Iron and Chronic Inflammation

in Obese and Lean Men with Colon Cancer: The Link with Hepcidin

BY

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THESIS

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LIST OF ABBREVIATIONS

ΔCt	Difference in threshold cycle
ACD	Anemia of chronic disease
ACTB	Beta-Actin
BMI	Body mass index
BMP	Bone morphogenic protein
COX	Cyclooxygenase
CRC	Colorectal Cancer
CRP	C-reactive protein
DCYTB	Duodenal Cytochrome b
DMT-1	Divalent metal transporter-1
ELISA	Competitive enzyme linked immuno-assay
EPO	Erythropoietin
FFQ	Food Frequency Questionnaire
FPN	Ferroportin
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
HAMP	Hepcidin
HB	Hemoglobin
HCT	Hematocrit
HIF	Hypoxia-inducible factor
ID	Iron deficiency
IL-6	Interleukin-6
IQR	Interquartile range

LIST OF ABBREVIATIONS (continued)

JAK	Janus kinases
METS	Metabolic Syndrome
mRNA	messenger ribonucleic acid
NHANES	National health and nutrition examination survey
NSAIDS	Non-steroidal anti-inflammatory drugs
RNA	Ribonucleic acid
rT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
STAT3	Signal transducer and activators of transcription
sTFR	Serum transferrin receptor
TFR	Transferrin receptor
TIBC	Total iron binding capacity
TSAT	Transferrin saturation
TNF-α	Tumor necrosis factor-alpha
UIC	University of Illinois at Chicago
WC	Waist circumference

SUMMARY

Obesity and alterations in iron metabolism are independently associated with increased risk for colorectal cancer (CRC). Hepcidin, a hormone produced by the liver, regulates systemic iron metabolism by controlling the iron efflux transporter ferroportin (FPN). Circulating and stored iron, inflammation, erythropoiesis and hypoxia influences hepcidin expression. Obese individuals have low iron status due to inflammation induced hepcidin which results in decreased iron absorption and release into circulation. Iron is associated with CRC due to the ability to generate reactive oxygen species and promote carcinogenesis. The link with obesity and iron regulation in CRC remains largely unknown.

The main objective of this study was to determine whether obesity induced alterations in iron metabolism are associated with increased risk for colorectal cancer (CRC) in men. Colon cancer cases (n=20) and controls (n=20) were recruited from the University of Illinois at Chicago (UIC) and John H. Stroger Jr. (Cook County) Hospital gastrointestinal (GI) clinics. Within colonic mucosa (tumors for cases; healthy mucosa for controls) iron accumulation was assessed by Perl's Prussian Blue staining. Additionally, messenger ribonucleic acid (mRNA) expression of hepcidin, iron influx transporter divalent metal transporter 1 (DMT-1), iron efflux transporter ferroportin (FPN) and inflammation, interleukin-6 (IL-6) was measured in colonic mucosa. We assessed iron status by serum hepcidin, serum transferrin receptor (sTfR), hemoglobin (Hb) and hematocrit (Hct). We also measured circulating inflammatory markers: IL-6, C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α) in serum. Obesity was measured with waist circumference (WC) and defined as WC$\geq$ 102 cm (Obese) or WC<102 cm (Lean). To control for the influence in iron status in the analysis, we matched cases (n=16) and controls (n=15) on hemoglobin and waist circumference.

By design, cases and controls had similar demographic characteristics and obesity status. Cases had increased iron accumulation in their colonic tumors (n=6) compared to

controls (n=1). Colonic mRNA expression of hepcidin was lower (2.9 fold; p=0.01) and IL-6 was higher (9.40 fold; p=0.001) in cases versus controls. No difference with DMT-1 was observed between groups. FPN appeared lower (1.4 fold, not significant) in cases versus controls. Cases had lower iron status (higher sTfR; p=0.004) and higher markers of inflammation (CRP, p=0.01; IL-6; p=0.06) compared to controls. While cases had lower serum hepcidin (p=0.02) compared to controls, levels were within the normal range for adult men. After stratifying by obesity, WC and systemic inflammation was positively correlated in controls (CRP: r=0.47, p=0.04; IL-6: r=0.71, p=0.71). Although serum hepcidin was similar between obese and lean controls, within the colonic mucosa obese had directionality for higher hepcidin (1.59 fold) and lower ferroportin (1.92 fold) expression compared to their lean counterparts. No association was found between obesity and serum hepcidin within cases. Advanced stages had directionality for higher colonic iron uptake, DMT-1 (2.45 fold) and inflammation, IL-6 (60.97 fold) compared to controls. These findings remained similar after matching on hemoglobin and waist circumference.

In conclusion we found that colon cancer cases have serum hepcidin levels not reflective of their low iron status compared to controls. The elevated serum hepcidin (normal range) in these subjects is likely due to the tumor induced inflammation which may reduce duodenal iron absorption and uptake into circulation resulting in low iron status. Colonic tumors had increased iron accumulation compared to controls thus any unabsorbed iron that enters the colonic lumen may promote carcinogenesis. Lower colonic hepcidin expression in tumors compared to healthy mucosa may influence increased iron uptake. The regulation of hepcidin in colonic tumors is likely influenced by stimuli that suppress its expression (hypoxia or erythropoietin (EPO)) rather than inflammation (IL-6). Obesity induced inflammation may contribute to early mucosal

changes with hepcidin and FPN to increase susceptibility for excess colonic iron exposure and CRC risk. Future studies need to confirm the mechanisms affecting hepcidin regulation in colonocytes and examine whether obesity in populations at risk for CRC (i.e. adenomas) contributes to similar alterations and increased mucosal iron accumulation.

I. INTRODUCTION

1. BACKGROUND

Obesity and iron are independent risk factors for colorectal cancer (CRC). Mechanistic explanations for their increased risks have centered on the low-grade, pro-inflammatory, hyperinsulinemic environment of obesity and the generation of reactive oxygen species (ROS) from high iron exposure. We propose the inflammation that accompanies obesity induces alterations in iron metabolism that stimulates hepatic secretion of the body's main regulator of systemic iron homeostasis, hepcidin. Hepcidin controls the iron flow into circulation by post-translational regulation of the body's only known iron efflux transporter, ferroportin-1. Acutely, high hepcidin inhibits both the absorption of iron from the diet and the release of iron out of storage sites by obliterating ferroportin-1 expression.

During normal conditions dietary iron (heme and non-heme) is poorly absorbed in the small bowel and ultimately enters the fecal stream (unabsorbed) to expose the colonic lumen to iron. We hypothesize the obesity induced elevations in hepcidin will further decrease iron absorption, trap iron in the small bowel and, when sloughed into the gut lumen, ultimately deliver this internalized, unabsorbed iron to the colonocyte, which has the same iron import proteins as the small bowel. The excess colonic iron generates ROS, damage DNA, migrate from the bottom of the crypt to the lumen and continue proliferating rather than inducing apoptosis.

2. SPECIFIC AIMS AND HYPOTHESES

Specific Aim 1: To identify the variability of iron accumulation, hepcidin, inflammation (IL-6), the iron influx transporter DMT-1, and the iron efflux transporter ferroportin-1 in the colonic mucosa in men with CRC (cases) and controls

Hypothesis 1: Men with CRC will have higher iron accumulation, IL-6, DMT-1, and hepcidin expression, and lower ferroportin-1 in their colonic mucosa than controls

Specific Aim 2: To determine circulating inflammatory markers and hepcidin levels in cases and controls

Hypothesis 2a: Men with CRC will have higher circulating hepcidin and inflammatory markers (IL-6, CRP, TNF-α) than controls

Hypothesis 2b: Among cases, obese men will have higher circulating hepcidin and inflammatory markers (IL-6, CRP, TNF-α) than lean men

Specific Aim 3: To identify how obesity modifies non-involved mucosal and tumor iron accumulation

Hypothesis 3a: Among cases (controlling for stage), ***tumor*** iron accumulation, IL-6, DMT-1, and hepcidin will be higher and ferroportin-1 will be lower in obese compared to lean men

Hypothesis 3b: Among controls, ***mucosal*** iron accumulation, IL-6, DMT-1, and hepcidin will be higher and ferroportin-1 will be lower in obese compared to lean men.

Specific Aim 4: To identify how cancer stage modifies mucosal and tumor iron accumulation

Hypothesis 4: Later stage tumors will have greater iron accumulation, higher expression of IL-6, DMT-1 and hepcidin and lower expression of ferroportin-1 in their colonic mucosa compared to early stage tumors.

II. RELATED LITERATURE

1. IRON REGULATION AND HEPCIDIN

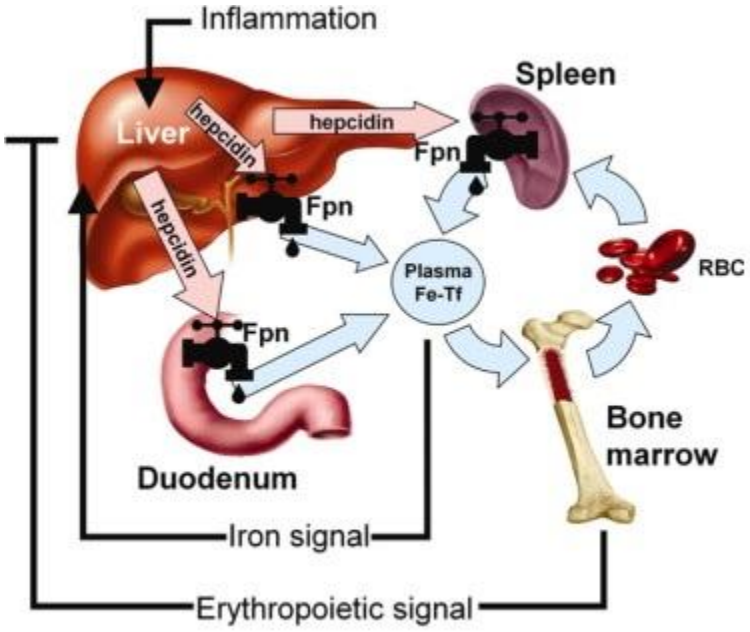
Iron is an essential element for cellular metabolism and key component of oxygen carrying proteins (1). A strict regulation of iron is required because iron is highly toxic and humans are unable to excrete large amounts. Cellular iron regulation is maintained by iron regulatory proteins (IRP) which bind to the iron responsive elements (IRE) within the mRNA of iron transporters to influence uptake, storage or release (2). When extracellular iron is high or low, systemic iron regulation is required to create a balanced concentration between the iron-transferrin complex in plasma and extracellular fluid which is achieved by controlling the iron release into plasma (1, 3). Iron can be introduced into the plasma by (1) absorption of dietary iron with duodenal enterocytes; (2) release of stored iron from hepatocytes; or (3) release of stored iron from reticuloendothelial macrophages. All three different cell types use ferroportin-1 (FPN), the only known iron export protein for the release of iron into the plasma. Disruption to FPN produces iron accumulation at any of these cell types and reduced iron bioavailability within the body (1, 4). These alterations could lead to either iron deficiency or toxicity.

Hepcidin, a peptide hormone primarily produced in the liver can act as a homeostatic regulator of iron metabolism and as a mediator of host defense and inflammation (Figure 1). During conditions of elevated circulating iron or when inflammation or infection is present, hepcidin is released into circulation and binds to FPN which triggers its internalization and degradation (5). The degradation of FPN results in increased iron sequestration within intestinal enterocytes, hepatocytes and iron-recycling macrophages reducing iron bioavailability (1, 4, 6). Conversely, during low iron status/anemia or hypoxic conditions, hepcidin expression is low or undetectable, thus ample FPN transporters are expressed to allow for maximal dietary iron absorption and mobilization from stores.

There are several pathways involved in the regulation of hepatic hepcidin expression (7).

When high concentrations of iron are in circulation, transferrin receptor-2 (TfR2) acts as an iron sensor and interacts with *HFE* to increase expression of hepcidin. During pro-inflammatory conditions interleukin-6(IL-6) increases hepcidin expression via the Janus Kinase(JAK)/Signal Transducer and transcription pathway (STAT-3) (8). Although less understood with iron status, Bone-Morphogenetic Protein (BMP) which can interact with hemojuvelin (*HJV*) at the plasma membrane to induce hepcidin expression.

Figure 1. Hepcidin in Systemic Iron Regulation



Adapted from Nemeth, E; Ganz, T. The role of hepcidin in iron metabolism. Acta Haematol. 2009 122 (2-3)..78–86.

2. IRON ABSORPTION

Daily only 1-2 mg of dietary iron is absorbed, primarily in the duodenum (9). This is approximately equal to the amount lost via sloughing of intestinal enterocytes, blood loss and sweat. Dietary iron exists in two forms: heme and non-heme, although, most dietary iron is present in the non-heme form. The mechanism for heme-iron absorption involves receptor mediated endocytosis (10). Non-heme iron absorption is initiated at the brush border of duodenal enterocytes via the reduction of ferric iron (Fe 3+) to ferrous iron (Fe 2+)via the membrane associated ferric reductase (Dcytb); the Fe+2 is then transported into the cell by divalent metal transporter-1 (DMT-1) at the apical membrane (11). Regardless of the mode of uptake of dietary iron, iron molecules absorbed within the enterocytes are either (a) stored as ferritin, (b) exported into the plasma by FPN at the basolateral membrane, or (c) lost with sloughing of the intestinal enterocytes (10, 12).

3. IRON DEFICIENCY

Iron deficiency (ID) is one of most prevalent nutritional deficiencies worldwide causing anemia. ID occurs due to poor dietary intake, increased iron demand required for growth and development, iron losses, and changes in blood volume (13-15). Iron deficiency develops in three stages beginning with depletion of iron stores (I), followed by diminished iron transport (II), and finally depletion of functional iron containing proteins and enzymes which results in ID with anemia (III) (16).

Hepcidin in those with ID is significantly low or undetectable (17, 18). This extremely low level allows for increased dietary iron absorption from enterocytes and release into circulation (19). Thus, diminished hepcidin concentrations in ID enable the body to maintain sufficient FPN concentrations and allow for increased iron uptake for erythropoiesis.

4. ANEMIA OF CHRONIC DISEASE

Unlike ID due to inadequate dietary iron intake, individuals with acute or chronic infection develop alterations in iron metabolism known as Anemia of Chronic Disease (ACD) (20). ACD is a host defense response mediated by pro-inflammatory cytokines (IL-6, CRP, tumor necrosis factor-α) that evolved to seize any iron from bacteria (1, 21-23). As a consequence to inflammatory conditions individuals with ACD have diminished erythropoiesis and decreased red blood cell half-life (24).

The ACD is characterized by low circulating iron, normal or increased iron storage and the presence of iron in bone marrow indicating impaired mobilization of iron from stores (25).. The use of serum transferrin receptor (sTfR) is preferred in individuals with inflammatory conditions unlike other more traditional markers for iron status (e.g. ferritin, serum iron, transferring) since it is unaffected by the acute phase response and accurately represents the cellular need for iron or rate of erythropoiesis (26). Increased sTfR levels are found in true iron deficient states. In addition, studies indicate that inflammation leads to hypoferremia and further into ACD though the IL-6 induced hepcidin mechanism (1, 6). Elevated hepcidin has been observed in individuals with ACD (17).

5. IRON STATUS AND OBESITY

An inverse relationship between adiposity and iron status has been well established in pediatric and adult populations (27-29). Overweight children and adolescents included in NHANES III were two times more likely to be iron deficient than those of normal weight (28). The obesity ID is associated with BMI and inflammation (measured by C-reactive protein) and not associated with age, race, dietary iron intake (heme and non-heme), factors affecting iron absorption, physical activity, or years since last menstruation (30, 31) . Additionally, excess dietary fat is associated with reduced iron absorption in mice which could be another reason for the association between obesity and low iron status, however the mechanisms remain unknown (32). Moreover, unlike traditional iron deficiency which occurs because of insufficient dietary iron

intake and/or blood loss, the iron depletion of obesity occurs through inflammation mediated iron metabolism dysregulation, resulting in decreased iron absorption and low iron status (30, 33, 34).

White adipose is a complex and active endocrine tissue with secretion of bioactive molecules from adipocytes, preadipocytes, and macrophages (35, 36). These bioactive pro-inflammatory molecules also referred to as adipokines include IL-6 and tumor necrosis factor alpha (TNF-α) among others (37). The release of these adipokines from macrophages lends to the systemic low-grade inflammatory state present in obesity (38).

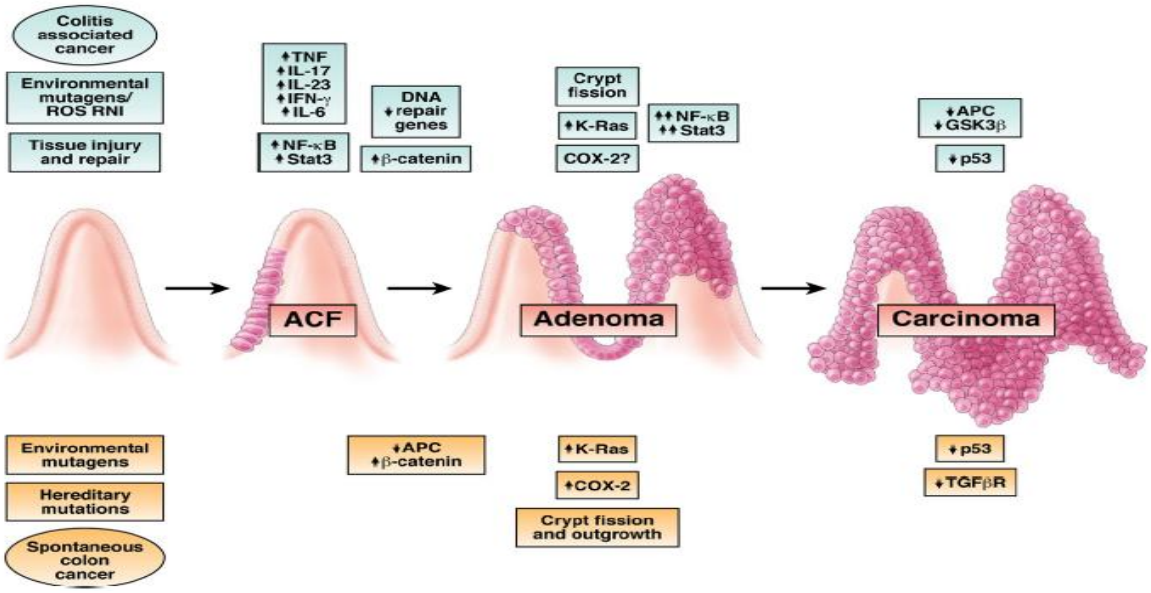
Yanoff et al. suggested that obesity presents a “mixed anemia” profile with characteristics of anemia of chronic disease superimposed on iron deficiency (33). Obese individuals have higher levels of serum hepcidin compared to lean individuals, but lower than the profile of anemia of chronic disease (27, 29, 39-41). Obese also have lower or sub-clinical iron status and not overt ID as indicated by the hemoglobin concentrations reported in the adult literature (18, 42). Hepcidin is the main determinant of the rate of iron absorption (21, 43, 44). The relationship with iron absorption and hepcidin is inversely correlated: at the duodenum, the mechanism may initiate with decreased iron efflux via hepcidin induced FPN downregulation, increased iron storage (ferritin), and subsequent decreased uptake (DMT-1) (45) . Young et al. reported in iron-replete women that serum hepcidin was inversely associated with iron absorption from supplemental and food-based non-heme iron sources (43). Elevated serum hepcidin concentrations appear to influence iron absorption, which may lead to a decreased uptake at the small bowel, or sloughing of iron rich enterocytes.

6. COLORECTAL CANCER

Colorectal Cancer is third in incidence and mortality of all cancers in the United States and worldwide rates parallel economic development reflecting an adaptation of a westernized lifestyle (46, 47). Only 10% of new cases are due to hereditary factors. The majority of CRC

occurs sporadically and environmental factors such as diet, exercise, lifestyle, are viewed as risk factors (48). These environmental factors can increase the susceptibility of mutations in genes responsible for the cell cycle growth, thereby allowing the development from adenomas or polyps to malignant adenocarcinomas (Figure 2). Several key genes play a role in the development of CRC: the *APC* gene, a tumor suppressor gene, is one of the first genes that may become mutated and is expressed in 80% of adenomas and adenocarcinomas (49, 50); the *K-ras* gene, an oncogene responsible for cell proliferation is also mutated in CRC; *p53* gene, a tumor suppressor, is believed to be the gene mutated that drives adenoma to adenocarcinoma (50) . These genes have been implicated to play a role in both sporadic CRC and inflammation associated CRC (51). Obesity may also increase the susceptibility of these gene mutations (52).

Figure 2. Sequence from Healthy Mucosa to Carcinoma



Adapted from Terzic J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer Gastroenterology. 2010;138(6)2101-14.

7. OBESITY AND COLORECTAL CANCER

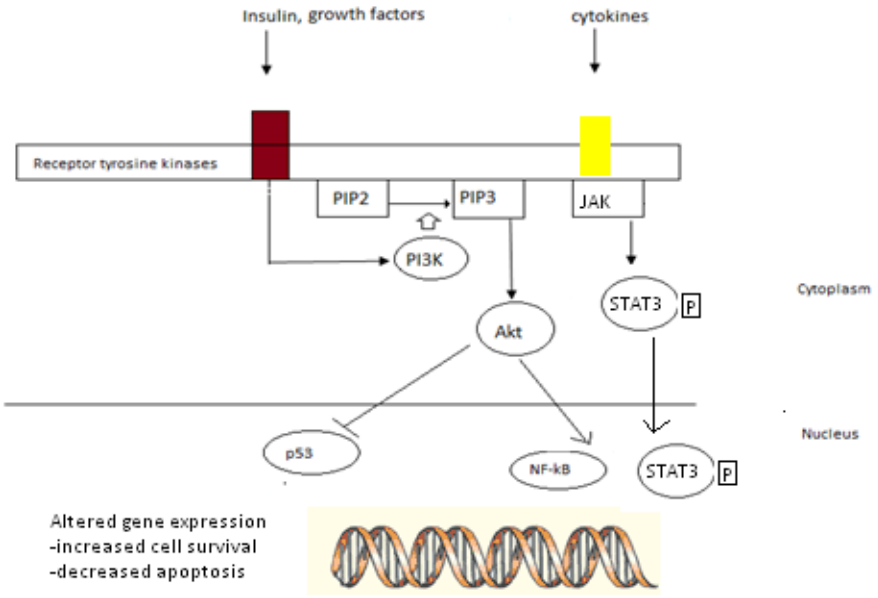
Several large prospective cohort studies have consistently demonstrated positive associations between obesity and CRC (53-55). A dose-response relationship with body mass index (BMI) or waist circumference (WC) exists with CRC (56). Men have greater risks than women which may be due to their greater abdominal circumference (57, 58). Risks for CRC increased 33% per 10 cm increase in WC and 43% per 0.1 unit increase waist-hip ratio (59). Both high circulating insulin (hyperinsulinemia) and low grade inflammation which accompanies obesity have been postulated to explain these increased risks (56, 59-61).

Elevated serum-C peptide, a marker of insulin secretion, is associated with increased

CRC risk (60, 62, 63). Insulin and insulin-growth-factor-1 (IGF-1) may bind to the tyrosine kinase receptors on colonic tissue and activate the protein kinase B (Akt) pathway (Figure 3). This binding activates signaling pathways for enhanced tumor growth and reduced apoptosis (52, 64). Initially, receptor tyrosine kinases (RTKs) at the cell surface have binding affinity for insulin, IGF-1, and other growth factors. When circulating IGF-1, insulin, or growth factors bind to RTKs, activation of the lipid kinase PI3K is induced which then phosphorylates PIP2 (Phosphatidylinositol 4,5-bisphosphate, a phospholipid) into PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate), which phosphorylates Akt (65). Once Akt is activated by phosphorylation, a cascade of other downstream targets in the cytoplasm become phosphorylated including the oncogenic protein Mdm2 which blocks the transcription of the tumor suppressor gene *p53* (66).

As illustrated in Figure 3 elevated levels of the pro-inflammatory cytokines TNF-α and IL-6 may also increase CRC risk (67, 68). It is hypothesized that elevations in these cytokines precipitate CRC via enhanced binding to their respective receptors on colonic tissue, resulting in upregulation of the pro-oncogenic transcription factors STAT3 and NF-κB (51, 69). Binding of these cytokines to their receptors on colonocytes allows the Jak enzyme to phosphorylate STAT3 to allow its translocation to the nucleus. Additionally, the Akt enzyme phosphorylates the cytoplasmic IκB complex (an inhibitor of NF-κB) which becomes degraded, thereby liberating NF-κB to enter the nucleus. Within the nucleus STAT3 and NF-κB can increase angiogenesis and invasiveness to allow tumors to become more resistant to apoptosis (69, 70).

Figure 3. Proposed Pathways for Obesity and Colorectal Cancer



8. COLONIC INFLAMMATION IN OBESITY

Obesity has recently been associated with chronic inflammation in the colonic mucosa (71, 72) characterized by elevated levels of circulating inflammatory cytokines, inflammation in the colon and upregulation of transcription factors STAT3 and NF- κB enhancing CRC risk. Furthermore, significant reductions in these inflammation and pro-oncogenic pathway expressions occurred following a 10% weight loss in these participants (72).

9. OBESITY AND ADENOMAS

The vast majority of colon adenocarcinomas develop from adenomas or polyps. Early detection and removal of adenomas are the standard of care for risk reduction of CRC (73). While obesity is associated with increased CRC risk, very few studies have explored if obesity contributes to adenoma development. Obesity has been associated with greater risks

adenomas in men but not women (74). A higher prevalence of adenomas for obese young males and premenopausal women has also been reported, however misclassification of adenomas and a small sample size limit interpretation of these findings (75). Investigation of circulating inflammatory cytokines IL-6 and TNF-α have been associated with adiposity and also an increased risk of colorectal adenomas (68).

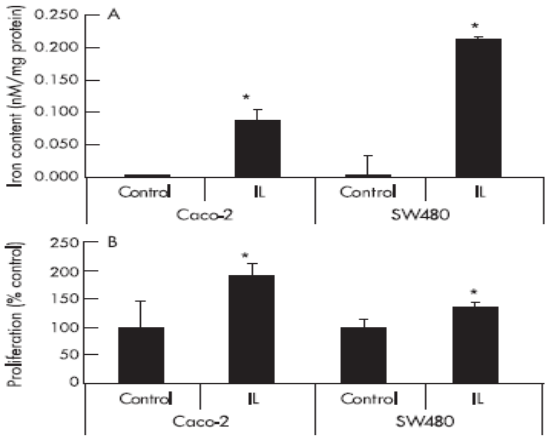
10. COLORECTAL CANCER AND IRON AT TISSUE LEVEL

Several animal and human studies have suggested that excess iron results in carcinogenesis (76-78). Brookes et al. examined normal and CRC samples from human colonic mucosa for iron accumulation and iron transporter expression (79). While they found no detectable iron staining (DAB enhanced Perl's staining) in normal colonic mucosa, the CRC samples had iron accumulation. Further, there was an increased expression of iron influx transporters (DMT-1, Dcytb, TfR1) and decreased efflux transporters (FPN, HEPH) in the tumors.

Iron has been proposed to play several key roles in the pathogenesis of CRC. Luminal iron may act as an initiator of CRC carcinogenesis with DNA damage as a result of the generation of reactive oxygen species (ROS) via the Fenton reactions. Colonic bacteria produce significant amounts of superoxide molecules. It has been postulated elevations of iron content in this superoxide rich environment may generate ROS which damage DNA and lead to mutations in key genes that regulate cell survival and proliferation (e.g. the *APC* gene and the β-catenin pathway) (51, 80).

Iron may also facilitate the promotion of cancer. Iron is a key nutrient required for cell growth via acting as a co-factor in DNA synthesis. Brookes et al examined the influence of iron on two cancer cell lines (Caco-2 and SW480) and found increased growth and proliferation in the iron loaded medium compared to those in the normal growth medium (Figure 4) (79).

Figure 4. Iron Content and Cell Proliferation in Colonic Tumors versus Controls



Adapted from Brookes MJ, Hughes S, Turner FE, Reynolds G, Sharma N, Ismail T, et al. Modulation of iron transport proteins in human colorectal carcinogenesis Gut. 2006 Oct;55(10)1449-60.

11. COLORECTAL CANCER AND IRON STATUS IN CIRCULATION

The prevalence of anemia (defined at baseline as < 12g/dL) in CRC has been reported to be about 44% (81, 82). Several mechanisms can contribute to the anemia seen in cancers, such as GI bleeding, chemotherapy, and the role of the immune system with hepcidin (83). However, the anemia or low iron status in colon cancer patients has not been well characterized.

12. HEPCIDIN AND COLORECTAL CANCER

The role of hepcidin in CRC has been limited in the literature. Since hepcidin acts to reduce FPN expression and iron export into circulation, it would be hypothesized that cancer cells exhibit more hepcidin expression to sequester iron for tumor needs. One study by Ward et al. (84) examined expression of hepcidin in both human CRC tissue and urine. They found higher mRNA hepcidin expression within the tumor (34 %;10 out of 34 tumor samples) compared to non-involved tissue (6%; 2 out of 34 non-involved samples) and that increased urine hepcidin was associated with advanced staging.

13. OBESITY, IRON AND COLORECTAL CANCER

While obesity and iron have been linked independently to the pathogenesis of CRC, the relationship between the obesity induced low-grade chronic inflammation and its alterations in iron metabolism and risks for CRC remains unexplored.

As described earlier, obesity precipitates low iron status via increases in hepatic hepcidin synthesis. Elevations in hepcidin traps iron in the small bowel, ultimately to deliver internalized, unabsorbed excess iron to the colonocyte when sloughed into the gut lumen. Genetic mutations occur via excess iron and ROS generation within the intestinal stem cell. Cells with damaged DNA migrate from the bottom of the crypt to the lumen and continue proliferating rather than inducing apoptosis (73). Furthermore, for existing tumors, elevated hepcidin may enhance iron retention by tumor cells, thus promoting tumor growth.

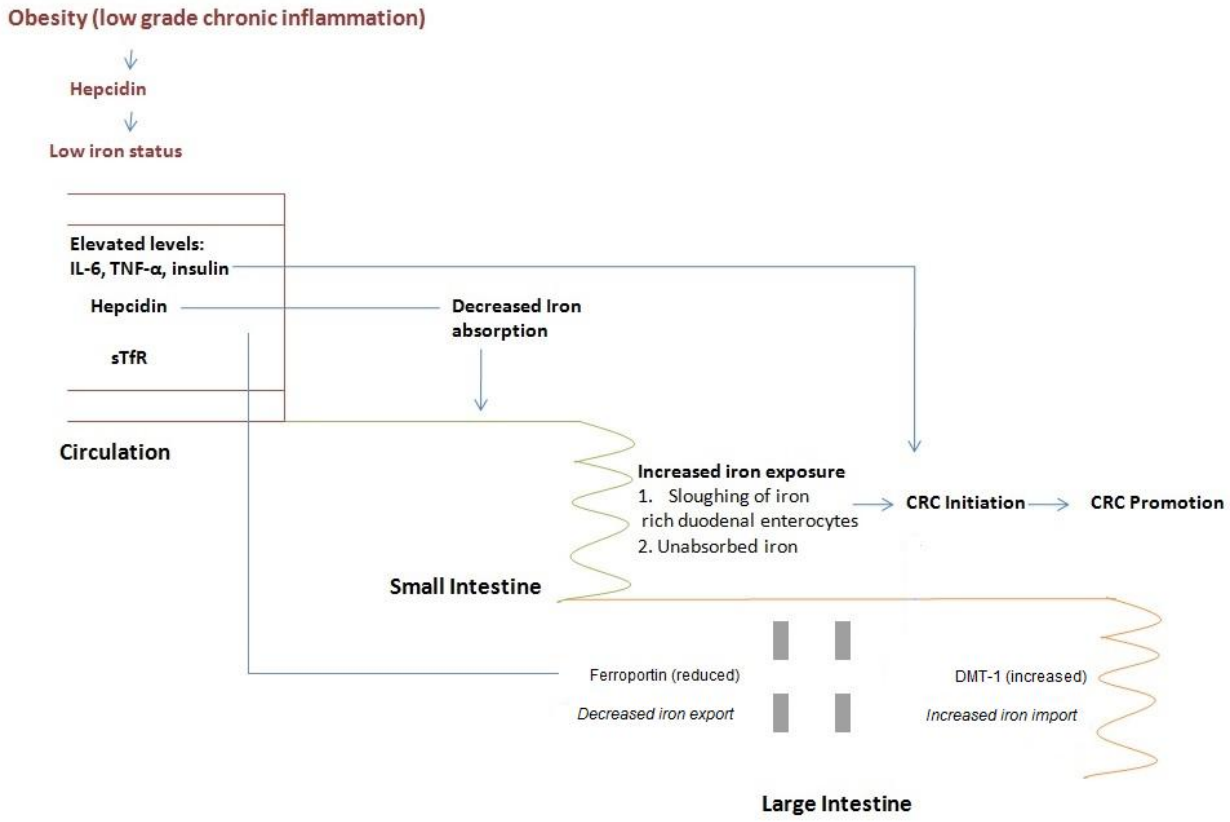
14. SUMMARY:

It has been demonstrated that (a) obesity alters iron metabolism resulting in iron sequestration within cells and reduced iron uptake within the GI tract (b) the colonic epithelium of obese individuals is inflamed (c) obesity and iron are each independently associated with increased CRC risks (d) tissue from colonic tumors have detectable iron and that iron accumulation is

higher with advanced staging. **The interaction between *the alterations in iron metabolism that accompany obesity* on CRC risks *remains unknown.***

This case-control study explores if obese men with CRC have more pronounced inflammation than lean men with CRC or lean or obese controls and if this heightened inflammatory state is accompanied by greater colonic inflammation, iron accumulation, hepcidin and higher iron influx and lower iron efflux proteins. Further, we explore if these characteristics become more pronounced in more advanced stages of CRC (Figure 5).

Figure 5. Proposed Pathway for Obesity, Inflammation and Iron in Colorectal Cancer



III. RESEARCH DESIGN AND METHODS

1. STUDY DESIGN AND POPULATION

A case-control design was created to assess the risks for CRC associated with chronic low grade inflammation present in obesity along with alterations in iron metabolism. A total of 40 (20 cases and 20 controls) adult men were included. These individuals were consented for recruitment from the patient scheduling chart for colonoscopies at the University of Illinois at Chicago (UIC) and John H. Stroger Jr. (Cook County) Hospital, gastrointestinal (GI) Clinics. Cases that were missed in endoscopy were approached on the day of surgery for recruitment. Cases are classified as sporadic adenocarcinoma; controls are classified as individuals with healthy colonic mucosa. This investigation is a component of the Chicago Colorectal Cancer Consortium (CCCC), a prospective study with Dr. Xavier Llor as the Principal Investigator, examining genetic and environmental risk factors for CRC.

Additional inclusion criteria include first occurrence of CRC for cases, English speaking, and no prior history of any cancers for controls.

Cancer staging in cases was defined by the American Joint Committee on Cancer (AJCC) TNM staging system: Stages I-IV.

2. EXCLUSION CRITERIA

Subjects were excluded if they are female, have rectal cancer, GI bleeding, a history of inflammatory bowel diseases (ulcerative colitis or Crohn's disease) or first degree family history of CRC.

Rational for exclusion: Individuals with colitis or IBD present an immune mediated disease state and have an increased risk of developing CRC. Individuals with family history of CRC (i.e. those with familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC),or first-degree relative with CRC) are a unique population with mechanisms for CRC that differ from the general populations risk factors. Due to differences in morphology between

colon and rectum, cancers of rectum origin were excluded. Active GI bleeders were excluded since this confounds the relationship between obesity and iron status. Females were excluded due to their differences in iron metabolism and reference ranges with iron parameters. Iron deficiency is more prevalent in women compared to men during the life cycle due to blood losses during menstruation and greater iron requirements in pregnancy. In addition, one of the mechanisms linking Obesity and CRC risk is the ability of high levels of insulin to bind to the receptor tyrosine kinases (RTKs) to upregulate cell proliferation. However the association of BMI and CRC seems to differ by menopausal status and hormone replacement therapy (85). In premenopausal women, where ovarian sources of estrogen are important, there is evidence that estrogen may upregulate insulin-growth-factor receptors thus increasing cell proliferation, while in post-menopausal women with hormone therapy there has been a decrease risk of CRC since the effect of estrogen and insulin appear to cancel each other (86).

3. SAMPLING PROCEDURE

The CCCC is IRB approved at multiple hospitals in Chicago for administering questionnaires, collecting blood and tissue samples. Subjects were approached prior to their endoscopies for recruitment. Those expressing interest had eligibility determined and written informed consent was obtained prior to any data collection.

Questionnaires (Medical History, Food Frequency Questionnaire) were completed in a designated private area of the clinics. Subjects were be fasting for their procedures, therefore due to timing and practicalities of drawing blood, blood samples were obtained after the procedure for endoscopy subjects and during the procedure for surgical patients; tissue biopsies (tumor and healthy mucosa) were obtained during the endoscopy for endoscopy subjects, and immediately after the tumor specimen has been removed for surgical subjects; paraffin-embedded tissue for cases will be obtained from the Department of Pathology at Stroger/UIC.

10 mL of whole blood collected in a serum separator tube was brought to Dr. Fantuzzi's lab at UIC within two hours and centrifuged at 1500 rpm for 10 minutes to allocate serum, which was kept frozen at -80 degrees Celsius until analysis. The tissue samples were stored in RNAlater and brought to Dr. Llor's lab at UIC within two hours; stored at +4 degrees Celsius for 24 hours; kept -80 degrees Celsius until analysis. For analysis RNA was extracted from the tissue samples at Dr. Llor's lab at UIC and sent to Dr. Nemeth's Lab at UCLA with dry ice to measure the gene expression of DMT-1, FPN, hepcidin, and IL-6. Categorization as a case or control occurred via the pathology report from the medical records.

4. SAMPLE SIZE ESTIMATION

Sample size calculations were based on hepcidin levels in colon cancer because these levels were hypothesized to be mechanistically linked to the heightened CRC risks with obesity. However, at the start of this study there was a lack of data for hepcidin levels in colon cancer patients versus healthy individuals. Therefore, initial sample size calculations was based on serum hepcidin levels for women undergoing bariatric surgery, then we re-assessed sample size calculations after analyzing 20 cases and 20 controls using the mean and standard deviations for mRNA expression of hepcidin in colonic tissue(tumors versus control tissue). The effect size for differences between cases (-10.28 $\pm$ 1.52 ΔCt, reference-target) and controls (-8.77 $\pm$ 1.06 reference-target ΔCt) was 1.51. A sample size of 12 in each group would be sufficient for identifying an effect size of this order of magnitude or larger with 80% statistical power at the 5% level of significance.

5. MEASUREMENT OF VARIABLES

a. Demographics, Medical History and Anthropometrics

Demographic and medical information: Demographic and medical information including date of birth, marital status, race/ethnicity, level of education, insurance type, family history of cancer, history of GI malignancies, medication use, smoking/alcohol history, and exercise were obtained from the CCCC interview questionnaire.

Height and Weight: Subjects were weighed and height measured upon check in to the endoscopy clinic. Weight is recorded closest to 0.1 pounds and height is recorded to 0.1 inches. Subjects were wearing gowns.

Body Mass Index: BMI was calculated using the height and weight readings obtained as described above using the formula BMI= (Weight in Pounds)/ ((Height in inches X Height in inches)) X 703. BMI was classified according to the guidelines for healthy weight, so males with a BMI of 18.5-29.9 were classified as lean or normal weight while those with a BMI of 30.0 or above were classified as obese (87).

Waist Circumference: Waist circumference was measured with participants in the standing position. An inelastic measuring tape with a metal tape measure was used. Three measurements were taken to the nearest 0.1cm with the subject wearing a gown. The measurement was taken as the midpoint between the ribs and iliac crest. If this section of the waist was difficult to identify, the smallest horizontal circumference was measured in this area. Waist circumference was classified according to the guidelines for healthy weight, so males with a waist circumference less than 102 cm were classified as lean, whereas individuals with a waist circumference greater or equal to 102 cm were classified as obese (87).

Tumor Localization/Staging: Location and staging of tumors was obtained from the surgical pathology report. Staging was based on the American Joint Committee on Cancer (AJCC) TNM staging system (e.g. Stage I, II, III, or IV). Localization may be left sided colon (splendic flexure,

descending, sigmoid, recto-sigmoid) or right sided (transverse, ascending, hepatic flexure, cecum).

b. Serum Measurements

Hemoglobin and Hematocrit: Hemoglobin (Hb) and Hematocrit (Hct) levels were recorded from the subject's medical records obtained near the date of their procedure. Hb was expressed as g/dL and Hct was expressed as %.

Serum transferrin receptor (sTfR): sTfR was measured using the Quantikine IVD Human sTfR Immunoassay ELISA (R&D Systems, Minneapolis, MN). 250 µl of serum was used to run this assay in duplicate by the Fantuzzi lab in the Department of Kinesiology and Nutrition at UIC. This assay uses a monoclonal antibody sandwich ELISA. Samples and controls were added to each to the wells, followed by a wash for any unbound substances, then an enzyme linked polyclonal antibody specific for sTfR was added, following a second wash a substrate was added, then after an incubation, an amplifier solution was added and color develops. The optical density (OD) was measured by a spectrophotometer to read at 450nm. The intensity of the color was directly proportional to the amount of sTfR in the plate. The manufacturers expected range is 8.7-28.1 nmol/L.

Serum hepcidin: Serum hepcidin levels were measured using an ELISA developed and patented by Intrinsic LifeSciences (La Jolla, CA). 100 µl of serum stored frozen at -80°C was shipped with dry ice to Intrinsic LifeSciences for analysis. For men with normal iron status, the 5-95% range for this assay is 29-254 ng/mL (17).

BMP-2: BMP-2 was measured using R&D Systems Quantikine Human BMP-2 ELISA (R&D Systems, Minneapolis, MN). 100 µl of serum stored at -80°C was shipped with dry ice to the Nemeth Lab at UCLA for analysis. This assay uses a monoclonal antibody sandwich ELISA.

Samples and controls were added to each to the wells, followed by a wash for any unbound substances, then an enzyme linked polyclonal antibody specific for BMP-2 was added, following a second wash a substrate was added, then after an incubation, an amplifier solution was added and color develops. The optical density (OD) was measured by a spectrophotometer to read at 450nm. The intensity of the color was directly proportional to the amount of BMP-2 in the plate. The manufacturers expected range is 62.5-4,000 pg/mL.

C-reactive protein: 100 µl of serum was used to measure CRP using this assay in duplicate by the Fantuzzi Lab in the Department of Kinesiology and Nutrition at UIC. CRP was measured using R&D Systems Quantikine Human CRP ELISA (R&D Systems, Minneapolis, MN). This assay uses a monoclonal antibody sandwich ELISA. Samples and controls were added to each to the wells, followed by a wash for any unbound substances, then an enzyme linked polyclonal antibody specific for CRP was added, following a second wash a substrate was added, then after an incubation, an amplifier solution was added and color develops. The optical density (OD) was measured by a spectrophotometer to read at 450nm. The intensity of the color was directly proportional to the amount of CRP in the plate. The manufacturers expected range is 0.78-50 ng/mL.

Interleukin-6: Interleukin-6 (IL-6) was measured using the R&D systems Quantikine High Sensitivity (HS) ELISA Human IL-6 (R&D Systems, Minneapolis, MN). 200 µl of serum was used to run this assay in duplicate by the Fantuzzi lab in the Department of Kinesiology and Nutrition at UIC. This assay uses a monoclonal antibody sandwich ELISA. Samples and controls were added to each to the wells, followed by a wash for any unbound substances, then an enzyme linked polyclonal antibody specific for IL-6 was added, following a second wash a substrate was added, then after an incubation, an amplifier solution was added and color develops. The optical density (OD) was measured by a spectrophotometer to read at 490nm. The intensity of the color was directly proportional to the amount of IL-6 in the plate. The manufacturers expected range is 0.156-10 pg/mL.

Tumor necrosis factor-alpha: Tumor necrosis factor-alpha (TNF-α) was measured using the R&D systems Quantikine High Sensitivity (HS) ELISA for Human TNF-α (R&D Systems, Minneapolis, MN). 200 µl of serum was used to run this assay in duplicate by the Fantuzzi lab in the Department of Kinesiology and Nutrition at UIC. This assay uses a monoclonal antibody sandwich ELISA. Samples and controls were added to each to the wells, followed by a wash for any unbound substances, then an enzyme linked polyclonal antibody specific for TNF-α was added, following a second wash a substrate was added, then after an incubation, an amplifier solution is added and color develops. The optical density (OD) was measured by a spectrophotometer to read at 490nm. The intensity of the color was directly proportional to the amount of TNF-α in the plate. The manufacturers expected range is 0.5-32 pg/mL.

Insulin: Insulin was measured using the ALPCO Diagnostics ELISA for Human Insulin (ALPCO Diagnostics, Salem, NH). 250 µl of serum was used to run this assay in duplicate by the Fantuzzi Lab in the Department of Kinesiology and Nutrition at UIC. This assay uses a monoclonal antibody sandwich ELISA format. The microplate was incubated twice and then the optical density (OD) was measured by a spectrophotometer to read at 450nm. The intensity of the color was directly proportional to the amount of insulin in the plate. The manufacturers expected range is 3 - 200 µIU/mL.

Glucose: Fasting glucose levels were recorded from the medical charts for blood work recorded near their procedure. Values were expressed as mg/dL.

c. Iron Staining

For cases, a paraffin-embedded tissue block was obtained from the Department of Pathology at UIC or Stroger. For controls, fresh tissue sections was harvested from endoscopy, placed in cassettes and fixed using formalin. Formalin fixed tissue was provided to the Research Histology and Tissue Imaging Core, who made tissue sections (tissue embedded

paraffinized histological slides). Tissue iron accumulation was measured using Perl's Prussian Blue Staining. The tissue section is treated with dilute hydrochloric acid to release ferric ions from binding proteins. These ions then react with potassium ferrocyanide to produce an insoluble blue compound (the Prussian blue reaction). The grading of the iron staining expression was as follows: 0=No iron presence, 1=Yes iron presence.

d. Measurement of Iron Transporters and Inflammation In Colonic Tissue

mRNA expression *(Divalent Metal Transporter-1, ferroportin, hepcidin, Interleukin-6)*: Fresh colonic tissue (adenocarincoma mass for cases, healthy mucosa for controls) was obtained in endoscopy or surgical clinics and immediately harvested in RNAlater stabilization reagent (Qiagen Inc., Hilden, Germany), then stored at +4° C for 24 hours, then transferred over to -80°C for storage. After the samples were collected and stored, RNA extraction was performed by the Llor Lab in the Department of Medicine at UIC, using the Maxwell 16 by Promega Instrument (Promega, Fitchburg, Wisconsin). RNA quality was assessed by Core Genomics Lab at UIC to insure adequate RNA band intensity. 800 ng of extracted RNA was sent over dry ice overnight to the Nemeth Lab at UCLA for gene expression using q RT-PCR. mRNA was converted to cDNA using BioRad iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) for reverse transcription with BioRad thermal cyclers, Controls: CFX Connect Real-Time System, Cases: CFX96 C1000 Touch. To measure mRNA expression of Divalent Metal Transporter-1(DMT-1), ferroportin, hepcidin, interleukin-6 (IL-6), and reference genes: Glyceraldehyde 3-phosphate dehydrogenase(GADPH), β-actin, this was performed by q-RT PCR using SsoAdvanced SYBR Green Supermix (BioRad CFX thermal cyclers). The following primers were used: human DMT-1 5'-TGAACCTAAAGTGGTCACGC -3' (forward-1), 5'-GGGTATGAGAGCAAAGGGAAG-3' (reverse-1), 5'-TCTACTTGGGTTGGCAATGTTT-3' (forward-2), 5'-GGCTACCTGCAGAAGACAGACT-3' (reverse-2); human ferroportin 5'-

CGGTGTCTGTGTTTCTGGTAGA-3' (forward-1), 5'-CTGGGCCACTTTAAGTCTAGC-3' (reverse-1), 5'-TTACCAGAA AAC CCC AGCTCTAG-3' (forward-2), 5'-AGTCTTTCACACCCATTAGATGAG-3' (reverse-2); human hepcidin 5'-CTGCAACCCCAGGACAGAG-3' (forward-1), 5'-TCTACGTCTTGCAGCACATCC-3' (reverse-1), 5'-CTTCCCCATCTGCATTTTCTG-3' (forward-2), 5'-CCAGCCATTTTATTCCAAGACC-3' (reverse-2); human IL-6 5'-CAC TCA CCT CTT CAG AAC GAA TTG A-3' (forward-1), 5'-GCC ATC TTT GGA AGG TTC AGG TTG-3' (reverse-1), 5'-CTT TTG GAG TTT GAG GTA TAC CTA GAG-3' (forward-2), 5'-GTC AGG GGT GGT TAT TGC ATC TAG-3' (reverse-2); human GADPH 5'-GAA GGT GAA GGT CGG AGT CAA-3' (forward), 5'-CAT GGG TGG AAT CAT ATT GGA ACA T-3' (reverse); human β-actin 5'- ACA CCT TCT ACA ATG AGC TGC GT-3' (forward), 5'- GATAGCACAGCCTGGATAGC-3' (reverse). The PCR cycling protocol was 1x 95°C for 3 minutes, 40x 95°C for 10secconds; 57°C for 30 seconds and the melt curve was 65 - 95°C in 0.5°C increments. The expression of DMT-1, ferroportin, hepcidin, and IL-6 were all normalized to β-actin and GADPH and the results are presented as difference in threshold cycle (ΔCt) using Ct(reference) - Ct(target), where reference equals to β-actin and GADPH and target equals gene of interest . For example, to calculate the expression of hepcidin mRNA: ΔCt(hepcidin)= Ct(β-actin/GADPH)- Ct(hepcidin). Using this method, the higher the value, the higher gene expression. We also calculated mRNA expression as 2 ^-delta delta (ΔΔCt), allows for an simplified interpretation as fold change or '*relative expression*' of cases (target) compared to controls (reference), where ΔΔCt= ΔCt(hepcidin controls)- ΔCt(hepcidin cases).

e. <u>Dietary Intake</u>

The Block Brief 2000 food frequency questionnaire (FFQ) was used to assess dietary intake. This questionnaire (88) is used to assess participants' recent dietary intake; it includes 70 items with commonly consumed portion sizes listed beside each food. Each subject was asked to recall what he has eaten and drank during past the 12 months. For each item,

frequency of consumption will be asked either per day, week or month.

6. STATISTICAL ANALYSIS

All data was entered and cleaned in Epi Info version 3.5.3 (Centers for Disease Control and Prevention (CDC), Atlanta, GA) and statistical analysis was performed by Statistical Analysis System (SAS) version 9.2 (SAS, Cary, NC). Level of significance was defined as $p<0.05$. Continuous variables were assessed for normal distribution and presence of outliers with normality plots and Shapiro-Wilk test. Since the majority of the continuous variables were not normally distributed we used non-parametric Spearman correlation coefficient analysis to examine the associations between continuous variables. To compare differences between continuous variables we used student's *t*-test for normally distributed variables and Mann-Whitney *U* test (or Wilcoxon-rank-sum test) for variables that were not normally distributed. The chi-squared test or Fisher's exact test was performed to compare categorical variables. Multivariable linear regression was used to adjust for any confounders.

a. Statistical Analysis for Subject Characteristics

This is a case-control study design, therefore controls should be representative of cases except disease state. Data on medical history (obtained with the CCCC questionnaire(Appendix A)) for each subject was presented to describe how cases and controls compare. For demographics, Age was defined as age at diagnosis; Education was classified into three groups as highest earned degree: College, High School, or below High School; Race was classified into four groups: Black/African American, White/Caucasian, Hispanic, or Asian/Pacific Islander; Martial Status was classified into three groups: Single, Married, or Divorced. For anthropometrics, we defined obesity dichotomously (i.e. obese=1 and lean=0) using two different methods as BMI $\geq$ 30 as obese and < 30 for lean, and using waist circumference $\geq$ 102

cm as obese and <102 cm as lean. Exercise was classified into five groups: Daily, 2-3 times per week, once per week, 1-2 times per month, or never. A dichotomous significant exercise variable was also created to account for duration and intensity of activity and defined as yes=1 or no=1. Medication use was also obtained. Each medication was classified dichotomously (i.e. yes=1 or no=0) for regular intake, which was at least three times per week within the last five years. Dietary intake data assessed with the Block Brief 2000 FFQ questionnaire is presented as intake per day.

For categorical variables, percentages are presented along with p-value for chi-squared test to compare differences between cases and controls. Continuous variables that were normally distributed are presented as mean $\pm$ standard deviations and the p-value for student's *t*-test else variables that were not normally distributed are presented as median and interquartile range (IQR) with the p-value for non-parametric Mann-Whitney *U* test.

b. Statistical Analysis for Colonic Tissue Findings

The goal of hypothesis 1 was to compare iron accumulation and expression of iron transporters and inflammation in colonic tissue between cases and controls. Iron accumulation was defined dichotomously for presence of iron in colonic tissue (i.e. yes=1 or no=0), and presented as percentages of expressed 'yes' within each disease state. We also calculated Odds Ratios and 95% Confidence Intervals for exposure of iron accumulation and colon cancer risk. To characterize the subjects for their iron accumulation, we stratified on iron accumulation presence and serum parameters and mRNA expression was compared within cases and controls. The parameter values were presented as mean $\pm$ standard deviations and the p-value for student's *t*-test otherwise we presented median and interquartile range (IQR) with p-value for non-parametric Mann-Whitney *U* test. Additionally, tumor location, cancer staging, medication use, and dietary iron intake were presented by iron accumulation presence and differences

between groups was assessed by Fisher's exact test. The mRNA data for tissue level expression of iron transporters and inflammation as presented in two methods. First, threshold cycle (ΔCt) for each parameter is expressed as continuous variable as mean ± standard deviations and the difference between cases and controls is performed by the student's *t*-test. The second method using the 2^-(ΔΔCt) conversion (explained in III.5.d.), where the fold-change for cases is presented compared to the controls as the reference.

c. Statistical Analysis for Serum Findings

The goal of hypothesis 2a was to compare the serum parameters (inflammatory cytokines, hepcidin, iron status, BMP-2, insulin, glucose) between cases and controls. These variables were all continuous and if they were normally distributed the results are presented as mean ± standard deviations and the p-value for student's *t*-test or for variables that were not normally distributed we presented median and interquartile range (IQR) with p-value for non-parametric Mann-Whitney *U* test. We attempted to log transform the serum data, however this did not change the distribution or direction of the associations. Since the majority of the serum variables were not normally distributed we also provided a table with only means ± standard deviations for all serum parameters. Findings are also presented by cases and controls matched on hemoglobin and waist circumference. We performed hemoglobin and waist circumference matching by removing the four lowest hemoglobins for cases and three highest hemoglobins for controls, as well as the three highest waist circumference for controls. Linear regression was performed to compare serum parameters between groups, after controlling for hemoglobin and waist circumference in separate models. The model was expressed as the serum parameter of interest as the dependent variable whereas the independent variables were disease status (case/control) and predictor controlling for (e.g. hemoglobin): serum hepcidin=β(disease) + β(Hb) + β(0).

d. Statistical Analysis for Obesity and Staging Findings

The goals of Hypotheses 2b, 3, and 4 were to compare how obesity and cancer staging modify the tissue and serum parameters in cases (only staging) and controls. For obesity, each cases and controls was grouped into obese or lean, therefore we had four overall groups: obese cases, lean cases, obese controls, and lean controls. For serum and mRNA (ΔCt), variables that were normally distributed are presented as mean ± standard deviations variables otherwise they are presented as median and interquartile range (IQR). For the obese versus lean comparison within cases, we performed multiple linear regression adjusting for staging (using stage 4 as a dummy variable). Within controls we used the student's *t*-test for normally distributed data or the non-parametric Mann-Whitney *U* test for variables. For the mRNA data we also calculated relative expression of the parameters with obesity using the 2^-(ΔΔCt) method with lean controls (healthiest group) as the reference. For cancer staging, cases were stratified into four groups and serum and mRNA data is presented as mean ± standard deviations for normally distributed variables or else median and interquartile range (IQR). A trend between groups was assessed by one-way analysis of variance (ANOVA) test with three degrees of freedom, else with variables that were not normally distributed we performed the Kruskal-Wallis test.

IV. RESULTS

1. SUBJECT CHARACTERISTICS

The primary analysis consisted of 20 cases and 20 controls, where all subjects had questionnaires administered (demographics, medical and medication history, dietary intake), and serum, colonic tissue (tumor for case, healthy mucosa for controls), paraffin embedded blocks collected.

a. Demographics and Anthropometrics

Demographics and anthropometrics of the cases and controls are presented in Table I. As designed the distribution of obese and lean participants were similar between cases and controls. Overall, the mean age, height, median weight, BMI and waist circumference, education, insurance type, race ethnicity, marital status and exercise frequency was similar between groups.

Table I. Demographics, Anthropometrics*

		Case(n=20)	Control (n=20)	P-value[a b]
Age(years)		61.2 ± 8.4	57.8 ± 6.6	0.16
Height(in)		69.6 ± 3.2	69.2 ± 4.3	0.70
Weight(lbs)		180.5 (35.0)	185.0 (54.0)	0.60
Body Mass Index (kg/m^2)		25.7 (4.9)	26.9 (9.0)	0.34
Waist Circumference(cm)		100.4 (11.5)	103.3 (18.2)	0.49
Obese by BMI				
	Obese (BMI≥30)	20%	30%	0.47
	Lean (BMI<30)	80%	70%	
Obese by WC				
	Obese (WC≥102)	50%	55%	0.75
	Lean (WC<102)	50%	45%	
Education				
	College	20%	15%	0.72
	HS	65%	75%	
	< HS	15%	10%	
Race				
	Black	50%	75%	0.16
	White	30%	5%	
	Hispanic	5%	10%	
	Asian/Pacific Islander	15%	10%	
Marital Status				
	Single	50%	35%	0.12
	Married	30%	60%	
	Divorced	20%	5%	
Exercise				
	Daily	25%	30%	0.33
	2-3 times/week	15%	35%	
	once/week	5%	5%	
	1-2 times/month	5%	10%	
	Never	50%	20%	
Significant Exercise				
	Yes	35%	30%	0.74
	No	65%	70%	

*Presented as mean ± SD, median (IQR), or percentage.
[a] Students *t*-test or non-parametric Mann-Whitney *U* test used for continuous variables.
[b] chi-squared test used for categorical variables.

b. Tumor Characteristics

Tumor characteristics of cases are presented in Table II. Staging was determined by the Llor lab at UIC based on pathology reports, CT scans and is presented with the American Joint Committee on Cancer (AJCC) staging system. There was a larger portion of right-sided tumors, n=13 (65%), compared to left-sided n=7 (35%). Of the cases collected, 11 (55%) were obtained in endoscopy clinic, while 9 (45%) were obtained during surgery.

Table II. Tumor Characteristics

	AJCC	n=20	%
Stage	I	3	
	II	8	
	III	6	
	IV	3	
Location	Right	7	35%
	Left	13	65%
Sample Type	Endoscopy	11	55%
	Surgery	9	45%

c. Medication Use

All subjects were asked about their medication use. Table III presents this information. Regular use (i.e. use at least 3 times a week, within last 5 years) of NSAID, COX-2 and Statins did not differ between groups. Aspirin use appeared borderline significant between groups (p=0.11). The dosages of Aspirin vary from 80mg ("baby aspirin") to 325 mg (regular).

Table III. Medication Use (Use 3+ times/week, within 5 years)*

		Case(n=20)	Control (n=20)	P-value[a]
Aspirin				
	Yes	40%	65%	0.11
	No	60%	35%	
NSAID				
	Yes	50%	40%	0.53
	No	50%	60%	
COX-2				
	Yes	10%	5%	0.55
	No	90%	95%	
Statins				
	Yes	21%	35%	0.33
	No	79%	65%	

* Values presented as percentages.
[a] chi-squared test used to assess differences between groups.

d. Dietary Intake

Dietary intake of macro- and micronutrients per day are presented in Table IV. Iron intake did not differ between groups: for non-heme iron cases typically consumed 6.7 (4.3) mg/per day compared to controls with 7.0 (4.3) mg/per day (p=0.62) and no differences were seen with heme iron (p=0.79). Only grain intake varied between groups as cases had lower intake compared to controls (p=0.04).

Table IV. Dietary Intake*

		Cases (n=20)	Controls (n=20)	P-value[a]
Energy	Kcals	1016.3 (958.41)	1100.1 (738.0)	0.95
Energy per body weight	kcals/kg	14.3 ± 5.8	13.8 ± 6.6	0.79
Carbohydrates	% of kcal	45.0 (9.1)	45.6 (12.2)	0.97
Fat	% of kcal	38.7 (12.2)	41.7 (9.1)	0.32
Protein	% of kcal	15.6 (4.8)	15.4 (3.4)	0.66
Fruits	Servings	0.70 (0.9)	0.93 (0.6)	0.48
Vegetables	Servings	0.94 (2.1)	1.0 (0.7)	0.55
Meat	Servings	1.5 (1.5)	1.6 (0.9)	0.91
Grain	Servings	2.6 (1.7)	3.4 (1.4)	0.04
Saturated Fat	G	15.4 (12.4)	18.5 (9.9)	0.99
Dietary Fiber	G	6.6 (10.8)	8.1 (7.6)	0.49
Iron	Mg	6.7 (4.3)	7.0 (4.3)	0.62
Heme iron	Mg	0.67 (0.7)	0.66 (0.64)	0.79
Calcium	Mg	348.6 (361.9)	403.6 (290.1)	0.90
Vitamin C	Mg	46.0 (78.1)	65.7 (57.8)	0.34
Vitamin D	IU	69.2 (97.2)	73.2 (57.5)	0.97
Vitamin E	mg α-te	3.6 (3.5)	4.7 (3.9)	0.37
β-carotene	Mg	1072.0 (1410)	1189.4 (1478)	0.64
Folate (total)	Mg	171.4 (212.4)	200.6 (139.9)	0.70
omega-3 FA	G	0.85 (0.86)	89.0 (0.39)	0.99
Alcohol	G	0.23 (2.0)	1.0 (2.1)	0.65
	*(values per day)			

* Presented as mean ± SD, or median (IQR).
[a] Differences between groups assessed by Students *t*-test or non-parametric Mann-Whitney *U* test.

2. SERUM FINDINGS

a. Inflammation and Iron Status Parameters

i. Crude Analysis

Serum parameters for inflammation and iron status are presented in Tables V, VI and VII. Even though the majority of the variables were not normally distributed with the exception of age glucose, Table V presents the findings by mean and standard deviations, while Tables VI-VII present median and interquartile range for non-normally distributed variables. To be more statistically precise, the results from Tables VI and VII were interpreted below.

Overall cases have higher levels of inflammation and lower iron status compared to controls. Specifically with inflammation, higher levels of CRP ($p<0.05$) and borderline significance for higher levels of IL-6 ($p=0.06$) and TNF-α ($p= 0.11$) were found in cases compared to controls. For iron status, cases had significantly higher levels of sTfR ($p<0.05$) and lower levels of hepcidin ($p=0.02$), hemoglobin ($p=0.01$) and hematocrit ($p=0.01$) compared to controls. To account for the influence of obesity, serum parameters were adjusted for waist circumference. After controlling for waist circumference, the direction of the associations did not change though several comparisons had improved significance for CRP, IL-6, sTfR, while TNF-α lost borderline significance ($p=0.75$) and hepcidin became borderline significance ($p=0.08$). To account for the influence of iron status, a separate model was used to adjust for hemoglobin with each serum parameter between cases and controls. Levels of CRP and sTfR maintained similar significance as seen in the crude and waist circumference adjustment models, however compared to the crude model, hepcidin became non-significant between groups ($p=0.52$) and IL-6 became borderline significant ($p=0.14$).

Table V. Inflammation and Iron Status Parameters in Serum

– Presenting Means ± Standard Deviations

	Case(n=20)	Control (n=20)
Age (years)	61.2 ± 8.4	57.8 ± 6.6
WC (cm)	102.1 ± 13.1	105.6 ± 15.2
CRP (µg/mL)	7.5 ± 2.1	4.1 ± 2.8
IL-6 (pg/mL)	2.8 ± 1.4	1.9 ± 1.3
TNF-α (pg/mL)	0.57 ± 0.63	0.65 ± 1.3
Hemoglobin(g/dL)	11.8 ± 2.1	13.3 ± 1.1
Hematocrit(%)	36.2 ± 5.5	40.1 ± 3.1
sTfR (nmol/L)	22.5 ± 12.0	12.5 ± 4.7
hepcidin (ng/mL)	66.8 ± 67.3	100.7 ± 52.1
BMP-2(pg/mL)	88.8 ± 11.2	85.0 ± 9.5
Insulin (µlU/mL)	3.0 ± 4.5	2.0 ± 2.0
Glucose (mg/dL)	97.8 ± 16.0	97.3 ± 13.4

Table VI. Inflammatory and Iron Status Parameters in Serum

– Presenting Medians (Interquartile Range)*

	Case(n=20)	Control (n=20)	P-value[a]	P-value[b]	P-value[c]
Age (years)	61.2 ± 8.4	57.8 ± 6.6	0.16	0.30	0.49
WC (cm)	100.4 (11.5)	103.3 (18.2)	0.49	-	0.81
CRP (µg/mL)	8.3 (3.5)	3.4 (4.7)	0.01	0.0001	0.003
IL-6 (pg/mL)	2.7 (1.4)	1.9 (1.3)	0.06	0.05	0.14
TNF-α (pg/mL)	0.32 (0.22)	0.24 (0.36)	0.11	0.75	0.68
Hemoglobin(g/dL)	11.8 (2.4)	13.2(1.3)	0.01	0.01	-
Hematocrit(%)	37.2 (4.5)	39.6 (3.7)	0.01	0.01	0.85
sTfR (nmol/L)	21.6 (17.0)	11.8 (6.1)	0.004	0.002	0.04
hepcidin (ng/mL)	58.6 (71.1)	96.0 (34.1)	0.02	0.08	0.52
BMP-2(pg/mL)	88.8 ± 11.2	85.0 ± 9.5	0.26	0.27	0.30
Insulin (µlU/mL)	1.3 (2.2)	1.4 (2.1)	0.48	0.22	0.37
Glucose (mg/dL)	97.5 ± 15.7	97.3 ± 13.4	0.96	0.96	0.60

* Presented as mean ± SD or median (IQR).
[a] Differences between groups assessed by Student's *t*-test or non-parametric Mann-Whitney *U* test.
[b] Adjusting for WC.
[c] Adjusting for Hb.

ii. Matching on Hemoglobin and Waist Circumference

Since hemoglobin and waist circumference may be potential confounders for serum hepcidin, Table VII presents the serum results with hemoglobin and waist circumference matching between groups. Matching consisted of removing the four lowest hemoglobins for cases and three highest hemoglobins for controls, as well as the three highest waist circumference for controls. As a result, the relationship between groups for hemoglobin (p=0.34) and waist circumference (p=0.95) became similar. Even after matching for these two variables, cases still had lower iron status (sTfR, p=0.07), lower hepcidin (p=0.06) and higher inflammation (CRP, p=0.001; IL-6, p=0.07; TNF-α, p=0.19). Overall, the associations remained similar across all parameters.

Table VII: Serum: Matching for Hemoglobin and Waist Circumference*

	Cases (n=16)	**Controls (n=15)**	**P-value[a]**
Age (years)	61.7 ± 9.2	58.5 ± 7.1	0.30
WC (cm)	98.4 (17.3)	101.4 (18.3)	0.95
CRP (µg/mL)	8.4 (3.0)	3.2 (3.7)	0.001
IL-6 (pg/mL)	2.5 (2.5)	1.6 (1.8)	0.07
TNF-a (pg/mL)	0.38 (0.36)	0.23 (0.45)	0.19
Hemoglobin (g/dL)	12.5 (2.4)	12.9 (0.8)	0.34
Hematocrit (%)	37.8 (4.9)	39.6 (3.5)	0.29
sTfR (nmol/L)	19.6 (11.8)	12.4 (6.3)	0.07
Hepcidin (ng/mL)	64.4 (59.0)	97.6 (43.6)	0.06
BMP-2 (pg/mL)	86.4 ± 10.4	83.9 ± 7.3	0.37
Insulin (µlU/mL)	1.3 (3.4)	1.3 (0.92)	0.35
Glucose (mg/dL)	97.9 ± 15.6	99.2 ± 14.0	0.83

* Presented as mean ± SD or median (IQR).
[a] Differences between groups assessed by Student's *t*-test or non-parametric Mann-Whitney *U* test.

3. TISSUE FINDINGS

a. Iron Staining

Perl's Prussian Blue staining for presence of iron accumulation in colonic tissue was expressed in six cases versus one control (Figure 6 A-D). Odds Ratio=8.14 (95% Confidence Interval: 0.88-75.45). Iron accumulation was remarkable in the cases and controls (6A and 6C) and distinguishable compared to cases and controls without iron presence (6B and 6D).

Analysis by serum is also presented in Table VIII. Overall, the cases with presence of iron report higher levels of serum hepcidin (+ Iron, cases median=89.8 ng/mL, IQR=120.3) versus cases without iron presence (median=51.4 ng/mL, IQR=77.1, p=0.10) with borderline significance. Other parameters between Iron +/- in cases did not appear different. In controls, the one subject with + Iron was older, had a lower waist circumference, lower inflammatory markers (CRP, IL-6, TNF-α), higher hepcidin and lower sTfR compared to controls without presence of iron in the colon.

mRNA data by iron staining is presented in IX. Overall there were no differences or trends between the Iron +/- groups within cases and similar to the crude values presented in Tables XI and XII. The one control for Iron + accumulation reported all lower values for the genes assessed in mucosa DMT-1, FPN, hepcidin and IL-6 compared to the means of the Iron - controls.

Table X presents other parameters by iron staining for cases: tumor location, staging, dietary iron, and aspirin use. The Iron + accumulation group appears to use less aspirin. Overall the distributions appear the same between Iron +/- accumulation groups.

Figure 6 A-D: Colonic Tissue Perl's Prussian Blue Staining for Iron Presence. 20X Magnification.

Figure 6A. Case with Iron Presence (+) (n=6)

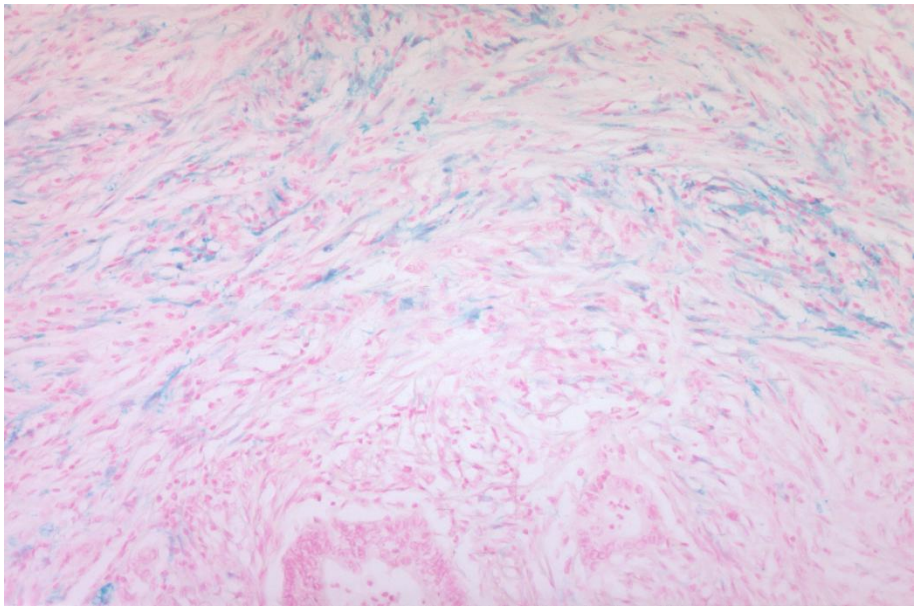


Figure 6B. Case without Iron Presence (-) (n=14)

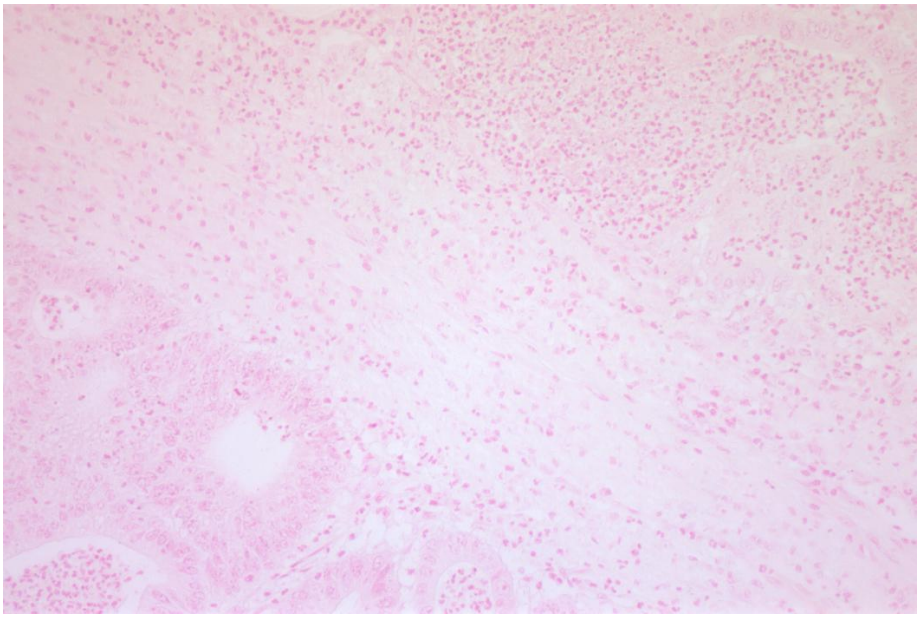


Figure 6C. Control with Iron Presence (+) (n=1)

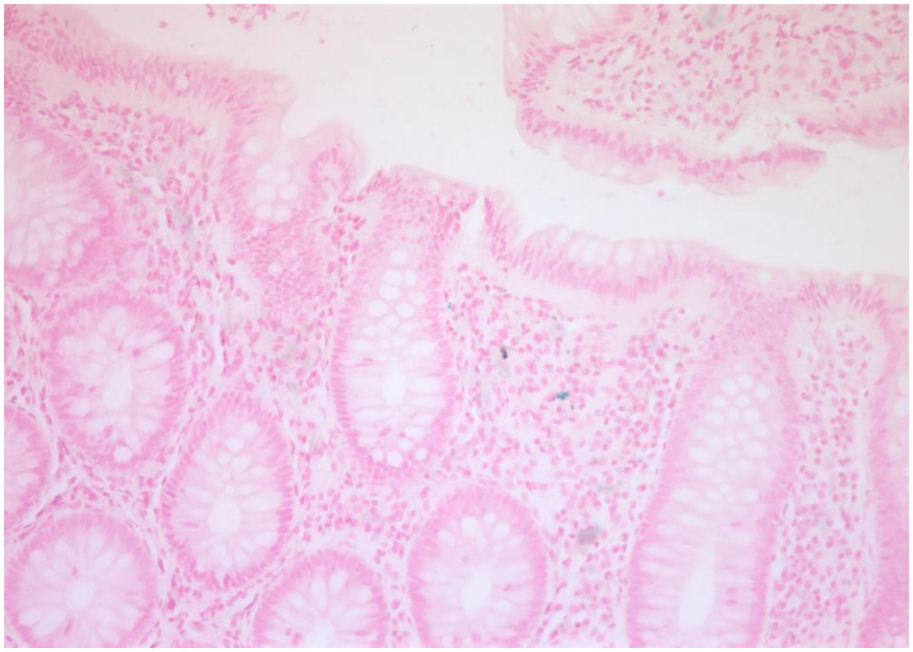
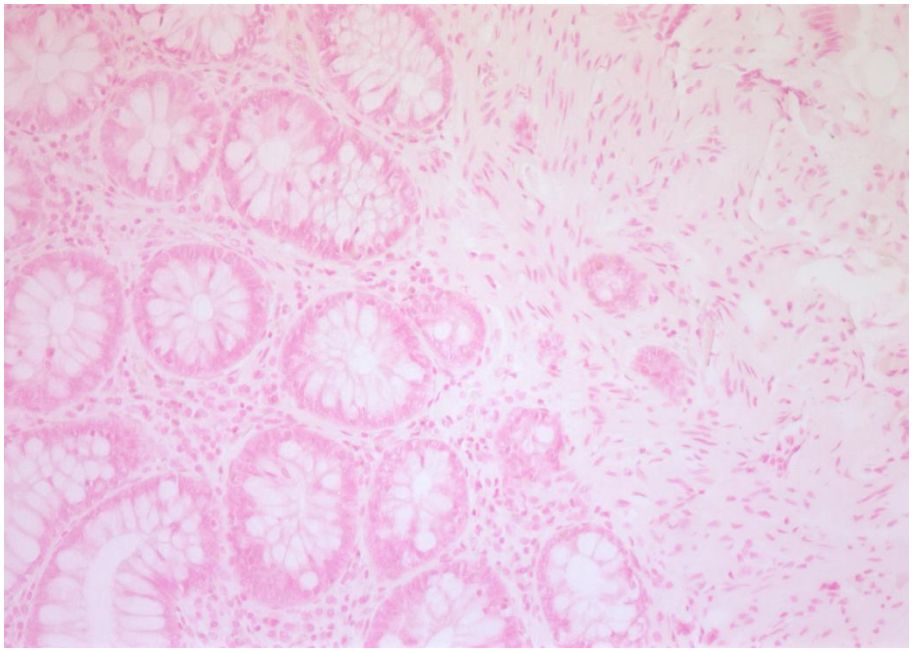


Figure 6D. Control without Iron Presence (-) (n=19)



i. Presented by Serum Findings

Table VIII. Serum Data: Iron Accumulation Presence, Yes (+ iron), No (- iron), in Colonic Tissue for Cases and Controls*

	Cases			Controls	
	Yes (n=6)	**No (n=14)**	**P-value[a]**	**Yes (n=1)**	**No (n=19)**
Age (years)	61.7 ± 4.7	60.5 ± 9.5	0.79	64	57.5 + 6.6
Waist Circumference (cm)	110.7 (25.4)	103.7 (10.5)	0.59	90.2	103.5 (18.0)
CRP (µg/mL)	6.7 (5.7)	8.2 (3.4)	0.73	0.07	3.55 (4.8)
IL-6 (pg/mL)	3.1 (3.0)	2.4 (2.0)	0.40	0.36	1.9 (1.7)
TNF-α (pg/mL)	0.36 (1.4)	0.33 (0.3)	0.73	0.21	0.25 (0.45)
Hemoglobin(g/dL)	12.7 (2.2)	12.0 (3.1)	0.89	13.1	13.2 (1.4)
Hematocrit(%)	37.5 (2.7)	37.8 (6.8)	0.58	41.5	40.2 (3.5)
sTfR (nmol/L)	20.1 (15.0)	21.4 (25.2)	0.88	6.36	12.4 (5.6)
hepcidin (ng/mL)	89.8 (120.3)	51.4 (77.1)	0.10	109.9	94.3 (43.6)
BMP-2 (pg/mL)	91.1 ± 9.8	87.8 ± 11.9	0.56	81.7	85.2 ± 9.7
Insulin (µlU/mL)	1.2 (0.93)	2.3 (3.7)	0.29	1.21	1.39 (2.8)
Glucose (mg/dL)	100.8 ± 18.9	98.9 ± 14.3	0.81	100	97.1 + 13.8

*Presented as mean ± SD or median (IQR).
[a] Differences between groups assessed by Student's *t*-test or non-parametric Mann-Whitney *U* test.

ii. Presented by Tissue Findings

Table IX. mRNA Data: Iron Accumulation Presence, Yes (+ iron), No (- iron), in Colonic Tissue for Cases and Controls*

	Cases					**Controls**		
	Yes(n=6)		**No(n=14)**		**P-value[b]**	**Yes(n=1)**	**No(n=19)**	
	ΔCt*	2^- ΔΔCt[a]	ΔCt*	2^- ΔΔCt[a]		ΔCt*	ΔCt*	2^- ΔΔCt[a]
DMT-1	-5.86 ± 0.83	1.35	-6.11 ± 1.3	1.13	0.71	-7.50	-6.22 ± 0.64	1.00
Ferroportin	-2.08 ± 1.9	0.57	-1.61 ± 1.5	0.79	0.59	-3.60	-1.14 ± 2.2	1.00
Hepcidin	-10.12 ± 1.8	0.39	-10.50 ± 1.2	0.29	0.63	-9.30	-8.74 ± 1.1	1.00
IL-6	-10.18 ± 3.2	7.06	-9.68 ± 2.8	9.99	0.76	-15.60	-12.83 ± 1.9	1.00

*Values presented as means ± SD.
[a] 'relative expression' compared to healthy colonic tissue.
[b] Difference assessed by *t*-test .

iii. Presented by Other Parameters

Table X. Other Parameters for Cases by Iron Presence (Yes/no): Staging, Tumor Location, Aspirin Use and Dietary Iron

	Cases		
	Yes(n=6)	**No(n=14)**	**P-value***
Location(n=)			
Right	3	4	0.61
Left	3	10	
Staging(n=)			
I	2	1	0.18
II	1	7	
III	1	5	
IV	2	1	
Aspirin Use (%)			
Yes	16.7	50.0	0.32
No	83.3	50.0	
Dietary iron	7.6 (3.2)	6.9 (10.5)	0.78
Dietary heme	0.56 (0.85)	0.75 (0.68)	1.0

*Differences assessed by Fisher's exact test or non-parametric Mann-Whitney *U* test.

b. mRNA Expression of Iron Transporters and Inflammation in Colonic Tissue

i. Crude Analysis

To understand iron metabolism at the tissue level between cases and controls we measured the expression of three genes involved in iron transport: DMT-1(iron influx, divalent metal transporter 1, FPN-1 (iron efflux, ferroportin-1), HAMP (hepcidin) and one gene for inflammation: IL-6 (interleukin-6). These findings are presented in Tables XI, XII, XIII, and XIV. Tables XI and XII present the raw ΔCt and ΔΔCt conversion, respectively, while Tables XIII and XIV presents the data after matching for hemoglobin and waist circumference.

Tissue from cases, i.e. the tumor, was compared to healthy colonic mucosa from controls. Values for each parameter for mRNA expression are presented as ΔCt (Ct reference- Ct target), therefore higher values implies a higher expression of the gene whereas lower values implies a lower expression of the gene. We used two independent sample student's *t*-test for DMT-1, FPN-1, HAMP and IL-6. For a simplified interpretation, Table XII presents the data as relative expression, treating the control group as the reference.

Cases had a 2.9-fold lower expression of hepcidin in their tumors compared to the healthy mucosa in controls (Cases -10.28 $\pm$ 1.5 to controls -8.77 $\pm$ 1.1 p=0.001). Also, cases had a 9.4-fold higher expression of IL-6 compared to controls. Cases (Cases -9.77 $\pm$ 2.8 to controls -13.0 $\pm$ 1.9, p=0.0001). While FPN-1 expression was 1.4-fold lower in cases versus controls, this was not statistically significant (cases -1.77 $\pm$ 1.5 to controls 1.27 $\pm$ 2.2, p=0.41). Cases had a 1.12-fold higher expression of DMT-1 compared to controls (cases -6.13 $\pm$ 1.1 to controls 6.29 $\pm$0.69, p=0.59), although this was not statistically significant.

Table XI. mRNA Expression of Iron transporters and Inflammation in Colonic Tissue*

mRNA*	Cases(n=20)	Controls(n=20)	p-value[a]	p-value[b]
DMT-1	-6.13 ± 1.1	-6.29 ± 0.69	0.59	0.79
FPN-1	-1.77 ± 1.5	-1.27 ± 2.2	0.41	0.51
HAMP	-10.28 ± 1.5	-8.77 ± 1.1	0.001	0.01
IL-6	-9.77 ± 2.8	-13.00 ± 1.9	0.0001	0.001

* Values presented as means ± SD
[a] Differences assessed by *t*-test.
[b] Adjusting for Hb.

Table XII. mRNA Expression of Iron Transporters, Inflammation in Colonic Tissue, Presented as Relative Expression Compared to Controls

mRNA*	Cases(n=20)	Controls(n=20)
DMT-1	1.12	1.0
FPN-1	0.71	1.0
HAMP	0.35	1.0
IL-6	9.38	1.0

ii. Matching on Hemoglobin and Waist Circumference

Tables XIII and XIV present the mRNA parameters after matching on hemoglobin and waist circumference. After including hemoglobin in the regression models, all directionality of genes remained the same i.e. hepcidin was still lower in cases versus controls (p=0.01) and IL-6 was higher in cases versus controls (p=0.001).

Table XIII. Matching Hemoglobin and Waist circumference: mRNA Expression of Iron Transporters and Inflammation in Colonic Tissue*

mRNA*	Cases(n=16)	Controls(n=15)	p-value[a]
DMT-1	-6.19 ± 1.1	-6.26 ± 0.65	0.84
FPN-1	-2.03 ± 1.5	-0.99 ± 2.4	0.16
HAMP	-10.53 ± 1.5	-8.75 ± 1.2	0.001
IL-6	-9.44 ± 3.0	-12.59 ± 2.0	0.002

* Values presented as means ± SD *.
[a] Differences assessed by *t*-test.

Table XIV. Matching Hemoglobin and Waist circumference: mRNA Expression of Iron Transporters and Inflammation in Colonic Tissue, Presented as Relative Expression Compared to Controls

mRNA*	Cases(n=16)	Controls(n=15)
DMT-1	1.05	1.0
FPN-1	0.49	1.0
HAMP	0.29	1.0
IL-6	8.88	1.0

4. OBESITY FINDINGS

a. Correlations of Waist Circumference and Serum Parameters

Correlations between waist circumference (WC) and each serum parameter by cases and controls are presented in Table XV. Within controls, CRP (r=0.47), IL-6 (r=0.71) and BMP-2 (r=0.46) are positively correlated with WC ($p<0.05$). No correlation was observed between WC and TNF-α ($p=0.82$). In contrast, cases did not have any significant correlations with WC and serum parameters, with the exception a positive correlation with insulin and glucose between waist circumference (both r=0.40, p=0.07) which was borderline significant. Moreover, the relationship with higher adiposity and inflammation seems to exist only in controls.

Table XV. Role of adiposity by disease state:

Correlations: Using waist circumference (WC) + each serum parameter*

	Cases(n=20)		Controls(n=20)	
WC	r*	p-value	r*	p-value
CRP	-0.16	0.57	0.47	0.04
IL-6	-0.19	0.41	0.71	0.001
TNF-α	-0.23	0.31	0.05	0.82
sTfR	-0.08	0.73	0.07	0.78
Hepcidin	-0.13	0.58	-0.14	0.56
Hb	0.14	0.55	-0.26	0.31
Hct	0.19	0.41	-0.39	0.12
BMP-2	-0.31	0.18	0.46	0.04
Insulin	0.40	0.07	0.13	0.58
Glucose	0.40	0.07	-0.16	0.54

*r= non-parametric spearman correlation coefficient.

b. Serum Parameters Stratified by Obesity

Serum parameters for inflammation and iron status were stratified by obesity (using waist circumference; obese $\geq$ 102 cm, lean <102 cm) and presented in Table XVI. Comparisons are presented as four independent samples. Comparisons between obese and lean within each disease state appears similar to the findings in Table X. Within controls, obese have significantly higher levels of CRP and IL-6 versus lean ($p<0.05$), however no differences in BMP-2. *We found that the distribution of obesity and cancer staging appeared different ($X^2=7.54$ for 3 degrees of freedom, p-value=0.06). Thus the comparisons within cases are adjusted for staging.* After adjusting for stage within cases, no differences in serum parameters between obese and lean were observed.

Table XVI. Role of Adiposity by Disease State, Stratified by Obesity *(Obese= WC> 102cm, lean=WC<102cm)**

	Cases				Controls			
	Crude(n=20)	Obese(n=10)	Lean (n=10)	P-value[a]	Crude(n=20)	Obese (n=11)	Lean (n=9)	P-value[b]
CRP (µg/mL)	8.3 (3.5)	8.2 (2.5)	9.2 (4.4)	0.70	3.4 (4.7)	6.4 (4.2)	2.2 (2.5)	0.03
IL-6 (pg/mL)	2.7 (1.4)	2.5 (1.4)	3.5 (2.7)	0.51	1.9 (1.3)	2.5 (2.0)	0.71 (0.9)	0.003
TNF-α (pg/mL)	0.32 (0.22)	0.32 (0.2)	0.43 (0.4)	0.78	0.24 (0.36)	0.25 (1.1)	0.21 (0.2)	0.48
Hemoglobin(g/dL)	11.8 (2.4)	11.7 (3.0)	12.0 (2.5)	0.23	13.0(1.2)	12.8 (1.5)	13.3 (1.0)	0.40
Hematocrit(%)	37.2 (4.5)	37.3 (5.2)	36.5 (4.9)	0.30	39.6 (3.7)	38.5 (5.1)	40.8 (3.5)	0.37
sTfR (nmol/L)	21.6 (17.0)	21.6 (22.1)	21.8 (14.0)	0.66	11.8 (6.1)	12.4 (3.8)	10.0 (8.3)	0.65
Hepcidin (ng/mL)	58.6 (71.1)	46.7 (48.4)	71.7 (71.0)	0.38	96.0 (34.1)	97.6 (28.4)	94.2 (37.5)	0.82
BMP-2 (pg/mL)	88.8 $\pm$ 11.2	87.3 $\pm$ 12.5	90.3 $\pm$ 10.2	0.47	85.0 $\pm$ 9.5	87.0 $\pm$ 9.7	82.6 $\pm$ 9.1	0.31
Insulin (µlU/mL)	1.3 (2.2)	2.4 (3.3)	1.0 (0.74)	0.17	1.4 (2.1)	1.4 (2.0)	1.3 (3.2)	0.65
Glucose (mg/dL)	97.5 $\pm$ 15.7	102.4 $\pm$ 12.2	92.4 $\pm$ 18.0	0.35	97.3 $\pm$ 13.4	92.8 $\pm$ 14.6	101.8 $\pm$ 11.1	0.19

* Presented as mean $\pm$ SD or median (IQR).
[a] Obese cases versus lean cases using multiple linear regression (adjusting for cancer staging).
[b] Obese cases versus obese controls using students *t*-test or non-parametric Mann-Whitney *U* test.

c. mRNA Expression Stratified by Obesity

mRNA expression of iron transporters and inflammation stratified by obese or lean status are presented in Tables XVII and XVIII. Table XVII presents the raw ΔCt data with significant differences between each groups for HAMP (hepcidin) and IL-6 comparisons. The directionality of the differences are the same as in the crude comparisons in Tables XI and XII, i.e. cases(obese or lean) have lower expression of hepcidin and higher expression of IL-6. Overall, there was no statistical significance for any mRNA expression parameter by obesity within each disease state. However within controls, obese had directionality for higher expression of hepcidin compared to lean.

Table XVIII presents the mRNA findings by relative expression using lean controls as the reference group. Within controls, obese had a 1.59-fold higher expression of hepcidin and 1.92-fold lower expression of ferroportin compared to lean. Additionally, obese controls had a 1.47-fold lower expression of IL-6 compared to lean counterparts. However within cases both obese and lean had lower expression of hepcidin and ferroportin compared to lean controls (*hepcidin*: obese 2.44-fold and lean 2.04-fold lower; *ferroportin* obese 2.38-fold and lean: 1.61-fold lower). Also within cases, obese expressed higher IL-6 presenting 3.97-fold and lean 14.12-fold higher expression compared to lean controls, however this is likely due to cancer staging (as mentioned before, differences were seen between obesity and staging). DMT-1 expression also seems to be in higher directionality in all groups compared to lean controls.

Table XVII. mRNA Expression by Obesity *(Obese= WC≥ 102cm, Lean=WC<102cm)**

	Cases				Controls			
	Crude (n=20)	Obese(n=10)	Lean(n=10)	P-value[a]	Crude (n=20)	Obese (n=11)	Lean (n=9)	P-value[b]
DMT-1	-6.13 ± 1.1	-6.15 ± 0.98	-6.11 ± 1.3	0.42	-6.29 ± 0.69	-6.22 ± 0.83	-6.38 ± 0.51	0.62
FPN-1	-1.77 ± 1.5	-2.05 ± 1.5	-1.48 ± 1.6	0.56	-1.27 ± 2.2	-1.51 ± 2.1	-0.97 ± 2.4	0.60
HAMP	-10.28 ± 1.5	-10.40 ± 1.3	-10.15 ± 1.8	0.97	-8.77 ± 1.1	-8.46 ± 0.97	-9.13 ± 1.4	0.17
IL-6	-9.77 ± 2.8	-10.68 ± 2.5	-8.85 ± 2.8	0.42	-13.00 ± 1.9	-13.22 ± 1.4	-12.67 ± 2.4	0.53

* Values presented as means ± SD .
[a] Obese cases versus lean cases using multiple linear regression (Adjusting for cancer staging).
[b] Obese controls versus lean controls using students *t*-test.

Table XVIII. mRNA Expression by Obesity *(Obese= WC≥ 102cm, Lean=WC<102cm), Presented as Relative Expression Compared to Lean*

	Cases			Controls	
	Crude(n=20)	Obese(n=10)	Lean(n=10)	Obese (n=11)	Lean(n=9)
DMT-1	1.12	1.17	1.21	1.12	1.0
FPN-1	0.71	0.42	0.62	0.52	1.0
HAMP	0.35	0.41	0.49	1.59	1.0
IL-6	9.38	3.97	14.12	0.68	1.0

5. STAGING FINDINGS

a. Serum Parameters by Staging

Serum parameters by staging are presented in Table XIX. Higher levels of serum IL-6 were observed in later stage compared to early stage (p=0.03).

Table XIX. Serum Parameters by Staging*

Stage	Controls(n=20)	Cases(n=20)	I (n=3)	II (n=8)	III (n=6)	IV (n=3)	P-value[a]
CRP (µg/mL)	3.4 (4.7)	8.3 (3.5)	5.3 (6.0)	8.5 (1.1)	5.8 (5.6)	9.0 (4.5)	0.15
IL-6 (pg/mL)	1.9 (1.3)	2.7 (1.4)	1.2 (1.2)	2.8 (1.1)	1.4 (1.4)	4.4 (0.04)	0.03
TNF-α (pg/mL)	0.24 (0.36)	0.32 (0.22)	0.33 (0.3)	0.26 (0.1)	0.27 (0.3)	0.40 (2.6)	0.61
Hemoglobin(g/L)	13.2(1.3)	11.8 (2.4)	13.4 (3.6)	11.8 (3.8)	12.9 (2.6)	11.1 (1.8)	0.47
Hematocrit(%)	39.6 (3.7)	37.2 (4.5)	40.7 (6.4)	37.1 (8.3)	38.9 (7.9)	35.7 (2.4)	0.38
sTfR (nmol/L)	11.8 (6.1)	21.6 (17.0)	15.5 (14.6)	21.4 (34.1)	17.4 (13.4)	22.7 (24.6)	0.89
hepcidin (ng/mL)	96.0 (34.1)	58.6 (71.1)	82.9 (20.2)	41.9 (48.4)	51.4 (94.2)	64.4 (249.1)	0.45
BMP-2 (pg/mL)	85.0 ± 9.5	88.8 ± 11.2	83.4 ± 8.6	91.6 ± 12.6	84.1 ± 9.7	87.5 ± 11.7	0.70
Insulin (µIU/mL)	1.4 (2.1)	1.3 (2.2)	2.7 (2.7)	1.4 (8.9)	1.9 (2.0)	1.1 (0.9)	0.46
Glucose (mg/dL)	97.3 ± 13.4	97.5 ± 15.7	109.3 ± 16.9	102.6 ± 13.9	98.2 ± 15.0	90.7 ± 13.1	0.45

*Presented as mean ± SD or median (IQR).
[a] Trend between groups assessed by one-way ANOVA or non-parametric Kruskal-Wallis.

b. mRNA Expression of Iron Transporters and Inflammation by Staging

mRNA expression of iron transporters and inflammation stratified by staging is presented in Tables XX and XXI. Overall, there were no significant differences across different stages compared to the raw ΔCt data in Table XIX. However with the relative expression comparisons we found directionality towards higher DMT-1 with advanced staging (with stage IV having a 2.45-fold higher level compared to controls). Additionally, IL-6 levels increased with advanced staging (stage IV 60.97-fold higher compared to levels in controls).

Table XX. mRNA Expression of Iron Transporters and Inflammation by Staging*

	Controls (n=20)	All Cases (n=20)	I (n=3)	II (n=8)	III (n=6)	IV (n=3)	P-value[a]
DMT-1	-6.29 ± 0.69	-6.13 ± 1.1	-6.50 ± 0.50	-6.29 ± 1.6	-6.15 + 0.69	-5.00 ± 0.61	0.36
FPN-1	-1.27 ± 2.2	-1.77 ± 1.5	-1.90 ± 2.7	-1.47 ± 1.4	-2.45 ± 1.2	-1.20 ± 1.8	0.66
HAMP	-8.77 ± 1.1	-10.28 ± 1.5	-10.87 ± 1.6	-10.34 ± 1.4	-10.50 ± 1.7	-9.63 ± 2.0	0.81
IL-6	-13.00 ± 1.9	-9.77 ± 2.8	-10.60 ± 3.8	-10.62 ± 2.3	-9.27 ± 1.8	-7.07 ± 4.4	0.28

***Values presented as means ± SD, or median (IQR).
[a]Trend between groups assessed by one-way ANOVA test.

Table XXI. mRNA expression by Staging, Presented as Relative Expression Compared to Control

	Controls (n=20)	All cases (n=20)	I (n=3)	II (n=8)	III (n=6)	IV (n=3)
Divalent Metal Transport-1*(DMT-1)*	1.0	1.12	0.86	1.0	1.10	2.45
Ferroportin *(FPN-1)*	1.0	0.12	0.65	0.87	0.44	1.05
Hepcidin *(HAMP)*	1.0	0.35	0.23	0.34	0.29	0.55
Interleukin -6*(IL-6)*	1.0	9.38	5.38	5.03	13.27	60.97

6. OTHER FINDINGS

While outside of our study aims, we included serum and tissue level findings by tumor location.

a. Tumor Location Findings

i. Serum Parameters by Tumor Location

Table XXII presents serum parameters by tumor location, left-sided or right-sided tumors. Overall, there were no significant differences between groups. Though, there are some trends. Left-sided tumors appear to have a larger waist circumference, slightly higher inflammation (CRP, IL-6), lower iron status (sTfR, Hb, Hct), and higher glucose levels.

Table XXII: Serum: Right-sided versus Left-sided tumors*

	Controls(n=20)	Cases (n=20)	Right(n=7)	Left (n=13)	P-value[a]
Age (years)	57.8 ± 6.6	61.2 ± 8.4	61.0 ± 10.6	61.3 ± 7.4	0.94
WC (cm)	103.3 (18.2)	100.4 (11.5)	96.0 (18.7)	103.4 (10.5)	0.45
CRP (µg/mL)	3.4 (4.7)	8.3 (3.5)	7.5 (3.4)	8.2 (6.0)	0.89
IL-6 (pg/mL)	1.9 (1.3)	2.7 (1.4)	1.7 (1.9)	2.6 (2.6)	0.78
TNF-α (pg/mL)	0.24 (0.36)	0.32 (0.22)	0.33 (1.0)	0.31 (0.24)	0.70
Hemoglobin(g/dL)	13.2(1.3)	11.8 (2.4)	12.2 (2.5)	11.6 (2.9)	0.67
Hematocrit(%)	39.6 (3.7)	37.2 (4.5)	37.2 (5.7)	35.8 (6.8)	0.56
sTfR (nmol/L)	11.8 (6.1)	21.6 (17.0)	18.4 (15.3)	21.4 (20.0)	0.72
Hepcidin (ng/mL)	96.0 (34.1)	58.6 (71.1)	52.8 (76.4)	64.3 (84.9)	0.40
BMP-2(pg/mL)	85.0 ± 9.5	88.8 ± 11.2	96.6 ± 8.8	86.8 ± 12.1	0.28
Insulin (µlU/mL)	1.4 (2.1)	1.3 (2.2)	2.6 (3.6)	1.4 (1.7)	0.36
Glucose (mg/dL)	97.3 ± 13.4	97.5 ± 15.7	92.4 ± 11.3	100.7 ± 17.8	0.28

*Presented as mean ± SD, or median (IQR).
[a] Difference between groups assessed by Student's *t*-test or non-parametric Mann-Whitney *U* test.

ii. mRNA Expression by Tumor Location

mRNA expression for iron transporters and inflammation by location, right or left-sided tumors are presented in Tables XXIII and XXIV. Overall, there were no significant differences except borderline higher expression of hepcidin in right-sided versus left-sided tumors (-9.49 $\pm$ 1.7 versus -10.70 $\pm$ 3.1, p=0.09) and a trend for higher IL-6 in left-sided versus right sided tumors.

Table XXIII: mRNA Expression by Tumor Location*

mRNA*	Controls(n=20)	Cases(n=20)	Right(n=7)	Left(n=13)	P-value[a]
DMT-1	-6.29 ± 0.69	-6.13 ± 1.1	-6.17 + 1.0	-6.11 + 1.2	0.91
FPN-1	-1.27 ± 2.2	-1.77 ± 1.5	-1.74 + 1.5	-1.78 + 1.6	0.96
HAMP	-8.77 ± 1.1	-10.28 ± 1.5	-9.49 + 1.7	-10.70 + 1.3	0.09
IL-6	-13.00 ± 1.9	-9.77 ± 2.8	-10.60 + 1.9	-9.32 + 3.1	0.33

Values presented as means ± SD.
[a] Differences between groups assessed by Student's *t*-test.

Table XXIV: mRNA expression by Tumor Location, Relative Expression Compared to Controls

mRNA*	Controls(n=20)	Cases(n=20)	Right(n=7)	Left(n=13)
DMT-1	1.0	1.12	1.09	1.13
FPN-1	1.0	0.12	0.72	0.70
HAMP	1.0	0.35	0.61	0.26
IL-6	1.0	9.38	5.28	12.82

V. DISCUSSION

The significance and implications of the results are presented in four sections. The first section focuses on *tissue* findings understanding the variability of iron accumlation, iron transporters and inflammation in the colonic mucosa in colon cancer cases and controls. In the second section *serum* findings on circulating inflammatory markers, hepcidin and iron status are discussed. In the third and fourth sections the role of *obesity* and *cancer staging* as modifiers are discussed, respectively. Finally, study strengths and limitations are presented.

1. DISCUSSION FOR TISSUE FINDINGS

We hypothesized that men with colon cancer would have higher colonic inflammation (IL-6), elevated levels of iron influx transporter (DMT-1) and diminished levels of iron efflux transporter (ferroportin) which would induce greater expression of colonic hepcidin, resulting in higher iron accumulation in their tumors compared to mucosal tissue of controls. In support of these alterations we found significant increases in IL-6, a trend for reduced ferroportin and higher colonic iron accumulation in cases versus controls. Unexpectedly, colonic (mRNA) hepcidin expression was significantly lower in cases than controls and DMT-1 was similar between groups. These findings for hepcidin remained when the analysis was repeated controlling for the participants obesity and Hb status. These findings suggest that hepcidin in colonocytes is regulated by low iron status and/or increased erythoid iron need of the patients with cancer rather than by inflammation.

a. Hepatic and Extra-hepatic Hepcidin Regulation

Iron regulation is determined by hepatic hepcidin production which is influenced by iron status (low iron status reduces synthesis), inflammation (increases synthesis) and hypoxia (reduces synthesis). The strength of all the parameters influence synthesis rates rather than a hierarchy of any individual signal (89). Conditions that elevate hepcidin production (excess iron

and/or inflammation) also reduce ferroportin expression at the basolateral membrane, limiting the release of iron from within the enterocyte into circulation. Conversely, signals which suppress liver hepcidin production (iron insufficiency or hypoxia) increase ferroportin expression and enhance the exit of iron from the enterocyte into the circulation.

While the conditions that stimulate and inhibit hepatic hepcidin production are well described, little is known of the control for extra-hepatic production. Extra-hepatic hepcidin has been found in the human adipose (18), colon (84) and rat heart (90). Adipose tissue production of hepcidin has been reported (29, 39), however in obese women it was not correlated with markers of iron status (i.e. transferrin saturation) and the expression of hepatic to adipose tissue hepcidin was 700-fold higher suggesting a diminished role for systemic iron regulation (18). Ward et al. reported hepcidin expression in the colon (84), however very little is known about the control of its production. Merle et al. investigated hepcidin production in the heart of a rat model and found both inflammation and hypoxia (contrary to liver) increased its expression. Collectively these studies demonstrate the limited understanding of extra-hepatic hepcidin and what influences its expression.

Expanding our understanding of the role of hepcidin expression in colonocytes during colon cancer will allow us to link iron regulation and cancer development. Iron metabolism is dysregulated during colon cancer, i.e. colonocytes have iron accumulation (79). Iron can act as a pro-oxidant nutrient, promote cell growth and proliferation, thus tight regulation is crucial (91). Hepcidin regulates iron availability in enterocytes and is influenced by iron status, inflammation and hypoxia (4, 6). Further, hypoxia and inflammation expression are increased during colon cancer (92, 93) . Therefore inflammation or hypoxia may influence hepcidin expression in colonocytes to alter iron exposure and affect cancer risk.

Colonic hepcidin expression and iron absorption

Our findings of significantly lower hepcidin coupled with a trend toward lower ferroportin expression and iron accumulation in tumors suggest that colonocyte-derived hepcidin does not play a role in tumor iron retention as tumor had increased iron despite decreased hepcidin mRNA. Tumors require higher iron uptake to meet the demands for cell proliferation and growth (79). Given the colon expresses similar iron transporters as in the small intestine (79), any alterations at the colonic level could affect uptake of luminal iron to meet the needs for tumor growth.

Our cross sectional study design prohibited detection of the cause of decreased expression of colonic hepcidin in cases compared to controls. The following two sections discuss hepcidin regulation and speculate on signals during cancer that may have resulted in its reduced expression

b. IL-6 and BMPs in Hepcidin Promoting Pathways in Colon Cancer

IL-6 is a pro-inflammatory cytokine that increases hepcidin signaling in the liver (8), heart (94) and adipose tissue (39). Our findings indicate IL-6 does not have this influence on hepcidin at the colonic level. The stimulation for hepcidin signaling occurs when IL-6 binds to its receptor to activate a signal transducer pathway, STAT-3 (not measured), which engages with the hepcidin promoter region in the nucleus to increase its expression (8, 95). Therefore one possibility for our findings is that STAT-3 was not activated and unable to induce hepcidin expression due to IL-6 receptor downregulation or inhibition. Alternatively, our findings could indicate IL-6/STAT-3 does not regulate hepcidin expression in colonic tumors. This IL-6/STAT-3 pathway is also activated in colon carcinoma to increase cell proliferation and reduce apoptosis (92). Bromberg et al. have suggested upregulation of STAT-3 target genes may increase expression and/or it may also signal the Wnt/β-catenin pathway (96). The Wnt/β-catenin signaling is a major oncogenic pathway in colon cancer. Thus, the elevated IL-6 observed in our

cases may reflect an increase in STAT-3 signal activation, which increases other pro-oncogenic genes rather than promoting hepcidin expression.

Bone morphogenic proteins (BMPs) are mediators that can increase hepcidin expression in the liver by several pathways (7). BMPs are in the family of transforming growth factor-beta (TGF-β) proteins and several classes exist including BMP-2, BMP-4 and BMP-6. Two mechanisms have been proposed for BMP-hepcidin regulation however the outcomes for hepcidin expression are similar for all of the BMP mediators (high BMP-high hepcidin expression). Within the liver iron stores directly regulate BMP-6 which influence hepcidin expression (i.e. low iron stores, low BMP and low hepcidin expression; high iron stores, high BMP and high hepcidin expression), although the mechanism is not well understood (7). BMP-2 and BMP-4 also influence hepcidin expression via their interactions with the BMP-HJV co-receptor which phosphorylates the SMAD-4 pathway to stimulate hepcidin expression (7). Although the BMP-hepcidin pathway was not a central component of our study we measured serum BMP-2 to explore its influence in non-inflammatory and non-iron pathways for circulating hepcidin regulation (Note: Serum Section discussed in Section 2). This is the first report of BMP-2 levels in circulation between colon cancer cases and controls and our findings of similar levels between groups suggest BMP-2 does not influence serum hepcidin.

If BMPs are responsible for the lower colonic hepcidin levels in cases then we would expect colonic BMP expression in tumors to be decreased. Unfortunately, we did not measure colonic BMP. BMPs are part of the TGF-β family of proteins which are associated with mutations in colon cancer (97). Thus, the BMP regulation of hepcidin expression suggests a potential link in colon cancer. Both increased and decreased expression of BMP expression in colonic tumors and its pathway have been reported (98-100). Kodach et al. investigated the role of BMP-2 in human colon cancer samples and found impaired BMP signaling, i.e. loss of BMP receptor or SMAD-4 (98). This agrees with previous studies which found that juvenile polyposis is associated with reduced BMP signaling (101, 102) and may suggest the alterations occur with

early onset of colon cancer (although Kodach et al. did not report staging). In contrast, Lorente-Trigos et al. found increased BMP-4 signaling in advanced human colon cancer samples (Stage III-IV) (100). The differences in tumor staging likely contributed to the discrepancies between the studies, however BMP regulation in colon cancer appears to be multifaceted (103). Future studies exploring the role of BMP: Hepcidin connection in colon cancer is warranted.

c. <u>Hypoxia/Erythropoietin Influence on Hepcidin</u>

The fate for the majority of iron that is absorbed in the diet is erythrocyte synthesis (21). During hypoxia hepcidin expression is suppressed to promote iron uptake (4, 104). Hypoxia-inducible factors (HIF-1,2α) are transcription factors in the liver and are associated with low hepcidin (105) . Previous studies using mouse models postulated that hypoxia directly reduces hepcidin expression in the liver (105, 106) however mechanisms for these findings were not explored. More recently, Liu et al. reported hypoxia induced expression of HIF-1,2 in mouse models directly reduced hepcidin expression and postulated this was regulated via erythropoietin (EPO) (107). EPO is a glycoprotein primarily produced by the kidneys and triggered during hypoxic conditions to increase erythropoiesis (108). The EPO induced erythropoiesis is thought to increase secretion of as yet unknown erythroid factor which in turn suppresses hepcidin expression (109) to facilitate increased iron availability/absorption.

Within the enterocyte hypoxia regulates iron absorption (110). Shah et al. induced hypoxia (via HIF-2-α) in the duodenum of mouse models and found it was associated with increased iron uptake via increased DMT-1 and Dcytb (111). Mastrogiannaki et al. confirmed these findings using similar methods and also found increased ferroportin levels further supporting the influence of hypoxic conditions within the small bowel on increasing iron absorption and export (112). The role of hypoxia on hepcidin levels was not explored, however given our understanding of hypoxia and increased iron uptake it is likely it would be accompanied by reduced hepcidin.

d. Circulating Iron Influence on Hepcidin

Low circulating iron may also be influencing lower expression of hepcidin in colonic tumors. Cases had lower iron status (Serum findings discussed in Section 2) demonstrated by higher sTfR compared to controls suggesting decreased iron availability and low serum iron (not measured). Low serum iron is associated primarily with low hepatic hepcidin expression (7), and a similar relationship may exist with low serum iron: colonic hepcidin. Thus, these findings suggest that colonocytes have a normal iron sensing mechanism.

e. Iron Transporter Dysregulation in Colon Cancer

Our findings are consistent with some but not all previous studies that explored iron accumulation and transporter expression at the colonic microenvironment. There is strong evidence that support excess iron accumulation results in carcinogenesis (77, 79, 113, 114). Brookes et al. (79) found higher iron staining and potential ferroportin dysregulation in human colon cancer tissue compared to their non-involved mucosa. They observed an increased expression of iron influx transporters DMT-1, Dcytb and TfR1. Radulescu et al. using human colon cancer cell lines (SW480 and RKO) also found significantly higher DMT-1 (mean 1.8-fold, $p = 0.021$) and TfR1 (mean 1.7-fold, $p = 0.036$) compared to controls (114). We compared the mucosal DMT-1 in men with colon cancer compared to men with healthy colonic tissue and found no differences between groups. We observed a 2.5-fold higher trend for DMT-1 expression in advanced tumors (Stage IV versus Stage I) (Note: Staging discussion in Section 4) which suggests higher iron uptake in our late stage cases. Brookes et al. did not provide staging for the mRNA expression of DMT1 in cases thus its role in their findings is unknown. Additionally, we only assessed one iron importer, DMT-1, however intestinal cells have other iron influx transporters including TR1 and heme-iron transporters (poorly understood). If TfR1 expression had been measured we hypothesize it would confirm previous findings (79, 114), i.e.

colonic tumors would have higher expression compared to controls. Unfortunately, none of these studies measured hepcidin expression in tumors. Our findings confirm that colonic tumors have iron accumulation and suggest increased iron uptake/decreased iron export contribute to the mechanism.

Colon cancer and colonic hepcidin expression

We found hepcidin expression is reduced in colonic tumors compared to healthy controls. Ward et al. measured colonic mRNA expression of hepcidin in 34 colon cancer cases compared to their non-involved mucosa (84). They detected elevated hepcidin within the tumor (34 %;10 out of 34 tumor samples) compared to non-involved tissue (6%; 2 out of 34 non-involved samples) and suggested an inverse relationship between hepcidin and ferroportin (data was not shown) (84). Differences between our findings and Ward et al. (84) are likely at least partly due to differences in the control groups used. Ward et al. compared tumor to non-involved tissue within cases; we used healthy controls. Different methods to measure mRNA expression including different primers and instruments also likely contributed to the variability of results and may explain the contrasting findings (115). Additionally, it is unknown if Ward et al. (84) participants were receiving chemotherapy or medication which could influence tissue levels and they did not measure factors that influence hepcidin signaling (e.g. inflammation, BMP). Similar to Ward's findings, we found that hepcidin expression in colonocytes is not modified by tumor location, staging, or anemia status. Our findings demonstrate that hepcidin expression is reduced in colon cancer cases versus a control group with healthy mucosa (similar population characteristics) and expand by controlling for inflammation, BMP, iron status, diet and obesity.

f. Excess Dietary Iron and Colon Cancer Risk/Progression

Dietary iron and supplementation in colon cancer

The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) has stated the evidence for dietary iron and risk for colon cancer was inconsistent (116), however none of these cited in review controlled for inflammation, hypoxia and iron transporters. Our findings suggest once colon cancer has developed and colonic mucosal hepcidin expression is reduced any excessive iron that it is exposed to would be absorbed and potentially promote cell proliferation. It has been shown that colon cancer cell lines (Caco-2 and SW480) exposed to increased iron increases Wnt/β-catenin signaling, a major oncogenic pathway, compared to controls (117). Recent evidence using mice with *APC* deletion (i.e. with colon cancer) demonstrated increased tumorigenesis when fed a high iron diet compared to a low iron diet (114). These findings support the role of excess luminal iron as a cancer promoter. Additionally, individuals with hemochromatosis have a mutation in the *HFE* gene and consequently lack a signaling in hepcidin expression, resulting in increased iron absorption and accumulation and have a increased risk for colon cancer (Odds Ratio = 1.40; 95% confidence interval = 1.07- 1.87) (118). Collectively, these studies coupled with our findings suggest prudence is warranted when prescribing high dietary iron or supplements in patients with colon cancer.

g. Therapeutic Methods to Reduce Iron Accumulation

Lower available iron may provide protection from cancer risk. The VA Cooperative Study #410 performed a clinical trial and found the group that was phlebotomized for iron reduction (n=636) (every six months during five years) had lower cancer incidence (Hazard ratio = 0.61, 95% confidence interval = 0.49 to 0.92; P-value=0.019) compared to the control group (n=641) (119). These findings suggest that reducing iron stores may provide protection from any iron-induced free radical damage. These patients were older men (not specified) and increasing age

is positively associated with increased iron stores (120) thus reducing any excess iron via phlebotomy may correct for these alterations.

Our cases may benefit from chelation therapy. These drugs interfere with iron delivery to the tumor (121) and reduce cell proliferation by blocking the pro-oncogenic Wnt/ β-catenin pathway (122). Iron chelation may benefit these patients by diminishing luminal iron delivery to the tumor. Future clinical trials need to demonstrate the feasibility of chelation as implementation to the human population is associated with side effects (123).

2. DISCUSSION FOR SERUM FINDINGS

Our men with colon cancer had elevated systemic inflammation which likely countered the influence of their iron-deficient state, resulting in normal rather than reduced serum hepcidin levels. Both serum CRP and IL-6 were significantly higher in cases versus controls. Although cases trended lower for serum hepcidin compared to controls, both levels were within the normal range for adult men, (29-254 ng/mL) and this remained after matching for hemoglobin. Normally low iron status/anemia suppresses hepcidin levels to near zero or undetectable. Our study is the first to report serum hepcidin levels in colon cancer patients. The next sections will discuss how inflammation contributed to normal serum hepcidin levels in cases and the implications this poses during colon cancer.

a. Iron Status and Inflammation in Colon Cancer

Colon cancer is associated with low iron status (124-126) and inflammation (127-130). Although costs constraints prohibited measurement of serum iron, ferritin or transferrin saturation, the higher sTfR coupled with lower Hb levels supports decreased iron availability within cases. Among colon cancer patients low iron status could be attributed to bleeding, treatment, nutritional deficiencies and inflammation (83) . We excluded patients that reported bleeding per rectum or undergoing chemotherapy/radiation and did not observe any differences with dietary iron intake between cases and controls. Our findings suggest inflammation induced low iron status in cases. Elevated serum IL-6 levels in cases likely induced higher hepatic hepcidin production via the IL-6/STAT-3 pathway. Further, increased serum hepcidin reduces duodenal iron absorption by reducing iron uptake and export into circulation (although DMT-1 and ferroportin were not measured in the duodenum). Overall our findings suggest that colon cancer is associated with serum hepcidin levels inappropriately high for the degree of iron restriction. We hypothesize this elevated serum hepcidin reduces duodenal iron absorption causing low iron status and exposes the colon to excess luminal iron within the fecal stream and

increased colon cancer risk. The inappropriately normal serum hepcidin may also further decrease ferroportin protein on tumor cells, promoting their iron retention and aiding their growth.

Other diseases with inflamed gastrointestinal mucosa and colon cancer

Patients with inflammatory Bowel Disease(IBD)/Ulcerative Colitis(UC) experience chronic colonic inflammation and are at an increased risk for colon cancer (51). These patients also have iron deficiency and elevated hepcidin (131). Oustamanolakis et al. found that UC patients (n=49) had elevated serum hepcidin levels compared to healthy controls (n=102) (UC median: 73.6 ng/mL range: (16.5–736), versus controls: 47.0 ng/mL (8.6–340.2); P-value<0.0001) and hepcidin was negatively correlated with hemoglobin (r= -0.36; P-value=0.0003) and positively correlated with CRP (r=0.29; P-value=0.04) (132). Similar to our men with colon cancer their inflammation induced serum hepcidin also likely reduces duodenal iron absorption and increased exposed of the colonic lumen to excess iron and cancer risk. Mouse models for UC demonstrated high dietary iron exacerbates colonic inflammation and tumor incidence (133, 134). Further, Gasche et al. (135) noted oral iron supplementation in IBD/UC patients did not improve their low iron status however when provided intravenously their low iron status resolved. Collectively, our findings and those of Gasche and Oustamanolakis support the hypothesis that inflammation induced elevations in hepcidin causes the link with excess luminal iron in the fecal stream and heightened exposure and neoplasm risk.

It is likely dietary iron and/or supplements would not correct the iron status of our cases because their serum hepcidin levels would prevent iron absorption and release into circulation. As mentioned in Section 1, any excess luminal iron exposure in the colon promotes carcinogenesis, thus a greater understanding of the role of serum and colonic level hepcidin, iron status and inflammation has the potential to expand options for prevention and treatment of colon cancer.

Aspirin, lower colonic iron exposure and colon cancer prevention

Aspirin use has been associated with lowering colon cancer risk via inhibiting pro-inflammatory enzymes cyclooxygenase (COX-1, COX-2). Rothwell et al. reported that long-term aspirin use (mean treatment 6.0 years) was associated with significantly reduced colon cancer incidence (Hazard ratio= 0.76, 95% Confidence Interval= 0.60-0.96; P-value=0.02) (136). Aspirin can also reduce risks for CRC by reducing hypoxia through its reduction of HIF-2α levels (137). As mentioned in Section 1, hypoxia within the tumors may increase iron uptake. We found the cases that stained positive for tumor iron reported less regular intake of aspirin compared to the cases that stained negative for iron, however we did not have adequate power to detect significance for this exposure. The aspirin associated risk reduction may involve its reduction in iron dysregulation, ultimately reducing luminal iron exposure.

b. <u>Iron Status Profile for Colon Cancer</u>

Overall our findings suggest that colon cancer is associated with a mixed anemia profile characterized by elevated sTfR (a marker of iron depletion) and inflammation (elevated CRP and IL-6). No previous reports of anemia in colon cancer have included measures of inflammation, despite its known influence on the iron parameters they used. We believe the inflammation present in our cases countered the signals to reduce hepcidin from the hypoxia within their tumor and low iron status. These simultaneous, opposing signals resulted in cases having serum hepcidin levels that were within the normal range, despite their iron depleted

status. These findings will enable clinicians to understand the iron status of their colon cancer patients and accurately prescribe treatment to correct their low iron status/anemia.

3. DISCUSSION FOR OBESITY FINDINGS

a. Obesity and Inflammation

The association between circulating inflammatory markers (CRP, IL-6, TNF-α) and waist circumference (WC) in a metabolic healthy population has been previously reported (138-140) and are quite similar to those observed with our controls. We did not however find significant correlations between TNF-α and obesity (141, 142). Our small sample size may have reduced our ability to detect the WC-TNF-α association. Collectively, our findings within controls confirm the traditional role of increased adiposity and low-grade chronic inflammation.

No association between obesity or WC and inflammation was found in our men with colon cancer. Numerous prospective studies demonstrate that a high WC, i.e. central adiposity is associated with an increased risk for colon cancer (54, 58, 59) . One of the mechanisms linking obesity and colon cancer has been the role of inflammation. Huang et al. postulated that the obesity related cytokines IL-6 and TNF-α produced by macrophages in adipose tissue and released into circulation could bind to its receptor on tumors and activate carcinogenesis pathways (Mechanisms discussed in II. 7.) (52). Independent of obesity induced inflammation, immune cells are present in the tumor microenvironment which can also secrete pro-inflammatory cytokines to increase signaling for cell proliferation (143). Our lack of detecting any influence of obesity markers of inflammation in our cases is likely due to their tumor induced inflammation (128, 129). Unfortunately, no previous studies included measures of obesity at diagnosis. These findings suggest in the presence of colon cancer obesity does not modify systemic inflammation instead obesity associated inflammation is likely a risk factor for cancer initiation.

b. Obesity and hepcidin

Tissue findings

As anticipated we observed directionality in colonic tissue for higher hepcidin and lower ferroportin expression in obese compared to lean controls. Although there were no differences with colonic IL-6 expression between groups, systemic inflammation (CRP, IL-6) was elevated in the obese group which likely stimulated higher colonic hepcidin expression. Hepcidin regulates iron availability via degradation of ferroportin which blocks export of iron from the iron containing cells (e.g. enterocytes, macrophages and hepatocytes) (1). Unexpectedly, the elevated colonic hepcidin in the obese did not result in any differences in mucosal iron accumulation between our groups. Only 1 of 20 controls stained positive for iron accumulation using Perl's staining which is a crude and reliable assessment (yes/no). One explanation for the null findings is that normal colonocytes do not absorb a significant amount of iron (from either the diet or circulation). Additionally, iron transporter alterations in pre-malignant mucosa likely requires more time to observe any changes. The transition from healthy mucosa to adenocarcinoma takes 10-15 years thus iron accumulation may increase with disease progression. To confirm this hypothesis a study in populations with high risk for colon cancer (i.e. patients with adenomas) should be conducted which measure colonic iron accumulation in obese versus lean participants using spectroscopy (quantitative method).

Serum findings

Our obese controls had higher markers of inflammation compared to their lean counterparts however they had similar serum hepcidin levels. Currently the obesity-hepcidin-low iron status relationship in adults has been limited to pre-menopausal women (18, 29). Women have an increased risk for iron deficiency compared to men. Our exclusion of women likely explains why we failed to observe a difference in serum hepcidin between obese and lean controls.

Supporting this idea, Galesloot et al. reported that BMI positively predicted 20.1% (r-squared) of the variation in serum hepcidin levels in females and only 3.9% (r-squared) of the variation in males (144). Additionally, Martinelli et al. reported that metabolic syndrome is positively associated with serum hepcidin, however this relationship was only found in females (145). Collectively our findings and these findings support the obesity: hepcidin relationship is not generalizable to the male population.

Although not assessed, testosterone could influence the obesity: hepcidin relationship. Population based studies have reported that obesity is associated with low testosterone levels (146, 147). Additionally, Bachman et al. found that administering testosterone in older men (mean=65.6 years old $\pm$ 4.3) suppressed hepcidin levels (148). Mechanistically, Guo et al. using hepatocytes (HepG2) observed that testosterone blocked BMP/SMAD signaling and resulted in decreased hepcidin expression (149). Thus it may be obese men with low testosterone (i.e. higher hepcidin inducing stimuli) have higher serum hepcidin compared to lean men. While our obese men were inflamed, they may have had similar testosterone levels compared to the lean men which prevented us to observe the obesity: hepcidin relationship. The link with testosterone, obesity and hepcidin remains to be explored.

Finally, the obesity: hepcidin association in our controls may have been due to a lack of comorbidities and IL-6-hepcidin signaling. The obesity: elevated hepcidin association found by Tussing-Humphreys et al. compared morbidly obese women (BMI mean=49.8, SD=11.0; WC: median=127.5 cm, IQR=22.5) versus lean HB-matched pre-menopausal women (BMI mean=22.5, SD=3.0; WC: median=77.9 cm, IQR=11.8) (P-value<0.0001) (18). Our groups did not have as large of an adiposity difference with obese controls (BMI mean= 31.5, SD=6.6; WC: median=109.7 cm, IQR=28.2) compared to lean controls (BMI mean= 24.2, SD=3.2; WC: median=91.4 cm, IQR=8.0) (P-value=0.001). These differences may have prevented IL-6-Hepcidin signaling. Mechanistically, IL-6 induces hepcidin expression by binding to its receptor (membrane receptor, sIL6mb or soluble receptor, sIL6R) which activates the STAT-3 pathway to

increase hepatic hepcidin expression (8). An explanation for our findings may be that higher IL-6 may not be functioning due to a lack of accompanying increases in its receptor (not measured). In agreement, Mohamed-Ali et al. found that metabolically healthy obese individuals had higher serum IL-6 compared to lean, however they did not have elevated IL-6 soluble receptor (150). A likely scenario affecting IL-6 and hepcidin signaling in obese individuals is the presence of other comorbidities. Zuliani et al. found that MetS individuals have higher IL-6 receptor compared to controls however after controlling for insulin resistance the levels were similar between groups (151). One hypothesis may be that morbidly obese men (i.e. BMI >40) with insulin resistance have higher serum hepcidin compared to metabolically healthy obese men. A future study should confirm this hypothesis before ruling out the obesity: hepcidin relationship in men.

4. DISCUSSION FOR STAGING FINDINGS

a. Association with IL-6 and Staging

Advanced staged colon cancers are associated with higher inflammation (152). Chung et al. found human colon cancer tissue (n=160) that expressed IL-6 was significantly associated with advanced staging, lymph node metastasis and venous invasion compared to tumors that did not express IL-6 (153). Several population based studies have also found that circulating IL-6 is associated with late stage colon cancer (154-156). IL-6 can act to increase cell proliferation and reduce apoptosis in tumors (92). Our findings confirm the hypothesis that advanced colon cancer staging is associated with higher IL-6 compared to early stage tumors.

Higher levels of IL-6 observed in our advanced staged cases within *tissue* and *serum* does not suggest increased expression of hepcidin. Similar to our findings Ward et al. also observed no modification for colonic tumor hepcidin expression by cancer staging (84). As discussed in Section 1, within colonic *tissue* hepcidin regulation is likely influenced by other factors e.g. hypoxia/EPO. Thus, these results are not surprising. In contrast, similar levels of *serum* hepcidin across staging was unexpected. Our normal serum hepcidin levels in cases (not reflective of iron their low iron status) suggests that inflammation induced higher circulating levels. One explanation of why a positive trend with IL-6 and hepcidin in advanced staging was not found may be due to our limited power. Another explanation could be that signals that decrease hepatic hepcidin expression (EPO/hypoxia) were increased across staging. Unfortunately, we did not measure EPO/hypoxia. Further, future studies should explore if circulating hepcidin is associated with advanced staging. Higher hepcidin levels in advanced colon cancer would suggest a role for tumor progression via a larger effect with decreased duodenal iron absorption and thus increased luminal exposure in the colon.

b. Role of Iron Transporters and Staging

Advanced colon cancer staging is accompanied with greater tumor iron sequestration. Numerous studies have shown that iron is required for tumor growth and proliferation (79, 91, 157) therefore alterations with its transporters across advanced staging seems plausible. We are the first to report DMT-1 mRNA expression in colonic tumors by staging and found a positive directionality with advanced staging. Brookes et al. analyzed the protein expression of DMT-1 in human colonic tumors and did not find any association with staging (data not shown) (79). Although mRNA and protein levels of DMT-1 are correlated (79), an explanation for the differing findings may be how the results were reported. We measured DMT-1 mRNA quantitatively (qRT-PCR) in contrast protein expression was measured qualitatively via staining (immunohistochemistry). Contrary to our hypothesis, we did not find a difference with staging and FPN expression. Brookes et al. found lower FPN protein expression with advanced staging (79). FPN expression can be modified post-translationally by inflammation to reduce its expression (158). Thus, it is likely the inflammation: staging relationship in our cases would also be associated with lower FPN protein expression. Collectively our findings coupled with Brookes et al. suggests that advanced staged colon cancer is associated with higher iron uptake and decreased iron efflux resulting in higher iron accumulation to progress carcinogenesis.

5. STUDY STRENGTHS AND LIMITATIONS

a. **Strengths**

Our strengths include measurements of hepcidin and inflammation in both serum and colonic tissue and simultaneously reporting iron status. Previous studies have reported low iron status and elevated inflammation in colon cancer patients however they did not account for the regulation by hepcidin. This is the first study that has measured both serum and colonic mRNA expression of hepcidin in colon cancer cases compared to metabolic healthy controls. Our findings are the first to demonstrate normal serum hepcidin levels in colon cancer subjects with low iron status. Additionally we are the first to report lower colonic hepcidin expression in tumors compared to control tissue. We also controlled for confounding with factors that influence iron status (hemoglobin, waist circumference, dietary iron, BMP) and our results remained unchanged.

b. Limitations

This study is presented with limitations. We were not able to account for other factors affecting hepcidin regulation such as EPO or hypoxia which are likely influencing our tissue level findings. Additionally we only measured BMP-2 and other classes of BMP exist including BMP-4, 6. These additional parameters were not measured due to cost constraints.

While our sample size (n=40) was sufficient to observe differences with colonic hepcidin expression between cases and controls, it posed challenges in other analyses. When we stratified our serum results by obesity or cancer staging our sample size comparisons became smaller and reduced statistical power. Thus this may have influenced our null findings with obesity or cancer staging as modifiers with serum hepcidin.

We suggested normal serum hepcidin levels during low iron status may be associated with reduced duodenal iron absorption. Unfortunately, we did not measure iron transporters in the duodenum or iron absorption. Small bowel biopsies are obtained during upper endoscopy procedures (EGD-esophagogastroduodenoscopy) and our subjects were only scheduled for colonoscopies or colorectal surgery. Iron absorption can be measured during specific time intervals by magnetic sector thermal ionization mass spectrometry (43) however our subjects were fasting and limited to blood collection at one time point.

Our analysis included only males. The primary reason why we excluded females was due to gender differences with iron metabolism. Thus we may be limited with generalizability to the entire population.

Finally, this study was cross-sectional thus we cannot assess causality.

VI. CONCLUSIONS AND FUTURE DIRECTIONS

In summary, we found that colon cancer patients exhibit iron dysregulation that is characterized by increased iron uptake for tumor needs rather than entering into circulation for hemoglobin synthesis. Dietary iron and supplementation likely exacerbates the tumor growth rather than correct the low iron status/anemia. Therefore, clinicians need to accurately understand the implications of the alternated GI tract during colon cancer before prescribing harmful treatment.

The link with obesity and iron for colon cancer risk requires further exploration by measuring additional parameters in colonic mucosa. Our findings suggest that the colonic mucosa in obese controls have increased hepcidin and decreased ferroportin expression which may be a risk factor for increased luminal iron exposure and neoplasm risk. To confirm this hypothesis that obesity induced inflammation is contributing to mucosal changes we would need to measure parameters unaccounted for in our analysis including factors that could increase iron uptake, TfR1 and a marker of DNA damage/oxidative stress, 8-hydroxyguanosine. A future study should also measure these parameters in adenoma tissue to determine if obesity and iron can ultimately influence iron dysregulation from the transition of healthy mucosa to adenocarcinoma.

Our findings may be generalizable to other epithelial tissue cancers including breast cancer. Obesity is associated with an increased risk for breast cancer in post-menopausal women (159, 160). Additionally, women with breast cancer have lower expression of ferroportin in their breast cancer tissue which is associated with a decrease in metastases–free prognosis (161) suggesting that decreased iron exposure within tumors reduces tumor growth. The link with obesity, iron and breast cancer remains to be explored.

Iron is an essential element however during colon cancer tumor cells appear to have iron homeostasis reprogrammed to benefit its needs. Recent advances with understanding the contribution of obesity and iron dysregulation may provide one of the pathways that increase

cancer risk. Characterizing these pre-malignant changes with obesity and iron could ultimately influence therapeutic and weight loss interventions for cancer prevention.

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Appendices

Appendix A

Interviewer Guide to be filled out by research staff only
Chicago Colorectal Cancer Consortium: Colorectal Cancer in African Americans
Pt. Study ID#: _____ **Date of Procedure:** _____
Date of Diagnosis (if different): _____
(Section for subjects ONLY): Please Attach Colonoscopy, Surgery, CT, and Pathology Reports
Tumor Localization: (Circle One)
Left Colon: Splenic Flexure Descending Sigmoid Rectum Recto-sigmoid
Right Colon: Transverse Ascending Hepatic Flexure Cecum
Check one: **[ ] Prevalent Tumor (diagnosis greater than 12 months ago) [ ] Incident Tumor**
Chemo prior to consent? [ ] Y [ ] N Date start:_____ Date end:_____
Type:_____
Synchronous Cancers: [ ] Y [ ] N If **YES**, Location 1_____Location 2_____
Sample Type Obtained: (Circle what applies)
Paraffin Endoscopic Biopsy Surgical Sample Blood ONLY
Personal Information: Sex: M F DOB: _____ Age: _____ Subject's Zip Code: _____
Height (in): _____ Weight (lbs): _____ Waist Circumference (cm):_____Marital Status:_____
Mother's Ethnicity: _____ Father's Ethnicity: _____
Insurance Type: [ ] Commercial [ ] Medicaid [ ] Medicare [ ] Non-insured [ ] VA Insurance
Ethnic group/Demographic Group:
African American Afro-Caribbean
Jewish Latin American
Caucasian/ N. European Native American
Hispanic - Caribbean Pacific Islander
Asian Other
Annual Household Income: (Taxable income for the previous year)
[ ] < $25,000 [ ] $25,000 - $ 75, 000 [ ] > $75,000
Number of residents living with subject (excluding subject): _____
Highest Level of Education:
[ ] Elementary School [ ] High School [ ] College Graduate (4-yr degree)
[ ] Middle School [ ] Some College [ ] Post-Graduate (e.g., MS, MD, PhD)
Occupation ***(if retired****, state occupation prior to retirement)*: _____
Personal Medical History: *Yes No Don't Know*
Has the subject ever had a colonoscopy? [ ] [ ] [ ]:
Where (Hospital):_____
When: _____How many: _____
IF YES, has the subject ever had colon polyps? [ ] [ ] [ ]
If YES, what were the diagnoses? (Mark all that apply):
[ ] Hyperplastic [ ] Tubular Adenoma [ ] Tubular Villous Adenoma [ ] Villous Adenoma
[ ] High-grade dysplasia [ ] Carcinoma in-situ [ ] Don't know

Yes No
Has the subject ever had ulcerative colitis? [ ] [ ]□□Confirmed by chart : Y
Has the subject ever had Crohn's disease? [ ] [ ]□□Confirmed by chart : Y **Has the subject ever had inflammatory bowel disease (IBD)?** [ ] [ ]□□Confirmed by chart : Y
Has the subject previously had Colon or Rectal cancer? [ ] Yes [ ] No
Age at CRC diagnosis_____ Location of cancer: _____
Where was the patient diagnosed (what hospital)? _____
(Attach pathology report of previous cancer diagnosis if you can.)
For subjects with CRC history & a current diagnosis of colon cancer CHECK ONE below to describe the new cancer:
____ **Metachronous** (two cancers of the colon at different times, not a recurrence, usually occurs within 6 mos.)
____ **Recurrence** (cancer in the same location as the previous tumor)
Any previous cancers? (List all below)
1. Type:_____Age at diagnosis_____ Hospital where patient was diagnosed: _____
2. Type:_____Age at diagnosis_____ Hospital where patient was diagnosed: _____

Family History of Cancer:
Information of the subject's close family members: **MOM or DAD, SISTERS or BROTHERS, AUNTS or UNCLES, GRANDPARENTS, and CHILDREN**
- Has anyone in the subject's *close* family had colon or rectal cancer? [ ] Yes [ ] No [ ] Don't know
If YES, please tell us which relative (maternal or paternal side) and about how old they were at diagnosis:
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
- Has anyone in the subject's *close* family had breast cancer? [ ] Yes [ ] No [ ] Don't know
If YES, please tell us which relative (maternal or paternal side) and about how old they were at diagnosis:
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
- Has anyone in the subject's *close* family had endometrial (uterine) cancer? [ ] Yes [ ] No [ ] Don't know
If YES, please tell us which relative and about how old they were at diagnosis:
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
- Has anyone in the subject's *close* family had ovarian cancer? [ ] Yes [ ] No [ ] Don't know
If YES, please tell us which relative (maternal or paternal side) and about how old they were at diagnosis:
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
- Has anyone in the subject's *close* family had stomach cancer? [ ] Yes [ ] No [ ] Don't know
If YES, please tell us which relative (maternal or paternal side) and about how old they were at diagnosis:
Relative: _____ Age: _____ Maternal or Paternal

Relative: _____ Age: _____ Maternal or Paternal
Relative: _____ Age: _____ Maternal or Paternal
-Is there any other family history of cancer? [ ] Yes [ ] No
Cancer type: _____ Relative: _____ Age: _____Maternal or Paternal
Cancer type: _____ Relative: _____ Age: _____ Maternal or Paternal
Cancer type: _____ Relative: _____ Age: _____ Maternal or Paternal

Medication and Supplement Use History:
1. Non-Steroidal Anti-Inflammatory Medications (NSAIDS) (circle type used): such as Advil, Motrin, Ibuprofen
a. Did he/she use these medicines in the past 5 years? [ ] Yes [ ] No
b. During the past 5 years, how often did he/she take these medicines? *(Check one)*
[ ] Rarely / Seldom (<1 time per month)
[ ] Occasionally (1 -10 days per month)
[ ] Regularly (3 days or more per week)
c. In total, how long did he/she take these? ____ years ____months ____weeks
2. COX-2 Inhibitors (Most often used for arthritis) (circle type used): such as Celebrex, Vioxx, Bextra
a.Did he/she use these medicines in the past 5 years? [ ] Yes [ ] No
b. During the past 5 years, how often did he/she take these medicines? *(Check one)*
[ ] Rarely / Seldom (<1 time per month)
[ ] Occasionally (1 -10 days per month)
[ ] Regularly (3 days or more per week)
c. In total, how long did he/she take these? ____ years ____months ____weeks
3. Aspirin (circle type used): such as "Baby" aspirin (81mg), Regular aspirin (325mg), Bufferin, Anacin, Excedrin, or Alka Seltzer
a. Did he/she use these medicines in the past 5 years? [ ] Yes [ ] No
b. During the past 5 years, how often did he/she take these medicines? *(Check one)*
[ ] Rarely / Seldom (<1 time per month)
[ ] Occasionally (1 -10 days per month)
[ ] Regularly (3 days or more per week)
c. In total, how long did he/she take these? ____ years ____months ____weeks
4. Fish Oil or Omega-3 Supplements
a. Did he/she use these supplements in the past 5 years? [ ] Yes [ ] No
b. How much was he/she taking _____ mg.
c. During the past 5 years, how often did he/she take these medicines? *(Check one)*
[ ] Rarely / Seldom (<1 time per month)
[ ] Occasionally (1 -10 days per month)
[ ] Regularly (3 days or more per week)
d. In total, how long did he/she take these? ____ years ____months ____weeks

Pt. Study ID#: _____

5. Cholesterol Medication (Statins) (circle type used): such as Levacor, Zocor, Pravachol, Lipitor, or Crestor

a. Did he/she use these medicines in the past 5 years? [ ] Yes [ ] No

b. During the past 5 years, how often did he/she take these medicines? *(Check one)*

[ ] Rarely / Seldom (<1 time per month)

[ ] Occasionally (1 -10 days per month)

[ ] Regularly (3 days or more per week)

c. In total, how long did he/she take these? ____ years ____months ____weeks

Smoking History: Does the subject currently smoke?

[ ] Yes → How much does the subject smoke per day?

- Cigarettes: __ __ - Other _____
- Cigars: __ __
- Pipes: __ __

[ ] No→ **Has the subject *ever* smoked?** [ ] Yes [ ] No

How long has it been since the subject quit? __ __ years __ __ months

How much did the subject typically smoke per day?

- Cigarettes: __ __ - Other _____
- Cigars: __ __
- Pipes: __ __

Overall, for how long has the subject smoked? __ __ years __ __ months

Alcohol History: Does the subject drink alcohol on a weekly basis?

____ YES → Quantify how much alcohol the subject consumes per week (on average)?

Beer (glasses / cans) __ __ Hard liquor (shots) __ __

Wine (glasses) __ __ Mixed Drinks __ __

____ NO → **Has the subject ever drank alcohol on a weekly basis?** [ ] Yes [ ] No

How long has it been since the subject quit? __ __ years __ __ months

How much did the subject typically drink per week (on average)?

Beer (glasses / cans) __ __ Hard liquor (shots) __ __

Wine (glasses) __ __ Mixed Drinks __ __

Overall, for how long has the subject drank alcohol? __ __ years __ __ months

Exercise: How often does the subject exercise?

Never 1-2 times per month 1 time per week 2-3 times per week Daily

What does the subject do for exercise?

1)_____2)_____3)_____

How many minutes does the subject exercise per day?

1)_____2)_____3)_____

Pt. Study ID#: _____
Genetic Testing:
1. Has the subject ever had genetic testing? [ ] Yes [ ] No [ ] Don't know
If YES, how long ago and what for?_____
Do you have a copy of your report that we can have? [ ] Yes [ ] No
Has the subject's parents, brothers, sisters, children or other family members ever had genetic testing? [ ] Yes [ ] No [ ] Don't Know
IF YES, who, how long ago, and what for: _____
Were any genetic abnormalities or mutations found? [ ] Yes [ ] No
Yes No Don't Know
2. Has the subject ever had Lynch Syndrome? [ ] [ ] [ ]
3. Is there a family history of familial adenomatous polyposis (FAP)? [ ] [ ] [ ]
3a. Has the subject ever been diagnosed with FAP? [ ] [ ] [ ]
4. Is there a family history of Gardner's Syndrome? [ ] [ ] [ ]
4a. Has the subject ever been diagnosed with Gardner's Syndrome [ ] [ ] [ ]
5. Has any of the subject's parents, brothers, sisters or children ever had colon polyps? [ ] [ ] [ ]
5a. If YES, were any of them under age 60 at diagnosis? [ ] [ ] [ ]

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ABSTRACTS: The Chicago Colorectal Cancer Consortium (CCCC), a step forward in understanding colorectal cancer in African Americans. Xicola RM, Gagnon M, Shaw J., Rodriguez G., Pusatcioglu CK, Clark J., Disharoon A., Janoski A., Morrissey K., Mraz K., Ravella S., Rawson J, Gluskin A., Brock J., Cerye K., Bhattacharya T., Guzman G, Chaudhry V, Kupfer S, Melson J, Freeman V, Xie H, Braunchweig C, Ellis N, Llor X. (Abstract-AACR Cancer Disparities Meeting 2011)

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