
AIN-76 vitamin mix	1.0	1.2	1.2
Additional Dietary lipid [4]	-	18.0	18.0

1. All values represent the percentage composition unless otherwise stated and are isocaloric.
2. Dietary groups are as follows: low fat corn oil (LFC), high fat corn oil (HFC), and high fat beef tallow (HFB).
3. Lovastatin treated groups were orally gavaged 20 mg/kg body weight per day for the last three weeks of the study.
4. Additional dietary lipid consisted of corn oil for the HFC group and beef tallow for the HFB group.

APPENDIX E: RIPA Buffer Recipe

From Current Protocols in Molecular Biology, Asubel et al. 1995 (vol. 2)

With the exception of SDS, all reagents were purchased from Sigma Chemical Company.

1% Triton-X 100
0.1% SDS
0.5% sodium deoxycholate
150 mM NaCl
5 mM EDTA
5 mM PMSF
10 ug/ml leupeptin
1 mM Na3VO4
5 mM NaPP
20 mM sodium phosphate
1 ul/ml aprotinin

Stored at 4^0C.

APPEDIX F: Buffers Used For Tissue Cell Lysate Preparation

Buffers Used for the Separation of Membranous and Cytosolic Fractions

Cytosolic Buffer:

25 mM Tris-HCl (pH 7.5)
5 mM EDTA
5 mM EGTA
0.25 M sucrose
12 mM mercaptoethanol.

Protein inhibitors were added just prior to use at the following levels: 0.5 μg/ml leupeptin, 2.0 ug/ml aprotinin, 10.0 ug/ml trypsin.

PMSF was added to each sample at a concentration of 1 ug/ml.

Membranous Buffer:

Cytosolic buffer with the addition of TritonX-100 at the level of 0.533%.

Whole Cell Lysate Buffer (RIPA Buffer):

50 mM Tris-HCl (pH 7.4)
150 mM NaCl
1.0% NP-40
1 mM EDTA
0.25% sodium deoxycholate
1 mM NaF.

Protein inhibitors were added just prior to use at the following levels: 1 μg/ml leupeptin, aprotinin, and trypsin.

PMSF and Na_3VO_4 (SOV) were added at a concentration of 1 mM.

APPENDIX G: Western Blotting/Immunoblotting Buffers and Acrylamide Gel Recipes

Based on the method described by Laemmli, 1976. All reagents and recipes are from Bio-rad.

Sample Buffer (SDS Sample Buffer)

Ingredient	mL
H_2O	3.8
0.5M Tris (pH 6.8)	1.0
Glycerol	0.8
10% Sodium Dodecyl Sulphate (SDS)	1.6
2-mercaptoethanol	0.4
1% (w/v) bromophenol blue	0.4
Total Volume	8.0

5X Electrode Running Buffer

Ingredient	Grams (g)
Tris Base	9.0
Glycine	43.2
Sodium Dodecyl Sulphate (SDS)	3.0

Brought up to 300mL volume with deionized water (stock) and stored at room temperature. Diluted to appropriate volume before use.

Transfer Buffer

Ingredient	Grams (g) / mL
Tris Base	3.03 g
Glycine	14.40 g
Methanol	200 mL

Both the Tris and glycine were mixed in 200 mL methanol and adjusted to a 1000 ml volume by addition of deionized water. Transfer buffer was stored at 4^0C.

TBST Solution

100mM Tris HCl
0.9% NaCl
0.1% Tween 20
Stored at room temperature

30% Acrylamide/Bis Mix

87.6 g acrylamide (29.2 g/100 mL)
2.4 g N'N-bis-methylene-acrylamide (0.8g/100 mL)

Brought to 300 mL volume with deionized water and stored in the dark at 4^0C.

10% Ammonium Persulphate (APS)

100 mg ammonium persulphate in 1 mL deionized water.

10% Sodium Dodecyl Sulphate (SDS)

10 g SDS dissolved (gentle stirring) in 90 mL deionized water. Brought to 100 mL volume with deionized water and stored at room temperature.

5% Stacking Gel

Ingredient	mL
H_2O	3.40
30% Acrylamide mix	0.83
0.5 M Tris HCl (pH 6.8)	0.63
10% SDS	0.05
10% APS	0.05
TEMED (N,N,N-tetramethylethylenediamine)	0.005
Total Volume	5.00

10% Separating Gel

Ingredient	mL
H_2O	3.30
30% Acrylamide mix	4.00
1.5 M Tris HCl (pH 8.8)	2.50
10% SDS	0.10
10% APS	0.10
TEMED (N,N,N-tetramethylethylenediamine)	0.004
Total Volume	10.00

Coomassie Blue Stain

0.1% Coomassie Blue R-250
40% methanol
10% acetic acid
50% deionized water

Stain for 30 minutes with gentle rocking.

Destain Solution

40% methanol
10% acetic acid
50% deionized water

Destain with several changes over at least one hour.

APPENDIX H: RT-PCR Buffer Recipes

Based on the methods from Current Protocols in Molecular Biology (vol.1), chapter 2.5. Aussubel et al., (eds), 1995. All reagents are from Gibco BRL.

5X First Strand PCR Buffer

Ingredient	mM
Tris HCl (pH 8.3)	250
KCl	375
$MgCl_2$	15

10X PCR Buffer

Ingredient	mM
Tris HCl (pH 8.4)	200
KCl	500

10X Sample Loading Buffer

Ingredient	Percent
Ficoll 400	20.00
SDS	0.10
Bromophenol blue	0.25
Xylene cyanol	0.25

10X TBE

Ingredient	Gram(s)/mL
Tris HCl	108 g
Boric acid	55 g
0.5 M EDTA	40 mL

Bring to one litre volume with deionized water.

2% Agarose Gel

Ingredient	Gram(s)/mL
H_2O	135.0 mL
Agarose	3.0 g
10X TBE	15.0 mL
Ethidium Bromide	4.5 uL

10X Gel Loading Buffer

Ingredient	Milligram(s)/mL
30% Glycerol	3.33 mL
TE	6.67 mL
0.25% xylene cyanol	25.00 mg
or	
0.25% Bromophenol Blue	25.00 mg

1kb DNA Ladder

Ingredient	microliters (uL)
1 kb DNA Ladder Stock	10
10X DNA sample buffer	100
Sterile Water	890

APPENDIX I: Hepatic Cyclin D1, and Caspase-3 Protein Expression Findings

The Amount of Dietary Lipid Affects Hepatic ERK-1 Protein Expression

A high fat diet resulted in increased hepatic ERK-1 protein expression compared to a low fat diet ($p<0.05$).

Lovastatin Treatment Increased Hepatic ERK-1 Protein Expression

In both the HFCα and HFBα treated groups, hepatic ERK-1 protein expression was significantly higher compared to the non-LOV treated group.

The Type of Dietary Fat affects Hepatic CD1 Protein Expression

In decreasing order, the level of CD1 protein expression in rat hepatic tissue was HFBα > HFB > LFC > HFCα > HFC (Table 34). The HFB dietary group had significantly ($p<0.0001$) higher CD1 protein expression compared to the HFC group. A significant ($p<0.0001$) main effect of saturated versus unsaturated dietary fat treatment was found for CD1 protein expression in rat hepatic tissue. In addition, a significant main effect of dietary fat on CD1 protein expression in hepatic tissue was found at $p<0.007$.

The Effect of Lovastatin Treatment on Hepatic CD1 Protein Expression Depends on the Type of Dietary Lipid

Lovastatin treatment in the HFBα group resulted in a significant ($p<0.0001$) increase in CD1 protein expression compared to the HFB group (Table 34). There was a significant ($p<0.01$) main effect of lovastatin treatment on CD1 protein expression in hepatic tissue. There was no difference in hepatic CD1 protein expression between the HFC and HFCα groups. There was a significant ($p<0.0001$) main effect of lovastatin by dietary fatty acid treatment on CD1 protein expression in hepatic tissue. CD1 protein expression was significantly ($p<0.0001$) higher in the HFBα group compared to the HFCα group.

The Amount of Dietary Lipid Affects Hepatic Caspase-3 Protein Expression

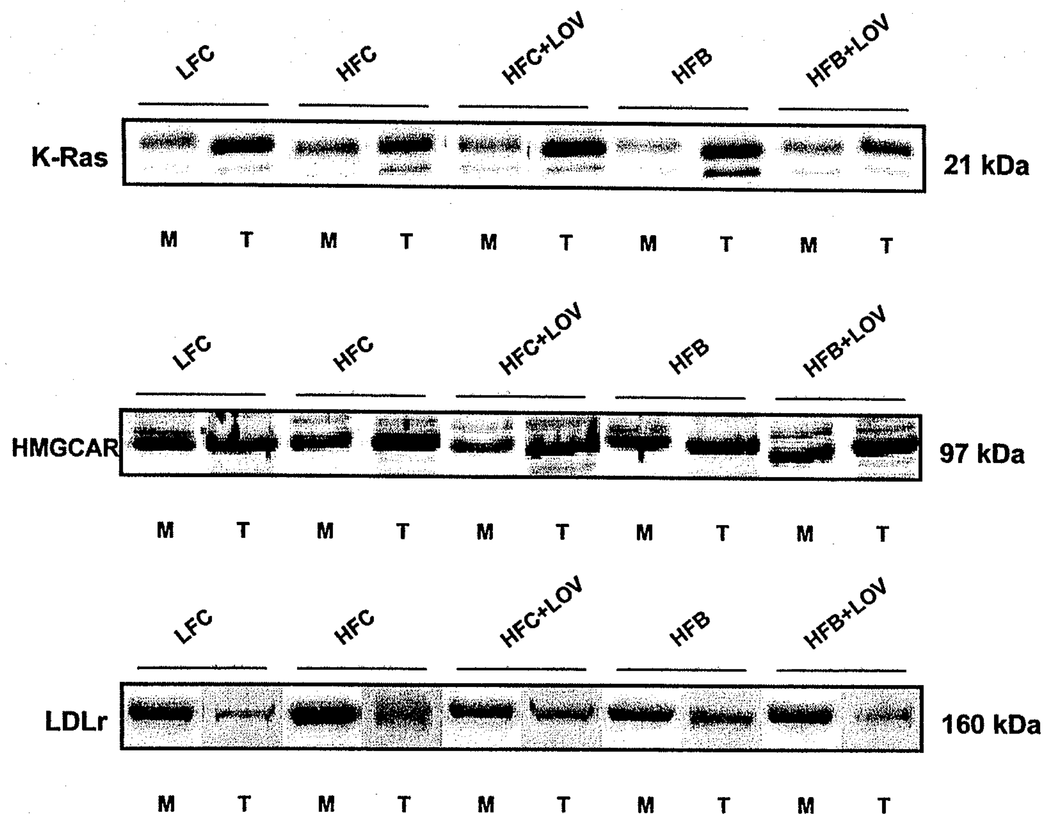
In decreasing order, caspase-3 protein expression in hepatic tissue was HFCα > HFBα > LFC > HFC > HFB (Table 35). A significant ($p<0.02$) main effect of low versus high dietary fat treatment on caspase-3 protein expression in the hepatic tissue was found whereby a low fat diet induced higher hepatic caspase-3 expression compared to a diet high in fat. Caspase-3 expression was similar between the HFC and HFB groups, as well as the LFC and HFC groups.

Lovastatin Treatment Significantly Increases Hepatic Caspase-3 Protein Expression

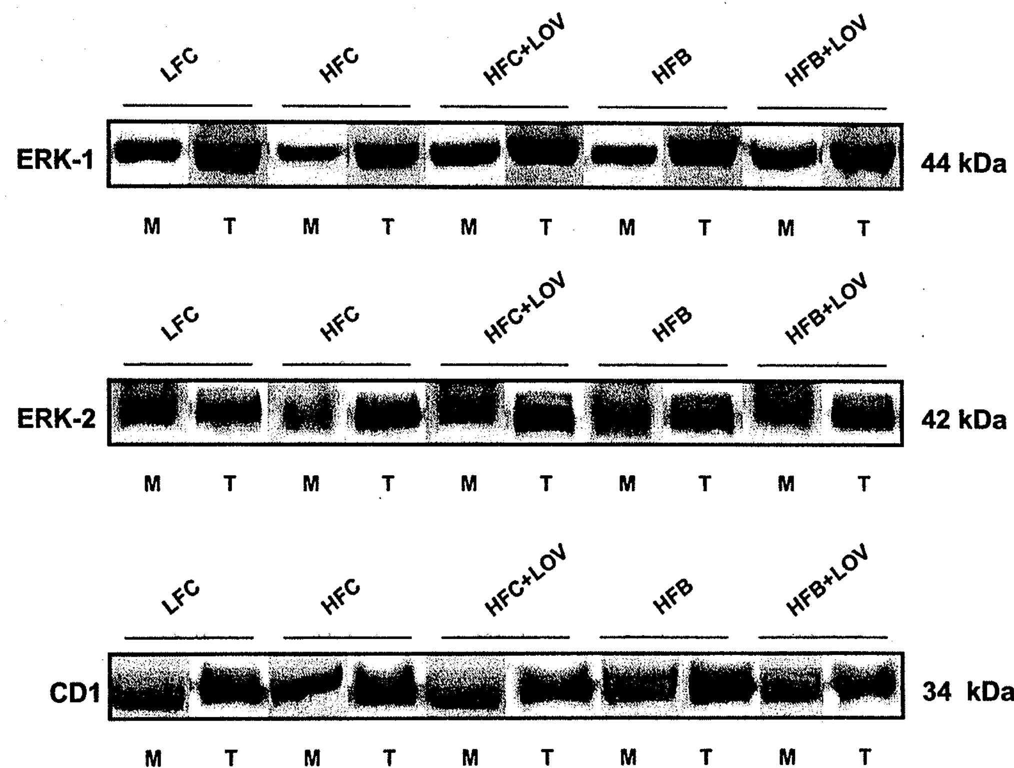
Both lovastatin treated groups, HFCα and HFBα, had significantly ($p<0.007$) higher hepatic caspase-3 protein expression compared to the non-lovastatin treated groups (Table 35). The HFCα and HFBα groups had similar levels of caspase-3 protein expression.

Appendix J

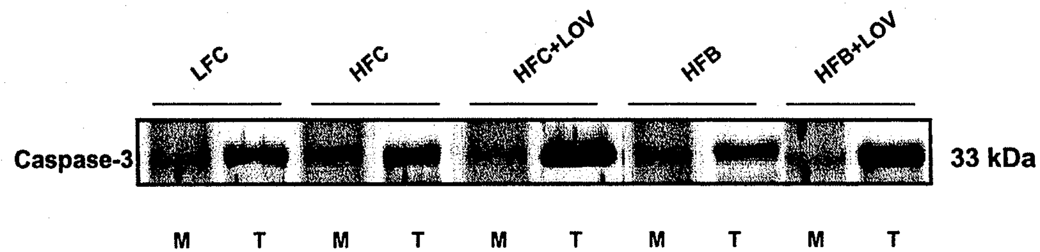
Representative Western Blot Images for $p21^{K\text{-}ras}$, HMGCAR, LDLR, ERK-1, ERK-2, CD1, and Caspase-3



Appendix J-1: Representative Western Blot Images for Experimental Molecules Involved in Cholesterol Biosynthesis and p21$^{K\text{-}Ras}$ Function. LFC, low fat corn oil; HFC, high fat corn oil; HFC+LOV, high fat corn oil plus lovastatin; HFB, high fat beef tallow; HFB+LOV, high fat beef tallow plus lovastatin; HMGCAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLr, low density lipoprotein receptor; ERK-1/2, extracellular-regulated kinase; CD1, cyclin D1; M, colonic mucosa; T, colonic tumor.



Appendix J-2: Representative Western Blot Images for Experimental Molecules Involved in Cell Signal Transduction and the Cell Cycle. LFC, low fat corn oil; HFC, high fat corn oil; HFC+LOV, high fat corn oil plus lovastatin; HFB, high fat beef tallow; HFB+LOV, high fat beef tallow plus lovastatin; HMGCAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLr, low density lipoprotein receptor; ERK-1/2, extracellular-regulated kinase; CD1, cyclin D1; M, colonic mucosa; T, colonic tumor.



Appendix J-3: Representative Western Blot Image for the Apoptotic Regulating Protein.
LFC, low fat corn oil; HFC, high fat corn oil; HFC+LOV, high fat corn oil plus lovastatin; HFB, high fat beef tallow; HFB+LOV, high fat beef tallow plus lovastatin; HMGCAR,3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLr, low density lipoprotein receptor; ERK-1/2, extracellular-regulated kinase; CD1, cyclin D1; M, colonic mucosa; T, colonic tumor.

Appendix K

A Preliminary Investigation into the Effect of Dietary Lipids and Genistein on $p21^{K\text{-}ras}$, HMGCAR, LDLr, ERK-1, ERK-2, CD1, and Caspase-3 in Normal and Neoplastic Colonic Tissue

In an attempt to gain further understanding into the role of the modulatory effects of dietary lipid on environmental factors (food components), as well as to make the most out of tissues harvested from experimental animals and experimental reagents purchased, Western blot (protein expression) and RT-PCR (gene expression) analysis were conducted on tissue samples from a parallel arm of the main thesis study in which Genistein was fed to a subset of animals from the HFC and HFB dietary groups (at the level of 250 mg/kg diet) for 12 weeks prior to the final termination of animals. The Tables and Figures below outline the preliminary findings from the analyses outlined above. For further details regarding experimental methods, see Chapter 3.

Appendix K-1. Plasma Total Cholesterol, Triacylglycerol, and LDL at 21 Weeks after Nine Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Total Cholesterol (mmol/L)$^{\pi, \lambda}$	3.31 ± 0.17^{a}	2.97 ± 0.12^{ab}	2.29 ± 0.19^{c}	2.60 ± 0.16^{bc}	2.26 ± 0.22^{c}
Triacylglycerol (mmol/L)$^{\Psi}$	1.86 ± 0.05^{a}	0.79 ± 0.02^{c}	1.37 ± 0.22^{b}	0.88 ± 0.07^{c}	0.93 ± 0.08^{c}
LDL (mmol/L)$^{\pi, \lambda}$	1.73 ± 0.12^{a}	1.86 ± 0.05^{a}	1.59 ± 0.13^{a}	1.49 ± 0.12^{a}	1.50 ± 0.12^{a}

1. Values are means ± SE.

2. Values in a row with different superscripts are significantly different at $p<0.05$; Ψ represents significant main effect of Genistein by fat interaction ($p<0.0008$); π represents significant main effect of dietary fat ($p<0.0009$) for total cholesterol and ($p<0.07$) for LDL; λ represents significant main effect of saturated versus unsaturated fat ($p<0.05$)

3. Animals were terminated during the day and 9 weeks after dietary intervention with the LFC, HFC, or HFB diets with a subset of animals from the HFC and HFB diet groups receiving Genistein treatment (250mg Genistein/kg diet). The plus and minus signs designate presence or absence of Genistein treatment, respectively. n=15-20 animals/group.

Appendix K-2. Plasma Total Cholesterol, Triacylglycerol, and LDL at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Total Cholesterol (mmol/L)	3.96 ± 0.08[a]	2.97 ± 0.11[c]	2.77 ± 0.08[c]	3.46 ± 0.13[b]	3.01 ± 0.09[c]
Triacylglycerol (mmol/L)	1.35 ± 0.04[a]	1.09 ± 0.08[b]	0.79 ± 0.07[c]	1.12 ± 0.13[ab]	1.24 ± 0.04[ab]
LDL (mmol/L)	1.76 ± 0.13[a]	1.63 ± 0.09[ab]	1.61 ± 0.05[a]	1.57 ± 0.06[ab]	1.74 ± 0.07[a]

1. Values are means ± SE.
2. Values in a row with different superscripts are significantly different at $p<0.05$.
3. Animals were terminated during the day and 12 weeks after dietary intervention with the LFC, HFC, or HFB diets with a subset of animals from the HFC and HFB diet groups receiving Genistein treatment (250mg Genistein/kg diet). The plus and minus signs designate presence or absence of Genistein treatment, respectively. n=15-20 animals/group.

Appendix K-3. Colonic Mucosa and Hepatic Cytosolic and Membrane-Associated p21$^{K\text{-}Ras}$ Protein Expression at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression[4]					
Colonic Mucosa					
Cytosol[*, 5, 6, 8, 9]	14.1 ± 1.1^{c}	21.2 ± 1.3^{a}	18.1 ± 1.0^{b}	12.1 ± 1.1^{cd}	10.4 ± 0.4^{d}
Membrane[7, 8]	3.5 ± 0.2^{bc}	2.9 ± 0.2^{c}	5.6 ± 0.4^{a}	3.0 ± 0.1^{c}	4.4 ± 0.6^{b}
Liver					
Cytosol[*, 6, 8, 9]	7.5 ± 0.8^{b}	10.4 ± 0.5^{a}	9.4 ± 0.2^{ab}	11.3 ± 1.3^{a}	7.8 ± 0.21^{b}
Membrane[6, 7]	3.3 ± 0.1^{ab}	2.2 ± 0.1^{c}	3.5 ± 0.5^{a}	3.9 ± 0.4^{a}	2.5 ± 0.2^{bc}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values represent K-Ras protein expression in the cytosolic and membrane fractions of the colonic mucosa and liver tissues. The amount of protein loaded for colonic mucosa cytosol and membrane fractions was 60 µg. The amount of protein loaded for liver cytosol and membrane fractions was 30 µg. See Materials and Methods for additional information.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=6-8 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.

4. Values in a row with different superscripts are significantly different at $p<0.05$; * represents significant differences between the cytosolic and membrane K-ras protein expression in both colonic mucosa and liver for all treatment groups ($p<0.0001$).

5. Significant main effect of dietary fat treatment ($p<0.0001$).

6. Significant main effect of saturated versus unsaturated fat for colonic mucosa cytosolic fraction ($p<0.0001$) and liver cytosolic ($p<0.02$) and membrane ($p<0.002$) fractions, respectively.

7. Significant main effect of Genistein x dietary fat interaction for colonic mucosa membrane fraction ($p<0.06$) and liver membrane ($p<0.0001$) fractions, respectively.

8. Significant main effect of Genistein for colonic mucosa cytosol ($p<0.03$) and membrane ($p<0.0001$) fractions as well as the liver cytosol fraction ($p<0.009$).

9. Significant main effect of low versus high dietary fat treatments for colonic mucosa cytosol ($p<0.04$) and liver cytosol ($p<0.001$) fractions.

Appendix K-4. **Colonic Mucosa and Tumor $p21^{K\text{-}Ras}$ Protein Expression at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein [1, 2, 3].**

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression[4]					
Colonic Mucosa[*, 5, 6, 7]	17.3 ± 1.8[c]	21.4 ± 0.5[b]	12.9 ± 1.0[d]	12.5 ± 0.6[d]	24.3 ± 0.3[a]
Colonic Tumors[6]	35.1 ± 5.6[ab]	27.5 ± 0.4[b]	42.2 ± 4.0[a]	35.8 ± 2.8[ab]	33.0 ± 2.8[ab]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Protein expression is for colonic mucosa and tumor whole homogenate. The amount of protein loaded for colonic mucosa and tumors was 30 µg. See Methods and Materials for additional information.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=6-8 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.

4. Values in a row with different superscripts are significantly different at $p<0.05$; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the LFC, HFC+, HFB-, and HFB+ dietary fat groups, respectively.

5. Significant main effect of saturated versus unsaturated fat for colonic mucosa ($p<0.0001$).

6. Significant main effect of Genistein x dietary fat interaction for colonic mucosa ($p<0.0001$) and tumors ($p<0.03$).

7. Significant main effect of Genistein for colonic mucosa ($p<0.07$).

Appendix K-5. Colonic Mucosa and Tumor p21$^{K\text{-}Ras}$ Activity at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
K-Ras Activity[4, 5, 6, 7]					
Colonic Mucosa*	5.56 ± 0.50^{c}	8.70 ± 1.50^{c}	45.06 ± 8.00^{b}	26.40 ± 2.30^{bc}	84.95 ± 13.0^{a}
Colonic Tumors	34.50 ± 4.10^{b}	18.70 ± 4.40^{b}	35.54 ± 6.0^{b}	39.60 ± 4.00^{b}	159.01 ± 10.7^{a}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.0001$; * represents significant differences between colonic mucosa and tumor K-Ras activity ($p<0.0001$) for the LFC and HFB+ dietary fat groups.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=3-5 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.
4. Significant main effect of Genistein x dietary fat interaction for colonic tumors ($p<0.0001$).
5. Significant main effect of saturated versus unsaturated fat for colonic mucosa ($p<0.04$).
6. Significant main effect of Genistein for colonic mucosa ($p<0.0001$) and tumors ($p<0.0001$), respectively.
7. Significant main effect of dietary fat for colonic mucosa ($p<0.0008$) and tumors ($p<0.0001$), respectively.

Appendix K-6. Colonic Mucosa and Tumor HMGCAR Gene Expression at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Three Weeks of Genistein[1,2,3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Gene Expression[4]					
Colonic Mucosa*	0.78 ± 0.05[a]	0.19 ± 0.06[a]	0.21 ± 0.17[a]	0.37 ± 0.08[a]	0.40 ± 0.30[a]
Colonic Tumors[5]	0.68 ± 0.05[a]	1.16 ± 0.10[a]	1.80 ± 0.47[a]	0.71 ± 0.06[a]	1.46 ± 0.45[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels).
2. Values in a row with different superscripts are significantly different ($p<0.05$). * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0008$) for the HFC-, HFC+, and HFB+ dietary fat groups, respectively.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=3-5 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.
4. HMGCAR gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.
5. Significant main effect of Genistein x dietary fat interaction for colonic tumors ($p<0.059$).

Appendix K-7. Colonic Mucosa and Tumor HMGCAR Protein Expression at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression[4]					
Colonic Mucosa[*, 7, 8]	19.5 ± 0.5[a]	16.7 ± 0.06[b]	15.3 ± 0.7[b]	18.6 ± 0.6[a]	11.8 ± 0.7[c]
Colonic Tumors[5, 6, 7, 9]	28.0 ± 1.1[bc]	33.8 ± 2.7[ab]	25.4 ± 1.6[c]	27.1 ± 1.3[c]	35.7 ± 3.4[a]
Liver[6, 7, 8]	40.5 ± 3.7[c]	63.7 ± 2.8[b]	110.1 ± 10.2[a]	45.0 ± 2.1[c]	62.2 ± 6.0[b]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.0002$ and $p<0.001$ for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for all treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=6-7 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.

4. HMGCAR protein expression is for colonic mucosa, tumor, and liver whole homogenate. The amount of protein loaded was 20μg. The 97 KDa band was assessed. See Materials and Methods for additional information.

5. Significant main effect of Genistein treatment for colonic tumors ($p<0.03$) and liver ($p<0.03$).

6. Significant main effect of dietary fat treatment for colonic tumors ($p<0.07$) and liver ($p<0.0001$), respectively.

7. Significant main effect of low versus high fat dietary treatments for colonic mucosa ($p<0.0001$), tumors ($p<0.001$), and liver ($p<0.009$), respectively.

8. Significant main effect of Genistein x dietary fat interaction for colonic mucosa ($p<0.0001$) and liver ($p< 0.0001$), respectively.

9. Significant main effect of saturated versus unsaturated dietary fat for colonic tumors ($p<0.02$).

Appendix K-8. Colonic Mucosa and Tumor LDL Receptor Gene Expression at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Gene Expression[4]					
Colonic Mucosa	0.35 ± 0.05[a]	0.47 ± 0.05[a]	1.30 ± 0.92[a]	0.55 ± 0.09[a]	0.63 ± 0.15[a]
Colonic Tumors	0.67 ± 0.06[a]	0.59 ± 0.13[a]	0.84 ± 0.16[a]	0.91 ± 0.26[a]	0.67 ± 0.15[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different ($p<0.05$). There was no significant difference between colonic mucosa and tumor whole homogenate for any dietary fat group.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=4-7 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.
4. LDLr gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.

Appendix K-9. Colonic Mucosa and Tumor LDL Receptor Protein Expression at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression [4]					
Colonic Mucosa[*, 5, 7, 8]	3.43 ± 0.16^{c}	5.25 ± 0.12^{a}	4.11 ± 0.03^{b}	3.77 ± 0.15^{bc}	3.63 ± 0.17^{c}
Colonic Tumors[5, 7, 8, 9]	1.78 ± 0.18^{c}	3.0 ± 0.23^{a}	3.32 ± 0.15^{a}	2.31 ± 0.05^{b}	2.91 ± 0.18^{a}
Liver [5, 6, 7, 8, 9]	1.25 ± 0.12^{b}	1.5 ± 0.08^{b}	1.47 ± 0.10^{b}	1.58 ± 0.11^{b}	2.38 ± 0.21^{a}

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.0002$ and $p<0.001$ for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for all treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=6-8 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.

4. LDLr protein expression is for colonic mucosa, tumor, and liver whole homogenate. The amount of protein loaded for colonic mucosa and tumors was 60µg. The amount of protein loaded for liver was 10µg. The 160 KDa band was assessed. See Materials and Methods for additional information.

5. Significant main effect of Genistein x dietary fat interaction for colonic mucosa ($p<0.0001$), tumors ($p<0.0001$), and liver ($p<0.02$).

6. Significant main effect of low versus high fat dietary treatments for liver ($p<0.04$).

7. Significant main effect of saturated versus unsaturated dietary fat for colonic mucosa ($p<0.03$), tumors ($p<0.002$), and liver ($p<0.006$).

8. Significant main effect of Genistein treatment for colonic mucosa ($p<0.01$), tumors ($p<0.02$) and liver ($p<0.0002$), respectively.

9. Significant main effect of dietary fat treatment for colonic mucosa ($p<0.0001$) and liver ($p<0.0003$).

Appendix K-10. Colonic Mucosa and Tumor ERK-1 Gene Expression in F344 Rats at 24 Weeks after Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Gene Expression[4]					
Colonic Mucosa[5]	1.89 ± 0.24[a]	1.13 ± 0.19[b]	2.02 ± 1.04[a]	1.36 ± 0.23[a]	1.16 ± 0.14[a]
Colonic Tumors[5]	0.77 ± 0.10[b]	1.47 ± 0.21[ab]	1.00 ± 0.19[ab]	1.34 ± 0.11[ab]	1.58 ± 0.22[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels).

2. Values in a row with different superscripts are significantly different ($p<0.05$). There was no significant difference between colonic mucosa and tumor whole homogenate for the any dietary fat group.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=5-6 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.

4. ERK-1 gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.

5. Significant main effect of low versus high fat dietary treatment for colonic tumors ($p<0.03$).

Appendix K-11. ERK-1 Protein Expression at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression [4]					
Colonic Mucosa*[, 5, 6, 7]	2.95 ± 0.24[b]	1.94 ± 0.06[d]	2.56 ± 0.18[bc]	2.25 ± 0.12[cd]	3.55 ± 0.24[a]
Colonic Tumors[5, 7]	6.70 ± 0.30[a]	4.30 ± 0.20[b]	6.05 ± 0.56[a]	6.13 ± 0.30[a]	6.92 ± 0.44[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at p<0.0001 and p<0.003 for colonic mucosa and tumors, respectively; * represents significant differences between colonic mucosa and tumor whole homogenate (p<0.0001) for all treatment groups.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=5-7 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.
4. ERK-1 protein expression is for colonic mucosa and tumor whole homogenate. ERK-1 was detected as a 44 kDa band. The amount of protein loaded for colonic mucosa and tumors was 15µg. See Materials and Methods for additional information.
5. Significant main effect of Genistein x dietary fat interaction for colonic mucosa (p<0.07) and tumors (p<0.007).
6. Significant main effect of low versus high fat dietary treatments for colonic mucosa (p<0.0001).
7. Significant main effect of dietary fat treatment for colonic mucosa (p<0.002) and tumors (p<0.01).

Appendix K-12. ERK-2 Protein Expression in Colonic Mucosa and Tumors at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression [4]					
Colonic Mucosa[*, 5, 6, 7]	1.94 ± 0.16[a]	1.44 ± 0.08[b]	1.96 ± 0.20[a]	1.56 ± 0.11[ab]	1.37 ± 0.02[b]
Colonic Tumors	2.51± 0.20[a]	2.56 ± 0.21[a]	2.94 ± 0.42[a]	3.02 ± 0.40[a]	3.24 ± 0.27[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.
2. Values in a row with different superscripts are significantly different at $p<0.008$ for colonic mucosa; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the HFC-, HFC+, HFB-, and HFB+ dietary fat treatment groups.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=5-7 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.
4. ERK-2 protein expression is for colonic mucosa and tumor whole homogenate. The amount of protein loaded for colonic mucosa and tumors was 15μg. ERK-2 was detected as a 42 kDa band. See Materials and Methods for additional information.
5. Significant main effect of dietary fat treatment for colonic mucosa ($p<0.002$).
6. Significant main effect of low versus high fat dietary treatments for colonic mucosa ($p<0.04$).
7. Significant main effect of saturated versus unsaturated dietary fat for colonic mucosa ($p<0.009$).

Appendix K-13. Cyclin D1 Gene Expression at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Gene Expression[4]					
Colonic Mucosa[*, 5]	0.52 ± 0.05[a]	0.39 ± 0.04[a]	0.57 ± 0.19[a]	0.37 ± 0.01[a]	0.28 ± 0.03[a]
Colonic Tumors[6]	0.57 ± 0.03[a]	0.80 ± 0.08[a]	0.99 ± 0.24[a]	0.91 ± 0.23[a]	0.87 ± 0.08[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels).
2. Values in a row with different superscripts are significantly different ($p<0.05$). * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.009$) for the HFB-, and HFB+ dietary fat treatment groups.
3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=5-7 rats/group). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.
4. Cyclin D1 gene expression is for colonic mucosa and tumor whole homogenate. See Materials and Methods for additional information.
5. Significant main effect of saturated versus unsaturated dietary fat treatment for colonic mucosa ($p<0.04$).
6. Significant main effect of Genistein treatment for colonic tumors ($p<0.03$).

Appendix K-14. Cyclin D1 Protein Expression in Colonic Mucosa, Tumors, and Liver at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression [4]					
Colonic Mucosa[*, 5, 6, 8]	2.78 ± 0.17[ab]	2.63 ± 0.09[b]	2.48 ± 0.08[b]	3.02 ± 0.06[a]	3.07 ± 0.13[a]
Colonic Tumors[5, 7, 8]	4.63 ± 0.11[bc]	5.80 ± 0.31[a]	5.21 ± 0.22[ab]	4.97 ± 0.18[bc]	4.57 ± 0.11[c]
Liver[5, 6, 8]	3.12 ± 0.07[ab]	2.75 ± 0.16[c]	2.65 ± 0.08[c]	3.23 ± 0.11[a]	2.91 ± 0.08[bc]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at p<0.007, p<0.003, and p<0.002 for colonic mucosa, colonic tumors, and liver respectively; * represents significant differences between colonic mucosa and tumor whole homogenate (p<0.0001) for all treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=5-8 rats/group for colonic mucosa and 5-6 rats/group for colonic tumors). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.

4. Cyclin D1 protein expression is for colonic mucosa, tumor, and liver whole homogenate. CD1 was detected as a 34 kDa band. The amount of protein loaded for colonic mucosa, tumors, and liver was 30μg. See Materials and Methods for additional information.

5. Significant main effect of Genistein x dietary fat interaction for colonic mucosa (p<0.007), tumors (p<0.002) and liver (p<0.006).

6. Significant main effect of saturated versus unsaturated dietary fat for colonic mucosa (p<0.001) and liver (p<0.02).

7. Significant main effect of dietary fat treatment for colonic tumors (p<0.03).

8. Significant main effect of low versus high dietary fat for colonic mucosa (p<0.04), tumors (p<0.05), and liver (p<0.004).

Appendix K-15. Caspase-3 Protein Expression in Colonic Mucosa, Tumors, and Liver at 24 Weeks after 12 Weeks of Treatment with LFC, HFC, HFB Diets and Genistein[1, 2, 3].

	Diets				
	LFC	HFC		HFB	
		-	+	-	+
Protein Expression [4]					
Colonic Mucosa[*, 7, 8]	2.75 ± 0.25[bc]	3.43 ± 0.03[a]	2.92 ± 0.10[ab]	3.12 ± 0.16[ab]	2.38 ± 0.17[c]
Colonic Tumors[5, 8]	4.78 ± 0.61[b]	6.09 ± 0.68[b]	6.36 ± 0.65[b]	4.12 ± 0.17[b]	9.33 ± 1.63[a]
Liver[5, 6, 8, 9]	3.75 ± 0.53[b]	3.28 ± 0.31[bc]	2.21 ± 0.04[d]	2.31 ± 0.25[cd]	6.12 ± 0.27[a]

1. Values are means ± SE. Values are given in arbitrary units (pixels) x 10^3.

2. Values in a row with different superscripts are significantly different at $p<0.004$, $p<0.002$, and $p<0.0001$ for colonic mucosa, colonic tumors, and liver respectively; * represents significant differences between colonic mucosa and tumor whole homogenate ($p<0.0001$) for the LFC, HFC-, HFC+, and HFB+ dietary fat treatment groups.

3. Animals were terminated 12 weeks after dietary intervention with the LFC, HFC, or HFB diets and Genistein treatment (250mg Genistein/kg diet) for a subset of the HFC and HFB animals (n=5-6 rats/group for colonic mucosa, 5-6 rats/group for colonic tumors and 6-7 rats/group for liver, respectively). The plus and minus signs designate the presence and absence of Genistein treatment, respectively.

4. Caspase-3 protein expression is for colonic mucosa, tumor, and liver whole homogenate. Caspase-3 was detected as a 33 kDa band. The amount of protein loaded for colonic mucosa and tumors was 60µg. The amount of protein loaded for liver was 10µg. See Materials and Methods for additional information.

5. Significant main effect of Genistein x dietary fat interaction for colonic tumors ($p<0.03$) and liver ($p<0.0001$).

6. Significant main effect of saturated versus unsaturated dietary fat for liver ($p<0.04$).

7. Significant main effect of dietary fat treatment for colonic mucosa ($p<0.02$).

8. Significant main effect of low versus high dietary fat treatment for colonic mucosa ($p<0.007$), tumors ($p<0.0005$) and liver ($p< 0.0001$).

9. Significant main effect of Genistein for liver ($p<0.0001$).