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The micronutrient profile of the typical American diet enhances colorectal carcinogenesis

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THE MICRONUTRIENT PROFILE OF THE TYPICAL AMERICAN DIET ENHANCES COLORECTAL CARCINOGENESIS

by

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ABSTRACT

The Micronutrient Profile of the Typical American Diet Enhances Colorectal Carcinogenesis

by

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The typical Western dietary pattern is characterized by the consumption of energy-dense, nutrient-poor foods and has been linked to increased risk of colorectal cancer (CRC). Our research group previously developed the total Western diet (TWD) that emulates typical human dietary intakes of macro- (carbohydrates, proteins, and fats) and micronutrients (vitamins and minerals) on an energy density basis for rodents. In the present study, we sought to determine the impact of TWD on biomarkers of metabolic syndrome and obesity in comparison to a commercial 45% fat diet used for models of diet-induced obesity (DIO diet) and the standard basal AIN93G diet, which is optimized for rodent health. Also, we included 2 additional test diets to evaluate the contribution of the micronutrient (vitamin- and mineral-modified diet, [VMM]) or macronutrient (macro-modified diet [MM]) contents of the TWD in development of cancer, obesity, and glucose intolerance. A chemical carcinogenesis model of inflammation-associated colon cancer was employed to evaluate impact of diets on colon cancer in mice. As expected,

mice consuming the DIO diet acquired an obesity/metabolic syndrome phenotype typified by increased food energy intake, greater rate of body weight gain, increased proportion of body composition as fat mass, higher fasting glucose, impaired glucose tolerance, and higher circulating levels of leptin. However, consumption of TWD did not alter any of these classic biomarkers of metabolic health, as these mice adjusted food intake so that energy consumption was similar to that for mice fed AIN93G. A different pattern was observed for colon carcinogenesis. Consumption of the TWD or VMM diet markedly increased colon tumor multiplicity and size compared to the AIN93G control, whereas consumption of the DIO or MM diets did not enhance colon tumorigenesis. Collectively, these observations point to a critical role of dietary micronutrients in colon carcinogenesis, and that this promoting effect is likely unrelated to the metabolic syndrome phenotype induced by a high fat diet. Moreover, our observations emphasize the need to take into account the micronutrient content of rodent basal diets when modeling typical U.S. nutrition in pre-clinical animal experiments in order to improve the translation of these studies to human nutrition and dietary intervention programs.

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PUBLIC ABSTRACT

The Micronutrient Profile of the Typical American Diet Enhances Colorectal Carcinogenesis

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Utah State University, 2013

The typical Western dietary pattern is characterized by the consumption of high-energy foods that are low in essential nutrients and has been linked to increased risk of colorectal cancer (CRC). Our research group previously developed the total Western diet (TWD) to emulate typical human dietary intakes of macro- (carbohydrates, proteins, and fats) and micronutrients (vitamins and minerals) based on food energy basis for rodents. In the present study, we sought to determine the impact of TWD on indicators of metabolic syndrome and obesity in comparison to a commercial 45% fat diet used for models of diet-induced obesity (DIO diet) and the standard basal AIN93G diet, which is optimized for rodent health. Also, we included 2 additional test diets to evaluate the contribution of the micronutrient (vitamin- and mineral-modified diet, [VMM]) or macronutrient (macro-modified diet [MM]) contents of the TWD in development of cancer, obesity, and glucose intolerance. We employed a chemical carcinogen model of colon cancer in mice with an agent to induce inflammation of the gastrointestinal tract to evaluate the impact of these experimental diets on formation of colon tumors. As expected, mice consuming the DIO diet gained excess weight and had symptoms typical

of metabolic syndrome, including greater rate of body weight gain, increased proportion of body fat mass, higher levels of blood glucose, impaired glucose tolerance, and higher circulating levels of the hormone leptin. However, consumption of TWD did not alter any of these biomarkers of metabolic health, as these mice adjusted food intake so that energy consumption was similar to that for mice fed AIN93G. Conversely, consumption of the TWD or VMM diet markedly increased colon tumor number and size compared to the AIN93G control, whereas consumption of the DIO or MM diets did not enhance colon tumor formation. Collectively, these observations point to a critical role of micronutrients in the development of colon cancer, and that this promoting effect is likely unrelated to the metabolic syndrome symptoms acquired by mice consuming strictly a high fat diet. Moreover, our observations emphasize the need to take into account the micronutrient content of rodent basal diets when modeling typical U.S. nutrition in pre-clinical animal experiments in order to improve the translation of these studies to human nutrition and dietary intervention programs.

DEDICATION

This work is dedicated to my parents, Nelson Perez and Karina Monsanto, who always encouraged me to follow my dreams and who, despite the distance, have been there for me throughout this entire journey. I also dedicate this work to my brother and sisters, Nelson, Allyson, Lilu, and Naia, who are a source of joy and the reason why I strive to give the best of me every day.

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LIST OF ABBREVIATIONS, DEFINITIONS, AND SYMBOLS

Abbreviations

$1,25(OH)_2D$	1,25-Dihydroxycolecalciferol
25(OH)D	25-Hydroxycholecalciferol
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACF	Aberrant crypt foci
AIN	American Institute of Nutrition
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
AUC	Area under the curve
BW	Body weight
CDC	Centers for Disease Control and Prevention
CO_2	Carbon dioxide
CRC	Colorectal cancer
CYP2E1	Cytochrome P450 2E1
DIO	Diet-induced obesity
DMH	1,2-Dimethylhydrazine
DSS	Dextran sodium sulfate
FAP	Familial adenomatous polyposis
GIP	Gastric intestinal peptide
HOMA	Homeostatic model assessment
IBD	Irritable bowel syndrome
IL	Interleukin
IQ	2-Amino-33-methylimidazo[4,5-f]quinolone
KW	Kidney weight
LARC	Laboratory Animal Research Center
LW	Liver weight
MAM	Methylazoxymethane
MAPK	Mitogen-activated protein kinase
MAP	*MUTYH*-associated polyposis
MCP-1	Monocyte chemotactic protein-1
MM	Macronutrient-modified
MNNG	N-Methyl-N'-nitro-N-nitrosoguanidine
MNU	Methylnitrosourea
NHANES	National Health and Nutrition Examination Survey
oGTT	Oral glucose tolerance test
PBS	Phosphate-buffered saline
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PI3K	Phosphatidylinositide 3-kinase
PLP	Pyridoxal-5'-phosphate

SD	Standard deviation
SEM	Standard error of measurement
SW	Spleen weight
TGF-β	Transforming growth factor-β
TNFα	Tumor necrosis factor alpha
TWD	Total Western diet
VMM	Vitamin- and mineral-modified

Definitions and alternate names

Vitamin B_6	Pyridoxine
Vitamin B_9	Folate
Vitamin B_{12}	Cobalamin
Vitamin D_3	Cholecalciferol
i.p.	*intraperitoneal*
ad lib.	*ad libitum*

Symbols

C	Celsius
cm	Centimeter
dL	Deciliter
g	Grams
g	Gravity
h	Hour
IU	International units
kcal	Kilocalories
kg	Kilograms
L	Liter
mg	Milligrams
min	Minutes
mm	Millimeter
mU	Milliunits
sec	Seconds
µg	Micrograms
µl	Microlitier
wk	Week

INTRODUCTION

Colorectal cancer is the third leading cause of cancer death and the third most commonly diagnosed cancer in men and women in the United States (1). In the last twenty years, global incidence rates of colorectal cancer have been increasing, reportedly due to an increase in the prevalence of certain cancer-associated practices, such as decreased physical activity and "westernized" diets (2). The typical Western Diet is a dietary trend characterized by increased consumption of highly processed foods that contain excess levels of fat, sodium, and refined sugars and are generally low in essential vitamins, minerals, and fiber (3-5). This dietary pattern is believed to play a critical role in the development of several chronic conditions that have increased in prevalence over the last 50 years, including obesity, type II diabetes, metabolic syndrome (5, 6) as well as colorectal cancer (7-9). Because of the increasing socioeconomic burden of colorectal cancer, a large body of research has been dedicated to the study of the role of nutrition on the development of this and other related conditions with the purpose of elucidating the mechanisms behind their pathology.

Statistics on colorectal cancer

Colorectal cancer (CRC) is the fourth most common cause of death worldwide, with approximately 608,000 deaths occurring each year (10). Global incidences of CRC have increased over the last twenty years, and this disease is more prevalent in economically developed countries, which account for up to 60% of reported cases (2, 10). The lifetime risk of developing CRC in the U.S. is about 5%, and the risk is slightly lower in women than in men. Based on the level of economic development of the region,

Central and Eastern Europe are estimated to have the highest mortality rates in both sexes (20.1 for male, 12.2 for female per 100,000), while Middle Africa is estimated to have the lowest mortality rate (3.5 for male, 2.7 for female). In the United States, significant improvements in colorectal screening and treatment methods have caused mortality rates to decrease 2.8% per year in men and 2.6% per year in women since 1998. However, according to data collected between 1975 and 2007 from the Surveillance, Epidemiology, and End Results (SEER) Program, colorectal cancer incidence rates have declined in the U.S. among adults 50 years and older, but have increased among adults younger than 50 years (1). Although the precise cause for this trend remains a topic of debate, current data point to several environmental factors as potential contributors to these apparent shifts in cancer risk.

Risk factors for colorectal cancer

CRC risk is influenced by external modifiable factors (lifestyle and diet) and intrinsic non-modifiable factors (genetic predisposition). Heredity accounts for approximately 30% of all the diagnosed cases of CRC, of which 5% are attributed to well-characterized heritable genetic defects (11, 12). The remaining cases are generally attributed to either single-gene mutations that are less pervasive than those observed in well-characterized syndromes, or mutations on multiple susceptibility loci that result in additive effects. However, several hereditary syndromes are well described and have been strongly linked with CRC development.

Lynch Syndrome (or hereditary nonpolyposis colorectal cancer) is an autosomal dominant cancer-susceptibility disorder caused primarily by germline mutations of

mismatch repair genes. Although it predisposes subjects to several other types of cancers, it accounts for about 3% of CRC cases and can increase CRC risk as much as 80% depending on the type of mutation (11, 13).

Familial adenomatous polyposis (FAP) is one of the best-studied polyp-forming syndromes, although it accounts for less than 1% of CRC cases (11, 14). FAP is an autosomal dominant disease that results from germline mutations in the *APC* gene. The severity of the condition varies depending on the location of the mutation within the *APC* gene, which produces other variants of the condition (*i.e.* Attenuated FAP, Gardner syndrome, and Turcot syndrome).

MUTYH-associated polyposis (MAP) is a germline inactivation of the base excision repair gene *MUTYH* (or *MYH*), which causes adenomatous polyposis of the colorectum and an increased risk of CRC. In patients carrying this mutation, the risk of CRC is nearly 100% by 60 years of age (11, 15).

Hamartomatous polyposis syndromes are a group of inherited conditions that exhibit varying individual characteristics, but commonly show hamartomatous rather than epithelial polyp histology. Disease-associated mutations occur in different genes, some of which are *STK11, SMAD4,* and *BMPR1A.* Individuals with any of the related conditions have an increased risk for several types of cancer, including CRC. These syndromes account for less than 1% of CRC cases (11, 16).

Familial non-syndromic colorectal cancer refers to any CRC cases involving an uncommon genetic defect that is sufficiently penetrant to give rise to an autosomal-dominant segregation pattern. Thus, specific genetic markers for these cases are not available. Risk of CRC is approximately 2- to 3-fold greater for an individual with a first-

degree relative that has been diagnosed with CRC after age 50. The disease can be caused by genetic polymorphisms, allelic variants, chromosomal instability, aberrant DNA methylation, and other environmental factors (11, 15, 17).

Finally, another critical non-modifiable risk factor for CRC is individual medical history. Individuals who have been previously diagnosed with CRC (even if they have recovered), who have had one or more adenomatous polyps, who suffer from inflammatory conditions (*e.g.* Crohn's disease, ulcerative colitis, and inflammatory bowel disease), or who suffer from a condition strongly associated with CRC (*e.g.* Type II diabetes or obesity), have an increased risk of developing CRC (1, 18). Additional conditions that have been strongly associated with an increased risk of CRC include obesity (9, 19, 20), metabolic syndrome (8, 21, 22), diabetes (23-25), and nonalcoholic fatty liver disease (7, 26, 27).

Lifestyle and diet are external modifiable factors that greatly influence CRC risk and disease progression, and global patterns of CRC incidence closely align with patterns of physical activity and dietary trends. High levels of physical activity have been associated with lower CRC risk and lower mortality (1, 28, 29), even after CRC diagnosis (30). Data for 2005 from the Centers for Disease Control and Prevention (CDC) show that more than half of U.S. adults fail to meet the amounts of physical activity recommended by the CDC and the American College of Sports Medicine (31), a figure that correlates with the high rates of CRC reported in the U.S.

Animal models of colorectal carcinogenesis

In general, most pre-clinical studies of CRC employ a either a chemical carcinogenesis animal model, in which the hydrazine compounds 1,2-dimethylhydrazine (DMH) or its metabolite azoxymethane (AOM) are used to induce sporadic tumors in the colon, or a transgenic animal model, wherein mice carry a heterozygous mutation in the adenomatous polyposis coli (*APC*) gene leading to development of tumors in the small intestine and colon (see 32 for a comprehensive review). The most widely used transgenic model of intestinal and colon cancer is the $APC^{Min/+}$ mouse (33). The *APC* gene regulates various cellular pathways, including Wnt-signaling (involved in cell proliferation), cell-cell adhesion, cell cycle progression, and apoptosis. When one or several of these pathways are disrupted via mutation, the cell could escape normal constraints on cell proliferation and acquire a survival advantage. For this reason, a mutation in the APC gene is considered one of the most important initiating events in sporadic CRC (34). The defining feature of this cancer model is a nonsense mutation that results in a truncated APC protein at amino acid 850, which induces the formation of polyps in the mouse intestine. The majority of these polyps develop in the small intestine, which is why this model is deemed more useful for the study of hereditary colon cancer types, such as FAP. However, others have developed modifications of this transgene to improve the cancer model so that it more closely emulates human CRC, including a) modifiers of *Min* (Mom), which comprise several loci located on different chromosomes which have an effect of tumor development (*e.g*. mom1, mom2); b) *Apc* variations, which include different truncations to the Apc protein (*e.g*. $Apc^{\Delta 716/+}$; $Apc^{\Delta 14}$); and c) compound mutants, which add a mutation to another gene (*e.g*. mismatch repair genes, Smad1) (33).

Chemically induced models of CRC are advantageous because they are highly reproducible, and the approach allows for testing on animals with different genetic backgrounds. Importantly, the pattern of cancer development in these models generally mimics the multistep process of carcinogenesis observed in human CRC, and most tumors develop in the distal colon. A number of chemicals have been show to effectively induce carcinogenesis in the colons of rats and/or mice, including the aforementioned hydrazine compounds DMH and AOM; some heterocyclic amines, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-33-methylimidazo[4,5-f]quinolone (IQ); and the alkylnitrosamide compounds methylnitrosourea (MNU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). AOM is now the most widely used chemical carcinogen for modeling sporadic colorectal cancer in rodents. AOM undergoes metabolic activation in the liver by the cytochrome P450 enzyme CYP2E1 to form the metabolite methylazoxymethane (MAM), which can readily generate a methyldiazonium ion that can alkylate DNA at the O^6 or N^7 position of guanine (35).

Disruption of the canonical Wnt/β-catenin signaling pathway is often observed in animals initiated with AOM (reviewed in 36, 37). The protein β-catenin aides in cell adhesion by associating with either cadherin or catenin to connect the actin cytoskeleton; it also acts as a co-transcriptional activator of genes that are under regulation of the Wnt signaling pathway. AOM exposure can lead to mutations in codons 31 and 41 of β-catenin, which prevents its degradation (37). Accumulation of the protein leads to transcriptional activation of a number of downstream gene targets that promote cell proliferation, such as cyclin D1 (38). AOM exposure also leads to mutations in the proto-oncogene K-ras, a small G protein that regulates both mitogen-activated protein kinase

(MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt intracellular signaling pathways, resulting in dysregulation of cell growth, proliferation, and glucose metabolism (39). One of the hallmarks of sporadic and AOM-induced colon cancer is perturbation of the transforming growth factor-β (TGF-β) signaling cascade. TGF-β is an important anti-inflammatory cytokine expressed in colon epithelial cells and serves as a key negative regulator of cell proliferation via control of cell growth and apoptotic pathways. The tumor suppressor function of TGF-β1 is often lost during tumorigenesis (32). Aberrant signaling of the TGF-β1 pathway has been shown in the AOM colon cancer model. Disruption of TGF-β1 signaling is characterized by decreased ratios of biologically active to inactive TGF-β1 in tumor cells (40), along with transcriptional repression of the TGF-β1 receptor TβR-II (41).

Sensitivity to AOM as a chemical carcinogen is highly influenced by the genetic background of the animal model used, as well as the route and frequency of administration (32, 42, 43). For example, SWR/J and A/J mouse strains are highly susceptible to AOM-induced tumors (16 to 36 tumors/mm colon following 8 repeated *i.p.* injections of 10 mg/kg AOM) compared to AKR/J mice, which are very insensitive (<1 tumor/mm colon) (43). Also, it should be noted that colon carcinogenesis does not appear to be influenced by gender, as similar incidence, multiplicity, and size of tumors in males and females have been observed previously (42).

Data from epidemiological studies have shown that CRC risk is strongly linked to long-term irritable bowel diseases (IBD), of which the most common types are Crohn's disease and Ulcerative colitis (44-46). The chronic intestinal inflammation that characterizes these conditions is now recognized as an important factor in CRC due to its

involvement in the disruption of the same oncogenic pathways that are disrupted in CRC (47). Both Crohn's disease and ulcerative colitis are caused by the mutation of several genes involved in the maintenance of the intestinal mucosal barrier that protects the intestinal wall from bacterial invasion. Dysfunction of this mucosal barrier leads to sustained damage of gut epithelial cells. This chronic injury to the gut triggers a compensatory immune response characterized by the up-regulation of cell proliferation and anti-apoptotic pathways that promote cell survival (48). Various molecules are involved in the activation of such pathways, including transcription factors (*e.g.* NF-κB, and STAT3), and various inflammatory cytokines (*e.g.* IL-6 and TNF- α), which are normally secreted during an inflammatory response. The resulting compensatory cell regeneration results in increased rates of mitosis that, when chronically active, increase DNA mutation rates (48, 49). Not only can this process promote the disruption of oncogenic pathways, but it can also provide cancerous cells with a nurturing environment due to the increased availability of proliferative and survival signals. This notion is supported by an animal study by Tanaka et al. (50), where they showed that a combined treatment of AOM with the inflammatory agent dextran sodium sulfate (DSS) significantly increased intestinal tumorigenesis in CD-1 mice. The AOM+DSS model yields a consistent, reproducible colon cancer outcome that is well defined in terms of mouse strain and AOM dosage (51). Moreover, this model recapitulates many of the molecular events that occur in spontaneous human colon cancer, such as β-catenin accumulation and K-ras mutations (37).

Dietary factors that promote colorectal carcinogenesis

Diet is one of the most influential factors affecting overall cancer incidence, with colorectal cancer being one of the most well established examples. The Western diet is characterized by high intakes of red and processed meats, fried foods, sweet foods (often in the form of soft drinks), and refined grains (5). Conversely, those consuming a western type diet typically have low intakes of fruits, vegetables, and fish. Overall, this food consumption pattern results in an unbalanced diet that is high in simple sugars, fats, and sodium yet low in many essential vitamins, minerals, and fiber (3). Evidence from epidemiological and pre-clinical animal studies supports the notion that the Western diet is positively associated to a higher risk and incidence of colorectal cancer (3, 5).

Many elements of a Western diet have been associated with higher rates of CRC. In developed countries, excess consumption of red meat has been linked to higher incidence of CRC (4, 52-54). Evidence from human case-control and cohort studies points to a positive correlation between high red meat consumption and CRC risk. A 6-year follow-up study using a cohort of 47,949 men from the Health Professionals study determined that consumption of beef, pork, and lamb had a strong positive association with CRC risk (53). Norat et al. showed that CRC risk was significantly associated with pork and lamb consumption in a separate 5-year cohort study of 478,040 men and women from Western Europe (54). This association between red meat consumption and CRC risk has been attributed, in part, to the presence of *N*-nitroso compounds, which are known alkylating agents (52, 55, 56). Also, red meat naturally contains high levels of heme, which generates reactive oxygen species (52, 57). Finally, well-cooked or preserved meats can contain high amounts of mutagenic heterocyclic amines (52, 56, 57).

Overall, evidence for a role of dietary fat in the etiology of CRC in humans is inconsistent. In the previously mentioned Health Professionals Follow-up Study cohort, fat from red meat was positively associated with higher CRC risk in men, while no association was observed for total fat or non-red meat fat intake (*i.e.* poultry, dairy, and vegetable) (53). Willett and coworkers reported that intakes of animal-derived saturated and monounsaturated fats were linked to increased risk of CRC in a prospective study of mid-age, healthy women (58). Alternatively, Lin et al. (59) reported that neither total fat intake nor intakes of different types of fats or major fatty acids were related to risk of CRC in healthy women. Moreover, in a combined analysis of 13 case-control studies including 5,287 cases of CRC and 10,470 controls, no evidence of increased risk was observed for any dietary fat variable after adjustment for total food energy intake or after subgroup analyses by sex, age, or anatomic location of the cancer (60).

In contrast to human data, the association between dietary fat intake and CRC promotion has been repeatedly demonstrated in different rodent models of colorectal carcinogenesis. As outlined above, chronic intestinal inflammation is a known risk factor for CRC. Researchers have repeatedly shown that intestinal inflammation is enhanced in C57BL/6J mice fed with diets with 20% (61), 45% (62), or 60% kcal from fat (63). In chemical carcinogenesis rodent studies, researchers often measure development of preneoplastic lesions, termed aberrant crypt foci (ACF), as a biomarker of colorectal carcinogenesis. Development of these lesions, as well as fully developed colon tumors, has been linked to specific sources of dietary fat. For example, ACF multiplicity was significantly higher in the colons of rats fed diets containing 10 or 20% corn oil compared to animals consuming diets with 10% or 20% fish oil (38, 64). Evidence from

other rat cancer studies suggests that tumor abundance was also greater in animals fed corn oil (38, 65) or beef fat (38, 66). On the other hand, consumption of fish or flaxseed oil provided protection against colon carcinogenesis in rodents (67-70). Collectively, these animal studies provide convincing evidence that the source of dietary fat, rather than total fat intake, is the driving factor for modulating colon carcinogenesis in rodents.

A number studies have indicated that CRC risk is inversely correlated to the consumption of vegetables, fruits, and/or fiber, which are essential components of a balanced diet (71). However, current data is not completely consistent, suggesting that other interacting factors are likely modulating their effects. For instance, a prospective study including 136,089 men and women from the Nurses' Health Study and Health Professionals' Follow-up Study found no correlation between fruit and vegetable consumption, and colon or rectal cancer incidence (72). A meta-analysis of data from 13 cohort studies analyzed in the Pooling Project of Prospective Studies of Diet and Cancer examined the association between dietary fiber intake and CRC risk. The authors observed that risk of colorectal cancer in an age-adjusted model was inversely associated with dietary fiber intake, although this association was no longer significant after adjusting for other risk factors (73). Conversely, a 5-year follow up study of 133,163 U.S. men and women examined the association between whole grains, fruit, vegetables, and dietary fiber and CRC (74). Although no independent association was observed between whole grains, fruit, or fiber and incidence of CRC, very low consumption of vegetables and fiber was associated with a 2-fold increase in the risk of colon cancer in men, while very low consumption of fruit was associated with a doubling of risk in women. In a separate meta-analysis of data from the Pooling Project of Prospective Studies of Diet

and Cancer, epidemiologists arrived at a similar conclusion, where the lowest intakes of fruits and vegetables (less than 200 g/day) were inversely associated with an elevated risk for distal colon cancer risk, but not proximal colon cancer (75). Finally, to examine the associations between food patterns and CRC incidence in older Americans, Wirfalt et al. (76) performed a cluster analysis in which individuals with similar characteristics were aggregated. Male subjects that consumed high amounts of fruits, vegetables, and low fat foods were less likely to develop CRC, even when considering other disease risk factors. On the other hand, female subjects in this "Vegetable and Fruit" cluster had lower CRC incidence, but not independently of other risk factors such as education, smoking, and ethnicity.

Much of the evidence for a role of specific micronutrients in modulation of CRC centers on calcium and vitamin D, the latter referring collectively to a group of secosteroid molecules that are critical for the uptake of calcium in the intestine. D vitamins include cholecalciferol (vitamin D_3), which is generally synthesized endogenously in response to ultraviolet light, usually in adequate amounts. However, some individuals do not obtain sufficient light exposure to maintain adequate levels of D_3, which is often fortified in staple foods, such as milk, to avoid deficiency (77). Overall, evidence from human epidemiological and animal laboratory studies points to lower risk of CRC for high intakes of calcium and vitamin D, particularly for senior women. Feskanich et al. (78), examined CRC risk in a nested case-control study among women participating in the Nurses' Health Study and determined that women $\geq$ 60 years of age with the plasma levels of 25-hydroxycholecalciferol (25(OH)D), a metabolite of D_3 and the precursor to the bioactive form 1,25-dihydroxycolecalciferol ($1,25(OH)_2D$),

had a much lower risk of CRC compared to subjects in the lowest quintile of 25(OH)D levels. Alternatively, this inverse relationship between circulating 25(OH)D and CRC risk was not observed in younger women. Lappe et al. (79) examined CRC in healthy, post-menopausal women receiving either a calcium supplement (1500 mg), a calcium supplement with 1100 IU vitamin D_3,or a placebo. Women receiving the dual calcium and vitamin D supplement or the calcium supplement alone were more likely to remain cancer-free (breast, colon, lung, uterus, and hematopoietic cancers were examined) during the 6-year analysis period. Park et al. (80) examined CRC risk as a function of dietary calcium and vitamin D intakes, dairy food consumption, and gender and ethnicity in the large Multiethnic Cohort Study of more than 215,000 participants. This group observed that total calcium intake and consumption of dairy foods were inversely correlated with CRC risk in both healthy men and women, and that this relationship for calcium was consistent across the ethnic groups examined (African Americans, Native Hawaiians, Japanese Americans, Latinos, and Whites). Vitamin D was also found to have protective effects in those individuals who do not supplement their diet with additional calcium. Evidence from animal studies supports these epidemiological observations. Depletion of calcium from the rodent diet increased spontaneous tumor formation while vitamin D supplementation inhibited tumorigenesis in C57BL/6J (81) and in $Apc^{1638N/+}$ mice (82).

Much of the research on B vitamins and CRC development has focused on vitamin B_9 (folate), vitamin B_6 (pyridoxal phosphate), and vitamin B_{12} (cobalamin), due to their function in the methyl donor pathway and one-carbon metabolism (83, 84). Folate is a required for the synthesis, repair, and methylation of DNA, but current data on folate

intake and CRC development are inconsistent. In human studies, higher folate intakes are generally associated with reduced CRC risk, but data from animal studies suggests that the dose and timing of folate intervention differentially modulate carcinogenesis. For instance, Sanjoaquin and coworkers (85) performed a meta-analysis that included 7 cohort and 9 case control studies and found that CRC risk was 25% lower among those in the highest category of food-derived folate intake compared with those in the lowest category. When analyzing total folate intake (food-derived and supplemented folate), only a 5% lower risk was observed. A separate meta-analysis that included 18 case control studies and 9 cohort studies from fourteen countries found that CRC incidence was decreased 13-18% in the highest quintile of dietary folate intake (86). However, in a study where AOM treated rats were given one of 3 diets containing 0, 2, or 8 mg folic acid/kg diet, the total number of colonic ACF increased with rising dietary folic acid concentration. The total number of colonic ACF was 54% higher in rats fed the 8 mg/kg diet than in the rats receiving the 0 mg folic acid/kg diet, suggesting that folic acid enhanced colorectal tumorigenesis (87). A separate study using C57BL/6J mice determined that colon carcinogenesis was largely unaffected by supplementation with dietary folate, although higher intakes of folic acid were linked to a higher proportion of relatively large tumors (88). Moreover, neither plasma nor colon folate levels were significantly correlated with tumor number (88).

The role of vitamin B_6 in the etiology or prevention of CRC is also unclear. Je et al. (89) performed a meta-analysis of 472 cases of CRC detailed in the Nurses' Health Study, the Health Professionals Follow-up Study, and the Physicians' Health Study and found no association with plasma levels of pyridoxal 5'-phosphate, the active form of B_6,

and CRC mortality. In a meta-analysis of 8 studies on vitamin B_6 intake and 4 studies on blood pyridoxal 5'-phosphate levels, Larsson et al. showed that both factors were inversely associated with the risk of colorectal cancer (90). Risk of CRC decreased by 49% for every 100-pmol/mL increase in blood pyridoxal 5'-phosphate level, while high vitamin B_6 intake was associated with a 20% decreased risk compared to low intake. Finally, in CD-1 mice, supplementation with 35 mg of pyridoxine HCl markedly reduced CRC incidence compared to a diet depleted in B_6 (91).

Because of its key role as a cofactor in the one carbon metabolism pathway, many researchers have included vitamin B_{12} in cancer prevention studies. However, like many other micronutrients, the role of this particular vitamin in colorectal carcinogenesis remains uncertain. In a follow-up study including 226 cases and double matched referents from the population-based Northern Sweden Health and Disease Study, Dahlin and colleagues found that plasma vitamin B_{12} concentrations were inversely associated with the risk of rectal cancer, while the association was less clear for colon cancer (92). These results are in agreement with an animal study by Choi and colleagues (93), where 30 Sprague-Dawley rats were fed with either a diet with 50 μg/kg diet of vitamin B_{12}, or a diet with no vitamin B_{12}. After 10 wk, the colonic DNA of the deficient rats displayed a 35% decrease in genomic methylation and a 105% increase in base substitution of uracil, which are anomalies that may increase the risk of colorectal carcinogenesis. In other studies, researchers often use a combination of vitamin B_{12} , folate, and vitamin B_6, since these are all essential components of the one-carbon metabolism pathway, and hence act in conjunction. In a randomized, double-blind, placebo-controlled trial, 1,470 female health professionals from the Women's Antioxidant and Folic Acid Cardiovascular Study

were randomly assigned to receive either a placebo or a combination pill of folic acid (2.5 mg), vitamin B_6 (50 mg), and vitamin B_{12} (1 mg). Researchers found no significant differences between the treatment and the placebo group (94). These variations and inconsistencies in the current data for B vitamins suggest interaction with other dietary factors may be modulating their impact on colorectal cancer.

Vitamins A (retinol), C (ascorbic acid), and E (tocopherols) are often studied in combination because of their known antioxidant activities, but their impact on CRC development has not been established conclusively. For instance, a recent study of 816 CRC cases and 815 controls from the Fukuoka Colorectal Cancer Study showed that women with the highest retinol intake had significantly lower risk, while risk for men was not significantly different (95). However, carotene, vitamin C, and vitamin E intakes were not associated with CRC risk in either men or women. These results contradict the findings of a separate study that used data from the North Carolina Colon Cancer Study-Phase II, which determined that the highest quartiles of β-carotene intake (another form of vitamin A) were inversely associated with reduced risk of distal CRC risk in whites, but not in African Americans (96). In this same study, a strong decrease in distal CRC was observed in whites in the highest quartile for vitamin C, selenium, and food-derived vitamin E intake, but not in the group that included vitamin E supplementation. In an xenograft study by Park and colleagues (97), nude immunodeficient mice were injected with human CRC cells (HCT-116) and then fed diets containing 2,400 or 200,000 IU vitamin A/kg. In this experiment, vitamin A supplementation caused a 4-fold reduction in metastatic tumor multiplicity, although tumor size and morphology were unaffected. A separate study examined the effects of combined vitamin excess or deficiency in

C57Bl/6J mice or $Apc^{Min/+}$ mice (98). Animals were fed either a control diet, a vitamin-depleted diet (reduced to 33% of the recommended daily allowance [RDA] for mice), or a vitamin-supplemented diet (vitamin content increased by 5-fold compared to RDAs for mice); the vitamins modified included all of the B vitamins as well as vitamins A, C, D, E, and K. Interestingly, these researchers observed that the number and size of colon polyps were significantly higher for both experimental diets, suggesting that both conditions of vitamin deficiency and excess supplementation were harmful in terms of CRC risk. Moreover, this work also underscores the notion that supplementation of diet with high levels of vitamins, either individually or in combination, may not be the best strategy for CRC prevention.

Modeling the Western diet in animal studies

As outlined above, many components of a typical Western style diet have been shown to modulate cancer risk. Yet, in the vast majority of pre-clinical animal studies to investigate mechanisms of carcinogenesis or cancer prevention or to identify new strategies for dietary cancer prevention, researchers routinely employ standard formulated diets that are generally balanced with respect to macro- and micronutrient levels to optimize animal health, such as the American Institute of Nutrition AIN93G diet formulation (99). However, these optimally formulated rodent diets are not relevant to most human nutrition patterns, especially for at-risk populations that frequently consume energy-dense, nutrient-poor foods. Thus, researchers have employed several different strategies to model the Western style diet in studies employing rodents to assess impact of diet on disease risk and/or development. One such approach is the “cafeteria” diet, in

which the animals are allowed free access to standard chow and water, and are concurrently offered highly palatable, energy dense, unhealthy human foods *ad libitum* (100). This dietary protocol promotes hyperphagia, rapid weight gain, increased fat pad mass, and biomarkers of metabolic syndrome and diabetes, such as glucose intolerance and insulin resistance. Some experts argue that the cafeteria diet has limited value as an experimental model because of difficulty in replicating the specific dietary conditions across experiments or laboratories and because it is poorly defined with respect to micronutrient composition (100, 101).

In a series of studies over the past twenty years, Newmark and colleagues have employed a selective approach in modeling the Western diet, wherein specific components of the diet are modified to emulate typical U.S. intakes (81, 102, 103). In their first study, Newmark et al. developed a "stress" diet, which was quite low in calcium and vitamin D_3, and modestly reduced in phosphate compared to the reference diet AIN76; also, the stress diet contained 20% fat as corn oil (40% of calories) compared to only 5% (12% of calories) in the reference diet (102). A subsequent study extended this stress diet to incorporate dietary components necessary for generation of methyl donors (folic acid, methionine, choline, and vitamin B_{12}) and determined that this new Western diet (NWD) also enhanced spontaneous tumor development in aged C57BL/6J mice, an effect that was reversed when calcium and vitamin D were added back to the stress diet (81, 103). Although this series of studies has convincingly demonstrated a role for dietary calcium and vitamin D_3 in modulating spontaneous colon carcinogenesis in mice, the scope of the diet remained limited in that it does not consider possible contribution of the dietary fat source, carbohydrates or proteins and does not reflect

typical human nutrition patterns for other key micronutrients, such as sodium, selenium, or vitamins A or E. Commercial Western diets have also been developed for the study of obesity; these diets (refered to as diet-induced obesity, or DIO, diets) typically contain 45% or 60% of energy as fat and differ from the AIN93G diets primarily in their high lard and sucrose content (104). Although these high fat diets effectively induce obesity in rodents (105), they are extreme in their sugar and fat compositions when compared to a typical Western dietary pattern and do not differ substantially from AIN93G diets in micronutrient content (104).

Importantly, none of the approaches described above for modeling typical Western nutrition appropriately considered the contribution of suboptimal micronutrient intakes in their disease models. To address this resource gap, our research team developed the new total Western diet (TWD) for rodents with energy and nutrient profiles that emulate a typical Western diet using available U.S. survey data (NHANES). The new TWD was formulated using a nutrient density approach, described in detail in a recent publication by members of our research group in the *Journal of Agricultural and Food Sciences* (106). Briefly, the amount of each macro- and micronutrient in the AIN93G basal diet, a diet routinely used in cancer studies today, was adjusted to match 50^{th} percentile intakes for Americans as reported in NHANES survey data. These mass amounts were then adjusted for caloric intake. Overall, the TWD is not necessarily extreme in the level of any given nutrient, but rather reflects the overall dietary pattern of the U.S. The TWD has fewer calories from protein and carbohydrate sources, and twice that from fat as compared to the AIN93G diet. The new diet contains more saturated and monounsaturated fats, less polyunsaturated fat, more complex carbohydrates, and twice

the level of simple sugars. The TWD has less calcium, copper, folate, thiamine, and vitamins B_6, B_{12}, D, and E, but much more sodium. This newly devised diet that better represents typical U.S. nutrition is highly useful for studies employing animal models of human cancer. For example, results of a preliminary experiment in A/J mice initiated with AOM indicate that the TWD markedly enhanced development of preneoplastic ACF compared to the reference diet AIN93G (107).

Project objectives and hypotheses

The objectives of this thesis project were as follows: 1) to investigate the impact of dietary macronutrients (protein, carbohydrate, and fat) and micronutrients (vitamins and minerals) on biomarkers of metabolic syndrome and 2) to determine the relative importance of the macro- and micronutrient composition of the typical Western diet on colon cancer risk using a mouse model of inflammation-associated colorectal carcinogenesis. This study was designed to test the following working hypotheses:

1. Mice consuming the TWD will acquire a metabolic syndrome phenotype, indicated by increased body weight, glucose intolerance, and insulin resistance, in a manner similar to the prototypical commercial high-fat diet (45% fat DIO diet) routinely used for diet-induced obesity pre-clinical studies.
2. TWD consumption will promote colon tumorigenesis in the C57BL/6J strain of mouse, which is highly susceptible to diet-induced obesity and metabolic syndrome.

3. Tumorigenesis in mice fed TWD will be more severe (greater number of and larger tumors) compared to mice consuming the standard basal diet AIN93G, which was formulated to be optimal for rodent health.
4. Tumor outcome will be more severe in mice fed TWD (reflecting typical U.S. nutrition with respect to both macro- and micronutrients) than in mice consuming a diet modified for macronutrient content only. In other words, a typical Western micronutrient profile will exacerbate the expected adverse effects of a high-fat, high-sugar diet on colon carcinogenesis.
5. The new TWD will be at least as effective, if not more so, in promoting colon carcinogenesis as the DIO diet (high fat only).

MATERIALS AND METHODS

Chemicals

AOM was purchased from Sigma-Aldrich (St. Louis, MO; CAS No. 25843-45-2), and reagent grade DSS was purchased from MP Biomedicals (Solon, OH; MW=36,000-50,000 Da). Phosphate buffered saline (PBS) solution was purchased from Caisson Labs (Logan, UT), and 10% buffered neutral formalin was purchased from VWR (Houston, TX). All other chemicals used were of reagent grade and purchased from general laboratory suppliers.

Animals

The Utah State University Institutional Animal Care and Use Committee approved all procedures for the handling and treatment of mice used in this study (protocols #2063 and #2114). Animals were housed in the Laboratory Animal Research Center (LARC) at Utah State University, which is an AAALAC approved facility. Mice were maintained in a pathogen-free vivarium at 18 to 23 °C with a 12:12 hour dark:light cycle and humidity between 20 to 50%. Mice were provided Bed-o'Cobs® 1/4 bedding (Andersons, Cincinnati, OH). Mice initiated with AOM were housed in HEPA-filtered cages on a IVC Air Handling Solutions ventilated housing system (Tecniplast, Buguggiate, Italy), while all other mice were housed in wire top cages within a ventilated Duo-Flo BioClean Unit (Lab Products, Inc.). Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 4 wk of age and acclimated to the vivarium for 1 wk, during which time mice were provided free access to food and water.

Experimental diets

Experimental test diets were obtained from Harlan-Teklad (Madison, WI). To determine the relative impact of dietary macro- and micronutrient composition on metabolic health and cancer outcome, we compared 5 different diet formulations as outlined in Table 1 and briefly described below:

1) AIN93G, the standard diet routinely employed in rodent cancer studies
2) The new total Western diet (TWD), as described in Hintze et al. (106)
3) A macronutrient-modified diet (MM), which contained the same carbohydrate, protein, and lipid content as in the TWD, yet retained the vitamin and mineral profile of the reference AIN93G diet
4) A vitamin- and mineral-modified diet (VMM), which matched the AIN93G diet for macronutrient content, yet contained a vitamin and mineral profile matching the TWD
5) A commercial diet-induced obesity diet (DIO) that contained 45% of energy as fat (primarily lard) and matched the AIN93G for micronutrient content.

Diets were obtained as a single lot from the vendor and maintained at 4 °C for the duration of the study. Fresh food was provided to mice twice per wk.

Experimental design

This study was performed in sequential parts, in which the treatment of sham mice preceding treatment of AOM+DSS-initiated mice. Sham mice were housed individually, whereas mice to be initiated with AOM were housed 5 animals per cage. At 5 wk of age, mice were randomly assigned to one of the experimental diet groups

TABLE 1. Composition of experimental diets

Nutrient	AIN93G	TWD	VMM	MM	DIO
Energy density (kcal/g)	3.8	4.4	3.7	4.4	4.6
Macronutrient					
Carbohydrates (g/kg diet)					
Corn starch	398	230	398	230	85
Maltodextrin	132	70	132	70	115
Sucrose	100	261	100	261	200
Cellulose	50	30	50	30	58
kcal (% of total)	*60.1%*	*54.5%*	*60.1%*	*54.5%*	*36.2%*
Proteins (g/kg)					
Casein	200	190	200	190	245
L-cystine	3	2.8	3	2.8	3.5
kcal (% of total)	*18.8%*	*15.4%*	*18.8%*	*15.4%*	*19.0%*
Fats (g/kg)					
Soybean oil	70	31.4	70	31.4	30
Anhydrous milk fat		36.3		36.3	
Olive oil		28.0		28.0	
Lard		28.0		28.0	195
Beef tallow		24.8		24.8	
Corn oil		16.5		16.5	
Cholesterol		0.4		0.4	
kcal (% of total)	*17.2%*	*34.5%*	*17.2%*	*34.5%*	*44.8%*
Micronutrients					
Minerals (mg/kg)					
Calcium	5000	2011	2011	5000	5000
Phosphorus	3000	2757	2757	3000	3000
Sodium	1019	7078	7078	1019	1019
Potassium	3600	5333	5333	3600	3600
Magnesium	507	589	589	507	507
Iron	35	31	31	35	35
Zinc	30	25	25	30	30
Copper	6	2.6	2.6	6	6
Selenium	0.15	0.2	0.2	0.15	0.15
Vitamins (unit/kg)					
Thiamin (mg)	5	3.5	3.5	5	5
Riboflavin (mg)	6	4.4	4.4	6	6
Niacin (mg)	30	50.6	50.6	30	30
Pyridoxine (mg)	6	3.9	3.9	6	6
Folate (mg)	2	1.3	1.3	2	2
Vitamin B_{12} (μg)	25	11	11	25	25
Vitamin A (IU)	4000	4300	4300	4000	4000
Vitamin D (IU)	1000	391	391	1000	1000
Vitamin E (IU)	75	24.6	24.6	75	75
Vitamin K (μg)	750	189	189	750	750
Choline (mg)	1027	648	648	1027	1027

Note: Abbreviations for experimental diets are as follows: total Western diet, TWD; vitamin- and mineral-modified diet, VMM; macro-modified diet, MM; and diet-induced obesity diet, DIO. Composition of the TWD was published previously (106). No data are available in NHANES for chloride, manganese, iodine, pantothenic acid, biotin, or ultra-trace minerals. Thus, levels of these components in the AIN93G diet were used in the formulation of all experimental diets.

described above (*N*=10 per diet group for sham mice, *N*=30 per diet group for AOM+DSS-initiated mice). For AOM mice, individual animals were tracked using ear tags and/or ear punch. Fresh food was provided twice per wk, and food consumption was monitored at each change. Individual body weights were recorded once per wk for all mice. Body composition of sham mice was determined by MRI scan (EchoMRI-700; EchoMRI, Houston, TX) every 4 wk. At 7 wk of age, mice were administered with either a subcutaneous injection of 10 mg/kg body weight AOM or sham with an equivalent amount of the PBS vehicle. Mice initiated with AOM were also provided 1% DSS via their drinking water for 4 wk, then tap water for the remainder of the study. Sham mice were provided tap water for the entire study period. After a total of 16 wk on the experimental diets, all mice were euthanized by CO_2 asphyxiation and necropsied.

At necropsy of all mice, the liver, kidneys, spleen, and heart were collected, weighed, and fixed in 10% buffered neutral formalin. Additionally, to assess fat distribution in sham mice, the subcutaneous, gonadal, mesenteric, and retroperitoneal fat pads were excised and weighed. Whole blood was obtained from sham mice by cardiac venipuncture, and serum was collected by centrifugation (10,000 × *g*) for 5 min using serum separation spin tubes (Sarstedt, Newton, NC). Serum was aliquoted into triplicate cryotubes, immediately frozen in liquid nitrogen, and stored at -80°C for analysis of plasma hormone concentrations. To assess tumor outcome in AOM+DSS-initiated mice, colon tissues were collected from 20 mice per diet group were collected. (Tissue samples from the remaining 10 AOM mice per group were preserved for other analyses not reported here). Colons were rinsed with PBS, slit open longitudinally from the cecum to the anus, placed flat between paper towels, and stored in 70% ethanol solution at 4°C.

Fasting glucose and glucose tolerance in sham mice

Fasting glucose was assessed in sham mice every 4 wk for the duration of the study. After a morning fasting period of 6 h, approximately 0.2 μl blood was drawn from a 1 mm cut on the tail tip. Glucose was measured using a standard glucose meter and glucose strips (Total Diabetes Supply, Boca Raton, FL) in triplicate for each animal. Oral glucose tolerance tests were performed on sham mice at wk 7 and 15 of the study. Following a morning 6 h fasting period, baseline glucose levels were obtained as described above. Then, mice were given a bolus of 10 mg/kg glucose delivered into the stomach by gavage needle (22 gauge, 3.81 cm long straight, 2.25 ball diameter). Plasma glucose was again measured in triplicate at 0, 15, 30, 60, and 120 min after oral gavage. The homeostatic model assessment (HOMA) method was used to assess insulin resistance in fasted sham mice using the formula, HOMA = [glucose × insulin]/405, where glucose is measured in mass units mg/dL and insulin is expressed as mU/L.

Plasma hormone levels in sham mice

In order to assess the impact of the experimental diets on biomarkers of inflammation and glucose metabolism, 17 plasma circulating hormones were selected for analysis: adiponectin, interleukin 2 (IL-2), IL-10, IL-6, monocyte chemotactic protein-1 (MCP-1), insulin, C-peptide, ghrelin, gastric intestinal peptide (GIP), glucagon-like peptide 1, glucagon, leptin, pancreatic polypeptide, peptide YY, resistin, tumor necrosis factor alpha (TNFα), and amylin. Serum samples from the sham mice were analyzed using a Bio-Plex suspension array system (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's supplied protocol with no deviation.

Assessment of tumorigenesis in AOM+DSS-initiated mice

To determine the effect of the experimental diets on colorectal carcinogenesis, colon samples were analyzed under a dissecting microscope and tumors were counted. The researcher was blinded to the sample identification at the time of assessment. Measurements of colon length and tumor volumes were obtained using a pair of electronic calipers. Tumors were identified and counted using the following criteria: 1) defined, generally round or oval shape, 2) defined, easily distinguishable edges, and 3) lighter color with respect to surrounding tissue. Each tumor was measured for length, height, and width. Tumor multiplicity was calculated as the number of tumors per mm colon for each tumor-bearing animal. Colon volume was estimated using the formula, tumor volume = $\pi/6(L \times W \times D)$, where L is the length, W is the width, and D is the depth of the tumor.

ACF were identified in using criteria described previously (32, 43). Briefly, ACF were characterized as crypts of larger size and often slit-shaped, with increased pericryptal area, and thickened layer of epithelial cells which sometimes resulted in greater staining around the crypt. Colon tissues were stained in methylene blue for approximately 30 sec to facilitate detection of ACF. Colon samples were coded so that the corresponding treatment group was not known by the researcher assessing the number of foci and the number of crypt cells per foci.

Statistical analyses

Statistical analyses for food intake, energy intake, body weight, relative organ weights, metabolic efficiency, percentage fat and lean mass, fasting glucose, glucose

tolerance, plasma hormone concentration, HOMA, tumor multiplicity, tumor volume (log transformed), ACF number, and total crypts were performed using mixed models analysis of variance (Mixed procedure, SAS 9.3) with the Satterthwaite degrees of freedom approximation when needed to account for unequal variances among experimental groups. Data for sham or AOM+DSS-initiated mice were analyzed independently, as the key experimental question of interest was not the impact of AOM exposure, but rather the impact of experimental diet on biomarkers of metabolic health and colon carcinogenesis. For analysis of data from AOM+DSS-initiated mice, the parameter cage was included as a random factor nested within each diet group to account for cage effects on outcomes. Analysis of colon tumor incidence was performed using a quasi-likelihood logistic regression (SAS genmod procedure). Finally, all analyses included the Bonferroni multiple comparison adjustment to account for multiple testing.

RESULTS

Food and energy intakes

Food intakes are reported on a per wk basis (Fig. 1A-B) or for the entire 16 wk feeding period (Fig. 1C-D) for individual sham mice or on a per cage basis for AOM+DSS-initiated mice. Results of the statistical analyses for food and energy intakes by mice fed different experimental diets are presented in Table 2. Over the course of the 16 wk feeding period, sham and mice fed the AIN93G or VMM diets consumed significantly more total food (9 to 16% increase for AIN93G group, 8 to 15% increase for VMM group) compared to their counterparts fed the TWD, MM or DIO diets ($P < 0.05$, Fig. 1). Although AOM+DSS-initiated mice were group housed, on a per cage basis, the same pattern was evident on a per-cage basis, where mice fed the AIN93G and VMM diets generally consumed more total food than mice provided TWD, MM or DIO diets (Fig. 1). Also of note was the observation that mice that were group housed consistently consumed less food than mice that were individually housed, even though food was maintained at sufficient amounts in the AOM+DSS cages throughout the study.

Energy intakes were calculated using the specific energy density for each experimental diet and the recorded food intake values on a per wk basis (Fig. 2A-B) and for the entire 16 wk feeding period (Fig. 2C-D). In sham mice, total energy intake for animals consuming AIN93G, TWD, or VMM diets was not significantly different (Fig. 2A,C). On the other hand, energy intakes for mice fed either the MM or DIO diets were markedly higher (>5%) compared to all other diet groups ($P < 0.05$) (Fig. 2A,C; Table 2). The pattern of energy intakes across diet groups was slightly different for

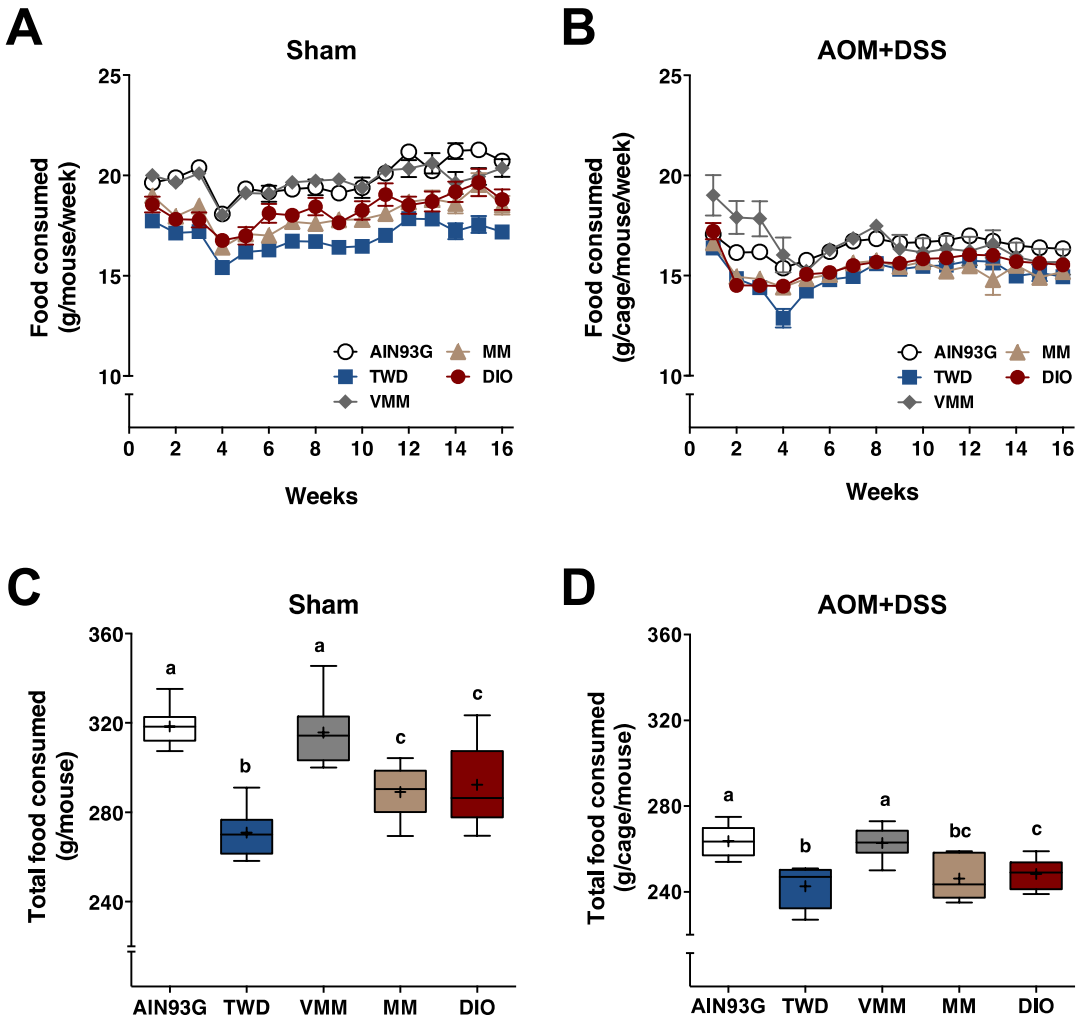

FIGURE 1. Food consumption in sham or AOM+DSS-initiated mice. Mean food intake per wk ± SEM is shown for sham mice (*N* = 10 individuals) (*A*) or mice initiated with AOM+DSS (*N* = 4 cages, data normalized by number of mice per cage) (*B*). Total food consumption data for each diet group are represented in box-and-whisker plots (plus symbol indicates the treatment group mean and whiskers are 1.5 times the interquartile range) for sham (*C*) or AOM+DSS-initiated mice (*D*). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation); Bonferroni-adjusted *P* values for multiple comparisons among diet groups for sham and AOM+DSS-initiated mice are provided in Table 2.

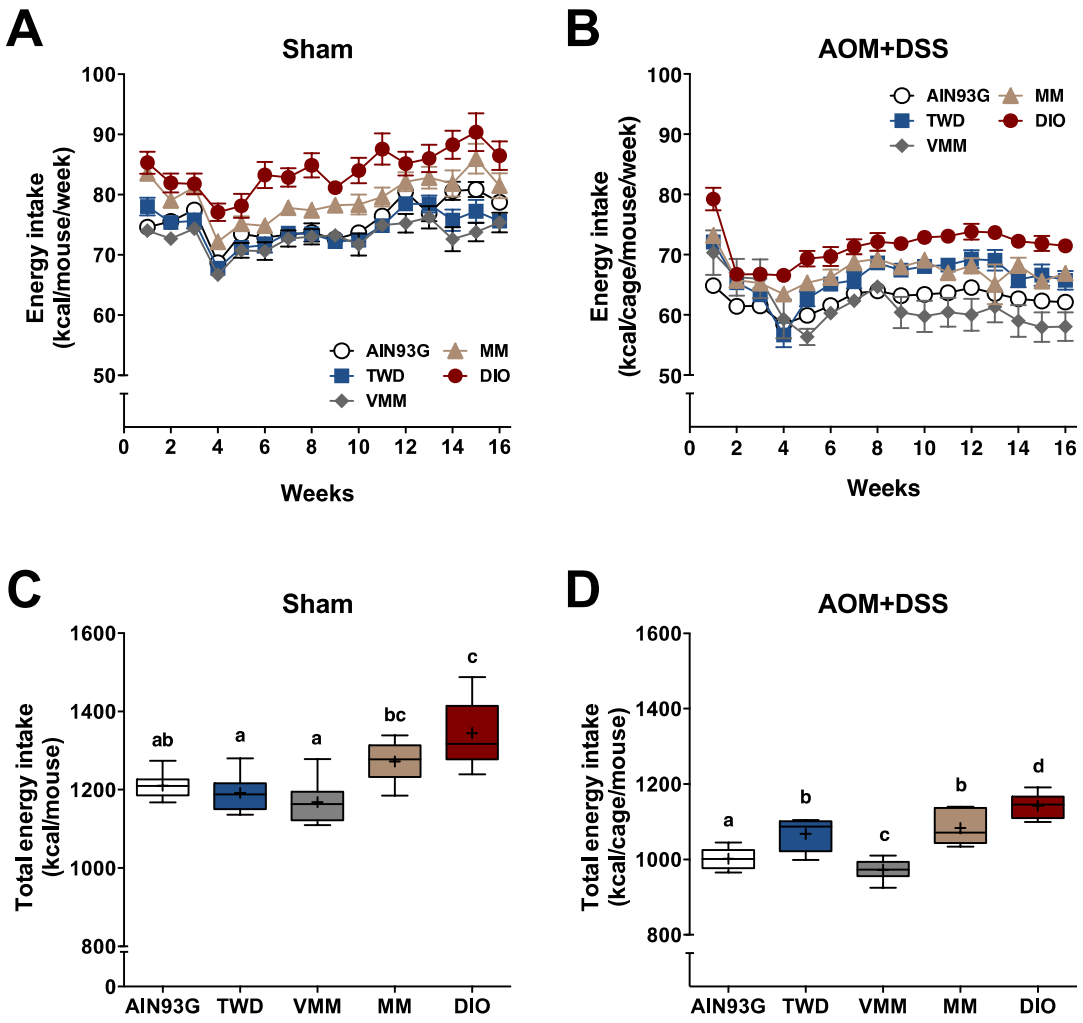

FIGURE 2. Energy intake in sham or AOM+DSS-initiated mice. Mean energy intake per wk ± SEM is shown for sham mice (*N* = 10 individuals) (*A*) or AOM+DSS-initiated mice (*N* = 4 cages, data normalized by number of mice per cage) (*B*). Total energy intake data for each diet group are represented in box-and-whisker plots (plus symbol indicates the treatment group mean and whiskers are 1.5 times the interquartile range) for sham (*C*) or AOM+DSS-initiated mice (*D*). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation); Bonferroni-adjusted *P* values for multiple comparisons among diet groups for sham and AOM+DSS-initiated mice are provided in Table 2.

TABLE 2. Comparisons among diet groups for food and energy intakes.

	Sham		AOM+DSS-initiated	
Comparison	**Food intake**	**Energy intake**	**Food intake**	**Energy intake**
AIN93G vs. TWD	**<0.0001**	1.0000	**<0.0001**	**<0.0001**
AIN93G vs. VMM	1.0000	0.9568	1.0000	**0.0165**
AIN93G vs. MM	**<0.0001**	0.1645	**<0.0001**	**<0.0001**
AIN93G vs. DIO	**0.0005**	**<0.0001**	**<0.0001**	**<0.0001**
TWD vs. VMM	**<0.0001**	1.0000	**<0.0001**	**<0.0001**
TWD vs. MM	**0.0309**	**0.0231**	1.0000	0.9118
TWD vs. DIO	**0.0062**	**<0.0001**	**0.0415**	**<0.0001**
VMM vs. MM	**0.0004**	**0.0013**	**<0.0001**	**<0.0001**
VMM vs. DIO	**0.0021**	**<0.0001**	**<0.0001**	**<0.0001**
MM vs. DIO	1.0000	0.0527	1.0000	**<0.0001**

Note: Analysis of each indicated parameter was performed by ANOVA (SAS mixed procedure) with the Satterthwaite method to approximate degrees of freedom to account for apparent unequal variances in parameter measurements among diet groups. For AOM+DSS-initiated mice, analyses of food and energy intakes were performed using cage data, rather than individual data for AOM+DSS-initiated mice; for all other parameters, cage was considered as a random factor nested within the diet treatment group. *P* values of the differences of least squares means are shown for each pair-wise comparison among diet groups, and significant differences are shown in bold.

AOM+DSS-initiated mice. As in sham mice, energy intake values (normalized to the number of mice per cage) were not significantly different between AIN93G and VMM groups, and both of these groups had lower energy intakes than mice fed MM or DIO diets (Fig. 2D). However, unlike sham mice, energy intake for AOM+DSS-initiated animals fed TWD and MM was similar and significantly higher than those fed AIN93G ($P < 0.0001$). These differences in the patterns of food and energy intake among diet groups could be attributed to food competition, as AOM+DSS mice were housed in groups of 4 to 5 mice during the study. Regardless, as with sham mice, the AOM+DSS-initiated mice fed the DIO diet consumed significantly more calories (>11%) than all

other groups (P<0.0001). Conversely, AOM+DSS-initiated mice fed the VMM consumed fewer calories than all other diet groups (P<0.05), reflecting the differences in energy density of the diets.

Body weight, organ weights, and metabolic efficiency

Sham and AOM+DSS-initiated mice gained weight steadily throughout the entire study period, although the rate of weight gain (and food consumption) for AOM+DSS-initiated mice was slightly lower for all diet groups in the week following carcinogen injection (Fig. 3A-B). At the conclusion of the study, final body weights for mice consuming TWD or VMM diets were not different for mice fed the reference AIN93G diet, yet they were significantly lower (P <0.05) than mice fed either the MM or DIO diets (Fig. 3C; Tables 3 and 4). A similar pattern was observed for mice initiated with AOM (Fig. 3D). Sham or AOM+DSS-initiated mice fed the DIO diet gained about 10 or 13% more weight, respectively, than their counterparts consuming the AIN93G reference diet. However, average final body weights were approximately 19% higher in DIO-fed mice (sham and AOM+DSS-initiated) than mice provided the VMM diet, representing the largest difference in body weight gain among the experimental diet groups.

In general, the weights of liver, kidney, and spleen with respect to body weight were not markedly affected by dietary treatment, with a few notable exceptions. The average relative liver weight for mice that consumed the DIO diet was marginally lower (0.47 to 0.58%) compared to all other diet groups (P < 0.05); this pattern was also evident in AOM+DSS-initiated mice, though only significant between DIO and AIN93G or TWD groups (P = 0.0208 and 0.0002, respectively). However, the absolute liver weights

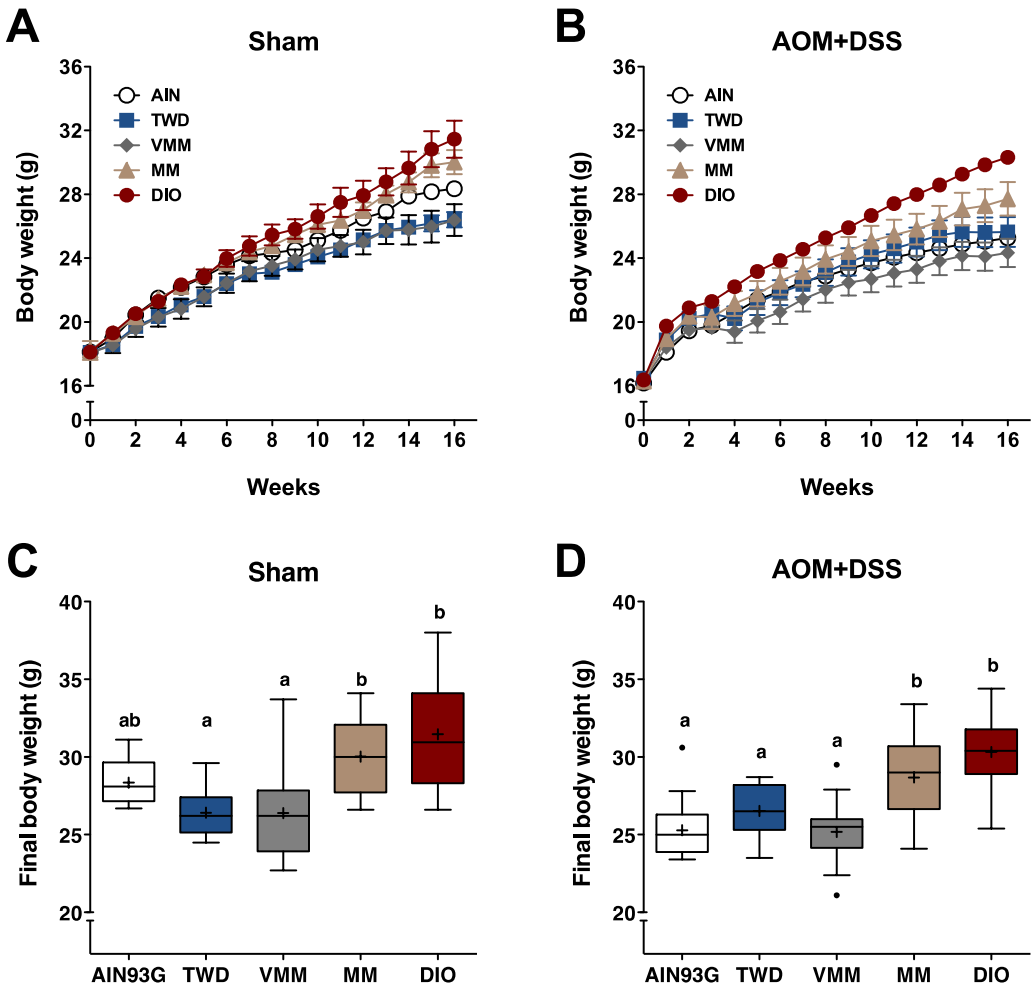

FIGURE 3. Body weight gain in sham or AOM+DSS-initiated mice. Values for body weight gain over time ± SEM are shown for sham (*N* = 10) (*A*) or AOM+DSS-initiated mice (*N* = 30) (*B*). Final body weight data for each diet group are represented in box-and-whisker plots (plus symbol indicates the treatment group mean, and whiskers are 1.5 times the interquartile range) for sham (*C*) or AOM+DSS-initiated mice (*D*). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation); Bonferroni-adjusted *P* values for multiple comparisons among diet groups for sham and AOM+DSS-initiated mice are provided in Table 4.

TABLE 3. Body organ weights for sham- and AOM+DSS-initiated mice.

Diet group	Body weight (g)	Liver weight (g)	Relative liver weight (%)	Kidney weight (mg)	Relative kidney weight (%)	Spleen weight (mg)	Relative spleen weight (%)
Sham							
AIN93G	28.4±1.4	1.06±0.086	3.74±0.22	367±23	1.30±0.10	74.0±9.7	0.261±0.031
TWD	26.4±1.5	1.00±0.082	3.77±0.18	387±24	1.47±0.12	68.0±9.2	0.259±0.040
VMM	26.4±3.2	0.993±0.12	3.78±0.50	366±37	1.39±0.071	78.0±13	0.297±0.052
MM	30.0±2.4	1.10±0.076	3.67±0.20	376±25	1.26±0.085	74.0±9.7	0.247±0.030
DIO	31.4±3.7	0.998±0.11	3.20±0.38	424±38	1.36±0.12	73.0±14	0.231±0.023
AOM+DSS-initiated							
AIN93G	25.3±1.7	1.09±0.12	4.30±0.42	341±33	1.35±0.11	85.7±15	0.340±0.063
TWD	26.5±1.6	1.20±0.15	4.51±0.48	382±41	1.44±0.11	188±104	0.711±0.40
VMM	25.2±1.7	1.08±0.13	4.29±0.51	358±40	1.42±0.14	148±101	0.590±0.42
MM	28.7±2.5	1.18±0.17	4.12±0.42	391±50	1.36±0.11	80.3±12	0.281±0.045
DIO	30.3±2.0	1.14±0.14	3.76±0.35	438±67	1.45±0.23	85.7±19	0.284±0.065

Note: Values shown are average body and organ weights or the relative liver, kidney and spleen weights (organ weight ÷ body weight × 100) ± standard deviation for each treatment group ($N = 10$ for Sham groups; $N = 30$ for AOM groups).

TABLE 4. Comparisons among diet groups for body and relative organ weights for sham- and AOM+DSS-initiated mice.

	Sham					AOM+DSS-initiated				
Comparison	**BW (g)**	**BW gain (g)**	**LW (%)**	**KW (%)**	**SW (%)**	**BW (g)**	**BW gain (g)**	**LW (%)**	**KW (%)**	**SW (%)**
AIN93G vs. TWD	1.0000	0.6970	1.0000	**0.0039**	1.0000	0.3025	1.0000	1.0000	1.0000	**<0.0001**
AIN93G vs. VMM	0.9905	0.6970	1.0000	0.4313	0.3080	1.0000	1.0000	1.0000	1.0000	**0.0010**
AIN93G vs. MM	1.0000	1.0000	1.0000	1.0000	1.0000	**<0.0001**	**0.0002**	1.0000	1.0000	1.0000
AIN93G vs. DIO	0.1141	**0.0413**	**0.0038**	1.0000	0.7296	**<0.0001**	**<0.0001**	**0.0208**	0.3986	1.0000
TWD vs. VMM	1.0000	1.0000	1.0000	0.8588	0.2176	0.2023	0.4877	0.8501	1.0000	0.7674
TWD vs. MM	**0.0331**	**0.0141**	1.0000	**0.0002**	1.0000	**0.0053**	**0.0054**	0.3606	1.0000	**<0.0001**
TWD vs. DIO	**0.0009**	**0.0001**	**0.0026**	0.1675	0.9821	**<0.0001**	**<0.0001**	**0.0002**	1.0000	**<0.0001**
VMM vs. MM	**0.0316**	**0.0141**	1.0000	**0.0450**	**0.0343**	**<0.0001**	**<0.0001**	1.0000	1.0000	**<0.0001**
VMM vs. DIO	**0.0008**	**0.0001**	**0.0016**	1.0000	**0.0019**	**<0.0001**	**<0.0001**	0.0880	1.0000	**<0.0001**
MM vs. DIO	1.0000	1.0000	**0.0183**	0.2856	1.0000	0.0570	0.1504	0.2683	1.0000	1.0000

Note: Analysis of each indicated parameter was performed by ANOVA (SAS mixed procedure) with the Satterthwaite method to approximate degrees of freedom to account for apparent unequal variances in parameter measurements among diet groups. *P* values of the differences of least squares means are shown for each pair-wise comparison among diet groups, and significant values are shown in bold. Abbreviations are as follows: body weight, BW; liver weight, LW; kidney weight, KW; and spleen weight, SW.

were not different among diet groups ($P > 0.05$), suggesting that the difference in relative liver weight values was a reflection not of a change in liver health status, but rather the body weight of mice consuming the DIO diet. Average relative spleen weight was markedly higher (up to 2-fold) in mice consuming TWD or VMM diets compared to other diet groups ($P \leq 0.001$), although this trend was not observed in sham mice (Tables 3, 4).

Metabolic efficiency was calculated for only sham mice, which were singly housed (Fig. 4). Animals fed the MM and DIO diets had significantly lower metabolic efficiency that TWD and VMM-fed mice, suggesting that they were less efficient and converting nutrients into energy. However, neither the MM nor DIO diet groups differed significantly from the AIN93-fed animals. Also, metabolic efficiency values for mice consuming diets modified in vitamin and mineral content (TWD and VMM diets) were not significantly different from mice fed AIN93G.

Body composition and fat distribution

To determine the impact of the experimental diets on fat accumulation, the body composition of sham mice was assessed periodically by MRI (Tables 5,6). In general, the rate of lean mass gain among any of the experimental diet groups was similar, and the average final lean mass was the same for all groups (Fig. 5A). However, when calculated as a percentage of body weight, lean mass at the end of the study was slightly lower in mice consuming the MM or DIO diets compared to the VMM diet ($P<0.05$), yet average percentage lean mass was not significantly different for any of the test diet groups compared to the reference AIN93G diet group (Fig. 5C). On the other had, a markedly

different pattern was observed for effect of diet on fat accumulation over time (Fig. 5B). At the end of the study, the average fat mass of mice fed either the MM or DIO diets was 7 to 12% higher compared to mice fed TWD or VMM (Fig. 5D). A comparison of the relative lean and fat mass gain among experimental groups shows that the apparent differences in body weight gain in MM and DIO animals was attributed entirely to an increase in fat mass.

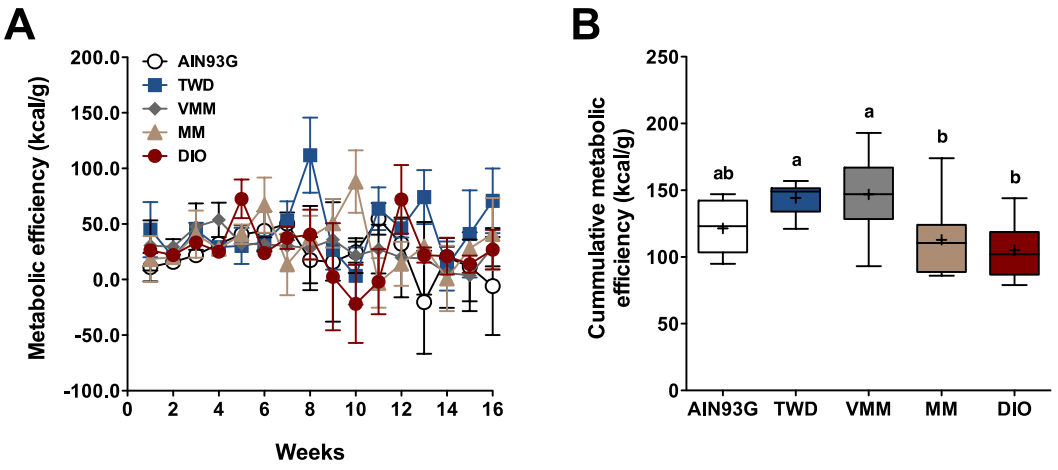

FIGURE 4. Metabolic efficiency in sham mice. *A*, Mean metabolic efficiency (calculated as weekly energy intake divided by weekly body weight gain) is shown ± SEM (*N* = 10 individuals). *B*, Values for cumulative metabolic efficiency (total energy intake divided by total body weight gain) are represented in box-and-whisker plots (plus symbol indicates the treatment group mean and whiskers are 1.5 times the interquartile range). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation to account for apparent unequal variances); Bonferroni-adjusted *P* values for multiple comparisons among diet groups are provided in Table 5.

TABLE 5. Body composition in sham mice.

Diet Group	Week 0	Week 4	Week 8	Week 12	Week 16
Fat mass					
AIN93G	1.88±1.06	2.53±0.43	3.09±1.31	3.64±0.64	5.34±1.38
TWD	2.22±0.51	2.58±0.22	2.43±0.35	3.63±0.60	4.52±1.06
VMM	2.16±0.64	2.59±0.41	2.85±0.70	3.33±1.01	4.14±1.41
MM	2.72±0.62	3.12±0.49	3.60±0.47	5.38±1.50	7.13±1.92
DIO	2.14±0.52	2.95±0.75	4.68±1.34	6.16±1.98	8.64±2.88
Lean mass					
AIN93G	15.32±1.13	17.70±0.79	17.97±1.68	20.63±0.40	19.99±2.11
TWD	15.33±1.00	16.75±0.74	18.26±0.51	19.75±0.59	19.52±0.93
VMM	15.03±1.55	16.86±1.59	18.97±1.36	19.36±1.67	19.99±1.35
MM	14.50±1.56	17.34±0.43	19.04±0.45	19.62±0.86	20.26±1.21
DIO	15.67±0.80	17.49±0.90	18.97±1.00	20.31±1.81	20.48±1.17

Note: Values shown are the average fat or lean mass (g) determined by EchoMRI ± SD for each treatment group (N = 10).

TABLE 6. Comparisons among diet groups for body composition in sham mice.

	Lean mass		Fat mass	
Comparison	**g**	**% of BW**	**g**	**% of BW**
AIN93G vs. TWD	1.0000	1.0000	1.0000	1.0000
AIN93G vs. VMM	1.0000	0.6791	1.0000	0.9477
AIN93G vs. MM	1.0000	1.0000	0.3509	0.4761
AIN93G vs. DIO	1.0000	0.5086	**0.0023**	**0.0082**
TWD vs. VMM	1.0000	1.0000	1.0000	1.0000
TWD vs. MM	1.0000	0.2837	**0.0282**	**0.0377**
TWD vs. DIO	1.0000	0.0628	**<0.0001**	**0.0003**
VMM vs. MM	1.0000	**0.0204**	**0.0074**	**0.0051**
VMM vs. DIO	1.0000	**0.0034**	**<0.0001**	**<0.0001**
MM vs. DIO	1.0000	1.0000	0.7317	1.0000

Note: Analysis of each indicated parameter was performed by ANOVA (SAS mixed procedure) with the Satterthwaite method to approximate degrees of freedom to account for apparent unequal variances in parameter measurements among diet groups. Effects of diet treatment on final lean or fat weights (g) and relative weights (%) are were determined separately. *P* values of the differences of least squares means are shown for each pair-wise comparison among diet groups, and significant values are shown in bold.

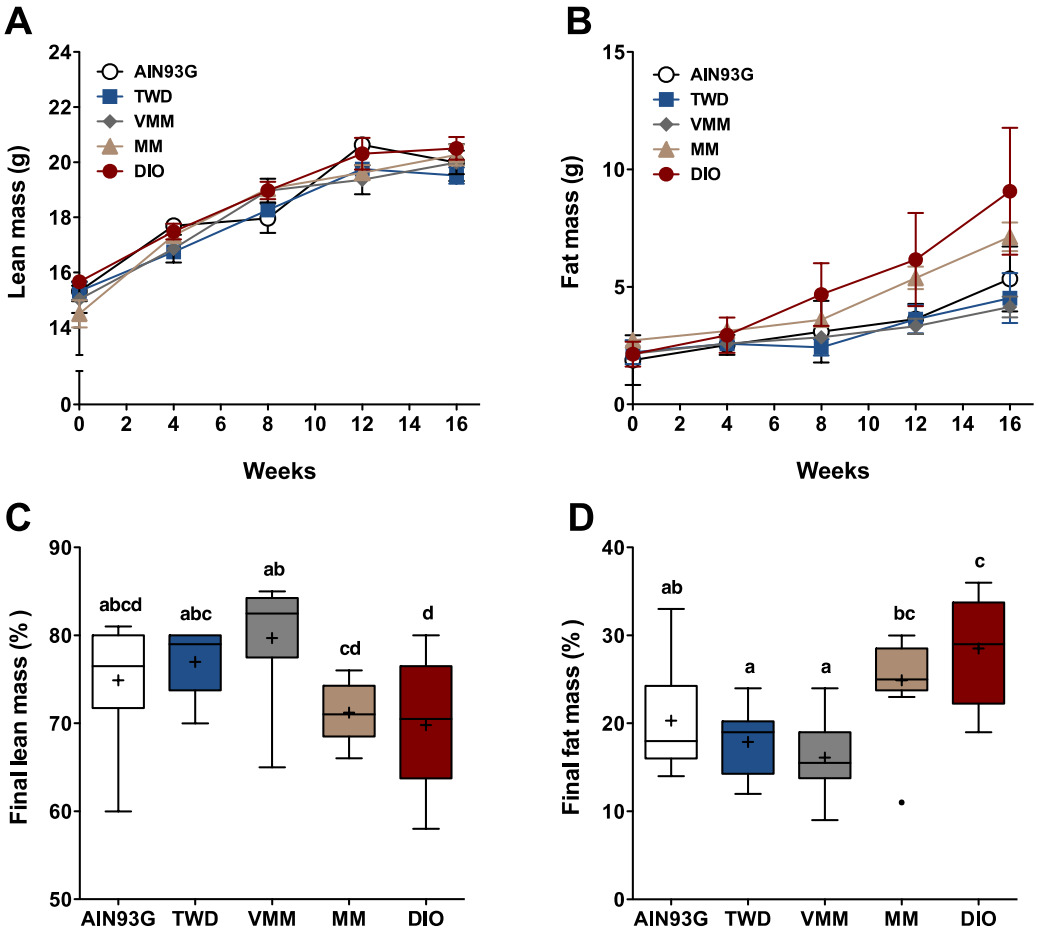

FIGURE 5. Body composition of sham mice. Body composition was assessed by MRI at wk 0, 4, 8, 12, and 16. The change in lean (*A*) and fat (*B*) mass for each diet group over time is shown ± SEM (*N* = 10 individuals). Values for the lean mass (*C*) and fat mass (*D*) with respect to final body weight (fat mass ÷ body weight × 100) are represented in box-and-whisker plots (plus symbol indicates the mean for the treatment group, and whiskers are 1.5 times the interquartile range). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation to account for apparent unequal variances); Bonferroni-adjusted *P* values for multiple comparisons among diet groups are provided in Table 8.

To determine the impact of experiment diet on fat distribution, we assessed the relative mass of subcutaneous, gonadal, mesenteric, and retroperitoneal fat pads with respect to final body weight of sham mice at the conclusion of the experiment (Tables 7, 8). A significant increase in the average percent fat mass was observed for all fat types, in animals that consumed either the MM or DIO diets, whereas no significant differences were apparent for mice fed AIN93G, TWD or VMM (Fig. 6). This effect of diet was most notable for subcutaneous fat, which was twice as abundant in mice fed DIO diet compared to the AIN93G, TWD or VMM diets ($P < 0.0001$). Additionally, a significant difference in the ratio of subcutaneous to visceral (gonadal, retroperitoneal, and mesenteric) fat was observed when comparing mice fed AIN93G (0.66 ratio) to those that consumed the DIO diet (0.91 ratio) ($P <0.01$), whereas this ratio was not different among mice fed either the TWD, VMM or MM diets.

Fasting glucose and glucose tolerance in sham mice

To assess the impact of experimental diets on biomarkers of metabolic syndrome, we measured fasting glucose and oral glucose tolerance periodically throughout the study in a subset of 6 mice randomly selected from each treatment group. At the start of the study, average fasting glucose levels were similar for all treatment groups (Fig. 7), and this trend among treatment groups was fairly consistent throughout the experiment (Table 9). At the conclusion of the study, average fasting glucose levels were not significantly different compared between the reference AIN93G diet and the other experimental diet groups (Fig. 7C). However, fasting glucose was elevated in DIO-fed mice compared to those that consumed the VMM diet ($P = 0.0043$).

TABLE 7. Fat deposition in sham mice.

	Subcutaneous		Gonadal		Mesenteric		Retroperitoneal		
Diet Group	**g**	**% of BW**	**g**	**% of BW**	**g**	**% of BW**	**g**	**% of BW**	**Ratio**
AIN93G	0.88±0.21	3.09±0.65	0.73±0.18	2.55±0.56	0.42±0.06	1.46±0.16	0.20±0.05	0.69±0.15	0.66±0.13
TWD	0.90±0.22	3.37±0.66	0.63±0.13	2.37±0.38	0.39±0.04	1.48±0.15	0.20±0.07	0.74±0.23	0.73±0.067
VMM	0.91±0.28	3.38±0.72	0.57±0.18	2.13±0.47	0.43±0.08	1.62±0.22	0.17±0.12	0.63±0.34	0.75±0.25
MM	1.51±0.57	4.93±1.81	1.09±0.29	3.58±0.77	0.53±0.09	1.75±0.19	0.38±0.12	1.26±0.31	0.77±0.052
DIO	2.09±0.71	6.50±1.41	1.20±0.43	3.70±0.97	0.62±0.14	1.95±0.24	0.49±0.18	1.53±0.40	0.91±0.11

Note: Values shown are the average weight or the average relative weight for each fat deposit (fat pad weight ÷ body weight × 100) ± SD for each treatment group (N = 10). The ratio of subcutaneous to visceral fat was calculated as the mass of subcutaneous fat ÷ (mass of gonadal + mesenteric + retroperitoneal fats).

TABLE 8. Comparisons among diet groups for fat distribution in sham mice.

Comparison	Subcutaneous (% of BW)	Gonadal (% of BW)	Mesenteric (% of BW)	Retroperitoneal (% of BW)
AIN93G vs. TWD	1.0000	1.0000	1.0000	1.0000
AIN93G vs. VMM	1.0000	1.0000	1.0000	0.6698
AIN93G vs. MM	**0.0079**	**0.0107**	**0.0011**	**0.0174**
AIN93G vs. DIO	**<0.0001**	**0.0007**	**<0.0001**	**<0.0001**
TWD vs. VMM	1.0000	1.0000	1.0000	1.0000
TWD vs. MM	**0.0389**	**0.0017**	**0.0033**	**0.0366**
TWD vs. DIO	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
VMM vs. MM	**0.0412**	**0.0001**	**0.0002**	**1.0000**
VMM vs. DIO	**<0.0001**	**<0.0001**	**<0.0001**	**0.0023**
MM vs. DIO	**0.0087**	1.0000	0.1211	0.1265

Note: Analysis of each indicated parameter was performed by ANOVA (SAS mixed procedure) with the Satterthwaite method to approximate degrees of freedom to account for apparent unequal variances in parameter measurements among diet groups. *P* values of the differences of least squares means are shown for each pair-wise comparison among diet groups, and significant values are shown in bold.

Glucose tolerance was assessed by calculating the area under the curve (AUC) for oral glucose tolerance tests performed at wk 7 and 15 of the study (Fig. 8). Values for AUC for the DIO group were significantly higher (by about 50%) than mice fed the AIN93G and VMM diets at both time points assessed (Fig. 8; Table 9), but other dietary treatment groups did not alter glucose tolerance in comparison to the reference AIN93G diet.

Plasma hormone levels in sham mice

At the conclusion of the study, levels of a variety of hormones critical to regulation of food intake, inflammation, and development of metabolic syndrome were measured in blood samples collected from sham mice. Seven in the multiplex assay were reliably detected in all samples, including adiponectin, resistin, leptin, insulin, GIP,

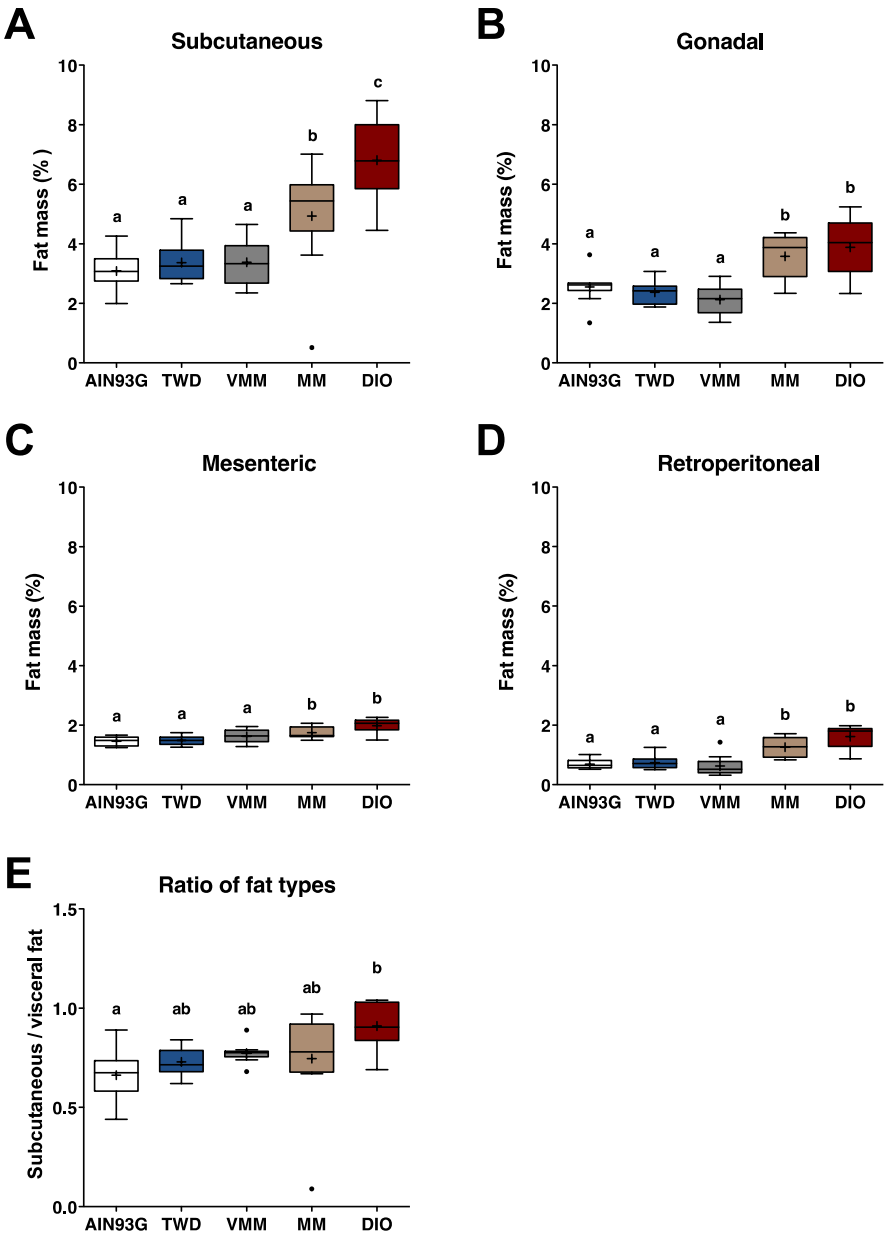

FIGURE 6. Fat distribution in sham mice. Values for the percentage subcutaneous (*A*), gonadal (*B*), mesenteric (*C*), and retroperitoneal fat (*D*) and the ratio of subcutaneous fat to visceral fat (including gonadal, mesenteric and retroperitoneal) (*E*) are represented in box-and-whisker plots (plus symbol indicates the mean for the treatment group, and whiskers are 1.5 times the interquartile range). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation to account for apparent unequal variances); Bonferroni-adjusted *P* values for multiple comparisons among diet groups are provided in Table 7.

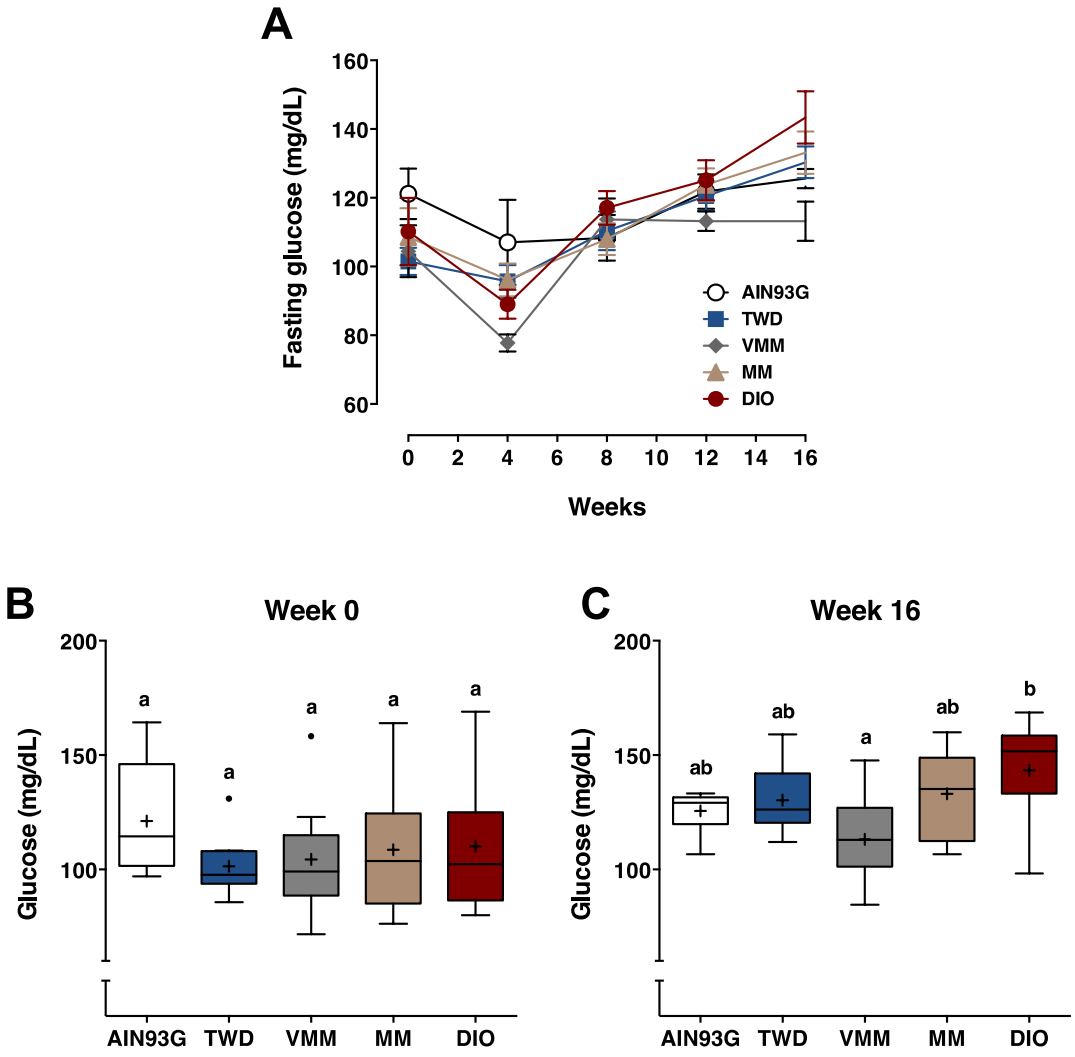

FIGURE 7. Fasting glucose levels in sham mice. Fasting glucose was determined at the start of the study, then again at wk 4, 8, 12, and 16 (*A*). The change in fasting glucose for each diet group over time is shown ± SEM ($N = 6$). Values for fasting glucose at wk 0 (*B*) and at wk 16 (*C*) are represented in box-and-whisker plots (plus symbol indicates the mean for the treatment group, and whiskers are 1.5 times the interquartile range). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation to account for apparent unequal variances); Bonferroni-adjusted *P* values for multiple comparisons among diet groups for each time point are provided in Table 9.

TABLE 9. Comparisons among diet groups for fasting glucose levels and oral glucose tolerance in sham mice.

	Fasting glucose					oGTT	
Comparison	**Wk 0**	**Wk 4**	**Wk 8**	**Wk 12**	**Wk 16**	**Wk 7**	**Wk 15**
AIN93G vs. TWD	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
AIN93G vs. VMM	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
AIN93G vs. MM	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
AIN93G vs. DIO	**0.0477**	**0.0395**	1.0000	1.0000	0.3022	**0.0477**	**0.0395**
TWD vs. VMM	1.0000	1.0000	1.0000	1.0000	0.3628	1.0000	1.0000
TWD vs. MM	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
TWD vs. DIO	0.1054	0.1202	1.0000	1.0000	1.0000	0.1054	0.1202
VMM vs. MM	1.0000	1.0000	1.0000	1.0000	0.1589	1.0000	1.0000
VMM vs. DIO	**0.0277**	**0.0137**	1.0000	0.7892	**0.0043**	**0.0277**	**0.0137**
MM vs. DIO	0.1403	0.2396	1.0000	1.0000	1.0000	0.1403	0.2396

Note: Analysis of each indicated parameter was performed by ANOVA (SAS mixed procedure) with the Satterthwaite method to approximate degrees of freedom to account for apparent unequal variances in parameter measurements among diet groups. *P* values of the differences of least squares means are shown for each pair-wise comparison among diet groups, and significant values are shown in bold.

C-peptide, and IL-6, while the levels of the other 10 hormones were below the detection limit of the assay. For the most part, the experimental diets did not significantly impact circulating hormone levels (Fig. 9). Two notable exceptions included leptin, which was elevated in mice fed DIO approximately 2-fold compared to mice provided the AIN93G, VMM or TWD diets ($P < 0.01$); plasma leptin levels were also elevated in mice fed the MM diet compared to mice consuming the AIN93G, TWD, and VMM diets (Fig. 9C). Lastly, plasma levels of GIP were significantly higher in mice consuming TWD compared to the VMM-fed animals, although neither group were significantly different from the reference AIN93G diet or the DIO diet group (Fig. 9D). Finally, the HOMA method was used to calculate insulin resistance, which was significantly higher in mice fed the DIO diet compared to those fed TWD ($P < 0.01$), although neither group was significantly different from the AIN93G, VMM or MM groups (Fig. 10)

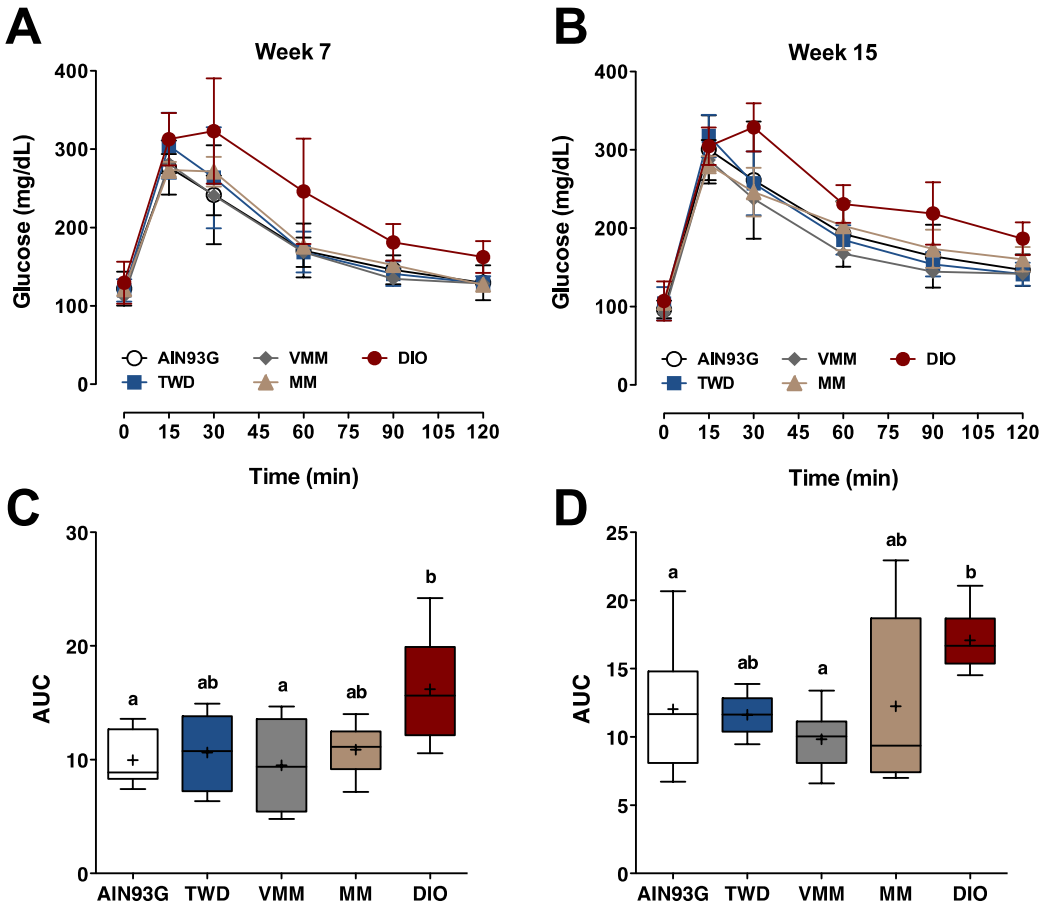

FIGURE 8. Oral glucose tolerance in sham mice. Results of oral glucose tolerance tests at wk 7 (*A*) and wk 15 (*B*) are shown. Symbols represent mean glucose levels ± SEM ($N = 6$). Glucose tolerance was assessed by calculating the area under the curve (AUC) with baseline set at 100 mg/dL. Calculated AUC values for oGTT at wk 7 (*C*) and at wk 15 (*D*) are represented in box-and-whisker plots (plus symbol indicates the mean for the treatment group, and whiskers are 1.5 times the interquartile range). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation to account for apparent unequal variances); Bonferroni-adjusted *P* values for multiple comparisons among diet groups for each time point are provided in Table 5.

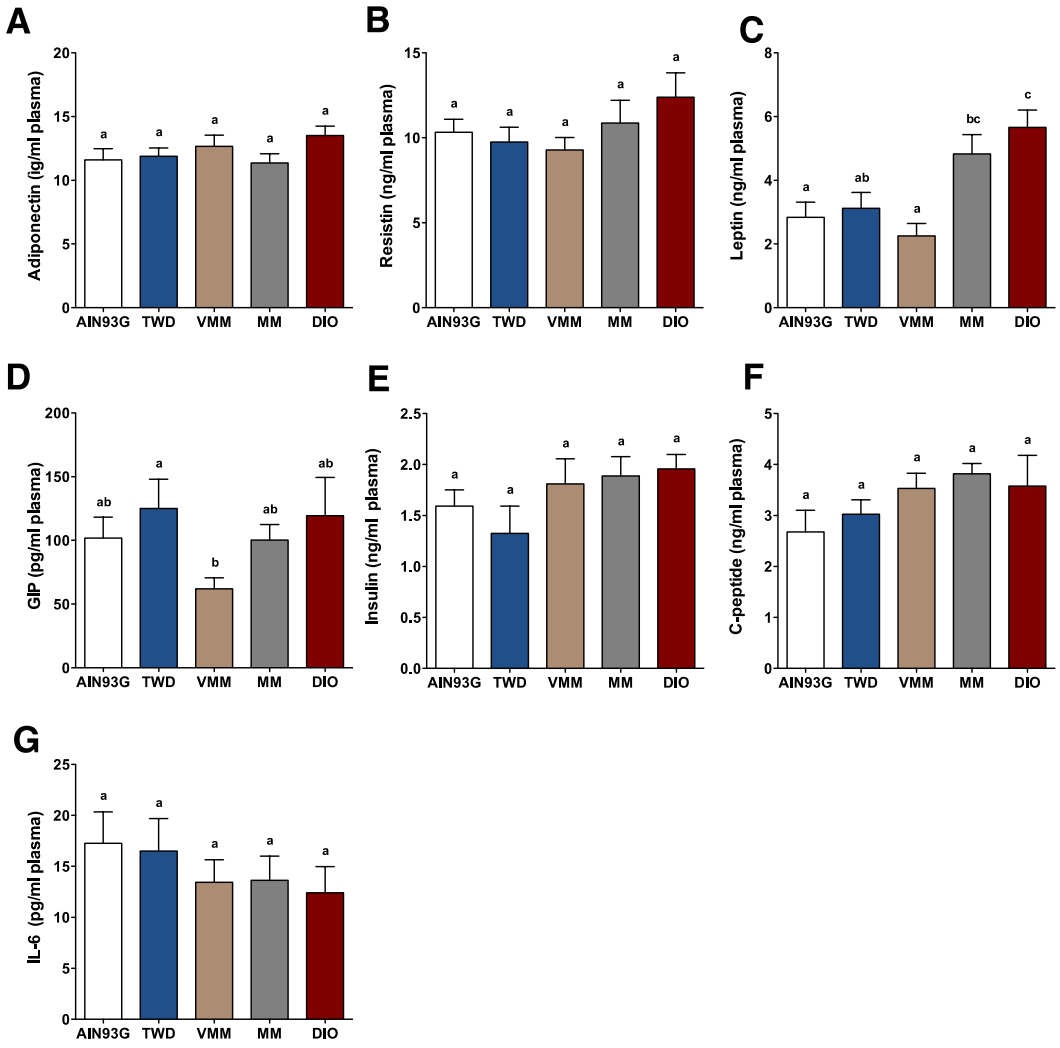

FIGURE 9. Plasma hormone levels in sham mice. Mean plasma concentrations of adiponectin (*A*), resistin (*B*), leptin (*C*), gastric inhibitory peptide (GIP) (*D*), insulin (*E*), c-peptide (*F*) and IL-6 (*G*) are shown ± SEM (*N* = 6 to 10). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with the Bonferroni adjustment for multiple comparisons).

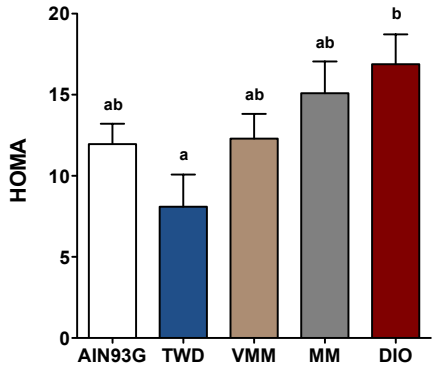

FIGURE 10. Insulin resistance by the homeostatic model assessment (HOMA) method. HOMA was calculated using matched values for fasting glucose and insulin at 16 wk as described in *Methods*. Average HOMA values are shown ± SEM (N = 6). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with the Bonferroni adjustment for multiple comparisons).

Impact of experimental diets on colon tumorigenesis

The most critical objective of this study was to assess the impact of experimental diet on colorectal carcinogenesis. At the end of the feeding trial, the number of preneoplastic lesions and the incidence, abundance and size of colon tumors were determined in a subset of mice initiated with AOM+DSS (N = 20) (Tables 10, 11). Values for all of these parameters are expressed on a colon length basis, as colon tissues with high tumor burden tend to be shorter in length and thus have less surface area. As expected with this initiation protocol, nearly all animals fed the reference AIN93G diet acquired colon cancer. However, tumors were detected in only 80% of the mice fed DIO diet, which was marginally significantly different (P=0.0479) from the 100% cancer incidence observed in mice fed TWD, VMM or MM diets (Table 10).

On the other hand, striking significant effects of experimental diet on tumor multiplicity and size were observed. Mice provided either the TWD or VMM diets had

nearly twice as many tumors as their AIN93-fed counterparts ($P< 0.01$), and about 3-times the number of tumors as animals provided the MM or DIO diets ($P <0.0001$) (Fig. 11A). However, tumor multiplicity was not significantly different in animals fed the MM or DIO diets compared to the AIN93G group.

A very similar pattern for tumor volume was also evident, as the average tumor size in mice provided TWD or VMM diets was about 3.6- to 2.5-fold higher than in mice fed AIN93G diet ($P <0.01$) (Fig. 11B). An even more pronounced increase in tumor volume was observed, a nearly 10-fold difference, when comparing the TWD and VMM groups to mice fed MM or DIO diets ($P < 0.0001$). Moreover, average tumor volumes in mice fed TWD and VMM were significantly greater than tumor volume for the reference AIN93G group ($P = 0.0009$ and 0.022, respectively).

To determine whether mice fed MM or DIO diets acquired a large number of preneoplastic lesions that failed to progress to tumors, we counted the average number of aberrant crypts and the number of ACF (Fig. 11C-D) (Table 10). No significant differences among diet groups were observed (Table 11), although the low variance evident in the TWD and VMM likely reflects that many of the preneoplastic in these diet groups progressed to form colon tumors.

TABLE 10. Colon tumorigenesis in mice initiated with AOM+DSS.

	Tumor outcome			Aberrant crypts	
Diet group	**Incidence ***	**Multiplicity †**	**Volume §**	**Aberrant crypt foci ¶**	**Total crypts ‡**
AIN93G	95% (19/20) [a,b]	0.0701±0.0089 [a]	1.53±0.24 [a]	0.0375±0.0110 [a]	0.0597±0.014 [a]
TWD	100% (19/19) [a]	0.124±0.014 [b]	5.49±0.76 [b]	0.0172±0.0033 [a]	0.0294±0.0064 [b]
VMM	100% (19/19) [a]	0.139±0.014 [b]	3.80±0.65 [b]	0.0353±0.0028 [a]	0.0382±0.0029 [b]
MM	100% (19/19) [a]	0.0308±0.0039 [a]	0.41±0.10 [c]	0.0318±0.0044 [a]	0.0508±0.0062 [a]
DIO	80% (16/20) [b]	0.0470±0.0041 [a]	0.51±0.13 [c]	0.0406±0.0081 [a]	0.0660±0.012 [a]

Note: Analysis of tumor incidence was performed using a quasi-likelihood logistic regression (SAS genmod procedure), while tumor multiplicity, tumor volume (log-transformed), number of aberrant crypt foci and number of total crypts were analyzed using a mixed model ANOVA (SAS mixed procedure) with the Satterthwaite method to approximate degrees of freedom to account for apparent unequal variances in parameter measurements among diet groups. Different letters indicate that the treatment groups are significantly different. *P* values of the differences of least squares means for each pair-wise comparison among diet groups are available in Table 7.

* Values are the percent incidence (cases/total) for each dietary group. Twenty mice were originally allocated to each diet group for assessment of tumor outcome, although one mouse each in the TWD, VMM, and MM diet groups died within 48 h of AOM injection due to the well-known toxic effects of this carcinogen.

† Tumor multiplicity was calculated as the average number of tumors per tumor-bearing animal normalized to the colon length. Values shown are mean tumor multiplicity per mm colon ± SEM.

§ Tumor size was estimated using the equation $Volume = \pi/6(L \times W \times D)$. Values shown are the mean tumor volume (mm^3) ± SEM.

¶ The average number of aberrant crypt foci was calculated as the number of foci per animal bearing aberrant crypts normalized to the colon length. Values shown are the average number of aberrant crypt foci per mm colon ± SEM .

‡ The average total number of aberrant crypts was calculated as the number of total crypts per animal bearing aberrant crypts normalized to the colon length. Values shown are the average number of total crypts per mm colon ± SEM.

TABLE 11. Comparisons among diet groups for colon tumorigenesis in mice initiated with AOM+DSS.

	Tumor outcome			Aberrant crypts	
Comparison	**Incidence**	**Multiplicity**	**Volume**	**Number foci**	**Total crypts**
AIN93G vs. TWD	1.0000	**0.0031**	**<0.0001**	0.6413	1.0000
AIN93G vs. VMM	1.0000	**<0.0001**	**0.0027**	1.0000	1.0000
AIN93G vs. MM	1.0000	1.0000	**0.0009**	1.0000	1.0000
AIN93G vs. DIO	0.2982	0.1177	**0.0220**	1.0000	1.0000
TWD vs. VMM	1.0000	1.0000	1.0000	1.0000	1.0000
TWD vs. MM	1.0000	**<0.0001**	**<0.0001**	1.0000	1.0000
TWD vs. DIO	**0.0479**	**<0.0001**	**<0.0001**	0.2880	0.1877
VMM vs. MM	1.0000	**<0.0001**	**<0.0001**	1.0000	1.0000
VMM vs. DIO	**0.0479**	**<0.0001**	**<0.0001**	1.0000	0.6011
MM vs. DIO	**0.0479**	1.0000	1.0000	1.0000	1.0000

Note: Analysis of tumor incidence was performed using a quasi-likelihood logistic regression (SAS genmod procedure), while tumor multiplicity, tumor volume (log-transformed), number of aberrant crypt foci and number of crypts per foci were analyzed using a mixed model ANOVA (SAS mixed procedure) with the Satterthwaite method to approximate degrees of freedom to account for apparent unequal variances in parameter measurements among diet groups. *P* values of the differences of least squares means are shown for each pair-wise comparison among diet groups, and significant values are shown in bold.

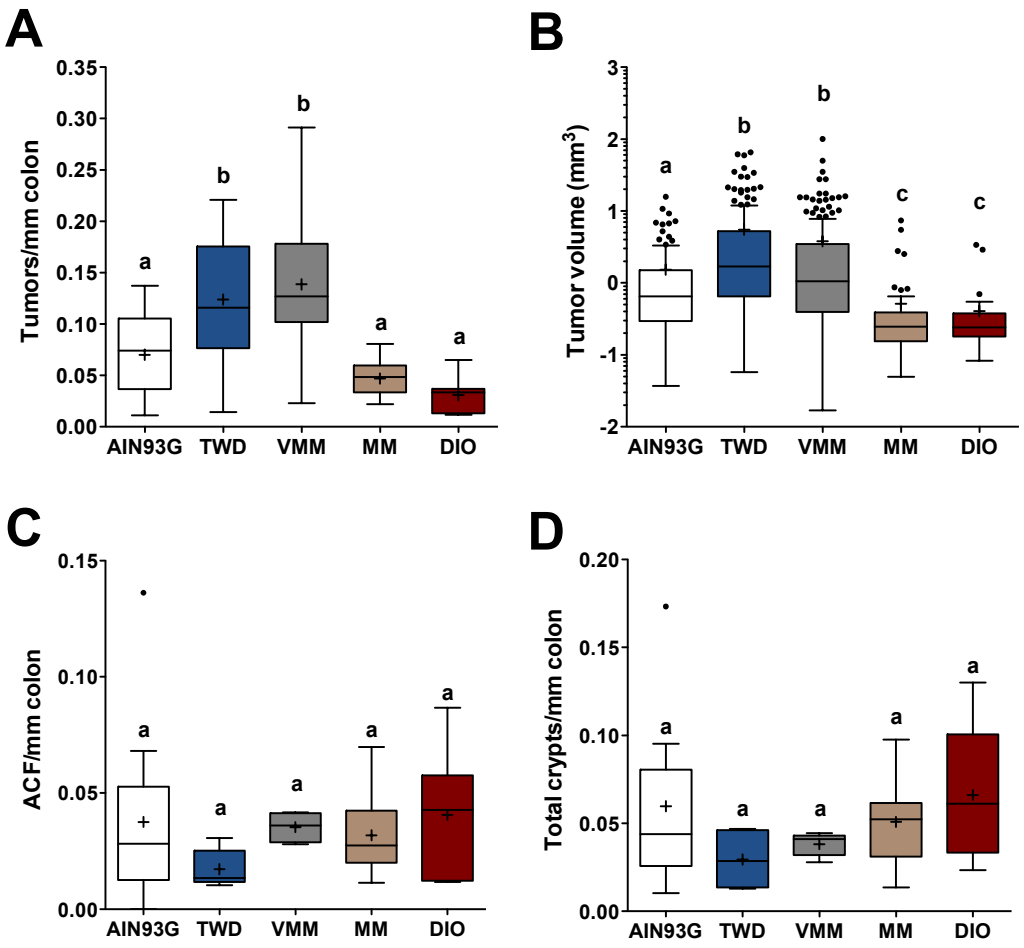

FIGURE 11. Colon tumorigenesis in mice initiated with AOM+DSS. Tumor multiplicity (number of tumors for tumor-bearing animals normalized by the colon length) (*A*), tumor volume (calculated as $V = \pi/6$ ($L \times W \times D$) where L is length, W is width, and D is depth) (*B*), aberrant crypt foci (number of foci for mice with foci) (*C*), and total crypts (total number of aberrant crypts for animals with foci) (*D*) are represented in box-and-whisker plots (plus symbol indicates the mean for the treatment group, and whiskers are 1.5 times the interquartile range). Different letters indicate that treatment groups are significantly different as determined by ANOVA (SAS mixed procedure with Satterthwaite approximation to account for apparent unequal variances); Bonferroni-adjusted *P* values for multiple comparisons among diet groups are provided in Table 10.

DISCUSSION

We report for the first time that a newly devised diet that recapitulates U.S. nutrition patterns with respect to macro- and micronutrient content markedly enhanced colorectal carcinogenesis in a mouse model of inflammation-associated colon cancer compared to a basal diet optimized for rodent health. Moreover, we determined that the micronutrient components of the TWD were primarily responsible for increased cancer susceptibility in these mice. This study is the second from our group to show that this diet functions to promote colon cancer in mice. Previously, we showed that A/J mice initiated with AOM and fed TWD for 16 wk developed significantly more ACF and total crypt cells than mice fed the AIN93G diet (107). Interestingly, green tea supplementation (0.2% Polyphenon E) markedly reduced the accumulation of aberrant crypts in animals fed the TWD, but did not alter colon carcinogenesis in animals consuming AIN93G. These observations pointed to a complex, yet critical interaction between basal diet and the activity of a well-established anticancer agent.

In an effort to understand better the impact of this westernized diet on cancer susceptibility, we sought to determine the relative impacts of the macro- and micronutrient components of the TWD on colon cancer as well as on biomarkers of metabolic syndrome and obesity in comparison to the commercial 45% fat DIO diet. As expected, mice consuming the DIO diet acquired an obesity/metabolic syndrome phenotype typified by increased food energy intake, greater rate of body weight gain, increased proportion of body composition as fat mass, higher fasting glucose, impaired glucose tolerance, and higher circulating levels of leptin. However, the TWD did not

significantly alter any of these classic biomarkers of metabolic health, a somewhat surprising observation considering that this diet contains substantially more fat than the AIN93G diet (35 compared to 17 percent of total kcal, respectively). Furthermore, our observations also suggest that the DIO diet does not enhance colon cancer in the AOM+DSS model, a finding somewhat contrary to prior observations for ACF in rats (66, 108). While this later finding was admittedly unexpected, it points to the critical need to employ a diet model that represents typical human nutrition. Had the experiment design included only DIO diet as the model obesogenic Western diet, we could have easily (and erroneously) concluded that colorectal cancer risk in mice was unaffected by the Western nutrition pattern. Moreover, we would have missed the more remarkable discovery that inappropriate micronutrient consumption strongly increased colon tumorigenesis in this mouse model, as evidenced by highly similar results for the TWD and the VMM diets, both of which had vitamin and mineral content that reflects typical American nutrition. Modification of macronutrient content alone (*i.e.*, DIO and MM diets) did not increase colorectal carcinogenesis, an observation that also points to a critical role for micronutrients in promoting colorectal carcinogenesis in this pre-clinical mouse model.

Numerous studies have shown that consumption of a high fat diet promotes a gain in relative fat mass in a dose-dependent manner (109). The higher adiposity observed in animals fed either the MM or DIO diets reflected the relatively high fat content of these diets (34 and 45%, respectively). However, new evidence points to a role in the source of dietary fat for influencing fat deposition and subsequent impacts on parameters of metabolic health. Catta-Preta et al. (110) reported that C57BL/6J mice fed a diet

containing 60% fat from lard or olive oil had higher levels of plasma insulin, resistin, and leptin, compared to mice fed diets with 60% sunflower oil or canola oil. Moreover, the ratio of subcutaneous to visceral fat was at least 40% smaller in animals fed the lard or olive oil diets compared to animals provided diets containing other lipid sources. The fat source for the DIO diet is primarily lard, whereas the fat content of the AIN93G and VMM diets is exclusively soybean oil. The MM and TWD diets incorporate a variety of fat sources relevant to typical Western nutrition, including soybean oil, milk fat, lard, olive oil, beef tallow, and corn oil. Interestingly, in this study, the ratio of subcutaneous to visceral fat was significantly higher in mice fed DIO compared to the reference AIN93G diet. However, we did not detect a difference in the fat type ratio between the DIO and MM diets, although these diets are not directly comparable due to their difference in total fat concentration. In another study by Huang et al. (111), higher levels of leptin and the pro-inflammatory hormones resistin, MCP-1 and CD192 were observed in C57BL/6J mice fed a 38% safflower oil diet rich in polyunsaturated fatty acids compared to isocaloric diets containing lard and milk fat. Our analysis of plasma hormones also included adiponectin, resistin, insulin, GIP, C-peptide, and IL-6. These hormones and cytokines, which are involved in energy balance and inflammation, are important biomarkers for systemic inflammation and colorectal cancer (19, 112). Unfortunately, the results of the hormone analysis for our study were limited because of the high variability in measurements among mice or because plasma concentrations of some hormones were below the assay limit of detection. However, blood plasma levels of leptin were closely correlated with body composition, as expected for this fat-derived hormone (113).

The consumption of a high fat diet is strongly linked to high rates of obesity due to increased energy intake, as opposed to greater food mass consumption (see review by 109). Observations from our study concur with this model, in that mice fed the DIO diet had significantly greater energy intake than their AIN93G-fed counterparts, yet lower food mass consumption. Importantly, these animals did not completely adjust their food consumption so that their energy intake was on par with animals consuming the reference diet. Moreover, this pattern was consistent throughout the study, indicating that DIO-fed mice did not adequately adapt over time to the high fat content of their diet. On the other hand, sham mice fed TWD consumed the least mass of food of any diet group, and this decrease was sufficient to normalize their total energy intake to that of the AIN93G-fed mice. As a consequence, mice fed the TWD did not gain excess weight nor develop biomarkers of obesity-induced metabolic syndrome. This difference in energy intake cannot be attributed solely to the modest 10% difference in fat content of these 2 diets, as sham mice fed the MM diet (matched to TWD for all macronutrients) had significantly greater food and energy intake compared to TWD-fed animals. That these observations were not entirely consistent for AOM+DSS-initiated mice could be attributed to competition among cage-mates or other behavior effects present in group-housed male mice.

The results of this study indicate that micronutrients contribute to colorectal carcinogenesis to a greater extent than do macronutrients in a mouse model of inflammation-associated colon cancer. For many endpoints evaluated, we observed that the data segregated into groups based on similarity in response: MM and DIO diets (with high fat the common feature); TWD and VMM diets (with modified vitamins and

minerals the common feature) and then the reference diet AIN93G. For endpoints associated with obesity and metabolic syndrome, significant effects were often observed for the MM and DIO diets, but not the others. The opposite pattern was evident for colon cancer outcome, with significant effects of diet apparent only for mice fed TWD or MM diets. Evidently, increased tumorigenesis in mice fed either of these diets was not linked to higher rates of obesity or metabolic syndrome. Because the TWD models the typical U.S. diet with respect to nearly all major micro- and macronutrient components, it is not possible to confidently single out one (or a few) constituents responsible for its tumor promoting effect. Nevertheless, our strategy is an important advance on prior studies that investigated the impact of only one or a select few components of the Western diet on colon cancer.

The nutrient density approach was previously employed by Newmark et al. (102) to construct a Western-type diet containing the levels of fat, phosphorous, calcium, and vitamin D observed in average American diets. Compared to the AIN76A diet, their Western diet contained higher levels of fat and phosphorous, and lower levels of calcium and vitamin D. C57BL/6J mice fed their Western diet promoted spontaneous hyper-proliferation and hyperplasia in colonic epithelium. In a subsequent study, this further modified this experimental diet by reducing levels of components critical for methyl donor generation, including folic acid, methionine, and vitamin B_{12} (103). Consumption of this modified western diet led to higher rates of spontaneous colon adenoma and carcinoma development in normal mouse colon. Other number of other rodent studies using the AOM model of CRC point to a role for high fat diet in enhancing colorectal

carcinogenesis, even though the fat composition in these experiments varied widely (38, 63, 97, 114, 115).

Alternatively, the importance of various dietary micronutrients on CRC development has also been shown in a number of animal and human studies. The TWD contains low amounts of several essential micronutrients when compared to both the DIO diet and RDA values for humans (106). On an energy density basis, amounts of vitamins B_6, B_{12}, and folate are about 33 to 50% of the TWD compared to the AIN93G control diet, while calcium and vitamin D levels are approximately 60% lower.

As outlined in the introduction, evidence from human epidemiological studies and experiments in animal models points to a role for both calcium and vitamin D in modulation of CRC (116-118). Calcium functions to regulate cellular signaling, cell proliferation and cell growth, while vitamin D is critical for adequate uptake of calcium in the small intestine. A 24-wk study on $Apc^{1638N/+}$ mice, which have a truncation on codon 1638 of the tumor suppressor *Apc* gene, found that feeding a modified AIN76A diet with half the content of vitamin D and 90% less calcium resulted in the formation of colonic adenomas and a carcinomas, and enhanced expression of cyclin D1 and anti-apoptotic protein Bcl-2, which are commonly overexpressed in colorectal cancer (82). In a different study using this same diet fed to C57BL/6J mice over a period of 3 and 6 months, researchers identified important transcriptome changes associated with induction of the oxidative stress response pathway (119). Moreover, calcium and vitamin D were determined to be important regulators of bile acid synthesis and excretion. Vitamin D has been shown to be involved in the detoxification of bile acids through the regulation of fibroblast growth factor 15 (120) and the vitamin D receptor (121) in the intestine, while

calcium has been shown to increase fecal fatty acid excretion (122, 123). Bile acids contribute to colorectal carcinogenesis by increasing cellular oxidative stress, and they have been associated with the promotion of cell populations resistant to their apoptotic effects (124). Bile acids are the main pathway for cholesterol catabolism in mammals, and hence they are generally found in high levels in individuals that consume a high-fat diet (125). Thus, the combined impact of excess fat with low calcium and vitamin D content in the TWD could explain the promoting effect of this diet on colon tumorigenesis observed in this study.

Choline, folate, and vitamins B_6 and B_{12} are essential in cellular biosynthetic pathways due to their roles as donors of methyl groups for one carbon-metabolism (84). These micronutrients are present in the AIN93G diet at substantially greater levels than the RDA value for humans, when compared on an energy density basis (106). Moreover, the TWD contains substantially lower levels of these micronutrients than are present in the AIN93G diet. A case-control study of subjects from the Multiethnic Cohort study found that people in the highest quartile for pyridoxal-5'-phosphate intake (active form of B_6) had a 48% reduction in CRC risk (126). A meta-analysis that included 7 cohort-studies and 9 case-control studies found a 25% lower risk of CRC among those in the highest category of dietary folate intake compared with those in the lowest category, while only a 5% lower risk was observed for total folate intake (including supplements) (85). An animal study using male Sprague-Dawley rats fed with either a diet with 50 μg/kg diet of vitamin B_{12} or a diet with no vitamin B_{12} found that, after 10 wk of feeding, rats fed the deficient diet displayed a 35 percent decrease in genomic methylation and a 105 percent increase in base substitution of uracil. Although some studies have found

weak or no association between CRC risk or development and dietary B-vitamins (87, 89, 127), the supporting evidence points to a complex interaction between other nutrients in the diet. Also, the low levels of B-vitamins in the TWD could explain the promoting effect on tumorigenesis observed in animals fed this diet.

In conclusion, evidence from this work supports the idea that a rodent diet more representative of the diet consumed by the majority of Americans is necessary to appropriately evaluate colon cancer risk and to develop specific and effective prevention strategies. Diets that modeled Western nutrition with respect to macronutrient content only yielded a greatly different disease phenotype than did a diet that also took into account suboptimal micronutrient consumption. The health consequences of chronic low intakes of multiple micronutrients are not well understood and should be studied in the context of modern dietary patterns. To extend upon this work, our research group plans to investigate the impact of TWD using the well-established $APC^{Min/+}$ genetic mouse model of gastrointestinal carcinogenesis. Also, our group will continue to investigate molecular markers of colorectal carcinogenesis in colon mucosa obtained from sham and AOM+DSS-initiated mice from this project using global gene expression microarrays and immunohistochemistry. Finally, given the profound impact of the TWD on colon carcinogenesis and the mounting evidence for perinatal or transgenerational impacts of diet on cancer risk (128-135), we plan to investigate the impact of TWD provided via the maternal diet on risk of cancer to offspring.

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