Anti-inflammatory Effects of Cruciferous and Apiaceous Vegetables in C57BL/6J Mice Colon

Rosa Moreno Narvaez

University of Arkansas, Fayetteville

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Anti-inflammatory Effects of Cruciferous and Apiaceous Vegetables in C57BL/6J Mice Colon

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Cell and Molecular Biology

by

Rosa Moreno Narvaez
Universidad Nacional Autónoma de Nicaragua, León.
Bachelor of Biomedical Sciences, 2012

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University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

________________________________________
Sabrina Trudo, Ph.D.
Thesis Director

________________________________________
Jae Kyeom Kim, Ph.D.  Gisela F. Erf, Ph.D.
Committee Member  Committee Member
Abstract

Previous studies have demonstrated chemo-preventive potential of cruciferous and apiaceous vegetables against colon cancer. Colon inflammation is one condition closely related with colon cancer initiation. Therefore, we wanted to compare if total western diet (TWD) was as pro-inflammatory as died-induced obesity (DIO) and a better dietary model for human conditions, and determine if diet supplementation with cruciferous (broccoli, watercress and cabbage) or apiaceous vegetables (celery and parsnip) could reduce dietary inflammation, and which vegetable was more effective.

Male CBL57/6J mice were fed chow for seven days, on day eight, mice were assigned to one of the following diets: American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation (AIN-93G), DIO, DIO+21% cruciferous (DC), DIO+21% apiaceous (DA), TWD, TWD+21% cruciferous (TC), and TWD+21% apiaceous (TA). On day 84 blood, colon, and liver were collected and stored at -80°C. Cytokine were measured in plasma (ARY006 array), and pro-inflammatory related genes were measured in colon tissue (PAMM-011Z array).

The DIO group did not differ in weight gain compared to any other group. Some cytokines signals in DA were likely to be increased when compared to DIO (G-CSF, IL-5, IL-6 , IL-7, I-TAC, TNF-α, TREM-1; p < 0.005) and when compared to DC (G-CSF, IL-2, IL-5, IL-7, IL-23, IP-10, I-TAC, M-CSF, TARC, TIMP-1, TNF-α; p < 0.005). Bmp2 had a higher mRNA expression in all diets compared to basal diet AIN-93G; p= 0.001. Ccl1 had similar mRNA expression in all diets compared to AIN-93G and DIO; p= 0.001. Ccl19 mRNA was equality expressed in all diets except for DIO; p= 0.000. Ccl4 had a higher mRNA expression in TC diet only when compare with DIO and DC; p= 0.058.
DIO and TWD did not induce inflammation or obesity. Vegetable-supplemented diets induced secretion of some pro-inflammatory cytokines. Cruciferous had a smaller inflammatory response compared to apiaceous vegetables. Although no beneficial effect was seen of vegetables on inflammatory pathways, we do not negate potential chemo-preventive effects by other mechanisms.

Colon Cancer, inflammation, cruciferous vegetables and apiaceous vegetables.
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Dedication

To Rosa Elba Morales Obando, an example of a life in service to others.
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Chapter 1: Introduction

Colorectal cancer (CRC) is the third most common cancer diagnosed worldwide and the second leading cause of cancer death in the United States (Thélin & Sikka, 2015). Worldwide epidemiological studies have documented the highest rates of colorectal cancer in developed countries with western-style diets, which are diets high in fats, red meats, and refined grains while low in fiber and fruit consumption (Arnold et al., 2017; Boyle & Langman, 2000). Furthermore, several studies show that migrants and their children acquired the colorectal cancer risk of their host countries, demonstrating that colorectal cancer is associated with environmental factors, diet being part of that environment (Haggar & Boushey, 2009; Santarelli, Pierre, & Corpet, 2008). Even with advances in the treatment of colorectal cancer, the survival rates improve too slowly. Therefore, colorectal cancer prevention is a major goal for public health and the understanding of the mechanism of colorectal cancer prevention would improve prevention strategies (Santarelli et al., 2008).

The effects of diet in the incidence of colorectal cancer is related to whether the diet is pro-inflammatory or anti-inflammatory. Several studies proposed possible pathways by which pro-inflammatory foods, such as high fat foods and red meat, may increase risk, these include the presence of heme iron and the production of carcinogens such as polycyclic aromatic hydrocarbons (Santarelli et al., 2008), but there are comparatively fewer studies about anti-inflammatory foods and even fewer about possible mechanisms responsible for their anti-inflammatory effects. One of these mechanisms include the suppression of NF-κB, (which is a central mediator of inflammation) by isothiocyanates present in cruciferous vegetables (Pahl, 1999).
This type of vegetable has also been studied for its protective effects against colon cancer and some mechanisms include: the regulation of epigenetic and the metabolism xenobiotics, inhibition of cell proliferation and tumorigenesis, and their antioxidant activity (Pan et al., 2018). Apiaceous vegetables rich in furanocoumarins and polyacetylenes, is another group that has also shown chemo-preventing effects against colon cancer by the inhibition of cytochrome P450 (Boyle & Langman, 2000; J. Kim, Gallaher, Chen, Yao, & Trudo, 2014; J. Kim et al., 2017; Peterson, Lampe, Bammler, Gross-Steinmeyer, & Eaton, 2006).

It is important to mention that most studies have been carried out in animal models (rats, mice), testing individual compounds, but not the use of whole vegetables or a combination of different classes of vegetables as would be consumed in real life. In addition, these studies have used a high-fat diet (60% fat) as the background diet of their studies. However, intake of 60% of calories from fat is extreme and unlikely in real life settings. For these reasons, the aims of our study were:

1) To compare the intestinal inflammatory response of two background diets, diet induced obesity (DIO) and total western diet (TWD) in C57BL/6J mice, and identify which one of these rodent diets would serve as a better dietary model of inflammation that reflects the human condition;

2) To determine if supplementation with two classes of vegetables, cruciferous (broccoli, cabbage, and watercress) and apiaceous (celery, and parsnips), can reduce the inflammatory response to an inflammation-producing diet, and which class of vegetable is more effective.
We hypothesized that TWD would be as pro-inflammatory as DIO, and therefore a better dietary model of inflammation that reflects the human condition. Furthermore, we also expected that vegetable supplementation would reduce the inflammatory response produced by diet, through the modulation of keys signaling pathways such as: NF-κB, Wnt/β catenin, or MAPK.
Chapter 2: Literature Review

Colon Cancer Worldwide and in the United States of America

Colorectal cancer continues to be the third most common cancer worldwide and the fourth most common cause of cancer death with nearly 1.4 million new cases and 700,000 deaths reported in 2012. It is predicted that the worldwide incidence of colorectal cancer will increase to 2.4 million by 2035. About two thirds of colorectal cancer cases in 2012 occurred in countries with high or very high development indices (World Cancer Research Fund/American Institute for Cancer Research, 2017).

In terms of geographical distribution, the Human Development Index (HDI) helps to compare the trends in incidence and mortality rates between developed and developing countries. There is an increasing incidence and mortality in countries with medium to high HDI, in particular Eastern Europe, Asia, and South America. Stabilizing or decreasing mortality trends are only observed in highly developed countries like USA, Australia, New Zealand, and several Western European countries. Females tend to have lower incidence rates than males, maintaining this pattern across the globe (Arnold et al., 2017).

In the United States, colorectal cancer is the third most common cancer and the second leading cause of death. In 2018, it was estimated to have caused 140,250 new cases representing 8.1% of all new cancer cases. From these, 97,220 cases corresponded to colon cancer and 43,030 to rectal cancer. As for mortality colorectal cancer, it is estimated to cause 50,630 deaths representing 8.3% of all cancer deaths in 2018. Colorectal cancer is more common in men than women and among those of African American descent (Noone AM, 2018).
What is Colon Cancer?

The National Cancer Institute Dictionary defines cancer as the term for diseases in which abnormal cells divide without control and can invade nearby tissues. These cells can also spread to other parts of the body through the blood and lymph systems. Colon cancer is a cancer that forms in the tissues of the colon. Rectal cancer and colon cancer are generally grouped together because of their many common features. In this case, the term colorectal cancer is used to refer to the cancer that starts in the colon or the rectum (American Cancer Society, 2017).

Precursor lesions of colorectal carcinoma.

There are many types of colorectal cancer, but around 96% of colorectal cancers are classified as adenocarcinomas. Some other less common types of colorectal cancer are: carcinoid tumors that start in hormone-making cells in the intestine; gastrointestinal stromal tumors that start in the interstitial cells of Cajal, and lymphomas that start in the lymph nodes, but can also start in the digestive system (for example, Non-Hodgkin Lymphoma). In addition, sarcomas, which are very rare, can be initiated in blood vessels, muscle, or connective tissues in the walls of the colon and the rectum (American Cancer Society, 2017). Since the majority of colorectal cancers are adenocarcinomas, we will focus on this type of cancer and its classification in this review.

Adenocarcinomas.

Adenocarcinomas start in the glandular epithelial tissues of the colon and the rectum. Polyps are the most common precursor lesion of this type of cancer. According to their shape, lesions can also be classified as (a) pedunculated, if it has a mushroom-like shape; (b) sessile, if
it is elevated but completely attached to the colon wall; (c) flat, if it is elevated less than half of the diameter of the lesion; and (d) depressed, if part of the lesion surface is below the level of the surrounding mucosa. Furthermore, precursor lesions can also include syndromes such as inflammatory bowel disease and hamartomatous polyposis syndromes. Colorectal epithelial polyps have a complex classification and are a broad group of dysplastic and nondysplastic neoplasms, many of which are widely recognized to confer risk of colorectal carcinoma.

*Nondysplastic serrated polyps.*

This type of polyp is divided in two groups (a) sessile serrated polyps with proliferative abnormalities and (b) hyperplastic polyps without proliferative abnormalities (American Cancer Society, 2017). The latter are the most common type of polyps in the colorectum. They are pale, sessile polyps with smooth contours and may display a layer of mucus on the surface (Waye JD, 1990). Occasionally, cells are multinucleated and likely to represent a degenerative phenomenon (Kambham, Troxell, & Longacre, 2005). Mutually exclusive KRAS and BRAF mutations (genes coding for proteins involved in cell signaling and that play an important role in the transformation of small to intermediate sized adenomas) are frequently present in hyperplastic polyps, therefore challenging the notion that they are nonneoplastic lesions and lack malignant potential. KRAS mutations are detected in up to 47% of hyperplastic polyps while BRAF mutations are detected in 40% to 76% (O'Brien et al., 2006). The other type of nondysplastic polyps are sessile serrated polyps, which are more common among women, unlike hyperplastic polyps. Sessile serrated polyps are present in 2% to 9% of endoscopically rejected polyps and 7% to 21% of all nondysplastic serrated polyps (Higuchi & Jass, 2004; Torlakovic, Skovlund, Snover, Torlakovic, & Nesland, 2003). The crypts of these polyps are dilated at their bases,
frequently with branching or budding above the muscularis mucosae (Torlakovic et al., 2003; Torlakovic & Snover, 1996).

**Conventional adenomas.**

This type of lesion is present in 40% to 60% of removed colorectal polyps. The number and size of the polyps are associated with the risk of cancer development (Winawer SJ, 1993). Adenomas can be defined as red, multilobulated masses that are either plaque-like or pedunculated (O’Brien et al., 2004).

According to their morphological appearances, adenomas are classified into three categories: tubular, villous, and tubulovillous. Tubular adenomas have at least a 75% volume of tubular or branched crypts, while villous adenomas have at least a 75% volume of slender fronds of a neoplastic epithelium. Lastly, tubulovillous adenomas only have a 25% to 75% volume limited to villous polyps (Winawer SJ, 1993). By definition, all adenomas are considered dysplastic and, depending on some cytologic and architectural features, the degree of dysplasia is classified as low-grade dysplasia and high-grade dysplasia.

In low-grade dysplasia, crypts that are prominent infoldings of the epithelium narrower at their bases compared to the surface have a variable size and are not oriented towards the muscularis mucosae. The nuclei of cells maintain their position at the base of the cell and nuclei are elongated with small nucleoli. Apoptotic cells are likely to be detected in the deep or superficial positions of the crypts. A rich neutrophils infiltrate may also be present. On the other hand, in high-grade dysplasia, crypts have a cribriform growth and irregular budding. The cells in this type of crypt are stratified, with prominent nucleoli, mitotic figures, and luminal necrosis (Fenoglio, Kaye, & Lane, 1973).
The majority of colorectal adenocarcinomas develop via the adenoma-carcinoma sequence. This is initiated by the adenomatous polyposis coli (APC) mutation, failing to participate in the complex that sequesters and facilitates β-catenin cytoplasmic degradation. Then, APC-β-catenin/Wnt signaling allows cytoplasmic accumulation of β-catenin, which is then translocated to the nucleus facilitating transcription of protooncogenes and driving cell proliferation. 80% of adenomas have an APC inactivating mutation, which nearly all show aberrant nuclear β-catenin immunoeexpression, indicating that abnormalities in the Wnt signaling pathway are universal in this type of lesion.

_Serrated adenomas._

This type of polyp is uncommon, accounting for about 1% to 2% of endoscopically removed colorectal adenomas (Longacre & Fenoglio-Preiser, 1990). Serrated adenomas have tubules and villous projections of the lamina propria that support crypts with convoluted architecture. Non-goblet epithelial cells are the most affected: the nuclei are elongated or penicillate with small nucleoli and smooth contours (Yantiss, Oh, Chen, Redston, & Odze, 2007). Abnormalities in the APC/β-catenin/Wnt signaling are found in 13% to 20% of serrated adenomas and up to 70% of serrated adenomas host KRAS- Mutations (O'Brien et al., 2006; Sawyer et al., 2002).

_Sessile serrated polyps with dysplasia._

This type of lesion is rare, accounting for 1% of all endoscopically removed polyps, and is more frequent in women than men aged 50 or older. The pathological features are heterogeneous, representing an intermediate stage in the progression of sessile serrated polyps to
invasive carcinoma (via DNA methylation and MSI). KRAS mutations are identified in less than 10% of mixed polyps, while BRAF mutations are identified in 40% to 89% (Chan, Zhao, Leung, & Yuen, 2003; Gala & Chung, 2011).

**Risk Factors of Colon Cancer**

**Nonmodifiable risk factors.**

*Family history.*

Roughly 10% of colorectal cancer cases are hereditary. They are caused by inherited gene mutations and can be grouped in two main categories: Hereditary Nonpolyposis Colorectal Cancer (HNPCC) and Familial Adenomatous Polyposis (FAP). HNPCC is caused by changes in DNA repair genes; for example, a mutation in one of the DNA repair enzyme genes like MLH1, MSH2, MLH3, MSH6, PMS1, or PMS2 (Kinzler & Vogelstein, 1996). The lifetime risk of colorectal cancer in people with HNPCC may be as high as 80%. A large portion of FAP is caused by a mutation in the APC gene (Feldman, Friedman, & Brandt, 2010). All individuals diagnosed with FAP will develop colon cancer if the colon has not been removed by the age of 40. Having a first-degree relative affected by colorectal cancer is another factor that can increase by two- to three-fold the risk of sporadic cancer. This risk can be even higher if the relative was younger than 45 years old when diagnosed or if more than one first-degree relative was diagnosed. A family history of adenomatous polyps also increases the risk of colorectal cancer (American Cancer Society, 2017; Haggar & Boushey, 2009).
**Personal history.**

Personal history plays an important role in colorectal cancer risk. Adenomatous polyps are precursor lesions of colorectal cancer and it has been reported that around 95% of sporadic colorectal cancer develops from these lesions (American Cancer Society, 2017). The risk of developing colorectal cancer is higher in individuals with a history of adenomatous polyps than in individuals with no personal history. Similarly, a personal history of inflammatory bowel disease represents a relative risk of colorectal cancer between 4- to 20-fold (Feldman et al., 2010; Haggar & Boushey, 2009).

**Age.**

The American Cancer Society recognizes an increase in the detection of colorectal cancer after the age of 40, which rapidly increases after the age of 50. Nonetheless, recent studies have reported an increasing incidence among younger people (O'Connell, Maggard, Livingston, & Cifford, 2004).

**Modifiable risk factors.**

**Body weight and physical activity.**

Excess body weight and physical inactivity are two interrelated risk factors that are responsible for about one-fourth to a third of colorectal cancers (Haggar & Boushey, 2009). Obesity can be responsible for the lack of physical activity in daily routines in men and women (Campbell et al., 2007; De Jong et al., 2005). On the other hand, higher overall levels of physical activity are associated with a lower risk of colorectal cancer. The increase of circulating estrogens and decrease of insulin sensitivity are some biological correlations of obesity that are
believed to increase cancer risk, particularly associated with excess abdominal adiposity independent of overall body adiposity. The increased risk of colorectal cancer associated with body weight and physical activity may not only be due to an increased energy intake but may reflect differences in metabolic efficiency (De Jong et al., 2005).

**Alcohol.**

An increased risk of developing colorectal cancer may be associated with regular alcohol consumption (Zisman, Nickolov, Brand, Gorchow, & Roy, 2006). Some of the mechanisms associated with alcohol consumption and cancer are: the production of acetaldehyde, as the first metabolite of ethanol oxidation (which can function as a carcinogen), the induction of cytochrome P-450E1 that leads to the generation of reactive oxygen species, and promotes the activation of various procarcinogens. Alcohol may also function as a solvent and enhance penetration of other carcinogens. In the presence of alcohol, some DNA mutations caused by smoking are less efficiently repaired (Pöschl & Seitz, 2004). Moreover, a diet low in essential nutrients is common in high alcohol consumers which can make tissues susceptible to carcinogenesis (Marmot et al., 2007).

**Cigarette smoking.**

Smoking is responsible for about 12% of colorectal cancer deaths (Zisman et al., 2006). Evidence has shown that smoking is an important factor in the formation and growth rate of adenomatous polyps which are documented precursor lesions of colorectal cancer (Botteri, Iodice, Raimondi, Maisonneuve, & Lowenfels, 2008). Long-term smoking has also been associated with larger polyps found in the colon and rectum (Tsong et al., 2007; Zisman et al., 2006). Alkaloids, phenolic compounds, volatile aldehydes, polycyclic aromatic hydrocarbons,
tobacco specific nitrosamines and heavy metals, are classes of cigarette compounds that are known to induce free radicals and possess toxic and carcinogenic activities (Zhang et al., 2012). These compounds could induce tumorigenesis and promote cancer development in various sections of the gastrointestinal tract through different mechanisms including, activation of nicotinic acetylcholine receptors, the formation of DNA adducts, the stimulation of tumor angiogenesis, and modulation of immune response (Li et al., 2014).

**Environmental risk factors.**

Cultural, social, and lifestyle factors are among the broadly defined “environmental factors.” Migration and urban residence have been reported to increase the risk of colorectal cancer. For example, the offspring of Japanese migrants to the United States have a three to four times higher incidence of colorectal cancer than the Japanese living in Japan (Boyle & Langman, 2000). Diets are part of the environmental factor, and the main focus of our research.

**Diet.**

Some diets are associated with a higher incidence of colorectal cancer. These diets include a high consumption of red or processed meat, a high consumption of fat, and a low consumption of calcium, fiber, fruit, and vegetables (Haggar & Boushey, 2009). The positive association of meat consumption is stronger for colon cancer than rectal cancer (Larsson & Wolk, 2006). Animal fat is the main fat source associated with colorectal cancer, while fiber intake may be responsible for the geographical differences in colorectal cancer rates observed between Africa and Westernized countries (World Cancer Research Fund International; Fund & Research, 2007; Haggar & Boushey, 2009; 2017; Larsson & Wolk, 2006).
Inflammation and Colon Cancer

Inflammation is a mechanism of the immune system as the first response to infection. In the context of tissue injury, inflammation could be defined as: “a multifactorial network of chemical signals to initiate and maintain a host response designed to heal the afflicted tissue.” The main components of inflammation are cells (neutrophils, eosinophils, monocytes, macrophages) and chemokines (Coussens & Werb, 2002).

Role of inflammation in colon cancer risk.

The relationship between inflammation and cancer is well accepted. This relationship has been studied for centuries. One can refer to Virchow in 1863, who associated inflammation with cancer (Fran Balkwill & Mantovani, 2001). In his hypothesis, he defined cancer as solely cell proliferation, caused by some irritants, tissue injury, and the inflammatory response that accompanied the injury. With the evolution of genetic technologies, many cancer pathways have been discovered, demonstrating that cancer is not only a matter of cell proliferation but includes a series of factors such as growth factors, an environment rich in inflammatory cells, active stroma, and DNA- damage promoting agents (Coussens & Werb, 2002).

The relationship between colon inflammation and the development of colorectal cancer has been studied in different types of colorectal cancer including sporadic and hereditary cancer. Colitis-Associated cancer (CAC) is a subtype of colorectal cancer that might be most relevant to this relationship and is associated with inflammatory bowel disease (IBD). Moreover, the molecular mechanism by which inflammation promotes cancer development can vary from CAC and other types of colorectal cancer. This mechanism can also vary depending on which step of colon tumorigenesis inflammation is taking place, and it can include tumor initiation, promotion,
progression, and metastasis (Terzić, Grivennikov, Karin, & Karin, 2010). Furthermore, chronic inflammation is responsible for oxidative damages to DNA, which lead to p53 mutations in inflamed epithelium and in tumor cells (Kraus & Arber, 2009). The mutations induced by inflammation can be a result of the direct oxidation of reactive oxygen species and the inactivation of mismatch repair enzymes at protein level (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009; Hussain, Hofseth, & Harris, 2003). Additionally, mouse and human studies have shown that DNA methyl transferases (Dnmt) 1 and 3 can be induced during inflammation silencing several target genes in colon cancer, but the inactivation of Dnmt resulted in the decrease of progression and suppression of tumor formation (Colotta et al., 2009; Hahn et al., 2008).

**The immune system in colon cancer.**

Both CRC and CAC tumors are formed by different types of immune cells. Cells of the innate immune system that can be detected in these tumors include: neutrophils, mast cells, natural killer cells, dendritic cells (DCs) and tumor-associated macrophages (Atreya, Atreya, & Neurath, 2008). In advance tumors, a specific subset of cells can be detected, named myeloid-derived suppressor cells, which help suppressing antitumor immune responses (Gabrilovich & Nagaraj, 2009). Cells from the adaptive immune system are also recruited and they may have pro- or anti-tumorigenic roles (Erdman et al., 2005).

**Cytokine Signaling and Tumor Promotion.**

The majority of tumor-promoting cytokines activate receptors on intestinal epithelial cells which activate oncogenic transcription factors and other oncogenic signaling pathways. Tumor Necrosis Factor (TNF), IL-6, and IL-1 are cytokines that promote colorectal cancer and CAC
tumor development. In contrast, IL-10 and TGF-β are cytokines that inhibit colorectal tumorigenesis (Christoph Becker et al., 2004; S. Wang, Liu, Wang, & Zhang, 2009). Frequently, in early states of CAC development tumor-promoting cytokines are produced by lamina propria macrophages and DC and by T cells in a late stage of progression (Christoph Becker et al., 2004; Grivennikov et al., 2009).

The activated protease ADAM17 mediates shedding of the membrane-bound IL-6 receptor. IL-6 receptor in its soluble form activates transcription factor STAT3 via trans-signaling. IL-6 stimulates proliferation of premalignant enterocytes and tumor growth (C Becker et al., 2005; Christoph Becker et al., 2004). STAT3 can also be activated by different cytokines (IL-11, IL-22), growth factors (hepatocyte growth factor), epithelial growth factor receptor ligands (TGF-α, EGF), and oncogenic tyrosine kinases (c-Met, Src) (Pickert et al., 2009). Once activated, STAT3 protects the gastrointestinal epithelium and stimulates its regeneration, promoting proliferation of pre-malignant cells (Grivennikov et al., 2009). The activation of STAT3 produces the expression of Bcl2 or Bcl-xL (which are antiapoptotic genes), Cyclin D1 or c-Myc (which are proliferative genes), and vascular endothelial growth factor (VEGF) (Naugler & Karin, 2008a). The activation of STAT3 also induces prolonged activation of the transcription factor NF-κB and both transcription factors activate genes required for every aspect of cancer development (Lee et al., 2009). IL-6 promotes Th17 cell differentiation and regulates the survival of proinflammatory Th1 cells while inhibiting T regulatory (Treg) cells (Bettelli et al., 2006; Dominitzki et al., 2007).

TNF is produced during initial inflammatory response. Some of its properties include production of other cytokines, chemokines and endothelial adhesion molecules, increase of vascular permeability, and recruitment of activated leukocytes. As a result, TNF can be
considered a tumor-promoting factor because it promotes inflammation angiogenesis and tumor dissemination (Frances Balkwill, 2009). TNF also activates transcription factors AP-1 and NF-κB (Frances Balkwill, 2009; Kruglov et al., 2008). Moreover, TNF is the main factor responsible for inactivation of transcription factor T-bet in DCs that led to spontaneous intestinal inflammation and CAC (Garrett et al., 2009).

Cytokines, that are upregulated in various types of cancers include IL-1 and IL-23. The mechanisms of action of IL-23 in CRC and CAC are not clear but it could be due to the effect of IL-23 on differentiation and propagation of Th17 cells or its effects in monocytes, memory T cells, and Tregs (Garlanda et al., 2007; Wirtz & Neurath, 2007).

TGF-β has a contradictory role in cancer development. It displays an antitumorigenic role by inhibiting proliferation, stimulating apoptosis, and by suppressing pro-tumorigenic cytokine expression (LI Yang & Moses, 2008). However, during malignant progression, TGF-β promotes epithelial-mesenchymal transition and suppresses the antitumor activity of immune cells to facilitate metastasis (Bierie & Moses, 2010; Linda Yang, Belaguli, & Berger, 2009).

Proposed Inflammatory pathways.

**NF-κB pathway**

NF-κB transcription factors are dimers assembled through five subunits: p105/p50, p100/p52, RelA (p65), c-Rel, and RelB. Active NF-κB signaling in premalignant and inflammatory cells are responsible for the activation of the majority of tumor-promoting cytokines (Karin, 2006). NF-κB activation can increase cell proliferation and angiogenesis, inhibit cell death, and promote cell invasion and metastasis, all of which supports tumorigenesis (Naugler & Karin, 2008b). Furthermore, the five subunits of NF-κB can be held inactive as
precursors in the cytoplasm or by the inhibitor of κB, Ikb protein. A simple explanation of the activation of NF-κB via the classical route would be: 1) pattern recognition receptors, TNF, IL1, and IL17 are the initial signal transduction pathways; 2) The initial signals activate the IKB kinase (IKK) complex (mostly IKK GAMMA and BETA), which phosphorylates the IKBs; 3) IKBs are targeted for ubiquitination and ultimate degradation in proteasome; and 4) after IKBs degradation, NF-κB is liberated, mainly p50/p65, to migrate to nucleus and regulate gene transcription (Vallabhapurapu & Karin, 2009). Another route to activate NF-κB is activation by cytokines such as RANKL and lymphotoxin-B that would activate IKK alpha homodimer which liberates mainly p52/ReIB NF-κB (Karin, 2006). In mouse studies, conditional ablation of IKKB in enterocytes decreased tumor formation by 80% but did not affect tumor size, suggesting that the activation of NF-κB is part of an early stage of tumor promotion rather than progression or growth (Greten et al., 2004).

**Wnt/β-catenin pathway**

The Wnt/β catenin pathway plays a central role in the development of colorectal cancer. Several molecules are involved in the positive or negative regulation of this pathway, with β-catenin being the most important and critical molecule among these.

In unstimulated cells free cytoplasmatic β-catenin is destabilized by a multiprotein complex formed by Axin, glycogen synthase kinase 3β (GSK3) and APC (a tumor suppressor). Interaction between Axin and GSK3 facilitates efficient phosphorylation of β-catenin by GSK3 and this phosphorylation event marks β-catenin for ubiquitination and ultimate destruction. On the other hand, the cytoplasmatic protein Dishevelled (Dsh) is recruited to the membrane when cells are stimulated by Wnt ligands. Dsh binds to Wnt through Frizzled (a transmembrane
receptor for the Wnt ligands), the Wnt signaling prompts the inhibition of the destabilization of free cytoplasmic β-catenin by the Axin complex. Now the stabilized β-catenin is released from the Axin complex and is translocated into the nucleus where it binds to T cell factor (TCF) to stimulate the transcription of Wnt target genes (Bienz & Clevers, 2000).

Additionally, APC is a protein normally expressed in nonproliferating colorectal epithelium and is essential for the control of normal growth and cell differentiation (MPOCC, 2013). About 85% of all sporadic and hereditary colorectal tumors show loss of APC function. Colon cancer cells with mutant APC contain high levels of free β-catenin which can be downregulated by exogenous wild type APC. The anti-tumorigenic effects of APC are not only limited to β-catenin degradation but also to the suppression of Wnt pathway by acting on promoter of Wnt responsive genes and the shuttling of β-catenin out of the nucleus (MPOCC, 2013).

**MAPK pathway**

The mitogen activated protein kinase (MAPK) pathway is part of the family of serine-threonine kinases and it has three major subfamilies for MAPK: the extracellular signal-regulated kinases (ERK MAPK, Ras/ Raf1/ MEK/ ERK), and the c-Jun N-terminal or stress-activated protein kinase (JNK or SAPK; and MAPK14). It is well established that in humans beings these three subfamilies can alter gene expression (Hommes, Peppelenbosch, & Van Deventer, 2003), but ERK MAPK is the most relevant subfamily, as there is evidence that this pathway is involved in the pathogenesis, progression, and oncogenic behavior of human colorectal cancer (X. Wang, Wang, Hu, & Evers, 2004). In general, protein kinase C (PKC) activity promotes the binding of GTP to the RAS family, which leads to Raf1 activation and subsequent MAPK
activation. Furthermore, there is plenty of evidence that the inhibition of this pathway can prevent tumor growth and therefore the pathway is potentially useful as targets for treatment of colorectal cancer (Fang & Richardson, 2005; Sebolt-Leopold et al., 1999).

**Diet and Colon Inflammation as a Risk of Colon Cancer**

**Diet constituents that increase colon cancer risk**

Epidemiological studies have shown geographic variations in the incidence of colorectal cancer. The disease is most frequent in developed countries with a western culture, accounting for over 63% of all colorectal cancer cases (Haggar & Boushey, 2009). Among these studies, the “migrant studies” have significant importance because they provide direct evidence that suggests that environmental factors play a major part in the etiology of colorectal cancer. Around 70 to 80% of colorectal cancers may owe their appearance to such factors (Boyle & Langman, 2000). In this type of study, populations migrating to a new and distant country and their offspring are monitored. The results indicate that the groups of migrants lose the risk associated with their country of origin to attain the patterns of their host country even after only one or two generations. The environmental factor related to colorectal cancer can be defined as the wide ill-defined cultural, social and lifestyles practices or in a general sense to everything we get in touch with and we introduce into our organism (i.e., foods) (Boyle & Langman, 2000; Haggar & Boushey, 2009; Mutanen & Pajari, 2011).

The influence of diet in colon cancer can go in two ways; diets that reduce the risk of developing colon cancer and diets that contribute to the risk of developing colon cancer. In respect to the diets that contribute to the risk of colon cancer a Western-style diet is positively associated with the development of colorectal cancer. This diet is characterized by being high in
meat, refined grains, and sugars while low in vegetables, and fiber (Slattery, 2000). Individual food parameters such as fat and red and processed meat have also been associated with the risk of colorectal cancer in ecological studies, animal experiments, and case-control and cohort studies (Boyle & Langman, 2000; Haggar & Boushey, 2009).

The concept of a typical western diet is linked with the implication of fat, especially animal fat, as a possible etiologic factor for colorectal cancer. It is believed that fats will favor the development of bacterial flora capable of degrading bile salts to N-nitroso compounds that have a carcinogenic potential (Haggar & Boushey, 2009; Larsson & Wolk, 2006; Santarelli et al., 2008). The examination of saturated fat was modestly associated with elevated C-reactive protein in 4,900 adult participants of the NHANES 99-00 (King, Egan, & Geesey, 2003). A study carried out in TLR4-deficient and wild type mice, compared a low fat diet (10 % kcal fat) to a high fat diet (60 % kcal fat) and found increased proinflammatory cytokines, increased plasma and fecal endotoxin levels, dysregulation of gut microbiota, and induced colitis in association with the high-fat diet (K.-A. Kim, Gu, Lee, Joh, & Kim, 2012).

Many studies have demonstrated the positive association of high consumption of red and processed meat with the development of colorectal cancer. Santarelli et al, reviewed different types of studies (cohort, case-control, and meta-analysis) in animals and in humans to gather evidence and to explain possible mechanisms for the development of colorectal cancer. They hypothesized that the potential mechanisms of this association are: the presence of heme iron in red meat and the production of heterocyclic amines and polycyclic aromatic hydrocarbons from meats cooked at high temperatures. They also found that processed meat has an excess risk of 20 to 50% in the highest category of processed meat-eaters compared to non-eaters. When comparing dose-response per gram of processed meat with grams of red meat in meta-analysis
studies, one gram of processed meat is at least twice and up to eleven times more promoting of colorectal cancer than red meat (Haggar & Boushey, 2009; Larsson & Wolk, 2006; Santarelli et al., 2008).

Furthermore, these studies only reflect the effect of individual foods but not the effect of whole diets as would be consumed in everyday life. Therefore Shivappa et al. studied the association between the inflammatory potential of overall diet and mortality with a focus on cancer, specifically digestive-tract cancer mortality, including colorectal cancer. The diet inflammatory index (DII) was previously developed with various inflammatory markers including C-reactive protein, IL-1β, IL-4, IL-6, IL-10, and TNF-α. The study estimated that subjects in a higher DII tertile were a 110% more likely to die from digestive-tract cancer than those subjects in a lower DII tertile 1. It is also relevant that this study found an increased intake of red meat in the lowest DII which is opposite to what was expected, but as this study took into account diet as a whole, read meat eaters could have a lower DII if they ate sufficient amounts of other anti-inflammatory components such as (fibers, vegetables, fruits) (Shivappa, Steck, Hussey, Ma, & Hebert, 2017).

**Diet constituents that decrease colon cancer risk**

There is growing evidence that groups of vegetables, such as cruciferous and apiaceous, may be beneficial in the prevention of colorectal cancer. A meta-analysis study evaluated 24 case-control studies and found an inverse association between high intake of cruciferous vegetables and colorectal cancer risk with similar results with cabbage and broccoli (Wu et al., 2013). Furthermore, there is also evidence of decreased inflammatory cytokines levels associated with the consumption of cruciferous vegetables, for example: a multiethnic study found an
inverse relationship between a vegetable dietary pattern (cruciferous and apiaceous vegetables included) and the levels of CRP and IL-6 (Nettleton et al., 2006) Similarly, a randomized trial in healthy adults used cruciferous vegetables and a combination of cruciferous plus apiaceous vegetables, and concluded that both classes of vegetables were responsible for reduced levels of IL-6 (Navarro et al., 2014). Moreover, Chinese middle-aged women had levels of TNF, IL1β, and IL-6 inversely correlated with highest intake of cruciferous vegetables (Jiang et al., 2014).

Therefore, in the present project, we hypothesize that TWD would be as pro-inflammatory as DIO, and therefore a better dietary model of inflammation that reflects the human conditions. Furthermore, we also expect that vegetable supplementation will reduce the inflammatory response produced by diet, through the modulation of keys signaling pathways such as: NF-κB pathway, Wnt/β catenin, or the MAPK.
Chapter 3: Materials and Methods

Animals and experimental design

An animal use protocol was submitted and approved by the University of Arkansas Institutional Animal Care & Use Committee (IACUC) to purchase a total of 84 male C57BL/6J mice (~4 weeks old) from Jackson Laboratory (two sets of 42 mice two weeks apart from each other).

Upon arrival, animals were housed at the Central Laboratory Animal Facility and adapted to a vegetable free-chow diet for 1 week. On day 8 mice were randomly assigned to one of the 7 different diets (n=6) for a total of 12 weeks. Mice were housed in the following conditions: 7 cages of 16.75” x 10.5” x 6”, 6 mice per cage, temperature of 22±0.5°C, 50% humidity, 12 hour light/dark cycle, free access to water and diets, and bedding and water refreshment every 3 days. Body weight and 24-hour food intake was measured once a week. The second set of mice was handled with the same protocol. During the study, four mice from the TWD group were euthanized; three due to skin problems related to one aggressive mouse in the cage and one with a broken leg.

At the end of their assigned diet intervention, mice were euthanized with CO₂ and by cardiac puncture. Blood samples were collected with EDTA anticoagulant, livers were split in two (one half preserved in liquid nitrogen and the other half in RNeater solution), and colons were washed with PBS and stored in RNeater solution (Life Technologies, Carlsbad, CA). Tissues stored in RNeater solution were left overnight at 4°C and moved to -80°C after 24 hours.
Study diets

Seven different types of diets were prepared for this study: AIN-93G (American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation) was used as control diet, DIO (diet-induced obesity), D+C (diet induced obesity plus 21% cruciferous vegetables), D+A (diet induced obesity plus 21% apiaceous vegetables), TWD (total western diet), T+C (total western diet plus 21% cruciferous vegetables), and T+A (total western diet and 21% apiaceous vegetables). All diet ingredients were purchased from Dyets.inc (Bethlehem, PA). Powdered ingredients were stored at -20°C, while liquid ingredients were stored at 4°C until needed. The formulation of AIN-93G was based on Reeves, Nielsen, and Fahey’s work (1993). The DIO was formulated to be a high fat diet (60% calories from fat) and TWD was also formulated based on a previous work (Hintze, Benninghoff, & Ward, 2012). Cruciferous vegetable-supplemented diets contained fresh watercress, broccoli, and green cabbage (21%, wet wt:wt; 7% per each vegetable). Apiaceous vegetable-supplemented diets included fresh celery and parsnip (21%, wet wt:wt; 10.5% per each vegetable). Furthermore, all vegetable supplemented diets were balanced for macronutrients and dry weights for each vegetable was taken from the United States Department of Agricultural nutrient database and summed according to each diet. Composition of the study diets are summarized in Table 1.

Organically grown vegetables were purchased in three batches from two local markets: broccoli, green cabbage, celery and parsnip at Ozark Natural Foods (Fayetteville, AR) and watercress at Whole Foods (Fayetteville, AR). Diets were prepared the same day of purchase. Vegetables were washed, dried with paper towels and peeled if necessary (parsnip), chopped into small pieces, and ground in a food processor (Cuisinart Deluxe, NJ) to further be mix with either DIO or TWD respectively using a mechanical mixer (Hobart, A-200-D) for 15 minutes.
Each diet was aliquoted in freezer bags (1 bag = 1 week of feeding) and stored at -80°C until fed. Aliquots were thawed at room temperature and fed to animals every three to four days.

**Plasma cytokine expression assay**

After collection, blood samples were kept on ice, and centrifuged at 11,180g for 15 minutes at 4°C, supernatant (plasma) was collected and stored at -80°C on the same day of sample collection. Before conducting the cytokine array, we determined the total protein concentration using the 23227 Pierce™ BCA protein assay kit from Thermo Fisher Scientific (Waltham, MA), and we diluted the samples to the lowest protein concentration to perform the array.

The ARY006 mouse cytokine array panel A (R&D Systems, Minneapolis, MN) was used to assess systemic effects of the dietary intervention on plasma cytokine expression. In brief, samples from two mice were pooled to obtain three pooled samples per diet group. Three replicas were performed, each containing one pooled sample per diet group. Nitrocellulose membranes with 40 selected capture antibodies spotted in duplicate were blocked with 2 mL of array buffer six for one hour on a rocking platform shaker. 100 µl of pooled plasma samples were mixed with 500 µl of buffer four, 900 µl of buffer six and 15 µl of biotinylated detection antibodies and incubated at room temperature for one hour. Buffer six was aspirated from membranes and samples mixture was added to membranes for overnight incubation at 2-8 °C on a rocking platform shaker. On the next day membranes were removed from the mixture and washed twice with 20 ml of wash buffer for ten minutes on a rocking platform shaker. After wash, the excess buffer was drained with paper towels and membranes were incubated 30
minutes with 2 mL of streptavidin-horse radish peroxidase at room temperature on a rocking platform shaker.

The washing procedure was repeated and the next steps were performed in a dark room: membranes were placed in a plastic sheet protector and one mL of chemiluminescent reagent mix was added to the membranes and incubated for one minute, the excess reagent was removed with absorbent lab wipes and membranes were place in an autoradiography film cassette. Membranes were exposed in duplicate to X-ray film for five and ten minutes. The light produced at each spot was proportioned to the amount of cytokine bound.

The signal intensity of the blot membranes was measured using the recommended manufacturer software HLImage++ (Western Vision Software, Salt Lake City, UT). Measurements were normalized with negative control spots and AIN-93G control diet.

**Colon cytokine expression array**

Moreover, we explored which inflammation-related genes may be differentially expressed by the experimental diets, we used the PAMM-011Z Inflammatory Cytokines and Receptors RT² Profiler PCR Array (Qiagen, Hilden, Germany). In brief, RNA from colon tissue was extracted and purified using the 73404 RNeasy Plus Universal Kit (Qiagen, Hilden, Germany). Quality of isolated RNA was monitored with the measured A260/280 ratio and RNA Integrity Numbers (Agilent 2100 Bioanalyzer, Santa Clara, CA). The assay was carried out in pooled RNA samples of equal amounts from two mice for a total of six mice and three samples per diet group: in brief, extracted RNA was reverse transcribed to cDNA following the manufactures instruction on the PCR array using the ABI StepOnePlus real time-PCR system
(Applied Biosystems, Waltham, MA). Data was normalized to housekeeping genes and analyzed based on \( \Delta \Delta C_T \) method.

**Statistical analysis**

The software SPSS 19.0 was used to perform data analyses. One-way analysis of variance (ANOVA) was used to determine statistical differences between basal diets (DIO diet vs TWD diet) and the effect of added vegetables (no vegetables vs vegetable added diets) for inflammatory responses. Individual group differences were inspected by Tukey test with a cut-off p-value of 0.05.
Table 1. Composition of basal diets (AIN-93G, DIO, TWD) and vegetable supplemented diets

<table>
<thead>
<tr>
<th>Ingredients (g/Kg)</th>
<th>AIN-93G*</th>
<th>DIO*</th>
<th>D+C*</th>
<th>D+A*</th>
<th>TWD*</th>
<th>T+C*</th>
<th>T+A*</th>
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<td>Cornstarch</td>
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<td>230</td>
<td>220.4</td>
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<td>253.96</td>
<td>256.48</td>
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<td>185.5</td>
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<td>151.93</td>
<td>139.53</td>
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<td>Sucrose</td>
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<td>L-cystine</td>
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<td>Choline bitartrate</td>
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<tr>
<td>Cruciferous vegetables*f</td>
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<td>---</td>
</tr>
<tr>
<td>Apiaceous vegetables*g</td>
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<td>---</td>
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<td>Total (g)</td>
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<td>1210.00</td>
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<td>1209.985</td>
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<td>Carbohydrate (%)</td>
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<td>Protein (%)</td>
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<td>25.18</td>
<td>19.00</td>
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<td>Fat (%)</td>
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<td>34.89</td>
<td>34.33</td>
<td>34.00</td>
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<td>6.36</td>
<td>6.30</td>
<td>3.00</td>
<td>2.95</td>
<td>2.92</td>
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</tbody>
</table>

*aAIN-93G (American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation), DIO (diet-induced obesity), D+C (diet induced obesity plus 21% cruciferous vegetables), D+A (diet induced obesity plus 21% apiaceous vegetables), TWD (total western diet), T+C (total western diet plus 21% cruciferous vegetables), and T+A (total western diet and 21% apiaceous vegetables).
*bAIN-93G mineral mix, purchased from dyets.inc catalog #210025. Custom TWD mineral mix, purchased from dyets.inc catalog #210107.
*cAIN-93G vitamin mix purchased from dyets.inc catalog #310025. Custom TWD vitamin mix, purchased from dyets.inc catalog #310088.
*dCustom fat blend for the University of Minnesota, purchased from dyets.in catalog #103860.
*eWatercress (70g), broccoli (70g), and green cabbage (70g), macronutrients peer group: carbohydrate 9.61g, protein 4.48g, fat 0.4g, fiber 3.90g.
*fCelery (105g) and parsnip (105g), macronutrients peer group:
carbohydrate 22.01g, protein 1.98g, fat 0.50g, fiber 6.80g. \textsuperscript{b}Percent of macronutrient after removing water weight of vegetables: 193.62g from cruciferous and 183.71g from apiaceous.
Chapter 4: Results

Food intake

There were no differences in food intake between groups on week one of the study. On week 6, mice in TWD (14.99 kcal ± 0.21), TC (14.22 kcal ± 0.52) and TA (14.35 kcal ± 0.65) had decreased food intake compared to AIN-93G (20.17 kcal ± 2.88). At the end of the study (week 12) mice in DIO group (26.79 kcal ± 5.15) had increased food intake compared to all other groups except DC group.

Body and Tissue weight

Initial body weight of animals was between 21.64g to 28.56g. There were some unexpected differences in weight gain between some diet groups. The DIO group did not differ in weight gain compared to any other group (Figure 2). Weight gain of mice in DC (19.44g ± 1.62) group was higher than the weight gained by mice on TWD (11.17g ± 1.75) and AIN-93G diet (11.41g ± 0.60), the latter having the lowest mean value. There were no differences in colon and liver weight between diet groups (Table 2).

Plasma cytokine expression assay

The mouse cytokine array panel A is able to detect 40 types of cytokines. This is a screening assay, therefore results are relative and not finite. In our study 18 cytokines (G-CSF, I-309, IL-2, IL-5, IL-6, IL-7, IL-17, IL-23, IP-10, I-TAC, KC, M-CSF, MIP-2, SDF-1, TARC, TIMP-1, TNF-α, and TREM-1) were differentially expressed between diet groups (Figure 3). Mice in AIN-93G, DIO, and TWD had similar cytokine secretion profile with the lowest means compared to the vegetable-supplemented diets. KC was the only cytokine that increased in DIO
compared with TWD (p = 0.036). Cytokine signals in DC had the same secretion profile as cytokines in DIO with lower means but no statistical difference, while some cytokine signals in DA were likely to be increased when compared to DIO (G-CSF, IL-5, IL-6, IL-7, I-TAC, TNF-α, TREM-1; p < 0.015) and when compared to DC (G-CSF, IL-2, IL-5, IL-7, IL-23, IP-10, I-TAC, M-CSF, TARC, TIMP-1, TNF-α; p < 0.026). Most cytokines in TC and TA had similar secretion profiles as TWD with slightly higher means, and with only three cytokines (G-CSF, I-309 and M-CSF; p < 0.005) in TA with increased secretion compared to TWD. Cytokine secretion of IL-17 was increased on TC compared to DC; p = 0.042. MIP-2 expression was higher on TWD compared to AIN-93G and DC; p = 0.019, while SDF-1 was decreased on all diets compared to AIN-93G diet; p = 0.00.

Colon cytokine expression array

Only four inflammation-related genes were statistically different between diets. Bmp2 had a higher mRNA expression in all diets compared to basal diet AIN-93G (p= 0.001). Ccl1 had similar mRNA expression in all diets compared to AIN-93G and DIO which had the lowest (p= 0.001). Ccl19 mRNA was equally expressed in all diets except for DIO which had a lower expression (p< 0.001). Ccl4 had a higher mRNA expression in TC diet only when compared with DIO and DC (p= 0.058).
Table 2. Weight of colon and liver tissues collected from C57BL/J6 mice by diet group.

<table>
<thead>
<tr>
<th>Diet*</th>
<th>Colon (g)</th>
<th>Liver (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-93G</td>
<td>15.87 ± 1.02</td>
<td>169.91 ± 8.05</td>
</tr>
<tr>
<td>DIO</td>
<td>16.64 ± 0.88</td>
<td>172.46 ± 8.71</td>
</tr>
<tr>
<td>DC</td>
<td>17.13 ± 1.28</td>
<td>214.39 ± 29.54</td>
</tr>
<tr>
<td>DA</td>
<td>15.75 ± 0.94</td>
<td>182.07 ± 17.32</td>
</tr>
<tr>
<td>TWD</td>
<td>15.60 ± 0.93</td>
<td>164.68 ± 20.40</td>
</tr>
<tr>
<td>TC</td>
<td>13.32 ± 0.46</td>
<td>160.92 ± 6.61</td>
</tr>
<tr>
<td>TA</td>
<td>13.53 ± 1.17</td>
<td>170.43 ± 17.51</td>
</tr>
</tbody>
</table>

*AIN-93G (American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation), DIO (diet-induced obesity), D+C (diet induced obesity plus 21% cruciferous vegetables), D+A (diet induced obesity plus 21% apiaceous vegetables), TWD (total western diet), T+C (total western diet plus 21% cruciferous vegetables), and T+A (total western diet and 21% apiaceous vegetables). Values are reported as least squares means ± SEM, n=6 per diet group.
Figure 1. 24-hour food intake of C57BL/J6 mice by week. Food cups were weighed on day of feeding (0 hour) and again 24 hours later. Cup weight at 0 hours was subtracted from cup weight at 24 hours and then divided by total animals on respective diets. This was measured once a week. AIN-93G (American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation), DIO (diet-induced obesity), D+C (diet induced obesity plus 21% cruciferous vegetables), D+A (diet induced obesity plus 21% apiaceous vegetables), TWD (total western diet), T+C (total western diet plus 21% cruciferous vegetables), and T+A (total western diet and 21% apiaceous vegetables). Values are reported as least squares means ± SEM, n=6 per diet group. Values within time points that do not share the same lowercase letters are different from each other (P<0.05). Food intake is expressed on dry weight basis as describe by Kim et al. 2017.
Figure 2. Weight gain of C57BL/J6 mice by diet group. Weight gain was monitored once a week. Initial weight (day 0) was subtracted from final weight (day 85). AIN-93G (American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation), DIO (diet-induced obesity), D+C (diet induced obesity plus 21% cruciferous vegetables), D+A (diet induced obesity plus 21% apiaceous vegetables), TWD (total western diet), T+C (total western diet plus 21% cruciferous vegetables), and T+A (total western diet and 21% apiaceous vegetables). Values are reported as least squares means ± SEM, n=6 per diet group. Bars that do not share the same lowercase letters are different from each other (P<0.05).
Figure 3. Effects of seven different diets on plasma cytokine secretion in C57BL/6J mice. AIN-93G (American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation), DIO (diet-induced obesity), D+C (diet induced obesity plus 21% cruciferous vegetables), D+A (diet induced obesity plus 21% apiaceous vegetables), TWD (total western diet), T+C (total western diet plus 21% cruciferous vegetables), and T+A (total western diet and 21% apiaceous vegetables). The relative level of each cytokine or chemokine was calculated using HLImage++ software (Western Vision Software, Salt Lake City, UT). Values are reported as least squares means ± SEM, n=4 per diet group. Bars that do not share the same lowercases letters are different from each other (P<0.05).
Figure 3. (Cont.)
Figure 4. Effects of seven different diets on mRNA expression of inflammation-relate genes in C57BL/6J mice. AIN-93G (American Institute of Nutrition, 1993 diet for growth, pregnancy and lactation), DIO (diet induced obesity), D+C (diet induced obesity plus 21% cruciferous vegetables), D+A (diet induced obesity plus 21% apiaceous vegetables), TWD (total western diet), T+C (total western diet plus 21% cruciferous vegetables), and T+A (total western diet and 21% apiaceous vegetables). mRNA from colon tissue was extracted, purified and reverse transcripted to cDNA following manufactures instruction, real time PCR was run with the RT² profiler PCR array, data was normalized to housekeeping genes and analyzed based on ΔΔC_T method. Values are reported as least squares means ± SEM, n=6 per diet group. Bars that do not share the same lowercase letters are different from each other (P<0.05).
Chapter 5: Discussion and Future Directions

Mice are one of the most useful models to study human diseases. Researchers are always trying to mimic human conditions; in this manner, the AIN-93G diet has widely been used to replicate an ideal human diet in mice models, and DIO to reproduce obesity, but none of these diets represent the larger group of Americans with an average diet. In an effort to replicate the average American diet, scientists developed the TWD as a background diet for rodent studies. TWD has not been compared to DIO regarding effects on colon inflammation. This is why in our study we wanted to compare these two diets in order to determine whether TWD is as pro-inflammatory as DIO and a better model for human diseases, especially colon cancer, as it is a model more translatable to human conditions. Moreover, previous studies have demonstrated the chemo-preventive potential of cruciferous and apiaceous vegetables in the prevention of colon cancer (Koushik et al., 2007; Tse & Eslick, 2014; Wu et al., 2013), and colon inflammation is one of the conditions closely related with colon cancer initiation. Therefore, we also wanted to investigate the effect of these two classes of vegetables on colon inflammation. In the following pages, we discuss our findings, the strengths and limitations of our study, and what would be the further direction of our research.

In our study, the similar plasma cytokine secretion profile found in AIN-93G compared to DIO, and TWD, differs from the study of Kyung-Ah Kim et al., where a high-fat diet (60% kcal % fat) produced higher levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) compared to a low-fat diet (10% kcal % fat) (2012), using the same animal model C57BL/6J as our study. In their study they also report an increased body weight of mice in the high-fat diet group compared to mice in low-fat diet group, which differs from our study where body weight gain of mice in DIO (high-fat diet) did not differ from any other diet, meaning DIO did not have
the effect we expected (i.e., the percent body weight gain in DIO statistically higher than AIN-93G). Another study using the same mouse model and a western style diet in cardiac hypertrophy reported increased body weight of mice in the western-style diet compared to the basal chow diet group (Mells et al., 2011), which was not the case with our western-style diet (bodyweight gain of mice on TWD was equal as the body weight gain of mice on AING-93G diet).

Even though we were not expecting a similar expression profile of any diet compared to the basal diet AING-93G, we were expecting similarities in DIO and TWD (i.e. the same cytokines increased or decreased at the same time in both diets).

To assess the systemic inflammatory response of mice to seven different diets, we measured 40 different cytokines in plasma samples. The higher levels of plasma cytokine secretion in all vegetable supplemented diets compared to the basal diets (AIN-93G, DIO TWD) also differs from the inverse relationship previous studies have found between vegetable consumption and inflammatory markers (Jiang et al., 2014; Navarro et al., 2014; Nettleton et al., 2006). Nonetheless, all these studies were carried out in humans.

Moreover, from the analysis of inflammation-related genes in colon samples, none of the four colon differentially expressed genes found in our study (Bmp2, Cccl1, Cccl4, Cccl19) were altered in the study of Liu et al. (2012) using colon ex vivo culture samples, where obese mice had 11 genes up-regulated and 18 genes down-regulated using the same animal and gene array as our study. However, they used older mice (6 to 18 weeks old) than our study (4 weeks old) and they fed the high fat diet for 12 weeks in one cohort and 17 weeks in the other.

It is widely accepted that adipose tissue expresses higher levels of pro-inflammatory cytokines (TNF-\(\alpha\), IL-1, IL-6) in diet-induced obesity models, and that the macrophages that
infiltrate the tissue are responsible for the expression of TNF-α and, IL-6, and the induction of nitric oxide synthase (Rajala & Scherer, 2003); several studies have reproduced these findings (K.-A. Kim et al., 2012; Liu et al., 2012). From the review of their methodology and their results, we have several discrepancies that may explain the failure of our study to induce obesity and inflammation in mice with high-fat diet, and therefore the ability to determine if there is a decrease in cytokine expression with vegetable-supplemented diets. Stanton et al. (2011) stated that C57BL/6 mice do not gain weight uniformly when fed a high-fat diet, and they decided to exclude from the study all mice with less than 7 gram weight gain on day 21 of high-fat diet feeding. It is important to mention that in this study cytokine expression was measured in serum samples and only IL-6 had a modest increased in the high-fat diet group, while GM-CSF, TNF-α, IL-12p70, and IL-1β did not have any increase (levels below the detection limit of the assay). Studies that did report elevated levels of TNF-α (K.-A. Kim et al., 2012; Liu et al., 2012), IL-1β, IL6 (K.-A. Kim et al., 2012) assessed levels in colon tissue. Concerning the vegetable-supplemented diets with higher cytokine expression, DC diet had a lowering effect on inflammation (with 11 decreased cytokines) compared to DA, which additionally had 7 other cytokines increased compared to DIO. The increase in cytokines such as TNF-α, IL6, IL17, and IL23, suggest the promotion of inflammation by the vegetable supplemented diets as these cytokines are responsible for the activation of the NF-κB transcription factor through the canonical pathway. IL-6 can also lead to the activation of STAT3 transcription factor, and induce expression of anti-apoptotic genes (Bcl2) and proliferative genes (Cyclin D1, c-Myc), all together promoting inflammation and angiogenesis, and inhibiting cellular apoptosis (Terzić et al., 2010). We focused our research on the chemo-preventive effect of cruciferous and apiaceous vegetables, nonetheless some studies have reported that some compounds (e.g. Indol-3-carbinol)
present in these types of vegetables are responsible for the promotion of colon cancer (Pence, Buddingh, & Yang, 1986; Temple & El-Khatib, 1987). Furthermore, a trial in healthy adults showed a 19% and 20% reduction of IL-6 level by cruciferous- and apiaceous-supplemented diets, but no effect of the vegetables on TNF-α or C-reactive protein as markers of inflammation (Navarro et al., 2014). All of the above is evidence of the variable effects diet can have on inflammation.

If we compare the plasma cytokine secretion of the ARY006 protein assay with the colon mRNA qPCR assay, one can observe that I-309 (Ccl1) was the only gene with increased levels in both assays. Nonetheless, protein secretion (I-309) was higher in TA compared to all basal diets (AIN-93G, DIO, TWD), but mRNA secretion was higher in TWD, TC, and TA compared to AIN-93G and DIO. This can be due to a post-transcriptional modification of the mRNA of the elevated cytokines identified with the protein array. In general, differentially expressed mRNAs are involved in the recruitment of cells of the immune system (neutrophils, monocytes, tumor-infiltrating lymphocytes) (Viola, Sarukhan, Bronte, & Molon, 2012), with lower levels on AIN-93G, DIO, and DC diet.

One of the main strengths of our study is the formulation of the vegetable supplemented diets DC and DA (TC, TA had been previously formulated by Kim et al. (2016)) for many reasons: diets were prepared in our lab, all ingredients were purchased from the same company and with the same lot number, we used a mix of whole vegetables (not purified phytochemicals) in quantities equivalent to a translatable amount of one cup per day for a human, and diets were adjusted for macronutrients so even with the supplementation of vegetables all diets had the same proportion of macronutrients.
The main limitation of our study was that mice on high-fat diets did not achieve obesity, which made it difficult to compare our results of the vegetable supplemented diets (DC, DA, TC, TA) to basal diets (AIN-93G, DIO, TWD). Another limitation is that we only measured the cytokine levels at the end of the feeding period, so we did not have baseline measurements of the cytokine levels to compare levels after the feeding trial. We had some difficulties measuring 24-hour food intake of the powder diets (AIN-93G, TWD), due to spillage of the diet, which we resolved somewhat with the use of followers (a special metal lid, with 6 small circular openings); but we also believe that the small openings of our food shields and the weight gain of mice over time may have cause some skin lesions, therefore we recommend using food cups with bigger openings. Moreover, testing the cytokine expression on plasma samples was a challenge due to the limited amount of sample per mouse, and the probability of protein degradation over time and per time the sample was thawed.

Based on the results of our research, we recommend following certain parameters to classify a mouse as obese and monitor their weight gain. Selection can be based on the percentage of body weight compared to basal diet (30-35% above basal diet), limiting the treatment (diet) to the time this parameter is achieved as was describe by Liu et al. (2012). They used 17 weeks for one cohort and 12 for the other. Also, have enough replicas of mice per group of diet so that once obesity is reached, some can be sampled at this time point and the others can separate into groups of diet supplemented with cruciferous or apiaceous vegetables for the time we consider that the vegetables would produce an anti-inflammatory and chemo-preventive effect. As the adipose tissue plays an important role in pro-inflammatory cytokine secretion, the measurement of total body fat could be a useful parameter to compare cytokine levels. If possible, measure the cytokine expression in blood, adipose tissue, and colon epithelial cells, as
cytokines can have a different expression pattern depending on the tissue type. Also, immuno-histological testing could be used to confirm macrophage infiltration of tissues, which cells are primarily responsible for pro-inflammatory cytokine secretion.

We did not determine that DIO and TWD both induce inflammation and both diets failed to induce obesity, nevertheless the cytokine expression profile of these diets was similar. Therefore, we did not discard the possibility for a future study which could monitor and achieve obesity by these diets and possibly still have a similar cytokine expression.

The stimulation of higher cytokine secretion by the vegetable-supplemented diets compared to basal diets only reassures the complexity and variable effects of vegetables on pathways in complex organisms.

Vegetable-supplemented diets did not have an anti-inflammatory effect but, on the contrary, induced the secretion of some pro-inflammatory cytokines. Nonetheless, cruciferous vegetables had a smaller response compared to apiaceous vegetables. This contradiction provides evidence of the complexity and variability of diet (vegetable) effects on inflammation, and the importance of studying their molecular mechanisms as seemingly negative effects on one type of pathway should not lead to discarding evidence of its chemo-preventive effects via other pathways.
References


Liu, Z., Brooks, R.S., Ciappio, E.D., Kim, S.J., Crott, J.W., Bennett, G., . . . Mason, J.B. (2012). Diet-induced obesity elevates colonic tnf-α in mice and is accompanied by an activation


Appendix

To: Sabrina Trudo  
FR: Craig Coon  
Date: October 17th, 2018  
Subject: IACUC Approval  
Expiration Date: March 2nd, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Modification to protocol #18103 "Anti-inflammatory effects of cruciferous and apiceous vegetables in mouse colon" to change twice weekly weighing to once a week.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond March 2nd, 2019 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp
To: Sabrina Trudo
Fr: Craig Coon
Date: December 10th, 2018
Subject: IACUC Approval
Expiration Date: March 2nd, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your personnel addition(s) of Jingsi Tang to protocol # 18103: Anti-inflammatory effects of cruciferous and apiceaeous vegetables in mouse colon.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond March 2nd, 2019 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp
To: Sabrina Trudo
Fr: Craig Coon
Date: March 16th, 2018
Subject: IACUC Approval
Expiration Date: March 2nd, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 18103: Anti-inflammatory effects of cruciferous and apiaceous vegetables in mouse colon.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond March 2nd, 2019 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Sabrina Trudo, Jae Kim, Jeong Pan, and Rosa Moreno. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subject.

CNC/trp
To: Sabrina Trudo  
Fr: Craig Coon  
Date: November 8th, 2018  
Subject: IACUC Approval  
Expiration Date: March 2nd, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your personnel addition(s) of Jianmin Chai and Xiaoyuan Wei to protocol #18103: Anti-inflammatory effects of cruciferous and apiaceous vegetables in mouse colon.

In granting its approval the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond March 2nd, 2019 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp