Snacking, Childhood Obesity, and Colon Carcinogenesis.

DISSERTATION

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By

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Abstract

Childhood obesity has been a serious problem in both developed and developing countries. Efforts at all levels of society have been made to reduce childhood obesity rates in the U.S., yet no significant reductions have been achieved over the past 10 years. Transitions in snacking over the past 30 years has been proposed as a risk factor for the increase in childhood obesity rates in the U.S. However, conflicting findings were reported by independent research groups. Recently, the long-term impacts of childhood obesity on chronic disease development have been acknowledged. Moreover, energy restriction has been established as an effective approach to lose weight and to reverse the harmful effects of obesity on chronic diseases in both human and animal models. However, the influences of lifetime transitions in energy intake on body weights as well as risks of cancer have not been fully examined. In the present research project, both risk factors of childhood obesity and its impacts on colon carcinogenesis have been investigated. To identify potential dietary risk factors for childhood obesity, 24-hour dietary recall data have been analyzed among a nationally representative population of U.S. children from ages 2-18. Among the selected population, snacking contributed more than 27% to total dietary fiber intake, while the fiber density in snacking was approximately 7 gm/1,000 kcal. Younger children (2-11 years old) had more fiber from snacking, compared to older children (12-18 years old), yet the fiber density in snack
foods remained the same. The inverse association between fiber density in snacking and the risk of being overweight/obese was limited to older children (12-18 years old, OR = 0.770, 95%CI: 0.59-0.998, p = 0.048), while the association among younger children was positive, but not statistically significant. To study the influences of lifetime alterations in energy intake on body weight and colon carcinogenesis, nine combinations of dietary energy intake were incorporated in a murine model of colon cancer. The observed detrimental impacts of a high fat diet on colon cancer risk were consistent with previous studies. The present project expands the understanding of energy restriction on colon carcinogenesis in that the protective effects of energy restriction on colon cancer development were limited to intervention in later life, while early life exposure to energy restriction increased the risk of colon cancer, especially with the combination of a high fat diet in later life. Colonic mucosal bacteria are proposed as a mediator of the long-term impacts of energy intake on body weight and colon carcinogenesis. Although an actual causal relationship has not been established, in this project the significant lingering impacts of dietary energy intake on gut microbiota structure and the relative abundances of certain phyla and genera supported the hypothesis that gut microbiota may be a mechanistic component underlying the causal pathway between diet and colon carcinogenesis. The present project not only identified a risk factor for childhood obesity to facilitate development of effective prevention strategies, but also expanded our knowledge of dietary energy intake in childhood as an important player in obesity development and colon carcinogenesis. Findings in this project emphasize the significant role of the gut microbiota on diet and colon health.
This dissertation is dedicated to my Heavenly Father and my beloved family.

"Ask, and it shall be given you;
seek, and ye shall find;
knock, and it shall be opened unto you."

Matthew 7:7
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# Table of Contents

Abstract ........................................................................................................................................... ii

Acknowledgments ............................................................................................................................. v

Vita ................................................................................................................................................... vi

List of Tables ..................................................................................................................................... viii

List of Figures .................................................................................................................................... ix

Chapter 1: Introduction .................................................................................................................... 1

Chapter 2: Fiber Content in Snacks and Childhood Obesity .......................................................... 23

Chapter 3: The impacts of lifetime alteration in dietary energy intake on experimental colon carcinogenesis ......................................................................................................................... 52

Chapter 4: The impacts of lifetime alteration in dietary energy intake on circulating cytokine levels ........................................................................................................................................ 76

Chapter 5: The impacts of lifetime alteration in dietary energy intake on gut microbiota structure ....................................................................................................................................... 101

References ...................................................................................................................................... 128
List of Tables

Table 1.1 Colon carcinogenesis related cytokine and their regulation by diet. .......................... 21
Table 1.2 Rodent models of colon carcinogenesis ..................................................................... 22
Table 2.1 Demographic distributions of children at 2–18 years old, NHANES 2005–2012 .......... 43
Table 2.2 Overweight/obesity rates. .......................................................................................... 44
Table 2.3 Snacking rates. ........................................................................................................... 45
Table 2.4 Total dietary fiber intake, contribution from snacking, and fiber density in snacking . 46
Table 2.5 Tertiles of dietary fiber from snacking and dietary fiber density in snacking .......... 48
Table 2.6 Associations of CRP levels with tertiles of contribution of fiber intake and fiber density in snacking ................................................................. 49
Table 3.1 Diet composition and calorie distribution ................................................................. 68
Table 4.1 Lower detection limits cytokines and their roles in TH1/Th2/Th17/Treg cell homeostasis ................................................................. 92
Table 4.2 Cytokine levels between diet groups in the 21-week study ........................................ 93
Table 4.3 Cytokine levels between diet groups in the 60-week study ...................................... 94
Table 5.1 Relative abundances of phyla and genera changed by diet intervention ............... 122
List of Figures

Figure 2.1 Odds Ratios (OR) of overweight/obesity ................................................................. 50
Figure 2.2 Odds Ratios (OR) of impaired glucose response .................................................. 51
Figure 3.1 Study design .......................................................................................................... 69
Figure 3.2 Weekly average body weights over time ............................................................... 70
Figure 3.3 Average body weights at weeks 15, 21, 33, and 60 ............................................... 71
Figure 3.4 Body fat distribution of representative mice in each group ................................. 72
Figure 3.5 Body fat mass at 15 and 33 weeks ....................................................................... 73
Figure 3.6 Blood glucose levels during glucose tolerance testing ........................................ 74
Figure 3.7 Dietary impacts on colonic ACF ........................................................................... 75
Figure 4.1 Study design .......................................................................................................... 95
Figure 4.2 Average body weights over time .......................................................................... 96
Figure 4.3 Average body weights at study end points by diet groups ................................. 97
Figure 4.4 Azoxymethane injections did not change the circulating levels of cytokines of interests ...................................................................................................................... 98
Figure 4.5 Changes in serum cytokine concentrations over time .......................................... 99
Figure 4.6 Circulating levels of IL-2 and IL-18 across different diet in promotion phase 100
Figure 5.1 Microbiota differs by diet .................................................................................... 125
Figure 5.2 Microbiota differs by anatomical location in the colon .................................... 126
Figure 5.3 Microbiota differs by cohorts .............................................................................. 127
Chapter 1: Introduction

1. Childhood Obesity Impacts and Risk Factors

According to the World Health Organization (WHO) in 2013, 42 million children under the age of 5 were overweight or obese worldwide. Moreover, in the past 30 years, the global prevalence of overweight or obese children and adolescents increased by nearly 50% \(^1\). The prevalence of overweight and obesity among children under 5 in developed countries is about double that of developing countries, however, the majority of affected children live in developing countries \(^2\). Furthermore, the rate of increase of childhood overweight and obesity has been more than 30% higher in developing countries than developed countries, which is partially due to the so-called "nutrition transition".

"Nutrition transition" describes the switch from traditional diets to high-fat, high-calorie diets as poor countries move up the income scale \(^3\). Currently, obesity is no longer a "non-communicable" disease; instead, it is an impending pandemic across the globe.

Similar to adults, obesity poses greater risk of insulin resistance and metabolic syndrome, Type 2 diabetes, joint problems, and psychological stress among children and adolescents \(^4,5,6,7,8\). Globally, Type 2 diabetes has been reported at an earlier age, primarily as a consequence of the increase in obesity and metabolic syndrome in early childhood \(^9\). The overall prevalence of metabolic syndrome among U.S. children and adolescents is less
than 5% however, the prevalence is much higher among overweight and obese children and is increasing with higher degrees of childhood obesity\textsuperscript{10,11}. Moreover, overweight children tend to have advanced maturation, determined by bone age, peak height velocity and age of menarche, which are all associated with increased fatness in adulthood\textsuperscript{12}. Therefore, obese children are more likely to be obese adults and have higher risks of adult health problems, such as heart disease, stroke, and several types of cancer, including cancer of the breast, colon, prostate, and endometrium\textsuperscript{13,14,15}. Along with the increased risk of multiple diseases in the short-term and long-term in the U.S., the estimated lifetime direct medical costs due to childhood obesity, including annual prescription medications, emergency room visits, inpatient bills and outpatient bills, is about $19,000 per person, or $12,660 per person when weight gain through adulthood among normal weight children is adjusted\textsuperscript{16}. Furthermore, obesity is associated with lower productivity while at work and obesity-related job absenteeism in the U.S. totals $4.3 billion annually\textsuperscript{17,18}. In the past few decades, no country has successfully reduced obesity rates. Lifestyle shifts driven by the rising incomes among low- and middle-income countries may worsen the scenario unless urgent steps are taken to address the issue.

Overweight and obesity, defined as abnormal excessive fat accumulation that may impair health, is a complex multifactorial and heterogeneous condition\textsuperscript{19}. Being obese secondary to genetic diseases or hormonal disorders, such as hypothyroidism, Albright’s Hereditary Osteodystrophy (AHO), and weight gain associated with medications, is far
less common\textsuperscript{20}. The major cause of overweight and obesity is the imbalance between energy intake and energy expenditure. The main causes of excess weight in youth are similar to those in adults, including excessive dietary intake and inadequate physical activity. Despite the similar root cause of childhood obesity in developed and developing countries, risk factors associated with childhood obesity are different. In developing countries, important determinants of childhood obesity includes high socioeconomic status, residence in metropolitan cities, female gender, unawareness and false beliefs about nutrition, marketing by transnational food companies, increasing academic stress, and poor facilities for physical activity\textsuperscript{9}. On the other hand, studies among school-age children from western developed countries found an inverse association between socioeconomic status and childhood adiposity\textsuperscript{21}. Moreover, children and adolescents are more vulnerable to community environmental causes, including limited access to healthy affordable food and greater availability of high-energy-dense foods, increasing portion size, and no safe and appealing places to play or be active\textsuperscript{22,23,24}. Given the large impact of lifestyle on obesity, behavioral changes are critical to include in any weight loss programs. To develop effective programs, it is first important to identify potential behaviors that are related to childhood obesity.

2. \textit{Snacking and Childhood Obesity}

The U.S. population eats more frequently throughout the day compared to three decades ago. There has been an increase from an average of 3 eating occasions/day in 1977 to approximately 5 eating occasions/day in 2006\textsuperscript{25}. In cross-sectional analyses on four
nationally representative surveys where snacking occasions were self-defined by respondents, the snacking prevalence increased significantly from 71% to 97% among adults and from 74% to 98% among children and adolescents between 1977 and 2006. Within the same time frame, the average snacking frequency increased by a full snacking occasion among both children and adults. Furthermore, the contribution of snacking to total daily energy intake increased to 24% among adults and 27% among children and adolescents in 2003-2006. The type of snack food consumed by U.S. children and adolescents also shifted to energy-dense, nutrient-poor foods over the past few decades. Although studies on snacking prevalence in developing countries are scarce, similar yet more dramatic changes have been documented. For example, between 1989 and 2011, the consumption of salty snacks and sweet snacks increased from 22% to 64% and 38% to 67%, respectively, in the Seychelles.

In developed countries, higher frequency of salty or sweet snacks consumption among children is associated with lower socioeconomic status and lower educated parents. On the other hand, in developing countries, consumption of highly processed snacks is significantly lower among children of lower educated mothers, compared to children of higher educated mothers; and children from higher income families and urban areas are more likely to consume energy-dense snack food, such as cookies, sweets, and ice cream. Despite the contradiction related to high consumption of energy-dense nutrient-poor snacks in developed and developing countries, the availability and accessibility of snack foods at home, in school, and in retail food stores in neighborhoods surrounding schools...
are the major causes underlying high consumption of energy-dense, nutrient-poor snack foods in both developed and developing countries.

Along with the shift in snack foods to energy-dense, nutrient-poor snacks among children over the past few decades, children are getting a big portion of their daily dietary intake from snack foods. The most recent analysis using the National Health And Nutrition Examination Survey (NHANES) showed that foods consumed at self-defined snacking occasions provided 19-32% of calcium, 27-37% of vitamin C, 14-29% vitamin D, and 26-34% of vitamin E of total dietary intake across age and gender groups among children and adolescents. However, more frequent snacking was associated with a higher total energy intake and a higher proportion of energy contributed by added and total sugar, which could result in increased body weight.

Multiple studies have examined the relationship between snacking behavior and weight status among children and adolescents with contradictory results. Among four cross-sectional studies and four longitudinal studies conducted in the U.S., only two of them used nationally representative samples, and none of these studies found a significant positive association between snacking and body weight status. Some found an inverse relationship between snacking frequency and weight status among subgroups of the population. Analyses on populations from other countries also saw inconsistent results, demonstrating either a positive or inverse association between snacking and childhood obesity.
Given the correlation between snacking and total dietary energy intake, total energy intake may be a confounding factor and neglecting to take this into account may result in a non-significant association between snacking and obesity. Thus, adjustment for total energy intake is appropriate in epidemiologic studies to control for confounding and to identify the effect of snacking on childhood obesity. The traditional method, and one used in previous studies to account for total energy intake, is the "nutrient density" approach, in which energy from snacking is divided by the total energy intake. The major problem with this method occurs when total energy intake is associated with disease, which holds true in the case of snacking and childhood obesity. In this situation, energy contributed by snacking will be confounded by total energy intake and dividing by total energy intake will simply induce confounding in the opposite direction.

Multiple different criteria were used by previous studies to define snacking occasion, including the types or amount of food consumed, time of the day, and self-identification by respondents. Therefore, the definition of snacking varies across studies and within the same study, especially in the case of self-identification of snacking by respondents. In those cases, nutrient-dense foods and energy-dense, nutrient-poor foods with the same calorie content will be treated the same in the analysis of energy contribution of snacking. For example, a medium sized banana may have the same amount of calories as a vending machine sized rice crisps, thereby contributing the same amount of energy, but they have
different impacts on health. The lack of a standard definition of snack occasions may partially explain the inconsistent findings in previous studies.

Another limitation of previous studies using NHANES data is that the estimation of usual intake is from a single 24-hour dietary recall. Food frequency questionnaire (FFQs) and 24-hour dietary recall are usually used in collecting dietary intake information in survey settings. Multiple-pass methods are used in collecting dietary intake information in a 24-hour time frame in NHANES, which captures rich details about daily intake of every item consumed. However, the trade-off of having less measurement errors than an FFQ is an increase in random error, which may affect the estimates of usual intake from one 24-hour recalls. A single recall does not reflect a person's usual daily intake; it represents only a "snapshot in time." Snack foods are usual episodically consumed, therefore, consumption in a specific day is subject to large within-person variation, and this may be greater than between-person variation, masking the association between snacking and childhood obesity.

Preventing obesity among children and adolescents is a public health priority in both developing and developed countries. Existing research does not indicate a positive association between snacking and childhood obesity, nor does it suggest that children should be discouraged from eating snack foods. However, the estimated average increase in energy consumed from snacking is 168 kcal/day \(^\text{42}\). In comparison, a recent study suggested that an average net reduction of 64 kcal/day would be required to achieve the
Healthy People 2020 target for reducing childhood obesity. Therefore, the significant increase in consumption of energy-dense nutrient-poor snack foods among children and adolescents urge a well-designed study to elucidate the association between snacking and childhood obesity.

3. Colorectal Cancer and Risk Factors of Carcinogenesis

Globally, colorectal cancer (CRC) is the third most common cancer, accounting for about 10% of all cancer cases. It is estimated that by 2035, there will be 2.4 million cases diagnosed annually. In both genders, over 50% of colon cancer cases are found in developed countries. Specifically, the age-standardized incidence rates are three times higher in developed countries compared to developing countries among men, and more than two times higher among women. High incidence rates have been documented in both developed countries and developing countries, however, the incidence rates in some newly developed or economically transitioning countries have exceeded the peak rates in longstanding developed countries. Furthermore, decreased colorectal cancer mortality rates have been observed in both genders, but this is largely confined to longstanding developed countries, such as the U.S. and New Zealand. On the other hand, increased mortality rates have been reported in economically transitioning countries, such as China, Korea, and Mexico. A population-based colorectal cancer screening program may increase the incidence rates in the short term through the detection of prevalent cases, but lower the incidence and mortality rates by capturing the disease at its early stage. The discrepancy of mortality rates between developed and developing countries may be
partially explained by the presence of colorectal cancer screening programs. In the United States, colorectal cancer continues to be the third leading cause of cancer-related deaths since 1998. It is projected that 134,490 individuals will be diagnosed with CRC in 2016 in the United States, 49,190 of whom will die from this disease. New technologies and treatments increase the survival rate of colorectal cancer, however, cancer care costs increase as new treatments are developed. Medical costs associated with colorectal cancer were projected to be $14 billion in 2010 and will be over $20 billion in 2020. The rising costs of colon cancer care illustrate the importance of advancing the science of cancer prevention to implement the most effective approaches in high risk populations.

Among all cancer cases, up to 10% of cases result directly from gene defects inherited from a parent. Up to 5% of all colon cancer cases are hereditary, with Lynch syndrome (LS) and familial adenomatous polyposis (FAP) being the most frequent forms. LS is associated with mutations in genes involved in the DNA repair pathway, namely the mutL homolog 1 (MLH1) and mutL homolog 2 (MSH2) genes, while FAP is caused by mutations in the tumor suppressor gene adenomatous polyposis coli (APC). In addition to the predisposed genetic mutations, non-modifiable risk factors for colorectal cancer also include older age, personal history of adenomatous polyps, inflammatory bowel disease, and family history of colorectal cancer or adenomatous polyps. Nevertheless, the majority of colorectal cancer cases occur in people without a family history or a predisposed illness. According to the American Institute for Cancer Research
(AICR), approximately 50% of colon cancer cases may be preventable by diet and lifestyle changes. The modifiable risk factors for colorectal cancer include nutrition practices, such as diet and physical activity, obesity, and heavy alcohol consumption. Specific nutrients have also been associated with colon cancer in both animal models and population studies. The protective effects of folate, vitamin A and carotenoids, selenium, and calcium in colon carcinogenesis have been demonstrated through preclinical models and epidemiologic studies. However, because the dose-response of these nutrients is not fully understood, dietary supplements are not recommended for colon cancer prevention. Instead, a healthy dietary pattern, featuring a high proportion of fruits and vegetables and a moderate-to-low proportion of processed and red meats, is recommended for colon cancer prevention and supported by published studies. Although total fat intake does not seem to alter colon cancer risk, higher intake of animal fat, the majority of which is saturated fat, increases the risk of colorectal cancer. On the other hand, as a nutritious source of fiber and micronutrients, fruit and vegetables consumption is associated with lower risks of colon cancer. Physical activity, another component of the lifestyle, is associated with lower colon cancer risk such that moderate daily activity lowers the risk of colorectal cancer and is recommended as a prevention measure by the AICR. Finally, obesity and body fat, which are interrelated with physical activity and dietary energy intake, are associated with elevated risks of colon cancer. Epidemiologic studies suggest that obese adults are more likely to be diagnosed with colon cancer, compared to their lean peers. Furthermore, gain in weights/body mass index (BMI) is positively associated with colorectal cancer risk. Numerous animal studies have documented that
genetic- and diet-induced obesity increases both the incidence and size of tumors in the colon \(^{61, 62}\). Therefore, maintaining a healthy weight is important to prevent colorectal cancer.

4. Dietary Energy Intake and Colon Carcinogenesis

In spite of the large amount of research on obesity and colorectal cancer, the mechanisms underlying the association between the two health conditions remain elusive. Adipose tissue, which is more than a means of energy storage, is an active endocrine organ that secretes numerous protein hormones, including leptin, adiponectin, and resistin. Leptin is known primarily for its role in food intake regulation. As a signal of energy sufficiency to the brain, increased leptin levels in obese subjects lead to the activation of the phosphoinositide 3-kinase/protein kinase B (PI3K-AKT) pathway, resulting in increased cell proliferation \(^{63}\). Furthermore, leptin-deficient Lep\(^{ob/ob}\) mice, which are genetically predisposed to obesity, are less sensitive to azoxymethane-induced polyp formation than are wild-type controls and are strongly protected from polyp formation induced by high fat diet \(^{64}\). Adiponectin is primarily known for its role in insulin sensitization of muscle and liver. Circulating adiponectin levels are decreased in both obese humans and animals, compare to their lean peers \(^{65, 66}\). Activation of the 5\(^{\prime}\) AMP-activated protein kinase (AMPK) pathway by adiponectin and the consequent suppression of the mammalian target of rapamycin (mTOR) and S6 kinase phosphorylation results in inhibition of cell proliferation, whereas low adiponectin levels favors cell proliferation \(^{67, 68}\). Mediated by low circulating adiponectin levels, hyperinsulinaemia (insulin resistance) and low serum
levels of insulin-like growth factor-binding protein (IGFBP)-3 are also risk factors for colon cancer development in obese individuals. In general, insulin-receptor-mediated signaling regulates metabolic pathways, whereas proliferation and cell growth are stimulated by insulin-like growth factor (IGF)-1-receptor-mediated signaling. Insulin increases IGF-1 levels through direct regulation of IGF1 transcription, whereas hyperinsulinaemia reduces IGFBP-1 levels. Insulin and bioavailable IGF-1 can cross-react with each other's receptor, and the IGF-1 receptor and the insulin receptor can form heterodimers. Hyper-activation of the insulin-IGF-1 pathway in obese individuals can then activate mitogen-activated protein kinases (MAPKs) and ultimately the PI3K-AKT pathway, promoting cell proliferation.

Chronic inflammation in obesity, exemplified by increased circulating free fatty acids, cytokines, and chemokines that attract immune cells into the local microenvironment, is another possible mechanism linking obesity with increased colon tumor burden. Emerging evidence suggests that tumor necrosis factor (TNF) is a key mediator of obesity-induced inflammation and colon cancer development. Mice fed a high fat diet had elevated levels of TNF, whereas TNF-neutralizing monoclonal antibodies decreased tumor incidence in azoxymethane (AOM) treated mice. On the other hand, findings in an azoxymethane-dextran-sodium-sulphate (AOM/DSS) model of intestinal inflammation in global interleukin (IL)-6-knockout mice suggests that the IL-6–Stat3 inflammatory axis contributes to colon tumor formation, however, the link between inflammation in diet-induced obesity and increased cancer risk is not supported by the study.
Given the deleterious impact of obesity on colon cancer development, energy restriction (ER) has been studied as a cancer prevention strategy for over a century \(^{75, 76}\). The anticancer effects of ER have not been widely studied in human cancer cases, especially colorectal cancer. ER interventions have generally been studied in cancer survivors, due to the long latency period of many obesity-related cancers. However, several clinical trials found a consistent reduction in leptin and insulin after short-term (6-month) or long-term (1-year) ER interventions among overweight or obese cancer survivors \(^{77, 78}\). Observational epidemiologic studies provide additional evidence that ER is associated with favorable cancer results. A retrospective study in Sweden found that women hospitalized for anorexia nervosa prior to age 40 had a 53% lower incidence of breast cancer \(^{79}\). Acute ER also reduced breast cancer risk in a cohort of Norwegians \(^{80}\). However, more severe and prolonged ER exposures increased risks for colon cancer and breast cancer, evidenced by cancer incidence among Israeli Jewish survivors of World War II \(^{81}\). Animal models, especially murine models of diet-induced obesity of cancer, have been commonly used to investigate the underlying mechanisms responsible for the observed effects of ER on cancer, which, unfortunately, are not yet clearly elucidated. As the opposite side of diet-induced obesity, ER attenuates growth signals and chronic inflammation through reduced glucose levels, adipose tissue, and levels of circulating and tissue pro-inflammatory cytokines \(^{73}\). Other emerging mechanisms underlying the anticancer effects of ER include the sirtuin family of proteins and autophagy in response to ER intervention. The specific roles of sirtuins in cancer development are not yet clear.
The sirtuin 1 (SIRT1) activator promoted tumor cell migration in a breast cancer model, whereas SIRT1 suppressed polyp formation in a transgenic intestinal tumor model. Furthermore, SIRT1-overexpression did not alter the anticancer effects of ER in a p53-deficient mouse model of cancer, indicating a limited role of SIRT1 in the anticancer effect of ER. However, it is possible that the role of SIRT1 and other sirtuins in cancer development depends on the type of cancer and disease stages. Furthermore, autophagy in cancer cells is promoted by AMPK as a response to energy stress, which is triggered by the TOR signaling regulated by ER. On the other hand, both normal cells and tumor cells utilize autophagy to survive. The specific role of autophagy in ER-mediated effects on tumor development remains elusive.

5. Cytokines as Mediators between Dietary Energy Intake and Colorectal Carcinogenesis

Inflammation, although necessary for damage repair, is now regarded as an enabling characteristic for the acquisition of the core hallmarks of cancer. Tumor-induced inflammation has a critical role in determining the fate of colorectal cancer. Antigen-driven immune responses have the power to curtail cancer progression, whereas nonspecific inflammatory activity can potently augment it. The distinct sides of tumor immunity are critically dependent upon cytokine networks that normally act to maintain gut homeostasis. The majority of research have demonstrated that ER results in decreased (or unchanged) concentrations of pro-inflammatory mediators, coupled with increased concentrations of anti-inflammatory mediators both at basal levels and following immune stimulation. Cytokines such as IL-6 and TNF-α are classically regarded as essential
players in colon carcinogenesis through activation of signal transducers and activation of transcription 3 (STAT3), and transcription factor nuclear factor-κB (NF-κB). More recently, the pro-inflammatory and anti-inflammatory effects of other cytokines in colorectal cancer have been appreciated in both human and mouse CRC models. However, only a few of them have been examined in the context of obesity-related colon cancer cases. Potential functions of each cytokine in colon cancer development and the regulation by ER and obesity have been listed in Table 1.1.

6. Microbiota as Mediators between Dietary Energy Intake and Colorectal Carcinogenesis

Another key player in gut homeostasis is the tens of trillions of microorganisms resident in our intestine. At any given time, the intestinal microbiota comprises over 100,000 billion bacterial cells. In the developing infant, maternal inoculation at birth and modified breastfeeding impact microbiota composition. After the first three years of life, the general structure of the microbiota is relatively consistent throughout healthy adult life. However, emerging data demonstrates aging, host physiology and health, pharmaceutical agents, and diet are important factors impacting variation in the microbiota structure and function. The commensal microbiota of the colon are hypothesized to be major determinants of health and disease, including chronic inflammatory syndromes and colon carcinogenesis.
Inflammatory bowel disease is regarded as a consequence of interruptions in mucosal unresponsiveness to gut microbiota\textsuperscript{95}. This concept is supported by animal models of colonic inflammation where inflammation is dependent on the presence of microbiota\textsuperscript{96}. Indeed, induction of regulatory T cells (Tregs) via the gut microbiota plays a vital role in maintaining gut homeostasis under normal conditions or in controlling inflammatory responses\textsuperscript{95}. Furthermore, certain bacteria are more effective than others in inducing Treg activity. For example, \textit{Bacteroides fragilis}, a commensal colonic organism existing in both mice and humans, induces forkhead box p3-positive (Foxp3\textsuperscript{+}) Tregs via direct interaction between the Toll-like receptor 2 (TLR2) on Foxp3\textsuperscript{+} Tregs and a polysaccharide produced by \textit{B. fragilis} known as polysaccharide A (PSA)\textsuperscript{95}. Increased Foxp3\textsuperscript{+} Tregs further suppress inflammation and reduce colitis burden. On the other hand, studies on fecal microbiota from \textit{Tbx21(-/-)Rag2(-/-)} ulcerative colitis (TRUC) mice provide evidence that \textit{Klebsiella pneumoniae} and \textit{Proteus mirabilis} are associated with colitis formation in these mice\textsuperscript{97}. The TRUC mouse is a transgenic model of T-bet deficiency, recombination activating gene 2 (RAG2) deficiency, and human ulcerative colitis (UC). The absence of T-bet leads to excessive production of TNF-\alpha by dendritic cells, which then synergize with IL-23 to drive IL-17 production by innate intestinal cells. In addition, the mice lack Tregs due to the RAG2 deficiency\textsuperscript{97,98}. Intriguingly, the fact that only administration of anti-TNF-\alpha, but not Tregs on TRUC mice diminishes \textit{K. pneumoniae} or \textit{P. mirabilis} levels, suggested that the colitogenic microbiota require the presence of an abnormal mucosal immunological microenvironment to cause disease. Therefore, the colitogenic potential of these organisms could be enhanced by interaction
with pro-inflammatory cytokines. In the light of the above studies specifying either the anti- or pro-inflammatory effects of the gut microbiota, it becomes of great interest to determine the microbiota in CRC patients and how risk factors of CRC may alter the gut microbiota.

Despite that one third of the microbiota is commonly present in most people, two thirds of the population are specific to each individual and are largely determined by environment and diet. For example, the composition of the gut microbiota in a newborn is dependent on the delivery mode (vaginal vs. cesarean) and breastfeeding practice (breastfed vs. formula)\textsuperscript{99,100}. By the age of 3, the microbiota becomes stable and similar to that of adults, continuing its evolution at a steadier rate throughout life. More recently, studies in animal models demonstrated that diet dominates host genetic factors in shaping the gut microbiota\textsuperscript{101}. In general, high-fat diet-induced obese animals had reduced Bacteroidetes and increased Firmicutes compared to control diet fed animals\textsuperscript{102,103,104}. The relative abundances of Firmicutes increased and Bacteriodetes decreased as calorie consumption increased\textsuperscript{105}. Conversely, one study found high-fat-fed mice, with or without restricted intake, had higher abundances of Bacteroidetes compared to low-fat-fed mice\textsuperscript{106}. Furthermore, a cross-sectional analysis on 98 healthy human subjects demonstrated that gut microbial enterotypes are linked with long-term dietary patterns\textsuperscript{107}. In spite of the quick response of the gut microbiota to dietary changes\textsuperscript{105,107}, the magnitude of the response is moderate and diet-induced changes in the gut microbiota are reversible\textsuperscript{107,108}. 
7. Early Life Dietary Energy Intake and Colon Carcinogenesis

Recently, the important role of early life exposures in disease development has been largely appreciated. Several epidemiological studies have reported long-term impacts of severe childhood/gestational ER on risks of diabetes, breast cancer, and colon cancer in adulthood. Specifically, severe ER during childhood and adolescence may reduce the risks for colorectal cancer in men and ovarian cancer in women\textsuperscript{109,110}. However, exposure to famine in gestation may increase the risk for breast cancer in women\textsuperscript{111}. Nevertheless, the impacts of early life dietary energy intake on colon carcinogenesis remain elusive, due to the limitations of prospective epidemiological studies. One limitation is that exposure to ER in early life is often measured by socioeconomic status, which lacks the details of dietary intake. Moreover, limited information on adulthood dietary intake may induce confounding in the results. Furthermore, long-term follow-up studies are not feasible, especially in the case of colon cancer. Regardless, research in diet and epigenetics suggested that epigenetic regulation could be one of the underlying mechanisms of which early life exposures may influence disease risks in adulthood, including colon cancer\textsuperscript{112}. In addition to alterations in gene expression via epigenetic regulation by diet and metabolites, the gut microbiota also plays an important role in the mechanisms underlying early life dietary exposure and colon cancer risk in adulthood. Indeed, butyrate, a metabolite of dietary fiber by the microbiota, exerts multiple beneficial effects at the intestinal and extraintestinal level, related at least in part to the epigenetic regulation of gene expression\textsuperscript{113}. Furthermore, several studies have reported
the interactions between microRNA and endogenous microbiota, as well as infectious bacteria \textsuperscript{114, 115, 116}. Therefore, it is of great interest to understand the regulation of microbiota by dietary intake in early life and its potential impacts on colon cancer risks.

8. Chemical-induced Rodent Model of Experimental Colon Cancer

To gain insight into the value of specific agents for prevention of human colon cancer, animal models of human colon cancer are widely used in dietary chemoprevention research. Rodent models of experimental colon cancer can be categorized into four groups: spontaneous cancer models, chemical induced models, transgenic models, and xenograft models. Advantages and limitations of each type of models are listed in Table 1.2.

Within chemical induced models, there are two different models. One is induced by AOM and the second by AOM/DSS. The first model induced by only AOM features distal tumors with mutations in the β-catenin gene (Ctnnb1), similar to LS \textsuperscript{117}. Additionally, tumor incidence and multiplicity can be altered by both genetic background and diet \textsuperscript{118}. The second model, induced by the combination of AOM and DSS, has proven to dramatically shorten the latency time for induction of colon cancer and to rapidly recapitulate the aberrant crypt foci-adenoma-carcinoma sequence that occurs in human CRC. However, inflammation induced by DSS may confound the pro-inflammatory effects of high fat diet/obesity on colon cancer development.
The climbing prevalence of obesity among both children and adults and its association with many chronic diseases, including diabetes, cardiovascular disease, and cancer, urge multidisciplinary research to identify risk factors of obesity and determine how obesity may influence chronic disease development, such as cancer. Consumptions of energy-dense, nutrient-poor snacks has increased over time among U.S. children. However, it is less clear whether snacking is associated with childhood obesity in the U.S. On the other hand, being obese in childhood accelerates the development of many chronic diseases, such as type II diabetes and cardiovascular diseases. However, it is unknown if childhood obesity is associated with increased risk of colorectal cancer in adults, which is the fourth most prevalent cancer in the U.S. Furthermore, the long-term impacts of childhood dietary energy intake on colonic inflammation and carcinogenesis are largely unknown. Therefore, I proposed a research project using multidisciplinary methods to better understand the associations between diet, obesity, and colon cancer. The proposed project used epidemiological methods to answer the question of whether or not snacking is associated with childhood obesity in the U.S. Furthermore, the proposed project used a well-designed animal model of colon cancer, simulating lifetime alterations in dietary energy intake, to investigate long-term impacts of childhood obesity on colon cancer and potential mechanisms. My dissertation project was significant in three aspects: (1) it expanded the knowledge of the associations between diet, obesity, and colon cancer; (2) it facilitated the identification of the most vulnerable population and development of targeted strategies to prevent obesity and colon cancer in the society; and (3) it generated new hypotheses for future grants for my professional development as a junior scientist.
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Function</th>
<th>Regulation by obesity</th>
<th>Regulation by ER diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>• Anti-tumor via reducing inflammation by appropriately handling bacteria challenge.</td>
<td>Increased by obesity in tumor microenvironment and circulating levels.</td>
<td>Reduced by ER diet induced weight loss.</td>
</tr>
<tr>
<td></td>
<td>• Pro-tumor via activation of NF-κB -dependent oncogenic pathways.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>• Anti-tumor at early stage, via inhibition of cell cycle and induction of apoptosis.</td>
<td>Increased by high-fat diet induced obesity.</td>
<td>Reduced by ER diet or Roux-en-Y gastric bypass.</td>
</tr>
<tr>
<td></td>
<td>• Pro-tumor at advanced stage, via angiogenesis promotion, anti-tumor immunity suppression, and epithelial-to-mesenchymal transition (EMT) induction.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Pro-tumor via pro-proliferation of cancer cells by activation of NF-κB and WNT-beta-catenin pathway.</td>
<td>Increased in obesity people, but reduced by high fat diet.</td>
<td>Decreased by ER diet induced weight loss.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Pro-tumor via driving proliferation, migration and angiogenesis through activation of STAT3 signaling.</td>
<td>Increased in obese patients, but may not altered by high fat diet.</td>
<td>Not altered by ER diet induced weight loss.</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Pro-tumor via activation of NF-κB in epithelial cells.</td>
<td>No relevant publication.</td>
<td>No relevant publication.</td>
</tr>
<tr>
<td>IL-8</td>
<td>Pro-tumor via promoting cell proliferation and vascularization.</td>
<td>Increased in obese patients.</td>
<td>Decreased by ER induced weight loss.</td>
</tr>
</tbody>
</table>

Table 1.1 Colon carcinogenesis related cytokine and their regulation by diet.
<table>
<thead>
<tr>
<th>Rodent model</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous cancer</td>
<td>• Capture much of the complexity underlying spontaneous colorectal carcinogenesis in humans.</td>
<td>• Molecular mutations are not clear</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Long study period</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Low calcium level may change physiology in ways that do not reflect the etiology of human colorectal cancer.</td>
</tr>
<tr>
<td>Chemically induced</td>
<td>• Moderate study period</td>
<td>• Exposure to chemical is irrelevant to human cases</td>
</tr>
<tr>
<td></td>
<td>• Tumor sites mimic human cases.</td>
<td>• Chemical has potential impacts on liver.</td>
</tr>
<tr>
<td>Genetic modified</td>
<td>• High incidence rate</td>
<td>• Most tumors are in small intestine.</td>
</tr>
<tr>
<td></td>
<td>• Short study period.</td>
<td></td>
</tr>
<tr>
<td>Xenograft</td>
<td>• Short study period.</td>
<td>• Less relevant to human colon cancer cases.</td>
</tr>
</tbody>
</table>

Table 1.2 Rodent models of colon carcinogenesis.
Chapter 2: Fiber Content in Snacks and Childhood Obesity

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Abstract

Childhood obesity has become a crucial public health issue in the U.S. since the 1980s. Currently, the obesity epidemic started expanding to developing countries, especially those experiencing a rapid success in economy. Along with the increase in childhood obesity rates, snack food consumption increased over the past three decades. Previous studies connecting snacking and childhood obesity found contradictory results. Simply targeting energy contribution from snacking limited the capacity of differentiating nutrient-rich snack foods from nutrient-poor snack foods. To identify the potential role of snacking in childhood obesity, we analyzed a nationally representative population of children ages 2-18 years from the National Health And Nutrition Examination Survey (NHANES) 2005-2012 data. Snack foods consumption was characterized by the contribution to total dietary fiber intake and the fiber density in snacking and was then further categorized into tertiles. A total of 10,585 children with reliable dietary recall data from day one and day two interviews were included in the analysis. Appropriate sample weights were used to address the complex design of NHANES. Consistent with national survey reports, about one third of the population was overweight or obese, according to the definition of childhood obesity by the Center for Disease Control and Prevention (CDC). Among children from 2-11 years of age, the contribution of fiber from snacking was significantly positively associated with the risk of childhood overweight and obesity.
(highest tertile compared to lowest tertile, OR = 1.28, 95%CI: 1.06-1.54, p = 0.010). As expected, the contributions of dietary fiber intake from snacking and fiber density in snack foods were significantly inversely associated with the risk of childhood overweight and obesity (highest tertile compared to lowest tertile, OR = 0.69, 95%CI: 0.49-0.97, p = 0.034; OR = 0.77, 95%CI: 0.59-0.998, p = 0.048, respectively). Analyses of glucose tolerance testing and fasting c-reactive protein levels among a subgroup did not find significant associations with either snacking contributions to total dietary fiber intake or fiber density in snack foods. The present study suggests that fiber density in snacks is significantly inversely associated with childhood obesity among 12-18 year olds. Therefore, recommending high fiber snack foods to 12-18 year olds could be a prevention strategy for childhood obesity.
Introduction

The worldwide prevalence of childhood overweight and obesity is estimated to reach 9.1% in 2020\(^2\). Despite that the rate of increase of childhood overweight and obesity has been more than 30% higher in developing countries than in developed countries, the percentages of overweight and obese children remain exceedingly high in the U.S., such that over one third of children and adolescents were overweight or obese in 2012\(^{136}\). Obese children and adolescents have to deal with not only non-medical complications, such as poor self-esteem and bullying, but also medical complications that were conventionally diagnosed in adults, such as cardiovascular disease, prediabetes, as well as bone and joint problems\(^{13,137}\).

Globally, Type 2 diabetes is being reported at an earlier age, primarily driven by the increase in obesity and metabolic syndrome in early childhood\(^9\). The overall prevalence of metabolic syndrome among U.S. children and adolescent is less than 5%, however, the prevalence is much higher among overweight and obese children and is increasing with higher degrees of childhood obesity\(^{10,11}\). In addition to impaired glucose metabolism, obese children have higher levels of high-sensitive c-reactive protein (hs-CRP) than their lean peers\(^{138}\). CRP is a substance produced by the liver in response to inflammation. Elevated circulating CRP levels have been linked to higher risks of cardiovascular
disease and several types of cancer\textsuperscript{139, 140}. Children and adolescents who are obese are more likely to be obese as adults and are at higher risks for adult health problems such as Type 2 diabetes, stroke, and cancer\textsuperscript{137, 141}. Therefore, identifying potential risk factors of childhood obesity is of great importance.

Dietary intake has been extensively studied as one of the risk factors of obesity. Snacking is an eating pattern with no concrete definition across studies\textsuperscript{42}. Nevertheless, the increase of childhood obesity coincides with a reported increase in snack foods consumption\textsuperscript{142}. In epidemiologic studies with moderate sample sizes, more frequent snacking has been associated with higher total energy intake, a higher proportion of energy contributed by added and total sugar, as well as higher body weight in children\textsuperscript{33, 35}. On the other hand, snacking contributes a fairly big proportion to total daily dietary intake among children. Recently, an analysis with NHANES data showed that foods consumed by children and adolescents at self-defined snacking occasions provided 19-32\% of calcium intake, 27-37\% of vitamin C intake, 14-29\% vitamin D intake, and 26-34\% of vitamin E intake across age and gender groups\textsuperscript{33}. Furthermore, the contribution of energy from snacking vegetables was significantly associated with lower BMI, while contribution of snacking energy from desserts and sweets was associated with a significantly higher BMI among adults\textsuperscript{143}. Therefore, the inconsistent associations between childhood obesity and snacking are partially due to the mixed definition of snacking.
Previously, studies using nationally representative populations of U.S. children reported a non-significant inverse relationship of BMI with snacking frequency and energy contribution from snacking. However, the energy contribution from snacking may not be an appropriate measure to differentiate nutrient-rich snack foods from nutrient-poor snack foods. Therefore, the true relationship between snacking and childhood obesity might be confounded by limitations in measuring dietary intake and describing snacking patterns. Considerable evidence supports that increasing consumption of dietary fiber is associated with lower risks of obesity. Often, the energy-dense nutrient-poor snack foods tend to contain less fiber than nutrient-rich foods with the same calories. Therefore, we targeted energy contribution from snacking and fiber density in snack foods to study the association between snacking and body weight status, as well as impaired glucose metabolism among 2-18 year olds.

**Materials and Methods**

*Study sample*

The present cross-sectional analysis was based on public domain data from NHANES, a continuing population-based survey that uses a complex, stratified, multistage probability sample design to create a representative sample of the non-institutionalised civilian U.S. population. Four biannual survey cycles were combined to produce estimates with greater precision and smaller sampling error. The NHANES protocol was approved by the National Center for Health Statistics Research Ethics Review Board, and written informed consent was obtained from all participants or proxies. Demographic, dietary,
questionnaire, and laboratory data from children and adolescents 2-18 years of age who participated in the combined survey years of 2005-2012 NHANES and reported reliable dietary intake in the two days interview were included in the analyses.

**Demographic Characteristics and Physical Activity Levels**

Demographic and physical activity levels were collected from the NHANES questionnaire data. To account for the different eating patterns, two age groups, 2-11 and 12-18 year olds, were created. Race and ethnicity were recoded as Hispanic, non-Hispanic White, non-Hispanic Black, or other. Family income to poverty ratios (PIR) were used to categorize household income levels where high income was defined as >3.5 of the PIR, medium income defined as 1.301–3.5 PIR, and low income defined as ≤1.3 PIR. Physical activity data were available among children and adolescents aged 12-18 years old and were categorized into three levels: "sedentary"-less than 150 minutes of moderate activity in a week, "moderate"-150 to 300 minutes of moderate activity in a week, and "active"- more than 300 minutes of moderate activity in a week.

**Dietary Assessment**

All NHANES survey participants completed two interviewer-administered 24-hour dietary recall surveys on nonconsecutive days: day one interviews were conducted in-person at the Mobile Exam Center (MEC); day two interviews were administered via telephone. Dietary interviews were conducted in English and Spanish. Translators were used to conduct interviews in other languages. Primary caregivers completed proxy
surveys for children younger than 12 years of age. Self-reported name of eating occasion type was used to identify occasions that were called a snack in either the English or Spanish version of the questionnaire. The Spanish translation of a snack included the following: merienda, entre comida, tentempie, bonana, and bocadillo. Drink, in both English and Spanish versions, was not categorized as snack. For this study, average energy and dietary fiber data in two days were calculated for each child with reliable dietary data in the surveys. Snack foods contribution to total dietary fiber intake (%) and dietary fiber density in children’s snacks was calculated (fiber density in snack foods = average grams of fiber per 1000 kcal total energy consumed in the snack). Those variables were used to create tertiles of snack food contribution to fiber intake and snack foods fiber density.

**Body Weight Status**

Detailed information about equipment, calibration, methods, quality control, and survey procedures can be found on the NHANES website (http://www.cdc.gov/nchs/nhanes). Anthropometric data were obtained when NHANES participants visited the MEC. Height and weight were measured using standard procedure. BMI-for-age z-score and percentile of BMI-for-age were calculated based upon 2010 Centers for Disease Control and Prevention (CDC) Growth Charts. For the purpose of this study, children who were underweight (less than the 5th percentil) were combined with those who had healthy body weight (5th–84th percentile), while children who were overweight or obese were combined (≥ 85th percentile of BMI-for-age).
Risk for Diabetes and Inflammation

Diabetic risk was evaluated with a two-hour oral glucose tolerance test among a subpopulation of 12-18-year-old adolescents. Oral glucose tolerance tests were performed in the morning session after a 9-hour fast. A fasting glucose level of at least 126 milligrams per deciliter (mg/dL) was used as the cut-point to establish impaired glucose metabolism. Pre-diagnostic circulating C-Reactive Protein (CRP) is a biomarker for systemic inflammation and is associated with increased risk of colorectal cancer. CRP levels were log-transformed due to a right skewed distribution and were analyzed among a subgroup of subjects. Detailed specimen collection and processing instructions are discussed in the NHANES Laboratory/Medical Technologists Procedures Manual (http://www.cdc.gov/nchs/data/nhanes/nhanes_09_10/lab.pdf).

Statistic Analysis

All analyses were performed on sample-weighted data with SAS software (SAS 9.4) to adjust for the complex sample design. An 8-year dietary two-day sample weight variable was created by assigning one-fourth of the 2-year dietary two-day sample weight (WTDR2D) for each person and used in dietary analysis. Similarly, an 8-year oral glucose tolerance test sample weight variable and an 8-year MEC sample weight variable were created and used in analysis of glucose tolerance testing and descriptive statistics, respectively.
The relationship between the dietary fiber level in children’s snacks and their risk for being overweight or obese was estimated using multiple logistic regression models. The independent variable was expressed as tertiles of fiber density in snacks (referent, lowest tertile) while the main dependent variable was coded as a dichotomous variable (overweight/obese (=1) or not (=0)). Analyses were stratified by age groups, controlling for age (as continuous variable), total energy intake (as continuous variable), gender, ethnicity, and family income. Results were reported as odds ratios (ORs) with 95% confidence intervals. Similarly, the relationship between dietary fiber intake level in snacks and the risk for impaired glucose metabolism was examined using logistic regression models where the independent variable was expressed as tertiles of fiber density (referent, lowest tertile) while the main dependent variable was coded as a dichotomous variable (impaired glucose metabolism (=1) or not (=0)). Analyses were stratified by age groups, controlling for age (as continuous variable), gender, ethnicity, and family income. The association between the dietary fiber level in children’s snacks and their risk for elevated inflammation was estimated using multiple linear regression models. The independent variable was expressed as tertiles of fiber density in snacks (referent, lowest tertile) while the main dependent variable, serum CRP levels, was coded as a continuous variable. Analyses were stratified by age groups, controlling for age (as continuous variable), gender, ethnicity, and family income. Results were reported as coefficient estimates. Significance level for all analyses was set at $p < 0.05$. 
Results

Demographic distribution of the population

The population characteristics are shown in Table 2.1. The total sample from four NHANES survey cycles included 13,418 children between 2-18 years old. Within the total population, 10,585 children, approximately 80% of the sample, had provided reliable intake reports in two-day interviews and were included in the analysis. Although more children were in the 2-11 age group, the demographic distributions of the two age groups were similar. However, in the younger age group (2-11 year-olds), more children were from low-income families, while more children were from high-income families in the older age group (12-18 year-olds). A physical activity questionnaire was asked among children age 12-18 years old with a 41% response rate. Among children who had reported physical activity, 60.2% of them were categorized as "active" while 26.8% were categorized as "sedentary".

Prevalence of overweight/obesity in the population

As shown in Table 2.2, the overall prevalence of overweight/obesity was significantly lower among 2-11 year-olds than 12-18 year-olds, with 27.4% and 34.7%, respectively (p< 0.0001). In both age groups, Non-Hispanic Whites children had significantly lower overweight/obesity rates than Hispanic children (24.6% vs. 35.3% in 2-11 year-olds, p<0.0001; 31.6% vs. 42.7% in 12-18 year-olds, p<0.0001). The difference of prevalence between children from high-income families and low-income families also reached statistical significance such that the difference was greater in the older age group (high-
income vs. low-income: 21.9% vs. 30.6% in 2-11 year-olds and 26.4% vs. 41.9% in 12-18 year-olds). The prevalence of childhood overweight/obesity among children with missing data in physical activity was not significantly different from children who had reported physical activity in the questionnaire.

Prevalence of snack-eaters in the population

Among all children who reported reliable dietary records, 96.1% reported energy-bearing snacks in either, or both, dietary interviews. Children in the older age group had a lower prevalence of snack-eaters compared to that in the younger age group (Table 2.3, p<0.0001). Only among the 2-11 year-olds were the prevalence of snack-eaters significantly different across subgroups such that Non-Hispanic White children had higher rates than Hispanic children (p=0.01) and children from high-income families were more likely to have snacks in a typical day compared to children from low-income families (p<0.0001). Similar to the prevalence of overweight/obesity in the population, the prevalence of children who had energy-bearing snacks in a typical day was not significantly different by physical activity levels (ps>0.05).

Total dietary fiber intake and fiber content in snacks consumed by the population

The dietary fiber intake in the population aged 2-18 is shown in Table 2.4. The average consumption of dietary fiber among children at 2-18 years old in the United States was 14.8 gm/day (data not shown). Children aged 12-18 years old had significantly higher consumption compared to 2-11 year-olds (16.0 and 14.0 gm, respectively, p<0.0001). In
both age groups, girls had significantly less dietary fiber than boys (p=0.0005 and p<0.0001 for 2-11 year-olds and 12-18 year-olds, respectively). Non-Hispanic Blacks had lower consumption compared to Hispanics in both age groups (ps<0.0001), while Non-Hispanic Whites had significantly lower consumption than Hispanics among 2-11 year-olds (p=0.0189). Children from high-income families had higher consumption than those from low-income families, although the difference was not statistically significant (p=0.264 and p=0.068 for 2-11 year-olds and 12-18 year-olds, respectively). Among 12-18 year-olds, the consumption of dietary fiber increased by physical activity levels, although this was not significant (p=0.273 and p=0.095 for moderate and vigorous levels, respectively).

The average contribution of snacks to total dietary fiber intake was 27.4% among children aged 2-18 (data not shown). Compared to younger children, children aged 12-18 had a greater proportion of fiber intake from snacks (26% and 29.3%, respectively, p=0.0035). Among children aged 2-11, girls had higher proportion of fiber from snacks than boys (p=0.023). In addition, Non-Hispanic Whites and Blacks had significantly smaller proportions of fiber from snacks, compared to Hispanics (p<0.0001 and p=0.032, respectively). In both age groups, children from high-income families had smaller proportions of dietary fiber intake from snacks, compared to those in low-income families (p=0.0013 and p=0.043 for 2-11 year-olds and 12-18 year-olds, respectively). Similar to total dietary fiber intake, the contribution of snacks to total dietary fiber intake
was not significantly different by physical activity levels (p=0.147 and p=0.712 for moderate and vigorous levels, respectively).

The overall fiber density in snacks was 7.0 gm/1000 kcal. The differences between the two age groups, gender, and family income levels were not significant (p=0.0932, p=0.178, p=0.126 (medium income versus low income), and p=0.485 (high income versus low income), respectively). In both age groups, fiber density in snacks consumed by Non-Hispanic Whites and Blacks was significantly lower than Hispanic children (p<0.0001 and p<0.0001 among 2-11 year-olds; p=0.039 and p=0.0001 among 12-18 year-olds, respectively). Interestingly, the fiber density of snacks consumed by physically active children was significantly higher than those consumed by sedentary children (8.0 and 6.3 gm/1000kcal, respectively, p=0.004).

**Associations between dietary fiber content in snacks and childhood obesity**

As shown in Table 2.1, physical activity measures were only available among a small group of children from age 12-18. The large within-person and between-person variations in estimating snack foods consumptions require a relatively big sample size to have adequate power. Considering the small sample size of reporters with physical activity measures and the absence of significant differences in obesity rates and snacking behaviors between reporters and non-reporters, we did not include physical activity in the following analyses.
Tertiles of contribution of snacks to total dietary fiber intake for 2–18 year old reporters are shown in Table 2.5. In 2–11 year old reporters, the contribution of snacks to total dietary fiber intake tertiles were 9.0%, 23.5%, and 45.5% in the lowest, medium, and highest tertiles, respectively. In 12–18 year-olds, tertiles of contribution of snacks to total dietary fiber intake were 7.4%, 23.9%, and 51.3% in the lowest, medium, and highest tertiles. Thus, the proportions of dietary fiber from snacks were widely varied in this population and the variation was larger in older age groups.

Similarly, fiber density in snacks greatly varied in the population. For children aged 2-18, the snack fiber density tertiles were 2.9, 6.3, and 14.6 gm/1000kcal in the lowest, medium, and highest tertiles, respectively. In 2-11 year old reporters, tertiles of fiber density in snacks were 3.0, 6.4, and 14.1 gm/1000kcal in the lowest, medium and highest tertiles. In 12–18 year-olds, tertiles of fiber density in snacks were 2.6, 6.1, 15.2 gm/1000kcal in the lowest, medium, and highest tertiles. The variation of fiber density in snacks was greater among the older age group in this population.

Odds ratios of being overweight/obese for children at 2–18 years old are shown in Figure 2.1. In 2–18 year old reliable reporters, the risk for overweight/obesity slightly decreased by 5% in the highest tertile of contribution of snacks to total dietary fiber intake compared to those in the lowest tertile (Figure 2.1a, OR = 0.95, 95%CI: 0.79-1.15, p = 0.609). Additionally, there was a trend of increasing risk for overweight/obesity with increasing contribution of snacks to total dietary fiber intake among respondents.
from 2–11 years old, where the risk for overweight/obesity slightly increased by 27.9% for children in the highest tertile compared to those in the lowest tertile (Figure 2.1a, OR = 1.28, 95%CI: 1.06-1.54, p = 0.010). Among 12–18 year-olds, there was a trend of decreasing risk of childhood obesity with increasing contribution of snacks to total dietary fiber intake such that the risk decreased by 30.7% among children in the highest tertile (Figure 2.1a, OR = 0.693, 95%CI: 0.49-0.97, p = 0.034). On the other hand, the risk of being overweight/obese for children at 2-18 years old was decreased by 7% in the highest tertile of fiber density in snacks compared to the lowest tertile, but the difference was not significant (Figure 2.1b, OR = 0.93, 95%CI: 0.79-1.09, p = 0.373). The increasing risk of being overweight/obese from lowest tertile to highest tertile of fiber density in snacks among 2-11 year-olds was not significant. However, among 12-18 year-olds, the risk decreased by 23.4% in children in the highest tertile (Figure 2.1b, OR = 0.770, 95%CI: 0.59-0.998, p = 0.048). Thus, eating snacks with high fiber density was associated with lower risks of childhood obesity among 12-18 year old children.

Associations between dietary fiber content in snacks and response to glucose tolerance testing

The risk for impaired glucose response among children from 12-18 years old was slightly elevated by increased contribution of snacks to total dietary fiber intake (Figure 2.2a, medium tertile: OR = 1.098, 95%CI: 0.484-2.492, p = 0.823; highest tertile: OR = 1.111, 95%CI: 0.456-2.707, p = 0.817), while slightly reduced by increased fiber density in snacks (Figure 2.2b, medium tertile: OR = 0.916, 95%CI: 0.403-2.082, p = 0.834;
highest tertile: OR = 0.892, 95%CI: 0.471-1.691, p = 0.727). Overall, dietary fiber in snacks was not significantly associated with impaired response to glucose tolerance testing among children at 12-18 years old.

**Difference in circulating c-reactive protein levels by dietary fiber content in snacks tertiles**

Circulating CRP levels were available among a subgroup of children from 2-18 years old from 2005-2010 NHANES. The differences in CRP levels by tertiles of contribution of snacks to total dietary fiber intake and fiber density in snacks are shown in **Table 2.6**. Overall, the adjusted association of CRP levels with tertiles of contribution of snacks to total dietary fiber intake and fiber density in snacks was not statistically significant.

**Discussion**

The National Health And Nutrition Examination Survey provides consecutive data on a nationally representative population to assess the health and nutritional status of adults and children in the United States. This study examined the associations between snacking and the risks of being overweight or obese among children in the U.S. To achieve the goal, NHANES data of 2-18 year-olds with reliable dietary recall in two interviews were analyzed and stratified by age group. Demographic characteristics were incorporated into the statistical model. Prevalence of overweight and obesity and snacking prevalence in this population are consistent with previous research\(^{146,142}\). The large contribution of snacking to total dietary fiber intake and the low fiber density in snack foods explains the
low dietary fiber consumption in the population. Results shown here indicate a lower risk of childhood obesity with increasing dietary fiber intake from snacking among U.S. children from age 12-18 years. A recent study on the association of total fiber intake with childhood obesity risk found similar effects in that a lower risk of childhood obesity was associated with higher total fiber intake.145

Data on snacking and weight status in the pediatric population are not consistent. A small number of studies found that greater consumption of energy-dense snack foods was associated with higher BMI among some groups of children.147 The majority of cross-sectional studies and a few longitudinal studies either found no evidence of a relationship between snacking behavior and weight status, or found evidence indicating an inverse association between snacking and childhood obesity.42 Despite important strengths in some of those studies, including large representative samples, the impact of total energy intake on weight status was often times neglected. In those studies, snacking frequency and the percentage of energy from snacking were often times used to assess snacking behavior.42 The association between weight status and nutrient intake, in this case the energy intake from snacking, was confounded by total energy intake and dividing by total energy intake to percentage of energy from snacking would induce confounding in the opposite direction.41 Furthermore, neither snacking frequency, nor the percentage of energy from snacking, can differentiate nutrient-rich snack foods from nutrient-poor snack foods. The large proportion of dietary intake of many nutrients from snacking among children suggests the important role of food choices in snacking in order to meet
the recommended dietary intake levels and to stay healthy. Therefore, previous studies on snacking frequency and energy contribution provided limited evidence in terms of advising the public to adapt a healthy dietary pattern.

Dietary fiber is the non-digestible carbohydrates and lignin that are intrinsic and intact in plants. Benefits of adequate fiber intake include lowering blood pressure, improving serum lipid profiles, reducing indicators of inflammation, improving bowel function, assisting weight loss, and lowering serum glucose levels. In weight control, fiber acts as a physiological obstacle to energy intake via displacing energy from the diet, limiting intake by promoting the secretion of saliva and gastric juice, and reducing the absorption efficiency of the small intestine. The mechanism behind the beneficial effects of fiber on diabetes has not been established, yet the mTOR pathway, glucagon-like peptide 1, and the increased viscosity by higher molecular weight fiber are likely to be involved. Therefore, consuming more fiber between meals via snacking may reduce the energy intake in the following meal and the total energy intake throughout a day.

One major strength of this study was the use of a nationally representative population with reliable dietary recall to explore the snacking-obesity relationship. Although the nature of cross-sectional surveys precludes any indication of causality, findings in this study suggested a potential prevention strategy for future research. A number of limitations were present in this study. The most critical issue was the random error in estimating usual intake from 24-hour recall. Although an average of two days intake was
used, the present study did not consider the variation in snacking throughout the week. The errors in estimating usual consumption of snack foods were not fully controlled. Furthermore, the absenteeism of physical activity levels in the regression model may result in residual confounding in the results. However, comparison between respondents and non-respondents in the physical activity questionnaire did not find significant differences in obesity rates or the fiber profile of snacking.

The American Dietetic Association delivered a position statement that the public should consume adequate amounts of dietary fiber from a variety of plant foods. Adequate dietary fiber intake not only has beneficial effects on risk factors for developing chronic diseases, but also reduces the chronic disease burden in the population. Dietary Reference Intakes recommend consumption of 14 g dietary fiber per 1,000 kcal, or 19-38 g/day for people in all age groups, except for infants younger than 1 year old. However, the usual intake of dietary fiber in this pediatric population was less than 15 g/day.

Snacking is an eating pattern that is prevalent among children in the U.S. Considering the large proportion of dietary fiber intake from snacking, recommending high fiber density snacks to children between the ages of 12-18 will benefit not only childhood obesity but also other chronic diseases in their adult life.

Additional Information

The authors declare no conflict of interest.
<table>
<thead>
<tr>
<th></th>
<th>Reliable dietary intake reporters at 2-11 years old (n = 6689, N(% ± SE))</th>
<th>Reliable dietary intake reporters at 12-18 years old (n = 3896, N(% ± SE))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>3389 (51.3 ± 1.0)</td>
<td>1957 (48.8 ± 1.5)</td>
</tr>
<tr>
<td>Females</td>
<td>3300 (48.7 ± 1.0)</td>
<td>1939 (51.2 ± 1.5)</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>2487 (21.7 ± 1.7)</td>
<td>1360 (18.4 ± 1.5)</td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>1978 (57.0 ± 2.2)</td>
<td>1096 (60.6 ± 2.0)</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>1618 (13.8 ± 1.2)</td>
<td>1114 (14.2 ± 1.2)</td>
</tr>
<tr>
<td>Other races</td>
<td>606 (7.5 ± 0.7)</td>
<td>326 (6.8 ± 0.8)</td>
</tr>
<tr>
<td>Household Income(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Income</td>
<td>2946 (33.6 ± 1.5)</td>
<td>1409 (28.6 ± 1.9)</td>
</tr>
<tr>
<td>Medium Income</td>
<td>2100 (36.3 ± 1.3)</td>
<td>1357 (36.2 ± 1.7)</td>
</tr>
<tr>
<td>High Income</td>
<td>1240 (30.1 ± 1.6)</td>
<td>883 (35.2 ± 2.0)</td>
</tr>
<tr>
<td>Physical Activity(^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedentary</td>
<td>-</td>
<td>615 (26.8 ± 1.9)</td>
</tr>
<tr>
<td>Moderate</td>
<td>-</td>
<td>219 (13.0 ± 1.4)</td>
</tr>
<tr>
<td>Active</td>
<td>-</td>
<td>769 (60.2 ± 2.3)</td>
</tr>
</tbody>
</table>

Table 2.1 Demographic distributions of children at 2–18 years old, NHANES 2005–2012.

\(^1\) n= 650 children had missing data on family income ratio.

\(^2\) Physical activity questionnaire targeted children at 12-18 years old and n= 2293 children had missing data.
<table>
<thead>
<tr>
<th></th>
<th>2-11 years old (n = 6630, % ± SE)</th>
<th>p value</th>
<th>12-18 years old (n = 3866, % ± SE)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>27.4 ± 0.8 (ref)</td>
<td>-</td>
<td>34.7 ± 1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>27.7 ± 1.0 (ref)</td>
<td>-</td>
<td>35.9 ± 1.6 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Females</td>
<td>27.0 ± 1.1</td>
<td>0.59</td>
<td>33.4 ± 1.4</td>
<td>0.25</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>35.3 ± 0.9 (ref)</td>
<td>-</td>
<td>42.7 ± 1.5 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>24.6 ± 1.3</td>
<td>&lt;0.0001</td>
<td>31.6 ± 1.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>32.9 ± 1.1</td>
<td>0.07</td>
<td>39.9 ± 1.8</td>
<td>0.26</td>
</tr>
<tr>
<td>Other races</td>
<td>17.4 ± 1.5</td>
<td>&lt;0.0001</td>
<td>29.6 ± 1.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Household Income³</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>30.6 ± 1.0 (ref)</td>
<td>-</td>
<td>41.9 ± 1.6 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Medium</td>
<td>28.4 ± 1.3</td>
<td>0.15</td>
<td>37.3 ± 1.9</td>
<td>0.11</td>
</tr>
<tr>
<td>High</td>
<td>21.9 ± 1.4</td>
<td>&lt;0.0001</td>
<td>26.4 ± 1.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Physical Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>-</td>
<td></td>
<td>35.0 ± 1.3</td>
<td>0.26</td>
</tr>
<tr>
<td>Sedentary</td>
<td>-</td>
<td></td>
<td>32.1 ± 2.1 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>-</td>
<td></td>
<td>41.4 ± 4.3</td>
<td>0.07</td>
</tr>
<tr>
<td>Active</td>
<td>-</td>
<td></td>
<td>33.6 ± 2.2</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Table 2.2 Overweight/obesity rates.
Overweight/obese rates among U.S. children at age of 2-18 in NHANES 2005-2012 were compared via a Chi-square test. Bolded text indicated significant difference from the reference group, p<0.05.

---

¹ n= 59 children at 2-11 at years old with missing data in body measurements were excluded.
² n= 30 children at 12-18 at years old with missing data in body measurements were excluded.
³ n= 650 children reported had missing data on family income ratio.
<table>
<thead>
<tr>
<th></th>
<th>2-11 years old (n = 6689, % ± SE)</th>
<th>p value</th>
<th>12-18 years old (n = 3896, % ± SE)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>98.4 ± 0.3 (ref)</td>
<td>-</td>
<td>92.4 ± 0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>98.2 ± 0.3 (ref)</td>
<td>-</td>
<td>91.6 ± 1.0 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Females</td>
<td>98.5 ± 0.3</td>
<td>0.53</td>
<td>93.2 ± 0.9</td>
<td>0.29</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>97.4 ± 0.4 (ref)</td>
<td>-</td>
<td>91.2 ± 1.2 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>98.9 ± 0.3</td>
<td>0.01</td>
<td>93.1 ± 1.0</td>
<td>0.28</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>97.2 ± 0.6</td>
<td>0.81</td>
<td>91.0 ± 1.2</td>
<td>0.91</td>
</tr>
<tr>
<td>Other races</td>
<td>99.5 ± 0.2</td>
<td>0.01</td>
<td>92.9 ± 1.7</td>
<td>0.54</td>
</tr>
<tr>
<td>Household Income¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Income</td>
<td>97.9 ± 0.4 (ref)</td>
<td>-</td>
<td>91.7 ± 1.0 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Medium Income</td>
<td>98.0 ± 0.6</td>
<td>0.88</td>
<td>92.1 ± 1.1</td>
<td>0.81</td>
</tr>
<tr>
<td>High Income</td>
<td>99.5 ± 0.2</td>
<td>&lt;0.0001</td>
<td>93.1 ± 1.3</td>
<td>0.43</td>
</tr>
<tr>
<td>Physical Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>-</td>
<td>-</td>
<td>92.2 ± 0.8</td>
<td>0.71</td>
</tr>
<tr>
<td>Sedentary</td>
<td>-</td>
<td>-</td>
<td>92.8 ± 1.3 (ref)</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>-</td>
<td>-</td>
<td>90.6 ± 4.1</td>
<td>0.56</td>
</tr>
<tr>
<td>Active</td>
<td>-</td>
<td>-</td>
<td>93.3 ± 1.1</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Table 2.3 Snacking rates.
Snacking rates among U.S. children from age of 2-18 in NHANES 2005-2012 were compared via a Chi-square test. Bolded text indicates a significant difference from the reference group, p<0.05.

¹ n= 650 children had missing data on family income ratio.
<table>
<thead>
<tr>
<th></th>
<th>Dietary Fiber (gm ± SE)</th>
<th>Snack Contribution to Dietary Fiber (% ± SE)</th>
<th>Fiber Density in Snacks (gm/1,000 kcal ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2-11 year-olds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* (n = 6689)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>14.0 ± 0.1 (ref)</td>
<td>26.0 ± 0.6 (ref)</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>14.5 ± 0.2 (ref)</td>
<td>25.4 ± 0.7 (ref)</td>
<td>6.8 ± 0.2 (ref)</td>
</tr>
<tr>
<td>Females</td>
<td><strong>13.6 ± 0.2</strong></td>
<td><strong>26.6 ± 0.7</strong></td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td><strong>Race/Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>14.8 ± 0.2 (ref)</td>
<td>28.5 ± 0.6 (ref)</td>
<td>7.9 ± 0.2 (ref)</td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td><strong>14.0 ± 0.2</strong></td>
<td><strong>24.7 ± 0.9</strong></td>
<td><strong>6.6 ± 0.2</strong></td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td><strong>13.2 ± 0.2</strong></td>
<td><strong>27.4 ± 1.1</strong></td>
<td><strong>6.6 ± 0.1</strong></td>
</tr>
<tr>
<td>Other races</td>
<td>13.8 ± 0.4</td>
<td><strong>24.9 ± 1.2</strong></td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td><strong>Household Income</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Income</td>
<td>14.0 ± 0.3 (ref)</td>
<td>27.1 ± 0.6 (ref)</td>
<td>7.0 ± 0.2 (ref)</td>
</tr>
<tr>
<td>Medium Income</td>
<td>13.7 ± 0.2</td>
<td><strong>25.8 ± 0.8</strong></td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>High Income</td>
<td>14.5 ± 0.3</td>
<td><strong>24.2 ± 1.1</strong></td>
<td>7.0 ± 0.3</td>
</tr>
</tbody>
</table>

*Table 2.4 Total dietary fiber intake, contribution from snacking, and fiber density in snacking.*

Total dietary fiber intake, contribution from snacking, and dietary fiber density in snacking among U.S. children at age of 2-18 were compared via a Chi-square test (NHANES 2005-2012). Bolded text indicated significant difference from the reference group, p<0.05.
### Table 2.4 Continued

<table>
<thead>
<tr>
<th></th>
<th>Dietary Fiber (gm ± SE)</th>
<th>Snack Contribution to Dietary Fiber (% ± SE)</th>
<th>Fiber Density in Snacks (gm/1,000 kcal ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12-18 year-olds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>(n = 3896)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td><strong>16.0 ± 0.2</strong>(^1)</td>
<td><strong>29.3 ± 0.6</strong>(^2)</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>17.9 ± 0.3 (ref)</td>
<td>30.0 ± 1.1 (ref)</td>
<td>6.9 ± 0.3 (ref)</td>
</tr>
<tr>
<td>Females</td>
<td><strong>14.2 ± 0.3</strong></td>
<td>28.4 ± 0.9</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>17.1 ± 0.6 (ref)</td>
<td>29.0 ± 1.6 (ref)</td>
<td>8.1 ± 0.5 (ref)</td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>16.3 ± 0.4</td>
<td>29.7 ± 0.8</td>
<td><strong>6.9 ± 0.3</strong>(^\text{ref})</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td><strong>13.8 ± 0.4</strong></td>
<td>29.6 ± 1.5</td>
<td><strong>6.1 ± 0.2</strong>(^\text{ref})</td>
</tr>
<tr>
<td>Other races</td>
<td><strong>15.1 ± 0.5</strong></td>
<td>26.6 ± 2.1</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>Household Income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Income</td>
<td>15.4 ± 0.6 (ref)</td>
<td>30.0 ± 1.7 (ref)</td>
<td>7.2 ± 0.3 (ref)</td>
</tr>
<tr>
<td>Medium Income</td>
<td>15.7 ± 0.4</td>
<td>30.8 ± 1.5</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>High Income</td>
<td>16.8 ± 0.5</td>
<td><strong>27.5 ± 1.1</strong></td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>Physical Activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>15.4 ± 0.4</td>
<td>29.4 ± 1.0</td>
<td>6.8 ± 0.3</td>
</tr>
<tr>
<td>Sedentary</td>
<td>15.9 ± 0.6 (ref)</td>
<td>28.4 ± 1.5 (ref)</td>
<td>6.3 ± 0.3 (ref)</td>
</tr>
<tr>
<td>Moderate</td>
<td>14.7 ± 0.8</td>
<td>26.6 ± 3.2</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>Active</td>
<td>17.5 ± 0.7</td>
<td>30.0 ± 1.4</td>
<td><strong>8.0 ± 0.4</strong>(^\text{ref})</td>
</tr>
</tbody>
</table>

\(^1\) Significantly different from 2-11 year-olds, p<0.0001.

\(^2\) Significantly different from 2-11 year-olds, p<0.0001.
<table>
<thead>
<tr>
<th>Age Group</th>
<th>Dietary Fiber from Snacking (%)</th>
<th>Dietary Fiber Density in Snacking (gm/1,000 kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-18 year-olds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowest Tertile</td>
<td>8.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Medium Tertile</td>
<td>23.7</td>
<td>6.3</td>
</tr>
<tr>
<td>Highest Tertile</td>
<td>47.9</td>
<td>14.6</td>
</tr>
<tr>
<td>2-11 year-olds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowest Tertile</td>
<td>9.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Medium Tertile</td>
<td>23.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Highest Tertile</td>
<td>45.5</td>
<td>14.1</td>
</tr>
<tr>
<td>12-18 year-olds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowest Tertile</td>
<td>7.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Medium Tertile</td>
<td>23.9</td>
<td>6.1</td>
</tr>
<tr>
<td>Highest Tertile</td>
<td>51.3</td>
<td>15.2</td>
</tr>
</tbody>
</table>

Table 2.5 Tertiles of dietary fiber from snacking and dietary fiber density.  
<table>
<thead>
<tr>
<th>Age Group</th>
<th>Contribution of snacks to total dietary fiber intake</th>
<th>Fiber density in snacks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated Coefficients</td>
<td>p value</td>
</tr>
<tr>
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Table 2.6 Associations of CRP levels with tertiles of contribution of fiber intake and fiber density in snacking.

Associations of CRP levels with tertiles of dietary fiber from snacking and fiber density in snacking among U.S. children at age of 2-18 via linear regression model, controlling for age, gender, race/ethnicity, and household income levels.
Figure 2.1 Odds Ratios (OR) of overweight/obesity.
(a) Odds ratios of overweight/obesity between tertiles of contribution of snacking to total dietary fiber intake, controlling for age, total energy intake, gender, ethnicity and family income levels. (b) Odds ratios of overweight/obesity between tertiles of fiber density in snacking, controlling for age, total energy intake, gender, ethnicity and family income levels.
Figure 2.2 Odds Ratios (OR) of impaired glucose response.
(a) Odds ratios of impaired glucose response among children at 12–18 years old between tertiles of contribution of snacking to total dietary fiber intake, controlling for age, gender, ethnicity and family income levels. (b) Odds ratios of impaired glucose response between tertiles of fiber density in snacking, controlling for age, gender, ethnicity and family income levels.
Chapter 3: The impacts of lifetime alteration in dietary energy intake on experimental colon carcinogenesis

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Abstract

Background: The obesity pandemic is spreading from developed countries to developing countries due to a nutrition transition in those countries. Adults in developing countries who used to be within the range of normal weight are now gaining weight and facing the problems of being overweight and obese. On the other hand, obesity also affects children in developing countries. Previous studies have established the causal relationship between obesity and several chronic diseases, including colon cancer. However, the impacts of childhood obesity on colon cancer development remain elusive. Furthermore, the risk of colon cancer in the case of a nutrition transition is unknown.

Objective: To better understand the role of lifetime alterations in energy intake on colon carcinogenesis, we examined colon cancer risks in a chemical-induced colon cancer model.

Design: The study contained two phases (the first was from 3 to 21 weeks of age and the second was from 22 to 60 weeks of age), C57BL6 female mice were fed a high fat, a control, or an ER diet, resulting in 3×3 groups at the end. From 16 to 21 weeks of age, all mice received weekly azoxymethane injections to induce colon cancer. Body weights were measured weekly throughout the study. Body composition and glucose tolerance testing were measured at 15, 33, 60 weeks of age and 13 and 31 weeks of age,
respectively. Aberrant crypt foci (ACF), a precancerous lesion in colon, were measured 60 weeks after necropsy.

Results: Besides the impacts on body weight over time, diet in both phases of the study significantly affected the risk of colon cancer. During promotion (22 to 60 weeks of age), a high-fat diet enhanced ACF formation compared to a control or an ER diet. In contrast, an ER diet during the initiation phase (3 to 21 weeks of age) enhanced ACF burden at 60 weeks, regardless of the diet in promotion phase.

Conclusion: An ER diet followed by high fat diet posed higher risks of colon cancer in female C57BL6 mice, therefore, future human studies identifying populations at risk of colon cancer should consider the impacts of dietary energy intake history.
Introduction

Globalization enables people in developing countries, especially those with higher socio-economic status, to shift from subsisting on traditional, low caloric diets to overeating on hypercaloric diets. The obesity pandemic originated in the U.S. and the world's other rich nations and remarkably spread to even the world's poorest countries especially in their urban areas. Nearly one in every seven adult women and one in every ten adult men are facing the problem of obesity and its accompanying non-communicable diseases. Remarkably, two-thirds of them are living in developing countries. The significant increase in obesity rates and the phenomenon of "nutrition transition" being a major causal factor in developing countries warranted an emerging research question on the impact of lifetime dietary energy intake on health and disease development.

Obesity has been associated with increased risks of diabetes, cardiovascular diseases, and many types of cancer, such as cancer of the colon and breast. The underlying mechanisms linking obesity and colon cancer are multifaceted, including insulin-like growth factor (IGF)-Akt signaling, adipocyte-derived cytokine-associated inflammation, and the emerging role of the gut microbiota and immune homeostasis. Furthermore, obese children are more likely to have an increased risk of prediabetes, risk factors of cardiovascular diseases, and are at higher risks of being obese in adult life.
Therefore, obese children are at higher risks for chronic diseases, such as Type 2 diabetes, heart disease, and several types of cancer. Energy restriction (ER), on the other hand, has been studied over decades as an established regimen for weight loss and extending the lifespan. Moreover, ER has shown protective effects on colon cancer development in rodent and Rhesus monkey studies\textsuperscript{152, 153}. However, previous studies found contradictory effects of early life ER diets on health outcomes in adult life. Severe ER during gestation and early childhood reduced the risk of colorectal cancer, especially in men, yet increased the risk of obesity and breast cancer\textsuperscript{109, 154, 155}. In addition to a lack of accurate estimates of actual dietary energy intake in gestation or childhood, most epidemiological studies failed to include dietary energy intake in adult life. Indeed, the Chinese Famine study further observed that offspring who were most affected by prenatal famine were those who, as their lives progressed, consumed a relatively high-fat Western diet and became obese in adulthood\textsuperscript{156, 157}.

In the context of adulthood hypercaloric diet, early life ER has been associated with exacerbated metabolic risks in female rats, compared to rats on a regular diet in early life\textsuperscript{158}. Previously, exposure to a high-fat diet in early life increased tumor multiplicity and precancerous lesion in a preclinical model of colon cancer\textsuperscript{159}. However, the influences of lifetime alterations in dietary energy intakes on colon carcinogenesis have not been investigated in the same model. In this study, we examined the impacts of energy balance, both diet restriction and an obesity-inducing high-fat diet, during the initiation (3
to 21-weeks of age) and promotion phases (22 to 60-weeks of age) of azoxymethane (AOM)-induced colon carcinogenesis.

Materials and Methods

Study Diets

Mice were assigned to one of three semi-purified diets (Research Diets, Inc. New Brunswick, NJ) (Table 3.1): 1) CON: a control diet (10% kcal from fat, D12450B); 2) HF: a high fat diet (45% kcal from fat, D12451); or 3) ER: a 30% ER diet (30% restricted compared to the control, D03020702).

Study design

To investigate the impact of alterations in dietary energy intake on colon carcinogenesis, we conducted a study where female C57BL/6N (Charles River, Wilmington, MA) mice were randomly assigned to either control, high fat or an ER diet, as described above, starting at weaning (3 weeks) until 21 weeks of age. Starting at week 22, until the end of the study at week 60 (modeling the promotion phase) mice were reassigned to one of the 3 diets. Thus, a total of 9 treatment groups were established in a 3×3 design (Figure 3.1, control- control (C-C, n=27), control-high fat (C-H, n=27), control-ER (C-E, n=27), high fat-control (H-C, n=17), high fat-high fat (H-H, n=13), high fat-ER (H-E, n=13), energy restriction-control (E-C, n=20), energy restriction-high fat (E-H, n=18), and energy restriction-ER (E-E, n=24)) (Figure 3.1). At the end of 60 weeks mice were necropsied after 10-12 hours fasting and colons were prepared for ACF examination.
Azoxymethane induced colon carcinogenesis

At 16 weeks of age, all mice received weekly intraperitoneal (i.p.) injections of AOM (Santa Cruz Biotechnology, Inc. Dallas, TX) at a dose of 10 mg/kg for 6 weeks. To manage the large sample size, the entire study was staggered and three identical cohorts of mice were obtained from Charles River (Wilmington, MA), where each cohort has mice representing all nine combinations of dietary patterns. The entire experimental procedures were conducted according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University (protocol #2011A0000074).

Mouse husbandry.

All mice were housed in a barrier room in the animal facilities at The Ohio State University (OSU) with controlled temperature at 23°C and a 12-h light/dark cycle. Mice arrived at 2-weeks of age with a dam and the dams were acclimatized on a CON diet for 1 week before weaning and initiating the study. Mice had free access to water. Mice on the CON or HF diets in either phase were group housed and fed *ad libitum*, while mice on the ER diet in either phase were individually housed and fed daily to ensure equal dietary energy intake. Standard approved enrichments were added to each cage to minimize environmental stress.
Body measurements.

Body weight was measured individually every week. Body fat was measured in a randomly selected cohort of mice (n=4~9) at 15, 33, and 60 weeks of age via magnetic resonance imaging (MRI) in the Small Animal Imaging Core (SAIC) at the Ohio State University. Briefly, the mouse body was segmented from the background using Otsu segmentation. A connected components algorithm was used to label the background objects in the image and ‘fill’ any holes in the segmented body image. A global threshold of 120 grey level intensity was chosen to segment fat from surrounding tissue. The whole body and segmented fat mask was then used to calculate the percentage of fat in the whole body.

Glucose tolerance test

Glucose tolerance testing was conducted at 13 and 31 weeks of age. A subset of mice (n=5~6) with body weights close to the average body weights in each diet group were fasted overnight (~10 hours). Baseline blood glucose levels were measured via a glucometer via a tail nick. Glucose solution was then intraperitoneally injected at a dose of 1.5 mg glucose/gm body weight. Blood was then collected via tail nick at 15, 30, 60, 90, and 120 minutes after the injection to measure glucose level via a glucometer.

Colon tissue harvest and ACF quantification.

At week 60, colonic tissue from all mice were harvested. Before euthanasia, all mice were fasted for 10-12 hours. During necropsy, the colon was excised under aseptic
conditions and gently rinsed in sterile cold phosphate buffered saline (PBS) (Fisher Scientific, PA). The entire colon was divided into three segments of equal length (proximal, medial, and distal). An approximately 15mm segment of each section was fixed in 10% neutral buffered formalin (Fisher Scientific, MA) and stained with 1% methylene blue (Fisher Scientific, MA) to identify ACF under a microscope. Total number of ACF in all three sections was recorded for each animal.

Statistical analysis
Weekly body weights (gm) were plotted as means and analyzed by repeated measure analysis of variance (RM-ANOVA) to compare the body weights across groups over time. Body weights (gm) at weeks 15, 21, 33, and 60, body fatness (%) at weeks 15, 33, and 60, the area under curve (AUC) in the glucose tolerance testing, and total number of ACF were reported as means ± SEM and analyzed using a two-way analysis of variance (ANOVA) controlled for cohort effect. To determine which groups were significantly different from one another, Bonferroni post hoc tests were conducted in SAS (version 9.4, Cary, NC).

Results
Body measurements altered by diet
Weekly body weights (means) of mice were significantly impacted by dietary intervention (Figure 3.2). Results from the RM-ANOVA showed significant effects of group by time, initiation phase diet by time, and promotion phase diet by time on body
weight. Specifically, average body weights (means ± SEM) at week 15, 21, 33, and 60 by diet are shown in Figure 3.3. At 21 weeks of age (end of initiation phase, Figure 3.3b), mice fed the CON (n=81), HF (n=43), and ER (n=62) diets weighed 22.71 ± 0.17 gm, 28.57 ± 0.76 gm, and 14.89 ± 0.12 gm, respectively (p<0.0001). At 60 weeks (end of promotion phase, Figure 3.3d), mice on the HF diet during the promotion phase had higher body weights compared to mice on the CON or ER diet, regardless of the diet they were fed during initiation (p<0.0001). The average body weight for the nine groups are as follows: H-H (n=13): 50.11 ± 1.48 gm; C-H (n=27): 48.80 ± 1.45 gm; E-H (n=18): 46.97 ± 2.03 gm; H-C (n=17): 32.62 ± 1.22 gm; C-C (n=27): 31.06 ± 0.68 gm; E-C (n=20): 31.00 ± 0.85 gm; H-E (n=13): 18.06 ± 0.55 gm; C-E (n=27): 18.14 ± 0.31 gm; and E-E (n=24): 18.06 ± 0.20 gm.

The diet-induced differences in body fatness as defined by MRI imaging, paralleled the body weights at each time point. MRI images of body fat for representative mice have been presented in Figure 3.4. Comparisons between groups at 15 and 33 weeks are illustrated in Figure 3.5. At 15 weeks of age, mice on the high-fat diet had 40.7% body fat, compared to 24.5% in control mice, and 13.3% in those on the energy restricted diet (Figure 3.5a, p<0.05). At 33 weeks, the diets during the promotion phase significantly influenced body fatness, regardless of the diets consumed during initiation (Figure 3.5b). However, among mice on the energy restricted diet during the promotion phase, mice on the energy restricted diet group in initiation continued to show a lower percentage of fat compared to mice fed the control or high fat diet group during initiation (Figure 3.5b,
EE=14.5%, CE=18.2%, and HE=17.3%, p<0.05). At 60 weeks, the impacts of the diet from the initiation phase on body fatness disappeared and a significant difference only existed based-upon the diets fed during the promotion phase (Figure 3.5c).

Responses to glucose tolerance testing altered by diet
Glucose tolerance testing was impacted by dietary patterns and showed a strong association with changes in body weight (Figure 3.6). After 10 weeks of diet interventions in initiation phase, the average area under the curve (AUC) in glucose tolerance testing was higher among mice on the high fat diet compared to mice on the caloric restricted diet (Figure 3.6a & c, p=0.0012). After 10 weeks of diet intervention during promotion phase, the average AUC differed significantly among mice on the different diets (Figure 3.6b & d, p<0.0001), while no difference due to diet in phase 1 was observed.

Dietary impacts on colonic ACF.
Given the unexpected observation that six weekly injections of AOM only induced tumors/polyps in six mice out of 186, we lacked sufficient power to examine the impact of dietary combinations on tumor formation. However, the number of ACF, a precancerous lesion, was carefully examined in segments of the proximal, medial and distal colon (Figure 3.7). Mice fed an ER diet during initiation had significantly higher ACF number than those on the CON or HF diet during the same period (Figure 3.7a). Mice on an ER diet during promotion, however, had significantly less ACF number than
those on a CON diet during promotion, while those on a HF diet during the same time period had the most ACF (Figure 3.7b). Among obese mice, mice with an ER or HF diet during the initiation phase had a greater burden of ACF compared to those on a CON diet (Figure 3.7c, 7.5, 5.4, 3.0 per mouse for E-H, H-H, and C-H, respectively, p<0.0001). However, the initiation diet did not change the burden of ACF among lean mice (Figure 3.7d). In comparisons among all nine groups, mice in the EH group (ER-HF) had the most ACFs compared to other groups (Figure 3.7e).

Discussion

We designed a study to examine the impact of dietary energy variation prior to, and during, initiation versus promotion on colon carcinogenesis in an AOM-induced colon cancer model. Unfortunately, the AOM dosing protocol previously described to induce colon cancer in FVB/N mice was not fully reproducible in C57/Bl6 mice and an insufficient number of colon tumors were detected to allow for statistical analysis. However, colonic ACFs, which is a precancerous colonic lesion and biomarker of colon cancer risk were carefully assessed. We have demonstrated several novel findings regarding the colonic precancerous lesions in female C57/Bl6 mice: (a) the impact of early dietary energy intake on the body weight and body fat is partially retained into adulthood; and (b) an ER diet during initiation phase enhances the carcinogenic impact of AOM while ER during promotion inhibits the carcinogenesis cascade. These findings have significant implications for preclinical research and human studies.
Findings regarding body weight change in this study were as expected. In both phases, the HF diet, with 45% kcal from fat, increased body weight, while the ER diet maintained the body weight at a significantly low level without symptoms of malnutrition. Previous studies on energy balance and body weight have reported controversial results because of the different nature of the diets (purified control vs. non-purified chow), different lengths of diet interventions, and different statistical analysis methods \(159, 163, 164\). Our study, which was more rigorous because of the purified diets with identical ingredients and the repeated measure ANOVA accounting for time effect, confirmed previous finding that an early exposure to a purified control or high-fat diet had no lasting effect on body weight in later life \(163\). The discrepancy with another study, where diet-induced obesity by a purified 60% high fat diet was persistent after temporary feeding, can be explained by the purity of the diet as well as the different fat content \(164\). Our study further supports efforts to use identical purified diets in order to eliminate unintended impacts from differences in dietary composition. The present study, for the first time, found a long-term effect of an ER diet on body weight after switching to a control or high-fat diet. After 12 weeks of feeding a control or a high fat diet, mice originally on caloric restricted diet had lower body weights than those originally on control or a high fat diet. Interestingly, the effect of initiation diet only existed when the promotion diet was controlled for in the statistic analysis, which indicated that mice on the caloric restricted diet in phase 1 had lower body weights than their peers on the other two diets if they were on the same diet in phase 2. Although the difference was no longer significant at the end point (39 weeks of feeding in phase 2), the average body weight of mice on the ER diet in phase 1 was lower
than that of mice on control or a high fat diet in phase 1. Collectively, our data supported the set-point theory, which can explain body weight regulation at the individual level. Its failure to explain the regulation at the population level might be attributed to insufficient information on people’s dietary histories. Moreover, mice on the ER diet in the early phase of life gained weight after switching to the HF diet. Their body weights were moderately lower than those on the HF or CON diet in the initiation phase (p>0.05) at the end of the study (60 weeks). This finding suggested that early-onset ER does not favor a lower body weight after a long period of time on a high-fat diet in adult life. Similar to the effects on body weights, an ER diet during initiation had a lasting effect on body fatness after the same length of intervention of the CON or HF diet during the promotion phase. However, the effects were not permanent, overridden by the impact of diets during the longer promotion phase, and disappeared at 60 weeks of age. It is clear that the duration of the change in energy balance and the magnitude of the change are both critical in their impact on body weight and adiposity. A greater effort to obtain dose-dependent results will enhance our ability to translate these finding to human investigations.

Consistent with previous studies, we did not observe a lasting impact of early exposure to control or a high fat diet. Mice on a control or high fat diet had ad libitum access to food, while mice on calorie restricted diet had 30% less calorie intake compare to the control. Therefore, the calorie gap between two phases among mice originally on a CON or HF diet would be less than that among mice originally on an ER diet. Our findings suggest
that the impact of dietary history on body weight depends on the duration and the extent of calorie discrepancy between two dietary patterns. Observational studies in humans found that severe ER in early life was associated with lower colon cancer risk. Our findings here suggest that the protective effect of early-onset ER on colon cancer may be mediated by lower body weight among those subjects compared to their peers with similar eating patterns in adult life.

The beneficial effects of the ER diet during promotion is consistent with previous studies, in which alterations in several biological pathways, including inflammation, have been proposed as possible mechanisms. Retrospective studies demonstrate that ER during childhood and adolescence reduces risk of colorectal cancer. However, we found that prior to, and during, initiation (AOM injections), an ER diet enhances development of subsequent precancerous lesions. The unexpected detrimental effects of an early life ER diet may perhaps be explained by the altered AOM metabolism due to changes in body composition. Perhaps the pharmacokinetics of AOM is altered and in conjunction with the usual dosing on a total body weight basis, we have changed the activation or degradation of the carcinogen. During promotion, a HF diet enhanced ACF burdens, which most certainly is mediated by different mechanisms.

Acknowledgements

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Additional information

The authors declare no conflict of interest.
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**Ingredient (per 1000 g)**

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**Table 3.1 Diet composition and calorie distribution.**

The formula\(^1\) to calculate the exact amount of food for mice on an ER diet in either phase was provided by the company (Research Diets, Inc. New Brunswick, NJ). To ensure 30% ER is equal for all animals on the ER in phase 2, reference animals were the CC (control-control) group.

\(^1\) The amount of ER diet for each animal/day = 0.71 × the average amount of CON diet consumed by the reference animals in the previous day.
In phase 1 (3-to 21-weeks of age) and phase 2 (22-to 60-weeks of age) of the study, C57BL/6N female mice were fed control (CON, 10% kcal from fat), a high fat (HF, 45% kcal from fat), or an energy restriction diet (ER, 30% caloric restricted compared to the control), leading to 3×3 groups of intervention at the endpoint (control-control (C-C), control-high fat (C-H), control-ER (C-E), high fat-control (H-C), high fat-high fat (H-H), high fat-ER (H-E), energy restriction-control (E-C), energy restriction-high fat (E-H), and energy restriction-ER (E-E)). From 16- to 21-weeks of age, all mice received a weekly intraperitoneal injection of AOM at a dose of 10 mg/kg for 6 weeks. All mice were euthanized at 60 weeks of age and the colon tissue was collected in paraffin for methylene staining.

Figure 3.1 Study design.
Figure 3.2 Weekly average body weights over time.
Average body weight (gm) of each diet group from 3 to 60 weeks of age. C57BL/6N female mice were weaned at 3 weeks of age and randomly assigned to a control, a high-fat, or an ER diet until 21 weeks of age (phase 1). From 22 to 60 weeks of age (phase 2), animals either remained on the same diet or switched to the other two diets they were not fed in phase 1, by which the study were expanded to 9 arms. C-C: control-control; C-E: control-energy restriction; C-H: control-high fat; E-C: energy restriction-control; E-E: energy restriction-energy restriction; E-H: energy restriction-high fat; H-C: high fat-control; H-E: high fat-energy restriction; H-H: high fat-high fat. Initiation diets were presented by different colors with red for CON, green for HF, and blue for ER. Promotion diets were presented by different line formats with solid line for CON, dot-dash line for HF, and dash line for ER. Comparisons between groups via a repeated measure analysis of variance found significant difference between groups over time.
Figure 3.3 Average body weights at weeks 15, 21, 33, and 60.
Body weights (mean ± SEM) at 15 (a), 21 (b), 33 (c), and 60 (d) weeks of age are shown between diet groups (n=13~27). Diet patterns are represented by letter C (Control diet, 10% kcal from fat), H (High-fat diet, 45% kcal from fat), and E (ER diet, 30% caloric restricted compared to control). Different letters on top of each bar denote significant differences (p < 0.05; two-way ANOVA and Bonferroni post hoc test) between diet groups.
Figure 3.4 Body fat distribution of representative mice in each group.
Body fat at 15 (a) and 60 (b) weeks of age are shown by Magnetic resonance imaging (MRI). Bright area indicates fat tissue, while dark grey area indicates lean tissue.
Figure 3.5 Body fat mass at 15 and 33 weeks.
Body fat (clear bar) and body weights (dark bar) of a subset of animals from at 15 (a, n=9), 33 (b, n=4–6), and 60 (c, n=4–6) weeks were shown between diet groups. Different letters in upper case denote significant differences in body weight. Different letters in lower case denote significant differences in body fatness. p < 0.05; two-way ANOVA and Bonferroni adjustment. Data are means ± SEM.
Figure 3.6 Blood glucose levels during glucose tolerance testing.
Response to glucose tolerance testing at 13 (a, c) and 31 (b, d) weeks are shown. Diet patterns are represented by letter codes C (Control diet, 10% kcal from fat), H (High-fat diet, 45% kcal from fat), and E (ER diet, 30% caloric restricted compare to control). Different letters in upper case denote significant differences in body weights. Different letters in lower case denote significant differences in AUC during glucose tolerance testing; p < 0.05.
**Figure 3.7 Dietary impacts on colonic ACF.**
(a) HF diet and ER diet in the initiation phase enhanced ACF numbers at the end of the study. (b) In the promotion phase, HF diet increased ACF burden at the end of the study, compared to the CON and ER diet. (c) Among obese mice, early life exposure to the ER diet increased ACF burden, compared to the CON diet. (d) Among lean mice, initiation diet did not change the burden of ACF. (e) Pair-wise comparisons among nine combinations of dietary patterns in the lifespan showed similar trends, with mice in E-H (ER-HF) group showing the most burden of ACF compared to other groups. (f) ACF under microscope. Different letters denote significant differences in total number of ACF. $p < 0.05$; two-way ANOVA and Bonferroni adjustment. Data are means ± SEM.
Chapter 4: The impacts of lifetime alteration in dietary energy intake on circulating cytokine levels

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Abstract

T helper (Th) lymphocytes play a key role in the adaptive immune system exerting a broad spectrum of biological function. The differentiation of various subsets of T cells depends on the expression of cytokines and receptors as responses to milieu changes. The plasticity of T lymphocyte subsets plays an important role in the initiation and development of pathological processes, including cancer and autoimmune diseases. The alterations of the T lymphocyte paradigm have been described in several autoimmune diseases, however, such studies in cancer are scarce. The present research measured Th1/Th2/Th17/Treg cytokines in an experimental colon cancer model with altered lifetime dietary energy intake. A cohort of three-weeks old C57/Bl6 female mice were fed with one of three different diets: control (CON), 30% energy restriction (ER), or high fat (HF) diet until 21 weeks of age. From 22 weeks to 60 weeks, mice were rerandomized to one of the three diets, yielding nine different combinations of dietary energy intake. Seven mice from each of the three diet groups were sacrificed at 21 weeks. The rest of the cohort received azoxymethane (AOM) from 16 weeks to 21 weeks of age to initiate colon tumor. Three mice from each of the three diet groups were sacrificed post injections (after 21 weeks), while another seven mice from each of the nine diet groups were sacrificed at the end of the study (at 60 weeks). Serum samples were collected during necropsy. Circulating levels of Th1/Th2/Th17/Treg cytokines were measured via
enzyme-linked immunosorbent assays and multiplex immunoassays. Interleukin-2 level was increased by AOM injection (p=0.023). Comparisons between samples from 21 weeks and 60 weeks detected elevated levels of interleukin-22, interleukin-13, interleukin-27, and interleukin-18, with reduced levels of transforming growth factor-β1, interleukin-4, and interleukine-1β. Cytokine levels did not change by diet groups at 21 weeks, however, interleukin-2 was significantly decreased by the ER diet during 22 weeks to 60 weeks, while interleukin-18 was increased, compared to the HF diet. This study, for the first time, described the circulating levels of Th lymphocyte-related cytokines in a mouse model of colon cancer with altered lifetime dietary energy intake.
Introduction

Chronic inflammation has been studied as a prominent promoter of tumor growth and metastatic spread of tumor cells for decades \(^{167,168}\). Regulated local inflammation for either wound healing, or pathogen clearance, results in the production of growth factors, chemokines, and pro-inflammatory cytokines \(^{169}\). Persistent inflammation encourages tumor formation by over-stimulating growth signaling \(^{170}\). Polarization of ordinary macrophages into type II macrophages (M2) suppresses their ability to present antigens to T cells while retaining their tissue-growth and pro-angiogenic abilities \(^{169,170}\). T cells are the primary means by which the immune system fights tumors in mice lacking a functioning adaptive immune system. The mice are at higher risks of spontaneous and carcinogen-induced sarcomas. \(^{171}\). T helper (Th) lymphocytes, identifiable by expression of the CD4 receptor, play an important role in the adaptive immune system. In response to cytokine signaling and other factors, naïve post-thymic CD4+ T cells differentiate into numerous lineages, including the Th1, Th2, Th17, and Treg lineages \(^{172}\). In spite of the increased frequency of circulating CD4+ T cells in patients with epithelial malignancies, the understanding of its impacts on tumor development remain elusive.

Differentiated from naïve CD4+ T cells by interferon γ (IFN-γ) and interleukin (IL)-12, Th1 cells produce IL-2, IL-12, IFN-γ and tumor necrosis factor α (TNF-α), assisting
cytotoxic lymphocytes and tumor-fighting M1 macrophages. Th2 cells, polarized by IL-4, can produce IL-4, IL-5, and IL-13, mediating an anti-inflammatory humoral response. However, Th2 cells may promote metastasis by stimulating M2 macrophages. Pro-inflammatory Th17 cells are polarized by the combination of transforming growth factor-β1 (TGF-β1) and IL-6, or IL-21. Th17 cells secrete IL-17, IL-21, IL-22, and granulocyte macrophage colony-stimulating factor (GM-CSF), promoting the spread of tumor cells via stimulating angiogenesis and fibroblasts and the migration of vascular endothelial cells. The anti-inflammation Treg cells produce IL-10 and TGF-β1 and act as immune suppressors, damping the adaptive immune response. Therefore, IL-10 and TGF-β1 could be used by tumors to suppress the efficacy of cytotoxic T cells and immune checkpoint inhibitors.

Imbalance of Th1/Th2/Th17/Treg cells in peripheral blood and specific tissues has been documented in several diseases, including cancer and autoimmune diseases. Reduced levels of Th1 cells and increased levels of Th2 cells have been reported in several types of cancer cases, including colon cancer. Serum levels of IL-17A, TGF-β1, IL-4 and IL-10 (Th2 response) were significantly higher while IL-2 and IFN-γ (Th1 response) were relatively lower in oral cancer patients. However, the alteration in circulating cytokines by altered dietary energy intake remains unclear. There is evidence that accumulation of adipose tissue promotes transition from a Th2 response to a Th1 response in adipose tissue, whereas ER which results in weight loss significantly reduced circulating Th1 cells in obese individuals. Furthermore, obesity produces
progressively more IL-17 in an IL-6-dependent process compared with lean counterparts, while the impact of ER on IL-17 level is unknown. In the present study, we identified circulating cytokine levels under different energy intake contexts.

**Materials and Methods**

**Study diets**

Mice were assigned to one of three diets as described in Chapter 3 (Research Diets, NJ) (Table 3.1): 1) CON: a control diet (10% kcal from fat, D12450B); 2) HF: a high fat diet (45% kcal from fat, D12451); or 3) ER: a 30% energy restriction diet (30% restricted compared to the control, D03020702).

**Study design**

As described previously, 3-week old female C57/Bl6 mice (Charles River, Wilmington, MA) were weaned and placed on one of the three diets until 21 weeks of age. At 16 weeks of age, all mice received weekly intraperitoneal (i.p.) injection of azoxymethane (AOM) (Santa Cruz Biotechnology, Inc. Dallas, TX) at a dose of 10 mg/kg body weight for 6 weeks. Mice were then fed either the same diet or one of the alternative two diets from 22-60 weeks of age. Thus, a total of 9 treatment groups were established as a 3×3 design (control-control (C-C, n=27), control-high fat (C-H, n=27), control-ER (C-E, n=27), high fat-control (H-C, n=17), high fat-high fat (H-H, n=13), high fat-ER (H-E, n=13), energy restriction-control (E-C, n=20), energy restriction-high fat (E-H, n=18),...
and energy restriction-ER (E-E, n=24)) (Figure 4.1a). At the end of 60 weeks mice were necropsied and serum samples were collected via cardiac puncture.

To collect samples from the initiation phase, a similar study was conducted and mice were euthanized at 21 weeks, with or without the 6-week AOM injection (Figure 4.1a, n=7 without AOM injection, n=3 with AOM injection).

Mouse husbandry

All mice were housed in a barrier room in the animal facilities at The Ohio State University (OSU) with controlled temperature at 23 °C and a 12-h light/dark cycle. Mice arrived at 2 weeks of age with dam and the dam was acclimatized on the CON diet for 1 week before weaning and initiating the study. Mice had free access to water. Mice on the CON or HF diet in either phase were group housed and fed ad libitum, while mice on the ER diet in either phase were individually housed and fed daily to ensure equal dietary energy intake. Standard approved enrichments were added to each cage to minimize environmental stress. To manage the large sample size, the entire study was staggered and three identical cohorts of mice were obtained from Charles River (Wilmington, MA) for the 60-week study. Each cohort had mice representing all nine combinations of dietary patterns. Another two cohorts were obtained from Charles River (Wilmington, MA) for the 21-week study. The entire experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University (protocol #2011A00000074).
Body weight measure

Body weight was measured individually every week at 12:00 pm on the same day of the week.

Serum sample preparation

Blood from each mouse was collected via cardiac puncture. After collection, the whole blood was allowed to clot by leaving it undisturbed for 20-30 minutes at room temperature. The clot was then removed by centrifuging at 13,000 x g for 10 minutes at 4°C. The supernatant (serum) was carefully aspirated, transferred into a clean polypropylene tube and stored at -80°C immediately until use. The samples were maintained at 2-8°C while conducting the enzyme-linked immunosorbent assay (ELISA).

Measurement of circulating cytokines

Circulating cytokine levels were measured among a subgroup of mice in the 60-week study (n=6~7) and all mice in the 21-week study (n=6~7). Circulating TGF-β1 levels were measured using a mouse TGF-β1 DuoSet ELISA kit (R&D System, MN). Serum samples were activated using a Sample Activation Kit 1 (R&D System, MN) per manufacturer's instruction. The levels of cytokines IL-2, IL-12p70, IL-18, IL-27, IFN-γ, IL-4, IL-5, IL-6, IL-13, IL-1β, IL-17A, IL-22, IL-23, GM-CSF, IL-10, and TNF-α in the serum were measured using a mouse Th1/Th2/Th9/Th17/Th22/Treg ProcartaPlex multiplex cytokine assay system (eBioscience, CA) per the manufacturer’s instruction.
In both the ELISA and multiplex assays, samples from all groups were assayed together in the same batch to minimize technical bias. All samples and standards were assayed in duplicates and mean values were used for data analysis. Standards were fitted in a 4-Parameter Logistic curve for the ELISA, and a 5-Parameter Logistic curve for the multiplex. The lower detection limits of each analyst are presented in Table 4.1. Undetectable readings were recoded as 0 in analysis.

Statistical analysis
The average weekly body weights of selected mice across groups were compared using repeated measure one-way analysis of variance (21-week study) and repeated measure mixed analysis of variance (60-week study). Bonferroni correction was used to decide if one group was significantly different than another group. Due to the skewness of the cytokine data, a non-parametric comparison was used to identify the effects of diet on cytokine levels. In the 21-week study, the comparison was conducted by a Kruskal–Wallis test by ranks and adjusted for the possible effect of AOM injection. In the 60-week study, the comparison was carried out via a Friedman Two-Way ANOVA, in which the initiation phase diet was considered as a "block" factor. All analyses were conducted in SAS (version 9.4). Significance level was defined as p<0.05.
Results

Body weights of selected mice

The average body weights of selected mice from the 21-week study and 60-week study are presented in Figure 4.2. In both studies, diet significantly changed body weights over time (Figure 4.2a, b, ps<0.0001 for 21-week and 60-week study). In the 21-week study, six-week AOM injections did not affect the body weights over time (Figure 4.2c, p=0.94). In the 60-week study, the average body weights of selected mice did not differ from the overall cohort (Figure 4.2d, p=0.94). However, the average body weights of mice in the 21-week study were significantly different from those selected in the 60-week study over time under repeated measure analysis (Figure 4.2e, p=0.001). Yet the differences were not significant in the last three weeks of the 21-week study (Figure 4.2f). The average body weights at the end point in each study are shown in Figure 4.3.

At the end point of the 21-week study, mice on the ER diet had significantly lower body weights compare to those on the HF and CON diet (Figure 4.3a, 15.3, 27.8, 20.7 gm for ER, HF, and CON, respectively; p<0.0001). At the end point of the 60-week study, mice on the same diet had similar body weights, regardless of the diet in initiation phase. However, mice switched from the CON diet to the HF diet had lower body weights than those that remained on HF (Figure 4.3b, p=0.0308).

Circulating cytokine levels

The circulating cytokine IL-10 was not detectable via the multiplex. Therefore, the average serum levels of IL-10 were not included in the following analysis.
1. Alterations by AOM injection

When comparing the cytokine levels in the 21-week study, we did not find a significant difference by AOM injections. However, IL-2 was significantly increased after six weeks of AOM injections (Figure 4.4, p=0.023).

2. Alterations by time

To investigate the impacts of time on serum cytokine levels in mice on an ER, HF, or CON diet, we compared cytokine concentrations in mice remaining on an ER, HF, or CON diet in the 21-week study and the 60-week study, controlling for the diet effects (Figure 4.5). Concentrations of IL-22, IL-13, IL-27, and IL-18 were elevated over time, however, the differences were not significant. However, we found significant reduction in the circulating levels of TGF-β1, IL-4, and IL-1β (p=0.036, p=0.0087, p=0.022, respectively), regardless of the diet.

3. Alterations by diet

In the 21-week study, p-values from a non-parametric ANOVA compared cytokine levels across diet groups (Table 4.2). We were not able to reject the null hypothesis that circulating levels of cytokines did not differ between diet groups (ps>0.05 for all cytokines). In the 60-week study, p-values from a non-parametric ANOVA compared cytokine levels across diet groups in the initiation phase, the promotion phase, and the overall nine combinations (Table 4.3). Consistently, different dietary energy intake in the initiation phase did not result in a change in the cytokine levels, even when the effects of diet in the promotion phase were controlled for. However, the serum levels of IL-2 and
IL-18 were significantly altered by different dietary energy intake in the promotion phase, even after the impacts of energy intake in initiation phase were controlled for (p=0.0096, p<0.0001, respectively). Surprisingly, we were not able to reject the null hypothesis that serum cytokine levels were not changed by different combination of dietary energy intake.

Significant alterations in the serum levels of IL-2 and IL-18 by diet in the promotion phase are shown in Figure 4.6. Specifically, mice on an ER diet during the promotion phase had significantly lower circulating IL-2, compared to those on a HF diet (Figure 4.6a, 0.2 and 0.9 pg/mL, respectively, p=0.0064). Compared to mice on an ER diet, mice on a CON and a HF during the promotion phase had significantly lower levels of serum IL-18 (Figure 4.6b, 331.5, 145.4, and 154.8 pg/mL, respectively, p=0.0002).

**Discussion**

In response to cytokine signaling and other factors, CD4+ T cells differentiate into distinct populations that are characterized by the production of certain cytokines\(^{174}\). Since the discovery of Th1 and Th2 cells in the late 1980s, the family of effector CD4+ helper T (Th) cell subsets has expanded into many additional Th subsets, including Th9, Th17, Th22, and Treg\(^{183}\). Each one of those subsets has a unique cytokine profile, functional properties, and presumed roles in promoting pathology in autoimmune and inflammatory diseases\(^{184}\). The classic view of immune cells in cancer is primarily one of tumor rejection, which has been supplanted recently by a more complex view of
leukocytes having both pro-and anti-tumor properties. Because the immune system allows for plasticity in subset differentiation and expression of “signature” cytokine(s) by other Th subsets, targeting a single cytokine or pathogenic T cell subset is not sufficient to understand the role of lymphocytes in cancer development. Recently, a panel analysis on frozen colorectal tumor species found that patients with high expression of the Th17 cluster had a poorer prognosis, whereas patients with high expression of the Th1 cluster had prolonged disease-free survival. The present study identified circulating cytokine patterns in an experimental colon cancer model with altered energy intake.

The present study did not find significant differences in circulating cytokine levels by diet at 21 weeks. However, previous studies consistently reported elevated pro-inflamatory cytokines in high fat diet fed mice. An inflammatory state characterized as a shift towards a Th1-skewed responsiveness was proposed by previous studies. On the other hand, the "signature" cytokines of Th1, Th2, and Treg cells (IL-12, IL-4, IL-10, respectively) all increased in mice on an ER diet, making a general pattern of immune changes difficult to determine. Despite the similar mouse strain as well as study design to the previous study, the contradictory results observed in the present study could be due to earlier intervention and a more moderate fat content in the high fat diet.
Diet in the promotion phase significantly changed the circulating levels of IL-2 and IL-18. IL-2 is a pleiotropic cytokine first identified as a T-cell growth factor that was subsequently shown to have a broad range of other actions as well. IL-2 is not a driving force for any type of T helper cell, but instead helps to augment or attenuate the signaling pathway essential for differentiation into various types of T helper cells. For example, IL-2 drives Th1 differentiation via promoting IFN-γ production and the expression of IL-12 receptor. On the other hand, the presence of IL-2 during the Th2 differentiation process is also important because IL-2 induces IL-4 receptor expression to allow cellular responsiveness to IL-4, the "signature" cytokine of a Th2 cell. Furthermore, the role of IL-2 in Th17 differentiation is more complicated in that IL-2 can both diminish Th17 differentiation and expand cells that produce IL-17, the "signature" of a Th17 cell. Although the manner in which IL-2 affects Treg function remains incompletely understood, IL-2 is required for Treg cell development. Therefore, the reduction of IL-2 by an ER diet in the second phase could indicate complex impacts on the T cell paradigm. On the other hand, the ER diet in the second phase was associated with increased IL-18. Similar to IL-2, IL-18 also has a complex role in immunity and inflammation, specifically in colon cancer. Produced by macrophages and endothelial cells, IL-18 induces production of chemokines, adhesion molecules, and pro-inflammatory cytokines. However, Il18−/− and Il18r−/− animals are highly susceptible to tumor formation in the dextran sodium sulfate (DSS)–AOM model. Moreover, IL-18 produced downstream of inflammasome activation in the intestinal epithelium attenuates experimental colitis through direct stimulation of Treg cells, suggesting that...
IL-18 could protect against oncogenic inflammatory processes\textsuperscript{131}. Therefore, the increased levels of IL-18 by an ER diet could explain the protective effects of the ER diet on colon carcinogenesis in this mouse model.

In addition to dietary energy intake, AOM injection and time also affected T lymphocyte cytokines levels in the serum. As discussed above, the increased level of IL-2 after AOM injection may indicate alterations in the immune system that cannot be explained in this study. Although data from murine studies generally support an age-related shift from a Th1-like (IL-2, IFN-γ) to a Th2-like (IL-4, IL-6) cytokine response, the present study did not observe the same transition\textsuperscript{196}. The discrepancy could be explained by the fact that serum samples of 21 weeks and 60 weeks were not from the same mice, but representative mice at each point.

The comprehensive design of the mouse model provided an opportunity to identify the T lymphocyte subsets in different combinations of energy intake, yet there was difficulty in interpreting the data. The observed changes in circulating cytokine may not be sufficient to determine the predominant T lymphocyte subsets under each condition of dietary energy intake, however, the multiplex immunoassay provided potential targets for future study.

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**Additional information**

The authors declare no conflict of interest.
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<th>Cytokine</th>
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<th>Th2 cells</th>
<th>Th17 cells</th>
<th>Treg cells</th>
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Table 4.1 Lower detection limits (LDL) of cytokines and their roles in TH1/Th2/Th17/Treg cell homeostasis.
D+: inducing cell differentiation; D-: inhibiting cell differentiation; P: produced by cells.
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Table 4.2 Cytokine levels between diet groups in the 21-week study. Analysis was conducted via a Kruskal–Wallis test by ranks for each cytokine, except for IL-10, which was under the lower detectable limit in the multiplex assay.
<table>
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<th>Effect of promotion phase diet (p value)</th>
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**Table 4.3 Cytokine levels between diet groups in the 60-week study.**

Analysis was conducted on each cytokine via a Friedman Two-Way ANOVA, in which the initiation phase diet was considered as a "block" factor. IL-10 was not included in the comparison, due to undetectable levels in the multiplex assay. Bolded text indicates a significant change by diet groups, p<0.05.
Figure 4.1 Study design.
In the 60-week study, C57BL/6N female mice were fed control (CON, 10% kcal from fat), a high fat (HF, 45% kcal from fat), or a energy restriction diet (ER, 30% caloric restricted compared to the control) in phase 1 (3-to 21-weeks of age) and phase 2 (22-to 60-weeks of age) of the study, leading to $3 \times 3$ groups of intervention at the endpoint. From 16- to 21-weeks of age, all mice received a weekly intraperitoneal injection of AOM at a dose of 10 mg/kg for 6 weeks. All mice were euthanized at 60 weeks of age and serum samples were collected via cardiac puncture.
In the 21-week study, C57BL/6N female mice were fed a CON, HF, or ER diet from 3-to 21 week. Three mice from each diet group received weekly i.p. injection of AOM for 6 weeks, as described previously. All mice were euthanized at 21 weeks and serum samples were collected via cardiac puncture.
Figure 4.2 Average body weights over time.
(a) and (b) Average body weights by diet in the 21-week study and 60-week study. (c) In 21-week study, AOM injection did not change the growth trends across diet groups. (d) In 60-week study, the average body weights of selected mice were not significantly different than the overall cohort. (e) Average body weights over time were significantly different between 21-week study and 60-study, (f) while not different in 19, 20, and 21 week. Means were compared via repeated measure analysis of variance with Bonferroni adjustment, significance level: p<0.05.
Figure 4.3 Average body weights at study end points by diet groups. 
In the 21-week study, mice on the ER diet had significantly lower body weights compared to those on the HF or the CON diet (a). In the 60-week study, mice on the same diet had similar body weights, regardless of the diet in the initiation phase, except for mice in H-H and C-H group (b). Means were compared via a non-parametric ANOVA with Bonferroni adjustment, different letters indicated significant difference, \( p < 0.05 \).
Figure 4.4 Azoxymethane injections did not change the circulating levels of cytokines of interests.
Th1/Th2/Th17/Treg cell related cytokines were not changed by AOM injections, except for IL-2 in which AOM injections elevated the serum IL-2 concentrations (a non-parametric ANOVA, p=0.023).
Figure 4.5 Changes in serum cytokine concentrations over time.
Serum cytokine levels did not change over time, except for IL-1β (p=0.022), IL-4 (p=0.0087), and TGF-β1 (p=0.036), where their circulating levels decreased over time. Comparisons between two studies were conducted via a non-parametric ANOVA, different letters indicated significant difference between two studies, $p < 0.05$. 
Figure 4.6 Circulating levels of IL-2 and IL-18 across different diet in promotion phase.
(a) The circulating levels of IL-2 and IL-18 were significantly altered by diet in the promotion phase, after adjusting for the effects of diet in the initiation phase. Mice on an ER diet during the promotion phase had significantly lower circulating IL-2, compared to those on a HF diet. (b) Compared to mice on an ER diet, mice on a CON or a HF diet during the promotion phase had significantly lower levels of serum IL-18. Friedman Two-Way ANOVA with Bonferroni adjustment was used to decide which group differed from the other group, significance level: p<0.05.
Chapter 5: The impacts of lifetime alteration in dietary energy intake on the gut microbiota structure

Authors:

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Abstract

Transitionally altered gut microbiota (GM) by short-term diet interventions may be restored by ceasing the intervention. However, it is unclear to what extent the impact of dietary history has on GM over a long-term diet intervention. Here we compared the colonic microbiota in C57Bl6 female mice on 3 diet interventions (control, energy restricted, and high fat) over two phases of life (1: 3-21 weeks of age and 2: 22-60 weeks of age). GM structures were significantly different by colonic site, regardless of the diet intervention. Moreover, GM structure was significantly altered by diet, with a higher proportion of Firmicutes and lower proportion of Bacteroidetes in mice on an ER diet in phase 2, compared to those on a HF diet, regardless of colonic site and the initiation diet. We further observed a substantial effect from the initiation diet on GM where mice on the ER diet had higher proportion of Bacteriodetes than those on a HF diet in phase 1, regardless of the promotion diet. This data suggests that early life dietary patterns have a sustained impact on the GM composition, even with a change in diet during a later phase of life.
Introduction

The commensal microbiota of the colon are hypothesized to be major determinants of health and disease, including chronic inflammatory syndromes and colon carcinogenesis. Recent advances in high throughput genomic analysis of the colonic microbiota are leading to the emergence of several key concepts. Evidence clearly indicates that the microbiota are defined by a complex and dynamic interplay between host genetic variables and multiple environmental factors. In the developing infant, maternal inoculation at birth and modified breastfeeding impact the microbial community composition. Critically, colonic microbiota community structure shows significant stability after the first three years of life. The general structure of the microbiota is relatively consistent throughout adult life, but emerging data demonstrates aging, host physiology and health, pharmaceutical agents, and diet being important factors impacting variation in the microbial community structure and function. Transient shifts have been observed by dietary interventions and more persistent changes by antibiotic administration on the gut microbial community. Moreover, within the same subject, the changes in GM by treatment varied between two courses of treatment with the same dose of ciprofloxacin. It is likely that the responses of microbial communities to a specific treatment depend upon not only the type and severity of the treatment, but also on the present community structure.
Many studies have found that the microbial community is responsive to diet changes, at both the compositional and functional levels. In general, high-fat diet-induced obese animals had reduced Bacteroidetes and increased Firmicutes compared to control diet fed animals\textsuperscript{102, 103, 104}. The relative abundances of Firmicutes increased and Bacteroidetes decreased as calorie consumption increased\textsuperscript{105}. Conversely, one study found high-fat-fed mice, with or without restricted intake, had higher abundances of Bacteroidetes compared to low-fat-fed mice\textsuperscript{106}. Furthermore, a cross-sectional analysis on 98 healthy human subjects demonstrated that gut microbial enterotypes are linked with long-term dietary patterns\textsuperscript{107}. In spite of the quick response of the gut microbiota to dietary changes\textsuperscript{105, 107}, the magnitude of the response is moderate and diet-induced changes in the gut microbiota are reversible\textsuperscript{107, 108}.

More than one-third of adults and 17\% of children and adolescents are obese in the United States\textsuperscript{136}. Obese youth are more likely to be obese as adults and have greater risks for cardiovascular disease, diabetes, and several types of cancer as an adult\textsuperscript{13, 137}. An increased accumulation of adipose tissue, resulting from excess energy intake and decreased physical activity, leads to obesity. Therefore, a western diet, characterized by a high intake of fat and calories, is one of the most well known risk factors for obesity and may thereby contribute to an increased risk of colon cancer\textsuperscript{198, 199}. ER that reduces body adiposity can improve insulin resistance, prevent heart disease, and reduce risk for several cancers\textsuperscript{165}. Retrospective studies demonstrate that ER during childhood and
adolescence reduces risk of colorectal cancer. ER may have impacts on reducing body weight and improving gut health via increased leptin secretion, which has been reported to favor several types of microbes. The diets introduced to children at the time of weaning may also influence microbial diversity in later life, however, the long-term impacts of diet during early life stages on the gut microbiota are not well understood. Therefore, in this study, we investigated the association of the colonic microbiota profiles with different combinations of a high fat, control, or calorie-restricted diets across the lifespan in a rodent colon cancer model. The study is designed to examine dietary impacts on AOM-induced colon carcinogenesis, although toxicity and cancer data will be presented in additional manuscripts.

**Materials and Methods**

*Study design*

As described in Chapter 3, to model the impact of dietary patterns on the GM during the carcinogenesis process, we conducted a study where female C57BL/6N (Charles River, Wilmington, MA) mice were randomly assigned to either a CON, a HF or an ER diet, as described below, starting at weaning (3 weeks) until 21 weeks of age. During weeks 16-21, animals received weekly AOM injections, therefore the initiation diets occurred prior to, and during, the initiation of the carcinogenesis process. Starting at week 22, through the end of the study at week 60 modeling the promotion phase, animals were reassigned to one of the 3 diets. Thus, a total of 9 treatment groups were established as a 3×3 design (control-control (C-C, n=27), control-high fat (C-H, n=27), control-ER (C-E, n=27), high...
fat-control (H-C, n=17), high fat-high fat (H-H, n=13), high fat-ER (H-E, n=13), energy restriction-control (E-C, n=20), energy restriction-high fat (E-H, n=18), and energy restriction-ER (E-E, n=24)) (Figure 6). At the end of 60 weeks animals were necropsied and colon prepared for ACF examination and colonic mucosal microbial analysis.

Azoxymethane induced colon carcinogenesis

At 16 weeks of age, all mice received a weekly intraperitoneal (i.p.) injection of azoxymethane (AOM) (Santa Cruz Biotechnology, Inc. Dallas, TX) at a dose of 10 mg/kg for 6 weeks. To manage the large sample size, the entire study was staggered and three identical cohorts of animals were obtained from Charles River (Wilmington, MA). Each cohort has animals representing all nine combinations of dietary patterns. All experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University (protocol #2011A0000074).

Mouse husbandry.

All mice were housed in a barrier room in the animal facilities at The Ohio State University (OSU) with controlled temperature at 23 °C and a 12-h light/dark cycle. Mice (with dam) arrived at 2 weeks of age, and dam were acclimatized on a CON diet for 1 week before weaning and initiating the study. Mice had free access to water during the study. Mice on a CON or HF diet in either phase were group housed and fed ad libitum, while mice on an ER diet in either phase were individually housed and fed daily to ensure
equal dietary energy intake. Standard approved enrichments were added to each cage to minimize environmental stress.

*Body measurements and ACF quantification.*

Weekly body weights and ACF numbers were measured as described in Chapter 3.

*Colon tissue harvest.*

At week 60, colonic mucosal tissues from one representative animal in each cage (n=1–9 per group in each cohort) were harvested for analysis to minimize cage effects on the microbiota. Before euthanasia, all animals were fasted for 10-12 hours. During necropsy, the colon was excised under aseptic conditions and gently rinsed in sterile cold phosphate buffered saline (PBS) (Fisher Scientific, PA). The entire colon was divided into three segments of equal length (proximal, medial, and distal). A 3 mm piece of tissue of each section was harvested and collected in sterile tubes for bacterial analyses. Tissue was frozen in liquid nitrogen and stored at −80°C for analysis.

*Bacterial analyses.*

DNA Isolation and Sequencing: DNA isolation and paired-end 2x300 Illumina MiSeq sequencing was performed by Molecular Research LP MR. DNA (Shallowater, TX). DNA was isolated from proximal, distal, and medial sections of colon tissues using the MoBIO PowerSoil kit (Valencia, CA). The V1-V3 16s rRNA gene primers 27F/534r were used in a 30 cycle PCR with the HotStarTaq Plus Master Mix (Qiagen, Carlsbad,
The thermoprofile used was: 1 cycle at 94 °C for 3 mins, 28 cycles at 94 °C for 30s, 53 °C for 40s and 72 °C for 60s, ending with an elongation step of 72 °C for 5mins. Amplicon quality was verified via agarose gel, then pooled and purified with Ampure XP beads. The Illumina TruSeq DNA library preparation protocol was used for library prep, then amplicons were loaded for sequencing in the Illumina MiSeq machine, following the manufacturer’s guidelines. MR. DNA filtering of final sequences included: removal of sequences <150 base-pairs long and removal of sequences with ambiguous base-calls. Samples were trimmed based upon a quality score of 25 and then joined. Final .fasta and .qual files were provided to the investigators.

Sequence Analysis: Fasta and .qual files and were filtered in QIIME (version 1.8.0) based upon: 200-650bp in length, maximum ambiguous bases set at 6, minimum qual score of 25, max. homopolymer run of 6, and zero allowed primer mismatches. 95.15% of sequences passed filtering, resulting in 65,294 sequences/samples (300 total samples). Operational taxonomic units (OTUs) picking was performed using tool set qiime-tools (http://github.com/smdabdoub/qiime-tools), a method that makes use of parallel BLAST OTU picking. In summary, qiime_tools splits .fasta files into smaller datasets for rapid picking using the Ohio Supercomputer with the parallel_blast_pick_otus.py command. OTUs were picked against the GreenGenes 13_8 97% OTUs reference database. After the resulting OTU results files were merged into one overall table, taxonomy was assigned based upon the gg_13_8 reference taxonomy.
Statistical analysis

Body weights (gm) and total number of ACF between selected mice and the entire cohort were reported as means ± SEM and analyzed using analysis of variance (ANOVA) controlled for cohort effect. To determine which groups were significantly different from one another, Bonferroni post hoc tests were conducted in SAS (version 9.4, Cary, NC).

Microbial sequence data were pooled for OTUs comparison and taxonomic abundance analysis but separated by batch in principle coordinates analysis (PCoA) to have clear PCoA figures. For even sampling, a depth of 10,000 sequences/sample was used. PCoAs were produced using Emperor. Community diversity was determined by the number of OTUs and beta diversity, measured by UniFrac unweighted and weighted distance matrices in QIIME. Microbiota composition analysis was determined by taxonomic abundances which were limited to genera with at least 1% abundance. The abundances were normalized by finding square root of proportion, then the arcsine of the square root.

Adonis, a permutational multivariate analysis of variance, was used to determine statistically significant clustering of groups based upon microbiota structure distances. Due to large impacts of cohort on the community structure, the impacts of initiation diet and promotion diet were analyzed on each individual cohort. ANOVA for unbalanced sample with multiple factors, including initiation diet, promotion diet, cohort, and colonic segment, was performed on taxonomic abundances with Bonferroni correction in SAS to
determine significant effects of those factors (version 9.4, Cary, NC). Significance was set at \( p < 0.05 \).

**Results**

*Body measurements altered by diet.*

The impacts of dietary energy intake during initiation promotion phases have been fully addressed in Chapter 3. Here we compared the body weights between selected mice and the overall cohorts. As expected, the body weights of selected mice were not significantly different from the overall cohort.

*Dietary impacts on colonic ACF.*

Similar to body measurements, the impacts of dietary energy intake on ACF burden have been comprehensively addressed in Chapter 3. Here we compared the ACF burden between selected mice with the overall cohorts. The null hypothesis was that mice selected for the gut microbiota analysis did not have significant differences from the overall cohort, in terms of ACF burden.

*Dietary impacts on colonic microbiota.*

In all three cohorts, diets during the initiation and promotion phases were independent significant factors in modeling the microbiota community structure \( p=0.006, p=0.001, p=0.003 \) for initiation phase diet, in three cohorts respectively; \( ps=0.001 \) for promotion phase diet in three cohorts; **Figure 5.1a & b**. The clustering for initiation phase diets is
less robust on the PCoA than those for promotion phase diets in the principle coordinate analysis (PCoA) figures (Figure 5.1a & b). At the phylum level, the initiation phase diet only affected the relative abundance of Bacteroidetes (Figure 5.1c). Mice on the ER diet during the initiation phase had a higher abundance of Bacteroidetes than those on the CON or HF diets (44%, 38%, 34%, respectively, ps<0.05), regardless of their promotion phase diet. At the genus level, after adjusting for cohort and sampling colonic location, the initiation phase diet had a long-term effect on the class Bacteroidia that the abundances of the genus Bacteroides and an unclassified genus in family S24-7 were higher in mice on the ER diet compared to mice on the CON or HF diets during this period (p<0.005 and p<0.0001, respectively; Figure 5.1d).

Furthermore, the promotion phase diet significantly changed the microbiota in four phyla and nine genera. The ER diet in the promotion phase reduced the relative abundances of Bacteroidetes compared to the HF diet (34%, 42%, respectively, p<0.05, Figure 5.1e). Mice on the ER diet in phase 2 also had higher relative abundances of Firmicutes and Deferrribacteres (57%, 6.9%, respectively) than those on the HF diet (51%, 2.7%, respectively, ps<0.05). The relative abundance of Verrucomicrobia did not differ between the ER diet and HF diet in the promotion phase, whereas it was significantly higher in mice on the CON diet (0.8%, 1.5%, and 2.7%, respectively, ps<0.001). The promotion phase diet also affected the abundances of nine genera with three from the phylum Bacteroidetes and five from the phylum Firmicutes after adjusting for the initiation phase diet, colonic location, and cohort effects (Figure 5.1f). The abundances of bacteria in the
genus *Prevotella*, an unclassified genus in *Lachnospiraceae*, and an unclassified genus in *Ruminococcaceae* were higher in mice on the HF diet in the promotion phase compared to those on the CON or ER diet (p=0.000002, p=0.016, p=0.0069, respectively). The adjusted abundances of genera *Ruminococcus* and *Allobaculum* were lower in mice on the HF compared to those on the other diets in phase 2 (p=0.000001, p=0.000004, respectively). The genera *Lactobacillus* and *Mucispirillum* were higher in mice on the ER diet compared to those on the CON or HF diets (p<0.000001, p=0.0015, respectively) in the promotion phase. Unclassified genera in *Rikenellaceae* and *S24-7* were lower in mice on the ER diet compared to those on the CON or HF diets in the promotion phase (p<0.000001, p=0.000004, respectively). When comparing across all 9 groups of dietary intervention, the relative abundances of 4 phyla and 13 genera were significantly changed by different combinations of dietary energy intake in early and adult life (means ± SE, Table 5.1). Genus *Prevotella* was significantly higher in C-H group compare to others (7.4 ± 15.2%, p<0.005). Genus *Ruminococcus* was significantly higher in C-E, E-C, and H-E groups (2.8 ± 2.0%, 4.4 ± 3.9%, 3.8 ± 2.9%, respectively, p<0.05). Genus *Bacteroides* showed the highest abundance in E-E group and lowest in H-H group (21.1 ± 12.7% and 9.5 ± 8.4%, respectively, p<0.05).

**Colonic microbiota differs by colonic sites.**

The overall microbiota varied by anatomical location in the colon (Figure 5.2). Samples from the proximal colon had the highest OTU numbers compared to those from the medial or distal colon (Figure 5.2a, p<0.0001). Beta diversity, measured by both
unweighted (p=.001, R²=.03798) and weighted UniFrac distances (p=.001, R²=.06269), was significantly different by anatomical site, as demonstrated by PCoA (Figure 5.2b, represented by cohort 2). The relative abundances of three phyla were significantly changed by location in the colon (Figure 5.2c). Firmicutes accounted for 59% of all sequences in the proximal colon, which was significantly higher than the medial colon (46%, p<0.0001) or distal colon (53%, p<0.05). The relative abundance of Bacteroidetes was lower (33%) in the proximal colon compared to the medial colon (44%, p<0.0001). Deferribacteres accounted for 2.5% of all sequences in the distal colon, which was significantly lower than the medial colon (6.4%, p<0.05), with the proximal colon showing 5.1% (p=0.269). A total of nine genera were changed by colonic location (Figure 5.2d). Five genera in the phylum Firmicutes were higher in the proximal colon compared to the medial and distal sections (ps<0.05), including two genera in the family Lachnospiraceae and two in the family Ruminococcaceae. Relative abundances of the genera Mucispirillum, Bacteroides, and Parabacteroides were higher in the medial section compare to the proximal and distal sections (ps<0.05).

Colonic microbiota differs by study cohort.

Although not a primary objective of the study, we chose to compare the three cohorts employed in the experiment to gain insight into issues that are very relevant to those pursuing similar studies where study animals may have a staggered start. The colonic microbiota was significantly different by cohort (Figure 5.3). The OTU numbers from samples in cohort 2 were higher than those in cohort 1 and 3 (Figure 5.3a, p<0.0001).
Initial analysis of Unifrac distance matrix in Adonis showed significant differences in microbiota across three cohorts (unweighted $p<0.001$, $R^2=0.03711$; weighted $p<0.005$, $R^2=0.02432$). The PCoA figure furthered showed that cohort 2 clustered separately from cohorts 1 and 3 (Figure 5.3b). Further investigation demonstrated that cohort 2 was from a different breeding facility than cohort 1 and 3. Moreover, relative abundances of 15 genera were significantly modified by cohort (Figure 5.3c). Genera that were significantly changed by initiation diet, promotion diet, and colonic sites were nested within those 15 genera (Figure 5.3d).

Discussion
The number of studies regarding the impact of diet and GM will increase rapidly as the technology improves and the cost declines. It has been demonstrated that the healthy adult GM is characterized as existing in a steady state that requires a major disturbance, such as antibiotic administration or significant dietary intervention (magnitude or duration), to permanently alter that state. Both human and animal studies show an acute response of microbiota to dietary challenge\textsuperscript{102}. However, it is unclear to what extent that energy balance over the life span influences the colonic microbiota after acute or chronic change. This raises a series of questions: (a) does early life dietary energy intake impose a community profile that remains stable in adulthood, regardless of diet change? (b) does a strong diet intervention (dramatic energy change and/or extended period of time) in adult life override the impact of early life dietary history? and (c) what are the health implications of the change in the GM resulting from alterations in energy balance? It is
possible that different dietary interventions in early life shape the colonization and in turn influence the responses of microbiota to diet in adult life.

In the present study, we did not have evidence to reject the null hypothesis that mice selected for microbiota analysis were the same as the overall cohort. Therefore, we are confident that mice selected for the gut microbiota analysis were representatives of the overall cohort.

After controlling for the impact of cohort and colonic site by adding these factors into the statistical model, we observed significant effects of the diet on microbiota profiles. Although dietary energy intake in early life did not show a lingering impact on the number of OTUs, the impact on taxonomic abundance was observed in adulthood. Specifically, exposure to a HF diet in early life was associated with a significantly reduced proportion of Bacteroidetes in adult life, regardless of the dietary intervention in adulthood. The positive association between the HF diet and reduced relative abundance of Bacteroidetes is consistent with a previous study. Given the lack of longitudinal analysis of the microbiota at the end of the initiation phase compared to the end of the promotion phase, we are unable to prove that the differentiated community structure was specifically retained from the earlier time point. However, it is clear that changes in energy balance early in life do have effects that are maintained for a long period of time in the mouse model. At the genus level, the relative abundance of Bacteroides was significantly higher in animals on an ER diet in the initiation phase compared to those on
a HF diet. Certain species in *Bacteroides* have protective effects from inflammation in experimental models of inflammatory bowel disease (IBD)\textsuperscript{210}, while some species induces colonic tumorigenesis in *Apc\textsuperscript{Min+/−}* mice\textsuperscript{211}. The proportion of *Bacteroides* also declined in animals challenged by psychological stressors\textsuperscript{212}. The disagreement between the potential protective effects of increased *Bacteroides* and the increased number of ACF in mice on the ER diet during early life suggests that either bacteria species, which drive the increased proportion of *Bacteroides*, promoted colon carcinogenesis in the model, or the diet-associated *Bacteroides* abundance change is independent of the observed diet-associated precancerous lesions alteration.

Most certainly the diet fed during the prolonged promotion phase has a profound influence on the colonic microbiota. The promotion diet did not change the number of OTUs, as noted for the diet during initiation. However, four phyla were significantly changed, regardless of past dietary energy intake history (initiation phase diet). Intriguingly, Bacteroidetes were higher in mice on the HF diet during the promotion phase, compared to mice on the ER diet with the same dietary history (CON, HF, or ER in the initiation phase). However, we found mice on the HF diet in both phases (H-H) still had lower proportion of Bacteroidetes compared to mice on the ER diet in both phase (E-E) (Table 1), which consistently demonstrated the association between the HF diet and reduced abundance of Bacteroidetes. Moreover, the relative abundances of Bacteroidetes in mice on the ER diet in the initiation phase were higher than those on the HF or CON in the initiation phase. Therefore, we hypothesized that the lower abundances of
Bacteroidetes in mice on the ER diet during the promotion phase may be largely due to mice fed the HF or CON diet during the initiation phase and the carry-on impacts of early life dietary patterns on Bacteroidetes abundances. On the other hand, the increased abundances of Firmicutes in ER-fed mice were likely due to the increased abundances of *Lactobacillus* (Table 5.1), which was previously reported to be inversely associated with social stress and inflammation\textsuperscript{213, 214}. An ER diet has shown suppressive effects on colon carcinogenesis, which were associated with alterations in several biological pathways, including inflammation\textsuperscript{165}. An ER diet has been shown to increase the *Lactobacillus* group counts in a group of overweight adolescents\textsuperscript{215}. Therefore, we hypothesized that the increased *Lactobacillus* by the ER diet during the promotion phase could be a mediator through which the suppressive effect of the ER diet can impact colon carcinogenesis. The differentiated effects of the ER diet in early and adult life on colonic microbiota indicated an interaction between the present colonic mucosal microbiota and the dietary intervention. The observed impacts of diet on colonic microbiota further supports the hypothesis that microbiota with similar structures may respond differently to specific dietary interventions while communities with different structures may respond differently to the same dietary interventions.

It is important for readers to understand that the dietary effects reported are in the context of the AOM-induced colon cancer model. This study was not designed to address the impact of the carcinogen on the microbiota structure and we do not have a parallel group of mice not exposed to AOM. However, one report indicates that six consecutive i.p.
injections of AOM did not significantly impact on the GM composition or richness, even with cancer presented in the treatment group \[1\]. However, based upon the analysis we can not rule out the possibility that our results reflected an interaction between diet and AOM on microbiota. Future studies are warranted and it is important to translate the current findings to studies addressing dietary changes on community structure in various models of colon carcinogenesis. In addition, we appreciate that the 16S rRNA sequencing technique is a valuable tool to demonstrate relative changes in the community, however, the tool is limited in its ability to interpret the functional changes of the colonic mucosal microbiota.

We detected significant differences in the microbiota profile in different segments of the murine colon with adjustment for cohort and diet. In rodents treated with AOM, the microbiota differed by habitats along the gastrointestinal tract \[2\]. However, the regional variation in microbiota along the colon has been scarcely reported in rodent models \[3\]. Human studies have not observed a quantitative or qualitative difference in bacteria from ileum to rectum \[4,5\], although one study reported a positive trend between distances within colon and quantitative differences \[6\]. The different OTU numbers as well as taxonomic abundances at the genus level by colonic sites observed in our study may be due to the fact that we examined samples from mice instead of human. The decreased diversity (measured by OTU numbers) in the distal colon could be explained by site-specific tumorigenesis in the AOM-induced colon cancer model. No gross tumors were observed along the colons in the mice, nor did AOM change the luminal microbial
composition in previous studies \(^{216}\). However, we cannot rule out the possibility that the decreased diversity and changed community structure between the anatomical locations along the colon were associated with morphological changes in the mucosa layer due to the toxigenic effect of AOM. The changes in taxonomic abundances along the colon and the underlying mechanisms warrant further study. In addition, researchers should be aware of those changes in study design and data analysis where colonic microbiota under investigation.

An unexpected observation we found was a significant cohort effect on the colonic microbiota. Indeed, study cohort independently altered all genera that were altered by either dietary intervention or anatomical location in the colon. Wildtype C57BL/6N mice from the same company (Charles River, Wilmington, MA) are thought to be genetically identical. Considering that all three cohorts in this study were identically handled from week 3 to 60 regarding food, water, room temperature, and chemical intervention, it is possible that different maternal/environmental exposures in the first 2 or 3 weeks affected microbial colonization, which was retained over a year. Indeed, ordering records showed that the first and third cohorts were from the same distributor facility in Raleigh, NC, while the second cohort was from Kingston, NY, which aligned with the PCoA figure showing that cohort 2 clustered separately from cohort 1 and 3. Another breeding study observed similar results, reporting that the cohort effect accounted for 26% of the variation in the taxa of a core measureable microbiota defined by quantitative pyrosequencing \(^{221}\). The diversity of the gut microbiota in pups decreased dramatically at
3 and 9 days after delivery in a mouse model, suggesting that the microbiota in those days is more susceptible to environmental factors which might impact colonization. Therefore, the differences of microbiota profiles by cohorts in our study could be explained by environmental factors. Although interindividually, variety has been largely addressed in previous studies, cohort effects were not widely appreciated. Our study suggests that cohort effects should be controlled in gut microbiota-related animal studies.

Many investigators examining colon diseases in rodent models utilize pharmaceutical agents, dietary patterns, or specific nutrients or bioactive phytochemicals to assess the impact on disease processes relevant to both prevention and therapy. Our work indicates that interventions altering dietary energy intake may act upon the colonic mucosal microbiota, changing both structure and possibly function, and thereby influencing biological or pathologic outcomes. Thus, energy intake, already known to be a powerful modulator of many host functions relevant to disease, is a crucial variable that should be addressed in all murine studies where microbiological outcomes are under investigation, as many interventions have an impact on food intake and growth. Furthermore, animal studies on the gut microbiota typically utilize fecal samples considering cost and invasiveness relative to human studies. Our work implies that the impacts of cohort and sampling location should be considered, especially in research on the mechanistic role of the gut microbiota in colon cancer, given that tumorigenesis may interplay with the colonic mucosal microbiota and that tumors are more likely to develop in the distal colon in both humans and the AOM-induced colon cancer model. Finally, dietary intervention
studies in humans targeting obesity and cancer prevention occasionally have had null results due to residual confounders. Our work suggests that the lingering impacts of early life dietary history should be considered, particularly in studies where the microbes were proposed as a mediator, as the emerging role of the gut microbiota in nutrition and health.

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**Additional information**

The authors declare no conflict of interest.
Table 5.1 Relative abundances of phyla and genera changed by diet intervention (% mean ± SEM).

Different letters denote significant differences in pair-wise comparisons (p < 0.05). p-value in ANOVA for unbalanced sample with Bonferroni adjustment.
Table 5.1 continued

<table>
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<tr>
<th>Phylum</th>
<th><strong>Initiation phase (wks 3 to 21)</strong></th>
<th><strong>High-fat (H)</strong></th>
<th><strong>Promotion phase (wks 22 to 60)</strong></th>
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<td></td>
<td><strong>C</strong></td>
<td><strong>H</strong></td>
<td><strong>E</strong></td>
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<td><strong>Bacteroidetes</strong></td>
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<tr>
<td>Bacteroidia</td>
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<td>12 ± 1.5</td>
<td>5 ± 0.9</td>
</tr>
<tr>
<td>Unknown genus in S24-7</td>
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<td>3.8 ± 0.0</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td><em>Bacteroides</em></td>
<td>15 ± 7</td>
<td>12 ± 2</td>
<td>16 ± 1.9</td>
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<td><em>Parabacteroides</em></td>
<td>3.7 ± 1.2</td>
<td>4.2 ± 1.0</td>
<td>7.1 ± 0.6</td>
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<tr>
<td><strong>Deferribacteres</strong></td>
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<tr>
<td><em>Mucispirillum</em></td>
<td>6.2 ± 3.5</td>
<td>3.7 ± 3.0</td>
<td>8.3 ± 1.8</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
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</tr>
<tr>
<td>Bacilli</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>2.2 ± 2.5</td>
<td>2.1 ± 2.2</td>
<td>8 ± 1.3</td>
</tr>
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<td><strong>Clostridia</strong></td>
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<tr>
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<td>41 ± 4.6</td>
<td>38 ± 4.0</td>
<td>25 ± 2.3</td>
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<td>5.3 ± 1.2</td>
<td>6.4 ± 1.1</td>
<td>4.1 ± 0.6</td>
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<tr>
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<td>1.2 ± 0.5</td>
<td>2.8 ± 0.4</td>
<td>2.3 ± 0.3</td>
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<tr>
<td><em>Ruminococcus</em></td>
<td>1.0 ± 0.7</td>
<td>0.9 ± 0.6</td>
<td>3.4 ± 0.3</td>
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<tr>
<td><strong>Erysipelotrichi</strong></td>
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<tr>
<td><em>Allobaculum</em></td>
<td>1.0 ± 2.4</td>
<td>0.3 ± 1.1</td>
<td>0.5 ± 1.0</td>
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<td><strong>Verrucomicrobia</strong></td>
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<tr>
<td><em>Akkermansia</em></td>
<td>1.9 ± 0.8</td>
<td>0.4 ± 0.7</td>
<td>1.3 ± 0.4</td>
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<tr>
<th>Phylum/Genus</th>
<th>Initiation phase (wks 3 to 21)</th>
<th>Promotion phase (wks 22 to 60)</th>
<th>Energy restriction (E)</th>
<th>P</th>
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<td><em>Prevotella</em></td>
<td>0.8 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 ± 1.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.1 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Unknown genus in Rikenellaceae</td>
<td>10 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13 ± 1.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6 ± 0.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>&lt; 0.0001</td>
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<tr>
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<td>8.1 ± 0.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.5 ± 0.9&lt;sup&gt;de&lt;/sup&gt;</td>
<td>3.5 ± 0.4&lt;sup&gt;de&lt;/sup&gt;</td>
<td>&lt; 0.0001</td>
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<td>15 ± 3.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><em>Parabacteroides</em></td>
<td>6.1 ± 0.9&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>6.9 ± 1.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.9 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.0021</td>
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<td><strong>Deferribacteres</strong></td>
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<tr>
<td><em>Mucispirillum</em></td>
<td>2.8 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4 ± 2.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.8 ± 1.3&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td><strong>Firmicutes</strong></td>
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<tr>
<td>Bacilli</td>
<td>46.4 ± 4.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.8 ± 4.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.4 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1.7 ± 2.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.7 ± 0.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>&lt; 0.0001</td>
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<td><strong>Clostridia</strong></td>
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<td>26 ± 3.8&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>4.1 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>1.5 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.1 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.0449</td>
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<tr>
<td><em>Ruminococcus</em></td>
<td>4.2 ± 0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.9 ± 0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.7 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Erysipelotrichi</strong></td>
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<td></td>
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<tr>
<td><em>Allobaculum</em></td>
<td>3.8 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.5 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>&lt; 0.0001</td>
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<tr>
<td><strong>Verrucomicrobia</strong></td>
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<tr>
<td><em>Akkermansia</em></td>
<td>3.4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

Table 5.1 Relative abundances of phyla and genera changed by diet intervention (% means ± SEM).
Figure 5.1 Microbiota differs by diet.
Diet interventions in phase 1 and phase 2 affected the microbiota structure in each individual cohort at the end of the study (60 weeks), showed in PCoA figures (a and b, respectively, not controlled for colonic sites). Each point corresponded to a sample colored by diet (CON: light green, HF: dark blue, and ER: orange). Initiation diet significantly changed the relative abundances of (c) one phylum and (d) two genera, while promotion diet significantly affected the relative abundances of (e) four phyla and (f) nine genera. Different letters in the bar chart indicate significant difference, p<0.05.
Figure 5.2 Microbiota differs by anatomical location in the colon.
(a) Significant differences by anatomical location were detected in OTU numbers. Beta diversity were significantly changed by location in the three individual cohorts. (b) The PCoA figure was presented cohort 2 (■: proximal colon, ●: medial colon, □: distal colon). Each point corresponded to a sample colored by colonic region (green: proximal; blue: medial; red: distal). (c) Proportions of Bacteroidetes, Firmicutes, and Determibacteres were changed along the colon. (d) Proportion of nine genera were changed along the colon. Different letters in the bar chart indicate significant different, p<0.05. OTU: Operational taxonomic units.
Figure 5.3 Microbiota differs by cohorts.
(a) OTU numbers of the colonic microbiota were significantly changed by cohorts, different letters indicate significant different, p<0.05. (b) Variation in community diversity by cohort was shown in a PCoA figure. Each point represented a sample colored by cohort (■: cohort 1; □: cohort 2; △: cohort 3). (c) The significantly changed relative abundances of 15 genera by cohorts were shown in heat map. (d) Numbers in the Venn represented the number of genus significantly changed by initiation diet, promotion diet, anatomical locations in colon, and cohorts. Numbers in overlapped area represented the number of same genus modified by multiple factors independently. OTU: Operational taxonomic units.
References


