

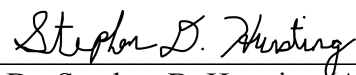
The impact of anti-inflammatory drug sulindac on obesity-associated colon tumor growth

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Abstract

Background: Obesity is a known risk factor for colon cancer, the second leading cause of cancer death in the United States. Excess visceral adipose tissue (VAT) associated with obesity stimulates pro-inflammatory and pro-tumorigenic signaling pathways that increases the risk for colon cancer. In this study we examine whether weight loss and/or treatment with the non-steroidal anti-inflammatory drug sulindac reduces obesity-associated tumor growth in a mouse model of colon tumorigenesis.

Methods: 10-week old male FVB mice (n=130) were given a chemical carcinogen, azoxymethane (10 mg/kg i.p.), for 5 weeks. Afterwards, the mice were randomized to a control (10% kcal from fat) or diet-induced obesity (DIO, 60% kcal from fat) diet for 15 weeks. Following an interim sacrifice of 5 mice/group, the DIO mice were further randomized to continue a DIO diet or switch to a control diet to induce weight loss and become formerly obese mice (FOb). Within each diet group (control, DIO, FOb) half the mice were randomized to receive sulindac supplementation (140 ppm in the diet). Eight weeks later, all mice were euthanized and tissues collected for analysis. Colon tumor gene expression was analyzed via microarray.

Results: The DIO mice, compared to controls, had significantly greater final body weight and body fat levels ($P<0.05$), while FOb mice had intermediate levels of adiposity. Treatment with sulindac did not affect final body weight or body fat percentage compared to groups that did not receive treatment. At interim sacrifice, DIO mice had a 2-fold greater tumor incidence and a 10-fold greater tumor burden compared to control mice. Across all groups, sulindac reduced tumor incidence, and in DIO and control mice, but not FOb mice, sulindac significantly reduced tumor burden ($P<0.05$). Several serum cytokines, including IL-6, CXCL1, VEGF, MCP-1, and G-CSF, were significantly elevated in DIO mice without sulindac treatment compared to control mice ($P<0.05$), but did not differ between FOb and control mice. Sulindac treatment significantly reduced serum levels of these proteins in DIO mice ($P<0.05$). Several adipokines, including leptin and PAI-1, were significantly increased in DIO mice in comparison to control and FOb mice ($P<0.05$). Sulindac treatment did not significantly reduce serum levels of these hormones in any of the diet groups. Tumor expression of matrix metalloproteinases and several genes involved in focal adhesion pathways was significantly higher in DIO mice versus the FOb and DIO + sulindac mice ($P<0.05$). *Ccl21* expression was significantly increased by sulindac treatment in DIO mice ($P<0.05$) but not affected by weight loss.

Conclusions: Weight loss and sulindac treatment completely reversed the effects of obesity on colon tumorigenesis, the latter without any change in adiposity. Tumoral gene expression suggests that these two interventions share some common anti-tumor mechanisms, but also may act through additional mechanisms specific to each intervention. Our findings indicate that research regarding the effects of NSAID treatment on colon cancer risk and/or progression in obese patients is warranted, especially in those who are unable to achieve moderate weight loss.

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Introduction

Colon Cancer Prevalence and Treatment

Colon cancer is the second most common cause of cancer death in the United States¹ and is characterized by the abnormal proliferation of cells in the colon. In 2017, there was an estimated 95,520 new cases of colon cancer with nearly equal distribution of incidence between men and women.² Since the 1980s, colon cancer survival rates have been rising due to increased awareness and early detection of polyps³. Current treatment for colon cancer is largely based on the stage of cancer, with earlier stages of cancer treated with polyp removal surgery and later stages using chemotherapy. If chemotherapy is used, FOLFOX (folinic acid, fluorouracil, and oxaliplatin) or CapeOx (capecitabine and oxaliplatin) are the most common treatment regimens⁴. The lifetime risk of developing colon cancer in men and women is approximately 4.5%, however this risk can increase with factors such as age, family history, and diet³.

Impact of Obesity on Colon Cancer

Obesity is a known risk factor for a number of cancers⁵ and can increase risk of developing colorectal cancer by 30% compared to individuals of normal weight, especially in men⁶. While there is an association between increased risk of colon cancer and increased body mass index (BMI), research suggests waist circumference is a more consistent risk factor, possibly partially independent of BMI⁷. The link between abdominal obesity and colon cancer suggests adipocyte hypertrophy and associated aberrant metabolic and inflammatory signaling in the colon-adjacent visceral adipose tissue (VAT) may be an intermediary in obesity-associated colon tumor growth.

The onset of obesity can cause chronic low-grade inflammation and adipocyte dysfunction. As an endocrine organ, adipose tissue releases both pro-inflammatory and anti-

inflammatory hormones known as adipokines. With obesity, adipose tissue modification can lead to malfunction of adipokine secretion as well as changes in the number, size, and phenotype of adipocytes⁸. Dysregulation of adipose tissue endocrine function and inflammation leads to a systemic inflammatory response and associated comorbidities of obesity¹¹.

Through adipocyte death, which increases in adipocyte hypertrophy, white adipose tissue (WAT) macrophages scavenge for cell debris and create the appearance of ‘crown-like structures’ (CLS) in adipose tissue⁹. With time, activation of receptors on the macrophages and adipocytes can induce pro-inflammatory cytokine and chemokine release leading to further tissue remodeling and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway important for carcinogenesis^{10, 14}. The pro-inflammatory proteins released during obesity include interleukin (IL)-6, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, leptin, and resistin⁸. These proteins, as well as other adipokines and cytokines, may promote the production of excess reactive oxygen species (ROS) within the colon, leading to DNA mutations and tumor initiation¹³. Furthermore, production of anti-inflammatory and anti-tumor adipokines, like adiponectin, is downregulated in this process. This change in metabolism and the hypoxic environment of excess adipose tissue can facilitate pro-tumorigenic conditions and activate signals used to promote cancer cell survival, proliferation, and migration¹².

Sulindac and Colon Cancer

A strong association between colon cancer risk and pro-inflammatory signaling has previously been established, and research demonstrates that the use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with decreased incidence and polyp formation in colon cancer¹⁵⁻¹⁷. One type of NSAID, sulindac, has been investigated as a chemotherapeutic

treatment for colon and other cancers. This drug is especially known for its pro-apoptotic and growth inhibitory activity and its ability to concentrate in the colonic epithelium, contributing to its efficacy in colon cancer¹⁸⁻¹⁹.

The mechanism through which sulindac targets cancer cells stems from its ability to inhibit both COX1 and COX2 pathways, which can promote tumor proliferation¹⁹, as well as its COX-independent anti-tumor mechanisms²⁰. Conventional NSAIDs, which solely target COX-dependent pathways, can be dangerous with prolonged use because they can lead to gastrointestinal tract bleeding, kidney failure, and cardio-toxicity due to prostaglandin inhibition²⁰. Sulindac's ability to further suppress tumor growth via COX-independent pathways, such as induction of EGR-1 protein expression, downregulation of EGFR signaling, and protection from E-cadherin loss, is one reason for its investigation as a cancer treatment²⁰. Given that sulindac acts through multiple anti-tumor mechanisms, it may be possible to achieve a response with a relatively smaller dose that does not induce the typical NSAID side effects. These studies suggest that reducing inflammatory signaling via sulindac treatment may be an effective strategy for decreasing obesity-induced colon tumor growth and incidence. However, further research is still necessary to fully understand the cell signaling pathways sulindac targets to reduce cancer progression.

Goal and Hypotheses

The primary goal of this study was to investigate whether weight loss and/or NSAID treatment reduces obesity-associated tumor growth in a mouse model of colon tumorigenesis. This was accomplished through four specific aims. The first was to characterize the impact of sulindac treatment on body weight, body composition, and serum metabolic hormone levels in obese and lean mice. We predicted that sulindac would not affect body weight and body composition, but may reduce some metabolic hormone levels.

Our second specific aim was to assess the effects of sulindac treatment in obese and lean mice and weight loss in obese mice on colon tumor incidence, multiplicity, and size. Due to the research indicating that sulindac reduces colon tumorigenesis, we hypothesized that colon tumor incidence, multiplicity, and size would diminish in groups treated with sulindac, including the obese mice, and also with weight loss in the formerly obese mice.

The third specific aim was to determine the effects of sulindac treatment and obesity reversal on systemic, colon tissue, and visceral adipose tissue inflammation by measuring markers of inflammation in the serum and tissues. Given that sulindac is an NSAID, we anticipated reduced levels of serum inflammatory cytokines in groups treated with the drug and also in formerly obese mice.

Our final aim was to determine and characterize the effects of sulindac treatment and obesity reversal on gene expression in tumors by analyzing microarray data. We hypothesized that the expression of genes with anti-tumorigenic activity would be increased and genes with pro-tumorigenic activity decreased in the mice treated with sulindac and in formerly obese mice that experienced weight loss.

Methods

Animal study design:

10-week old male FVB mice (n=130) were given a chemical carcinogen, azoxymethane (10 mg/kg i.p.), for 5 weeks. Afterwards, the mice were randomized to a control (10% kcal from fat) or diet-induced obesity (DIO, 60% kcal from fat) diet for 15 weeks. Following an interim sacrifice of 5 mice/group, the DIO mice were further randomized to continue a DIO diet or switch to a control diet to induce weight loss and become formerly obese mice (FOb). Within each diet group (control, DIO, FOb) half the mice were randomized to receive sulindac supplementation (140 ppm in the diet). Eight weeks later, all mice were sacrificed by carbon dioxide euthanasia and cervical dislocation. The samples collected from the mice during the study included serum, colon rolls (paraffin-embedded), tumor (flash-frozen), normal colon epithelium (flash-frozen), mesenteric adipose tissue (paraffin-embedded and flash-frozen), and fecal samples (flash-frozen) at baseline, before diet switch, and endpoint of the study.

Body Weight and Fat Composition Analysis:

Body weight of mice was taken at baseline and then weekly until euthanization. Body composition was measured using dual energy X-ray absorption (DEXA) after euthanization.

Tumor Incidence, Multiplicity, and Size Analysis:

Investigator enumerated the colon tumors for each mouse at interim sacrifice and at the endpoint of the study. Digital calipers were used to measure two dimensions of each tumor to calculate the total tumor cross-sectional area.

Serum Cytokine Analysis:

Serum cytokines were analyzed using Bio-Plex Multiplex Immunoassay on a Bio-Plex® Magpix Multiplex Reader (Bio-Rad).

Serum Metabolic Hormone Level Analysis:

Serum metabolic hormones were analyzed using Bio-Plex Multiplex Immunoassay on a Bio-Plex® Magpix Multiplex Reader (Bio-Rad). Serum IGF-1 was measured via a Mouse Magnetic Luminex Screening Assay (R&D Systems).

Microarray Analysis:

First, RNA isolation was performed on flash-frozen colon tumors using TRIzol Reagent (Sigma-Aldrich) according to manufacturer's instructions. RNA integrity was assessed on an Agilent 2100 Bioanalyzer, and RNA was analyzed via Clariom S Microarray (Affymetrix) by UNC's Functional Genomics Core. Genes of interest from the microarray were then validated by quantitative RT-PCR. RNA was reverse-transcribed to cDNA using MultiScribe Reverse Transcriptase (ThermoFisher Scientific), then analyzed using Taqman™ Gene Expression Assays (Applied Biosystems) on a ViiA™ 7 RT-PCR System (Applied Biosystems).

Statistical Analysis:

Animal study data is presented as mean $\pm$ SD. All data except the microarray analysis was analyzed by two-way ANOVA, followed by Tukey's post hoc test, using GraphPad Prism 7 software. Microarray data was analyzed using the Transcriptome Analysis Console (TAC) software from Life Technologies. In all figures, different letters indicate significant differences, and $P < 0.05$ was considered statistically significant.

Results

Body Weight and Composition Analysis:

Each diet group (Control, DIO, FOb) had significantly different final body weights in comparison to each other (Figure 1A, 1B). Specifically, DIO mice, compared to controls, had significantly greater body weight ($P<0.0001$), while FOb mice had intermediate levels of final body weights that were significantly different from both Control ($P=0.006$) and DIO ($P<0.0001$) mice. However, sulindac treatment did not significantly affect body weight within each diet group (Figure 1A, 1B). Body fat percentage was significantly different between DIO mice and control mice ($P<0.0001$) that received the same treatment, but sulindac treatment within each diet group did not significantly affect body composition (Figure 1C). Body fat percentage of FOb mice did not significantly differ from control groups, but did differ from DIO mice with sulindac treatment ($P=0.0026$; Figure 1C).

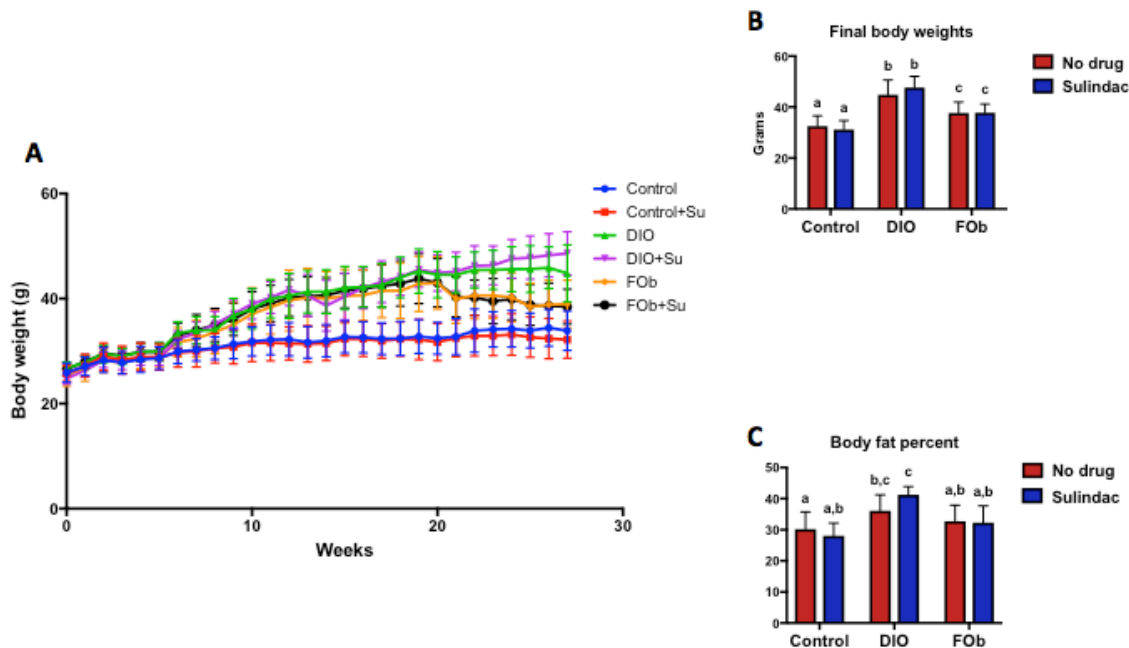


Figure 1. (A) Body weight over time. **(B)** Body fat percent. **(C)** Final body weights. Different letters indicant significant differences, $P < 0.05$.

Tumor Incidence, Multiplicity, and Size Analysis:

At interim sacrifice, DIO mice had a non-significant 2-fold greater incidence of tumor compared to control mice (Figure 2A). At the end of the study, sulindac reduced tumor incidence across all groups ($P=0.014$; Figure 2B). At interim sacrifice, DIO mice had a non-significant 10-fold greater tumor multiplicity compared to control mice (Figure 2C). Sulindac significantly reduced tumor multiplicity in both control ($P=0.044$) and DIO ($P<0.0001$) mice, but not FOb mice (Figure 2D). Tumor cross-sectional area was significantly increased in DIO mice relative to all other groups ($P<0.01$ for all comparisons, Figure 2E).

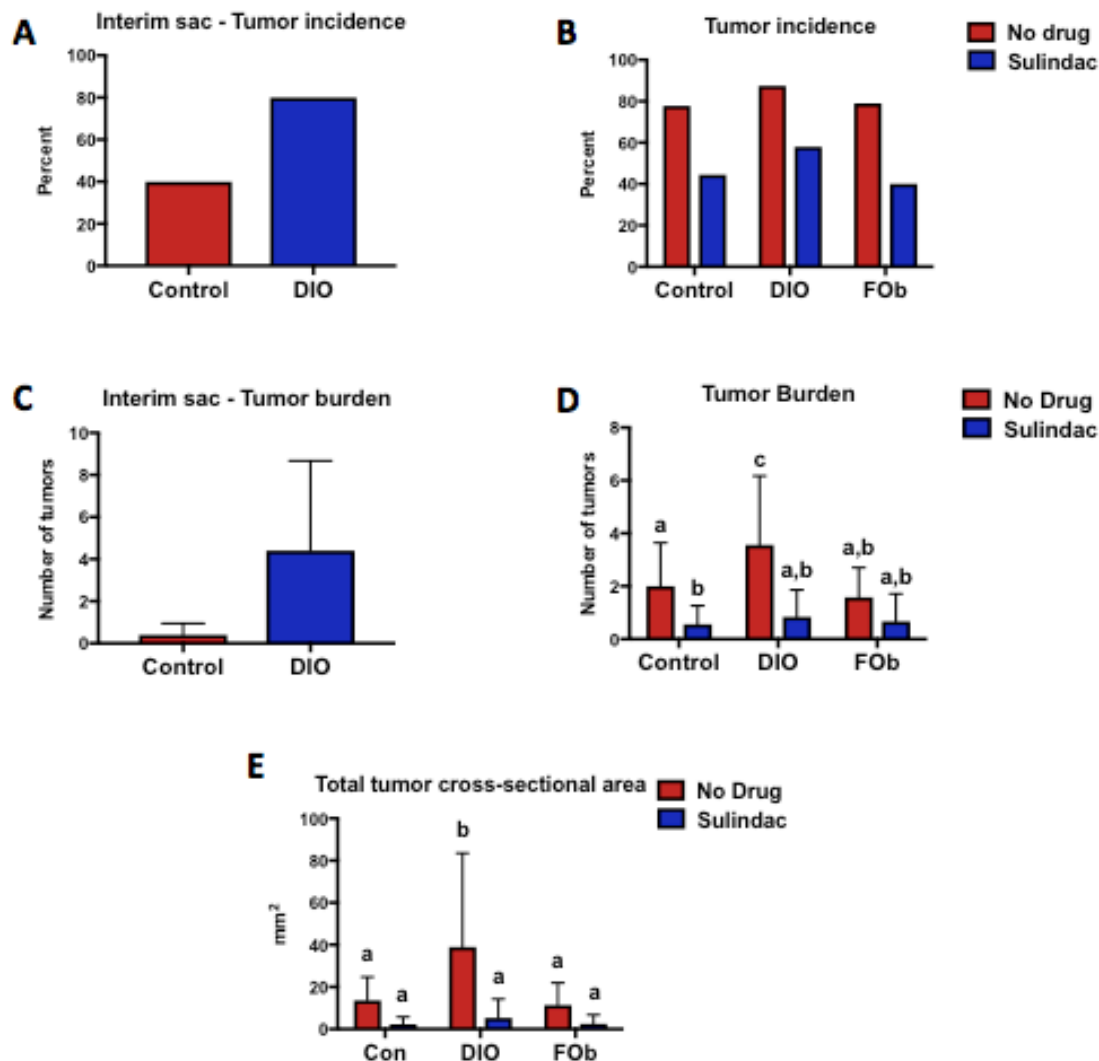


Figure 2. (A) Tumor incidence at interim sacrifice (sac). (B) Final tumor incidence. (C) Tumor burden at interim sac. (D) Final tumor burden. (E) Final total tumor cross-sectional area. Different letters indicant significant differences, $P < 0.05$.

Serum Cytokines Analysis:

Serum levels of interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), chemokine (C-X-C) ligand 1 (CXCL1), and granulocyte colony-stimulating factor (G-CSF) were significantly elevated in DIO mice without

sulindac treatment in comparison to control mice with and without sulindac treatment ($P<0.05$ for all comparisons; Figure 3A-E). Serum levels of all cytokines measured did not differ between FOb and control mice that received the same treatment (Figure 3A-J). Sulindac treatment significantly reduced serum levels of IL-6, VEGF, MCP-1, CXCL1, G-CSF, and IL-1 β in DIO mice ($P<0.05$ for all comparisons; Figure 3A-F). In control and FOb mice groups, sulindac treatment non-significantly reduced all serum cytokine levels (Figure 3A-J) with the exception of Interferon gamma (IFN γ), which exhibited a significant decrease with sulindac treatment in the control group ($P=0.0186$; Figure 3G) and CXCL1, which was slightly higher with sulindac treatment in the control group (Figure 3D). Treatment with sulindac in DIO mice reduced serum cytokines to a level that did not significantly differ from control and FOb groups treated with sulindac (Figure 3A-J). There were no significant differences across all groups in serum cytokine levels of IL-17 and IL-10 (Figure 3H-I).

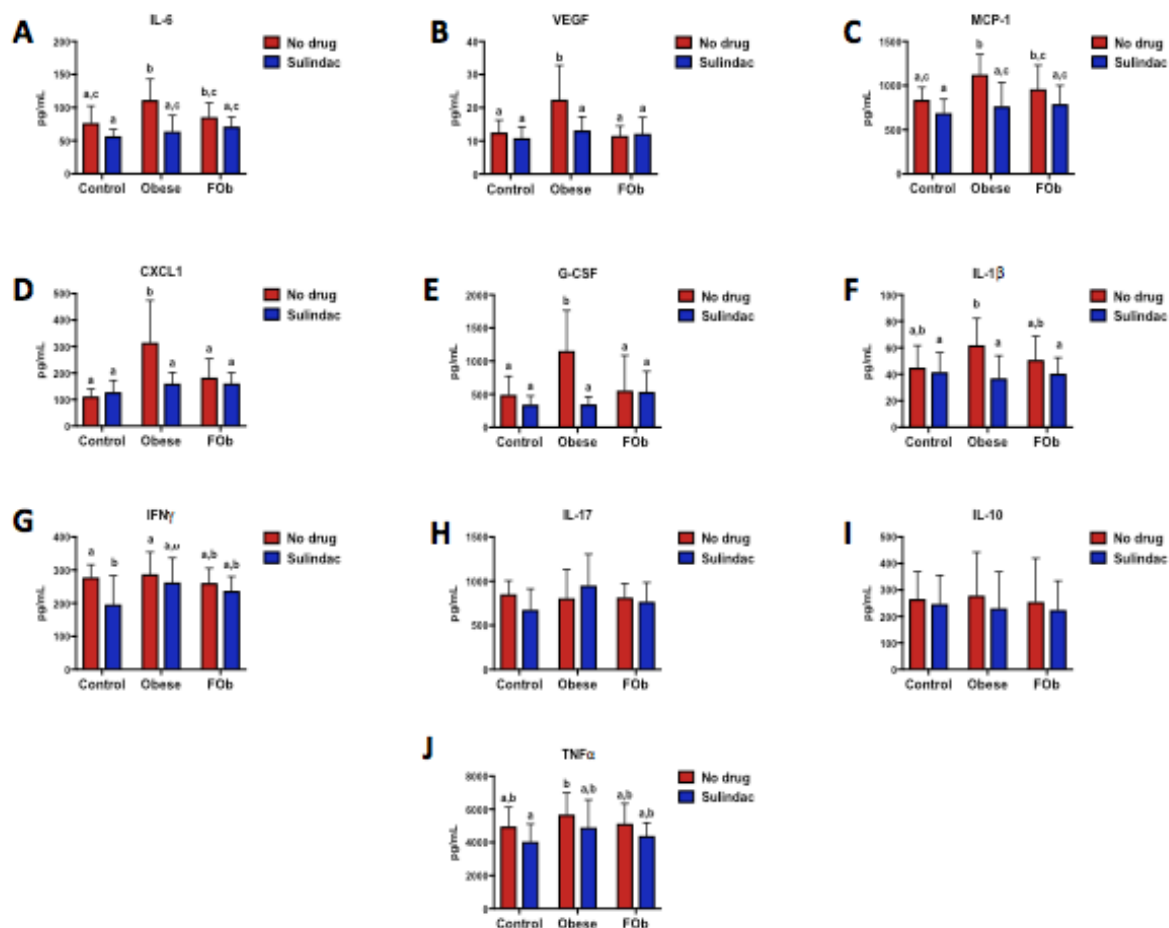


Figure 3. Serum levels of (A) IL-6, (B) VEGF, (C) MCP-1, (D) CXCL1, (E) G-CSF, (F) IL-1 β , (G) IFN γ , (H) IL-17, (I) IL-10, (J) TNF α . Different letters indicate significant differences, $P < 0.05$.

Serum Metabolic Hormone Analysis:

There was a significant increase in leptin and insulin-like growth factor 1 (IGF-1) levels in all DIO mice compared to all control mice ($P < 0.05$ for all comparisons; Figure 4A-B). Serum levels of adiponectin, glucagon-like peptide-1 (GLP-1), glucagon, ghrelin, insulin, gastric inhibitory polypeptide (GIP), plasminogen activator inhibitor-1 (PAI-1), and resistin were not significantly different between DIO mice and control mice (Figure 4C-J). Glucagon, ghrelin, insulin, GIP, and resistin were not significantly different between DIO mice and FOb mice that

received the same treatment (Figure 4E-H, J). Leptin levels were significantly increased in DIO mice compared to control and FOb mice ($P<0.05$ for all comparisons; Figure 4A), and there was a significant increase in leptin:adiponectin ratio in DIO mice compared to the other groups ($P<0.05$ for all comparisons; Figure 4K). Sulindac treatment did not significantly change serum levels of leptin, IGF-1, adiponectin, GLP-1, ghrelin, insulin, GIP, PAI-1, or resistin (Figure 4A-D, F-J) within any of the diet groups with the exception of glucagon levels in DIO mice, which were significantly decreased with sulindac treatment ($P=0.006$, Figure 4E). There were no significant differences across any of the groups in serum hormone levels of insulin, GIP, and resistin (Figure 4G-H, J).

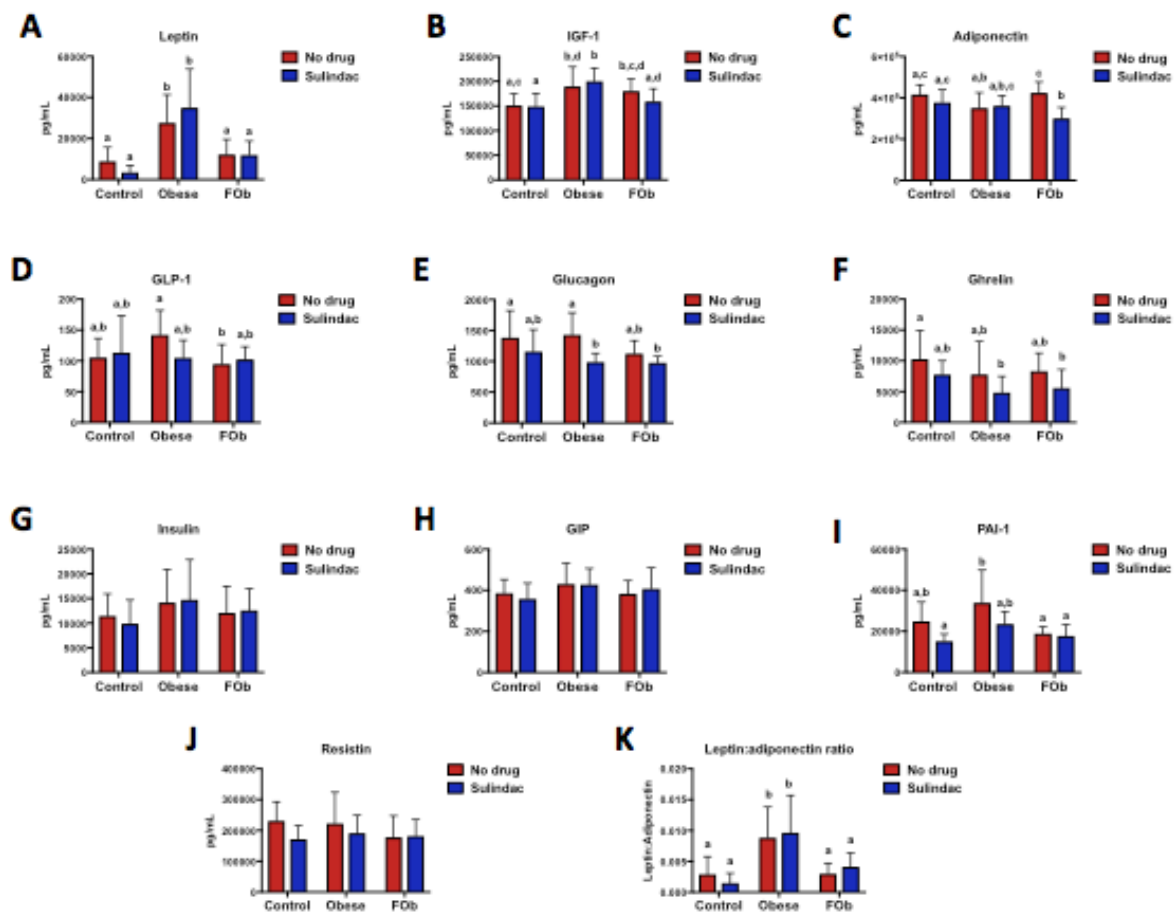


Figure 4. Serum levels of (A) Leptin, (B) Leptin, (C) Adiponectin, (D) GLP-1, (E) Glucagon, (F) Ghrelin, (G) Insulin, (H) GIP, (I) PAI-1, (J) Resistin, (K) Leptin:adiponectin ratio. Different letters indicate significant differences, $P < 0.05$.

Microarray Analysis:

Hierarchical clustering of the microarray data demonstrates that tumoral gene expression clusters by group (Figure 5). This data indicates that tumoral gene expression is linked to and varies by the diet and treatment received.

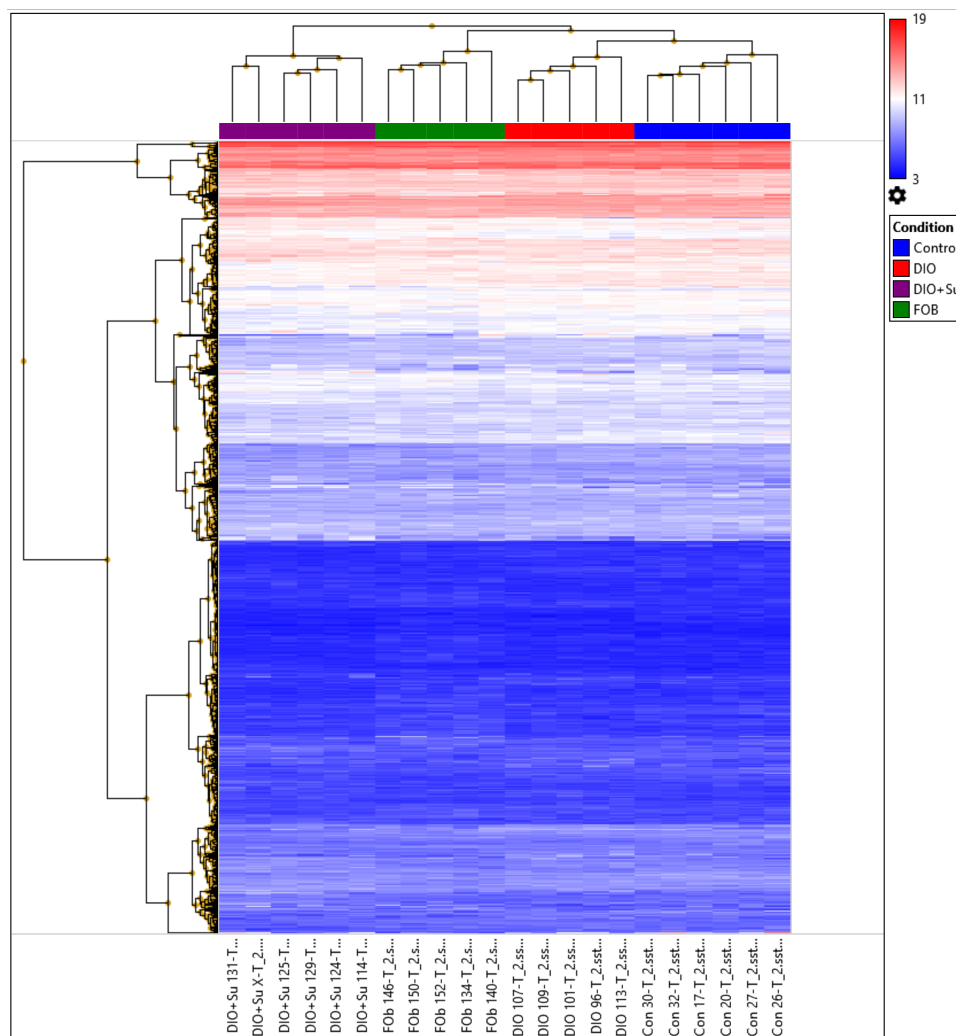


Figure 5. Hierarchical clustering of gene expression.

Analysis of tumoral gene expression by microarray revealed that in control and DIO mice there were significant differences in gene enrichment ($P < 0.05$), primarily for metabolism-related pathways. We chose to further explore pathways that were associated with cancer progression, such as oxidative stress, estrogen metabolism, Keap1-Nrf2, and focal adhesion, which led to validation by qPCR of *Nqo1* and *Ngf* gene expression (Table 1). When comparing DIO with

DIO+Sulindac mice, pathways of interest included matrix metalloproteinases, Keap1-Nrf2, chemokine signaling, and estrogen metabolism. This led to validation by qPCR of *Mmp10*, *Mmp3*, *Mmp13*, *Ccl11*, *Ccl21*, and *Nqo1* gene expression (Table 2). Furthermore, when analyzing differences between DIO and FOb mice, significant pathways of interest involved in cancer progression included matrix metalloproteinases, focal adhesion, and TGF beta signaling, which led to validation through qPCR of *Colla2*, *Col5a2*, *Flt1*, *Fnl*, *Pgf*, *Zeb2*, *Tfibr2*, *Mmp9*, and *Mmp12* (Table 3). Gene expression levels of the pro-inflammatory, pro-tumor cytokine *Il11* were also found to be significantly higher in DIO ($P=0.0027$) and Control ($P=0.017$) mice relative to DIO+Sulindac mice in our microarray analysis, so *Il11* expression was validated by qPCR as well.

Pathway	Genes Upregulated	Genes Downregulated	P-value
Oxidative Stress	Nqo1	Mgst1	0.0017
Adipogenesis genes		Lpl, Cebpd, Frzb	0.0028
Retinol metabolism		Lpl, Rbp4	0.0031
Alzheimers Disease		Lpl, Casp12	0.012
Alanine and aspartate metabolism		Gad2	0.025
Estrogen metabolism	Nqo1		0.027
Keap1-Nrf2	Nqo1		0.029
Biogenic Amine Synthesis		Gad2	0.031
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	Efna3	Ngf, Pfkfb3	0.032
Statin Pathway		Lpl	0.042

Table 1. Top ten most significant pathways for Control vs. DIO mice.

Pathway	Genes Upregulated	Genes Downregulated	P-value
Matrix Metalloproteinases	Mmp10, Mmp3, Mmp13		0.00050
Keap1-Nrf2		Gsta2, Nqo1	0.0025
Endochondral Ossification	Frzb, Mmp13, Plau		0.0046
Spinal Cord Injury	Gadd45a, Gja1	Tacr1	0.017
Chemokine signaling pathway	Gm13306, Ccl11	Ccl21a, Ccl21c	0.019
Complement and Coagulation Cascades	Serpind1, Plau		0.044
Lung fibrosis	Ccl11, Plau		0.047
Heme Biosynthesis	Alas2		0.047
Alanine and aspartate metabolism	Gad2		0.063
Estrogen metabolism		Nqo1	0.068

Table 2. Top ten most significant pathways for DIO vs. DIO+Sulindac mice.

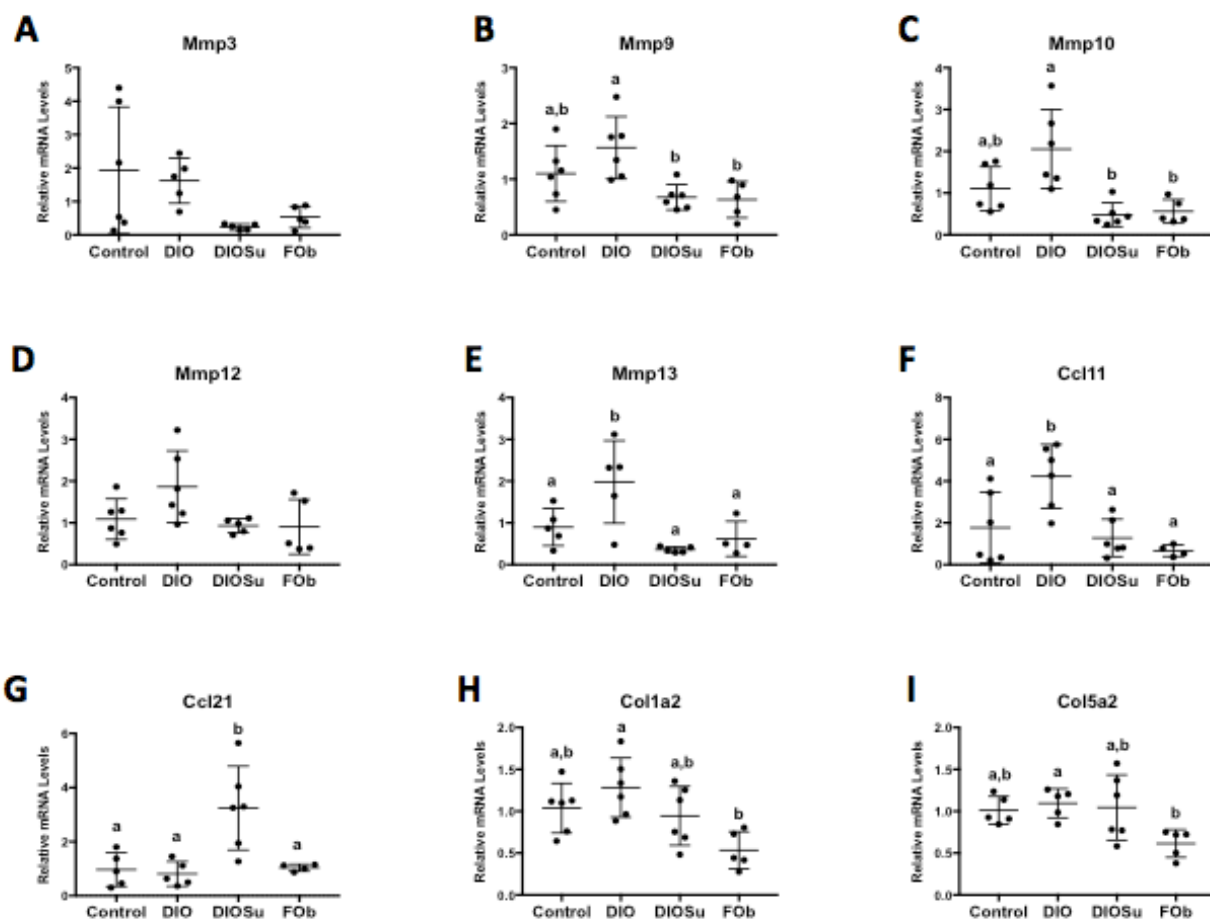
Pathway	Genes Upregulated	Genes Downregulated	P-value
Spinal Cord Injury	Ptprz1, Mmp12, Mmp9, Pla2g5, Selp		0.00068
XPodNet - protein-protein interactions in the podocyte expanded by STRING	Trf, Ltbp1, Flt1, Ngf, Zeb2, Fn1, Csf1, Wif1, Sulf1,		0.00070

	Angptl2, Tgfb3, Tcf21, Sparc, Nrp1, Tgfbr2		
PodNet: protein-protein interactions in the podocyte	Wif1, Sulfl, Angptl2, Tgfb3, Tcf21, Sparc, Nrp1, Tgfbr2		0.0016
Focal Adhesion	Col1a2, Col5a2, Flt1, Fn1, Pgf	Tesk2	0.0017
TGF Beta Signaling Pathway	Tgfbr2, Ltbp1, Zeb2		0.0055
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	Col1a2, Col5a2, Fn1, Csf1, Pgf, Ngf, Flt1		0.0075
Endochondral Ossification	Pthlh, Frzb, Mmp9		0.0090
Matrix Metalloproteinases	Mmp9, Mmp12		0.017
Inflammatory Response Pathway	Col1a2, Fn1		0.018
Prostaglandin Synthesis and Regulation	Anxa5	Hpgd	0.019

Table 3. Top ten most significant pathways for DIO vs. FOb mice.

Sulindac treatment and weight loss significantly reduce relative mRNA levels for matrix metalloproteinase genes *Mmp9*, *Mmp10*, and *Mmp13* compared to DIO mice ($P<0.05$ for all comparisons; Figure 6B-C,E) Additionally, sulindac treatment and weight loss non-significantly reduced relative mRNA levels of *Mmp3* and *Mmp12* (Figure 6A, D). Sulindac treatment

significantly increased relative mRNA levels for *Ccl21* ($P=0.0032$, Figure 6G), while weight loss and sulindac treatment significantly reduced mRNA levels of *Ccl11* compared to DIO mice ($P<0.05$ for all comparisons; Figure 6F). Relative mRNA levels for genes involved in the focal adhesion pathway, *Colla2*, *Col5a2*, *Csfl*, *Fnl1*, *Pgf*, and *Ngf*, were significantly reduced in the FOb mice compared to DIO mice ($P<0.05$ for all comparisons; Figure H-J, N, P, R). No significant differences between groups were seen in *Il11*, *Zeb2*, *Flt1*, and *Tgfbr2* expression (Figure 5K-M, O). *Nqo1* expression was significantly reduced in DIO relative to control mice ($P=0.0480$), but did not significantly differ between FOb and DIO+Su mice (Figure 5Q).



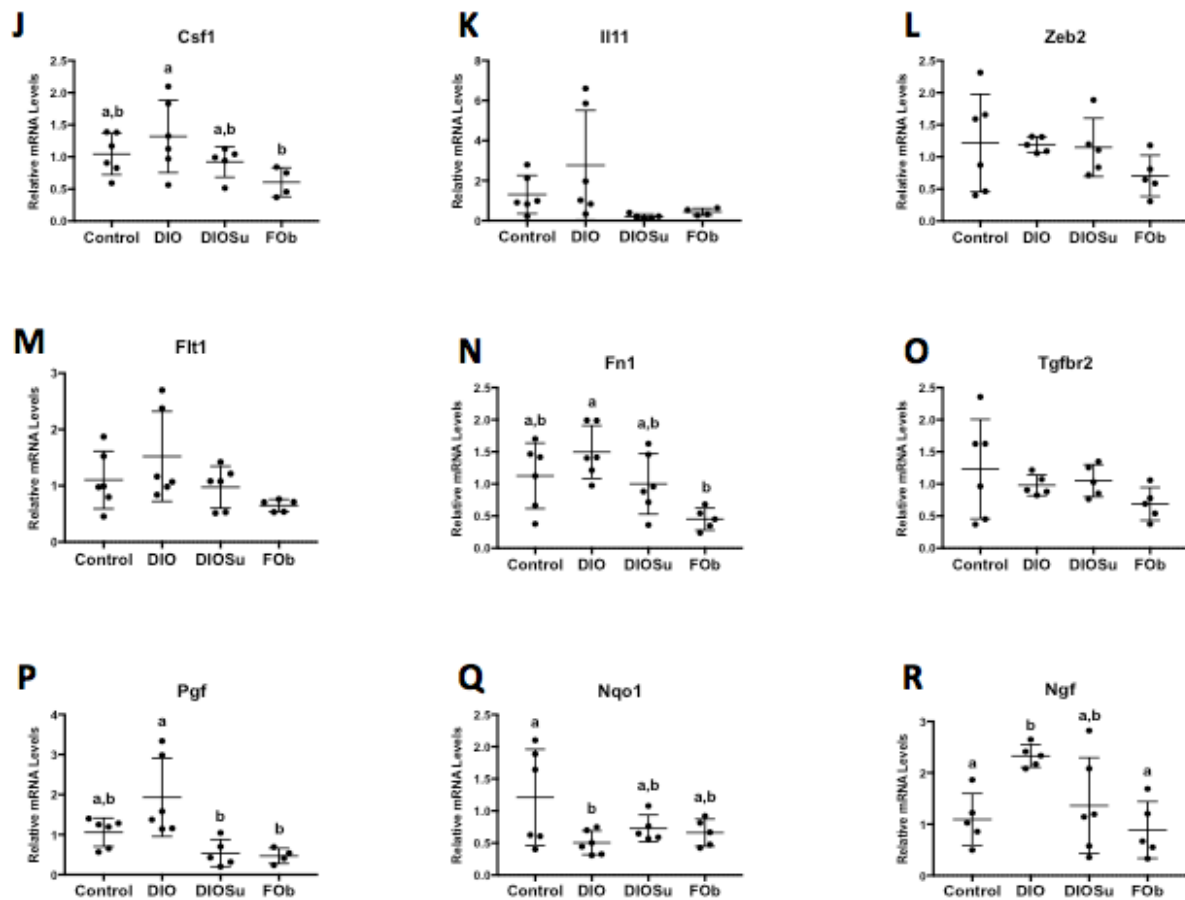


Figure 6. Tumoral gene expression levels, measured by qPCR, of (A) Mmp3, (B) Mmp9, (C) Mmp10, (D) Mmp12, (E) Mmp13, (F) Ccl11, (G) Ccl21, (H) Col1a2, (I) Col5a2, (J) Csfl, (K) Il11, (L) Zeb2, (M) Flt1, (N) Fn1, (O) Tgfbr2, (P) Pgf, (Q) Nqo1, (R) Ngf. Different letters indicate significant differences, $P < 0.05$.

Discussion

This study aimed to understand whether the tumor-promoting effects of obesity could be reversed by an NSAID and/or weight loss in a mouse model of colon cancer. The study produced substantial evidence indicating that moderate weight loss and treatment by sulindac both reverse the pro-tumor effects of obesity. There is evidence that weight loss and sulindac may reduce tumor growth through a decrease in matrix metalloproteinase and focal adhesion pathways. Matrix metalloproteinases are important regulators of tumor growth and contribute to extracellular remodeling, angiogenesis, cellular differentiation, proliferation and apoptosis. These zinc dependent endopeptidases work by degrading extracellular matrix (ECM) proteins that are used to prevent tumor invasion and metastasis²¹. In groups treated by sulindac and weight loss, gene expression of matrix metalloproteinases (*Mmp3*, *Mmp9*, *Mmp10*, *Mmp12* and *Mmp13*) were reduced compared to the untreated obese mice, suggesting a decrease in MMP-related tumor progression activity.

Additionally, expression of genes used in focal adhesion pathways (*Colla2*, *Col5a2*, *Flt1*, *Fnl1*, *Pgf*, *Csfl*, *Ngf*) were also reduced in weight loss and drug treated groups compared to untreated obese mice. Focal adhesion pathways are activated during cancer development to allow signaling between cancer cells and the tumor microenvironment²². Cancer cells work with ECM proteins to form intracellular complexes named focal adhesions, which use signaling proteins to reorganize the cytoskeleton. This rearrangement in the tumor environment is important for tumor angiogenesis and metastasis, which enables cancer progression²². This reduction in focal adhesion-related genes in the tumors may be another mechanism by which sulindac and weight loss are able to reduce tumor growth.

However, weight loss and sulindac treatment also differentially affected tumor gene expression. Evidence for differences between the sulindac and weight loss-treated mice are especially apparent in the differences in *Ccl21* gene expression. CCL21, also known as chemokine (C-C motif) ligand 21, is a chemokine that regulates cell recruitment and T-cell activation following localization of lymphocytes and stimulated dendritic cells²³⁻²⁴. In the literature, there is evidence that CCL21 is able to induce infiltration of immune cells into the tumor microenvironment and suppress the growth of the tumor by producing a tumor-specific immune response. In studies that sought to evaluate the prognostic significance of *Ccl21* gene expression in colorectal cancer patients, results demonstrate that high expression of *Ccl21* was an independent predictor of more favorable survival²⁴. The data from our study demonstrate that *Ccl21* gene expression is significantly increased in sulindac mice in comparison to the obese, control, and weight loss groups. This difference in gene expression is indicative not only of differences in the mechanisms by which sulindac and weight loss affect tumor progression, but also reveals a mechanism beyond changes in COX activity by which sulindac acts as an effective anti-tumor drug.

Our data indicates that the manner by which sulindac inhibits tumor growth utilizes both COX dependent and independent pathways. Literature suggests that prostaglandin E2, a key pro-inflammatory eicosanoid produced by COX-2²⁵, can induce expression of MMP9²⁶, which along with other metalloproteinases acts to enhance cancer progression. One reason for a decrease in metalloproteinase expression in sulindac mice without changes in adiposity levels is sulindac's ability to inhibit the byproducts of COX pathways from forming. However, based on sulindac's association with increased CCL21 levels, it is also evident that the drug is able to reduce tumor progression through COX independent pathways. For CCL21-induced signal transduction and

recruitment of dendritic cells to produce a tumor suppressing response, prostaglandin E2 is required²⁷. Since prostaglandin E2 is a by-product of COX-2, there is evidence that sulindac treatment is also effective in reversing the pro-tumor effects of obesity through COX-independent mechanisms. This finding particularly reveals sulindac's versatility compared to other NSAIDs because it is able to act in a COX dependent and independent manner to inhibit the progression of colon cancer.

Conclusions

Moderate weight loss and sulindac treatment both completely reverse the effects of chronic obesity on colon tumorigenesis. Sulindac treatment reverses the effect of obesity independent of any change in adiposity. These two interventions may share some common anti-tumor mechanisms, but also have different treatment-specific effects based tumoral gene expression analysis. The findings suggest that research regarding the effects of NSAID treatment on colon cancer risk and/or progression in obese patients is warranted, especially in those who are unable to achieve moderate weight loss.

Future Directions

Future goals of study include determining the effects of sulindac treatment on visceral adipose tissue (VAT) inflammation by measuring the incidence of crown-like structures, and determining tumor grade and inflammation in the colon through tissue analysis by veterinary pathologist. We also aim to measure prostaglandin E2 in the blood and adipose tissue due to the results of our study.

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