Effects of a Mediterranean Diet on the Prostaglandin E_2 Pathway in Individuals at High Risk for Colon Cancer

by

Elkhansa Sidahmed

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Doctoral Committee:

Professor Zora Djuric, Co-Chair
Associate Professor Peter Mancuso, Co-Chair
Associate Professor Ana Baylin
Assistant Professor Grace Yi Chen
Assistant Professor Rafael Meza
Associate Professor Ananda Sen

DEDICATION

I dedicate this work to my parents, Nour and Hassan, for all of their love and support

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TABLE OF CONTENTS

DEDICATION ii
ACKNOWLEDGEMENTS.....iii
LIST OF TABLES.....viii
LIST OF FIGURES.....x
ABSTRACT.....xi
CHAPTER 1. Introduction.....1
1.1Colorectal Cancer: Overview.....1
1.2 Colorectal Cancer Risk Factors.....2
1.3 Prostaglandin E2 Enzymatic Pathway Biomarkers for Colorectal Cancer Prevention.....3
1.4 Strategies for the Prevention of Colorectal Cancer.....5
1.4.1 Non-steroidal Anti-inflammatory Drugs (NSAIDs) for the Prevention of Colorectal Cancer 5
1.4.2 Mediterranean Diet and Colorectal Cancer Prevention.....6
1.5 Effects of the Mediterranean Diet on the Prostaglandin E2 Enzymatic Pathway.....7
1.6 Study Description 8
1.7 Research Objectives 10
CHAPTER 2. Evaluations of Changes in Diet, Serum and Colon Tissue Biomarkers during Six Months of Dietary Intervention.....16
2.1 Abstract.....17
2.2 Introduction.....18
2.3 Methods.....21
2.4 Results.....31

2.5 Discussion 37
CHAPTER 3. Relationships of Prostaglandin E_2 (PGE_2) with Fatty Acid Concentrations and Gene Expression in Colon of Individuals at Increased Risk of Colon Cancer.....51
3.1 Abstract.....51
3.2 Introduction.....52
3.3 Methods.....54
3.4 Results.....58
3.5 Discussion.....62
CHAPTER 4. Effects of a Mediterranean Diet Intervention on the Prostaglandin E_2 (PGE_2) Pathway in Colon Tissue of Individuals at High Risk for Colon Cancer.....77
4.1 Abstract.....77
4.2 Introduction.....78
4.3 Methods.....79
4.4 Results.....86
4.5 Discussion.....89
CHAPTER5. Conclusions.....103
5.1 Significance.....103
5.2 Objectives.....104
5.3 Limitations and Strengths of the study.....107
5.4 Summary and Implications.....107
5.5 Bibliography.....109

LIST OF TABLES

Table 1.1: Epidemiology of colorectal cancer worldwide.....12

Table 2.1: Dietary Goals Tracked on Self-monitoring Forms …….....43

Table 2.2: Adherence with Dietary Counseling for Subjects who Completed 6 Months of Study 44

Table 2.3: Predictors of Dietary Goal …….....45

Table 2.4: Dietary Intakes over Time in the Two Study Arms.....46

Table 2.5: Serum Concentrations of Nutrient Biomarkers by Diet Arm... 48

Table 2.6: Colon Tissue Concentrations of Nutrients by Diet Arm.....49

Table 3.1: Characteristics of 114 Study Participants.....68

Table 3.2: Partial Correlation Coefficients between Demographic Factors with Colon PGE_2 Concentrations and with mRNA Expression Levels of Enzymes and Receptors in the PGE_2 Pathway 70

Table 3.3: PGE_2 Concentrations and Relative Expression of Enzymes and Receptors in PGE_2 Pathway in High Risk Individuals 71

Table 3.4:PGE_2 Concentrations and Normalized mRNA Expression of Enzymes and Receptors in the PGE_2 Pathway by Medication Use …….....72

Table 3.5: Spearman Correlation Coefficients of PGE_2 Concentrations with Normalized mRNA Expression Levels of Enzymes and Receptors in the PGE_2 Pathway 73

Table 3.6: Spearman Correlation Coefficients of Colon Tissue Fatty Acid Concentrations with Colon PGE_2 Concentrations and with Normalized mRNA Expression of Enzymes and Receptors Levels in the PGE_2 Pathway 74

Table 3.7: Predictors of Colon PGE_2 Concentrations in Linear Regression Models.....75

Table 3.8: Beta Coefficients for Predictors of Colon PGE_2 in the Final Linear Regression Model 76

Table 4.1: Antibodies and Experimental Conditions for Immunohistochemical (IHC) Staining……....94

Table 4.2: Characteristics of the Study Participants in Each Study Arm.....95
Table 4.3: Concentrations of Prostaglandins and Relative Expression of Genes in Colonic Mucosa over Time 96
Table 4.4: Concentrations of Prostaglandins and Relative Expression of Genes in Colonic Mucosa over Time for non-users of NSAIDs.....97
Table 4.5: Concentrations of Prostaglandins and Relative Expression of Genes in Colonic Mucosa over Time by Body Weight Status.....98
Table 4.6: Spearman Correlations Coefficients of Changes in Colon Tissue Fatty Acids with Changes in Prostaglandins and Gene Expression.....99
Table 4.7: Spearman Correlation Coefficients of RNA Expression with Protein Expression in Colon. Tissue ….....100

LIST OF FIGURES

Figure 1.1: Recruitment and Retention of Subjects.....13

Figure 1. 2: Potential Mechanisms by which the Mediterranean Diet could Affect Formation of Prostaglandin E_2 (PGE_2).....14

Figure 1. 3: Checklists for Tracking Food Group Exchanges. 15

FIGURE 2.1: Changes in Select Parameters for the Healthy Eating and Mediterranean study arms.....50

Figure 3.1: Schematic Showing PGE_2 Metabolism and its Signaling Pathway.....67

Figure 3.2: Normalized mRNA Expression of Enzymes and Receptors in the PGE_2 Pathway for all Study Participants.....69

Figure 4.1: RNA and Protein Expressions of Biomarkers in the PGE_2 Pathway.....101

Figure 4.2: Immunohistochemically-stained colon tissue.....102

ABSTRACT

Prostaglandin E_2 (PGE_2) is a pro-inflammatory mediator in the colon, and high levels of PGE_2 increase the risk of colon cancer. PGE_2 production can be inhibited by the use of non-steroidal anti-inflammatory drugs, but these agents have unacceptable side effects for long-term use in a cancer prevention setting. Dietary approaches for prevention therefore are an attractive, non-toxic alternative. In particular, the Mediterranean diet may be ideal, and it appears to have preventive and anti-inflammatory effects. The central hypothesis of this thesis research was that adherence to a Mediterranean dietary intervention would favorably affect the expression of the enzymes that degrade and synthesize PGE_2. The study recruited 120 subjects at increased risk of colon cancer, and they were randomized to a Mediterranean or a Healthy Eating diet. Dietary data, blood and colon tissue biopsies were collected at baseline and after six months. At baseline, there were strong, positive, associations of colon PGE_2 concentrations with cyclooxgenase-1 expression and with saturated fatty acid concentrations in the colon biopsies. Study subjects showed excellent adherence to the dietary interventions. This was reflected in serum nutrient biomarkers but change in colon tissue nutrient biomarkers was modest. Surprisingly, the Mediterranean diet intervention showed no significant effects on colon PGE_2, but a prostaglandin formed from omega-3 fatty acids, PGE_3, was increased in the Mediterranean arm, as was cytoplasmic prostaglandin E synthase 3. This indicates that dietary intervention in healthy persons may work to increase preventive compounds such as PGE_3 without affecting PGE_2. In addition, the results indicated that cyclooxygenase (COX)-1, and not the inducible COX-2, was a major determinant of colon PGE_2. Since neither of the interventions changed COX-1 expression

nor saturated fatty acid concentrations in the colon, further research should investigate the biological factors that contribute to inter-individual variability in these two significant determinants of colonic PGE_2.

CHAPTER 1

Introduction

1.1 Colorectal Cancer: Overview

Colorectal cancer is the third most common cancer and has the fourth highest mortality rate world-wide. It accounts for 9% of all cancer-related deaths with more than one million new cases reported globally each year. Incidence rates of CRC are lowest in Africa, Middle East and the Mediterranean region. In comparison, the highest rates occur in developed countries such as Australia/New Zealand, Western Europe, and the USA (Table1.1) [1]. An individual's chance of developing CRC in his/her lifetime is 1 in 20, on average, in Western industrialized countries [2].

In the United States, CRC is the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women. The American Cancer Society estimates that approximately 132,700 new cases will be diagnosed with CRC, and 49,700 deaths will be expected by the end of 2015 [3, 4]. All these together make CRC a major public health concern globally, especially in the developed countries including USA.

Colorectal cancer (CRC) is the term given to the development of cancer within the large intestine, more specifically, the colon and rectum. Cancer develops less often in the small intestine. CRC is normally found in the inner lining of the large intestine. In its later stages, CRC can grow inside the walls of the colon or rectum and may even penetrate into the bloodstream and lymph vessels, which can metastasize to other organs of the body such as the liver and lungs.

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Colorectal cancer is thought to develop through a multi-step process over a decade or more, with increased epithelial cell proliferation leading to the development of aberrant crypts, benign polyps, adenomas, and finally, invasive cancer. Approximately 96% of colorectal cancers originate from adenocarcinomas [5]. CRC is a heterogeneous and complex disease with at least three major forms: hereditary, colitis-associated CRC, and sporadic forms. The hereditary form of colorectal cancer is caused by inherited mutations in genes and accounts for approximately 2–7% of all CRC cases. Colitis is a chronic inflammatory state of the inner lining of the colon, which affects individuals at considerably younger ages than CRC and results in approximately 15% of all cases. Sporadic form of CRC represents about 60-70 of all cases, and many of these may be associated with environmental and dietary risk factors [6-8].

1.2 Colorectal Cancer Risk Factors

There are many risk factors that are known to contribute to CRC risk. The most prominent risk factors include: 1) being 50 years or older, 2) having a personal or family history of CRC and 3) carrying a genetic predisposition such as familial adenomatous polyposis (FAP) or hereditary nonpolyposis colorectal cancer (HNPCC).

Other important contributing risk factors are behavioral such as lack of regular exercise, obesity, alcohol consumption and smoking[9]. Several dietary factors also are linked to colorectal cancer risk. For example, Western diets, which are rich in saturated fats, refined sugars and low in fruits, vegetables and fiber, are associated with increased risk of colorectal cancer [10]. In fact, it is estimated that diet could be responsible for 30-50% of worldwide incidence of

CRC cases [11]. Because of this, it is important to identify dietary interventions that can prevent or reduce the chance of developing colorectal cancer.

1.3 Prostaglandin E_2 Enzymatic Pathway Biomarkers for Colorectal Cancer Prevention

Prostaglandin E_2 (PGE_2), a pro-inflammatory mediator, is a well-established biomarker for determining the risk for both colon and rectal cancer development. It plays a significant role in colonic crypt cellular expansion, as well as the consequent formation of adenoma [12, 13]. PGE_2 is formed from arachidonic acid (AA), an essential fatty acid, via action of the cyclooxygenases, namely constitutive COX-1 and inducible COX-2 in the colonic mucosa.

Increased levels of PGE_2 in the colon tissues are strongly linked to colon cancer progression [14]. In normal tissue, COX-2 expression is highly regulated and the level is very low, which poses challenges in its measurement [14, 15]. On the other hand, cyclooxygenase-1 (COX-1), which is responsible for the basal production of PGE_2, may have an active role in early stage of colon cancer development among people with risk factors [15]. For example, experimental animal studies demonstrated that COX-1 is involved in intestinal polyp formation [16, 17]. Additionally, inhibition of COX-1 by the selective blocker mofezolac was found to suppress colonic aberrant crypt foci formation, suggesting COX-1 may play an important role in early stages of colon cancer [17, 18]. Clinical studies have found it possible to reduce the number of intestinal polyps in familial adenomatous polyposis patients by inhibiting COX-1 and COX-2 with use of NSAIDS [19].This inhibition appears to be more effective than using selective inhibitor drugs such as Celecoxib, to suppress COX-2 only [20, 21]. Recent findings from a clinical trial showed that protein levels of COX-1 in colon of subjects at high risk of colon cancer were two times higher compared with COX-1 in colon of a normal risk group [22]. All

these findings strongly suggest that the possible effects of COX-1 in PGE_2 formation and involvement in colon cancer progression, especially for individuals at high risk or with early lesions.

In addition to cyclooxygenases there are other enzymes involved in the PGE_2 metabolic pathway that may play a role in colon cancer development and progression. Such enzymes also may be promising targets for colon cancer prevention [23]. [23]. For example, the prostaglandin E synthases (PTGES) work together with the cyclooxygenases to form PGE_2 from arachidonic acid [24].There is three forms of PTGES. Cytosolic PTGES (cPTGES) is constitutively expressed and complexes with COX-1. Microsomal PTGES-1 (mPTGES) is induced by pro-inflammatory stimuli, is constitutively expressed, and co-localizes with both COX-2 and COX-1 [25]. Additionally, in Min mouse models, deletion of mPTGES-1 inhibited intestinal carcinogenesis in one study but not in another [26, 27].

Another important enzyme involved in the PGE_2 pathway is 15- Prostaglandin dehydrogenase (15-PGDH). 15-PGDH is a rate-limiting enzyme responsible for prostaglandin E 2 degradation [28]. Deletion of 15-PGDH was found to be associated with increased expression of COX-2, increased PGE_2 and tumor development [28, 29]. Other findings also suggest that this enzyme may play a critical role in cellular transformation by degrading PGE_2 [28, 30]. It is therefore important to consider all the major enzymes in the PGE_2 pathway along with cyclooxygenases.

In addition to the important role of PGE_2 synthetic and degradation pathways in colon cancer, PGE_2 exerts its action through four receptors EP 1-4. These EP receptors are also recognized as a potential target for colon cancer prevention and treatment [31]. For example, EP4 has been proposed as a safer alternative target in the chemoprevention of CRC instead of

COX-2 inhibition [32]. In the normal colon, expression of EP2 and EP4 is much higher than that of EP1 and EP3[33]. In addition, EP2 has been implicated in colon carcinogenesis in experimental models and EP4 appears to have a role in intestinal permeability [34, 35]. We therefore focused this study on EP2 and EP4. Targeting both the PGE_2 enzymatic as well as downstream pathway will allow us to better understand the association between PGE_2 formation and its metabolic pathway in the normal colon. It will also allow us to evaluate if these are suitable targets for dietary prevention.

1.4 Strategies for the Prevention of Colorectal Cancer

1.4.1 Non-steroidal Anti-inflammatory Drugs (NSAIDs) for the Prevention of Colorectal Cancer

Several pharmacological prevention strategies for colorectal cancer target the prostaglandin signaling pathway, specifically to block the formation of PGE_2. One established pharmacological approach is the use of non-steroidal anti-inflammatory drugs (NSAIDs), which have been demonstrated to decrease the risk of developing colon cancer by 40-50% [36, 37]. Despite the protective effect of NSAIDs in CRC prevention, their prolonged use is unfortunately associated with development of gastrointestinal bleeding and ulcers [37-39]. This is likely due to the inhibition of COX-1, which is normally responsible for maintaining the integrity of the gastric mucosa [40]. Removal of these anti-inflammatory inhibitors after prolonged use, however, caused polyps to reoccur [41, 42]. In order to overcome the gastrointestinal adverse effects of NSAIDs, selective COX-2 inhibitors were developed. Unfortunately, data from clinical trials demonstrated an increased cardiovascular risk associated with these drugs, limiting their therapeutic value [43]. Therefore, there is a need for the development of more suitable

prevention strategies that have fewer or no side effects. This also makes a dietary approach a safer option for targeting the PGE_2 metabolic pathway.

1.4.2 Mediterranean Diet and Colorectal Cancer Prevention

Dietary patterns around the world are associated with cultural traditions and regional availability of food resources. Industrialized nations tend to follow some variation of a the so-called Western diet, which is characterized by high intakes of fat, red meat, refined grains, and sugar, and a low intake of fruits, vegetables and whole grains. The Western dietary practices have been shown to increase the risk of colorectal cancer (CRC) [44]. In contrast, the traditional Mediterranean diet includes lower intakes of red meat, refined grains, and sugar, and higher intakes of olive oil, fish, fruits, and vegetables.

Interestingly, despite the fact that both diets are high in fat, the Mediterranean diet has been demonstrated to have a preventive effect on the risk of colorectal cancer [45]. This may be due to the fact that the type of fat, namely olive oil, consumed in a Mediterranean diet is different from that in a Western diet. Olive oil is rich in mono unsaturated fatty acids (MUFA) and relatively low in both saturated fatty acids (SFA) and omega-6 polyunsaturated fatty acids (PUFA). The beneficial effects of MUFA on colorectal cancer have, however, been difficult to assess. Prospective studies have not yielded evidence of prevention with dietary MUFA, but case-control studies and studies of tissue fatty acids have shown preventive effects of higher MUFA [46-49]. These discrepancies could be due to the fact that there are many different dietary sources of MUFA, including red meat. Fish, another component of the Mediterranean diet, is a good source for omega-3 fatty acids.

A recent meta-analysis of fish consumption and colorectal cancer risk in humans indicated that fish consumption had protective effects [50]. Other aspects of the Mediterranean dietary pattern may be important as well, as are higher intakes of fruits, vegetable and grains. Case-control studies of the Mediterranean diet showed that relatively high intakes of fruit, vegetables, and whole grains are preventive and decrease colorectal cancer risk [51, 52]. Moreover, in the European Prospective Investigation into Cancer and Nutrition (EPIC) study, recurrence of adenomatous polyps was decreased in subjects who followed a Mediterranean dietary pattern [53].

A randomized trial designed to determine the effectiveness of early detection of cancer found that adherence to a Mediterranean dietary pattern was associated with reduced risk of colorectal adenoma in men [54]. Furthermore, increased blood carotenoids levels, a marker of fruit and vegetable consumption, provided protection against adenomatous polyps [55-57]. These findings highlight the multi-pronged role that the Mediterranean diet can have on colon cancer prevention.

1.5 Effects of the Mediterranean Diet on the Prostaglandin E_2 Enzymatic Pathway

A unique aspect of the Mediterranean diet is that it is rich in omega-3 (fish oil) and omega-9 (olive oil) fatty acids, while low in omega-6 (corn oil) fatty acids. The omega-6 fatty acid, arachidonic acid, is a substrate for production of eicosanoids including PGE_2. Following a Mediterranean dietary pattern should help to lower the omega-6: omega-3ratios. For example, when dietary omega-6 fatty acid intakes were low, colon tissue levels of 20:5 (omega-3) or 20:3 (omega-9) were much higher after omega-3 or omega-9 fatty acid supplementation, as opposed to when omega-6 fatty acid intakes are high [58, 59]. In addition to providing dietary omega-9

fatty acids, olive oil, unlike corn oil, contains many phenolic compounds with antioxidant and anti-inflammatory properties that together with oleic acid (18:1, n-9) can suppress COX-2 [60-62].

A study in mice showed that increased dietary olive oil decreased COX-2 protein levels in the colon of mice with colitis [63]. Oleocanthal from olive oil was shown to inhibit both COX-1 and COX-2 [64]. Supplementation with flaxseed, which contains high levels of omega-3fatty acids reduced colon tumor development and COX-2 expression relative to dietary supplementation with omega-6 rich corn meal [65, 66]. Finally, plentiful phytochemicals with antioxidant properties in plant foods would be expected to affect eicosanoids since oxidative stress induces COX-2 [67, 68]. This makes a Mediterranean dietary pattern approach attractive for prevention and for targeting the PGE_2 pathway (Figure 1.2).

1.6 Study Description

The Healthy Eating for Colon Cancer Prevention Study is a randomized clinical trial conducted at the University of Michigan, Ann Arbor, MI Institutional Review Board (HUM00007622). Subjects at increased risk of colon cancer enrolled in this trial between July 2007 and November 2010. Increased risk was defined as having one first degree relative or two second degree relatives with adenomatous polyps or colon cancer, or having a personal history of early stage colon cancer. Dietary, demographic and anthropometric data as well as serum and tissue samples were obtained and used for this thesis research.

A dietary eligibility criterion was designed to exclude persons already following a Mediterranean diet or a low-fat diet. Eligible subjects were randomized to either a Healthy Eating based on the U.S. Healthy People 2010 recommendations [69] or a modified

Mediterranean diet for 6 months (Figure 1). Information on recruitments, eligibility and dietary assessment as well as the interventions were discussed in details and published in [70, 71]. Briefly, dietary assessments were done using written records and 24-hour recalls, which were collected at baseline, 3 and 6 months. A study questionnaire captured demographic characteristics, such as employment, education, physical activity, medication use, colon cancer risk factors and other demographic characteristics at baseline. A health update questionnaire was used at 3 and 6 months to capture changes in medication use, and health. The dietary counseling used Bandura's social cognitive theory to address self-efficacy, self- monitoring, social support, goal setting and developing problem solving strategies. Subjects were asked to track food group exchanges consumed from each targeted food category, and this differed in each diet arm (Figure 1.3).

Blood samples were obtained at baseline and six months. Plasma was used for analysis of high sensitivity C-reactive protein (CRP) using a latex immunoturbimeteric assay. Serum fatty acids were extracted and measured using gas chromatography with mass spectral detection. Carotenoids were extracted and measured using measured by high pressure liquid chromatography with visible detection as described previously [72].

Eight colon biopsies were collected from each individual at baseline and at six months using flexible sigmoidoscopy procedure without prior preparation of the bowels. Five of these biopsies were used for analysis of fatty acids, carotenoids, prostaglandins, other eicosanoids and quantifications of gene expression at each time point. Two biopsies were fixed in formalin and slides were prepared for immunohistochemistry to quantify the protein levels of enzymes and receptors in the PGE_2 pathway. The fixed tissues were also used for Ki-67 staining to quantify the epithelial cell proliferation.

1.7 Research Objectives

Colon cancer remains a leading cause of cancer deaths worldwide, especially among developed countries including the U.S. [2, 3]. This makes it important to identify prevention strategies for this disease. A diet-based approach should offer an advantage over pharmacological approaches since it can alleviate toxicity concerns. The goals of this proposal was to develop a better understanding of the effects of diet on formation of PGE_2 in the colon of high risk individuals since colon PGE_2 is a biomarker of CRC risk.

The effects of a Mediterranean diet on the eicosanoid pathway remains largely unknown. The relationships between expression of individual enzymes in the PGE_2 pathway and PGE_2 levels is also not well described, especially in humans. The objective of this dissertation research was to evaluate these pathways in persons at increased colon cancer risk before and after dietary change. We hypothesized that adherence to a Mediterranean dietary intervention will be associated with reduction in PGE_2 production by modulating the expression of the enzymes that degrade and synthesize PGE_2 in the normal colon tissue. The specific aims were:

1) Aim 1, presented in Chapter 2, was to assess the effects of a Mediterranean intervention on dietary changes over six months. These assessments included a) analysis of food and nutrient intakes from food records and unannounced 24 hour recalls at baseline, three, six months; b) ability to comply with food group goals after six months of intervention; and c) serum and colon tissue levels of key nutrients biomarkers.

2) Aim 2, presented in Chapter 3, was to evaluate factors that affect PGE_2 concentrations and expression of key enzymes and receptors involved in its pathway before dietary intervention at study entry. This included considerations of demographic factors, such as gender, obesity, use

of non-steroidal anti-inflammatory drugs, and physical activity as well as substrate availability, namely colon tissue fatty acid concentrations.

3) Aim 3, presented in Chapter 4, was to evaluate whether the Mediterranean dietary intervention changed PGE_2 concentrations and gene expression of enzymes and receptors involved in its pathway. To achieve this aim, we examined changes in gene expression by diet arm assignment over six months in the entire study group and in subgroups defined by use of non-steroidal anti-inflammatory medications, and overweight status. We also evaluated the relationships between changes in gene expression and changes in colon tissue fatty acids after six months. This included validating RNA expression results with semi-quantitative immunohistochemical analysis. In Chapter 5, a summary of the major findings and implications of this research is given

Table 1.1: Epidemiology of colorectal cancer worldwide*:* Data from GLOBOCAN 2012 [1]

Parameter	Males	Females	All Persons
Incidence			
Number of new cases	746,298	614,304	1,360,602
Number of new cases per 100,000 population	21.0	17.6	19.3
Proportion of all newly diagnosed cancers	10.0%	9.2%	9.7%
Rank among all newly diagnosed cancers	3rd	2nd	3rd
Mortality			
Number of deaths	373,639	320,294	693,933
Number of deaths per 100,000 population	10.5	9.2	9.8
Proportion of all cancer-related deaths	8.0%	9.0%	8.5%
Rank among all cancer-related deaths	4th	3rd	4th

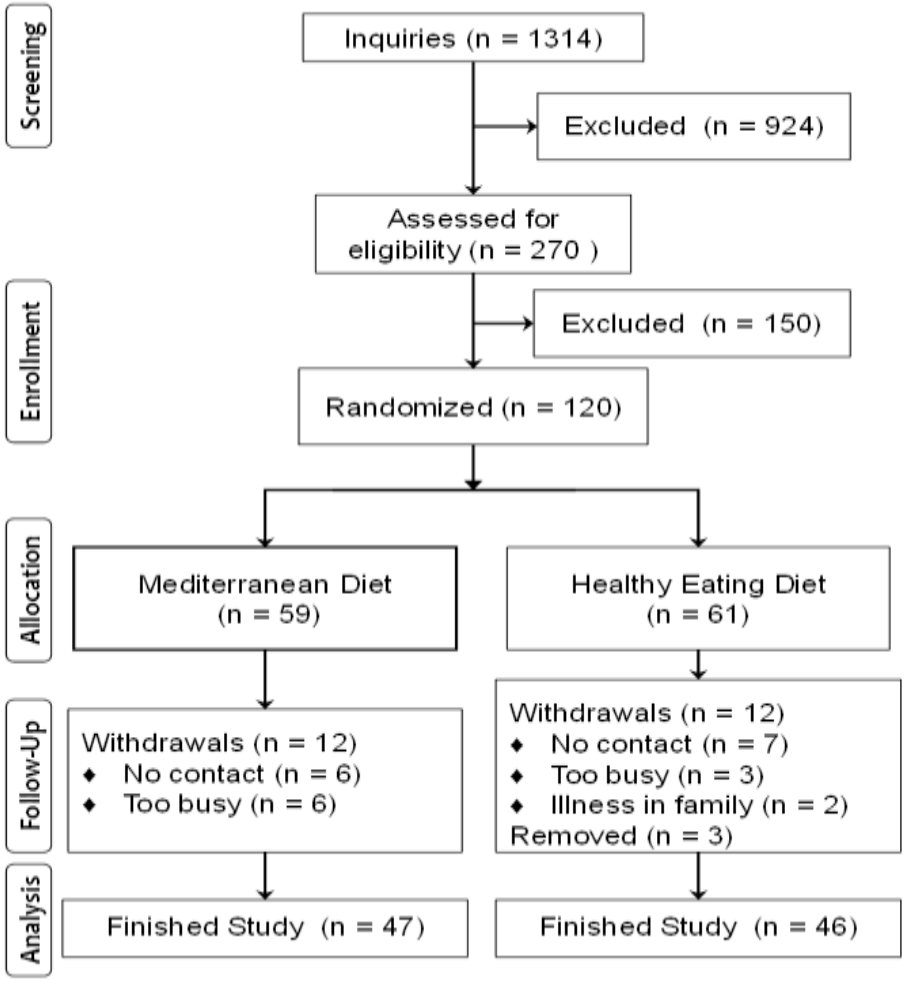

Figure 1.1: Recruitment and Retention of Subjects
The figure shows subject flow in the study according to CONSORT criteria. Three individuals were removed from study either due to initiation of supplement use (fish oils, high level thiamine) or diet change (increased sodium intake recommended by a physician).

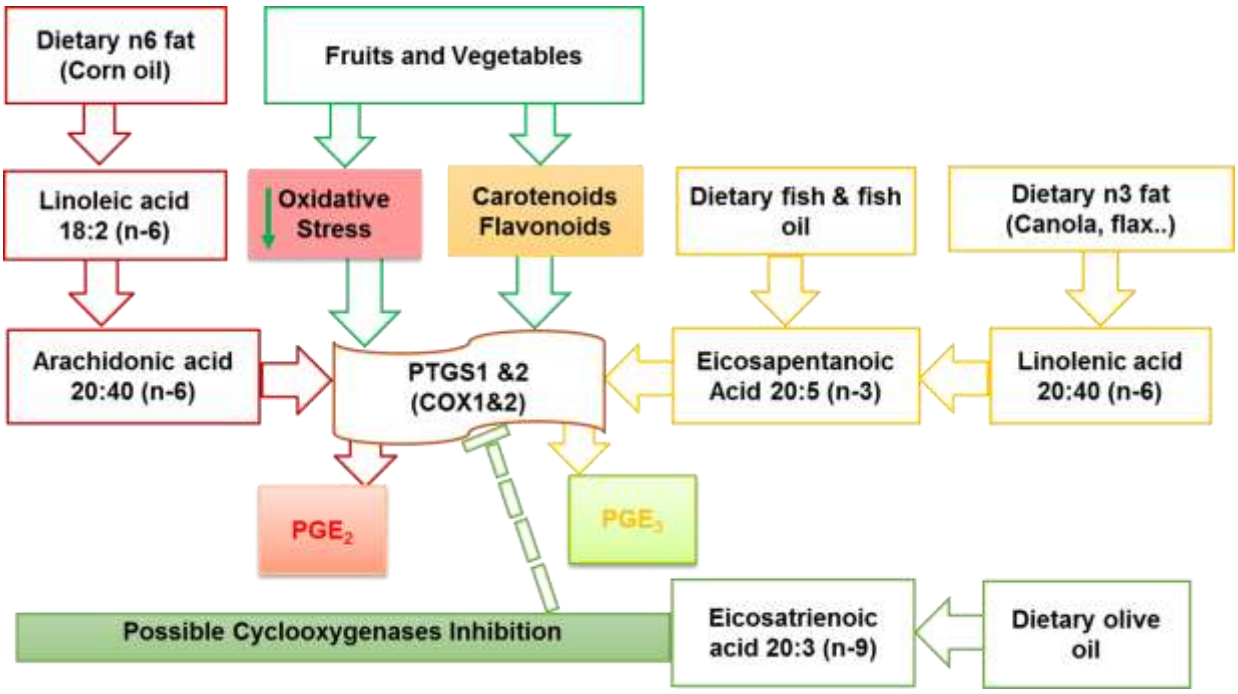

Figure 1. 2: Potential Mechanisms by which the Mediterranean Diet could Affect Formation of Prostaglandin E_2 (PGE_2)

A)

MY STUDY GOALS	
FOOD CHOICE	MY GOAL
FRUIT	
VEGETABLES Dark Green or Orange	
Other Veggies	
WHOLE GRAINS	
SATURATED FAT GRAMS	

DATE	FRUIT		VEGETABLES			WHOLE GRAINS			Grams Saturated FAT								
			Green Orange	Other													
	1	2	1	2	3	1	2	3	g	g	g	g	g	g	g	g	Total

B)

MY STUDY GOALS:

FOOD CHOICE	MY GOAL
MUFA Fat	
Omega-3 Fats	
Dark Green Veg	
Red Vegetables	
Orange/Yellow Vegetables	
Other Vegetable	
Allium	
Herbs	
Fruit	
Whole Grains	

DATE	MUFA FATS	OMEGA-3 2 per week	VEGETABLES						FRUIT	WHOLE GRAINS		
			Dark Green	Red	Orange & Yellow	Other	Allium	Herbs	Vitamin C & Other			
mo. day	7 - 10 per day		1 - 2	1 - 2	1 - 2	1 - 2	1	1	1 - 4	1	2	3

Figure 1. 3: Checklists for Tracking Food Group Exchanges
Different tracking forms were used by the two diet arms: **A)** Healthy Eating Diet, and **B)** Mediterranean diet

CHAPTER 2

Evaluations of Changes in Diet, Serum and Colon Tissue Biomarkers during Six Months of Dietary Intervention

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2.1 Abstract

Mediterranean diets appear to have preventive properties but intervention studies have not been done with cancer endpoints. Since cancer has a long timeline for development, studies with biomarker endpoints might be important to evaluate preventive potential. The objective of this study was to develop exchange list diets for Mediterranean and Healthy Eating goals, and to evaluate adherence to dietary goals, dietary intakes and serum and colon tissue biomarkers of dietary intakes in study subjects. This study recruited 120 persons at increased risk of colon cancer. Subjects were randomized to a Mediterranean versus a Healthy Eating diet for six months. Dietary adherence was similar in both diet arms with 82-88% of goals being met at 6 months, but subjects took more time to achieve the Mediterranean goals than the Healthy Eating goals. The relatively modest fruit and vegetable goals in the Healthy Eating arm were exceeded, resulting in fruit and vegetable intakes of about 8 servings per day in each arm after six months. A significant ($P<0.05$) weight loss and a decrease in serum C-reactive protein concentrations was observed in the overweight/obese subgroup of subjects in the Mediterranean arm. Increases in dietary intakes of fruit and vegetables were reflected in the blood carotenoid levels in both diet groups. The Healthy Eating arm increased serum lutein, β-, and α-carotene significantly ($P < 0.05$). In the Mediterranean arm, the significant increases were in serum lutein, β-cryptoxanthin, β-carotene. Serum monounsaturated fatty acids and omega-3 fatty acids were increased only in the Mediterranean arm. In colon tissue, there were few changes noted indicating that metabolic factors may limit changes in the colon over 6 months of intervention. In conclusion, notable dietary changes appear to have minimal effects on colon nutrient concentrations, at least over six months of intervention. Whether or not changes in circulating concentrations of these nutrients can affect colon biology remains to be determined.

In conclusion, the Healthy People 2010 goals resulted in a higher consumption of fruits and vegetables, which reflected an increase in serum and colon tissue carotenoids; these carotenoids may protect against colon cancer.

2.2 Introduction

Research into discerning the health effects of specific dietary patterns is challenged by the availability of methods to elicit defined dietary changes. A large number of studies have designed interventions using group, school or worksite based approaches or electronic media, but these have generally resulted in very modest increases in fruit and vegetable intakes [73-76]. Studies that have utilized intensive one-on-one counseling combined with self-monitoring have generally shown larger dietary changes, and this includes two cancer prevention research studies that targeted increases in fruit, vegetable and fiber intakes combined with decreases in total fat [77, 78]. None of these intervention studies used an exchange list approach for improving diet quality.

The Exchange Lists for Meal Planning booklet was first developed by the American Dietetic Association and the American Diabetes Association as a tool for diabetic meal planning [79-81]. The exchange lists have been modified for use in other countries, but there has been surprisingly little research done to evaluate their effectiveness [80, 82-85]. Only a handful of studies have modified the exchange lists to achieve low-fat diets and/or diets that target increased variety of fruits and vegetable [72, 86, 87]. The exchange list approach is potentially a method to achieve the USDA dietary recommendations [88], but this has not been tested. The exchange list approach was used in this study to design two different diets that might be useful for colon cancer prevention.

Diet appears to plays a role in modulating risk of many cancers, and colon cancer is among the cancers for which diet has the biggest impact [89]. One observational study of a large screening cohort found that persons who consumed diets with relatively more features of either the Mediterranean diet, the USDA Food Guide recommendations or the Dietary Approaches to Stop Hypertension (DASH) Eating Plan, all were preventive of colorectal adenomas in men, but only the USDA Food Guide pattern was preventive in women [54].

Although research on USDA-recommended diet has been more limited, extensive international research on Mediterranean diets has indicated its' cancer prevention potential [90-92]. Prior to the 1950's, risk of colorectal cancer was low in Greece, but the incidence has increased with the westernization of the diet and incidence is higher among Greek immigrants to the U.S. and Australia [90, 93-95]. In the U.S., rates of colon cancer are among the highest in the world [96]. All the major components of the traditional Greek diet appear to be protective for colorectal cancer, including olive oil, fish, legumes, whole grains, and fruits and vegetables [51]. In comparison to the American diet, the Mediterranean diet has higher intakes of omega-3 and omega-9 fatty acids and lower intakes of omega-6 polyunsaturated fats [97]. The Mediterranean diet also contains much higher intakes of plant-based foods and monounsaturated fats (MUFA), and lower red meat intake [97].

In most of the Mediterranean diet intervention studies that have been done, the population being studied was living in southern Europe and a high MUFA food was provided. This is exemplified in two of the larger studies that were done with disease endpoints [98, 99]. There has been relatively few intervention studies reported using a Mediterranean diet in American populations [100]. Well-designed intervention studies can isolate and identify the effects of diet

versus that of other lifestyle factors on health endpoints. Initial studies began to develop an exchange-list approach to elicit multiple dietary changes consistent with Mediterranean intakes [101]. This approach was further expanded in the present study to include goals for dark green herbs (e.g. parsley, basil) and omega-3 fats obtained from fish and flax seeds to more fully mimic Greek-Mediterranean dietary intakes. In addition, an exchange list was devised to target Healthy People 2010 recommendations for fruits, vegetables, whole grains and saturated fats [102]. With an exchange list, foods are classified into categories, and there are daily goals for consuming foods from each category. Any food within a category, in the specified serving size, can be used (or exchanged) to meet the daily intake goal for that category. Such an approach offers an individual flexibility in food choices for meeting dietary goals. Serum and colon tissue nutrients markers can be used to assess if subject adherence meet the dietary goals of an intervention.

In the Polyp Prevention Trial, the intervention diet that targeted decreased fat intakes and increased intakes of fiber, fruits and vegetables had no effect on polyp recurrence, and the increase in plasma carotenoids was modest [6]. Subjects with excellent adherence, however, did have decreased polyp recurrence, had relatively higher carotenoids levels and they also exhibited better dietary intakes at baseline [34]. Additionally, relatively higher concentrations of α-carotene and vitamin A at baseline or averaged over time, were protective that study [35]. Similar findings have been observed in the Women's Health Initiative: women with higher serum β-carotene concentrations averaged over time had lower risk of colon cancer [36]. These results indicate that long-term exposures to fruits and vegetables may be necessary for prevention and that blood concentrations are important to evaluate.

Blood concentrations can reflect carotenoid absorption and metabolism, in addition to dietary exposures. Moreover, although serum concentrations of carotenoids have been shown to be useful markers of fruit and vegetables intakes, there is relatively much less information available on colon concentrations of carotenoids. In addition, it was important to evaluate the effects of dietary change on fatty acids concentrations. Increased omega-3 or fish oil fatty acids and conversely decreased omega-6 fatty acids have been associated with decreased colon cancer in experimental models and humans [39, 40]. Increased fruits, vegetables, and omega-3 fats and decreased omega-6 fats could work together to suppress colonic inflammation via fatty acid substrate competition for cyclooxygenase enzymes and inhibition of cyclooxygenases by phytochemicals from plant-based foods [41].

The purpose of this research was to develop intervention methods that would result in adherence to a modified Mediterranean diet or a standard Healthy Eating. Adherence to the dietary goals was evaluated by food records and recalls. Additionally, serum and colon tissue fatty acids and carotenoids were measured as a more objective indicator of dietary fat, fruit and vegetable intakes.

2.3 Methods

Participants and Eligibility

The Healthy Eating for Colon Cancer Prevention Study was approved by the University of Michigan Institutional Review Board (HUM00007622). The study was listed on the ClinicalTrials.gov website maintained by the National Institutes of Health (registration number NCT00475722). A total of 120 subjects were recruited as previously described [70]. There were

61 participants in the Healthy Eating arm and 59 in the Mediterranean Diet arm, in the Ann Arbor, MI and surrounding areas from July 2007 to November 2010 [70].

The overall objective of the Healthy Eating for Colon Cancer Prevention study was to design and evaluate implementation of novel exchange list diets that could be used in a biomarker study for individuals at high risk of colon cancer. The study collected blood and colon biopsy samples for investigation of cancer biomarker endpoints such as prostaglandins, epithelial proliferation and epithelial nuclear morphology. In a prevention study, one would target individuals at increased risk and it was therefore important to test the intervention in a high risk population.

Subjects at increased risk of colon cancer were eligible for the study. Eligibility was defined as having one first-degree or two second-degree relatives with colon cancer or a personal history of adenomatous polyps or early stage colon cancer in the past if they were at least two years post cancer treatment. Other inclusion criteria included good, general health, being at least 21 years old, body mass index (BMI) at least 18.5 and less than 35 kg/m^2. It was felt that it would be inappropriate to prescribe a diet that seeks to maintain current body weight in persons with class II obesity and higher. Exclusion criteria included being on a medically prescribed diet, which would require extensive counseling to correct nutritional deficiencies or taking supplements or medications. Some factors may interfere with intervention effects, such as taking supplemental vitamins and minerals. The limit for eligibility was above 250% of the Recommended Daily Allowance since most common multi-vitamin supplements are below this level.; for this reason, those who already met high consumption of these nutrients were excluded

from the study. The study also excluded those who consumed high doses of other supplements with potential antioxidant function such as glucosamine and chondroitin.

Dietary eligibility criteria were designed to exclude persons already following a Mediterranean diet or a low-fat diet. Eligible diets were at least 23% calories from total fat with no more than 48% of fat as MUFA. Fruit and vegetable intakes to meet eligibility criteria were below two-thirds of the 2005 USDA recommended servings/day [70]. This was enumerated excluding white potatoes after the first serving and iceberg lettuce. These vegetables can be consumed in large quantities, but since they are low in carotenoids, subjects were not excluded from participation if intakes of fruits and vegetable were too high because consumption of these two foods.

Eligible participants were stratified into categories based on gender, body mass index (less than 25 versus at or above 25 kg/m^2), regular use of non-steroidal anti-inflammatory drugs (yes/no), and colon cancer risk status (prior colon cancer, prior adenoma or a positive family history of colon cancer) prior to randomization to a Healthy Eating or Mediterranean diet for 6 months. Stratification was important to assure equal representation of participants with these characteristics in the two study arms. The full details of recruitment and retention to the Healthy Eating Study have been published elsewhere [70].

Dietary Assessment

Dietary eligibility for recruitment to the study was assessed using two days of written records and one un-announced 24-hour recall. Subjects were given written and verbal instructions on how to maintain a complete food record with sufficient detail for analysis. If

details were missing, staff called the subject to verify details of foods eaten. The ability to provide a complete and plausible food record was part of the eligibility determination. Dietary recalls and food records also were collected at baseline, 3 and 6 months. Food records were completed by subjects on a Sunday and Monday, and subjects were called for an un-unannounced 24-hour recall on one further weekday. All the dietary recalls were conducted using the 5-pass method [103]. The recalls were done by trained staff but not by the study dietitian since it were felt that this would maximize objectivity in data collection. An additional 24-hour recall was obtained at the first study visit, and all four days were averaged to obtain an estimate of baseline diet.

The same assessments were repeated at six months. At three months, two days of written records and one un-announced 24-hour recall were analyzed before the visit to give each participant feedback on their progress. Mean nutrient intakes from the in-person recalls were similar to those calculated for the average of the three other days [70]. It should be noted that an average of at least three days is generally required for accurate estimation of energy intake, but even a single recall can provide estimates of energy, fat and fruit/vegetable servings that were not significantly different from that of four days of food records [104, 105].

For 5% of the 406 records completed, one to two days of data was missing due to inability to obtain a recall or due to failure to collect a written record. The food records and recalls were analyzed using the Nutrition Data System for Research (NDSR) software (version 2010, Nutrition Coordinating Center, University of Minnesota). Records entered with previous versions of the software (2007-2009) were re-analyzed with the 2010 nutrient database at study completion. Double entry of a random sample of 30 records was done for quality control and

these revealed average differences of 10% or less for intakes of energy, vitamin E, vitamin C, calcium, percent of calories from fat, total carotenoids, and whole grain servings.

Questionnaires and Anthropometric Assessments

A study questionnaire designed for this study captured demographic characteristics of subjects. A Health-Update Questionnaire was used at 3 and 6 months to capture changes in medication use, health and physical activity levels. Physical activity was assessed using a validated questionnaire and metabolic equivalents (MET) were calculated [106]. This questionnaire asked respondents about time spent walking at various speeds and performing mild, moderate and strenuous activities.

Self-efficacy for making dietary changes was assessed in all subjects at baseline and 3 months using seven behaviors targeted by both interventions, and answers were given on a Likert-type 5-point scale [107]. The seven items asked about confidence to find a way to eat a variety of fruits and vegetables, finding way to meet fat goals, finding time to buy needed foods, finding time to prepare foods, finding ways to stick to goals when others around you make it difficult, controlling the home environment, and meeting goals when eating out. Internal consistency of the scale was good with an overall Cronbach alpha of 0.85.

Anthropometric measures were obtained at baseline, 3 and 6 months by trained staff of the Michigan Clinical Research Unit using a written protocol. Body weight was measured in light clothing, without shoes and rounded to the nearest quarter pound with a Scale-Tronix model 5005 Stand on Scale (White Plains, NY). Height was measured to the nearest 0.1 cm with a stadiometer and BMI was calculated as kg/m^2. Waist and hip circumference was measured to the

nearest 0.1 cm. Blood pressure was measured using a sphygmomanometer by auscultation of the upper arm. All measures at baseline and 6 months were obtained in the morning after an overnight fast but the 3-month visits were scheduled at the subject's convenience.

Dietary Interventions

The Mediterranean and Healthy Eating interventions were delivered using individualized counseling with a registered dietitian. The schedule for counseling was weekly for the first month, biweekly for the next two months and monthly for the last three months. The counseling at baseline and 3-months was done face to face, and the remained of the scheduled counseling was done by telephone calls that were structured to last about 20 minutes. All individual diet goals were based to maintain energy intake reported at baseline.

At the baseline visit, subjects were presented with exchange booklets written by study staff that listed foods in categories together with serving sizes, and their own individual goals were written in the booklet. The booklet information was also provided in an abbreviated form on a single, laminated page. Other printed materials provided were for buying fruits and vegetables, estimating portion sizes, and reading food labels. Subjects randomized to the Mediterranean diet treatment arm received study recipes, sample menus for seven days and flax recipes from the Flax Council of Canada. Subjects in the Mediterranean arm were asked to keep food diaries until they became adept at meeting exchange goals, as determined by the dietitian from review of self-monitoring records, after which they could use a checklist format to track exchanges consumed from each targeted food category. Subjects in the Healthy Eating diet arm received only checklists from the start. These checklists were available both in printed format

and as excel files. Each group received a bimonthly newsletter written for that diet arm with news of the study progress, and information on seasonal foods and recipes.

The dietary counseling used Bandura's social cognitive theory that addresses self-efficacy, self- monitoring, social support, goal setting and developing problem solving strategies [108]. At every counseling session after baseline, a review of dietary intakes in the previous period was the main subject of discussion between the dietitians and the study participant, and this formed the basis for short-term goal setting. If a participant's intake of any vitamin or mineral was <67% of Dietary Reference Intake values, however, they were given a list of foods that are rich in that nutrient to correct the deficiency.

Study participants were requested to keep self-monitoring records for 5-7 days before each counseling call and to mail them to the dietitian. The counseling session at by which a participant achieved all of their food exchange goals was recorded by the dietitian using a review of each participant's self-monitoring logs. The number of goals met was also recorded at six months.

Dietary Goals

The goals for the Healthy Eating diet were based on the U.S. Healthy People 2010 recommendations [102].The specific dietary goals are shown in Table 2.1.The saturated fat goal was given in grams per day, based on baseline energy intake, and subjects enumerated grams of saturated fat in the foods that they consumed on the tracker. Reducing saturated fat intake resulted in a small decrease in total fat intake by study participants, and participants therefore were able to reduce total fat intake to less than 30% of calories without additional counseling for

maintaining total fat intake to below 30% of calories. A food list of high salt foods that participants should avoid was provided, but subjects were not asked to track sodium intake.

The number of goals was greater in the Mediterranean arm (Table 2.1). The 'fat' goal was to maintain 30% of calories from fat while reducing PUFA and SFA intakes by about 50% and 30%, respectively, and increasing MUFA intake by about 50%. Subjects in this group were asked to consume foods high in omega 3 fatty acids at least twice a week. The 'whole grain' goal was the same as in the Healthy Eating treatment arm. 'Fruit and vegetable' goals were for consumption of at least 7-9 FDA servings per day, depending on energy intake, and to include culinary herbs and allium vegetables, as shown in Table 2.1.

Blood Sample Assays

Blood samples were obtained at baseline and six months following after an overnight fast. Measures of high sensitivity C-reactive protein (CRP), were measured using a latex immunoturbidimetric assay (laboratory analysis and all assays were done by the Michigan Diabetes Research and Training Center Core Chemistry Laboratory).

Total serum fatty acids were extracted with Folch reagent and measured as fatty acid methyl ester by gas chromatography with mass spectral detection. Carotenoids were extracted with hexane and measured by high pressure liquid chromatography [72]. There was not enough blood for carotenoid analysis from one overweight/obese subject in the Healthy Eating arm at baseline. Colon mucosal biopsies were obtained circumferentially 15–25 cm above the anal sphincter by flexible sigmoidoscopy without any prior preparation of the bowels. Six biopsies were frozen in liquid nitrogen within 5 seconds of incision and stored at −70°C until analysis.

Colon samples were analyzed for carotenoids and fatty acids in a similar way as serum except that a colon tissue homogenate was prepared first. A total of 4 frozen biopsies were homogenized together, using pulverization under liquid nitrogen, a technique described previously [109]. A portion of the homogenate that was equivalent to one biopsy (150µl) was used for carotenoid analysis and an equal portion was used for fatty acid analysis. Samples were diluted with 50µl PBS prior to extraction. There was one tissue sample missing at 6 months from an overweight/obese complete in the Mediterranean arm that refused flexible sigmoidoscopy.

Statistical Analyses

Alcohol intake was calculated from the study questionnaire using USDA values for standard sizes of wine (15.4 g/glass), beer (13.9 g/beer) and spirits (15.9 g/drink). All analyses were done in SPSS version 18 (PASW Statistics, IBM Corporation, Chicago, IL, USA). Various aspects of dietary counseling were compared across the two dietary treatment arms using two-sample t-test or Fisher's exact test depending on whether the variable of interest was continuous or categorical (Table 2.2). Linear regression was used to evaluate predictors of the percentage of goals met at the end of the trial (Table 2.3).

To evaluate changes over time in the dietary intakes, serum analytes and tissue nutrients, regression analyses were carried out under a linear mixed models framework. The linear mixed model regression analysis with an intent-to-treat analysis was used, which provides valid results in presence of drop-outs and incorporates all available data at every given time point. Separate models were used for each of the nutrients as outcome, with a 3-level variable time (baseline, 3 months, and 6 months) as the primary within-subject factor and diet group assignment as the primary between-subject factor. The variable of interest was the group*time interaction that

indicates any difference in the pattern of change over time across groups. Regression models were controlled for covariates that can affect dietary intakes including age, gender, and BMI. To isolate the effect of diet quality, energy intake was used as a time-dependent covariate for nutrient intakes. Residuals were checked for normality of the distribution and the outcome was appropriately transformed as needed prior to the final model fit (as indicated in the footnote of Table 2.4) on which the inference is based. Clustering within subjects was incorporated by means of an unstructured variance-covariance matrix. Models also were constructed using the data stratified by baseline weight status (normal or overweight/obese). In order to evaluate and compare average changes over time in fatty acids, carotenoids, and other nutrients found in serum across two groups, linear mixed regression models were also used with time, diet group assignment and the group-by- time interaction as the primary predictors. The models were controlled for baseline age, BMI and smoking as non-time-dependent covariates. Age was slightly higher in the Mediterranean group than the Healthy group (means of 55 versus 50, respectively).

The prevalence of baseline smoking status was slightly different in the two study arms (11% in the Healthy arm versus 17% in the Mediterranean arm), although the difference was not statistically significant at 5% level ($p = 0.06$ based on Fisher's exact test). Since smoking status may potentially affect the fatty acid and carotenoid concentrations, it was used as a covariate in the regression models BMI did not differ appreciably between the two groups at baseline, but it is known to affect carotenoid concentrations [110]. Further, the samples were analyzed in several analytical batches in the laboratory, which was a potential source of variation, and so batch was used as an additional covariate. SFA was square root transformed. Log transformation was used for all other variables except for MUFA, which required no transformation.

Similar models were used for concentrations of fatty acids and carotenoids obtained from colon tissue samples. Apart from baseline age, BMI, and smoking status, the regression models were controlled for variation across laboratory analysis batches. All outcomes required a natural logarithmic transformation with the exception of SFA, which required a square transformation for analysis, and MUFA, which required no transformation.

2.4 Results

Study subjects

Recruitment and retention of subjects to this study was described previously [70]. Briefly, 59 subjects were randomized to the Mediterranean arm of the study and 60 subjects to the Healthy Eating arm. Most of the subjects were Caucasian (88%), mean age was 53 years and most were female (72%). Only one subject had a personal history of colon cancer and the rest of the subjects either had a strong family history of colon cancer (64%) or a previous adenoma (27%) or both (9%) [70]. None of these characteristics differed significantly between the two diet arms [70]. There were 93 participants of the original 120 who completed the whole 6 months of study participation (46 in the Healthy Eating arm and 47 in the Mediterranean arm).

Counseling adherence

Measures of adherence and dietary goal achievement are shown in Table 2.2. Subjects in both arms received a similar number of contacts over six months of study, as shown in Table 2.2. Adherence with the recordkeeping requirements were similar in both arms, with an average of 80% of the requested records being returned. The period that elapsed before the counseling session at which subjects were able to achieve all of their dietary goals, however, differed

significantly by study arm (Table 2.2). The time required to meet goals was, on average, 48 days in the Healthy arm and 82 days in the Mediterranean arm.

At the 6-month time point, the food records and 24-hour recalls were used to assess the number of dietary goals met for each participant. The percent of goals met at 6 months in each arm was good, at about 80% in each arm, but the number of subjects meeting all goals at 6 months was considered by the team to be low, especially in the Mediterranean arm. This could be due in part to the fact that the omega-3 food group goal was for a weekly, not a daily, intake, making it difficult to discern goal- meeting from four days of diet data. There also was some deterioration of dietary intakes of target nutrients in the Mediterranean arm from 3 to 6 months. Since dietary goals were just being met by 2½ months, on average, better adherence to this diet might require a greater frequency of counseling contacts or more time for subjects to become adept at it.

Subjects of normal weight did not differ from subjects who were overweight or obese with regard to session number at which goals were reached, number of calls or minutes of counseling time (not shown). The percent of dietary goals met at 6 months was, however, greater for the 33 normal weight subjects (90% goal met, SD 13) versus the 60 overweight or obese subjects (82% goals met, SD 24%, p=0.036 by a two-sample t-test). Recordkeeping was also slightly greater in the normal weight versus overweight or obese subjects (86% versus 78%, respectively, p= 0.082). There were no significant differences by gender in counseling adherence, although the session number at which goals were reached in the Mediterranean arm was borderline different for men (5.9 sessions) versus women (7.3 sessions, p = 0.053 determined by the two-sample t-test).

A brief scale was used to measure self-efficacy for making dietary changes (see Methods). This scale was devised to measure the seven behaviors targeted by both interventions, and this revealed no significant differences in mean scores by diet arm (Table 2.2). In addition, there were no significant differences from baseline to 3 months in either diet arm, as determined from paired t-tests (not shown). Although self-efficacy for making dietary changes did not change appreciably over time, it was a significant predictor of reaching dietary goals in a linear regression model. Self-efficacy at baseline and record-keeping percentage over 6 months were significant predictors of goal attainment at six months (Table 2.3). Diet arm assignment, number of counseling calls, length of time spent on telephone counseling, gender, education, current smoking status, age, marital status, baseline intake of fruits and vegetables, baseline BMI, and baseline obesity were not significant predictors of meeting dietary goals at 6 months.

Food and nutrient intakes over time

Changes in nutrient intakes were evaluated using mixed linear regression models consistent with intention to treat principles (Table 2.4). Variables that exhibited significant fixed effects of diet group assignment and group*time interaction are annotated in Table 2.4. Significant fixed effects of BMI status (normal weight or overweight/obese) were evident for saturated fat, trans fats, carotenoids, fiber and calcium. Significant fixed effects of gender were evident for energy, saturated fat, omega-6 PUFA and fiber. There was a significant group*time interaction for several dietary variables. Energy was significantly decreased from baseline in only the Healthy Eating group and carbohydrate intakes were significantly increased only in the Mediterranean group. MUFA intake decreased in the Healthy Eating group and increased in the Mediterranean group. Trans fats, total fruit and vegetable servings, glycemic load and sodium all

changed in the same direction in both arms, but the pattern of change in the Mediterranean group was different over time, which resulted in a significant interaction effect (Table 2.4).

Subjects in the Healthy Eating arm reported a reduction in total energy, percent of energy from fat and saturated fat intake over 6 months of intervention that was maintained quite well from 3 to 6 months. In the Mediterranean arm, there was some deterioration of diet in the last three months of study. It is interesting to note that MUFA intakes decreased in the Healthy Eating arm and increased in the Mediterranean arm. Intakes of omega-6 and omega-3 fatty acids also differed by diet arm, with significant interaction effects being present in each case. Although trans fats were not targeted by the intervention, there was a significant decrease in the Mediterranean arm only.

Both diet groups reported increased intakes of whole grains and fiber, and a decrease in red meat intake. Glycemic load decreased significantly in both diet arms. Carbohydrate intake increased in the Healthy Eating arm significantly over time in the mixed regression model, although this was not reflected in the simple means of all available data shown in Table 2.4. The Mediterranean arm was unique in the significant decrease in sodium and the increase in calcium, even though these were not specifically targeted by the intervention. Sodium intake was not decreased in the Healthy Eating arm.

The goal for consuming five servings of fruits and vegetables per day in the Healthy Eating arm was surpassed resulting in statistically similar fruit and vegetable intakes in the two study arms (7.6 vs. 8.2 servings/day in the Healthy and Mediterranean arms, respectively, at six months). The significant group* time interaction indicated that total fruit and vegetable intake changed over time differently in the two study arms, perhaps due to the decrease in the

Mediterranean arm from 3 to 6 months. The somewhat higher total fruits and vegetable intakes in the Mediterranean arm were mainly due to vegetable intakes (not shown).

Variety of fruit and vegetable intakes was scored and assessed by adding one point for each different type of fruit or vegetable that was consumed in a quantity that was at least half of a serving/day. The variables included in the variety count were six kinds of fruit intake (citrus, citrus juice, other fruits, other fruit juice, avocado, and fried fruit) and eight different kinds of vegetable intake (deep green, deep yellow, tomato, white potato, other starchy vegetables, other vegetables, fried vegetables not including potatoes, and vegetable juice). Variety of fruit and vegetables intakes appeared to be similar between diet arms as well, but enumeration of allium vegetables and herb intakes was not available in the NDSR program. Increases in dark green and yellow vegetables were similar between the two arms and significant in each case, but citrus intake increased significantly only in the Mediterranean arm (data not shown). Tomato intakes did not differ significantly over time, although there was a trend for a decrease in the Healthy Eating arm and an increase in the Mediterranean arm (not shown).

Changes in anthropometric variables and blood markers of health risks

There was little change in anthropometric variables. There was a small mean weight loss in both arms, 0.92 and 1.58 kg in the Healthy Eating and Mediterranean arms, respectively, but this was not statistically significant. In stratified analyses, there was a significant weight loss in overweight/obese subjects randomized to the Mediterranean arm, $p<0.05$ (Figure 1.1). Mean hip circumference decreased in the Mediterranean arm from 41.1 to 40.0 inches. Diastolic blood pressure decreased significantly in the Healthy Eating arm (from 76 to 72 mm mercury).

There were no significant effects of either intervention on blood lipids, growth hormone or measures related to insulin status. In stratified analyses, HDL decreased and LDL did not change significantly Figure 1.1. C-reactive protein, however, decreased at 6 months in overweight/obese subjects randomized to the Mediterranean arm (Figure 1.1).

Changes in serum markers of dietary intakes

Serum fatty acids and carotenoids were measured since they can be useful biomarkers of changes in dietary intakes of fat, fruits and vegetables. With regard to the major classes of fatty acids, there were no significant changes in serum concentrations of saturated fatty acids, but concentrations of MUFA, omega-3 PUFA and the ratio of omega-3:omega-6 PUFA changed significantly in the expected directions in the Mediterranean arm (Table 2.5).

For total serum carotenoids, changes were similar in both diet arms, but the increase in total carotenoids was significant only in the Healthy arm. Specific fruit and vegetable goals in the Healthy arm were for 5 servings per day and including at least one carotenoid-rich dark orange or green vegetable. Increases in lutein were significant in both arms of the study, β and α-carotene increased significantly only in the Healthy arm, and β-cryptoxanthin increased significantly only in the Mediterranean arm. The fruit and vegetable goals in the Mediterranean arm were to consume at least one serving from each of seven categories of fruits and vegetables (18). There were no statistically significant changes in lycopene or zeaxanthin concentrations in either arm. Changes that approached statistical significance with $p < 0.10$ were in β-cryptoxanthin in the Healthy arm and in β and α-carotene in the Mediterranean arm.

Changes in fatty acids and carotenoids in the colon

Changes in carotenoids and fatty acids in the colon biopsy tissue were in the same direction as in blood, but the changes were smaller and fewer differences were statistically significant (Table 2.6). Interestingly, concentrations of omega-3 fatty acids increased in the Healthy arm, but the change was small. Significant increases in several carotenoids were also found in the Healthy arm only (Table 2.3). Changes in the Mediterranean arm that approached significance with $p < 0.10$ were for omega-3PUFA, omega-3/omega-6 ratio, β-cryptoxanthin and α-carotene.

2.5 Discussion

Dietary interventions that target the entire eating pattern as a whole have good potential for prevention of many cancers and can deliver a combination of preventive compounds. This may be important since interventions with single food components have not had consistently beneficial results [111-114]. In the present study, exchange lists were derived to target either Healthy Eating or Mediterranean patterns. Goal attainment was reasonably good for participants on both diets and large dietary changes were observed in both study arms. However, it did take individuals more time to meet the dietary goals in the Mediterranean versus the Healthy arm, perhaps because the Mediterranean diet had more goals and therefore required larger changes from baseline (Table 2.2). Predictors of adherence to dietary goals were record-keeping and baseline self-efficacy for making dietary changes (Table 2.3). It therefore may be important to increase counseling efforts directed at self-efficacy and record-keeping to improve adherence. Adherence is always a concern in clinical trials. In the Polyp Prevention Trial, for example, there was no significant effect of a low-fat, high fiber intervention overall, but the subset of subjects with excellent adherence did have a lower polyp recurrence rate [115].

The study presented here yielded several unexpected results. The Mediterranean intervention resulted in an increase in calcium, which is encouraging since a recent supplementation trial has indicated a cancer preventive potential for calcium [116]. In the Mediterranean diet, primary calcium sources were derived from low-fat dairy, such as cheese, yogurt, and milk. The Mediterranean diet also showed decreases in both trans fats and sodium (Table 2. 4). These decreases could result from lower use of ready-made, food products, many of which have a dietary fat content that is not consistent with the Mediterranean goals. One of the other interesting results was that the higher goals for fruit and vegetable intakes in a greater variety in the Mediterranean diet arm did not result in significantly higher intakes than the more modest goals fruit and vegetable goals in the Healthy arm (Table 2.4). This indicates that the exchange list goals derived in this study for fruit and vegetable consumption that are consistent with Healthy People 2010 goals might be sufficient to increase both quantity and variety of intakes.

Given the similarity between the two diets arms in fruit and vegetable intakes, the major difference between the two interventions was found in dietary fat intakes. The Mediterranean intervention uniquely increased mean dietary intakes of both MUFA and omega-3 fats, with decreases in omega-6 fats. This is potentially important since prostaglandin E_2 (PGE_2) is formed from arachidonic acid (omega-6 fatty acid) via cyclooxygenase -2 (COX-2), which is induced by high omega-6 fatty acid diets [117]. PGE_2 is strongly and positively associated with colon cancer risk [118]. In contrast, omega-3 and omega-9 fatty acids, the main types of fats found in Mediterranean diet, have protective effects and have been associated with decreased PGE_2 levels and COX-2 expression [119-121]. The Mediterranean intervention also achieved relatively large

increases in dietary MUFA, possibly due to consumption of olive oil, a main component of the Mediterranean diet [122, 123].

The other important difference between the two interventions was a significant weight loss in overweight or obese subjects randomized to the Mediterranean diet. This was achieved despite the facts that the dietary counseling was designed to maintain baseline weight and mean reported energy intakes did not change significantly in the Mediterranean study arm (Table 2.4). The reasons for the observed weight loss with the Mediterranean diet are not clear. One of the factors might be related to increased post prandial oxidation of MUFA versus SFA, which would favor weight loss in the Mediterranean versus the Healthy diet [124, 125]. Other Mediterranean interventions were typically done with individuals who had cardiovascular or diabetes risks, and counseling for energy restriction was provided for individuals who were not of normal weight, such as the study of Esposito et al. [122, 126]. In the Medi-Ravage study, energy restriction was not used and there was a slight, non-significant weight loss [127]. The weight loss in the overweight and obese group was associated with a significant decrease in C-reactive protein in the Mediterranean arm, although we cannot determine if this was due to weight loss or to the change in dietary composition (Figure 2.1).

We also used biomarkers of dietary intakes to evaluate the effects of the two interventions. Counseling for the Healthy Eating diet, namely 2 servings per day of fruit and 3 servings per day of vegetables, with at least one being dark green or orange, was sufficient to increase concentrations of several serum and colon carotenoids (Table 2.5). The Mediterranean group with goals for consuming at least 7 servings per day of fruits and vegetables in 7 different categories did not display relatively larger increases in serum carotenoids. One could speculate

that subjects in the Healthy arm met their goals with a variety of foods to increase palatability, resulting in a broad spectrum of carotenoid intakes in the Healthy Eating arm. The only serum carotenoid that was increased significantly in the Mediterranean arm, but not the Healthy arm, was β-cryptoxanthin, which is found mainly in fruits. Whether or not other phytochemicals are increased by a Mediterranean diet needs to be investigated. This would include flavonoids such as quercetin, which is high in onions and apples, and phenolic compounds from [128, 129].

The Mediterranean diet also was distinctive in increasing serum concentrations of MUFA and omega-3 fatty acids, but this may reflect recent diet since phospholipids were not isolated. Changes in micronutrients were smaller in the colon than in the serum, and the only significant changes were noted in the Healthy Eating arm (Table 2.6). This could be due to: 1) the short time frame of the intervention since tissue stores may require more time to reach equilibrium than blood, 2) errors inherent in dietary assessment, especially when only 4 days are used at each time point, and 3) to the role of metabolic factors. Most dietary nutrients are absorbed in the small intestine, and colonic exposures to nutrients, therefore are likely to occur at the basolateral, not luminal, side via the systemic circulation. This is especially true for the distal colon that was sampled in this study.

It was disappointing that colon carotenoids were not changed to a larger extent. In experimental models, many individual carotenoids have been shown to be protective of colon cancer including lutein and lycopene [130, 131]. A pooled analysis of 11 cohort studies indicated that of the dietary carotenoids, only lutein and zeaxanthin, which were measured together in foods, displayed weak protective effects for colorectal or colon cancer (2). Colon lutein or lycopene concentrations, however, were not significantly increased after six months of either

intervention (Table 2.6). Lycopene in both serum and adipose tissue has been previously found to be poorly related to dietary intakes, and supplementation may be a more feasible method to increase concentrations of lycopene [132, 133].

Changes in dietary fats and serum fatty acids were also not reflected in the colon. This is potentially important since fatty acid availability is a key determinant of the types of prostaglandins and other eicosanoids that are produced in cells [134]. There were few significant changes in colon fatty acids other than a slight increase in omega-3 PUFA in the Healthy arm. This indicates the possible importance of metabolic processes in regulating tissue fatty acids concentrations, and these may be genetically determined.

A limitation of this study is that subjects recruited for the study were largely well-educated and Caucasian, limiting generalizability of the results. Another limitation of the study is that persons at increased colon cancer risk might be more motivated than the general population to adhere to dietary recommendations. On the other hand, intensive interventions such as this may be most appropriate in such populations with defined cancer risk. Strengths of the study include the randomized design and novel intervention methods with good participant adherence. Weaknesses include the fairly short time frame of intervention (6 months) and a reliance on self-reporting for dietary assessments.

In conclusion, this study implemented two different exchange-list dietary intervention strategies in persons at increased risk of colon cancer. The intervention that was based on Healthy People 2010 goals resulted in similar increases in quantity and variety of fruits and vegetables as the more elaborate Mediterranean intervention, indicating that more modest goals for fruit and vegetables could be adequate to increase consumption. Whether or not other

phytochemicals are affected to a relatively greater extent with Mediterranean diets remains to be established.

The Mediterranean diet arm did not have weight loss goals, but it resulted in a significant weight loss and a decrease in serum C-reactive protein in the subjects who were overweight or obese at baseline. Given the difficulty in achieving and maintaining weight loss, the present results indicate that the Mediterranean exchange list approach should be more fully explored in studies of weight loss and weight loss maintenance.

Lastly, the Mediterranean intervention was unique in increasing intakes of MUFA and omega-3 fatty acids, but this did not affect colon levels of fatty acids over six months of study. These results point to the possible role of metabolic factors and genetic variability in regulating colon tissue nutrient and fatty acids, which may impact PGE_2 concentrations. It also highlights the importance of understanding the effect of inter-individual variability in PGE_2 concentrations.

Table 2.1: Dietary Goals Tracked on Self-monitoring Forms

Diet Arm	Dietary Goal	Method of Enumeration
Healthy Eating[a]	Saturated Fat < 10% of calories	Saturated Fat grams per day
	Fruit	two servings per day[b]
	Vegetables	two servings per day
	Dark green or orange vegetable	one serving per day
	Whole grains	at least three servings per day
Mediterranean[c]	High MUFA foods	7-10 exchanges per day (5 g per exchange)
	High omega-3 food	twice a week, 3 ounce serving size (with limits on fish with higher mercury)
	Dark green vegetable	one to two servings per day
	Orange and yellow vegetable	one to two servings per day
	Red vegetable	one to two servings per day
	Other vegetable	one to two servings per day
	Dark green culinary herbs	one serving per day,1 TB fresh or 1 tsp. dried
	Allium vegetables	use liberally at least once a day
	Fruit	one serving per day Vitamin C Fruit and one serving per day Other Fruit
	Whole grains	at least three servings per day

[a] The exchange book for the Healthy Eating diet included a list of sodium content of various types of foods, but sodium intake was not tracked.
[b] For both diets, one serving for fruits and vegetables was defined as 1 medium, 1 cup fresh, 2 cups leafy greens, ½ cup canned or cooked, ½ cup juice or ¼ cup dried. For grains, serving sizes were 1 ounce (12 chips or 6 crackers), 1 slice bread, ½ cup cooked grain, ¾ cup dry cereal, or 3 cups popcorn.
[c] The exchange book for the Mediterranean diet included lists of foods high in omega-6 fats to either avoid, limit to twice a week or limit to twice a day and a high MUFA list. The total fruit and vegetable goal was 7-9 servings/day, depending on baseline energy intake, and variety was defined by use of five exchange groups for vegetables and two exchange groups for fruit.

Table 2.2: Adherence with Dietary Counseling for Subjects who Completed 6 Months of Study[a]

Variable	Healthy Eating (n=46)	Mediterranean (n=47)	P-value[a]
Number of counseling Calls	10.3, 0.6	10.6, 1.0	0.106
Total minutes counseling	212, 67	245, 45	0.008
Number of sessions to meet goals[c]	5.2, 1.8	6.9, 2.2	<0.001
Record-Keeping[d]	81%, 22%	80%, 22%	0.883
Self-Efficacy score at baseline	31, 4	31, 3	0.802
Self-Efficacy score at 3 months	31, 3	31, 3	0.399
Percent of goals met at 6 months	88%, 23%	82%, 18%	0.159
Participants meeting ≥ 70% of goals at six months, number and percent	41, 89%	40, 85%	0.759
Participants meeting 100% of goals at six months, number and percent	31, 67%	15, 32%[e]	0.001

[a] Data shown is mean and SD, or number and percent for subjects who completed 6 months.

Table 2.3: Predictors of Dietary Goal

Predictor of Dietary Goal Attainment	Beta[a]	P-value
Record-keeping	0.476	<0.001
Self-efficacy at baseline	0.342	<0.001

[a]The model was controlled for diet arm assignment, gender, baseline age and baseline BMI status (normal weight or not), all of which were not significant predictors of goal attainment. The two factors showed accounted for 37% of the variance in goal attainment ($p < 0.001$).
[a] Data shown is mean and SD, or number and percent for subjects who completed 6 months.

Table 2.4: Dietary Intakes over Time in the Two Study Arms

Nutrient or Food	Healthy Eating			Mediterranean		
	Baseline (n=61)	3 months (n=49)	6 months (n=47)	Baseline (n=59)	3 months (n=50)	6 months (n=47)
Energy (kcal per day)[a]	2144, 649	1828, 509[b]	1899, 454[b]	2001, 574	2087, 665	2030, 657
Total fat, (% of energy)[a,c]	35, 6	27, 6[b]	28, 7[b]	35, 6	36, 6	33, 6
Total Protein, (g per day)	84, 24	77, 24	81, 22	77, 22	84, 29	83, 24
Carbohydrate, (g per day[a,c])	261, 83	260, 69[b]	265, 71[b]	247, 81	256, 93	261, 96
Saturated fat, (g per day)	30, 13	18, 10[b]	18, 7[b]	26, 9	19, 9[b]	19, 8.5[b]
MUFA, (g per day)[a,c]	32, 12	22, 10[b]	24, 10[b]	30, 12	46, 16[b]	39, 18[b]
Omega-6 PUFA, (g per day)[c,d]	15, 6	12, 5	13, 2	16.0, 7.6	13.2, 6.7[b]	11.8, 4.7[b]
Omega-3 PUFA, (g per day)[c,d]	1.8, 0.8	1.7, 1.1	1.8, 0.8	1.9, 1.1	2.9, 2.4[b]	2.4, 1.7
Long chain omega-3 fats, (g per day)	0.13, 0.20	0.26, 0.52	0.24, 0.32	0.15, 0.26	0.41, 0.57	0.35, 0.43
Trans fats, (g per day)[a,c]	3.6, 2.0	2.3, 1.6[b]	2.1, 1.4[b]	3.6, 2.7	1.5, 1.4[b]	1.9, 1.9[b]
Fruit and veg., (servings per day)[a]	4.55, 1.84	7.64, 3.17[b]	7.60, 3.43[b]	4.47, 1.72	9.42, 3.12[b]	8.20, 3.32[b]
Total Carotenoids, (mg per day)	11.0, 6.0	22.3, 13.5[b]	19.2, 8.6[b]	11.2, 6.4	25.5, 13.4[b]	22.1, 13.3[b]
Variety fruit and (servings per day)	3.2, 1.2	4.5, 1.8[b]	4.4, 1.8[b]	3.4, 1.5	5.3, 1.4[b]	4.9, 1.7[b]
Whole grains, (servings per day)	1.8, 1.b	3.3, 1.7[b]	3.4, 1.6[b]	1.9, 1.7	3.4, 1.8[b]	3.4, 2.1[b]
Fiber, g per day	22, 8	29, 11[b]	30, 10[b]	22, 8	36, 16[b]	33, 13[b]
Red meat, (servings per day)	1.9, 1.6	1.4, 1.5	1.0, 1.2[b]	1.5, 1.3	1.2, 1.6	0.9, 1.1[b]
Legumes, (servings per day)	0.25, 0.38	0.16, 0.27	0.30, 0.44	0.20, 0.27	0.35, 0.49	0.41, 0.55
Glycemic Load[a,c]	200, 69	188, 54[b]	189, 58	190, 73	168, 82[b]	179, 79[b]
Sodium, (g per day)[a,c]	3.48, 1.17	3.05, 1.19	3.04, 0.98	3.30, 1.13	2.77, 1.39[b]	3.06, 1.12[b]
Calcium, (mg per day)	934, 340	921, 376	978, 387	843, 36	1041, 403[b]	1026, 331[b]

Data shown is raw mean and SD for all available data.

[a] A significant group*time interaction was present for indicated variables from mixed linear regression models using variables transformed to achieve normality, as described in methods. Covariates in the analyses were energy intake (except in the case of energy and percent fat), gender, baseline BMI status (normal weight or not) and baseline age. One subject in the Healthy arm who completed food records for the 6 month visit did not attend the 6 month study visit. The transformations used before analysis were log for saturated fat, PUFA, fiber, sodium, calcium, legumes and glycemic load; square root for whole grain servings, fruit and vegetable servings, and total carotenoids; fourth root for MUFA, and long chain omega-3 fats; and the reciprocal square root for energy.

[b] Significantly different than baseline for that diet arm.

[c] A significant fixed effect of group was present in the model.

[d] The omega-6 PUFA intake was the sum of 18:2 and 20:4.The omega-3 PUFA intake was the NDS output variable "omega-3 fatty acids" which is the sum of 18:3, 18:4, 20:5, 22:6, and 22:5.

[e] Significantly different than 3 months for that diet arm.

Table 2.5: Serum Concentrations of Nutrient Biomarkers by Diet Arm. Data shown is raw mean and SD for all available data at each time point

Serum Nutrient	Healthy Eating		Mediterranean	
	Baseline (n = 61)	6 Months (n = 47)	Baseline (n = 59)	6 Months (n = 47)
SFA (%)	34, 5	34, 5	34, 5	33. 5
MUFA (%)[a]	24, 6	24, 5	24, 5	27, 4[b]
Omega-6 PUFA (%)	36, 7	36, 7	37, 7	34, 6[b]
Omega-3 PUFA (%)	3.8, 1.2	4.1, 1.5	3.7, 1.5	4.2, 1.4[b]
Omega-3/Omega-6 fatty acid ratio	0.11, 0.04	0.12, 0.05	0.11, 0.07	0.13, 0.05[b]
Total carotenoids (pg per mL)	959, 508	1240, 873[b]	1033, 807	1154, 746
Lutein (pg per mL)	170, 84	200, 85[b]	174, 104	219, 152[b]
Zeaxanthin (pg per mL)	40, 22	46, 21	40, 19	47, 44
β-Cryptoxanthin (pg/mL)	79, 51	115, 106	88, 74	118, 114[b]
β-Carotene (pg per mL)	229, 227	382, 507[b]	303, 472	367, 436[b]
α-Carotene (pg per mL)	78, 70	164, 244[b]	97, 136	110, 86
Lycopene (pg per mL)	363, 262	333, 236	331, 271	294, 168

[a] A significant group by time interaction was present at $p < 0.05$. Significance testing is based on the model for transformed outcome, with transformations as described in methods.

[b] Significantly different than baseline for that diet arm, $p < 0.05$. All models had analysis batch, baseline age, BMI, and smoking status as covariates.

Table 2.6: Colon Tissue Concentrations of Nutrients by Diet Arm. Data shown is mean and SD for all available data

Nutrient Level	Healthy Eating		Mediterranean	
	Baseline (n = 61)	6 Months (n = 47)	Baseline (n = 59)	6 Months (n = 47)
SFA (%)	32, 5	32, 4	32, 4	32, 4
MUFA (%)	31, 4	31, 4	32, 5	33, 5
Omega-6 PUFA (%)	32, 7	31, 5	31, 5	31, 5
Omega-3 PUFA (%)	4.8, 2.4	5.1, 2.3[2]	4.4, 2.3	4.6, 2.2
Omega-3/Omega-6 ratio	0.16, 0.10	0.17, 0.08[2]	0.15, 0.08	0.16, 0.08
Total carotenoids	17, 15	29, 50[2]	17, 19	21, 19
Lutein (pg per ml)	8.1, 7.7	14.7, 34.3	8.0, 8.1	10.7, 11.4
Zeaxanthin (pg per ml)	0.79, 0.83	1.03, 1.46	0.74, 0.73	0.83, 0.61
β-Cryptoxanthin (pg per ml)	0.93, 0.68	1.42, 1.48[2]	1.01, 0.84	1.32, 1.14
β-Carotene (pg per ml)[a]	2.2, 2.9	3.8, 4.7[2]	2.8, 6.8	3.2, 4.1
α-Carotene (pg per ml)[a]	1.1, 1.7	2.9, 4.2[2]	1.3, 1.7	1.9, 2.1
Lycopene (pg per ml)	3.5, 5.2	4.9, 11.2	3.3, 3.9	3.0, 3.5

[a]Significant group-by-time interaction was present from mixed linear regression models, after transformation of variables to achieve normality, as described in Methods.

[a] Significant different than baseline for that diet arm, $p < 0.05$. All models included analysis batch, baseline age, BMI, and smoking status as covariates.

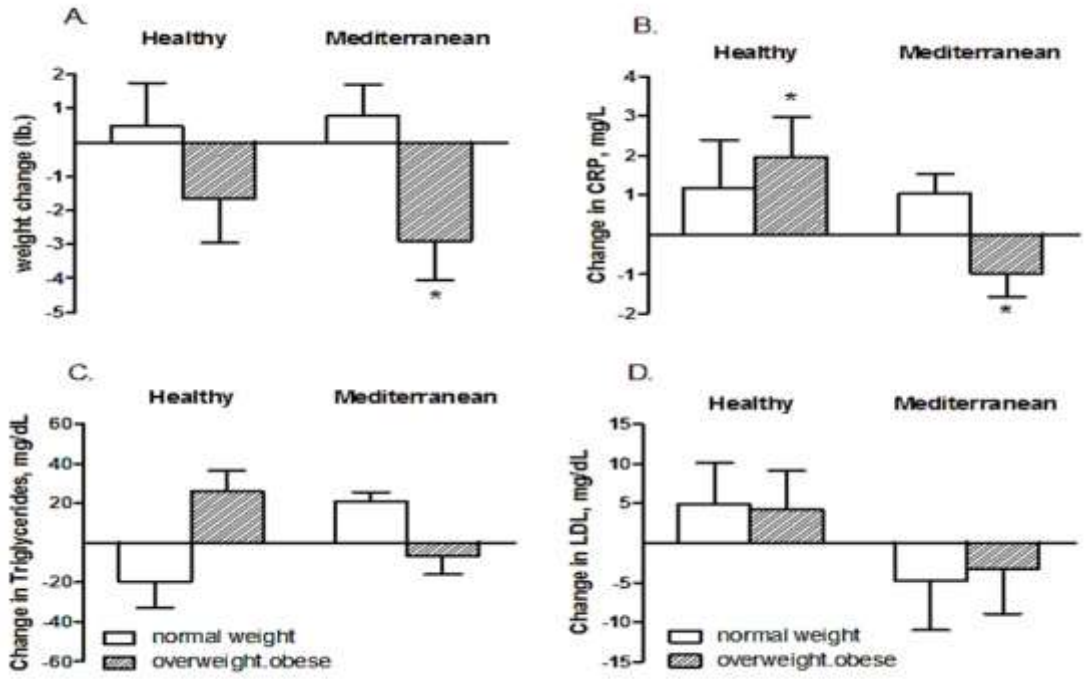

Figure 2.1: Changes in Select Parameters for the Healthy Eating and Mediterranean study arms

The data shown is mean and SE for: A. body weight, B. C-reactive protein, C. triglycerides and D. low density lipoprotein (LDL), given as change from baseline to 6 months in subjects who were either normal weight or overweight/obese at baseline. *Mixed models regression indicated that the decrease in body weight and C-reactive protein in the Mediterranean arm for overweight/obese subjects (starred) was statistically significant after controlling for baseline age and gender ($p<0.05$).

CHAPTER 3

Relationships of Prostaglandin E_2 (PGE_2) with Fatty Acid Concentrations and Gene Expression in Colon of Individuals at Increased Risk of Colon Cancer

3.1 Abstract

It is important to better understand inter-individual differences in colonic PGE_2 concentrations since PGE_2 is closely associated with colon cancer risk. This can be done by evaluating the influence of substrate availability (fatty acids), medication use, baseline demographics and colon cancer risk factors on PGE_2 concentrations and expression of enzymes and receptors in its pathway.

In order to evaluate the effects of these factors on the PGE_2 pathway, colon biopsies were obtained from 120 individuals at high risk for colon cancer. PGE_2 concentrations in the colon tissue were measured using reverse-phase liquid chromatography with tandem mass spectral detection (LC-MS/MS). Fatty acids were measured using gas chromatography. Quantitative Real Time PCR (QrtPCR) was used to measure mRNA expression of genes in the PGE_2 pathway: PTGS1 and 2, PTGES-1 and 3, HPGD and PGE_2 receptors 2 and 4. Correlations, t-test and regressions models were used to determine the associations between these factors and PGE_2 concentrations. The most highly expressed genes were HPGD and PTGS1. PTGS1 expression was significantly and positively associated with PGE_2 but not the other enzymes. There was no association of PGE_2 with demographic factors, or substrate fatty acids (arachidonic acid or eicosapentaneoic acid). Use of non-steroidal anti-inflammatory agents decreased colon PGE_2

concentrations and expression of PTGS2 but not expression of PTGS1. Saturated fatty acid concentrations were positively associated with PGE_2 concentrations. In multivariate linear regression models, both SFA and PTGS1 were significant positive predictors of PGE_2 after controlling for non-steroidal anti-inflammatory drugs uses, gender, age, and smoking status. These results demonstrate the potentially important role of PTGS1 as a biomarker for colon cancer prevention. It also highlights the significant effect of both PTGS1 expression and saturated fatty acid concentrations along with PTGS2 in regulating PGE_2 concentrations in healthy individuals at high risk for colon cancer.

3.2 Introduction

Colorectal cancer is a term used collectively to denote colon and rectal cancer (CRC). In the United States, CRC is the third most common type of cancer and the third leading cause of death in both men and women [135].The American Cancer Society estimates that approximately 132,700 new cases will be diagnosed with CRC, and 49,700 deaths will be expected by the end of 2015 [4]. About 60-70% of colon cancer cases are sporadic, i.e. not due to known genetic factors, and this potentially points to an important role of modifiable risk factors in the prevention of colon cancer [136-138].These factors include dietary behaviors and lifestyle choices, such as lack of regular physical activity, obesity, alcohol consumption and smoking [139-142].

Prostaglandin E_2 (PGE_2) is a pro-inflammatory mediator and is strongly associated with colon cancer risk and development [143, 144]. PGE_2 is synthesized in a stepwise manner by initial release of the fatty acid arachidonic acid (AA) from the cell membrane phospholipids by phospholipase A2. This is followed by metabolism of AA by constitutive COX-1 and inducible

COX-2, to form an unstable endoperoxide intermediate, prostaglandin H2 (PGH2) and isomerization of PGH_2 to PGE_2 via prostaglandin E synthases (PGES) (Figure 3.1). There are three forms of PGES: cytosolic PGES (cPGES) and microsomal PGES-1 and 2 (mPGES1 and 2). Both cPGES and mPGES-2 are constitutively expressed and believed to be associated with COX-1 [145], while mPGES-2 co-localizes with COX-2 [146]. In contrast, mPGES-1 is induced by pro-inflammatory stimuli and is found to be overexpressed in colon cancer [24, 147, 148]. The synthesized PGE_2 can be catabolized by 15-prostaglandin dehydrogenase (15-PGDH) or can exert its actions in autocrine and paracrine manner by binding to its receptors in the cell membrane (PTGER 1-4), as shown in Figure 3.1.

It is well established that inducible COX-2 plays a critical role in colon cancer development by increasing PGE_2 concentrations [149, 150]. Interestingly, animal studies also indicate the important role that constitutive expression of COX-1 plays in colon cancer development [151, 152]. Additionally, several epidemiological and experimental studies have indicated that NSAIDs inhibit both COX-1 and COX-2 [153]. In colon tumors, microsomal-prostaglandin E-synthase (mPGES-1) and COX-2 are overexpressed [148]. In contrast, 15-PGDH, an enzyme that degrades PGE_2, is shown to be down-regulated during tumorigenesis [28]. The PGE_2 receptors (PTGER) are also a potential target for colon cancer prevention and treatment[31].

In addition to the role of the enzymatic pathway on PGE_2, growing research points to the effects of dietary fatty acids in controlling PGE_2 levels. For example, higher intakes of dietary omega-6 fatty acid increase arachidonic acid (AA omega-6, substrate fatty acid) levels in colon tissue [154]. On the other hand, intake of eicosapentaneoic acid (EPA n-3), increases the ratio of

EPA to AA, which in turn decreases PGE_2 levels due to the inhibition of COX-1 activity by EPA [155]. Similarly, intake of monounsaturated fatty acids (MUFA n-9, non-substrate fatty acids) may decrease PGE_2 levels by reduction of COX-2 expression during tumor genesis [121]. In contrast, formation of PGE_2, COX-2 expression and inflammation were found to be enhanced by saturated fatty acids (SFA) through activation of toll-like receptors [156].

The aim of the present study was to better understand inter-individual differences in colon PGE_2 concentrations by evaluating the factors that affect expression of genes in the PGE_2 pathway.

3.3 Methods

Study Participants, Eligibility and Design

Individuals at increased risk of colon cancer were eligible for the Healthy Eating for Colon Cancer Prevention Study. The study was approved by the University of Michigan Institutional Review Board (HUM00007622) and information on the recruitments, eligibility criteria, dietary assessment and intervention were described previously in detail [70, 71]. All subjects were given a written informed consent to participate in the study. In brief, the study recruited 120 individuals with a strong family history of colon cancer, or a personal history of adenomatous polyps or early stage colon cancer. Other inclusion criteria included being in good general health, being at least 21 years old, and having a body mass index (BMI) of ≥ 18.5 and < 35 kg/m^2. Eligible subjects were randomized to one of two dietary interventions, a Healthy Eating diet or a Mediterranean diet, for 6 months. The data shown here is focused on baseline measures made in this study. A dietary recalls and two days of food records were collected prior

to baseline. An additional 24-hour recall was obtained at the study visit to obtain an average estimate of baseline diet. A study questionnaire captured medical history and behavioral factors such as age, gender, education, physical activity, and medication use as well as colon cancer risk factors (Table 3.1).

Medication use

Medications that were commonly used by study participants were evaluated for effects on the biomarkers quantified in colon biopsies. The most prevent medication was regular use of NSAIDs for cardiovascular disease prevention by 24 subjects which was aspirin with a dose of 81 mg/day or 325 mg every other day. Cholesterol medications were used by 19 subjects and this included Crestor, Ezetimibe, Lipitor, Lovastatin, Mevacor ,Simvastatin, Vytorin, and Welchol. Lastly, blood pressure medications were used by 21subjects and these included Acebutolol, Atenolol, Hydrochlorothiazide, Losartan, Lisinopril and Metoprolol or combinations. Occasional use of other medications was not analyzed.

Flexible Sigmoidoscopy and Tissue Collection

Colon tissue biopsies were collected by flexible sigmoidoscopy. This was done without prior preparation of the bowels. Six biopsies were immediately flash frozen in liquid nitrogen within 30-50 seconds after removal from the subject and then stored at −70°C for further biomarkers analysis.

RNA Extraction and Reverse Transcription (cDNA Synthesis)

A frozen colonic biopsy specimen, approximately 5 mg, was pulverized to a fine powder using a liquid-nitrogen-cooled mortar. RNA was extracted from the tissue powder using the TRIzol method for extraction, according to the manufacturer's protocol (Invitrogen, cat. no. 15596-026). Briefly, 1mL of TRIzol was added to each sample prior to homogenize on ice by using an Ultrasonic Processor (Misonix, Farmingdale, NJ). Isopropanol 100% (0.5 mL) was used for RNA precipitation in the presence of 50 µl of glycogen for each 1mL of TRIzol homogenate.

The RNA pellet was washed three times with 75% ethanol, and resuspended in 20µl RNAse free water after drying. The concentration and purity of RNA were determined using ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA); all samples used had a ratio of 260/280 and value greater than 1.7. The RNA was diluted to achieve 1ug RNA/9.9ul RNase free water. The Reverse Transcription System was used to synthesize the cDNA following the manufacturer protocol (Promega, cat. no. A3500). The samples were in the thermal cycler (BIO-RAD, cat. T100), for 1 minute at 25°C, 1.5 hours at 42°C, 5 seconds at 95°C, and then left at 4°C until cool.

Quantitative Real-Time PCR (RT-qPCR)

Real-Time PCR was performed by using TaqMan® Environmental Master Mix 2.0 (Life Tech, cat #4398044). A 26 µl PCR reaction was prepared using 5µl of cDNA (RT product), a 1.25 µl gene specific primer, and a 13.5 µl primer mixture from Life Tech TaqMan® Gene Expression Assays. A mixture of cDNA from the sample pool was used to construct the standard curve. All samples and standard curves were run in duplicate in the real-time PCR reactions. The primers and probes used for real time PCR were purchased from Applied Biosystems (Foster City, CA, USA). The primers used were as follows: COX-1 (PTGS1), Hs00377726_ml), COX-2

(PTGS2, Hs00153133_m1), mPGES1 (PTGES1, Hs01115610_ml), cPGES (PTGES3, Hs00832847_gH), 15 HPGD (HPGD, Hs00168359_ml), EP2 (PTGER2, Hs04183523_m1), and EP4 (PTGER4, Hs00168761_m1). The cytokeratin 20 Krt20, a marker of colonic epithelial cell mass, was used as an internal control for normalization (Hs00300643_m1) [157]. The real-time PCR thermal conditions were: 50°C 2 min, 95°C 10 min followed by 40 cycles of 95°C 15 sec and 60°C 1 min. The mean efficiency for the PCR standard curve for each primer was: PTGS1 (95%), PTGS2 (90.4%), PTGES (105%), PTGES3 (97.2%), HPGD (99%), PTGER2 (93%), PTGER4 (94%), and Krt20 (94%). For quantification, the standard curve method was used, and the average amount of each target mRNA expression and Krt20 mRNA expression was established from the standard curve. The target gene expression was then normalized by the Krt20 expression.

PGE_2 and Fatty Acids Quantification

The method used to quantify PGE_2 was reverse-phase liquid chromatography with tandem mass spectral detection (LC-MS-MS), as previously established for rodent tissues [109]. In brief, tissue homogenates were prepared from two frozen colon biopsies, which were about 5 mg of tissue each. Ether was used for extraction of PGE_2 prior to LC-MS-MS analysis. The analysis was performed using deuterated internal standards (Cayman Chemical, Ann Arbor, MI) and a Luna Phenyl-Hexyl analytical column (2 x 150 mm, 3 μm particle size, Phenomenex, Torrance, CA). Since deuterated compound was not available for PGE_3, PGE_2-d_4 was used for quantifying PGE_3. A small portion of the homogenate was also used for analysis of total protein by the Bradford assay. The PGE_2 values were expressed as nanogram (ng) of PGE_2 per milligram (mg) of protein.

The fatty acid measures of colon homogenates were performed using GC-MS analysis , as previously published [158]. In brief, frozen colon tissue biopsy weighing approximately 5mg was pulverized and added to a tube of 150 µl of ice-cold phosphate buffered saline containing 0.1% BHT and 1mM EDTA for sonication by an Ultrasonic Processor and processed twice 30 seconds. One ml of chloroform and methanol with a ratio of 1:1 was used for total lipid extraction and fatty, acid methyl esters were prepared with METH-PREP II derivatization reagent prior to GC-MS analysis (Alltech, Deerfield, IL) [158].

Statistical Analysis

All statistical analyses were conducted using IBM SPSS software version 22 (PASW Statistics, IBM Corporation, Armonk, New York). All variables were checked for normality of the distribution before analyses and transformed as needed. Natural log transformation was used for normalizing gene expression of enzymes and receptors in the PGE_2 pathway, and the square root was used for concentrations of PGE_2 and arachidonic acid. Descriptive statistics were used to present the subject characteristics. To evaluate the effects of the baseline factors on PGE_2, two-sample t-tests and partial correlations were used. Spearman correlation coefficients were used to determine the associations between PGE_2, gene expression and colon tissue. Generalized linear model regression (GLM) models were used to determine the predictors of colon PGE_2 concentrations. Although known risk factors for colon cancer of age, gender and smoking status did not show significant effect on colon PGE_2 concentrations, they were nonetheless entered as covariates in the models.

3.4 Results

Effect of the Demographic Factors on PGE_2 and Its Pathway

The characteristics of the enrolled study participants are shown in Table 3.1. Demographic factors such as age gender, alcohol intake, obesity and physical activity did not show significant associations with PGE2 or gene expression of enzymes and receptors in its pathway (Table 3.2). However, medication use showed significant effects on PGE_2 concentrations and gene expression (Table 3.3). NSAIDs use (in 24 subjects), significantly reduced both PGE_2 concentrations and PTGS2 gene expression. The significant effects of regular medication use (given for medication users and nonusers, respectively) for NSAIDs on concentrations of PGE_2 were (11 vs. 20 ng/mg protein) and PTGS2 (0.16 vs. 0.23); for cholesterol medications on PTGES1 (0.11 vs. 0.19, n=19 users); and for blood pressure medication on PTGS2 (0.16 vs. 0.22, n=21 users) and on PTGR2 (0.2 5 vs 0.31).

Some individuals were taking more than one of these medications. To determine the effect of specific types of medication use, subgroup analysis was conducted excluding individuals using more than one type of medication (Table 3.4). For NSAIDs users, after excluding subjects with cholesterol and blood pressure medication users (n=11), the significant difference in PGE_2 remained. However, the significant effect of NSAIDs use on PTGS2 disappeared, though the mean for users stayed lower vs non users (0.19 vs. 0.26) in users and nonusers, respectively). For seven cholesterol medications users, after excluding subjects taking NSAIDs and blood pressure medications, the difference in PTGES1 remained significant (0.11 vs. 0.20 in users and nonusers, respectively). For the 11 individuals who used blood pressure medication, after excluding those subjects taking NSAIDs and cholesterol medications , the effect on PTGS2 (0.16 vs. 0.27, in users and nonusers, respectively) was almost significant

(p=0.06), whereas the difference in PTGR2 (0.27 vs 0.38 in users and nonusers, respectively) was no longer significant.

Gene expression of enzymes and receptors in the PGE_2 Pathway

Gene expression of the enzymes and receptors involved in the PGE_2 pathway are shown in Figure 3. 2. The highest relative mean mRNA expression in colon biopsies was 1.31 for HPGD and 1.12 for PTGS1 (1.12). PTGS1 expression was almost five-fold higher in comparison to PTGS2 levels (0.2), which appear to have a very low expression. PTGES3 mRNA expression (0.5) was almost two-fold higher than PTGES1 expression (0.17). Lastly, among the PGE_2 receptors, PTGER4 was expressed almost three fold higher in comparison to PTGER2 (0.90 vs. 0.34).

Associations of PGE_2 Concentrations with Gene Expression

Table 3.5 shows the Spearman correlation coefficients between PGE_2 concentrations and relative mRNA expression of enzymes and receptors. Correlations were significant at the $p< 0.01$ level. PGE_2 was significantly correlated with PTGS1 ($\rho=0.27$). PTGS1 was significantly correlated with PTGS2 ($\rho=0.50$), as well as the synthase enzyme PTGES1 ($\rho=0.58$) but not with the degradation enzyme HPGD. PTGS1 was also correlated with the PGE_2 receptors PTGER2 ($\rho=0.28$) and PTGER4 ($\rho=0.51$). Although PTGS2 was not found to be associated with PGE_2, it showed significant association with PTGES1 ($\rho=0.61$), HPGD (0.36), PTGER2 ($\rho=0.58$) and PTGER4 ($\rho=0.48$). For the synthase enzymes, PTGES1 was also significantly correlated with HPGD ($\rho=0.34$), PTGER2 ($\rho=0.53$) and PTGER4 ($\rho=0.45$). However expression of PTGES3 was not associated with expression of any enzymes and receptors. HPGD was significantly

correlated with PTGER2 (ρ=0.53) and PTGER4 (ρ=0.30). Finally, PTGER2 was significantly correlated with PTGER4 (ρ=0.51).

Associations of PGE_2 Concentrations, Gene Expression of Enzymes and Receptors and Colon Tissue Fatty Acids

To understand the relationships between PGE_2, gene expression and colon tissue fatty acids, Spearman correlations were conducted (Table.3.6). Initial correlations were explored between colonic PGE_2 pathway and dietary intakes to determine if there were any relationships between the two. None of the correlations were significant with $p < 0.05$ (data not shown). Next, correlations with colon nutrient concentrations with gene expression were explored (Table 3.6). There were significant positive associations between PGE_2 and colon tissue saturated fatty acids (ρ=0.31). For gene expression, there were significant positive associations between PTGS1 and EPA (ρ=0.24). For PTGS2 there was a negative association with monounsaturated fatty acids MUFA (ρ=0.28), and PTGS2 tended to positively associate with AA. PTGER4 had a significant positive association with AA (ρ=0.44) and EPA (ρ=0.39), whereas it showed a negative significant association with MUFA (ρ=0.38).

Predictors of PGE_2 levels in Linear Regression Models

Finally, linear regression models were utilized to determine if effects of colon fatty acids on gene expression are significant after controlling for NSAIDs use, age, smoking status and gender (Table 3.7a). Interestingly, SFA and PTGS1 were both significant in the model as predictors for PGE_2, and both remained significant predictors of PGE_2 in the final model. The

beta coefficients for females were higher than for males, and current smoking predicted higher PGE_2 as did higher tissue SFA and higher PTGS1 (Table 3.7 b).

3.5 Discussion

Epidemiological and clinical studies have emphasized the importance of surrogate end point biomarkers in understanding colon cancer development and prevention. Hence, identification of the factors that affect these markers is important, especially for those among those at high risk for the disease [159, 160]. Although PGE_2 has been well-recognized as a biomarker of colon cancer risk, few studies have been investigated expression of genes in its synthesis, degradation and signaling pathways [19, 161]. Furthermore, to our knowledge, no studies have been undertaken to look at the associations between gene expression and colon tissue fatty acids among individuals at high risk for colon cancer. This study provided us with the ideal opportunity to investigate these associations.

In Figure 3.1, the mean level of mRNA expression of 15 PGDH (HPGD) and COX-1 (gene name PTGS1) were high, as could be expected for normal colon tissue. PTGS1 is constitutively produced [162, 163]. In contrast, PTGS2 and PTGES1 (microsomal prostaglandin E synthase) had the lowest mean levels of mRNA expression among those quantified. Both PTGS2 and PTGES1 enzymes are known to be overexpressed during carcinogenesis, and in normal tissue expression may be quite low [14, 164].

It is interesting to note the inter-individual variability of gene expression that was found among subjects, especially for 15 PGDH. This finding is supported by recent data showing that expression of 15-PGDH gene expression varied 4.4 fold between individuals but remained stable

within the colon of the individual, regardless of colon location [157, 165]. This variability on theses biomarkers among subjects at high risk for colon cancer highlights the potential to further categorize individuals with regard to risk based on these biomarkers levels. For example, a recent publication on the relationship between 15-PGDH expression levels and aspirin treatment found that higher expression of 15-PGDH levels in normal colon tissue were a potential predictor in determining the efficacy of aspirin treatment for colon cancer prevention [166].

Next, we evaluated the impact of demographic factors on PGE_2 concentrations and expression of genes in its pathway. Age, gender, alcohol consumption, smoking status, obesity, and physical activity as well as history for colon cancer did not significantly impact on PGE_2 and gene expression (Tables 3.2 and 3.3). However, medication use did influence expression of genes in the PGE_2 pathway. NSAIDs are known to lower colon cancer risk by decreasing PGE_2 levels via inhibition of the cyclooxygenase activity of both prostaglandin endoperoxide H synthase-1 and 2 [12, 19, 167]. In this study NSAID use was found to reduce PTGS2 but not PTGS1 mRNA expression levels (Table 3.4). PTGS-2 must be contributing at least somewhat to PGE_2 in the normal colon since NSAID use did significantly decrease PGE_2 concentrations [168].

Cholesterol medication use was significantly associated with reduced mRNA expression of PTGES1 (Table 3.4). The significant reduction in PTGES1 by cholesterol medication use is consistent with published data that suggest statins as a potential pharmacological approach for prevention [169]. PTGES1 is the synthase that is known to associate with PTGS2. Previous research also has indicated that combination use of NSAIDs with cholesterol medications may be useful for CRC prevention and treatment [170]. A significant effect of blood pressure

medication on lowering PTGS1 was also found in this study, but there is no published data showing associations of blood pressure medications with CRC risk.

Although the influence of cholesterol and NSAID medication use on gene expression indicates a role for the PTGS2 pathway in colon PGE_2, colon PGE_2 was correlated with expression of PTGS1 (COX-1) but not PTGS2 (COX-2), as shown in Table 3.5. This could be due to the higher level of PTGS1 versus PTGS2 expression level. Therefore, PTGS1 may be seen as a potential target marker for colon cancer prevention among high risk individuals. These results also agree with previous findings of a ginger prevention trial. In that trial, it was found that protein levels of PTGS1 in colon of subjects at high risk of colon cancer were two-fold higher in comparison to colon PTGS1 in the normal with no risk group [22]. This ginger trial did not quantify PTGS2 protein expression since the low expression of this enzyme makes it hard to measure in healthy colon tissue [15].

In addition to demonstrating a positive association between PGE_2 and PTGS1, our data in Table 3.5 also show that PTGS1 has a strong positive association with PTGES1 but no association with PTGES3. PTGES3 is known to be constitutively expressed in normal individuals and is thought to work with PTGS1 to produce basal PGE_2 [145]. Therefore, the lack of linear correlation between PTGS1 and PTGES3 may be due to the fact that the study subjects were at high risk for colon cancer or that other regulatory mechanisms exist. Such individuals may differ versus healthy individuals with low risk.

Other significant findings were that HPGD showed a strong positive association with PGE_2 receptors (PTGER2 and 4) and synthases enzymes (PTGs2 and PTGES1) but not PTGS1. It can be speculated that HPGD expression may work to counteract the effects of increases in

synthetic pathways in normal colon. HPGD could be induced to degrade the high level of PGE2 caused by increased expression of synthetic enzymes.

Finally, we found that the colon tissue monounsaturated fatty acids (MUFA) tended to have a non-significant negative association with PGE_2 and to have a significant negative association with PTGS2 (often referred to as COX-2 activity). A reduction in PGE_2 and PTGS22 levels may be responsible for the previously observed anti-inflammatory effects of MUFA found in olive oil [63]. In contrast to the relationship of high MUFA with lower PGE_2 concentrations, saturated fatty acids (SFA) showed a positive relationship with PGE_2 concentration (Table 3.6). High dietary intake of SFA has been suggested to increase colon cancer risk [171]. Alternatively, SFA non-substrate fatty acids may allosterically stimulate the cyclooxygenase activity of PTGS2 [172, 173]. The effects of both PTGS1 and SFA on increasing PGE_2 concentrations were further confirmed in linear regression analysis (Tables 3.7 A and B). These results are consistent with the known pro-inflammatory effects of SFA via activation of toll-like receptors (TLR) that in turn set off an inflammatory cascade [172, 174]. COX-2 expression is known to be induced by activation of TLR, although some reports indicate that COX-1 can be induced as well [175, 176]. Saturated fatty acids also may increase the production of PGE_2: although non-substrate saturated fatty acids slightly inhibit COX-1 they stimulate COX-2 [172].

In summary, the findings of this study point to evidence of significant positive associations between COX-1 expression, colon SFA and PGE_2. There was, however, a role for PTGS2 discerned from the effects of NSAID use despite low PTGS2 expression levels in normal colon tissue. These results suggest that PTGS1 and PTGS2, as well as SFA are potential targets for colon cancer prevention among individuals at high risk for colon cancer. This is consistent

with previous research in humans at high risk for colon cancer [22] but differs from in vivo data on chemically induced colon carcinogenesis, which suggested that only PTGS2 may be a prevention target. The metabolic or dietary differences that determine saturated fatty acid concentrations in colon, together with PTGS1 and COX-2 expression and/or activity, appear important in regulation of colon PGE_2 concentrations and therefore colon cancer risk.

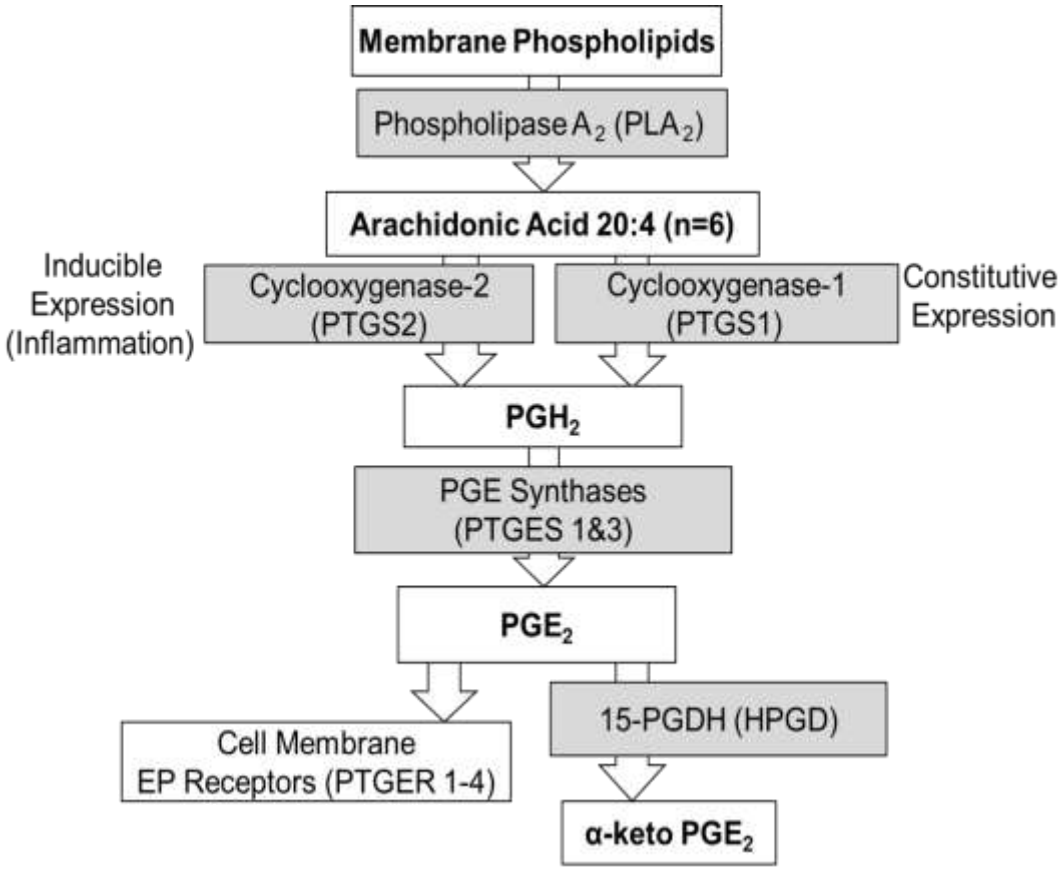

Figure 3.1: Schematic Showing PGE_2 Metabolism and its Signaling Pathway

Table 3.1: Characteristics of 114 Study Participants [a]

Characteristics	
Demographic Factors	
Age, Years	53 ,12
Female, Gender	81, 71%
Healthy Eating Diet, Group	57, 50%
Caucasian, Race	99, 87%
Behavioral Factors	
Alcohol Consumption, g/day	6.3, 8.5
Current Smokers	12, 10%
Physical Activity, METs per day[b]	19.1 ,13.9
Anthropometrics Measurements	
Body Mass Index, kg per m^2	27, 4
Waist to Hip Ratio	0.9, 0.1
Medical History	
History of Adenomas	5, 4.4
Family History of Colon Cancer	86,75.4
Both Risk Factors	11, 10%
Medication Use	
Regular NSAIDs Users	24, 21%
Cholesterol Medication	19, 16.7%
Blood Pressure Medication	21, 18.4%
Dietary Intakes	
Energy, kcal per day	2085, 624
Omega-6 Polyunsaturated fat, g per day	38, 7
Omega-3 Polyunsaturated fat, g per day	4, 1.2
Monounsaturated fat, g per day	31, 13
Saturated fat, g per day	28.4, 11.5

[a] Data is shown in mean, SD, or in number and percentage.
[b] Metabolic equivalents per day.

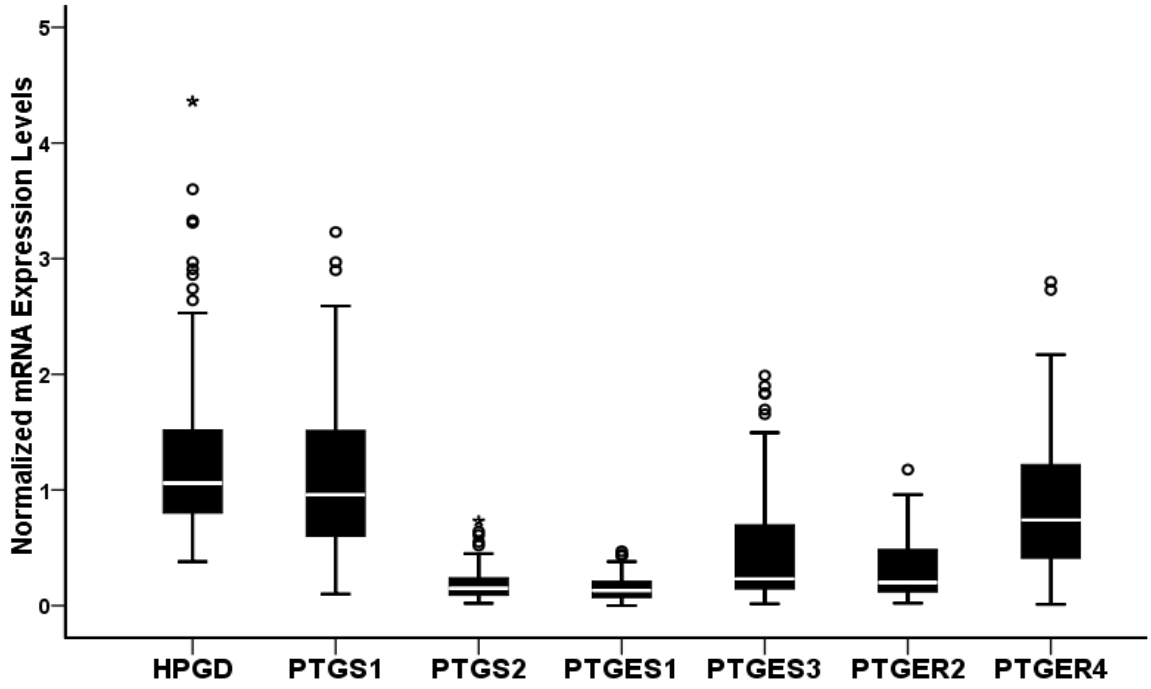

Figure 3.2: Normalized mRNA Expression of Enzymes and Receptors in the PGE_2 Pathway for all Study Participants. Data shown is mean, SD, minimum and maximum

Table 3.2: Partial Correlation Coefficients between Demographic Factors with Colon PGE_2 Concentrations and with mRNA Expression Levels of Enzymes and Receptors in the PGE_2 Pathway

Variable	PGE2	HPGD	PTGS1	PTGS2	PTGES1	PTGES3	PTGER2	PTGER4
Age (years)[a]	-0.128	0.091	0.149	0.042	0.039	-0.145	0.206	0.108
Alcohol Intake[b] (g/day)	-0.088	-0.027	-0.130	-0.075	-0.101	0.081	-0.040	-0.055
Physical Activity[c] (MET/day)	-0.029	0.086	0.049	-0.007	0.207	0.133	0.161	0.080

[a] Control variables for age were Body Mass Index, Gender, and use of NSAIDs, cholesterol medications and blood pressure medications use.
[b] Control variables for alcohol Intake were gender, age and body mass index NSAID regular use, cholesterol medications, blood pressure medications and arthritis.
[c] Control variables for physical activity were: gender, age, body mass index, arthritis, and use of NSAID , cholesterol medications, and blood pressure medications. MET is a metabolic equivalent per day.

Table 3.3: PGE_2 Concentrations and Relative Expression of Enzymes and Receptors in PGE_2 Pathway in High Risk Individuals. Data are given as mean, SD

Demographic Factors	N	PGE_2	PTGS1	PTGS2	PTGES1	PTGES3	HPGD	PTGER2	PTGER4
Gender									
Female	82	19, 13	1.4, 1.0	1.1, 0.7	0.2, 0.16	0.44, 0.44	0.19, 0.15	0.34, 0.31	0.9, 0.6
Male	33	15, 14	1.4, 1.0	1.2, 0.7	0.2, 0.14	0.51, 0.53	0.15, 0.1	0.36, 0.26	1.0, 0.7
Smoking Status									
Yes	13	21, 12	1.1, 0.9	0.2, 0.11	0.15, 0.1	0.7, 0.6	1.3, 0.7	0.19, 0.15	0.8, 0.6
No	72	16, 12	1.2, 0.7	0.22, 0.18	0.19, 0.14	0.4, 0.4	1.3, 0.9	0.36, 0.31	0.9, 0.6
Past	30	19, 14	1.1, 0.6	0.2, 0.13	0.15, 0.13	0.5, 0.5	1.7, 1.1	0.38, 0.3	0.9, 0.6
BMI									
<25 kg/m^2	39	19, 11	1.1, 0.7	0.2, 0.15	0.19, 0.14	0.46, 0.46	1.4, 1.04	0.38, 0.33	0.9, 0.1
≥25 kg/m^2	76	17, 14	1.15, 0.7	0.22, 0.15	0.17, 0.13	0.47, 0.47	1.4, 1.0	0.32, 0.28	0.7, 0.1
NSAID Users									
Yes	24	**11, 9**[a]	1.1, 0.7	**0.16, 0.1**	0.15, 0.12	0.6, 0.7	1.7, 1.4	0.34, 0.32	0.9, 0.5
No	91	**20, 14**	1.1, 0.7	**0.23, 0.2**	0.18, 0.14	0.42, 0.38	1.3, 0.9	0.34, 0.3	0.9, 0.1
Cholesterol Medications									
Yes	19	17, 15	1.03, 0.6	0.16, 0.15	**0.11, 0.1**	0.4, 0.5	1.3, 0.8	0.28, 0.31	0.83, 0.7
No	96	18, 13	1.14, 0.7	0.22, 0.16	**0.19, 0.14**	0.5, 0.5	1.4, 1.1	0.35, 0.30	0.91, 0.61
Blood Pressure Medications									
Yes	21	14, 11	0.9, 0.6	**0.16, 0.11**	0.16, 0.12	0.6, 0.6	1.6, 1.2	0.25, 0.21	0.78, 0.62
No	94	19, 14	1.2, 0.7	**0.22, 0.17**	0.18, 0.14	0.44, 0.44	1.4, 0.9	0.31, 0.31	0.92, 0.61

[a] Bolded pairs differ significantly ($p<0.05$) from the 2-sample t-test

Table 3.4: PGE2 Concentrations and Normalized mRNA Expression of Enzymes and Receptors in the PGE2 Pathway by Medication Use
Data are given as mean, SD

Colon Markers	Aspirin[a]		Cholesterol Medications[b]		Blood Pressure Medications[c]	
	Yes= 11	No= 71	Yes= 8	No= 71	Yes= 21	No= 94
PGE_2	**11, 8**	**20, 14[d]**	23, 17	20, 14	15, 11	20, 14
PTGS1	1.3**,0**.7	1.2, 0.7	1.0, 0.5	1.2, 0.7	**0.8, 0.45**	**1.2, 0.7 [d]**
PTGS2	0.19,0.1	0.27, 0.28	.18, 0.2	0.27, 0.28	0.17, 0.04	0.27, 0.28
PTGES1	0.19**,0**.15	0.2, 0.17	**0.11, 0.1**	**0.2, 0.17[d]**	0.19, 0.05	0.21 0.17
PTGES3	0.65**,0**.7	0.52, 0.6	0.3, 0.3	0.5, 0.6	0.51, 0.38	0.53, 0.61
HPGD	1.8, 1.4	1.4, 1.05	1.2, 0.7	1.4, 1.1	1.3, 0.7	1.4, 1.1
PTGER2	0.5, 0.4	0.38, 0.39	0.5, 0.4	0.38, 0.4	0.27, 0.25	0.38, 0.39[d]
PTGER4	1.0, .5	1.1, 1.1	0.9, 0.9	1.1, 1.1	0.82, 0.7	1.01, 1.1

[a] Regular use of aspirin was defined as 81 mg/day or 325 mg every other day.
[b] Cholesterol medications were Rosuvastatin, Ezetimibe, Atorvastatin, Lovastatin and Simvastatin.
[c] Blood pressure medications were Acebutolol, Atenolol, Hydrochlorothiazide, Losartan, Lisinopril and Metoprolol or combinations.
[d] Significantly different between users and non-users of medications with at $p<0.05$.

Table 3.5: Spearman Correlation Coefficients of PGE_2 Concentrations with Normalized mRNA Expression Levels of Enzymes and Receptors in the PGE_2 Pathway

Colon Markers	PGE_2	PTGS1	PTGS	PTGES1	PTGES3	HPGD	PTGER2
PTGS1	**0.27****	1.00					
PTGS2	0.16	**0.50****	1.00				
PTGES1	0.12	**0.58****	**0.61****	1.00			
PTGES3	0.04	0.08	0.02	-0.17	1.00		
HPGD	0.11	0.18	**0.36****	**0.34****	0.02	1.00	
PTGER2	0.04	**0.28****	**0.58****	**0.53****	-0.01	**0.53****	1.00
PTGER4	0.17	**0.51****	**0.48****	**0.45****	0.08	**0.30****	**0.51****

****** Correlation is significant at the $p<0.01$ level (2-tailed).

Table 3.6: Spearman Correlation Coefficients of Colon Tissue Fatty Acid Concentrations with Colon PGE_2 Concentrations and with Normalized mRNA Expression of Enzymes and Receptors Levels in the PGE_2 Pathway

Colon Markers	PGE_2	PTGS1	PTGS2	PTGES1	PTGES3	HPGD	PTGER2	PTGER4
AA	0.10	0.15	0.21	0.18	0.08	0.17	0.22	**0.44****
EPA	0.19	**0.24****	0.05	0.16	0.06	**0.20***	0.03	**0.39****
MUFA	-0.11	-0.18	**-0.28****	-0.17	0.07	-0.15	-0.23	**-0.38****
SFA	**0.30****	0.11	-0.02	0.03	0.06	0.03	0.01	0.10

****** Bolded and starred coefficients are significant at the $p < 0.01$ level (2-tailed).

Table 3.7.: Predictors of Colon PGE_2 Concentrations in Linear Regression Models

Model	Adjusted R Square	AIC	P-value for F Change
NSAID use + Age+ Smoking status + Gender	0.104	440.446	0.002
NSAID use + Age+ Smoking status + Gender **+ PTGS1 expression**[a]	0.181	425.739	0.000
NSAID use + Age+ Smoking status + Gender **+ Colon SFA**	0.163	433.788	0.000
NSAID use + Age+ Smoking status + Gender **+ Colon SFA + PTGS1 expression**	0.230	419.737	0.000

[a] Bolded predictors were significant in the linear regression model

Table 3.8. Beta Coefficients for Predictors of Colon PGE_2 in the Final Linear Regression Model

Predictors	β Coefficients	STD. Error	P-value
NSAID user	-0.68	1.387	**0.060**
Gender/ Female	0.64	0.310	**0.033**
Age	-0.02	0.012	**0.109**
Current smoker	0.88	0.438	**0.044**
PTGS1	0.65	0.198	**0.001**
Tissue SFA	0.10	0.034	**0.004**

CHAPTER 4

Effects of a Mediterranean Diet Intervention on the Prostaglandin E2 (PGE2) Pathway in Colon Tissue of Individuals at High Risk for Colon Cancer

4.1 Abstract

Prostaglandin E_2 (PGE_2) is a pro inflammatory mediator that is well known to increase risk of colon cancer. PGE_2 production can be decreased by the use of non-steroidal anti-inflammatory drugs (NSAIDs). Unfortunately, the prolonged use of NSAIDs show unacceptable side effects, which makes it necessary to look for safer chemoprevention strategies such as diet. The Mediterranean diet has been shown to have systemic anti-inflammatory effects. In this study, dietary data and colon mucosal biopsy samples from a dietary intervention trial were used to evaluate the preventive effects of a Mediterranean diet versus the Healthy Eating diet in the colon. After six months of intervention, PGE_2 did not show a significant change in either diet arm. PTGES3 did show a significant increase both diet arms and PGE_3 showed a significant increase in the Mediterranean diet arm only. Additionally, we evaluated correlations between changes in tissue fatty acids with changes in gene expression. Change in arachidonic acid (AA) concentration in the colon showed a significant, strong positive association with changes in PTGES1 Interestingly, change in colon MUFA showed a significant negative association with change in PTGES1 in the Mediterranean diet arm, while showing negative association with PTGES3 in the Healthy Eating arm. Finally, we confirmed the relationships between RNA gene expression protein expressions by immunohistochemical analysis using quantitative image

analysis. These results indicate that the dietary intervention had little effect on PGE_2 and gene expression of enzymes in the pro-inflammatory pathway.

4.2 Introduction

Prostaglandin E_2 (PGE_2) is a pro-inflammatory mediator that is strongly associated with colon cancer risk [143, 144]. It is well established that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the colon cancer risk via inhibition of PGE_2 formation and inhibition of both cyclooxygenases enzymes, COX-1and 2 [12, 19, 167]. However, prolonged NSAID use may lead to long-term gastrointestinal side effects, such as bleeding and ulcer development, as well as an increase in cardiovascular risks [177, 178]. Therefore, alternative and safer chemoprevention strategies need to be investigated, especially among subjects at high risk for colon cancer.

Epidemiological studies have indicated that diet could be useful in the prevention of 30-50% of colorectal cancer [11]. A number of epidemiological studies have yielded information to identify what types of diets might be preventive. A Western diet, which is consumed in the more developed countries, is generally associated with increased risk of colon cancer [179, 180]. This type of diet is characterized by high intakes of fat, red meat, refined grains and sugar. In contrast, the traditional Mediterranean diet includes high intakes of fruits, vegetables, fish and whole grains. Although the traditional Mediterranean diet is also high in fat, this type of fat is derived from olive oil and fish. Both monounsaturated fat and omega-3 fats have been associated with lower risk of chronic diseases, such as cardiovascular disease and most types of cancers, including colon cancer [90, 181].

One important mechanism by which a Mediterranean diet can prevent colon cancer is via modulation of eicosanoids synthesis, particularly PGE_2 formation [150]. The mechanisms behind prevention by the Mediterranean diet include a decrease in omega-6 and an increase in omega-3 fatty acids, which is expected to lower PGE_2 and COX-2 expression [109, 119, 182]. The effects of a Mediterranean diet may also due to the antioxidant effects of higher intakes of fruits, vegetables, and olive oil that will reduce COX expression [183].

In this study, we compared the effect of the Mediterranean diet to a Healthy Eating diet recommended by the U.S. Department of Agriculture (USDA) on colonic prostaglandin pathways. The Healthy Eating diet encouraged increased intakes of fruits, vegetables and whole grains and a decreased saturated fat intake. The Mediterranean diet had a higher content of mono-unsaturated fatty acids (MUFA) and n3 fatty acids combined with lower total poly-unsaturated fatty acids (PUFA) [90]. The objective of this study was to investigate the potential benefits of a Mediterranean diet on risk of colon cancer via regulation of key enzymes in the PGE_2 pathway. Changes in dietary intakes, colon tissue fatty acid and prostaglandin concentrations, and gene expression of enzymes that regulate PGE_2, were quantified. These included RNA expression of prostaglandin H synthase (PTGS) -1 and -2, microsomal-prostaglandin E-synthase (mPGES-1), and 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which degrades PGE_2. In addition, we evaluated the PGE_2 receptors PTGER2 and 4. Gene expression was verified by quantitative immunohistochemical analysis of proteins. Finally, we established the relationships between gene expressions in the colon with concentrations of PGE_2.

4.3 Methods

Study Participants, Design and Dietary Intervention

The Healthy Eating Study was a randomized dietary intervention trial that was approved by the University of Michigan Institutional Review Board (HUM00007622). The study recruited 120 individuals at high risk for colon cancer, and individuals were randomized into Healthy Eating or Mediterranean Diet arms for six months. Detailed information on the recruitments, eligibility criteria, dietary assessment and intervention were previously described [70, 71].

Dietary eligibility was assessed using two days of written records and one unannounced 24-hour recall. Dietary recall and food records were collected at baseline, 3 and 6 months. An additional 24-hour recall was obtained at the first study visit, and all four days were averaged to obtain an estimate of baseline diet and the same assessments were done at six months using the Nutrition Data System for Research software (NDSR), version 2010.

The Healthy Eating study implemented individualized counseling with a registered dietitian to help subjects achieve study goals using Bandura's social cognitive theory. The theory focuses on social support, goal setting, self-efficacy, self-monitoring and constructing strategies for problem solving [108].

Both the Healthy Eating and Mediterranean diet arms were designed to increase the intakes of fruit, vegetable and whole grain; however, goals for fat intake differed in each diet arm. The Healthy Eating diet, which was based on the U.S. Healthy People 2010 recommendations [102], limited saturated fat (SFA) intake to 10% of an individuals' total energy intake while the Mediterranean diet goals sought to decrease PUFA intake by 50%. The Mediterranean group also were asked to consume foods high in omega-3 fatty acids, such as fish, at least twice a week and increase monounsaturated fat intake from plant sources by 50% [71].

Colon Biopsies Collection and Processing

A flexible sigmoidoscopy procedure without prior preparation of the bowels was performed at baseline and at six months to obtain colon tissue biopsies. Eight mucosal tissue biopsy specimens from each individual were collected in the distal sigmoid colonic mucosa at each time point. Of these, six biopsies were immediately placed in liquid nitrogen exactly 20 seconds after removal from the individual. Biopsies were stored at -70°C until biomarkers analysis and genes expression could be quantified. The other two biopsies were submerged in ice-cold, phosphate-buffered saline (pH 7.4) and fixed in formalin (10% formaliomega-90% phosphate buffered saline pH 7.4). Biopsies were kept for 18-24 hours in formalin before being transferred to 70% ethanol. Biopsies were kept in 70% ethanol for no more than one week before embedding in paraffin.

RNA Extraction, cDNA Synthesis and qrt PCR

One biopsy of approximately 5 mg tissue from each participant at each time point was used for RNA extraction. The tissue was soaked in 100 µL of RNAlater-ice stored overnight at -20ºC. On the second day, the tissue was removed from the -20ºC freezer. A liquid-nitrogen-cooled mortar was used to pulverize the tissue biopsies into a fine powder. The fine tissue powder was then added to a tube with 1 ml TRIzol for RNA extraction following the manufacturers' protocol (Invitrogen, cat. no. 15596-026). The biopsy tissue was homogenized on ice using an Ultrasonic Processor (Misonix, Farmingdale, NJ). The tissue homogenate was placed in a sterile 1.5 ml tube containing isopropanol 100% (0.5 mL) and 50 µl of glycogen for RNA precipitation.

After the RNA was precipitated, the pellet was washed three times with 75% ethanol, dried, and then resuspended in 20µl RNAse free water. RNA concentration and purity were determined using ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). cDNA constructs were made for samples that showed a ratio of 260/280 of greater than 1.7. Samples were diluted as follows: one µg RNA diluted in 9.9 µl RNase free water. cDNA was synthesized using the Reverse Transcription System following the manufacturer protocol (Promega, cat. no. A3500). The cDNA samples were placed in a thermal cycler (BIO-RAD, cat. T100), for 1 minute at 25°C, 1.5 hours at 42°C, 5 seconds at 95°C, and left at 4°C until cool.

A TaqMan® Environmental Master Mix 2.0 (Life Tech, cat #4398044) was used for Real Time PCR quantification. 5 µl of cDNA were added to 21 µl PCR reactions to make the PCR reaction mix (1.25 µl gene specific primers, 13.5 µl primer mixtures from Life Tech TaqMan® Gene Expression Assays, and 6.25 µl of RNA free water). The standard curve was constructed from a mixture of cDNA from the sample pool. All samples at two time point baseline, six month and standard curves were run in each PCR plate in duplicate. Primers and probes from Applied Biosystems (Foster City, CA, USA) were used for PCR as follows: COX-1 (PTGS1), Hs00377726_ml), COX-2 (PTGS2, Hs00153133_m1), mPGES1 (PTGES1, Hs01115610_ml), cPGES (PTGES3, Hs00832847_gH), 15 HPGD (HPGD, Hs00168359_ml), EP2 (PTGER2, Hs04183523_m1), and EP4 (PTGER4, Hs00168761_m1). The internal control for normalization was cytokeratin 20 Krt20, a known marker of colonic epithelial cell mass (Hs00300643_m1) [157]. Real-time PCR thermal conditions were as follows: 50°C for 2 minute, 95°C for 10 minute followed by 40 cycles of 95°C for 15 seconds and 60°C for1 minute. The mean efficiencies of the PCR standard curves calculated for each primer were as follows: PTGS1 (95%), PTGS2 (90.4%), PTGES (105%), PTGES3 (97.2%), HPGD (99%), PTGER2 (93%),

PTGER4 (94%), and Krt20 (94%). The standard curve method for quantification was used to calculate the average amount of each target mRNA expression to Krt20 mRNA expression from the standard curve.

Reverse-phase liquid chromatography with tandem mass spectral detection (LC-MS-MS) was used to quantify PGE_2 and PGE_3 as previously described [109]. In short, tissue homogenates were prepared from two frozen colon biopsies. Eicosanoids were extracted with ether prior to LC-MS-MS analysis. Deuterated internal standards (Cayman Chemical, Ann Arbor, MI) and a Luna Phenyl-Hexyl analytical column (2 x 150 mm, 3 μm particle size, Phenomenex, Torrance, CA) were used. Because deuterated internal standard was not available for PGE_3, both were quantified using PGE_2-d_4. Protein content of the homogenate was determined by the Bradford assay. Eicosanoids were expressed as nanogram (ng) of PGE_2 or PGE_3 per milligram (mg) of protein.

GC-MS analysis was used to measure fatty acids from colon homogenates as previously published[158]. Pulverized colon tissue corresponding to one 5 mg biopsy was added to a tube containing 150 μl of ice-cold phosphate buffered saline with 0.1% BHT and 1mM EDTA. Tubes were placed in an ultrasonic processor for two intervals of 30 seconds. A ratio of 1:1 chloroform and methanol was used for extraction of lipids. Prior to GC-MS analysis, methyl esters were prepared with METH-PREP II derivatization reagent (Alltech, Deerfield, IL) [158].

Tissue MicroArray (TMA)

Formalin-fixed, paraffin-embedded tissue blocks (FFPE) of colon biopsy specimens that remained from the study were used to construct a tissue microarray using the methodology of Nocito et al. [184]. Each sample was represented by a single 1 mm diameter core and 32 slides

were made from each TMA. The first and last slides were stained with hematoxylin and eosin (H&E).

Immunohistochemistry Staining and Protein Quantification

Immunohistochemical (IHC) staining was conducted using slides that had the largest number of full-length crypts, as predicted from the H&E stained slides. The IHC was done for the enzymes and receptors that showed higher RNA expression. The PTGS2 protein is known to be expressed in normal colon tissue in very low or undetectable levels. The enzymes selected for IHC quantification were HPGD (n=46), PTGS1 (n=43), PTGES3 (n=24), PTGER2 (n=32) and PTGER4 (n=35). The sample size for each of the antibodies varied depending on the availability of sections with full-length colon crypts suitable for quantification. A total of 32 slides were cut from a tissue micro-array (TMA) created from all available colon tissues. The first and last (1 and 32) slides were stained with H&E to visualize crypts. Immunohistochemical staining for protein of interest was done sequentially using two slides per antibody (one from each side of the TMA). IHC staining was performed on a DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO LSAB+ or Envision+ as detailed in Table 4.1 and diaminobenzidine (DAB) as the chromogen. De-paraffinized TMA sections were labeled with antibodies listed in Table 4.1 at ambient temperature. Microwave epitope retrieval was used prior to staining. Appropriate negative (no primary antibody) and positive controls (tumor tissue) were stained in parallel with each set of antibodies studied. A light counterstain with H&E was used.

The slides were imaged in a microscope with a high resolution Leica Biosystems scanner to generate whole-slide digital scans of all TMA slide. Images were transferred to files for quantification of staining with Aperio ePathology image analysis software (Leica Biosystems).

Although some tissue samples did not show full-length crypts due the direction of cutting or amount of tissue available, those slides were still quantified for the whole tissue reading. For each tissue sample, the whole tissue, the mucosal layer, and the submucosa under the epithelium, using a thickness equivalent to the thickness of the mucosa, were quantified for positive and negative staining. Aperio Image analysis algorithms based on color partitions and intensity of positive staining were used to quantity the proteins of interest. The percentage of the total positive staining for all three areas of the tissue was analyzed for each gene.

Statistical Analysis

After completing quality control checks for all data saved in excel files, the data were transferred into SPSS files. All statistical analyses were conducted using IBM SPSS software version 22 (PASW Statistics, IBM Corporation, Armonk, New York). All variables were checked for normality of the distribution before analyses, and data were transformed as needed. Descriptive statistics were used to evaluate subject characteristics. For making comparisons of interest between treatment arms, two-sample t-tests were used for continuous variables, and chi-square tests were used for categorical variables.

To calculate changes over 6 months in PGE_2, PGE_3 and RNA expression of genes in the PGE_2 pathway using intention to treat principles, linear regression analyses with a random intercept (mixed models) were used. Time, diet group, and the group-by-time interaction were the primary predictors in the mixed models controlling for covariates that can affect gene expression including select baseline variables (age, regular use of non-steroidal anti-inflammatory drugs or NSAIDs, BMI) and percent of dietary goals met. The same statistical analyses were carried out for subgroups defined by NSAID use and overweight/obese status at

baseline. To determine the relationships between changes in PGE_2, PGE_3 and expression of genes in PGE_2 pathway with changes in colon tissue fatty acids over the six months, Spearman correlations coefficients were determined. Spearman correlations were also used to evaluate the relationships between RNA expression and protein expression of the enzymes and receptors in the PGE_2 pathway.

4.4 Results

Characteristics of Subjects

The characteristics of subjects who had tissue biopsies available for RNA quantification are shown in Table 4.2. There were no significant difference in baseline characteristics between subjects in the Mediterranean diet and the Healthy Eating diet groups except that the mean age in the Mediterranean diet arm (55 years) was significantly different from the mean age in the Healthy Eating diet (50 years). Tobacco smoking also was higher in the Mediterranean versus Healthy Eating (9 versus 3 percent), but this was not significantly different between the two dietary groups. However, the detrimental effects of tobacco use on the colon may have negated some of the benefits of this intervention.

Changes in Prostaglandin Levels and Gene Expression over Six Months

Changes in PGE_2, PGE_3 and gene expression were evaluated to determine the effect of the dietary intervention over six months (Table 4.3). After adjusting for age, regular use of non-steroidal anti-inflammatory drugs or NSAIDs, BMI using linear regression analyses with a random intercept (mixed models effects), changes in PGE_2 did not show significant difference in either dietary arms. However, PGE_3 levels showed a significant increase in the Mediterranean

diet arm from baseline to 6 months. PTGES3 was the only biomarkers in the PGE_2 pathway that showed significant increase in RNA expression in both dietary arms over the 6-month period, and it was increased.

NSAID use showed significant effects on PGE_2 and PTGS2 gene expression at baseline as shown in Chapter 3 (Table 3.4).NSAID use was therefore used as a covariate in mixed effects models that evaluated changes in PGE_2 over time ($p=0.002$ for the NSAID effect) (Table 4.3). Therefore, a subgroup analysis for non-NSAID users was conducted to evaluate the effect of the dietary intervention. The results for non-NSAID users were essentially the same as for the entire study group. There was an increase in PGE_3 in the Mediterranean diet group, and an increase in PTGES3 in both dietary groups (Table 4.4). BMI also was a significant covariate in the mixed models for change in PGE_3 ($p=0.016$). In order to evaluate the effects of BMI weight status, a subgroup analysis for normal weight versus overweight/obese subjects was also conducted. The results in the subgroups were the same as in the whole study group (Table 4.5).

Associations between Changes in Prostaglandin Levels and Gene Expression with Changes in Colon Tissue Fatty Acids

We also evaluated relationships between changes in PGE_2, PGE_3 and expression of genes in the PGE_2 pathway with changes in colon tissue fatty acids over the six months. In the Mediterranean diet arm, colon tissue arachidonic acid (AA) concentration showed a significant and strong positive association with PTGES1 ($\rho=0.43$, $p<0.01$). Also, AA tended to associate positively with PTGS2 (($\rho=0.28$, $p=0.06$). EPA was negatively associated with PTGER4 ($\rho=-0.37$, $p<0.05$), while SFA tended to have positive association with PTGS2 ($\rho=0.27$, $p=0.07$). Interestingly, decrease in MUFA showed a significant negative association with increase in

PTGES1 in the Mediterranean diet arm, and decrease in MUFA tended to have negative association with increase in PTGE3 in the Healthy Eating arm. Also, in the Healthy Eating arm, increased AA was associated with an increase in PTGS1, while a decrease in SFA was associated with an increase in PGE_3 (Table 4.6).

Associations between RNA Gene Expression and Protein Levels

In the final part of this study, we aimed to confirm and validate the RNA gene expression results performed by the qrtPCR analysis. First, we quantified protein expression in the whole colon tissue on each slide utilizing quantitative image analysis of slides for which IHC was done to show expression of proteins of interest. HPGD (n=46) showed the highest mean protein level followed by PTGS1 (n=43), PTGES3 (n=24), PTGER4 (n=35), and PTGER2 (n=32), respectively (Figure 4.1). These results matched the RNA gene expression quantification data (Figure 3.2).

We also calculated the association between RNA expression of each gene with its corresponding protein levels in whole tissue in the surface mucosa (epithelium) and the sub mucosal regions, using a depth equivalent to the epithelial layer. The HPGD gene showed significant association with whole tissue, mucosa and submucosa, with the highest association being found with staining in the mucosal region. This likely may be due to the fact that HPGD is highly expressed in the outer mucosa. PTGS1 expression showed significant associations with protein expression all three areas, with the strongest association being found in the submucosa. In contrast, PTGES3 RNA expression showed a strong positive association with whole tissue protein expression, but not when evaluated in the sub regions. RNA expression of both PGE2 receptors: PTGER2 and 4, showed significant associations with their protein expression in all

areas of the colon tissue. However, RNA expression of PTGER2 had stronger associations with protein expression in the submucosa than PTGER4, as shown in Table 4.7 and Figure 4.2 (A-J).

4.5 Discussion

Epidemiological and observational studies have indicated that a Mediterranean diet increases longevity and lowers the risk for most chronic diseases, including colon cancer and other cancers [185, 186]. However, little research has been conducted to understand the mechanisms by which this diet can have a positive effect on lowering colon cancer risk. One hypothesis is that the fat intake component of the Mediterranean diet may affect the PGE_2 metabolic pathway. PGE_2 is an inflammatory mediator strongly associated with colon cancer risk [187].

In this study, we had the opportunity to evaluate the effect of a Healthy Eating diet based on recommendation of the U.S. Department of Agriculture (USDA), versus a Mediterranean diet over a 6 month period on colon biomarkers with a focus on pathways. The major difference between the two diets was in fat intake.

Although recent literature reviews have emphasized the benefits of components of the Mediterranean diet in decreasing the risk for colon cancer [123], this study found that there was little change in the prostaglandin E_2 pathway in the normal colon tissue of individuals at increased risk of colon cancer. Based on the study hypothesis, it was expected that the Mediterranean diet would decrease the genes involved in PGE_2 synthesis (prostaglandin H and E synthases) and increase the gene involved in PGE_2 degradation (PG dehydrogenase). However, in linear mixed models adjusting for age, regular use of non-steroidal anti-inflammatory drugs or NSAIDs, BMI, the only change in the Mediterranean diet arm was an increase in PGE_3. PGE_3 is

derived from eicosapentanoic acid (EPA), omega-3 fatty acids found mainly in fish oil [155, 188]. PGE_3 is a less inflammatory than PGE_2. In lung cancer cells, PGE_3 inhibited tumor cell proliferation and antagonized the effect of PGE_2 [189]. Also, PGE_3 derived from EPA shows differential effects through its ability to diminish colonic stem cell expansion and self-replication relative to PGE2 derived from AA, which promotes colon tumorigenesis [190].

PTGES3 (cPGES3) was the only gene in the PGE_2 metabolic pathway that changed significantly in both dietary arms after six months, and its expression was induced. Cytoplasmic prostaglandin E synthase 3 (cPGES3) is a constitutively expressed enzyme, which has a synergistic relationship with PTGS-1, to produce PGE_2 from arachidonic acid. Although PGE_2 is a critical molecule in colon tumorigenesis, PGE_2 also appears important for normal colon tissue homeostasis and maintenance [15, 145]. In the immune system, PGE_2 generally works to dampen inflammation, but such an effect could allow a tumor to evade immune surveillance. This makes it difficult to evaluate the effects of changes in PGE_2 production on colon tumorigenesis and it may be important to develop methods to study cell-specific PGE_2 production. In addition, stress-induced responses might be more important than basal levels of PGE_2 with spikes in PGE_2 resulting in biological changes leading to carcinogenesis.

There may be several possible reasons for the unexpected lack of change in PGE2 with dietary intervention. It may be difficult to rectify the effects of a diet that is followed for a decades before an intervention is instituted. It is therefore possible that the six months intervention timeframe was not long enough to make a significant impact on the biomarker measured if biological changes have already taken place. Colon epithelial cells have a limited lifetime of less than a week, but nutrient partition into adipose stores and may take many months to equilibrate after dietary change. Since most individuals required 2-3 months to make all the

requested changes, they were only following the intervention diets for 3-4 months. Another potential reason could be due to the role of metabolic and genetic factors in controlling PGE_2 levels, and perhaps diet will have limited impact relative to those. In the normal state, PGE_2 has an important role in tissue repair and it may not be beneficial to reduce the basal level of PGE_2 beyond a threshold level [15].

In our previous findings in Chapter 3, at baseline we found positive relationships between colon PGE_2 concentration, PTGS1 gene expression and saturated fatty acids (SFA) concentrations in colon tissue. We also found that some of the genes in the PGE_2 pathway were associated with colon fatty acid concentrations, including a negative association between PTGS2 and MUFA. Here we evaluated these relationships after dietary intervention. Our results showed there was a significant positive association between changes in arachidonic acid levels and changes in PTGES1 gene expressions in the Mediterranean arm. In addition, we found there were significant negative associations between changes in PGE_3 with changes in SFA in the Healthy Eating diet group, and there was also a negative association between changes in PTGER4 with changes in EPA in the Mediterranean arm (Table 4.6). Changes in MUFA, a non-substrate fatty acid for cyclooxygenases that is consumed at high levels in the Mediterranean diet, showed a negative and significant association with changes in PTGES1 in the Mediterranean diet arm, and significant positive association with changes in PTGES3 in the Healthy Eating arm. Both PTGES enzymes (1 and 3) are responsible for producing PGE_2; however, PTGES1 is an inducible enzyme that works with COX-2 to produce PGE_2 during colon cancer progression [191].

Although the changes in the two enzymes were negatively associated with changes in MUFA, the main dietary source of MUFA in both diet arms were different. MUFA sources in the

Mediterranean diet derived primarily from olive oil and may have anti-inflammatory effects, while the MUFA sources in the Healthy Eating diet may derive from meat and dairy, which may have pro-inflammatory effects on PGE_2 pathway. Whether diet source of MUFA from olive oil (vegetable) or from red meat (animal) exerts different health effects is not known, and further research is needed to investigate such associations. These results highlight the importance of substrate and non- substrate fatty acid availability in the colon tissue for influencing PGE_2 production.

Finally, we confirmed the association between RNA expression and the expression of the corresponding proteins using immunohistochemical (IHC) staining. This was important to address since not all mRNA may be transcribed to its protein [192, 193]. The correlations of relative mRNA expression and positive staining by IHC were all statistically significant when quantifying protein in the whole tissue (Table 4.7). This is understandable since it was evident that each enzyme was expressed in different regions of the colonic mucosa. Evaluation of gene expression, however, could not be done in specific cell types. In addition, the IHC staining was dependent on the nature of the tissue section obtained with regard to presence of full-length crypts. This is a limitation of this study. Another limitation of this study was that only 114 of 212 tissue samples were available for immunohistochemistry.

Strengths of the study are the randomized design and excellent adherence to dietary goals, as reported previously [71]. Although the Mediterranean diet without calorie restriction has been shown to prevent diabetes among subjects with high risk for cardiovascular disease [194-196] no intervention studies have been completed for evaluating cancer risk. The studies that have been done with Mediterranean diets and cancer risk were focused on the

epidemiological associations. The present study is the only study where colon tissue from humans was available for analysis of biomarker of colon cancer risk before and after dietary change.

In summary, the study results showed that both the Healthy Eating and Mediterranean dietary interventions had little effect on PGE_2 concentrations and the expression of genes in its pathway, with the possible exception of PTGES3, which increased with both diets. In the Mediterranean dietary arm, we found that PGE_3 was significantly increased in the colon tissue, which is encouraging since PGE_3 does appear to have anti-inflammatory effects and may prevent colonic tumorigenesis. There was limited evidence that changes in colon fatty acids could affect the prostaglandin pathway, but changes in colon fatty acids in this study were small. These results indicate that other factors not related to overall diet quality may govern inter-individual differences in colon fatty acids and the PGE_2 pathway.

Table 4.1: Antibodies and Experimental Conditions for Immunohistochemical (IHC) Staining

Antibody Name	Manufacturer	Catalog Number	Species	Concentration	Incubation time (minutes)	Epitope retrieval	Detection	Positive? Control Tissue
PTGS1	Abcam, Cambridge, MA	Ab-109025	Rabbit	1:500	60	HIER pH6[a]	LSAB+[c]	Skin
PTGES3	Novus, Littleton, CO	NBP2-19998	Rabbit	1:500	30	HIER pH6[b]	Env+[d]	Gastrointestinal cancer
HPGD	Santa Cruz Biotechnologies, Dallas, TX	SC-48908	Goat	1:50	60	HIER pH6	LSAB+	Cerebellum
PTGER2	R&D systems, Minneapolis, MN	MAB6656	Mouse	1:100	30	HIER pH9	Env+	Gastrointestinal cancer
PTGER4	Cayman Chemicals, Ann Arbor MI	101775	Rabbit	1:500	60	HIER pH6	LSAB+	Kidney

[a] Heat-induced epitope retrieval (HIER) in 10 mM Citrate buffer at pH 6.
[b] HIER in10 mM Tris HCl/1 mM EDTA at pH 9.
[c] Liquid streptavidin biotin plus horseradish peroxidase (LSAB+).
[d] Envision plus horseradish peroxidase (Env+).

Table 4.2: Characteristics of the Study Participants in Each Study Arm[a]

Characteristics	Healthy Eating Diet n=57	Mediterranean Diet n=57
Age, years[b]	50, 14 (range 22–72)	55, 10 (30–82)
Female gender	39, 68.4%	42, 73.7%
Caucasian race	51, 89.5%	48, 84.2%
Married/committed	39, 68.4%	38, 66.7%
College graduate	45, 78.9%	44, 77.2%
BMI, kg/m^2	27, 4 (range 19–34)	27, 4 (range 18–35)
Physical activity	21, 15 (range 1–68)	18, 13 (range 0–55)
Tobacco users[c]	3, 5.3%	9, 15.8%
Alcohol consumption g/day	7, 10 (range 0–46)	6, 7 (range 0–30)
Regular aspirin user	12, 21.1%	12, 21.1%
Cholesterol medication use	6, 10.5%	13, 22.8%
Blood Pressure medication use	7, 12.3%	14, 24.6%
Family history of colon cancer	40, 70.2%	36, 63.2%
History of adenomas	11, 19.3%	13, 22.8%
Both risk factors	6, 10.5%	8, 14.0%

[a] Data are given as mean and SD or number and percent
[b] Significantly different between the two groups.
[c] Tobacco user were marginally significant different p=0.06.

Table 4.3: Concentrations of Prostaglandins and Relative Expression of Genes in Colonic Mucosa over Time

Markers	Healthy Eating Diet		Mediterranean Diet	
	Baseline n=57	Six months n=45	Baseline n= 56	Six months n=45
PGE_2	17,15	17,15	19,13	19, 16
PGE_3	1.2,1.2	1.5,1.7	**1.3,1.1**	**1.8,1.7**[b]
PTGS1	1.1,0.7	1.3,0.9	1.1,0.7	1.2,0.9
PTGS2	0.2,0.2	0.3,0.3	0.2,0.2	0.2,0.2
PTGES1	0.2,0.2	0.2,0.2	0.17,0.12	0.23,0.23
PTGES3	**0.7,1.0**	**0.9,1.2**[b]	**0.5,0.7**	**0.7,1.0**[b]
HPGD	1.4,1.0	1.3,1.0	1.5,1.0	1.5,1.1
PTGER2	0.4,0.31	0.4,0.5	0.3,0.3	0.4,0.5
PTGER4	0.9,0.8	1.1,0.9	0.9,0.7	1.2,0.7

[a]Data shown is for study participants for whom tissue biopsies for RNA quantification were available.
[b] Significantly different than baseline for that diet group, $p < 0.05$. Data shown is mean and SD

Table 4.4: Concentrations of Prostaglandins and Relative Expression of Genes in Colonic Mucosa over Time for non-users of NSAIDs

Markers	Healthy Eating Diet		Mediterranean Diet	
	Baseline n=45	Six months n=34	Baseline n= 44	Six months n=36
PGE_2	20,15	20,314	21,14	21,16
PGE_3	1.2,0.92	1.5, 1.6	**1.4, 1.2**	**2.1, 2.00[a]**
PTGS1	1.2,0.7	1.3, 0.93	1.02, 0.63	1.2, 0.94
PTGS2	0.22, 0.17	0.27, 0.30	0.22, 0.17	0.23, 0.19
PTGES1	0.21, 0.19	0.18, 0.018	0.17, 0.13	0.19, 0.13
PTGES3	**0.7, 1.1**	**0.95, 1.3[a]**	**0.5, 0.7**	**0.75, 1.0[a]**
HPGD	1.3, 0.7	1.4, 1.0	1.3, 0.79	1.3, 0.7
PTGER2	0.34, 0.29	0.47, 0.57	0.35, 0.29	0.39, 0.44
PTGER4	0.97, 0.84	1.14, 1.0	091, 0.68	1.17, 0.74

[a] Significantly different than baseline for that diet group from mixed models, $p < 0.05$. Data shown is actual mean and SD.

Table 4.5: Concentrations of Prostaglandins and Relative Expression of Genes in Colonic Mucosa over Time by Body Weight Status. Data shown is mean and SD

Normal Weight Individuals				
Markers	Healthy Eating Diet		Mediterranean Diet	
	Baseline n=20	Six months n=16	Baseline n=18	Six months n=17
PGE_2	17,10	15,14	21,13	20,13
PGE_3	1.4,1.0	2.0,2.2	**2.0,1.5**	**2.6,2.5** [a]
PTGS1	1.2,0.7	1.1,0.7	1.0,0.7	1.1,1.1
PTGS2	0.19,0.15	0.23,0.13	0.21,0.17	0.2,0.13
PTGES1	0.19,0.14	0.18,0.18	0.19,0.15	0.19,0.15
PTGES3	**0.63,0.77**	**0.83,0.83** [a]	**0.32,0.51**	**0.90,1.32** [a]
HPGD	1.40,0.85	1.5,1.30	1.40,1.10	1.50,1.01
PTGER2	0.37,0.30	0.49,0.73	0.38,0.38	0.38,0.49
PTGER4	0.76,0.55	0.91,0.73	1.0,0.7	1.2,0.9
Overweight or Obese Individuals				
Markers	Healthy Eating Diet		Mediterranean Diet	
	Baseline n=18	Six months n=16	Baseline n=18	Six months n=17
PGE_2	18,16	18,14	18,13	19,17
PGE_3	1.1,1.3	1.2,1.3	**0.9,0.6**	**1.4,1.0** [a]
PTGS1	1.1,0.7	1.2,0.9	1.2,0.7	1.3,0.95
PTGS2	0.22,0.16	0.25,0.3	0.22,0.17	0.24,0.19
PTGES1	0.2,0.19	0.15,0.17	0.16,0.11	0.24,0.26
PTGES3	**0.7,1.1**	**0.95,1.4** [a]	**0.5,0.7**	**0.7,0.7** [a]
HPGD	1.3,1.0	1.2,0.7	1.5,0.9	1.6,1.1
PTGER2	0.34,0.32	0.37,0.35	0.31,0.24	0.43,0.45
PTGER4	1.1,0.9	1.1,0.95	0.9,0.64	1.2,0.64

[a] Significantly different than baseline for that diet group, $p < 0.05$.

Table 4.6: Spearman Correlations Coefficients of Changes in Colon Tissue Fatty Acids with Changes in Prostaglandins and Gene Expression. Change was calculated as the percent changeover 6 months of dietary intervention

	Healthy Eating	Mediterranean	Healthy Eating	Mediterranean	Healthy Eating	Mediterranean	Healthy Eating	Mediterranean
	AA	AA	EPA	EPA	SFA	SFA	MUFA	MUFA
PGE_2	0.21	0.103	-0.01	-0.01	-0.06	0.07	0.16	-0.03
PGE_3	0.12	0.23	0.23	0.09	**-0.30**[a]	0.01	0.18	0.01
HPGD	-0.11	0.12	-0.05	0.00	0.02	0.12	0.15	-0.20
PTGS1	0.28	0.18	-0.02	-0.13	0.14	0.03	-0.06	-0.17
PTGS2	0.08	0.28,	-0.01	-0.001	-0.10	0.27,	0.03	-0.23
PTGES1	0.17	**0.43**[b]	-0.01	-0.01	0.08	0.22	0.06	**-0.32**[a]
PTGES3	0.07	0.04	0.06	-0.13	0.02	0.15	-0.29,	0.15
PTGER2	0.23	0.22	-0.06	-0.12	-0.12	0.27	0.18	-0.19
PTGER4	0.25	0.18	0.07	**-0.37**[a]	0.03	0.20	0.1	0.09

Table 4.7: Spearman Correlation Coefficients of RNA Expression with Protein Expression in Colon. Tissue

	Whole tissue	Mucosa	Submucosa
PTGS1(n=45)	**0.33**[a]	**0.31**[a]	**0.37**[a]
HPGD (n=46)	**0.34**[a]	**0.38**[a]	**0.34**[a]
PTGES3 (n=24)	**0.50**[a]	-0.12	0.21
PTGER2 (n=32)	**0.62**[b]	**0.36**[a]	**0.71**[b]
PTGER4 (n=35)	**0.45**[b]	**0.31**[a]	**0.43**[a]

[a] Correlation is significant at 0.05 level (2-tailed).
[b] Correlation is significant at 0.01 level (2-tailed).

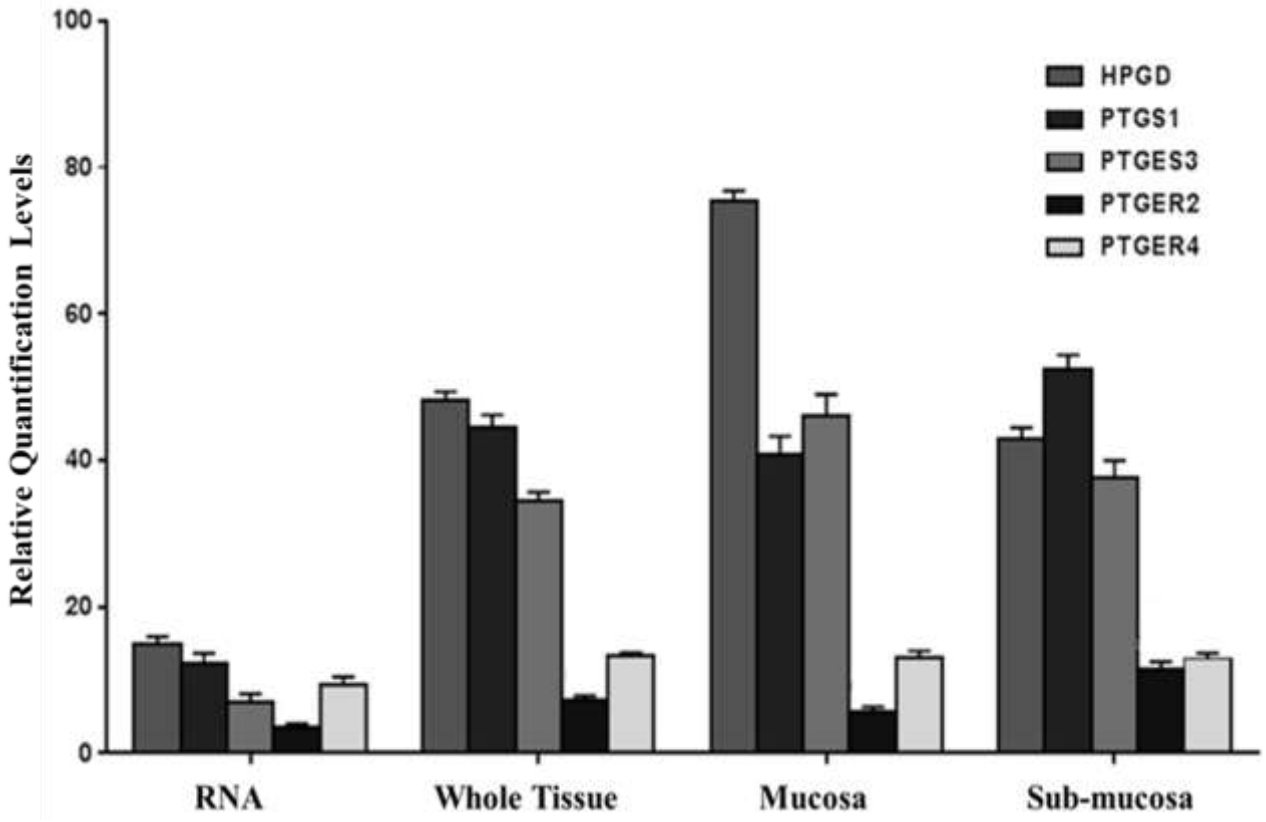

Figure 4.1: RNA and Protein Expressions of Biomarkers in the PGE_2 Pathway

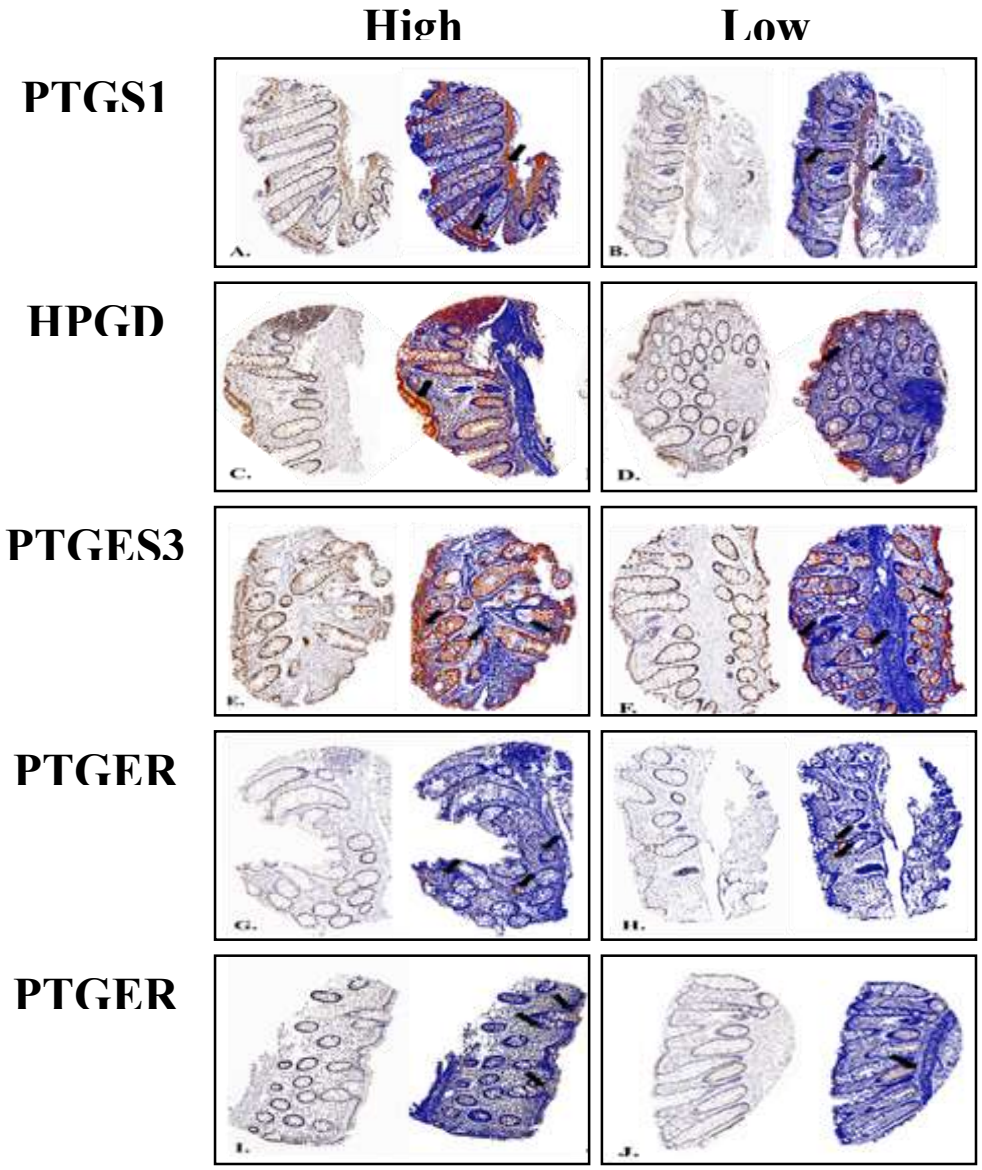

Figure 4.2: Immunohistochemically-stained colon tissue

(A, B), HPGD (C, D), PTGES3 (E, F), PTGER2 (G, H), and PTGER4 (I, J). Examples of biopsies with high expression (left panels) and low expression (right panels) are shown. Each panel shows the original and the false-color image generated using orange, red and brown for light, medium and highly positive pixels, respectively, and blue for negative pixels. Black arrows indicated the area where protein is most highly expressed for each gene: PTGS1 in the outer epithelium and submucosa, HPGD in the outer epithelium, PTGES3 in the epithelial cells, and PTGER2 in the stroma and PTGER4 in the epithelial cells.

CHAPTER 5

Conclusions

5.1 Significance

Despite the fact that colorectal cancer is one of the most preventable diseases, it is still the third most common cancer with the fourth highest mortality rate, and more than one million annual new cases reported worldwide [1]. In the United States, colorectal cancers are also the third most common cancer type; therefore, this is a major public health concern [3]. Identification of cancer preventive strategies is difficult due to the long time frame of cancer induction. In this regard, use of biomarkers of cancer risk is helpful.

Prostaglandin E_2 (PGE_2) is a well-established pro-inflammatory mediator that can serve as a biomarker of colon cancer risk. Problems with measuring PGE_2 in human tissue are that it is unstable compound and that its' production fluctuates rapidly upon tissue injury. Our study controlled for these factors by using timed sample processing protocols, but evaluating expression of genes in the prostaglandin pathway might be more stable to measure and might predict PGE_2 production.

This study evaluated the utility of using expression of genes involved in the PGE_2 as predictors of PGE_2 formation, and this study also evaluated if a Mediterranean diet could affect PGE_2 production in the colon. Dietary change would be a non-toxic alternative to continuous use of non-steroidal anti-inflammatory drugs (NSAIDs) that show gastric toxicity. The Mediterranean diet has been shown to have systemic anti-inflammatory effects and to reduce risk

of CRC [90, 91, 197, 198]. It is also a "whole-diet" approach that may be more beneficial than modulating individual dietary nutrients. For example, dietary fiber has had consistent preventive effect on risk of colon cancer while dietary fiber supplementation has not [113, 114]. The impact of the Mediterranean diet as a whole on the PGE_2 pathway and other biomarkers related to cancer remains unexplored.

5.2 Objectives

The purpose of this dissertation research was to evaluate the effect of a Mediterranean diet, on PGE_2 and expression of genes in its pathway in the colon of persons at increased risk for colon cancer. The hypothesis was that adherence to a Mediterranean diet would be associated with a reduction in formation of PGE_2 and corresponding changes in the expression of genes involved in its metabolic pathway.

Aim 1

The first aim was to establish that adherence to two dietary interventions, Mediterranean and Healthy Eating, was achieved using the exchange lists diets that were developed for both interventions (Chapter 2). We evaluated dietary adherence and both serum and colon tissue biomarkers of dietary intakes over six months of intervention. We found that subjects in both study arms largely achieved their food group goals. However, individuals in the Mediterranean diet arm took longer to achieve their dietary goals than in the Healthy Eating diet arm. This most likely was due to the fact that the Mediterranean diet arm had a greater number of dietary goals.

Another interesting finding was that subjects in the Healthy Eating arm exceeded their goals for fruit and vegetables, and this resulted in similar fruit and vegetable intakes in both

study arms. This indicates that the more modest exchange list goals for fruit and vegetable consumption in the Healthy Eating arm, derived from the Healthy People 2010 guidelines, were sufficient to increase both quantity and variety of fruit and vegetable intakes similar to that in the Mediterranean arm with more complex goals. This is significant because the reported dietary changes were accompanied by increased concentrations of several serum and colon carotenoids. These findings have important implications for the design of dietary guidelines since increased concentrations of colon carotenoids are found in normal versus polyp tissue and appear to have beneficial effects on colon cancer recurrence [56, 199, 200].

The Mediterranean diet arm was unique in increasing the intake of monounsaturated fatty acids (MUFA) and Omega-3 fatty acids as compared to the Healthy Eating diet arm. These increases were found to be reflected in serum fatty acid concentrations [122, 123]. Carotenoids and fatty acids in colon tissue were, however, were less responsive to reported changes in diet than serum carotenoids [201]. One possible reason could be that the 6-month time frame was inadequate for accumulation of carotenoids in tissues. Another factor may be the role of metabolic and genetic factors in regulating colon tissue micronutrients.

Unexpectedly, we found there was a significant weight loss in the overweight and obese subjects randomized to the Mediterranean diet. This may be due to the high intakes of MUFA. Data on post-prandial fat oxidation indicates that MUFA is oxidized more readily than SFA and therefore would be less likely to increase adipose stores when energy intakes are equal [124, 125]. This may be one reason why high MUFA diets have been associated with lower abdominal obesity [202].

Aim 2

Aim 2 evaluated the factors that contributed to inter- individual variability in PGE_2 concentrations and in expression of genes in its pathway before dietary intervention, at study entry (Chapter 3). The highest mRNA expression was for 15-PGDH (HPGD) and COX-1 (PTGS1), as could be anticipated for normal colon tissue. In contrast, we found that COX-2 (PTGS2) and mPGES1 (PTGES1) had the lowest mean levels of mRNA expression among the quantified genes. High variability of gene expression was found among subjects, especially for 15-PGDH, and future work could evaluate the etiology of this variability. Expression of 15-PGDH, however, did not predict PGE_2 concentrations in the colon. The most important finding was that PTGS1 mRNA expression and saturated fatty acids (SFA) positively predicted colon PGE_2 in linear regression models that included NSAID use, age, gender and smoking. This finding is consistent with studies showing that saturated fatty acids activate inflammatory pathways through toll-like receptors (TLR) [174, 203, 204]. SFA also can activate COX through an allosteric mechanism [173]. As a result, we propose that high dietary intake of SFA may be linked to increased colon cancer risk in part through elevation of colon PGE_2.

Aim 3

In Aim 3, we evaluated whether the Mediterranean dietary intervention affected PGE_2 concentrations and gene expression of enzymes and receptors in the PGE_2 metabolic pathway (Chapter 4). We hypothesized that the Mediterranean diet would act to reduce PGE_2 formation. However, we found that after six months of dietary intervention, there was very little change in PGE_2. On the other hand, PGE_3 was increased in the colon of subjects randomized to the Mediterranean diet group. PGE_3 is derived from COX-mediated metabolism of eicosapentaenoic acid (EPA), an omega3 fatty acid found mainly in fish oil [155, 205]. PGE_3 has anti-inflammatory properties, inhibits colon epithelial cell proliferation, induces colon tumor cell

apoptosis and inhibits colon stem cell self-replication [188-190, 206, 207]. We also evaluate the relationships between changes in PGE_2, fatty acids and gene expression after six months of intervention. We found a positive association between changes in colon arachidonic acid and changes in PTGS1, and a negative association between changes in SFA and changes in PGE_3 in the colon. This suggests involvement of substrate and non-substrate fatty acid availability in regulating PGE_2 synthesis. Finally, we confirmed the association between mRNA gene expression and its corresponding protein levels by IHC to validate our results.

5.3 Limitations and Strengths of the study

Limitations of this work included the fact that subjects tended to be well-educated and Caucasian, which is not representative of the entire U.S. population. Another limitation was that only 114 of 212 tissue samples were available for protein quantification. Furthermore, the duration of the study was only six months and this may not be sufficient time to allow dietary intervention to fully affect changes in colon tissue fatty acids and carotenoids.

Strengths of the study included the randomized design and the novel intervention methods that resulted in good participant adherence. Additionally, it is one of the few studies where colon tissue from humans was available for analysis before and after dietary change. This was also the first study to evaluate relationships between dietary intakes, tissue nutrient concentrations, PGE_2 concentrations and expression of genes in the PGE_2 pathway among persons at high risk for colon cancer.

5.4 Summary and Implications

This thesis research indicated significant associations of PGE_2 concentration with expression of PTGS1 and with the saturated fatty acids in human colon tissue. PTGS2 has often been utilized as a target for colon cancer prevention, and this was based on observations of the induction of PTGS2 during colon carcinogenesis. In persons with normal tissue however, these results indicate that PTGS1 would be a target for prevention. The relationships of colon PGE_2 with SFA is also potentially important and intriguing since dietary intervention did not greatly affect tissue fatty acids. Future studies could look at metabolic and genetic factors that govern the nature of the fatty acids that are stored in colon tissue. Furthermore, intervention studies with a longer duration more than six months may be implemented to allow enough time for the diet to affect stores of fat-soluble nutrients[208].

The findings also revealed that mRNA expression of genes in the PGE_2 pathway was highly variable among individuals. This points to future research needs aimed at deriving a better understanding of these inter-individual differences. The methods developed here for inducing dietary changes and for obtaining colon biopsy tissues in a consistent way open the possibility for these and other types of research questions pertaining to colon cancer prevention to be answered in human studies.

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