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Brief Statement of Findings: Through experimental studies involving *Apc*$^{Min/+}$ mice differentially expressing *nitric oxide synthase-2 (Nos2)*, we show that dietary arginine-increases the grade of intestinal tumors in a *Nos2*-dependent manner. Additionally, our epidemiologic analysis of 511 colorectal cancer (CRC) cases over a 10-year period reveals that familial CRC cases in the highest quartile of meat consumption (i.e. a food group with high arginine content) prior to diagnosis have decreased survival compared to those in all other meat consumption quartiles. Potential mechanisms for these findings are analytically and experimentally explored.

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Abstract

Elevated polyamine and nitric oxide levels (both derived from arginine) promote tumorigenesis, whereas NSAIDs inhibit colorectal cancer (CRC) incidence in experimental and epidemiologic studies. We investigated dietary arginine-induced intestinal tumorigenesis and NSAID-inhibitory effects in *Apc*$^{Min/+}$ mice differentially expressing *nitric oxide synthase-2* (*Nos2*). We also studied effects of estimated arginine exposures through meat consumption on tumor characteristics and survival in human CRC cases. Dietary arginine increased high-grade colon adenoma incidence in *Apc*$^{Min/+}$ *Nos2*$^{+/+}$ mice, but not in *Nos2* knockout mice. Additionally, celecoxib suppressed intestinal steady state ornithine decarboxylase RNA levels ($P < 0.001$), induced steady state spermidine/spermine N^1-acetyltransferase RNA levels ($P = 0.002$), decreased putrescine levels ($P = 0.04$), and decreased tumor number in the small intestines of *Apc*$^{Min/+}$ *Nos2*$^{+/+}$ mice ($P = 0.0003$). 511 cases from our NCI-supported CRC gene-environment study were analyzed based on self-reported meat (as a surrogate for arginine) consumption. Familial CRC cases (n = 144) in the highest meat consumption quartile (Q4) had no statistically significant differences in tumor grade compared to cases in Q1-Q3 ($P = 0.32$), however they were observed to have decreased overall survival (OS) (10-year OS = 42% vs. 65%; $P = 0.017$), and increased risk of death in an adjusted analysis (hazards ratio [HR] = 2.24; $P = 0.007$). No differences in tumor grade, OS, or adjusted HR were observed for sporadic CRC cases (n = 367) based on meat consumption. Our results suggest important roles for arginine and meat consumption in CRC pathogenesis, and have implications for CRC prevention.

Introduction

Extensive epidemiologic studies have investigated the relationship between meat consumption and CRC risk. Meat consumption was shown not to be associated with CRC in a large meta-analysis of 34 case-control and 14 cohort studies published during 1973-1999, while red meat and processed meat intake were associated with moderate increases in CRC risk(1). A large cohort study has recently confirmed an increased risk of developing CRC associated with consumption of red meat and processed meats, and demonstrated a protective effect for fish and poultry(2). While many epidemiologic studies have assessed the risk of meat consumption on developing CRC(1;3), there is a paucity of data addressing the risk of meat consumption on outcomes in CRC patients. Meat consumption is the major source of dietary arginine in humans – with high quantities of arginine found not only in red meat but also in pork, fish, and chicken(4). Meat and meat products accounted for 37-38% of total daily arginine in two European studies, where subjects were estimated to have similar daily arginine intake to U.S. men (i.e. 4.2 – 5.0 g/day)(5;6). In cell culture, normal cells become quiescent in an arginine-deficient diet, whereas most transformed or malignant cells die(7;8). Arginine supplementation(8), particularly L-arginine(9), has been shown to increase tumor growth in rodents whereas D-arginine may have tumor-inhibitory properties(9). In human breast cancer tissue, dietary arginine has been shown to stimulate tumor growth(10).

Importantly, arginine is the key substrate for two competing metabolic pathways believed to be involved in carcinogenesis: the nitric oxide (NO) synthase pathway and polyamine synthesis. Arginine is catabolized by NO synthase 2 (NOS2) and other NO synthases to form nitric oxide. Inducible isoforms of NOS2 are abundant in human colorectal adenomas(11). NOS2 is also overexpressed in rat colon carcinoma tissues after azoxymethane treatment(12;13), an effect that is inhibited by aspirin(12). *Apc*$^{Min/+}$ *Nos2* knockout mice provided with dietary arginine were noted to have a decreased

number of intestinal and colonic adenomas compared to *Apc*$^{Min/+}$ *Nos2*$^{+/+}$ mice(14;15). Thus it has been suggested that NOS2-selective inhibitors may have a future role in CRC chemoprevention.

Arginine is catabolized by the enzyme arginase to form ornithine, the substrate for putrescine synthesis. This is the first step in polyamine biosynthesis and is catalyzed by the enzyme ornithine decarboxylase (ODC)(16). Multiple abnormalities in the control of polyamine contents results in increased polyamine levels that can promote tumorigenesis(17). APC, a gene that is mutated in FAP and in the majority of sporadic colon cancers, regulates ODC and therefore polyamine synthesis(18). In accordance, Loss of APC increases ODC expression, polyamine content, and small intestinal tumor formation(19). Treatment with the chemopreventive agent α-difluoromethylornithine (DFMO), a suicide inhibitor of ODC, has been shown to decrease polyamine levels and suppress tumorigenesis in the small intestine and colon of *Apc*$^{Min/+}$ mice (an FAP mouse model)(19), and decrease colorectal tissue polyamine levels in human patients with colon polyps(20).

Together, DFMO and non-steroidal anti-inflammatory drugs (NSAIDs) inhibit intestinal cancer development in mouse models(21). Sulindac(22) and aspirin(23) are NSAIDs that have been demonstrated to activate the polyamine catabolic pathway via increased expression of spermine spermidine acetyltranferase (SSAT), polyamine acetylation and subsequent export. It is not known whether this mechanism exists for the selective cyclooxygenase-2 (COX-2) inhibitors.

In the experimental portion of this investigation, we examine the role of *Nos2* and dietary arginine in murine carcinogenesis via gene knockout mouse model systems. We also investigate the effects of celecoxib on *Odc* and *Ssat*, and test whether celecoxib treatment can inhibit intestinal tumorigenesis in FAP mouse models. In the epidemiologic portion of this study, we investigate the effects of dietary meat

consumption on tumor characteristics at presentation and survival in CRC cases. We hypothesized that any differential effects of meat consumption on these clinical outcomes might be mediated through gene-environment interactions, and thus manifest predominantly in cases with multifactorial inherited susceptibility to CRC (i.e. familial CRC cases).

Materials & Methods

Mouse experimental procedures.

Animals and experimental conditions: C57BL/6J-*Apc*^{Min} mice were acquired at 6 weeks of age from the Jackson Laboratory (Bar Harbor, ME) and bred in The University of Arizona's Animal Care Facility. Affected *Apc*$^{Min/+}$ mice were obtained by crossing C57BL/6J-*Apc*^{Min} males with C57BL/6J females. For assessment of dietary arginine effects, C57BL/6J-*Apc*$^{Min+/}$-*Nos2*$^{-/-}$ mice (*Apc*$^{Min+/}$-*Nos2*$^{-/-}$) were produced by crosses between C57BL/6J-*Apc*^{Min} male and C57BL/6J-*Nos2*$^{tm1Lau/j}$ female mice for more than 10 generations. Offspring were genotyped using allele-specific polymerase chain reaction (PCR) as described in genotyping protocols from the Jackson Labs website (http://jaxmice.jax.org/). All animals were raised in cages under non-sterile microisolator conditions and were put on the various treatments at age 5-6 weeks. Animals were fed with an AIN-93G diet (Harlan Teklad), a defined diet which includes 1.08 grams arginine/kg mouse body weight per day. Mice were sacrificed at age of 110 days by CO_2 inhalation and were analyzed by tumor scoring, tumor grade evolution, and Northern blotting. Throughout the experiment, animals had access to drinking water and food *ad libitum*.

Assessment of dietary arginine effects on tumor number and grade in Apc$^{Min/+}$ *Nos2*$^{+/+}$ *mice:* For assessment of dietary arginine effects on tumor grade, the diet was

supplemented with arginine in the drinking water (0-0.2%, Sigma-Aldrich, St. Louis, MO). Mice have a 7.5 times larger metabolic rate compared to humans (24). Therefore, mouse dietary arginine concentrations (1.08 – 1.38 grams arginine/kg mouse body weight per day, as described previously (15)) were adjusted for differences in metabolic rates, i.e. concentrations in the upper range of that consumed by human (121 mg/kg per day, as described (6)). After termination, whole small intestine and colon segments were removed, flushed with buffered saline, opened longitudinally and spread out with the mucosal surface up. A Swiss gut roll of small intestine, colon, and all grossly evident colon tumors were fixed in 10% buffered formalin. Processed, paraffin-embedded 5-micron sections were stained with hematoxylin and eosin prior to evaluation by a veterinary pathologist. The grade of dysplasia for each adenoma examined histologically was determined on the basis of criteria outlined previously(25).

Celecoxib effects in intestinal tissue of Apc$^{Min/+}$ mice. To determine the effect of celecoxib on gene expression and tumor number, beginning at the age of 5 weeks, C57BL/6J-*Apc^{Min}* mice were fed the semi-purified AIN-93G diet containing 150 or 500 ppm celecoxib.

Odc and Ssat expression: *Odc* and *Ssat* expression in small intestinal tissue of *Apc$^{Min/+}$* mice was measured after 60 days of treatment via Northern blot analysis. After termination, the intestinal ileum was removed, opened, and flushed with ice-cold saline. The 1 cm tumor-free portions of the ileum were placed in TRIzol® reagent (Gibco, BLR) homogenized using a Polytron homogenizer, and processed for the isolation of total cellular RNA [46]. Twenty μg of total RNA was separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Hybon-N, Amersham). The cDNA probes for Northern blotting were prepared using [^{32}P]-dCTP and a random priming technique (Roche Molecular Biochemicals, Indianapolis, IN). The probes were

purified with G-50 Sepharose columns (Roche Molecular Biochemicals, Indianapolis, IN) and quantitated using a scintillation counter. Membranes were hybridized with ^{32}P-labeled cDNA encoding for mouse *Odc* (1.38 kb EcoRI-EcoRI fragment) and human *Ssat* (0.67 kb EcoRI-EcoRI fragment). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (0.75 kb PstI-XbaI fragment) was used to control for variations in RNA loading. Northern blot autoradiograms were quantitated by densitometric analysis (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

Polyamine Analysis: We measured polyamine content in the intestinal tissue of animals treated with 500ppm celecoxib, as this represents a valuable endpoint of drug effect on the polyamine pathway. The 1 cm tumor-free portions of the ileum were collected, flushed with ice-cold saline and stored frozen at -80^{o} C. Samples were processed and assayed for polyamine (putrescine, spermidine, spermine and N^{1}-acetylspermine) content by reverse-phase high performance liquid chromatography with 1,7-diaminoheptane as an internal standard (26). Protein content in each sample was determined by the BCA protein assay kit (Pierce, Rockford, IL). Data are expressed as nmol polyamine per mg protein.

Combined effects of DFMO and celecoxib on tumorigenesis in Apc$^{Min/+}$ mice. To assess the combined effect of celecoxib and DFMO on intestinal tumorigenesis, DFMO was administered alone, or in combination with celecoxib (500 ppm in the diet) at two different doses of 0.5% and 2% in the drinking water. The independent effects of celecoxib (500 ppm in the diet) on intestinal tumorigenesis were also tested. After termination, whole small intestine and colon segments were removed, flushed with buffered saline, opened longitudinally and spread out with the mucosal surface up. Tissues were fixed in 10% neutral buffered formalin for 24 hours and then placed in 70% ethanol. Tumor number per mouse was evaluated using a LSGA epi-illuminator

dissecting microscope (Olympus Optical Co., Ltd, Tokyo, Japan) at 20x magnification. Tumors larger than 0.5 millimeter were counted and their diameter was measured. All assessments were done by the same observer who was blinded to the mouse treatment group. Animal weight at the time of sacrifice was recorded to determine if they absorbed similar amounts of food and water after the aforementioned treatments.

Epidemiologic CRC gene-environment study.

Study population: For the present study, all incident colorectal cancer cases diagnosed between 1994 and 1996 were obtained from a previously described study investigating gene-environment interactions in colorectal cancer (27). Family history of cancer was ascertained via telephone interview. Familial (non-HNPCC non-FAP) CRC cases were identified as those having at least one first degree relative with CRC. Amsterdam criteria were used to define HNPCC families(28). HNPCC and FAP cases, familial cases, and sporadic CRC cases were identified using available family history data. 1256 cases were consented for the study during the defined period, including 1007 sporadic CRC cases, 228 familial CRC cases, and 21 HNPCC cases, as described elsewhere(29). All HNPCC cases were subsequently excluded from the analysis. 511 of the sporadic and familial CRC cases completed a food frequency questionnaire (FFQ).

Clinical and demographic data: Clinical and demographic data from consented cases were obtained from the Cancer Surveillance Programs of Orange County, Imperial County, and San Diego County, California (CSPOC/SANDIOCC databases)(27). Recorded data included demographic information (age, gender, ethnicity), histology, tumor site, tumor grade, stage at presentation, survival status, and treatment with surgery, radiation therapy, or chemotherapy. These data were abstracted from medical and laboratory records by trained tumor registrars according to *Cancer Reporting in*

California: Vol. 1, Abstracting and Coding Procedures for Hospitals(30). Tumor site and histology were coded according to criteria specified by the World Health Organization in *International Classification of Diseases for Oncology* (ICD-O)(31). Primary site code was searched using the SEER site code for colon (21041, 21043-21048) and rectum (21051-21052). Appendiceal cancers were excluded. Histology codes included invasive cases of: adenocarcinoma (8140, 8144, 8210, 8260-8263, 8380, 8490), mucinous adenocarcinoma (8470, 8480, 8481), carcinoma (8010, 8020, 8070, 8071, 8124, 8240), and not otherwise specified (8041, 8042, 8120, 8130, 8243, 8246, 8560, 8570, 8722). Staging was grouped into three broad categories that could be classified from clinical and pathologic records, and defined according to SEER summary staging as localized disease, regional disease, and remote disease (localized or regional disease with distant spread). Hospital registrars contacted cases annually, and CSPOC/SANDIOCC staff annually reviewed state death certificates to identify deceased registry cases. Follow-up data through December 2003 were available for analysis. The last date of follow-up was either the date of death or the last date the individual was contacted.

Meat and other food consumption: Food consumption was self-reported via a validated 100-item NCI-Block food-frequency questionnaire (FFQ), where patients were asked to report their usual eating habits during the one year prior to diagnosis of CRC(32;33). Micronutrient data (i.e., calcium, iron), total daily fiber intake, total daily energy intake and body mass index (BMI) were calculated from the self-reported FFQ responses. The number of medium-sized servings consumed per week for each meat item was calculated by multiplying the number of servings consumed per week by the estimated serving size (i.e. 0.5 for small, 1.0 for medium, and 1.5 for large). Meat consumption quartiles were subsequently calculated. Cases in the highest quartile of meat consumption (Q4) were compared to those in all of the other meat consumption quartiles

(Q1-Q3). Arginine intake for each FFQ food item was calculated by estimating the quantity of arginine per serving from widely available computer-based food composition tables(4), and multiplying this value by the consumption frequency and serving size for each food item. As an internal control measure, manual chart review was conducted on all cases reporting total daily energy intake of less than 500 kcal (n = 15) or greater than 5000 kcal (n = 6), to ensure valid FFQ reporting, including one patient reporting extremely high meat intake (61.5 medium-size serving equivalents per week). All such cases were determined to contain complete and valid data, and were included in the analysis.

Statistical analysis.

Animal experiments: A non-parametric test (Wilcoxon rank sum or Mann-Whitney) was performed for the analysis of *Odc* and *Ssat* gene expression. For the difference in the number of tumors between control and treated mice, the Poisson regression analysis was used. Logistic regression was used to analyze the difference in low and high grade adenoma incidence.

Epidemiologic studies: Comparisons of demographic, clinical, and pathologic variables between cases were performed using Pearson chi-square statistic or Fisher's exact test for nominal variables and Student *t*- test for continuous variables. For the analysis of continuous variables by meat consumption quartile, a non-parametric one-way analysis of variance (ANOVA) using Wilcoxan (Kruskal-Wallis) test was performed. Linear regression analysis was used to assess dependent relationships between continuous variables. Univariate survival rate analyses were estimated using the Kaplan and Meier method, with comparisons made between groups by the log rank test. Cox proportional hazards modeling using time since diagnosis were performed. Each variable in the

model was coded using dummy variables. All statistical analyses were conducted using SAS 9.1 statistical software (SAS Institute, Cary, NC). Statistical significance was assumed for a two-tailed *P* value less than 0.05.

Ethical considerations. Cases signed a consent form allowing for release of medical information, including pathology reports, tissue blocks, and linkage to the aforementioned Cancer Surveillance Programs. This study has been approved by the University of California, Irvine Institutional Review Board (No. 1993-257).

Results

Mouse experimental studies – the role of arginine and its catabolic product, polyamines, in intestinal tumorigenesis

Supplemental dietary arginine effect on incidence of high-grade adenomas in $Apc^{Min/+}$ $Nos2^{+/+}$ mice.

We examined the effect of dietary arginine on colonic tumor incidence in mice. $Apc^{Min/+}$ $Nos2^{+/+}$ mice or $Apc^{Min/+}$ *Nos2* knockout mice were fed an AIN-93G diet supplemented with dietary arginine in the drinking water (0, 0.02% and 0.2%). Supplementing the diet with arginine did not affect animal weight. All mice weighed 20-23 grams, i.e. they absorbed similar amounts of food and water in the different treatment groups. $Apc^{Min/+}$ mice expressing *Nos2* showed an arginine-dependent increase in high-grade adenoma incidence in the colon (Fig. 1). Supplementing the diet with 0.2% arginine resulted in a more than 2-fold increase in tumor incidence ($P = 0.042$). In contrast, low-grade adenoma incidence in mice expressing *Nos2* was not affected by dietary arginine. In *Nos2* knockout mice, adenoma incidence was not affected by arginine; therefore, the increase in colon tumor grade with dietary arginine was *Nos2*-dependent.

Celecoxib effect on polyamine metabolic enzymes in intestinal tissue of Apc$^{Min/+}$ mice.

We tested whether celecoxib affects polyamine metabolism in the intestinal tract of Min mice. After 60 days of treatment, celecoxib suppresses the steady state RNA levels of *Odc*, and induces steady state RNA levels of *Ssat* in a dose dependent manner (Figs. 2A and 2B). Celecoxib effects on *Odc* and *Ssat* expression were observed in the small intestine but not in the colon, and had statistically significant values for *Odc* at both 150 and 500 ppm, concentrations ($P < 0.001$) and for *Ssat* at the concentration of 500 ppm ($P = 0.002$). We found a statistically significant decrease in the concentration of putrescine, the product of ODC activity, in the small intestine of mice treated with 500 ppm of celecoxib ($P = 0.04$) (Table I). We found also a marginal decrease in tissue levels of N1-acetylspermidine (the product of the SSAT enzyme activity, $P = 0.07$). This decrease was small and not statistically significant possibly due to luminal secretion of acetylated spermidine.

Combination of DFMO and celecoxib: effect on tumorigenesis in Apc$^{Min/+}$ mice.

DFMO at a concentration of 0.5% did not affect tumor number in *Apc$^{Min/+}$* mice fed with a defined AIN93G diet (Fig. 2C). A higher dose of DFMO (2%) reduced the tumor number per mouse by about 50%. We did not observe a significant tumor location dependency with the DFMO treatment (data not shown). Celecoxib (500 ppm) reduced tumor number by about 50% with little location dependency ($P = 0.0003$). The combination of 0.5% DFMO and 500 ppm of celecoxib reduced tumor number by over 80% ($P < 0.0001$) and was more effective in suppressing distal intestinal tumor number, compared to proximal intestinal tumor number (data not shown). The combination of a higher dose of DFMO with celecoxib did not improve the efficacy of these agents to suppress tumorigenesis ($P < 0.0001$). We found a statistically significant increase in body weights

of mice treated with celecoxib ($P = 0.02$, data not shown). Mice treated with 2% DFMO alone or in combination with celecoxib had a statistically significant decrease in body weights compared with control animals ($P = 0.005$ and $P = 0.04$, respectively). No effect on body weight was found in mice treated with 0.5% DFMO in combination with celecoxib implying that the water consumption and the caloric intake of the treatment mice was similar to these of the control mice.

Epidemiologic Studies – effect of meat and arginine intake on clinical outcomes

CRC study population.

The 511 incident CRC cases identified included 367 sporadic CRC cases and 144 familial CRC cases. Demographic comparisons for these groups (Table II) reveal significant baseline differences. Familial patients were older at presentation, and had a greater proportion of localized tumors and tumors in the proximal colon. While the proportion of cases treated surgically or with radiation was similar for familial and sporadic CRC cases, fewer familial CRC cases received chemotherapy. However, this may be related to the (expected) finding that chemotherapy treatment was strongly associated with increased stage at presentation (i.e. recorded use of chemotherapy was 16%, 68%, and 81% among local, regional, and remote stage cases, respectively; $P < 0.0001$). Types of meat, and corresponding medium serving sizes as listed in the FFQ are depicted in Table III, along with estimated arginine contents. Due to the baseline differences between sporadic and familial CRC cases, meat consumption quartiles were constructed separately for each group: familial CRC cases: Q1 (0 to 4.3 servings per week), Q2 (4.4 to 7.0 servings per week), Q3 (7.1 to 10.7 servings per week), Q4 (10.8 to 27.4 servings per week); sporadic CRC cases: Q1 (0 to 5.3 servings per week), Q2 (5.4 to 8.2 servings per week), Q3 (8.3 to 12.5 servings per week), Q4 (12.6 to 61.5 servings per week). Comparisons for each of the investigated variables across all four

meat consumption quartiles are presented in Table IV, for familial and sporadic CRC patients.

Relationship between meat consumption and total daily arginine intake

The relationship between meat consumption and total daily arginine intake was investigated analytically. For the overall group (n=511), mean arginine intake from all foods was estimated at 23.2 grams/week (i.e. 3.3 grams/day). The median meat-derived arginine intake was estimated at 11.7 grams/week (1.7 grams/day). Forty-nine percent of total daily arginine intake in this study was due to meat consumption. Meat consumption was revealed to be a predictor of total daily arginine intake in linear regression analysis (R-square = 0.77, P<0.0001) (Fig. 3).

Effects of meat intake on clinical outcomes.

For familial cases, no differences in tumor grade (*P* = 0.32), or stage at presentation (*P* = 0.48) were observed based on meat consumption quartile. Similarly, among sporadic CRC cases, no differences in tumor grade (*P* = 0.75), or stage at presentation (*P* = 0.18) were noted based on meat consumption quartile (Table IV). Among familial CRC cases, overall survival (OS) was significantly decreased for cases in the highest meat consumption quartile (n = 36) vs. cases in all other meat consumption quartiles (n = 108) (quartile 4: 10-year OS = 42%; Q1-Q3: 10-year OS = 65%; *P* = 0.017) (Fig. 4). No statistically significant differences in OS were detected for cases in meat consumption quartiles Q1-Q3 (*P* = 0.34). Subset analysis revealed that among the 101 colon cancer cases, borderline significant decreased OS was observed for cases in the highest meat consumption quartile (n = 74) vs. cases in all other meat consumption quartiles (n = 27) (Q4: 10-year OS = 44%; Q1-Q3: 10-year OS = 70%; *P* = 0.052). No statistically significant differences were noted for the 43 familial rectal cancer cases based on meat

consumption group (Q4: 10-year OS = 33%; Q1-Q3: 10-year OS = 60%; *P* = 0.19), however these subset analyses were underpowered as there were only 9 rectal cancer patients in meat consumption Q4. Among sporadic CRC cases, OS was similar for cases in the highest meat consumption quartile (n = 91) compared to cases in all other meat consumption quartiles (n = 276) (Q4: 10-year OS = 62%; Q1-Q3: 10-year OS = 62%; *P* = 0.89.

Variables known to predict survival in CRC were included into the multivariate survival model, including age, gender, and stage at presentation. The following variables were highly correlated with meat consumption quartile but were not significantly associated with survival: daily fiber intake, daily calcium intake, daily iron intake, and total daily energy intake - thus they were not included in the final multivariate analysis. For familial CRC cases, the highest meat consumption quartile was associated with an increased hazard ratio (HR) of 2.24, 95% C.I. 1.25-4.03, (*P* = 0.007) compared to familial CRC cases in all other meat consumption quartiles after adjustment for age, gender, and stage at presentation (Table V). The trend test for meat consumption quartile among familial CRC cases was not statistically significant (HR = 1.08, 95% CI 0.84-1.39; *P* = 0.57). For sporadic CRC cases, no statistically significant increased hazard was noted for the highest quartile of meat consumption group (HR = 1.01, 95% CI 0.67-1.51; *P* = 0.98) compared to sporadic CRC cases in all other meat consumption quartiles after adjustment for age, gender, and stage at presentation (Table V).

Discussion

The results presented in this study demonstrate that meat and its major component, arginine, increase tumor grade in mice and are associated with adverse outcomes among familial CRC cases. Among familial CRC cases, those with the highest quartile

of meat consumption prior to diagnosis (i.e. 10.8 to 27.4 servings per week) had significantly decreased survival compared to cases in all other meat consumption quartiles in univariate, and adjusted analyses. The different pathways involved in arginine metabolism are presented in Fig. 5. As depicted, arginine can be converted directly to polyamines by intestinal bacteria(6), but this route is not essential, as the hepatic and extra-hepatic arginases catabolize arginine to ornithine, which is itself converted by ODC to form polyamines. The arginase enzymes compete with NOS, which converts arginine to nitric oxide and citrulline.

Arginine supplementation increased high grade intestinal tumor incidence in *Apc*$^{Min/+}$ *Nos2*$^{+/+}$ mice, but not in *Nos2* knockout mice. Thus, *Nos2* is involved in the arginine-dependent increase in colon adenoma grade. In accordance, a separate study from our laboratory described dietary arginine-induced increases in overall colon tumor incidence and number that were *Nos2*-dependent(15). In that study, dietary arginine was found to primarily affect colonic tumor initiation in *Apc*$^{Min/+}$ mice. Similarly, our present findings suggest that supplemental arginine-induced tumorigenesis is mediated through NO synthase. Assessment of polymorphisms in the NOS genes and promoters could potentially help explain our epidemiologic findings, which implicate gene-environment interactions between meat intake and improved survival for familial CRC cases but not sporadic CRC cases. Currently, *Nos2* single nucleotide gene polymorphisms with functional promoter activity have been described (34-36), but have not been reported in CRC patients.

As shown in Table III, fish, poultry, and pork contain comparable amounts of arginine to red meat. We have demonstrated in this study that meat consumption represents a reasonable estimator of total arginine intake (Fig. 3). Others have shown that arginine estimates from nutrient composition tables and short-term food intake records correlate with serum arginine levels(6). Even so, the poor outcomes associated

with the highest quartile of meat consumption noted in our study may reflect the effects meat constituents other than arginine, or different cooking methods that could not be accounted for in this analysis. Heterocyclic amines (HCAs) formed in meat during cooking(37-40), polycyclic aromatic hydrocarbons (PAHs)(41), and N-nitroso compounds (NOCs, i.e. from processed meat)(42) are known carcinogens that have been implicated in colorectal carcinogenesis, and could account for the observed survival effects of the present study. Red meat intake, and also intake of heme iron has been associated with high NOC levels, thus implicating a role for iron in carcinogenesis(43). The FFQ utilized in our study is not adequately designed to assess cooking methods or level of doneness, thus we were unable to assess the effects of HCAs, PAHs, or NOCs. However a FFQ model has recently been developed by others to assess intake of these carcinogenic compounds, and future outcomes-based studies using this type of model may reveal the effect of these compounds on survival in CRC(44). Genotypic differences in *UDP-glucuronosyltransferase-1A7* have been reported to modify the risk of colon cancer to dietary carcinogen exposures(45), and single nucleotide polymorphisms in *N-acetyltransferase-2* have been reported to modify the metabolic activation of heterocyclic amine carcinogens(46). Therefore the observed survival differences based on reported meat consumption among familial CRC cases may be related to different distributions of such genes involving heterocyclic amine metabolism.

Researchers form our group have shown that arginine increases intestinal polyamine levels in $Apc^{Min/+}$ mice, which can be inhibited by treatment with DFMO(47). Additionally, DFMO inhibits the arginine-induced increases in colonic tumor burden and tumor grade in $Apc^{Min/+}$ mice. DFMO has been shown previously in clinical trials to decrease colorectal mucosa polyamine content in humans(20), and such findings serve as the basis for ongoing colorectal cancer clinical trials using DFMO as a chemopreventive agent (48). Our experimental data revealed that celecoxib induced

expression of *Ssat*, lowered *Odc* levels, and suppressed putrescine levels in the intestines of *Apc*$^{Min/+}$ mice. Additionally, celecoxib significantly decreased the intestinal tumor number in *Apc*$^{Min/+}$ mice - an effect augmented by DFMO. *Ssat* induction by celecoxib promotes polyamine catabolism, and *Odc*-repression by celecoxib and DFMO leads to decreases in polyamine synthesis, as depicted in Fig. 5, with a resultant decreased tumor incidence. An important finding in this study is that the celecoxib- and DFMO- inhibitory effects on tumorigenesis in *Apc*$^{Min/+}$ mice operate in an additive manner. This reflects the inhibitory effects of DFMO and celecoxib on separate pathways involved in polyamine synthesis. This finding correlates well with reports on NSAIDs and DFMO in experimental models of tumorigenesis, and with the design of phase II chemoprevention clinical trials incorporating the use of NSAIDs and DFMO for CRC prevention(22;48).

The exact mechanisms for celecoxib-induced transcriptional activation of *Ssat* are unknown. However, transcription of *Ssat* has been demonstrated to occur by COX-dependent and COX-independent mechanisms(22;23). This may involve activation via peroxisome-proliferator-activated receptor-γ (PPARγ), decreased polyamine levels, and apoptosis in colon cancer cells(22). Alternatively, celecoxib treatment may result in activation of NFκB signaling and subsequent binding to *Ssat* promoter sites(50).

Our findings implicate gene-environment interactions between meat consumption, and presently unknown genotypes among affected individuals with multifactorial inherited susceptibility to CRC. Our *in vivo* results showing the additive benefits of DFMO and celecoxib in mouse intestinal tumorigenesis models combined with epidemiologic data demonstrating poor survival for familial CRC cases that were the highest consumers of meat, support further molecular epidemiologic investigations into genotypic analysis of CRC cases. Our *in vivo* results showing the additive benefits of

DFMO and celecoxib combined with epidemiologic data demonstrating poor survival for familial CRC cases that were the highest consumers of meat support further molecular epidemiologic investigations into genotypic analysis of CRC cases. Furthermore, these findings add to the growing support for development of therapeutic colorectal cancer clinical trials involving arginine depletion via dietary modification, NOS2 inhibition using targeted molecular therapies, and multi-targeted polyamine inhibition in select patient populations.

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Figure and Table Legends

Table I. Polyamine content in the small intestine of $Apc^{Min/+}$ mice treated with 500 ppm celecoxib. *, *P* = 0.04 indicates statistically significant difference vs. control group.

Table II. Descriptive comparisons for familial and sporadic CRC cases. *indicates that some observations had missing data for this variable.

Table III. Types of meat queried in the food frequency questionnaire with medium serving size descriptor. Estimated arginine contents are derived as described in "Materials and Methods".

Table IV. Descriptive variables for familial and sporadic CRC cases by meat consumption quartile, Q1 (lowest) - Q4 (highest). *indicates that some observations had missing data for this variable.

Table V. Adjusted survival analysis using Cox proportional hazards model. HR = hazards ratio.

Figure 1. Dietary arginine effect on colon tumor grade in $Apc^{Min/+}$ mice. Dietary arginine was added to the drinking water in different concentrations; no arginine added (□, n = 22 for $Apc^{Min/+}$ $Nos2^{-/-}$ mice and n = 39 for $Apc^{Min/+}$ $Nos2^{+/+}$ mice), 0.02% arginine (▩, n = 14 for $Apc^{Min/+}$ $Nos2^{-/-}$ mice and n = 11 for $Apc^{Min/+}$ $Nos2^{+/+}$ mice), 0.2% arginine (■,, n = 11 for $Apc^{Min/+}$ $Nos2^{-/-}$ mice and n = 12 for $Apc^{Min/+}$ $Nos2^{+/+}$ mice). The incidence of colon tumor was measured as described in "Materials and Methods". *, *P*=0.042, statistical significance relative to $Apc^{Min/+}$ $Nos2^{+/+}$ mice not supplemented with arginine.

Figure 2. Celecoxib effects on polyamine metabolic enzymes, *Odc* and *Ssat*, and on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. **A** - *Odc* expression in small intestine of control animals (n=10) and animals treated with 150 ppm (n=5) or 500 ppm celecoxib (n=5). Intestinal tissues were collected and processed for total RNA isolation and Northern blotting as described in "Materials and Methods". Steady state *Odc* levels in small intestinal tissue of *Apc*$^{Min/+}$ mice were normalized to the level of expression of the GAPDH gene in the same sample. *, $P<0.001$ statistical significance relative to the control group. **B** – *Ssat* expression in small intestine of control animals (n=10) and animals treated with 150 ppm (n=5) or 500 ppm of celecoxib (n=5). Intestinal tissues were collected and processed for total RNA isolation and Northern blotting as described in "Materials and Methods". Steady state *Ssat* levels in small intestinal tissue of *Apc*$^{Min/+}$ mice were normalized to the level of expression of the GAPDH gene in the same sample. *, $P=0.002$, statistical significance relative to the control group. **C** - Effect of DFMO and celecoxib on intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse model. Mice were treated with DFMO (0.5% or 2%), or with 500 ppm celecoxib alone, or in combination with DFMO (0.5% or 2%) (n=10 for all groups except for the 2% DFMO group, n=8). Tumor number per mouse in small intestine was counted as described in "Materials and Methods". *P* values between control and different treatment groups are presented in the figure.

Figure 3. Regression analysis plot depicting the dependent relationship of total dietary arginine intake on meat consumption quantity (n = 511; R-square = 0.77).

Figure 4. Overall survival (OS) for familial CRC cases, as determined by meat consumption quartile. Solid line, lowest meat consumption quartiles Q1-3 (0 to 10.7

servings per week; n = 108; 10-year OS = 65%); dashed line, highest meat consumption quartile (10.8 to 27.4 servings per week; n = 36; 10-year OS = 42%).

Figure 5. Arginine synthesis, metabolism and catabolism. Alternate pathways, and inhibitors of these pathways are indicated. NOS, nitric oxide synthase; DFMO, difluoromethylornithine; ODC, ornithine decarboxylase; NSAIDs, non-steroidal anti-inflammatory drugs; SSAT, spermidine/spermine N^1-acetyltransferase; OAT, ornithine aminotransferase; HCAs, Heterocyclic amines; PAHs, polycyclic aromatic hydrocarbons; NOCs, N-nitroso compounds.

Reference List

(1) Norat T, Lukanova A, Ferrari P, Riboli E. Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. Int J Cancer 2002; 98(2):241-256.

(2) Chao A, Thun MJ, Connell CJ, McCullough ML, Jacobs EJ, Flanders WD et al. Meat consumption and risk of colorectal cancer. JAMA 2005; 293(2):172-182.

(3) Giovannucci E, Willett WC. Dietary factors and risk of colon cancer. Ann Med 1994; 26(6):443-452.

(4) U.S. Department of Agriculture, Agricultural Research Service. USDA National Nutrient Database for Standard Reference, Release 18. Nutrient Data Laboratory Home Page, http://www nal usda gov/fnic/foodcomp 2005.

(5) Oomen CM, van Erk MJ, Feskens EJM, Kok FJ, Kromhout D. Arginine intake and risk of coronary heart disease mortality in elderly men. Arteriosclerosis Thrombosis and Vascular Biology 2000; 20(9):2134-2139.

(6) Venho B, Voutilainen S, Valkonen VP, Virtanen J, Lakka TA, Rissanen TH et al. Arginine intake, blood pressure, and the incidence of acute coronary events in men: the Kuopio Ischaemic Heart Disease Risk Factor Study. American Journal of Clinical Nutrition 2002; 76(2):359-364.

(7) Scott L, Lamb J, Smith S, Wheatley DN. Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. British Journal of Cancer 2000; 83(6):800-810.

(8) Yeatman TJ, Risley GL, Brunson ME. Depletion of Dietary Arginine Inhibits Growth of Metastatic Tumor. Archives of Surgery 1991; 126(11):1376-1382.

(9) Szende B, Tyihak E, Trezl L. Role of arginine and its methylated derivatives in cancer biology and treatment. Cancer Cell Int 2001 Dec 17;1(1):3 2001; 1:3.

(10) Park KG, Heys SD, Blessing K, Kelly P, McNurlan MA, Eremin O et al. Stimulation of human breast cancers by dietary L-arginine. Clin Sci (Lond) 1992; 82:413-417.

(11) Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM et al. Frequent nitric oxide synthase-2 expression in human colon adenomas: Implication for tumor angiogenesis and colon cancer progression. Cancer Research 1998; 58(2):334-341.

(12) Escribano M, Molero L, Lopez-Farre A, Abarrategui C, Carrasco C, Garcia-Mendez A et al. Aspirin inhibits endothelial nitric oxide synthase (eNOS) and Flk-1 (vascular endothelial growth factor receptor-2) prior to rat colon tumour development. Clinical Science 2004; 106(1):83-91.

(13) Takahashi M, Fukuda K, Ohata T, Sugimura T, Wakabayashi K. Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat

colon tumors induced by azoxymethane. Cancer Research 1997; 57(7):1233-1237.

(14) Ahn B, Ohshima H. Suppression of intestinal polyposis in Apc(Min/+) mice by inhibiting nitric oxide production. Cancer Research 2001; 61(23):8357-8360.

(15) Yerushalmi HF, Besselsen DG, Ignatenko NA, Blohm-Mangone KA, Padilla-Torres JL, Stringer DE et al. The role of NO synthases in arginine-dependent small intestinal and colonic carcinogenesis. Mol Carcinog 2005; 45:93-105.

(16) Wu GY, Morris SM. Arginine metabolism: nitric oxide and beyond. Biochemical Journal 1998; 336:1-17.

(17) Thomas T, Thomas TJ. Polyamine metabolism and cancer. J Cell Mol Med 2003; 7(2):113-126.

(18) Fultz KE, Gerner EW. APC-dependent regulation of ornithine decarboxylase in human colon tumor cells. Mol Carcinog 2002; 34(1):10-18.

(19) Erdman SH, Ignatenko NA, Powell MB, Blohm-Mangone KA, Holubec H, Guillen-Rodriguez JM et al. APC-dependent changes in expression of genes influencing polyamine metabolism, and consequences for gastrointestinal carcinogenesis, in the Min mouse. Carcinogenesis 1999; 20(9):1709-1713.

(20) Meyskens FL, Emerson SS, Pelot D, Meshkinpour H, Shassetz LR, Einspahr J et al. Dose De-Escalation Chemoprevention Trial of Alpha-Difluoromethylornithine

in Patients with Colon Polyps. Journal of the National Cancer Institute 1994; 86(15):1122-1130.

(21) Reddy BS. Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. Environmental and Molecular Mutagenesis 2004; 44(1):26-35.

(22) Babbar N, Ignatenko NA, Casero RA, Jr., Gerner EW. Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. J Biol Chem 2003; 278(48):47762-47775.

(23) Martinez ME, O'Brien TG, Fultz KE, Babbar N, Yerushalmi H, Qu N et al. Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. Proceedings of the National Academy of Sciences of the United States of America 2003; 100(13):7859-7864.

(24) Hawk CT, Leary SL, Morris TH. Formula for Laboratory Animals, 3rd Ed. Ames, IA: Blackwell Publishing, 2005.

(25) Boivin GP, Washington K, Yang K, Ward JM, Pretlow TP, Russell R et al. Pathology of mouse models of intestinal cancer: Consensus report and recommendations. Gastroenterology 2003; 124(3):762-777.

(26) Seiler N, Knodgen B. High-performance liquid chromatographic procedure for the simultaneous determination of the natural polyamines and their monoacetyl derivatives. J Chromatogr 1980; 221(2):227-235.

(27) Peel DJ, Ziogas A, Fox EA, Gildea M, Laham B, Clements E et al. Characterization of hereditary nonpolyposis colorectal cancer families from a population-based series of cases. J Natl Cancer Inst 2000; 92(18):1517-1522.

(28) Vasen HFA, Mecklin JP, Khan PM, Lynch HT. The International-Collaborative-Group-On-Hereditary-Non-Polyposis-Colorectal-Cancer (Icg-Hnpcc). Diseases of the Colon & Rectum 1991; 34(5):424-425.

(29) Zell JA, McEligot A, Ziogas A, Nguyen AV, Anton-Culver H, Holcombe RF. Wine consumption prior to diagnosis improves survival in familial colorectal cancer patients. AACR Colorectal Cancer: Molecular Pathways and Therapies - Conference Proceedings 2005; Abstract B21.

(30) Cancer Reporting in California.Vol.1. Abstracting and coding procedures for hospitals. Emeryville, CA. California Tumor Registry, Department of Health Services, Cancer Surveillance Section 1986.

(31) Fritz A, Percy C, Jack A, Shanmugaratnam K, Sobin L, Parkin DM et al. International Classification of Diseases for Oncology. 3rd ed. Geneva: World Health Organization, 2000.

(32) Block G, Hartman AM, Dresser CM, Carroll MD, Gannon J, Gardner L. A Data-Based Approach to Diet Questionnaire Design and Testing. American Journal of Epidemiology 1986; 124(3):453-469.

(33) Block G, Woods M, Potosky A, Clifford C. Validation of A Self-Administered Diet History Questionnaire Using Multiple Diet Records. Journal of Clinical Epidemiology 1990; 43(12):1327-1335.

(34) Coia V, Juliger S, Mordmuller B, Kreidenweis A, Stroh AL, Ortega C et al. Analysis of polymorphic sites in the promoter of the nitric oxide synthase 2 gene. Biochemical and Biophysical Research Communications 2005; 335(4):1123-1131.

(35) Hobbs MR, Udhayakumar V, Levesque MC, Booth J, Roberts JM, Tkachuk AN et al. A new NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children. Lancet 2002; 360(9344):1468-1475.

(36) Kun JF, Mordmuller B, Perkins DJ, May J, Mercereau-Puijalon O, Alpers M et al. Nitric oxide synthase 2 (Lambarene) (G-954C), increased nitric oxide production, and protection against malaria. Journal of Infectious Diseases 2001; 184(3):330-336.

(37) Keating GA, Bogen KT. Methods for estimating heterocyclic amine concentrations in cooked meats in the US diet. Food and Chemical Toxicology 2001; 39(1):29-43.

(38) Sinha R, Rothman N, Salmon CP, Knize MG, Brown ED, Swanson CA et al. Heterocyclic amine content in beef cooked by different methods to varying

degrees of doneness and gravy made from meat drippings. Food and Chemical Toxicology 1998; 36(4):279-287.

(39) Sinha R, Knize MG, Salmon CP, Brown ED, Rhodes D, Felton JS et al. Heterocyclic amine content of pork products cooked by different methods and to varying degrees of doneness. Food and Chemical Toxicology 1998; 36(4):289-297.

(40) Murtaugh MA, Ma KN, Sweeney C, Caan BJ, Slattery ML. Meat consumption patterns and preparation, genetic variants of metabolic enzymes, and their association with rectal cancer in men and women. Journal of Nutrition 2004; 134(4):776-784.

(41) Kazerouni N, Sinha R, Hsu CH, Greenberg A, Rothman N. Analysis of 200 food items for benzo[a]pyrene and estimation of its intake in an epidemiologic study. Food and Chemical Toxicology 2001; 39(5):423-436.

(42) Knekt P, Jarvinen R, Dich J, Hakulinen T. Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: a follow-up study. Int J Cancer 1999; 80(6):852-856.

(43) Cross AJ, Pollock JRA, Bingham SA. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. Cancer Research 2003; 63(10):2358-2360.

(44) Sinha R, Cross A, Curtin J, Zimmerman T, McNutt S, Risch A et al. Development of a food frequency questionnaire module and databases for compounds in cooked and processed meats. Molecular Nutrition & Food Research 2005; 49(7):648-655.

(45) Butler LM, Duguay Y, Millikan RC, Sinha R, Gagne JF, Sandler RS et al. Joint effects between UDP-glucuronosyltransferase 1A7 genotype and dietary carcinogen exposure on risk of colon cancer. Cancer Epidemiology Biomarkers & Prevention 2005; 14(7):1626-1632.

(46) Hein DW, Fretland AJ, Doll MA. Effects of single nucleotide polymorphisms in human N-acetyltransferase 2 on metabolic activation (O-acetylation) of heterocyclic amine carcinogens. Int J Cancer 2006; 119(5):1208-1211.

(47) Yerushalmi HF, Besselsen DG, Ignatenko NA, Blohm-Mangone KA, Padilla-Torres JL, Stringer DE et al. Role of polyamines in arginine-dependent intestinal and colonic carcinogenesis in *Apc*$^{min/+\ \text{mice}}$. Mol Carcinog 2006; [Epub ahead of print] PMID: 16705737.

(48) Gerner EW, Meyskens FL. Polyamines and cancer: Old molecules, new understanding. Nature Reviews Cancer 2004; 4(10):781-792.

(49) Ignatenko NA, Babbar N, Mehta D, Casero RA, Gerner EW. Suppression of polyamine catabolism by activated Ki-ras in human colon cancer cells. Molecular Carcinogenesis 2004; 39(2):91-102.

(50) Babbar N, Gerner EW, Casero RA, Jr. Induction of Spermidine/Spermine N1-Acetyltransferase (SSAT) by Aspirin in Caco-2 Colon Cancer Cells. Biochem J 2006;394(Pt 1):317-324.

Table I.

	Polyamine content, nmol/mg protein (Mean+SD)			
	Putrescine	Spermidine	Spermine	N^{1}-Acetyl spermidine
Control	0.54±0.25	8.14±1.41	5.55±0.86	0.22±0.06
Celecoxib, 500 ppm	0.18±0.12*	6.58±1.12	6.98±1.16	0.14±0.03

Table II.

	Familial CRC (n=144)	Sporadic CRC (n=367)	*P*
Median Age (in years, with range)	64 (32-90)	57 (22-95)	<0.0001
Gender			
Male	72 (50%)	190 (52%)	0.72
Female	72 (50%)	177 (48%)	
Ethnicity			
Caucasian	130 (90%)	306 (83%)	0.16
African-American	1 (<1%)	2 (<1%)	
Hispanic	9 (6%)	31 (8%)	
Asian	3 (2%)	27 (7%)	
Other	1 (<1%)	1 (<1%)	
Stage at Presentation			
Local	71 (50%)	139 (38%)	0.049
Regional	53 (38%)	172 (48%)	
Remote	17 (12%)	51 (14%)	
Colon Site			
Proximal and Transverse	64 (44%)	106 (29%)	0.002
Descending	8 (6%)	14 (4%)	
Sigmoid	29 (20%)	104 (29%)	
Rectosigmoid	22 (15%)	46 (13%)	
Rectum	21 (15%)	87 (24%)	
Large Intestine-Other	0 (0%)	10 (3%)	
Histologic Subtype			
Adenocarcinoma	128 (89%)	336 (92%)	0.30
Mucinous Adenocarcinoma	11 (8%)	18 (5%)	
Carcinoma	5 (4%)	8 (2%)	
Not Otherwise Specified	0 (0%)	4 (1%)	
Tumor Grade			
Grade 1	25 (19%)	43 (13%)	0.36
Grade 2	87 (66%)	237 (71%)	
Grade 3	20 (15%)	55 (16%)	
Grade 4	0 (0%)	1 (<1%)	
First Course of Treatment			
Surgery	136/144 (94%)	339/366 (93%)	0.46
Radiation Therapy	16/144 (11%)	62/366 (17%)	0.10
Chemotherapy	50/137 (37%)	182/350 (52%)	0.002
Fiber Intake (mean grams/day)	11.7 +/- 0.5 SE	12.2 +/- 0.4 SE	0.41
Calcium Intake (mean mg/day)	707 +/- 53 SE	710 +/- 26 SE	0.96
Iron Intake (mean mg/day)	10.7 +/- 0.4 SE	12.0 +/- 0.3 SE	0.026
Energy Intake (mean kcal/day)	1477 +/- 70 SE	1695 +/- 49 SE	0.015
Body Mass Index (mean BMI, kg/meters^2)	26.0 +/- 0.4 SE	26.4 +/- 0.3 SE	0.42
Meat Consumption (mean number of medium servings per week)	8.1 +/- 0.5 SE	9.5 +/- 0.3 SE	0.022
Mean Weekly Arginine Intake (grams)	21.1 +/- 1.0 SE	24.0 +/- 0.7 SE	0.022
Mean Weekly Meat-derived Arginine Intake (grams)	10.1 +/- 0.6 SE	12.4 +/- 0.4 SE	0.006

Table III

Food Item	*Medium Serving Size*	**Estimated Arginine Content (per medium serving)*
Bacon	2 slices	0.40 g
Sausage	2 patties or links	0.34 g
Beef (includes steaks, roasts, beef sandwiches)	4 ounces	1.94 g
Beef stew or Pot pie	1 cup	0.68 g
Hamburger (includes cheeseburger, meatloaf, beef burritos, tacos)	1 medium sandwich or 4 ounces	1.01 g
Hot dogs	2 hot dogs	0.64 g
Liver (includes chicken liver)	4 ounces	2.28 g
Liverwurst	2 slices	0.45 g
Ham (includes bologna, salami, other lunch meats)	2 slices or 2 ounces	0.58 g
Pork (includes pork chops, roasts)	2 chops or 4 ounces	1.87 g
Chicken or Turkey (includes sandwiches, also roasted/stewed/broiled)	2 small or 1 large piece	1.85 g
Fried chicken	2 small or 1 large piece	1.39 g
Fried fish or fish sandwich	4 ounces or 1 sandwich	1.12 g
Tuna (includes tuna salad, tuna casserole)	½ cup	1.26 g
Oysters	5 pieces, ¼ cup, or 3 ounces	0.36 g
Shellfish (includes shrimp, crab, lobster, etc.)	5 pieces, ¼ cup, or 3 ounces	1.58 g
Other fish (includes broiled or baked fish)	2 pieces or 4 ounces	1.63 g

Table IV

	Familial CRC patients (n=144)				*P*	Sporadic CRC Patients (n=367)				*P*
	Meat Q1 (n=36)	Meat Q2 (n=36)	Meat Q3 (n=36)	Meat Q4 (n=36)		Meat Q1 (n=92)	Meat Q2 (n=92)	Meat Q3 (n=92)	Meat Q4 (n=91)	
Median Age (range)	68 years (40-90)	66 years (32-82)	63.5 years (37-87)	62 years (37-89)	0.08	59 years (22-95)	57 years (30-93)	58 years (31-77)	53 years (29-64))	0.0005
Gender										
Male	9 (25%)	20 (56%)	20 (56%)	23 (64%)	0.005	35 (38%)	40 (43%)	47 (51%)	68 (75%)	<0.0001
Female	27 (75%)	16 (44%)	16 (44%)	13 (36%)		57 (62%)	52 (57%)	45 (49%)	23 (25%)	
Ethnicity										
Caucasian	33 (92%)	34 (94%)	35 (97%)	28 (78%)	0.22	71 (77%)	80 (87%)	81 (88%)	74 (81%)	0.34
All others	3 (8%)	2 (6%)	1 (3%)	8 (22%)		21 (23%)	12 (13%)	11 (12%)	17 (19%)	
Stage										
Local	19 (54%)	18 (51%)	21 (58%)	13 (37%)	0.48	40 (44%)	40 (43%)	36 (40%)	23 (26%)	0.18
Regional	11 (31%)	14 (40%)	10 (28%)	18 (51%)		37 (41%)	41 (45%)	42 (46%)	52 (58%)	
Remote	5 (14%)	3 (9%)	5 (14%)	4 (11%)		13 (14%)	11 (12%)	13 (14%)	14 (16%)	
Colon Site										
Colon	24 (67%)	28 (78%)	22 (61%)	27 (75%)	0.39	55 (60%)	57 (62%)	57 (62%)	55 (60%)	0.99
Rectum	12 (33%)	8 (22%)	14 (39%)	9 (25%)		34 (37%)	32 (35%)	33 (36%)	34 (37%)	
NOS	-	-	-	-		3 (3%)	3 (3%)	2 (2%)	2 (2%)	
Histology*										
Adenoca	35 (97%)	32 (89%)	32 (89%)	29 (81%)	0.23	84 (91%)	85 (92%)	84 (92%)	83 (91%)	0.74
Other	1 (3%)	4 (11%)	4 (11%)	7 (19%)		8 (9%)	7 (8%)	7 (8%)	8 (9%)	
Tumor Grade*										
Grade 1	7 (21%)	4 (13%)	7 (21%)	7 (21%)	0.32	14 (16%)	11 (13%)	11 (13%)	7 (8%)	0.75
Grade 2	21 (64%)	25 (78%)	23 (70%)	18 (53%)		60 (70%)	61 (71%)	57 (70%)	59 (72%)	
Grade 3/4	5 (15%)	3 (9%)	3 (9%)	9 (26%)		12 (14%)	14 (16%)	14 (17%)	16 (20%)	
Treatment*										
Surgery	34 (94%)	35 (97%)	33 (92%)	34 (94%)	0.79	86 (94%)	88 (96%)	83 (91%)	82 (90%)	0.49
Radiation	2 (6%)	3 (8%)	6 (17%)	5 (14%)	0.42	11 (12%)	15 (16%)	17 (19%)	19 (21%)	0.42
Chemo	11 (34%)	11 (31%)	13 (37%)	15 (44%)	0.69	42 (49%	35 (38%)	52 (60%)	53 (61%)	0.007
Fiber (g/day)	9.0 +/- 0.9	9.5 +/- 0.7	12.3 +/- 0.9	15.9 +/-1.2	<0.0001	9.5 +/- 0.6	11.0 +/- 0.5	12.3 +/- 0.5	16.3 +/- 1.1	<0.0001
Calcium (mg/d)	448 +/- 48	542 +/- 53	867 +/- 86	972 +/- 169	<0.0001	524 +/- 45	660 +/- 42	724 +/- 52	934 +/- 62	<0.0001
Iron (mg/d)	6.8 +/- 0.6	8.8 +/- 0.6	11.9 +/- 0.6	15.1 +/- 0.9	<0.0001	8.1 +/- 0.4	9.8 +/- 0.4	12.1 +/- 0.4	18.1 +/- 0.9	<0.0001
Energy (kcal/d)	853 +/- 72	1161 +/- 83	1712 +/- 92	2183 +/- 174	<0.0001	1079 +/- 52	1365 +/- 45	1709 +/- 56	2634 +/- 123	<0.0001
BMI (kg/m^2)	25.4 +/- 0.8	27.0 +/- 0.8	26.6 +/- 0.9	24.9 +/- 0.7	0.31	25.8 +/- 0.5	25.6 +/- 0.6	26.3 +/- 0.5	27.9 +/- 0.7	0.046
Total arginine (g/wk)	10.7 +/- 0.9	15.3 +/- 0.9	23.5 +/- 0.9	34.8 +/- 2.1	<0.0001	12.6 +/- 0.7	19.3 +/- 0.5	25.5 +/- 0.7	38.6 +/- 1.5	<0.0001
Meat-derived arginine (g/wk)	3.2 +/- 0.3	7.1 +/- 0.3	11.0 +/- 0.3	19.2 +/- 0.9	<0.0001	4.0 +/- 0.2	9.1 +/- 0.2	13.7 +/- 0.3	22.8 +/- 1.0	<0.0001

Table V.

	Familial CRC (n=144; deaths=61)			Sporadic CRC patients (n=367; deaths=142).		
	HR	95% HR Confidence Limits	*P*	HR	95% HR Confidence Limits	*P*
Age (years)	1.05	(1.02-1.08)	0.0001	1.03	(1.01-1.05)	0.0007
Gender						
Female	1.00	-	-	1.00	-	-
Male	1.33	(0.78-2.27)	0.30	1.33	(0.94-1.89)	0.11
Stage						
Local	1.00	-	-	1.00	-	-
Regional	1.52	(0.83-2.78)	0.18	1.66	(1.09-2.55)	0.019
Remote	10.57	(5.18-21.60)	<0.0001	14.06	(8.75-22.58)	<0.0001
Meat Consumption Quartile						
Quartiles 1-3	1.00	-	-	1.00	-	-
Quartile 4 (highest)	2.24	(1.25-4.03)	0.007	1.01	(0.67-1.51)	0.99

Figure 1.

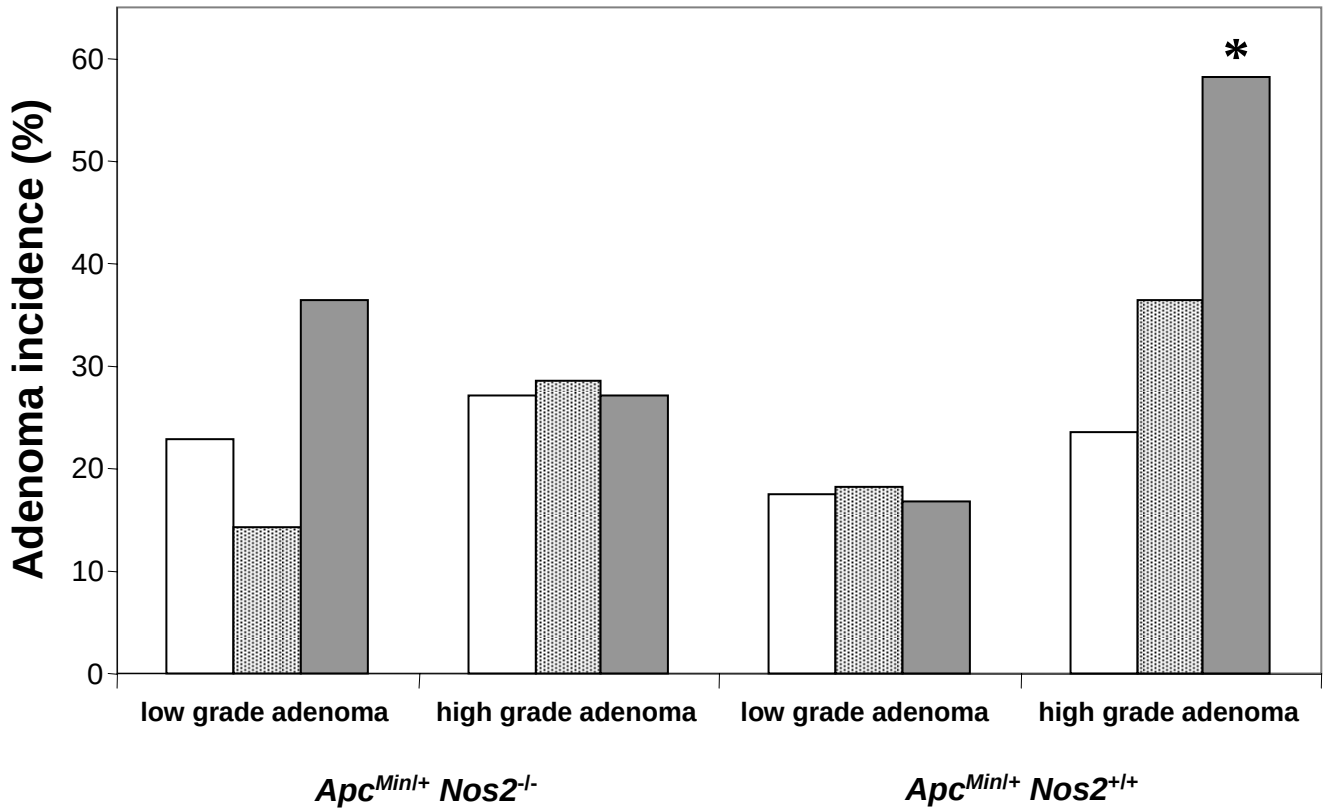

Adenoma incidence (%)
0
10
20
30
40
50
60
*
low grade adenoma
high grade adenoma
low grade adenoma
high grade adenoma
Apc^Min/+ Nos2^-/-
Apc^Min/+ Nos2^+/+

Figure 2A.

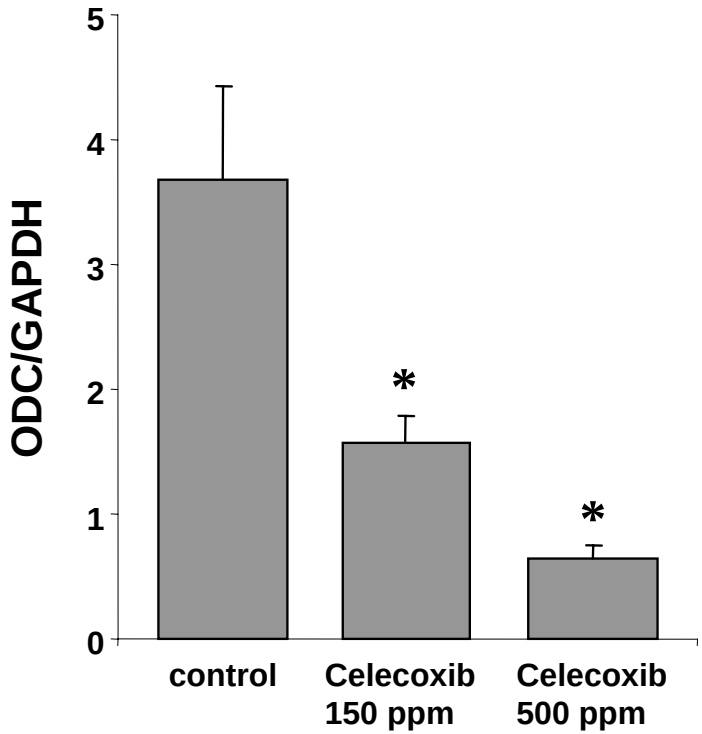

ODC/GAPDH
5
4
3
2
1
0
*
*
control
Celecoxib
150 ppm
Celecoxib
500 ppm

Figure 2B.

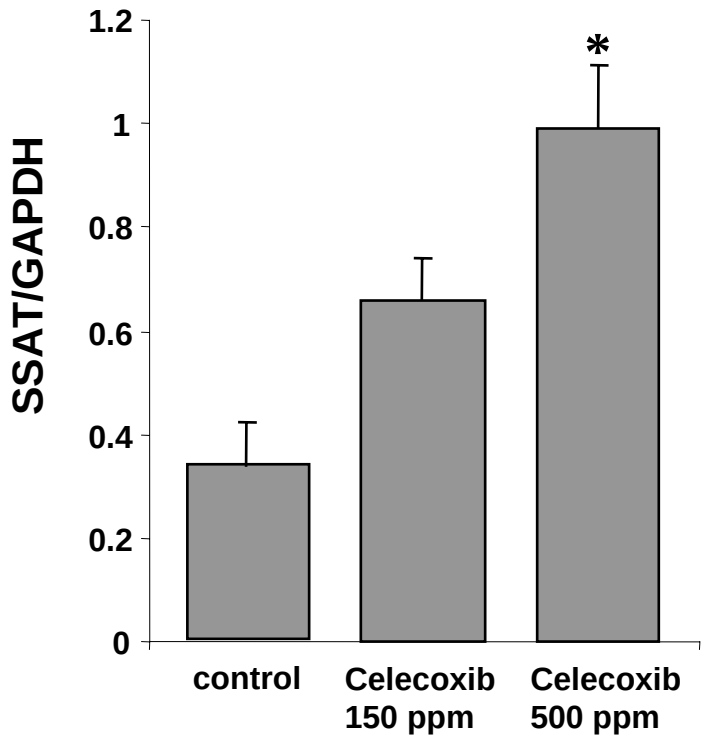
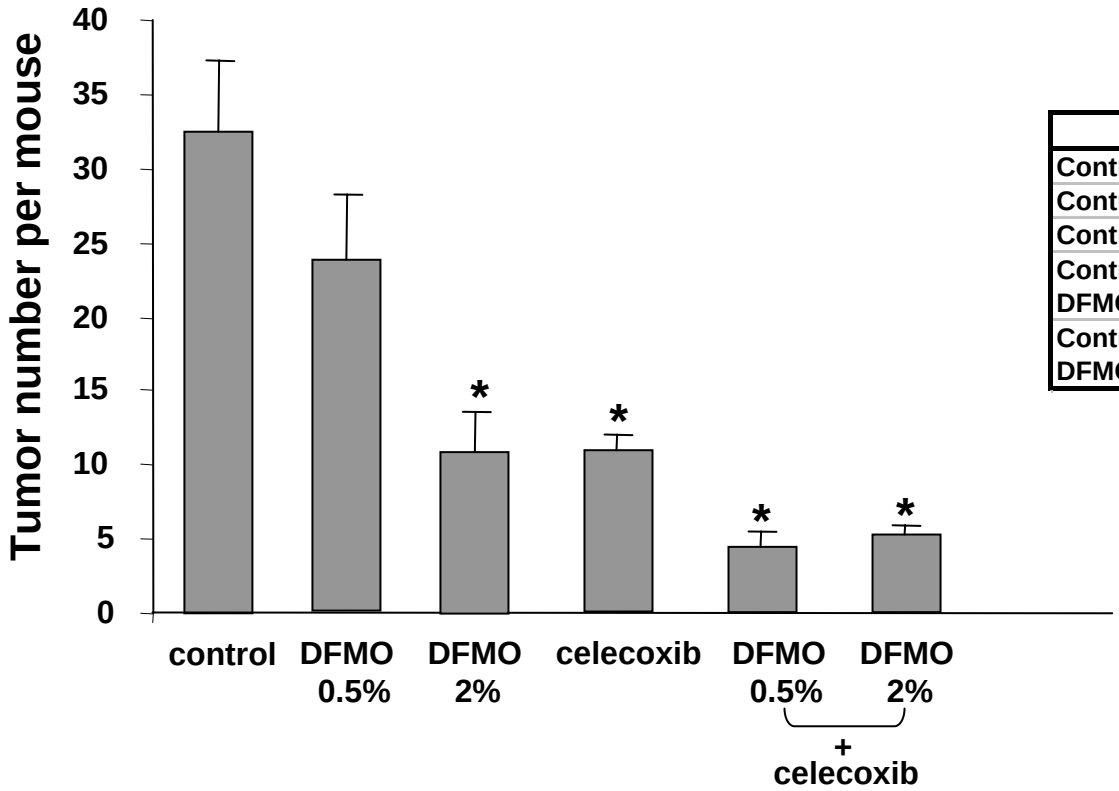

SSAT/GAPDH
1.2
1
0.8
0.6
0.4
0.2
0
*
control
Celecoxib 150 ppm
Celecoxib 500 ppm

Figure 2C.



Group	*P value
Control vs DFMO 0.5%	0.19
Control vs DFMO 2%	0.002
Control vs Celecoxib	0.0003
Control vs DFMO 0.5% & Celecoxib	<0.0001
Control vs DFMO 2% & Celecoxib	<0.0001

Figure 3.

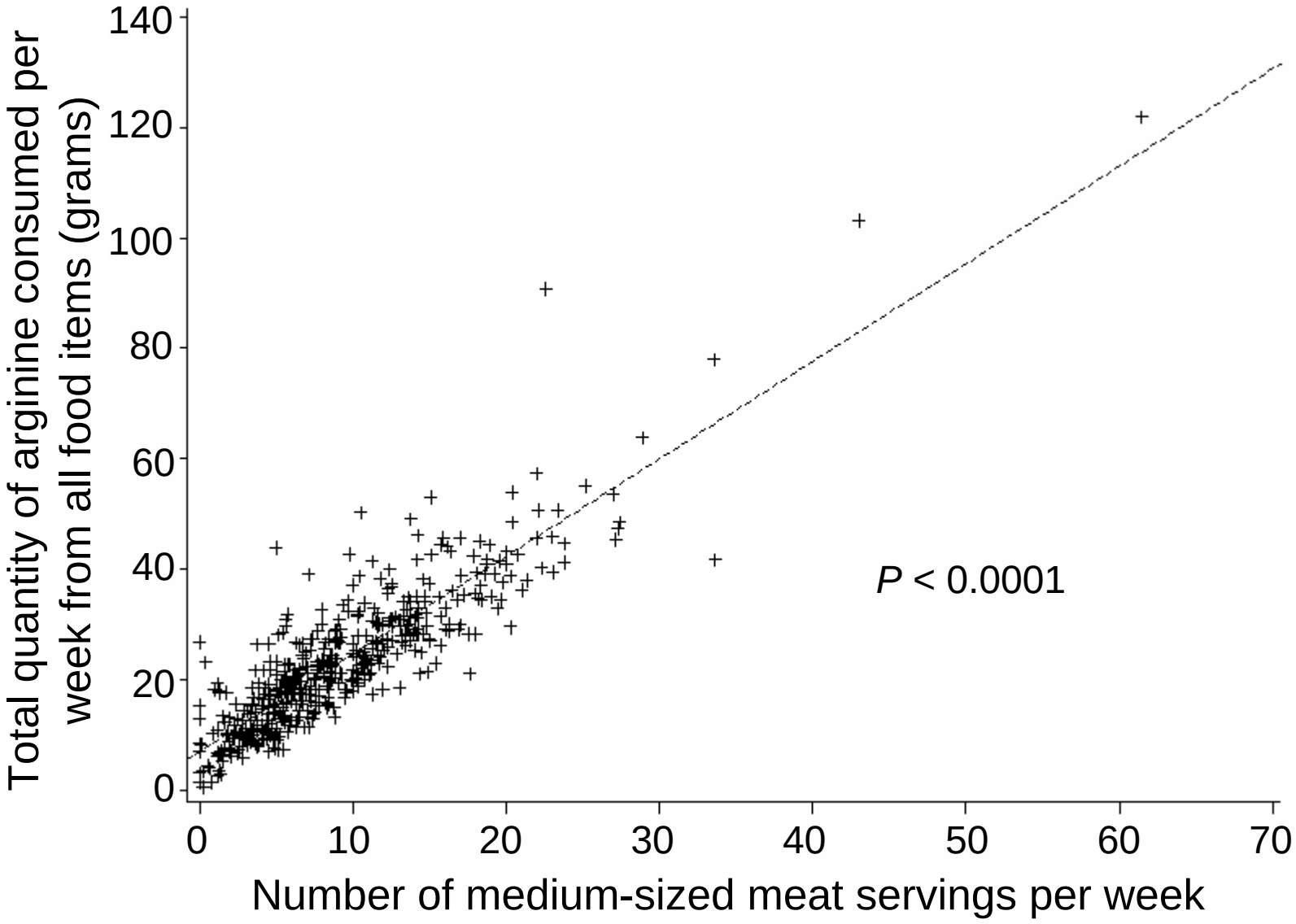

Total quantity of arginine consumed per week from all food items (grams)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Number of medium-sized meat servings per week
P < 0.0001

Figure 4.

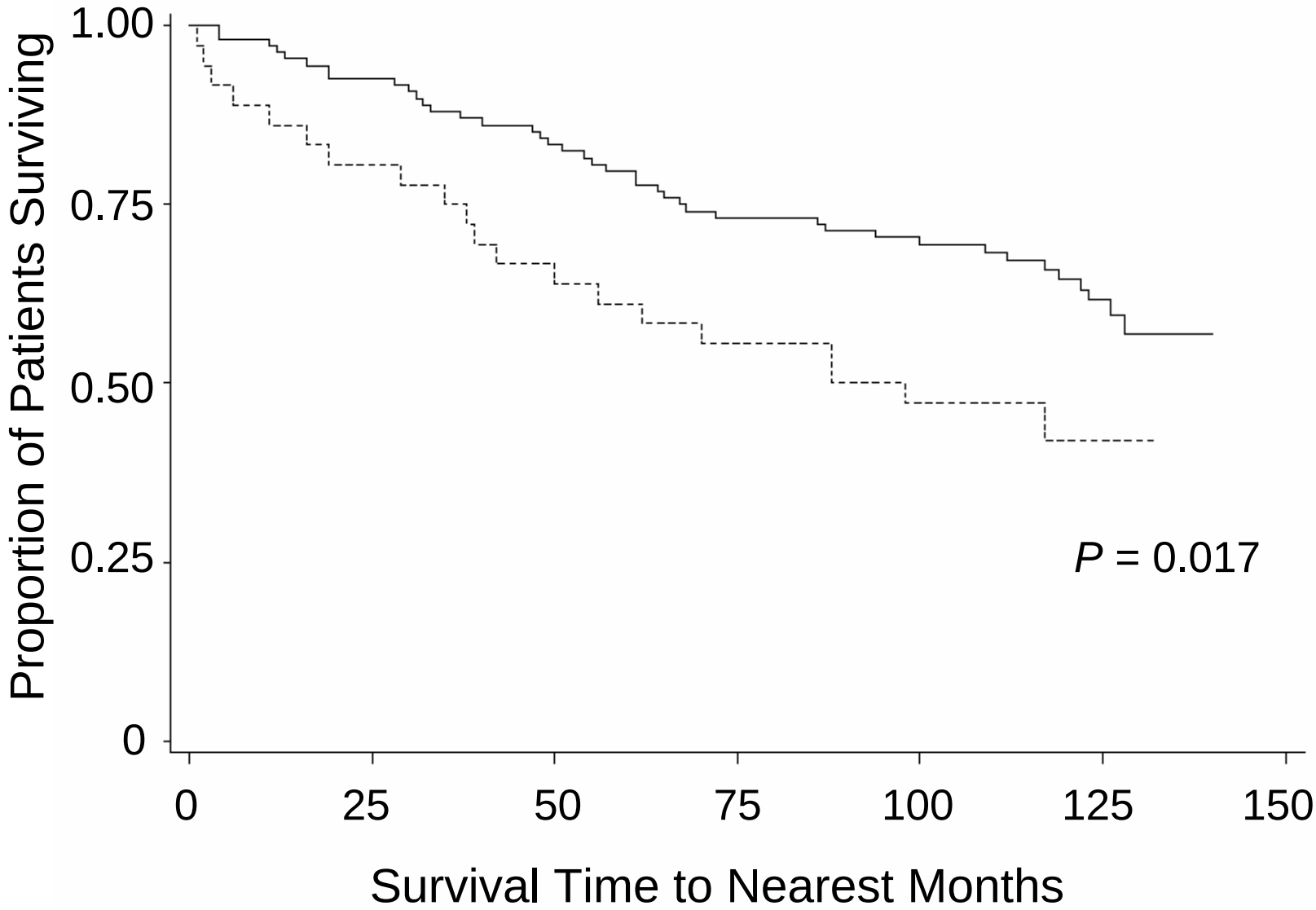
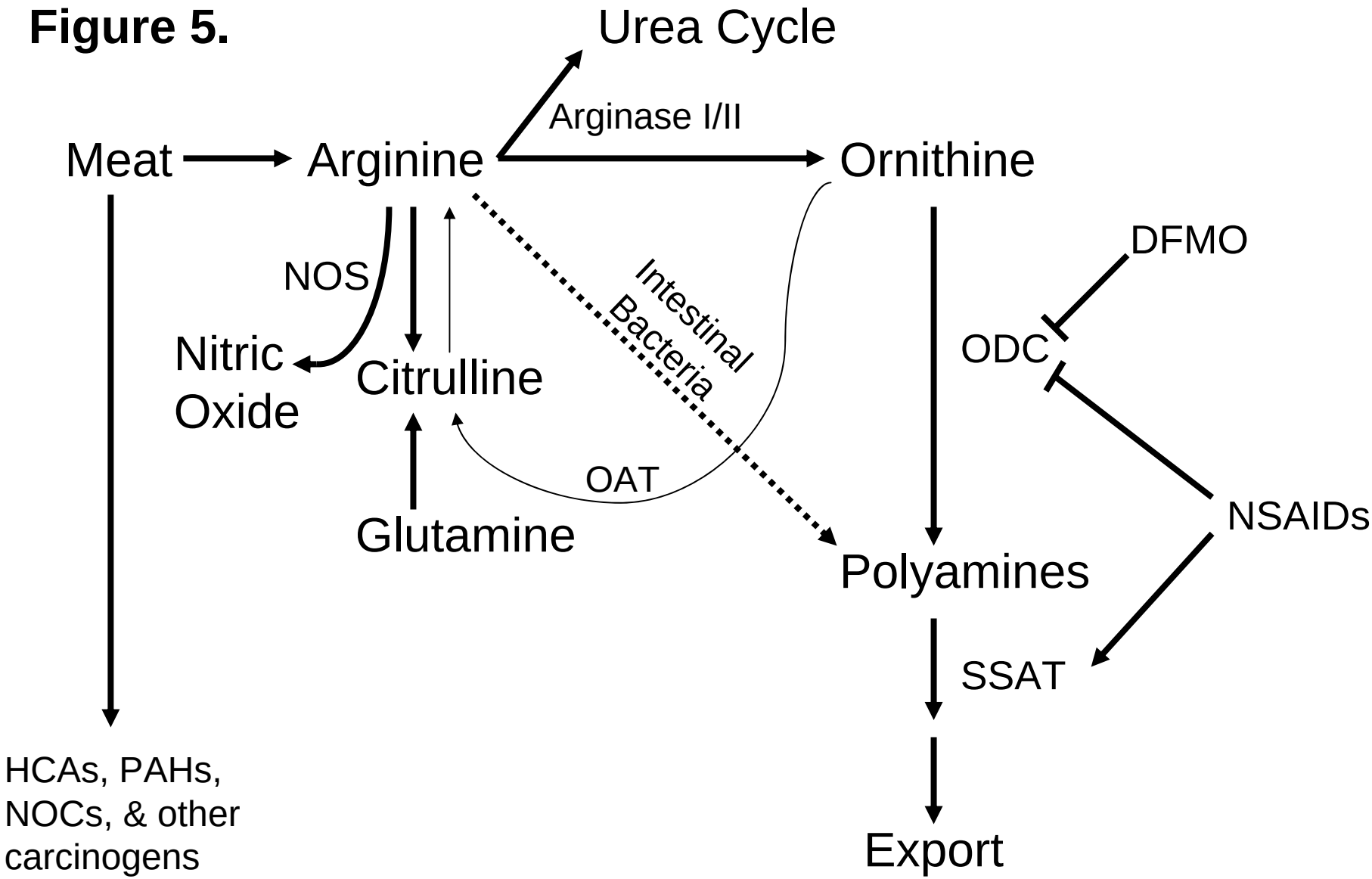

1.00
0.75
0.50
0.25
0
Proportion of Patients Surviving
P = 0.017
0
25
50
75
100
125
150
Survival Time to Nearest Months

Figure 5.


Urea Cycle
Arginase I/II
Meat
Arginine
Ornithine
NOS
Nitric Oxide
Citrulline
Intestinal Bacteria
DFMO
ODC
OAT
Glutamine
NSAIDs
Polyamines
SSAT
HCAs, PAHs, NOCs, & other carcinogens
Export