Effect of Obesity-associated Iron dysregulation on Breast Cancer Risk in Postmenopausal Women

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THESIS

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# TABLE ON CONTENTS

## I. INTRODUCTION
   1. OVERVIEW
   2. SPECIFIC AIM AND HYPOTHESES

## II. BACKGROUND AND LITERATURE
   1. POST-MENOPAUSAL BREAST CANCER
   2. OBESITY AND POSTMENOPAUSAL BREAST CANCER RISK AND PROGNOSIS
   3. MECHANISTIC LINK BETWEEN OBESITY AND POSTMENOPAUSAL BREAST CANCER
   4. OBESITY AND IRON METABOLISM
   5. IRON AND CANCER
   6. OBESITY, IRON AND POSTMENOPAUSAL BREAST CANCER

## III. RESEARCH DESIGN AND METHODS
   1. STUDY DESIGN AND POPULATION
   2. SAMPLING PROCEDURE
   3. SAMPLE SIZE ESTIMATIONS

## IV. MEASUREMENT OF VARIABLES
   1. DEMOGRAPHICS AND BASELINE INFORMATION
   2. BODY MASS INDEX
   3. BIOCHEMICAL MEASURES
   4. BREAST TISSUE ANALYSIS

## V. STATISTICAL ANALYSIS
   1. STATISTICAL ANALYSIS FOR SPECIFIC AIM 1
   2. STATISTICAL ANALYSIS FOR SPECIFIC AIM 2
   3. STATISTICAL ANALYSIS FOR SPECIFIC AIM 3

## VI. RESULTS
   1. RESULTS FOR SPECIFIC AIM 1
   2. RESULTS FOR SPECIFIC AIM 2
   3. RESULTS FOR SPECIFIC AIM 3

## VII. DISCUSSION
   1. DISCUSSION FOR SPECIFIC AIM 1
   2. DISCUSSION FOR SPECIFIC AIM 2 AND 3
   3. STUDY’S STRENGTHS AND LIMITATION

## VIII. CONCLUSIONS AND FUTURE DIRECTIONS

## IX. APPENDICES
   APPENDIX - A
   APPENDIX - B
   APPENDIX - C

## X. CITED LITERATURE
LIST OF TABLES

Table VI-I: Anthropometric and Background Information in cases and controls (n=88) ................................................................. 30
Table VI-II: Tumor Characteristics among cases (n=44) ......................................................................................................................... 31
Table VI-III: Plasma levels in cases and controls (n=88) ...................................................................................................................... 32
Table VI-IV: Comparison of parameters in obese vs non-obese within and between cases (n=44) and controls (n=44) ................................................................. 34
Table VI-V: Spearman correlations for plasma hepcidin with BMI and selected biochemical measures among overall participants, within cases and controls and by obese and non-obese groups ............... 36
Table VI-VI: Final linear regression model for log hepcidin in overall participants, controls, cases and ER, PR-double positive tumors (Log hepcidin = log CRP  log sTFR) .................................................................................................. 38
Table VI-VII: Tissue parameters in cases and controls ............................................................................................................................ 40
Table VI-VIII: Comparison of parameters in obese vs non-obese cases ........................................................................................... 41
Table VI-IX: Correlations of plasma hepcidin with iron and FPN1 content in overall participants, cases and controls ................................................................. 45
LIST OF FIGURES

Figure II-1: Established mechanistic links between obesity and breast cancer............................................................... 6
Figure II-2: Obesity induces iron insufficiency due to increase in pro-inflammatory peptide hormone, hepcidin ......................................................................................................................................................... 14
Figure II-3: The hypothesized link between obesity, iron metabolism and breast cancer ............................................ 18
Figure VI-1: Perls’ staining indicating non-heme iron (Fe (III)) in blue color, photographed at 200x ....................... 42
Figure VI-2: Breast tumor showing mononuclear inflammatory infiltrates, photographed at 200x ...................... 42
Figure VI-3: Ferroportin (IHC- depicted by brown color) indicating decrease in breast cancer tissue, photographed at 200x ........................................................................................................................................ 43
Figure VI-3: Breast tumor showing smaller adipocytes on the invasive front of cancer, photographed at 4x ........ 44
LIST OF ABBREVIATIONS

BMI  Body Mass Index
CLS  Crown-like structures
CRP  C-reactive protein
DCIS Ductal carcinoma in-situ
DFS  Disease-free survival
DXA  Dual-energy X-ray absorptiometry
ELISA Competitive enzyme linked immuno-assay
ER+, PR+ Estrogen receptor positive, Progesteron receptor positive
FPN1 Ferroportin
H & E Hematoxylin and eosin
HRT  Hormone Replacement Therapy
ID   Iron deficiency
IDC  Invasive Ductal Carcinoma
IHC  Immunohistochemistry
IL-6 Interleukin-6
IQR  Inter Quartile Range
IRP2 Iron regulatory protein-2
JAK  Janus Kinase
mRNA Messenger ribonucleic acid
NHANES National health and nutrition examination survey
ROS  Reactive oxygen species
SD   Standard deviation
SHBG Sex hormone binding globulin
STAT3 Signal transducer and activator of transcription
sTFR Soluble Transferrin Receptor
TfR1 Transferrin receptor-1
UIC  University of Illinois at Chicago
I. INTRODUCTION

1. Overview
In the US, one out of every eight women will be diagnosed with breast cancer in her lifetime [1]. Obesity, a major epidemic problem, is an established risk factor [2] and cause of poor prognosis [3] for postmenopausal breast cancer. The chronic low-grade inflammation induced by obesity is the primary mechanistic link for this enhanced risk via distinct, yet intertwined, pathways. Specifically, these pathways include (a) elevated adipocyte estrogen synthesis [4, 5], (b) enhanced insulin resistance and hyperinsulinemia, which affect cell metabolism, decrease sex hormone binding globulin (SHBG) and elevate free estrogen [6], and (c) adipokines, leptin and adiponectin, secreted by adipose tissue [7]. Breast tumors grow in proximity with adipose tissue, and recent studies have focused on the effect of tumor-surrounding adipocytes on breast cancer [8].

Excess iron is pro-oxidant and can generate reactive oxygen species (ROS), causing oxidative stress and DNA damage, which is critical for carcinogenesis. Iron is also necessary for tumor growth, as rapidly dividing cells have high iron requirements. Administration of iron in animal models of cancer increased tumor development [9, 10]. Studies have shown that breast cancer tumors have more iron [11] and express higher ferritin [12] and higher transferrin receptor-1 (TfR1), indicating increased tumor iron needs [13] than normal breast tissue. Postmenopausal women with elevated iron concentrations in breast tissues have a higher risk of developing breast cancer [14]. Increased hepcidin and decreased ferroportin-1 (FPN1) mRNA expression (two main iron regulatory proteins) in breast tissue correlate with poor prognosis of breast cancer [15]. Incubation of breast cancer cell lines with hepcidin down regulates FPN1 and increases the iron labile pool [15]. A recent study found iron regulatory protein-2 (IRP2) elevated in breast cancer and associated with decreased ferritin, increased TfR1 and the labile iron pool [16].

Obesity-induced low-grade inflammation also alters iron metabolism [17]. Obese children [18, 19] and premenopausal women [20] are iron-insufficient due to their increased production of the hepatic peptide, hepcidin [16]. Hepcidin’s primary role is regulation of systemic iron homeostasis by binding to the only known iron exporter,
The hepcidin-FPN1 complex is internalized and degraded within the lysosomes, preventing the iron within the absorptive enterocytes, splenic macrophages and hepatocytes from exiting these cells and entering the plasma. In conditions of iron overload and inflammation hepcidin levels are elevated to reduce iron absorption and availability. In iron deficiency and hypoxemia hepcidin levels are extremely low to maximize iron availability [22]. It has been hypothesized that the elevated hepcidin that accompanies obesity results in iron sequestration in adipose tissue-associated macrophages and in other tissues like endothelium [23].

Obesity increases cancer risk among postmenopausal women. Due to menstrual cessation postmenopausal women are generally iron sufficient. Excess iron is carcinogenic. Obesity is also associated with dysregulated iron metabolism. However, it is unknown if obesity-induced alterations in iron metabolism among postmenopausal women causally contributes to increased breast cancer risk along with other established pathways.

2. **Specific aim and hypotheses**

The purpose of this study was to determine if dysregulated iron metabolism contributes to the obesity-enhancing risk for breast cancer in postmenopausal women. A case-control design was used to explore the effect of obesity on hepcidin levels and iron status among postmenopausal women. Further, we assessed association of plasma hepcidin and a marker of iron status (soluble transferrin receptor (sTFR)) with established plasma biomarkers of obesity-related breast cancer risk, breast cancer occurrence and breast tissue iron and FPN1 content.

**SPECIFIC AIM 1:** To describe the influence of obesity on plasma hepcidin levels and sTFR in postmenopausal women without (controls) and with (newly diagnosed cases) breast cancer and to assess the association of these iron-status parameters with established breast cancer risk factors (inflammation, adipokines and estrogen) and breast cancer occurrence.

**Hypothesis 1a:** Obese postmenopausal women (cases and controls) will have significantly higher plasma hepcidin and sTFR (indicator of altered iron metabolism) than their non-obese counterparts.
Hypothesis 1b: Iron insufficiency and elevated hepcidin among obese women (cases and controls) will be associated with higher markers of inflammation, leptin and estrogen and lower levels of adiponectin.

Hypothesis 1c: Systemic hepcidin will be positively associated with breast cancer after controlling for other established risk factors.

SPECIFIC AIM 2: To determine if breast tissue’s iron content, FPN1 content (the exclusive cellular iron exporter) and mammary adipocyte cell size vary between cases and controls and to assess the impact of obesity on these parameters.

Hypothesis 2a: Cases will have higher iron content, lower FPN1 content and decreased mammary adipocyte cell size compared to controls.

Hypothesis 2b: Breast tissue of obese participants (cases and controls) will have higher iron content, lower FPN1 content and bigger adipocyte cell size than tissue from their non-obese counterparts.

SPECIFIC AIM 3: To discern the association between plasma hepcidin and breast tissue iron and FPN1 content in cases and controls.

Hypothesis 3a: Plasma hepcidin will be positively associated with iron content and negatively associated with FPN1 content in breast tissue and these associations will be stronger in cases than controls.
II. BACKGROUND AND LITERATURE

1. Post-menopausal breast cancer

The third most frequent cancer in the world is breast cancer, which is also the most common malignancy and cause of death among women [24]. Postmenopausal breast cancer is much more common than premenopausal breast cancer (441 vs. 75/10000 women) [25]. The classic model of neoplastic progression in breast tissue initiates in normal epithelium cells and progresses in the multistep process of (a) flat epithelial atypia, (b) atypical ductal hyperplasia (c) ductal carcinoma in situ /lobular carcinoma in situ and finally to (d) invasive ductal cancer (IDC)/ invasive lobular cancer [26]. Genetic (Breast Cancer gene (BRCA), Phosphatase and tensin homolog (PTEN) mutations) and epigenetic factors (DNA methylation and histone modification) account for a majority of post-menopausal breast cancer development; however, there are other established environmental factors such as Body Mass Index (BMI) and weight gain, reproductive history (parity, age at menarche, age at first birth, age at menopause), lactation, postmenopausal hormone and alcohol consumption that also considerably increase risk [27].

2. Obesity and postmenopausal breast cancer risk and prognosis

Almost 40% of women 40 years or older are obese in the United States [28] and this excess adiposity increases their risk of breast cancer by 250 % compared to their lean counterparts [2]. A pooled analysis of seven cohorts exploring the BMI and breast cancer risk association among postmenopausal women found increased risk with increasing BMI (p trend=.0001) [29]. Another cohort, the Malmo diet and cancer study, studied Swedish postmenopausal women with 5.7 years of follow-up (n=246 cases; 11913 controls) and also found a positive association between BMI and breast cancer (p trend=0.01) [30] further confirming elevated BMI as an established risk factor for postmenopausal breast cancer.

Obesity also leads to poor prognosis of breast cancer [3]. Obese women with breast cancer have significantly larger tumors and higher rates of lymph node metastasis compared to non-obese counterparts [31, 32]. Sparano et al. reported that obesity without any significant co-morbidities was associated with reduced disease-free
survival (DFS) (HR, 1.24(1.1-1.5); p=.0008) and overall survival (HR, 1.37(1.1-1.7); p=.002) among women with breast cancer [33]. A prospective cohort of postmenopausal women (N=5629) in England followed for seven years for cancer mortality (Million Women study) found almost half of the postmenopausal breast cancer deaths in England and Scotland were attributable to obesity (p=.02) [34]. Another cohort study on early stage breast cancer in Denmark (N=53,816, 2/3rd of study population included postmenopausal women) reported obesity as an independent prognostic factor for distant metastasis and death when followed for ten and thirty years, respectively [35]. Overall, studies have indicated increased risk of poor prognosis and higher mortality among obese women with breast cancer.

3. **Mechanistic link between obesity and postmenopausal breast cancer**

The established links between obesity and postmenopausal breast cancer are illustrated in figure II.1. These include: 1) Inflammatory signaling 2), Enhanced estrogen bioavailability due to increased estrogen production and decreased SHBG due to hyperinsulinemia, and 3) Adipocytokines — Leptin and Adiponectin. Various arms of this diagram depict different mechanistic links between obesity and breast cancer and also include detailed pathways based on different types of studies (in vitro, in vivo, animal and human studies). Explanations for each of these pathways are provided.
3.1 Inflammatory signaling

The relationship between inflammation and cancer is well known. Neoplastic cells produce various cytokines and chemokines to promote tumor friendly environment via recruiting inflammatory cells and inducing cell proliferation. Infiltration of macrophages, mast cells, fibroblasts and endothelial cells in the tumor microenvironment specifically in response to inflammation further stimulates angiogenesis and promotes tumor growth [36, 37].

In vitro studies indicate important roles of cytokines and chemokines (interleukin-6 (IL-6), interleukin-8, tumor necrosis factor -α, chemokine (C-C motif) ligand 2) in breast cancer through promotion of vascularization, endothelial cell proliferation and angiogenesis [38-40]. Obesity is characterized by adipocyte hyperplasia and hypertrophy, which leads to high levels of circulating pro-inflammatory cytokines [36, 41]. These cytokines
promote infiltration of macrophages and T cells and further increase local and systemic cytokines [42]. Thus, adipose tissue in obesity is both an endocrine and immune organ that creates a chronic low grade inflammation. It is hypothesized that obesity is a risk factor for breast cancer and worsens disease progression due to cross-talk between immune cells, macrophages, adipocytes and breast epithelial cell [43].

**IL-6 & breast cancer**

One of the pleiotropic cytokines produced by adipose tissue is IL-6 [44]. Serum IL-6 is significantly positively correlated with elevated BMI and adiposity [45, 46]. The relationship between IL-6 and breast cancer has been studied in various population-based and in vitro studies. Women with breast cancer have elevated systemic IL-6 compared to healthy women and these levels are associated with the extent of tumor invasion and TNM staging (cancer staging notation system) of the disease [47]. Gonollu et al. [48] reported obese and overweight postmenopausal women with early stage breast cancer have elevated endogenous IL-6 compared to healthy BMI-matched controls. Women with metastatic breast cancer and recurrent breast cancer included in two different prospective cohorts had elevated IL-6 at diagnosis, an independent predictor for poor prognosis [49, 50]. A study by Zhang et al. [51] reported breast cancer patients with increased plasma IL-6 have poor therapeutic success. At the tumor tissue level the association between IL-6 and breast cancer outcomes is inconsistent. Some studies showed elevated tissue IL-6 as a negative prognosis marker (i.e. higher expression in the advanced tumors than the in-situ) [52, 53], while others showed the opposite results [54, 55]. Thus, while population studies show significant associations between elevated IL-6 and breast cancer, in vitro studies have inconsistent results. Interleukin-6 works in a paracrine and/or autocrine manner to alter the function of its target cells. Sullivan et al. [56] reported over expression of IL-6 in MCF-7 cells (breast cancer cell line) induced epithelial-mesenchymal transition as well as increased invasiveness. Shen et al. [57] reported IL-6 inhibits insulin growth factor-1 induced cell proliferation in breast cancer cell line which signifies protective effect of IL-6 at tissue level. Therefore, at the tissue level IL-6 may act as a double-edged sword for breast carcinogenesis and there may be other local environmental factors dictating its action.
**CRP & breast cancer**

C-reactive protein (CRP) is an acute phase protein, is primarily produced by hepatocytes in response to tissue injury, infection or inflammatory conditions. It is a sensitive marker for systemic inflammation [58]. Analysis of NHANES data reported a correlation between obesity and CRP levels, which was stronger in women than men [59]. A meta-analysis of 51 studies looking at the association between CRP and obesity found similar results [60]. Serum CRP has also been significantly linked with breast cancer poor prognosis in large prospective cohorts (Copenhagen breast cancer study [61] and Health Eating, Activity and Lifestyle (HEAL) study [62]). A meta-analysis of 10 studies comparing high and low CRP levels in breast cancer patients reported CRP to have critical prognostic value (HR = 1.62) [63], whereas a nested case-control study from a large cohort demonstrated CRP was not an independent predictor of breast cancer risk when controlling for BMI and other obesity-related factors (leptin, insulin and vitamin D) [64]. Collectively these findings indicate that CRP is a marker of inflammation in breast cancer, although we have limited studies comparing lean and obese cases to identify its role as a mediator for obesity-induced breast cancer risk.

**Local breast inflammation in obesity with increase in breast adipocyte cell size**

Obesity leads to changes in the physiological functions of adipose tissue. Expansion of adipose tissue, coupled with production of chemotactic factors, enhances infiltration of macrophages within adipose tissues [65]. Female breast predominantly contains adipose tissues and recent studies emphasize its role in altering the local microenvironment favoring tumor development. Dirat et al. reported altered morphology of breast adipose tissue on the invasive front of tumor [66]. A recent gene expression study found healthy breast tissue of obese women had greater local inflammation and macrophage infiltration compared to normal breast tissue [67]. Breast tissue lesions identified in mammary adipose tissue of obese and overweight women with breast cancer were found to have necrotic adipocytes rimmed by macrophages, known as Crown-like Structures (CLS) [5]. Such CLS in the breast tissue represent inflammatory foci and result in nuclear factor kappa-light-chain-enhancer of activated B cells activation in the nucleus. Breast tissue of obese women has increased adipocyte size and local inflammation, which is associated with elevated estrogen synthesis when compared to tissue from non-obese women [4]. These findings suggest obesity may predispose women to an inflammatory microenvironment in the breast that increases their cancer risk.
3.2 Enhanced estrogen synthesis

Systemic estrogen and breast cancer risk

Estrogen is a well-established risk factor for breast cancer among postmenopausal women. A nested case-control study within the European Prospective Investigation into Cancer and Nutrition study (n= 677 postmenopausal breast cancer cases and 1309 age matched controls) reported women in the highest quintile of free estradiol (active estrogen) levels had two times higher risk of developing breast cancer compared to women in the lowest quintile [68]. Similar results were reported in a pooled analysis of nine prospective studies assessing the effect of endogenous estrogen on breast cancer risk in postmenopausal women (n=663 cases and 1765 controls) [69]. Of these nine studies, six were conducted in the US and the remaining three were in the UK, Italy and Japan. They found the relative risk for breast cancer for women in the highest quintile of estradiol levels was 2.6 (95% CI, 1.8 – 3.9) compared to those in the lowest quintile and there was a significant dose response. Data from two cohorts, the New York University Women’s Health Study (NYUWHS) and the Nurses’ Health Study (NHS), both of which were included in the pooled analysis mentioned above, have reported additional results that strengthen these findings. A nested case-control study within postmenopausal participants of NYUWHS (N= 297 cases and 563 controls) had blood samples collected at baseline and a second sample within five years of diagnosis. They found a significant association between elevated baseline estrogen and breast cancer risk. Plasma estrogen at the follow-up visit confirmed the association was not due to tumor-related hormone production; rather baseline circulating levels (i.e. prior to tumor development) were independent risk factors for postmenopausal breast cancer [70]. Another study of postmenopausal women that participated in the NHS with 10-years of follow-up (N= 418 cases and 817 controls) investigated the estrogen-breast cancer association considering their overall risk for the disease. They found baseline elevated serum estrogen levels were significantly associated with developing breast cancer, independent of genetic and epigenetic factors included in other risk prediction models for breast cancer (Gail scores, Rosners models etc.) [71]. Together these studies support the independent role of elevated estrogen levels in enhancing postmenopausal breast cancer risk.

Obesity-induced estrogen synthesis and breast cancer

Ovarian estrogen production decreases after menopause; however in obesity the decline is much less pronounced because excess adipose tissue continues to act as a primary source of estrogen [72]. Adiposity
increases systemic free estrogen via (a) increased adipocyte estrogen production and (b) decreased SHBG levels [73], a plasma glycoprotein produced in the liver which has high affinity for estrogen. In obesity there is decreased hepatic production of SHBG [74], which leads to elevated unbound estrogen. A cross sectional study of randomly selected postmenopausal women (n=267) from the Women’s Health Initiative Dietary Modification Trial found BMI was positively associated with estrogen and negatively associated with SHBG [75]. These results were corroborated by a report on healthy women participating in the EPIC study (n=1217 postmenopausal women) [76]. Another cross sectional study of 1092 women enrolled in the EPIC study analyzed the relation of estradiol (active estrogen) and SHBG with other breast cancer risk factors. Among postmenopausal women (n=456) BMI had a significant positive linear relationship with estradiol and a significant negative linear relationship with SHBG in dose response manner. Other breast cancer risk factors, including alcohol consumption, smoking, family history and reproductive history, had no effect on estradiol or SHBG in these women [77]. These studies indicate that increased breast cancer risk with increasing BMI is at least partly attributable to endogenous estrogen exposure.

**Estrogen synthesis in normal and malignant breast tissue**

Various animal and human studies support mammary adipose tissue as the primary site for aromatase expression and estrogen production [4, 5]. Morris et al. reported that breast tissue of obese women had elevated levels of inflammatory mediators that activate cyclooxygenase (COX)-2 derived Prostaglandin E2 (PGE2) [4]. PGE2 stimulates the cyclic AMP/PKA signal transduction pathway that activates aromatase [5]. It has been hypothesized that paracrine interaction between estrogen produced by adipose tissue and breast epithelial cells predisposes women to breast hyperplasia and cancer. Increased estrogen interacts with the estrogen receptor-α present in neighboring epithelial cells and activates transcription of specific genes that enhance carcinogenesis [73].

**3.3 Adipokine hormones – leptin and adiponectin**

Leptin and adiponectin are adipokine hormones synthesized and secreted by adipocytes and have been recently studied for their influence on breast cancer. Biological activities of leptin and adiponectin and their effect on breast neoplastic cells are largely opposite to each other [78]. Plasma leptin levels increase, whereas adiponectin decreases, proportionally to BMI and nutritional status. Tessitore et al. reported that women with breast cancer
have higher plasma leptin and elevated expression of leptin mRNA in adipose tissue when compared to healthy subjects [79]. A large prospective study (n=561 cases; 561 controls) of Swedish women reported BMI correlated positively with leptin (r=0.72, p =<.01) and negatively with adiponectin (r=-.23, p=<.01) at baseline [80]. After eight years of follow-up a non-significant positive association of baseline leptin levels among advanced breast cancer cases was found; no association of adiponectin was reported. Unfortunately these studies did not report their findings separately for postmenopausal women. Very recently a nested case-control within the Multiethnic Cohort (MEC) study reported postmenopausal women in the highest quartile of circulating leptin at baseline had almost twice the risk of developing breast cancer (OR=1.9; 1.4-2.7) compared to women in the lowest quartile (n=706 cases; 706 controls) [81]. The leptin/adiponectin ratio was also found to be significantly associated with breast cancer risk (OR=1.9; 1.4-2.7). No association was detected independently for adiponectin. The positive association of leptin and leptin/adiponectin ratio was dose dependent and remained significant after adjusting for BMI. Three case-control studies reported similar findings supporting the leptin-breast cancer link [78, 82, 83]. Two out of these three studies also analyzed serum adiponectin and found it was negatively associated with breast cancer risk (p <0.05) [82, 83]. Miyoshi et al. reported that women in the lowest vs. highest tertile of serum adiponectin had almost four times the risk of developing breast cancer (OR=3.9; 1.2-12.3) [84]. A nested case-control within NHS cohort reported a significant negative association between adiponectin and breast cancer (RR=0.073; 0.55-0.98) controlling for hormone replacement therapy (HRT) [85]. Leptin is also associated with poor prognosis of breast cancer. Among postmenopausal women with breast cancer (n=98) both BMI and leptin were significantly associated with pathological tumor size and TNM stage [31]. As a group these studies indicate a significant role of increased leptin and decreased adiponectin levels as a risk for postmenopausal breast cancer.

**Mechanisms mediating the involvement of leptin and adiponectin in breast cancer**

Both endocrine and paracrine actions of leptin have been hypothesized to induce breast carcinogenesis. Invasive breast tumors have higher expression of leptin and leptin receptors compared to healthy mammary tissue [86]. Genetically obese leptin-deficient (MMTV-TGF-α/Ob-Ob-) female mice do not develop mammary tumors, supporting a significant role for leptin in obesity-induced mammary carcinogenesis [87]. Various in vitro experiments demonstrated that leptin influences intracellular messengers (STAT3; PI3K/Akt; ERK2; MAPK)
involved in cell proliferation, differentiation and survival. These messengers activated via leptin are also involved in cell migration and angiogenesis through activation of vascular endothelial growth factor gene transcription) [88].

Elevated leptin is also linked with increased estrogen production. A cross-sectional study (n=87) on women with endometrial and breast cancer reported higher leptin levels with increased plasma estradiol among postmenopausal women [89]. Serum leptin levels also showed a positive association with increased expression of estrogen and progesterone receptor in breast tumors [90]. Leptin amplifies estrogen signaling by increasing aromatase gene expression [91]. An in vitro study found that breast cancer cell lines exposed to estrogen and leptin together develop larger tumors than when exposed to estrogen alone [92]. These findings indicate a synergistic effect exists between leptin and estrogen. Increased leptin production in breast tissue also results in tumor-associated macrophages accumulation [93] and enhanced expression of cadherin-1, an adhesion molecule implicated in cell proliferation and survival [92]. Thus leptin affects tumor growth via multiple concurrent pathways.

The mechanism of adiponectin’s protective effect on breast cancer is not fully understood. Two proposed mechanisms include: (1) direct effect of adiponectin on breast cancer cells via mitogen-activated protein kinase, adenosine monophosphate-activated protein kinase, Wnt/β-catenin and estrogen receptor signaling pathways that suppresses growth and promote apoptosis as shown by in-vitro studies, and (2) indirect effect of adiponectin via enhancing insulin sensitivity at breast epithelium and regulation of inflammatory responses [94, 95]. Peroxisome proliferator-activated receptor gamma (PPAR-γ) metabolic pathways help to enhance insulin sensitivity and DNA repair mechanisms. It has been shown that PPAR-γ act as a protective factor for sporadic breast cancer by up-regulating Breast Cancer-1 (BRCA1) gene expression (tumor suppressor) [96]. As adiponectin gets activated via PPAR-γ, it may also be playing some role in decreasing sporadic breast cancer incidence although more studies are needed to understand the mechanism. Therefore, obese women with low adiponectin levels may have elevated breast cancer risk. Although most studies appear to indicate a role for low adiponectin in breast cancer risk in conjunction with high leptin levels, more work needs to be done in this area.
4. Obesity and iron metabolism

4.1 Normal iron regulation with hepcidin

Iron is an essential nutrient for overall health maintenance. One of the key regulators of iron is hepcidin, a small peptide hormone primarily produced in the liver. Hepcidin regulates iron entry in the plasma by down regulating the iron exporter protein, FPN1, in enterocytes, hepatocytes and splenic macrophages: hepcidin binding to FPN1 is followed by lysosomal internalization and degradation. Synthesis of hepcidin is upregulated primarily by elevated body iron stores and inflammation; and downregulated by hypoxia/erythropoiesis [21]. Therefore, when iron stores are elevated or when inflammation or infection is present, hepatic hepcidin production increases and hepcidin binds to FPN1, resulting in its internalization and degradation in the lysosomes. FPN1 is the only known cellular iron exporter, thus its degradation leads to sequestration of intracellular iron within intestinal enterocytes, hepatocytes and iron-recycling macrophages [97].

4.2 Obesity influences iron regulation

Epidemiological studies support the presence of inflammation-induced iron deficiency among obese populations [18-20]. A cross-sectional study in a racially diverse sample of men and women (n=670) showed that serum hepcidin was inversely correlated with iron levels (r=-0.23, p<.0001) BMI [98]. Data from NHANES III showed that obesity is associated with elevated inflammatory status, decreased serum iron and increased serum ferritin [99]. It was concluded in the study that obesity-induced inflammation was responsible for low serum iron. Unfortunately it was suggested in the earlier study that iron sufficiency could be assessed solely on elevated serum ferritin. Ferritin being an acute phase protein could get elevated from obesity associated low grade inflammation thus may not serve as a good marker for iron status in obese. Similar findings of elevated ferritin were reported by Yanoff et al. among 234 obese participants [100]. They also reported significantly higher sTFR in obese individuals compared to non-obese controls. No variation was found in their dietary iron intake of obese and non-obese participants suggesting diet was not modulating their iron status A case-control study among postmenopausal women (n=50 obese; 50 lean) reported high sTFR (indicating higher iron needs) and similar ferritin levels comparing obese vs. lean subjects [101]. BMI was found to be linearly correlated with sTFR (r=0.48; p<.001), suggesting the presence of iron-deficient erythropoiesis with increasing obesity. These findings suggest important correlations between obesity-induced low grade inflammation and low iron availability for erythropoiesis. They
also demonstrated serum ferritin is an acute phase protein and supported sTFR as a better marker of iron status than ferritin among obese population.

The low grade inflammation of obesity elevates hepcidin levels in children [18, 19] and premenopausal women [20] via increased IL-6, which activates the JAK-STAT3 pathway [102]. Increased hepcidin reduces iron absorption, iron export from hepatocytes and iron-recycling macrophages and leads to iron insufficiency (Figure II.2) [97]. A cross-sectional study conducted in obese women having bariatric surgery found elevated CRP, hepcidin and sTFR levels compared to their hemoglobin-matched lean controls [20]. Six months after surgery they found weight loss was accompanied by reduced inflammation and hepcidin and improved iron status [103]. The influence of obesity on hepcidin levels has not been reported in postmenopausal women. Bekri and colleagues reported subcutaneous and visceral adipose tissue from obese women expresses elevated hepcidin when compared to tissue from their lean counterparts, and hepcidin expression was positively correlated with BMI and IL-6 [23]. However, this association was not found in a similar study by Tussing-Humphreys et al. [104]. The role of elevated hepcidin levels in obesity-induced iron insufficiency among children, adolescent and premenopausal women is well known; however, hepcidin production by adipose tissues is still questionable.

Figure II-2: Obesity-induced iron insufficiency due to increase in hepatic hepcidin production
5. **Iron and cancer**

Iron is an essential element for both physiological cellular functions and neoplastic cell growth [105]. Different iron-regulatory proteins have been studied in various cancers, including hepcidin, FPN1, lipocalin, members of the six trans-membrane epithelial antigen of the prostate and iron regulatory protein (IRP). Various studies support the role of these proteins in modifying bioavailable iron, which increases the risk of cancer [106].

Iron serves as an important nutrient for tumor growth due to its regulation of various molecules that control cell cycle progression e.g. p53 [105]. Some iron-containing proteins are also involved in energy metabolism and DNA synthesis. Iron balance is very important, as excess levels catalyze the formation of free radicals, which increase oxidative tissue damage [107]. Various animal models demonstrated excess iron administration is carcinogenic [9, 10]. Hemochromatosis is a condition of iron overload and women with this condition have increased risk of breast cancer (Hazards ratio: 2.39 (1.22-4.25)) [108]. It is postulated that this elevated risk is due to increased oxidative damage and activation of proliferative pathways [109]. Iron chelators inhibit the growth of aggressive tumors in vitro and in vivo [110, 111]. Thus, there are at least two aspects to the iron-breast cancer link: (1) iron may promote carcinogenesis via oxidative stress and/or (2) tumor cells modify their iron regulation to acquire more iron that helps tumor to grow irrespective of overall iron status.

5.1 **Iron and breast cancer**

Various studies in normal and malignant breast tissue have examined iron and its regulatory proteins in mammary carcinogenesis. Early studies in this area from the mid-70s and 80s were focused mainly on two iron parameters, ferritin and transferrin receptor. Tissue ferritin content of mammary carcinoma was reported to be six times higher than in benign breast tissue [12] and expression of Tfr1 progressively increases with advancement of the disease from benign to DCIS to invasive carcinoma [112]. In more recent years, other iron parameters, such as iron content and the critical iron regulatory proteins hepcidin and FPN1, have been analyzed. Breast cancer tissue has higher iron (p<.0001) compared to normal tissue [11]. A nested case-control study (n=125 cases; 138 controls) found a positive association between tissue iron concentration and breast cancer (OR=2.77 (1.25-6.13) for highest versus lowest quintile; p trend=0.008) [14]. Pinnix et al. analyzed genetic expression of FPN1 among women with breast cancer using a publicly available microarray data set from five cohorts (n=800) [15]. They
found decreased FPN1 was an independent prognostic factor for poor outcome. Further, combined analysis from three cohorts (n=504) showed elevated hepcidin along with decreased FPN1 was associated with significantly lower distant metastasis-free survival. In another study, an iron regulatory gene signature comprised of 16 iron-related genes that predominantly facilitate cellular iron accumulation via iron acquisition or decreased iron export, has been associated with poor prognosis of breast cancer [113]. Mainly, two gene expression dyads that facilitated iron accumulation were associated with poor prognosis. These genes include an anti-export dyad (high hepcidin with low FPN1) and a pro-import dyad (high transferrin receptor with low HFE). Mannello et al. examined nipple aspirate fluid in women with and without cancer (n=16 cases; 19 controls) to determine the influence of ductal and lobular cell microenvironment [114]. They found significantly higher CRP and iron binding proteins (ferritin and transferrin) among cases than normal subjects. Collectively these findings suggest a significant role of iron and its regulatory proteins in the cross-talk between tumor cells and their environment. These results also support acquiring more iron promotes a proliferative advantage for tumor cells.

There are limited studies looking at plasma markers of iron status association with breast cancer. Some studies have reported elevated preoperative plasma ferritin levels among women with breast cancer compared to normal women [115-117]. Panis et al. reported elevated serum ferritin in breast cancer cases, along with high oxidative stress and inflammation (CRP), compared to healthy women [115]. However, ferritin being an acute phase reactant, its levels may not reflect the actual iron stores in women with breast cancer. Plasma sTFR is considered a better marker to study iron sufficiency/insufficiency among patients with elevated inflammation [118]. Only one study, reported 20 years ago, examined plasma sTFR among women with breast cancer (n=19 cases; 16 controls) and found no difference between cases and controls [119]. Serum iron is reported to be positively associated with postmenopausal breast cancer in a Swedish population (HR=1.09; 95% CI 1.02-1.15) [120]; however, another study has shown no association [121]. A recent analysis for trace elements in plasma of pre-and postmenopausal women with high breast cancer risk (BRCA1 mutation carriers, n=48 cases; 96 controls) contradicts positive findings. Women in the highest tertile of plasma iron had 57% (p trend=0.06) lower risk of breast cancer compared to those in the lowest tertile [122]. The authors postulated the low levels of iron may have been due to 51% of participants being premenopausal. We propose the lower plasma levels among these high-risk women may indicate more iron sequestered in the breast tissue, which elevated their cancer risk. Two
very recent studies demonstrated elevated systemic hepcidin levels were associated with breast cancer [123, 124]. The first study included 65 invasive cases, 88 benign lesions and 120 healthy controls and found hepcidin as a significant predictor of malignant breast cancer (OR=1.42; 1.18-1.70)[123]. However, controlling for other breast cancer risk factors was not done. Zhang et al. recently reported a case-control study of both pre- and post-menopausal women with breast cancer (n=90 cases and 90 controls) and found elevated hepcidin levels among cases compared to age-matched controls (p=0.02) [124]. However, this study did not account for BMI. To establish a link between systemic markers of iron metabolism and breast cancer, more work is needed using appropriate plasma markers of iron status coupled with hepcidin and markers of inflammation.

Several studies have investigated the effect of dietary iron on breast cancer risk, however findings have been inconsistent. Breast cancer risk was significantly associated with meat consumption in some [125, 126] but not in other studies [127]. However, a case-control study by Bae et al. reported decreased dietary iron intake among women with breast cancer (n=121) when compared to controls (n=149) [128]. The inconsistent results can be due to different tools used for dietary iron assessment. A commentary by Huang suggested that because gut iron absorption represents roughly 0.05% of total body iron store (4-5 g) the role of dietary iron may be quite limited, and account for the discordant results reported for its association with breast cancer [129].

It is possible that iron availability does not promote breast tumor development. Rather, regulatory changes within tumors occur that allow them to acquire iron successfully, regardless of iron availability. Greater ability to acquire and retain iron seems to allow a more aggressive growth of the tumor.

6. **Obesity, iron and postmenopausal breast cancer**

Obesity precipitates low iron status via elevated hepcidin. Elevated hepcidin may help the tumor retain more iron by degrading any FPN1 tumor cells may express. Indeed, incubation of breast cell lines with hepcidin increases the intracellular labile iron pool [15]. Whether the same is true with high systemic hepcidin levels among obese women is unknown. Obesity and iron, which are interrelated, have been independently linked with pathogenesis of breast cancer. Postmenopausal women are more likely to be iron sufficient due menstruation cessation. They also have a greater incidence of breast cancer and obesity is a well-established risk factor for this population.
Expanding our understanding of the obesity-induced alterations in iron metabolism among postmenopausal women, the purpose of this study is to examine the effect of systemic hepcidin on breast cancer via its effect on breast tissue iron and ferroportin levels (Figure II.3.) Other obesity-related pathways that enhance breast cancer risk among this population may have a synergistic effect with iron dysregulation.

Figure II-3: The hypothesized link between obesity, iron metabolism and breast cancer
III. RESEARCH DESIGN AND METHODS

1. Study design and population

To address our hypothesis we conducted a nested case-control study from Dr. Kent Hoskins’s ongoing cohort “Blood Based Biomarkers of Breast Neoplasia”. This prospective cohort has been established to examine genome-wide DNA methylation changes in peripheral blood of women with breast cancer and has IRB approval at ten clinics in the Chicago area, including UIC. Participants enrolled include women having breast biopsies following an abnormal mammogram or palpable abnormality upon physical examination. Participants with a prior history of DCIS, any other malignancy within the last ten years, pregnant or lactating are excluded. As part of the cohort, plasma is collected prior to biopsy and formalin-fixed breast tissue is also obtained. For our study, we included only postmenopausal women (obese and non-obese cases and controls) recruited in the cohort.

Rationale for the study population

Postmenopausal women were selected because (a) the exposure of interest, obesity, is a well-established risk factor for postmenopausal breast cancer, (b) dietary iron deficiency occurs much less frequently after menopause than during the child-bearing years, rendering detection of an obesity-related influence of iron levels more likely, and (c) postmenopausal breast cancer is much more common than premenopausal breast cancer. Menopausal status was confirmed from participants’ medical records.

(i) Inclusion criteria: Only women with incident breast cancer were included as cases. The majority of incident cases recruited in the original cohort had IDC (almost 95%). Thus to minimize heterogeneity, “cases” were limited to this category.

(ii) Exclusion criteria: Some studies have shown a protective effect of obesity on breast cancer risk among premenopausal women [130]. To enable detection of the influence of obesity premenopausal women were therefore excluded. The effect of obesity on breast cancer risk in African American women is mixed [131]. Over 90% of the participants in the Hoskins cohort study were Caucasian at the time of study initiation. Iron
parameters [132] and the obesity-breast cancer relationship [131] in postmenopausal women are influenced by race, thus the current study was restricted to Caucasian women.

2. Sampling procedure

Non-obese (BMI between 18.5 to 29.9) and obese (BMI ≥ 30) postmenopausal women recruited for the cohort were selected as participants for this case-control study. Cases included were women with newly diagnosed IDC. Controls included women with non-proliferative benign lesions matched for age (up to 5 years) and BMI (up to 1 unit) to the selected cases. Cases and controls were identified using the pathology report from medical records.

3. Sample size estimations

Sample size calculations were based on serum hepcidin levels because these levels were hypothesized to be mechanistically linked to the heightened breast cancer risk of obesity. Initially, determination of sample size was based on serum hepcidin medians and Inter Quartile Range (IQR) from previous data obtained in our lab from a baseline and six month follow-up study of obese premenopausal women undergoing bariatric surgery [20, 103]. These two studies are the only data reporting the impact of weight loss on hepcidin concentrations in obese populations. There is no study describing obese postmenopausal hepcidin levels.

Upon analysis of our first 44 controls (obese and non-obese), re-powering for sample size was done using the mean effect size (27.6) for differences between obese (113.16 ± 34.80 ng/mL serum hepcidin) and non-obese women (85.55 ± 30.89 ng/mL serum hepcidin). A sample size of 18 women/obesity group (36 cases and 36 controls) provided > 80% power to detect a 0.05 significant difference. Thus, overall 44 cases (22 obese and 22 non-obese) and 44 age and BMI-matched controls (22 obese and 22 non-obese) were included in this study.
IV. MEASUREMENT OF VARIABLES

1. **Demographics and baseline information**

Demographic parameters assessed include age, usage of (HRT) and birth control pills, and reproductive history (age of menarche, parity, age of first live birth), family history of breast cancer and ovarian cancer. All this information was obtained as part of the original cohort. Questionnaires were completed in a designated private area of the respective clinics.

**Tumor Receptor Status/Staging (TNM):** Information on receptor status and staging of tumors was obtained from the pathology report. Tumors were estrogen receptor +/-; progesterone receptor +/- or human epidermal growth receptor 2/neu +/- as tested using IHC. Staging was based on the AJCC staging manual 7th edition for TNM classification (T=tumor size; N=Nodal status; M=Metastasis).

2. **Body mass index**

Body mass index was calculated using the height and weight obtained during the clinical visit and classified according to the guidelines for healthy weight (Normal weight (BMI = 18.5 - 24.9), overweight (BMI = 25.0 - 29.9) and Obese (BMI ≥ 30.0)).

3. **Biochemical measures**

Whole blood was collected in ethylenediaaminetetraacetic acid (EDTA) tubes and centrifuged for 15 minutes at room temperature within 30 minutes of collection to separate plasma and cellular elements. Plasma was immediately frozen at -20C and placed at -80C within 72 hours of collection.

**Soluble transferrin receptor**

**Rationale:** sTFR levels reflect iron status and are not influenced by inflammation. Levels are elevated when erythroid precursors are low in iron and/or expanded in number (e.g. as a response to anemia), and sTFR is
reduced when erythroid cells are iron replete. There is limited information of sTFR levels among women with breast cancer evaluated for breast biopsies.

**Technique:** Serum transferrin receptor was measured using 40 μl of plasma for each sample (assay done in duplicate) with the Quantikine IVD Human sTFR Immunoassay ELISA (R&D Systems, Minneapolis, MN) in the Fantuzzi lab in the Department of Kinesiology and Nutrition at UIC. The minimum detectable concentration of sTFR is less than 0.5 nmol/L. For this assay manufacturer's expected reference range is 8.7–28.1 nmol/l, any value greater than 28.1 nmol/l is indicative of Iron deficiency. This cut point was used to dichotomize our participants as iron depleted (sTFR >28.1 nmol/L) or iron sufficient (sTFR ≤ 28.1 nmol/L).

### 3.1 Hepcidin

**Rationale:** Serum hepcidin is elevated by inflammation and iron overload and reduced in iron deficiency [97]. The exposure of extracellular hepcidin to human breast cell lines increased the labile iron pool within the cells [15]. No study to date reported hepcidin levels in obese postmenopausal women. Association between systemic hepcidin levels and breast cancer considering obesity status has never been explored before.

**Technique:** Plasma hepcidin was measured using an ELISA developed and patented by Intrinsic LifeSciences (La Jolla, CA) in duplicate. One-hundred μl of plasma was shipped to Intrinsic LifeSciences for analysis. This assay's sensitivity is 0.5 ng/ml, intra-assay coefficient of variation (CV) is 5-19% and median inter-assay CV is 12%. For women without inflammation and with normal iron status (n=882), the 5-95% range for this assay is 17-286 ng/ml [133].

### 3.2 High sensitivity c-reactive protein

**Rationale:** CRP is an established marker of inflammation. Elevated CRP is associated with poor prognosis of breast cancer [61].

**Technique:** Twenty micro-liters of plasma was used to measure CRP in duplicate using the R&D systems Quantikine® Elisa kit for human CRP (R&D Systems, Minneapolis, MN) at the Fantuzzi lab in the Department of
Kinesiology and Nutrition at UIC. The sensitivity of the assay is .00001 µg/ml, intra-assay CV is 4.4 -8.3% and median inter-assay CV is 7%.

3.3 Interleukin-6

Rationale: IL-6 is known to have two sided effect on breast cancer growth (tumor promoting and inhibitory effects) depending on presence of other modulating factors at the tissue level. Systemic IL-6 is significantly associated with breast cancer [38]. Systemic low grade inflammation present in obesity is associated with elevated IL-6 and IL-6 is associated with increased hepcidin production by the liver [102].

Technique: Interleukin-6 was measured using the R&D systems Quantikine® ELISA kit for IL-6 (R&D Systems, Minneapolis, MN) at the Fantuzzi lab in the Department of Kinesiology and Nutrition at UIC. The assay was done in duplicate using 200 µl of plasma. The sensitivity of the assay is .039 pg/ml, intra-assay CV is 6.9 -7.8% and median inter-assay CV is 7.2%.

3.4 Estrogen

Rationale: Elevated estrogen levels are one of the key established obesity-related risks for breast cancer in postmenopausal women [73]. An in vitro study suggested an iron-reducing effect of estrogen, which may enhance reactive oxygen species production and induce oxidative stress [107].

Technique: Estrogen/estradiol was assessed using an Estradiol ELISA Kit (Alpco Diagnostics, USA) using 100 µl of plasma. The assay was done in duplicate at the Fantuzzi lab in the Department of Kinesiology and Nutrition at UIC. The sensitivity for this assay is 1 pg/ml.

3.5 Leptin

Rationale: Leptin has an angiogenic effect and is considered an obesity-related cause for breast cancer [88]. Non-fasting leptin is associated with fasting insulin levels. It has been suggested that non-fasting leptin along with
adiponectin levels and their ratio (leptin/adiponectin) can give an approximate picture of insulin resistance when fasting blood is not available for analysis [89].

**Technique:** Plasma Leptin was assessed in duplicate using R&D systems Quantikine® ELISA kit for Leptin with 20 µl of plasma. The sensitivity for this assay is 7.8 pg/ml, intra-assay CV is 3.0 -3.3% and median inter-assay CV is 4.2%.

### 3.6 Adiponectin

**Rationale:** Adiponectin has anti-angiogenic effects and reduced levels in obesity are associated with breast cancer risk [134].

**Technique:** Plasma Adiponectin was assessed in duplicate using R&D systems Quantikine® ELISA kit for Adiponectin with 20 µl of plasma. The sensitivity for this assay is 0.25 ng/ml, intra-assay CV is 2.8 -5.0% and median inter-assay CV is 5.9%.

### 4. Breast tissue analysis

To assess tissue levels of iron and ferroportin, formalin-fixed paraffin-embedded tissue blocks of biopsy samples, collected as part the original cohort, were used. From selected cases and controls (n=88), majority (92%, 82/88) were from three study sites of prospective cohort. Due to cost constraints and ease of access to medical records and tissue blocks, only participants from these three study sites were included in specific aim 2. Out of 82 subjects identified, 84% (69/82) had tissue blocks available for analysis. Biopsy tissue samples without detectable tumor epithelium (n= 1) or presence of blood clots (n=4) were excluded, leaving a final sample of 64 (n=34 cases, n=30 controls) for tissue analysis.

All samples were sectioned at 5-micron thickness and mounted on Starfrost/ Plus slides for Perls’ staining and Immunohistochemistry (IHC). For subjects who had multiple tissue blocks collected at biopsy, Hematoxylin and eosin (H&E) slides were also prepared and the most appropriate section was chosen by the pathologist for staining. All tissue staining was done at the Research Resource Center at UIC.
4.1 Iron staining

**Rationale:** Hepcidin increases iron in breast cells [15]. Measuring iron content of breast tissue will allow us to understand the effects of alterations in plasma levels of hepcidin and the potential link between obesity and breast cancer via altered iron metabolism.

**Technique:** Perls’ Prussian blue staining was used to quantitatively assess iron accumulation in breast tissue. Slides were de-paraffinized and hydrated to distilled water. They were incubated in a working solution of 2% hydrochloric acid and 2% potassium ferrocyanide (pK4fe (CN)) for 20 minutes. This helps to release ferric ions from binding proteins and produce an insoluble blue compound (the Prussian blue reaction). Slides were then washed in running water until sections were clear. Then were counterstained with Nuclear Fast Red, rinsed in water, dehydrated and mounted with Micromount. Two pathologists blinded to the experimental group graded the iron stained slides from 0-3: 0 = undetectable staining, 1 = weak staining, 2 = intermediate staining and 3 = strong staining.

4.2 Ferroportin staining

**Rationale:** Pinnix et al. reported decreased FPN1 content in breast cancer tissue using IHC analysis and decreased FPN1 content in breast cancer cell lines using western blotting [15]. It was also reported that normal ductal structures in the breast of cancer patients had the highest FPN1 expression while the invasive tissue had lowest expression of FPN1 [15]. None of the studies have looked at how FPN1 concentrations may vary with BMI among breast cancer cases and controls.

**Technique:** Immunohistochemical analysis was performed using an anti-FPN1 antibody (Alpha Diagnostic MTP11-A, similar to the antibody used in earlier study on breast cancer [15]) at the RRC. Tissue slides were hydrated through a xylene and alcohol gradient. Antigen was unmasked via an EDTA-based retrieval solution, and then rinsed in wash buffer for 5 minutes. For the demonstration of anti-FPN-1, tissue sections were blocked with H2O2 blocking reagent for 20 minutes at room temperature. Slides were rinsed with PBS, and then the antibody was applied at a titer of 1:100 for 60 minutes at room temperature. Slides were further rinsed and then treated with High Def blue IHC chromogen (Cell Marque, Rockland CA) for 20 minutes at room temperature. Anti-
FPN-1 staining was detected by Diaminobenzidine (Cell Marque, Rockland, CA) for 10 minutes. Slides were rinsed in distilled water, counterstained, dehydrated through an alcohol gradient and mounted with micromount.

Blinded to participants’ disease status (cases/controls) two pathologists scored three different components on the slides: 1) epithelial tissue (tumor and normal, if present, for cases and normal for controls); 2) adipose tissue and macrophages. Results for epithelial and adipose tissues were expressed using an H-score according to (a) staining intensity: 0- undetectable staining, 1- weak staining, 2- intermediate staining and 3- strong staining), and (b) percent of positive staining cells (0 to 100%). The resulting H score (0 – 300 scale) = staining intensity * percent of positive staining cells. FPN1 in macrophages was scored using the staining intensity only, as mentioned above.

4.3 Average breast adipocyte size

**Rationale:** Breast cancer grows in proximity to adipose tissue. Adipocytes influence the tumor micro-environment in various ways [8]. Breast adipocyte cell size is positively associated with presence of CLS and IL-6 levels in women with breast cancer [4]. To determine its association with iron or FPN1 content average breast adipocyte size was calculated.

**Technique:** Histological sections were used to measure adipocyte size. Slides were photographed at 20X, utilizing a Nikon digital camera. Images were store in Jpeg format and adipocyte size was calculated using Image J software. Specifically, the surface area in μm² was measured from a total of 100 cells per subject in three separate fields. Biopsy samples which had less than 100 adipocytes were excluded. To avoid investigator bias with cell selection, only adjacent cells were measured. The average value calculated for each section was considered.

4.4 Mononuclear inflammatory cell infiltrate

As most of the iron staining was found in mononuclear inflammatory cells, all tumor histological slides were assessed for presence of infiltrating cells inside and around the tumor. The score given by the pathologist varied from 1–3: 1-no or scarce infiltration, 2-moderate infiltration, and 3- severe infiltration.
V. STATISTICAL ANALYSIS

All statistical analysis was performed using SAS 9.3 (Cary, NY, USA). The significance level was set at \( p < 0.05 \).

1. **Statistical analysis for specific aim 1**

The goal was to describe the effect of obesity on various plasma markers among cases and controls and to assess the association of iron status parameters with established breast cancer risk factors (inflammation, adipokines and estrogen), and breast cancer occurrence.

Mean, standard deviation (SD), median and IQR were used to assess continuous variables and frequency was used for categorical variables. Variables that were not normally distributed were log-transformed prior to analysis. Comparisons between cases and controls were done using Student's paired t-test; Wilcoxon sign rank test or chi-square test (categorical variables). The within-group comparisons between obese and non-obese were done using a Student's unpaired t-test or Wilcoxon rank sum test. Due to multiple comparisons, a Bonferroni correction was also applied. Spearman’s test was used to assess relationships between plasma hepcidin, sTFR, CRP, IL-6, leptin, adiponectin, estrogen and BMI. Variables that were significantly correlated with hepcidin or are a known predictor of hepcidin were considered as potential confounders in the simple regression. These variables were considered to develop a best fit multivariable model to predict log plasma hepcidin among cases and controls. Best-fit unadjusted and multivariable models using conditional logistic regression were developed with disease status (yes/no) as the dependent variable and hepcidin as primary independent variable. Hepcidin was considered as continuous and dichotomized variable (high hepcidin (upper quartile \( \geq 152 \) ng/ml), low hepcidin (<152 ng/ml)). Established risk factors for breast cancer, including family history of breast and ovarian cancer, age of first live birth (<20, 21-24, 25-29, >30 years, nulliparous), age of menarche (<12, 13-14, >14 years), ever use of HRT (yes/no), ever use of birth control pills (yes/no), biochemical measures (CRP, IL-6, sTFR, leptin, adiponectin and estrogen) were examined as potential confounders and effect modifiers.
2. **Statistical analysis for specific aim 2**

Aim 2 of the study was to evaluate tissue parameters between cases and controls and assess the impact of obesity. The iron accumulation score, FPN1 scores and average adipocyte size of breast tissue were assessed for normality. Iron score and score for FPN1 in macrophages were analyzed as dichotomized variables (high vs. low status). Descriptive statistics included medians, IQRs and frequencies (for categorized variables). Comparisons between the groups were done using Wilcoxon rank test or Fisher exact-test. Associations among tissue parameters were assessed using Spearman’s test.

3. **Statistical analysis for specific aim 3**

The goal was to determine the association between systemic hepcidin and tissue parameters among cases and controls. The associations between plasma and tissue parameters were assessed using Spearman’s test. Additionally cases were dichotomized by iron score (high vs. low) and differences in plasma levels and FPN1 content were assessed using Student’s t-test or Wilcoxon rank test. Further, a logistic model was used for disease status as dependent variable and plasma hepcidin, tissue iron and FPN1 as independent variables.
VI. RESULTS

1. Results for specific aim 1

The overall background and demographic information of participants are presented in Table VI.I. By design cases and controls were similar for BMI and age; no other significant differences between groups were found for breast cancer risk factors.
Table VI-I: Anthropometric and background information in cases and controls (n=88)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases (n=44)</th>
<th>Controls (n=44)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD) years</td>
<td>60.9 (7)</td>
<td>59.6 (7.3)</td>
<td>0.40 ~</td>
</tr>
<tr>
<td>Weight, mean (SD) kg</td>
<td>79.1 (18.7)</td>
<td>78.1 (18.4)</td>
<td>0.80^</td>
</tr>
<tr>
<td>Height, mean (SD) cm</td>
<td>164.2 (8.8)</td>
<td>163 (7.7)</td>
<td>0.21^</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>29.3 (6.3)</td>
<td>29.3 (6.4)</td>
<td>0.95 ^</td>
</tr>
<tr>
<td>Age of menarche n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12 years</td>
<td>6 (15)</td>
<td>7 (16)</td>
<td>0.98</td>
</tr>
<tr>
<td>12-13 years</td>
<td>24 (55)</td>
<td>23 (54)</td>
<td></td>
</tr>
<tr>
<td>&gt;=14 years</td>
<td>9 (21)</td>
<td>9 (21)</td>
<td></td>
</tr>
<tr>
<td>Don’t remember/Missing</td>
<td>5 (9)</td>
<td>5 (9)</td>
<td></td>
</tr>
<tr>
<td>Age of first live birth n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20 years</td>
<td>21 (47)</td>
<td>14 (33)</td>
<td>0.51</td>
</tr>
<tr>
<td>20-24 years</td>
<td>10 (23)</td>
<td>12 (29)</td>
<td></td>
</tr>
<tr>
<td>25-29 years</td>
<td>1 (2)</td>
<td>2 (5)</td>
<td></td>
</tr>
<tr>
<td>30 + years</td>
<td>6 (14)</td>
<td>10 (24)</td>
<td></td>
</tr>
<tr>
<td>Never gave birth</td>
<td>6 (14)</td>
<td>4 (9)</td>
<td></td>
</tr>
<tr>
<td>Missing (n=2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth control pills n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>26 (59)</td>
<td>21 (50)</td>
<td>0.40</td>
</tr>
<tr>
<td>Missing (n=2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT (ever used) n (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>19 (43)</td>
<td>17 (44)</td>
<td>0.97</td>
</tr>
<tr>
<td>Missing (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history of breast cancer n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20 (45)</td>
<td>25 (58)</td>
<td>0.12</td>
</tr>
<tr>
<td>No</td>
<td>20 (45)</td>
<td>15 (32)</td>
<td></td>
</tr>
<tr>
<td>Don’t know/Missing</td>
<td>4 (10)</td>
<td>4 (10)</td>
<td></td>
</tr>
<tr>
<td>Family history of ovarian cancer n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>28 (64)</td>
<td>19 (49)</td>
<td>0.32</td>
</tr>
<tr>
<td>No</td>
<td>4 (9)</td>
<td>7 (18)</td>
<td></td>
</tr>
<tr>
<td>Don’t know/Missing</td>
<td>12 (26)</td>
<td>13 (33)</td>
<td></td>
</tr>
</tbody>
</table>

- Except for the four continuous variables age, weight, height and BMI all other characteristics are compared using chi-square test between cases and controls
- ~ Paired t-test comparing the means between two groups
- ^Wilcoxon sign rank test comparing the means between two groups
**Tumor Characteristics:** As presented in Table VI.II, the majority of cases (73%) had ER+,PR+ (Double positive) breast tumors. Almost 50% of the tumors were larger than 2 cm and a similar number of tumors had positive nodal status. There was only one case with metastasis.

**Table VI-II: Tumor characteristics among cases (n=44)**

<table>
<thead>
<tr>
<th>Tumor characteristics</th>
<th>Cases (n=44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double positive tumors n (%)</td>
<td>Yes 32 (73)</td>
</tr>
<tr>
<td></td>
<td>No 9 (20)</td>
</tr>
<tr>
<td></td>
<td>Don’t know 3 (7)</td>
</tr>
<tr>
<td>Tumor size &gt; 2 cm n (%)</td>
<td>Yes 21 (48)</td>
</tr>
<tr>
<td>Nodal status n (%)</td>
<td>Yes 21 (48)</td>
</tr>
<tr>
<td>Metastasis n (%)</td>
<td>Yes 1 (1)</td>
</tr>
</tbody>
</table>
Biochemical characteristics:

(i) **Comparison between cases and controls:** Plasma levels of hepcidin, sTFR, inflammatory markers (CRP and IL-6), adipokines (leptin and adiponectin), and estrogen in newly diagnosed cases and controls are presented in Table VI.III. Hepcidin trended higher in cases (p=0.1) and adiponectin was significantly elevated in cases (p=0.02) compared to controls; no other significant differences were found between the groups.

### Table VI-III: Plasma levels in cases and controls (n=88)

<table>
<thead>
<tr>
<th>Plasma variables</th>
<th>Cases (n=44)</th>
<th>Controls (n=44)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTFR (nmol/l)</td>
<td>22.27 (6.99)</td>
<td>23.2 (9.19)</td>
<td>0.80</td>
</tr>
<tr>
<td>Hepcidin (ng/ml)</td>
<td>121.32 (110.71)</td>
<td>100.78 (41.73)</td>
<td>0.10</td>
</tr>
<tr>
<td>CRP (µg/ml)</td>
<td>2.57 (4.18)</td>
<td>1.86 (4.58)</td>
<td>0.10(^\dagger)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.84 (1.75)</td>
<td>1.73 (1.86)</td>
<td>0.65(^\dagger)</td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>6.26 (8.48)</td>
<td>5.94 (8.64)</td>
<td>0.88(^\dagger)</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>12.79 (15.56)</td>
<td>10.37 (15.12)</td>
<td>0.15</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>12.38 (7.58)</td>
<td>8.14 (8.03)</td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td>Iron deficiency n (%)</td>
<td>9 (20)</td>
<td>12 (27)</td>
<td>0.45</td>
</tr>
</tbody>
</table>

- All the values are median (IQR); Variables compared using paired t-test; \(^\dagger\) compared using Wilcoxon Sign Rank test.
(ii) Within and in-between group comparison of obese and lean (cases and controls): To further explore the effect of disease status, cases and controls were compared stratifying by obesity (Table VI.IV). No differences between obese cases and controls were found for any plasma measurement. Among non-obese women, cases had significantly higher CRP (p=0.05), IL-6 (p=0.006) and a trend toward higher hepcidin (p=0.08) compared to controls. Obesity-related changes within cases and controls were examined comparing obese vs non-obese subgroups (Table VI.IV). Healthy obese participants had significantly higher CRP, IL-6, leptin, estrogen and hepcidin than their non-obese counterparts. Among cases only CRP and leptin were significantly higher in obese compared to their non-obese counterparts. Functional iron insufficiency (sTFR > 28.1 nmol/L) was present in 24% of the overall study population and 23% of obese controls. Overall iron insufficiency was significantly associated with lower hepcidin levels (hepcidin categorized in quartiles; chi square p value=0.05) but was not associated with obesity or disease status.
Table VI-IV: Comparison of parameters in obese vs non-obese within and between cases (n=44) and controls (n=44)

<table>
<thead>
<tr>
<th>Plasma variables</th>
<th>Cases</th>
<th>Controls</th>
<th>P Value b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obese (n=22)</td>
<td>Non-obese (n=22)</td>
<td>P valueª</td>
</tr>
<tr>
<td></td>
<td>Obese (n=22)</td>
<td>Non-obese (n=22)</td>
<td>P valueª</td>
</tr>
<tr>
<td>BMI</td>
<td>35.0 (7.47)</td>
<td>24.3 (3.53)</td>
<td><strong>&lt;.0001</strong></td>
</tr>
<tr>
<td>sTfR (nmol/l)</td>
<td>20.90 (6.46)</td>
<td>23.87 (6.53)</td>
<td>0.45</td>
</tr>
<tr>
<td>Hepcidin (ng/ml)</td>
<td>146.98 (116.78)</td>
<td>107.63 (86.36)</td>
<td>0.46</td>
</tr>
<tr>
<td>CRP (µg/ml)</td>
<td>3.43 (4.48)</td>
<td>1.49 (2.0)</td>
<td><strong>.002</strong></td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>2.42 (1.67)</td>
<td>1.48 (0.75)</td>
<td>.10</td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>6.93 (7.89)</td>
<td>5.24 (9.87)</td>
<td>0.34</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>22.96 (19.21)</td>
<td>7.40 (4.21)</td>
<td><strong>&lt;.0001</strong></td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>9.94 (6.74)</td>
<td>14.11 (8.56)</td>
<td>0.12</td>
</tr>
<tr>
<td>Iron deficiency n(%)</td>
<td>4 (18)</td>
<td>5 (23)</td>
<td>0.71#</td>
</tr>
</tbody>
</table>

- All the values are median (IQR) except iron deficiency;ª Compared using Student’s t-test; b compared using Paired t-test; ^ compared using Wilcoxon Sign Rank test for cases vs. controls and Wilcoxon Rank Sum test for obese vs. lean; # Iron deficiency was compared using chi-square test.
- Due to multiple comparisons Bonferroni correction was applied. At α < 0.0016, most of the significant differences were preserved except CRP (obese vs. non-obese case; non-obese cases vs. control) and hepcidin (obese vs. non-obese control).

(iii) Correlations between plasma variables: Overall hepcidin was significantly negatively correlated with sTFR (r=-0.22, p=0.04) and positively correlated with IL-6 (r=0.21, p=0.05) and CRP (r=0.28, p=0.007). Among controls a significant positive correlation was found between hepcidin and BMI (r=0.36, p=0.02), IL-6 (r=0.33, p=0.03), (CRP r=0.42, p=0.0005) and a weak correlation was observed with leptin (r=0.25, p=0.09). No
significant correlations between hepcidin and selected variables (BMI, IL-6, CRP, sTFR and leptin) were found in cases (Table VI.V). Further, when cases/controls were stratified by obesity status CRP was significantly correlated with hepcidin levels only in non-obese women ($r=0.44$, $p=0.04$). The highest mean value for plasma hepcidin (Table VI.IV) occurred in obese cases and was negatively associated with BMI ($r=-0.41$, $p=0.04$). Only non-obese controls had a negative correlation between sTFR and hepcidin ($r=-0.41$, $p=0.06$). Among obese controls, sTFR was positively correlated with CRP and IL-6 (both $p$ values=0.002). As expected BMI in controls was positively correlated with CRP ($p=0.0001$), IL-6 ($p=0.0001$), leptin ($p=0.001$) and estradiol ($p=0.01$), and negatively correlated with adiponectin ($p=0.008$). Among cases BMI was positively correlated only with CRP ($p=0.0085$) and leptin ($p=0.0001$).
Table VI-V: Spearman correlations for plasma hepcidin with BMI and selected biochemical measures among overall participants, within cases and controls and by obese and non-obese groups

<table>
<thead>
<tr>
<th>Plasma variables</th>
<th>Overall (n=88)</th>
<th>Cases (n=44)</th>
<th>Controls (n=44)</th>
<th>Cases (n=22)</th>
<th>Controls (n=22)</th>
<th>Cases (n=22)</th>
<th>Controls (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI r (p value)</td>
<td>0.14 (0.18)</td>
<td>-0.01 (0.93)</td>
<td>-0.41 (0.04)</td>
<td>-0.02 (0.92)</td>
<td>0.36 (0.02)</td>
<td>-0.01 (0.96)</td>
<td>0.24 (0.27)</td>
</tr>
<tr>
<td>sTfR r (p value)</td>
<td>-0.22 (0.04)</td>
<td>-0.25 (0.09)</td>
<td>-0.13 (0.55)</td>
<td>-0.32 (0.14)</td>
<td>-0.17 (0.27)</td>
<td>0.12 (0.58)</td>
<td>-0.41 (0.06)</td>
</tr>
<tr>
<td>CRP r (p value)</td>
<td>0.28 (0.007)</td>
<td>0.16 (0.31)</td>
<td>-0.19 (0.39)</td>
<td>0.44 (0.04)</td>
<td>0.42 (0.005)</td>
<td>0.27 (0.23)</td>
<td>0.37 (0.09)</td>
</tr>
<tr>
<td>IL-6 r (p value)</td>
<td>0.21 (0.05)</td>
<td>0.14 (0.37)</td>
<td>-0.04 (0.87)</td>
<td>0.20 (0.36)</td>
<td>0.33 (0.03)</td>
<td>0.17 (0.44)</td>
<td>-0.06 (0.79)</td>
</tr>
<tr>
<td>Leptin r (p value)</td>
<td>0.09 (0.37)</td>
<td>-0.01 (0.96)</td>
<td>-0.26 (0.24)</td>
<td>-0.01 (0.74)</td>
<td>0.25 (0.09)</td>
<td>0.04 (0.84)</td>
<td>0.06 (0.80)</td>
</tr>
</tbody>
</table>
(iv) **Linear regression models:** Multiple linear regression models indicated log CRP ($p=0.0002$) and log sTFR ($p=0.019$) were independent predictors of log hepcidin (Table VI.VI). These associations remained when assessed separately in controls (adj $r^2=0.30$), however these correlations were not found in cases. To further explore hepcidin association within the cases, participants were categorized as with or without double-positive tumors. In cases with both ER and PR positive tumors CRP ($p=0.016$) and iron status (sTFR, $p=0.014$) predicted hepcidin (adj $r^2=0.23$). Leptin, adiponectin, estradiol, IL-6 and disease status were not significantly associated with hepcidin in univariate or multivariate analysis in any of the models. Log hepcidin ($\beta=-0.14$; $p=0.02$), log CRP ($\beta=0.05$; $p=0.04$) and log estrogen ($\beta=-0.05$; $p=0.01$) were significant predictors of sTFR as independent variable (adj $r^2=0.09$, data not shown in the table). Among controls all associations remained (adj $r^2=0.24$), whereas in cases there were no significant associations.
Table VI-VI: Final linear regression model for log hepcidin in overall participants, controls, cases and ER+, PR+ tumors (Log hepcidin = log CRP  log sTFR)

<table>
<thead>
<tr>
<th></th>
<th>Final models</th>
<th>β</th>
<th>SE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (n=88)</td>
<td>(R²= 0.15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>5.85</td>
<td>0.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log CRP</td>
<td>0.13</td>
<td>0.03</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Log sTFR</td>
<td>-0.41</td>
<td>0.19</td>
<td>0.036</td>
</tr>
<tr>
<td>Controls (n=44)</td>
<td>(R²= 0.30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>6.76</td>
<td>0.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log CRP</td>
<td>0.17</td>
<td>0.04</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Log sTFR</td>
<td>-0.68</td>
<td>0.24</td>
<td>0.0074</td>
</tr>
<tr>
<td>Cases (n=44)</td>
<td>(R²= 0.02)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>5.62</td>
<td>0.99</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log CRP</td>
<td>0.08</td>
<td>0.07</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Log sTFR</td>
<td>-0.31</td>
<td>0.31</td>
<td>0.33</td>
</tr>
<tr>
<td>Cases (ER + PR + tumors, n=32)</td>
<td>(R²= 0.24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>6.48</td>
<td>0.88</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Log CRP</td>
<td>0.17</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Log sTFR</td>
<td>-0.58</td>
<td>0.28</td>
<td>0.05</td>
</tr>
</tbody>
</table>
(v) **Logistic regression model:** Unadjusted conditional logistic regression results indicated hepcidin significantly predicted disease status as a continuous variable (OR=1.009, 95% CI= 1.00 to 1.018, p=0.048). Hepcidin as a dichotomized variable (high levels (>=151 ng/ml) vs low levels (<151 ng/ml)) was also significantly associated with disease status (OR=3, 95% CI= 1.09 to 8.25, p=0.033). Final multivariate conditional logistic model for disease status after considering all the other variables included hepcidin (OR=4.41, 95% CI= 1.37 to 14.22, p=0.013) and adiponectin (OR=1.73, 95% CI= 1.08 to 2.79, p=0.024) as significant predictors.

2. **Results for specific aim 2**

Tissue samples were available for 73% (64/88) of the participants included in specific aim 1 (34 cases and 30 controls). Obesity occurred in 19 cases and 13 controls. Baseline characteristics and plasma levels were again compared between cases and controls for this subset (n=64), as presented in Appendix I, II and III. For baseline characteristics, family history of breast cancer was the only significant difference between cases and controls in this smaller group. Stratifying by obesity status revealed this difference was present only in obese women: 83% (10/12) of obese subjects had the disease in the absence of breast cancer family history vs. 43% (7/16) who had the disease in the presence of breast cancer family history (p=0.05). All the plasma comparisons for these subjects were similar to the overall comparisons presented in the tables for specific aim 1. Slight increases in p-values reflect the loss in power due to the smaller sample size.

(i) **Iron content of breast tissue:** Cases had significantly more iron accumulation (iron dichotomized as high score >=1 or low score as <1) compared to controls (21% vs 3% respectively, p=0.05) (Table IV.VII). As shown in Figure VI.1, most of the iron accumulation was within the mononuclear inflammatory infiltrating cells, and there was no iron staining in epithelial cells. These inflammatory cells were presumably macrophages due to their foamy appearance (although cluster of differentiation 68 (CD68) staining was not done, CD68 is expressed on macrophages and can be used for macrophage identification). Macrophages infiltrate breast tumors [135] and are considered a storage site for iron [136]. Among the cases iron staining was present in 28% of the ER+, PR+ tumors (7/ 25) vs. 0% in other tumor types (p=0.15). To further explore the effect of obesity on iron accumulation stratified analysis was done. Among the cases, non-obese subjects had significantly higher iron accumulation than obese counterparts (40 % vs 3%, p=0.03) (Table VI.VIII).
Assessment for level of mononuclear cell infiltrates in cases showed obese and non-obese had similar mean scores (p=0.25) (Figure VI.2). Overall, non-obese cases had more iron-stained inflammatory cells whereas obese cases had minimal iron staining in these cells.

Table VI-VII: Tissue parameters in cases and controls

<table>
<thead>
<tr>
<th>Tissue parameters</th>
<th>Cases (n=34)</th>
<th>Controls (n=30)</th>
<th>P value¹</th>
<th>P value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron score (high/low; n (%))</td>
<td>7 (21)</td>
<td>1(3)</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>FPN1 in macrophages (high/low; n (%))</td>
<td>6 (18)</td>
<td>12 (40)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>H-score for FPN1 in adipose tissue (0-300)</td>
<td>120 (101.2)</td>
<td>129.8 (110)</td>
<td>0.91^</td>
<td>0.90</td>
</tr>
<tr>
<td>H-score for FPN1 in epithelium (0-300)</td>
<td>127.5 (83.7)</td>
<td>171.5 (84.5)</td>
<td>0.15^</td>
<td>0.16</td>
</tr>
<tr>
<td>Average breast adipocyte size; µm²</td>
<td>846.7 (469.1)</td>
<td>926.9 (540.6)</td>
<td>0.1^</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Note: All the values are median (IQR) or frequency n(%). Comparisons were done using Fisher exact test or ^ Wilcoxon Rank test. ¹ unadjusted p values; ² adjusted for BMI using logistic regression model
Table VI-VIII: Comparison of parameters in obese vs non-obese cases

<table>
<thead>
<tr>
<th>Plasma variables</th>
<th>Cases (n=34)</th>
<th>Controls (n=30)</th>
<th>P Value b</th>
<th>P Value a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obese (n=19)</td>
<td>Non-obese (n=15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>33.0 (5.21)</td>
<td>24.6 (2.45)</td>
<td>&lt;.0001 ^</td>
<td></td>
</tr>
<tr>
<td>Iron score – (high/low) n(%)</td>
<td>1 (3)</td>
<td>6 (40)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>FPN1 in macrophages – (high/low) n(%)</td>
<td>2 (10)</td>
<td>4 (27)</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>H-score for FPN1 in adipose tissue</td>
<td>117.5 (72.5)</td>
<td>125.0 (117.5)</td>
<td>0.35 ^</td>
<td></td>
</tr>
<tr>
<td>H-score for FPN1 in epithelium</td>
<td>150.0 (77.5)</td>
<td>125.0 (80.0)</td>
<td>0.46 ^</td>
<td></td>
</tr>
<tr>
<td>Average breast adipocyte size (µm²)</td>
<td>939.31 (678.81)</td>
<td>637.65 (385.76)</td>
<td>0.006 ^</td>
<td></td>
</tr>
<tr>
<td>Tumor size &gt;2 cm, n(%)</td>
<td>13(68)</td>
<td>4 (27)</td>
<td>0.04</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note: All the values are median (IQR) or frequency (n,%); * Compared using Student’s t-test; ^ compared using Wilcoxon Rank test; # Iron score, FPN1 in macrophages and tumor size was compared using Fisher exact test.
Figure VI-1: Perls’ staining indicating non-heme iron (Fe (III)) in blue color, photographed at 200x
As shown by arrows iron accumulation was in mononuclear inflammatory cells presumably macrophages
(a) Strong iron staining in (IDC) (score=3);
(b) Intermediate iron staining (score=2) in IDC;
(c) Weak iron staining (score=1) in IDC;
(d) Undetectable staining (score=0) in benign tissue

Figure VI-2: Breast tumor showing mononuclear inflammatory infiltrates, photographed at 200x
(a) High infiltration
(b) Low infiltration
(ii) Ferroportin content of breast tissue: The iron exporter protein FPN1 was assessed in three major tissues of the tumor microenvironment (epithelial cells, adipose tissue and macrophages). Macrophages in cases had significantly lower FPN1 compared to controls (p=0.05) (Table VI.VII). However, when stratified by obesity this difference remained only in the obese (p=0.04). Tumor epithelium of cases was insignificantly lower for FPN1 compared to normal epithelium of controls (p=0.15) (Table VI.VII, Figure VI.3). Some of the tumor slides (n=22) with detectable normal epithelial cells were scored separately (data not shown in the tables). There was no significant difference for either score (normal vs. tumor epithelial).

Figure VI-3: Ferroportin (IHC- depicted by brown color) is decreased in breast cancer tissue. Photographed at 200x
(a) High staining in normal epithelium of benign tissue;
(b) Low staining in tumor epithelium of tumor tissue;
(c) High staining in macrophages;
(d) Low staining in macrophages.
(iii) **Breast adipocyte size**: Histological examination of biopsy tissue indicated smaller adipocytes at the invasive front of the tumor (Figure VI.4). At similar BMI cases trended to have lower average breast adipocyte size compared to controls (p=0.1). In both cases and controls obese women had larger adipocytes compared to non-obese women (p<.0006).

![Figure VI-4: Breast tumor showing smaller adipocytes on the invasive front of cancer, photographed at 4x](image-url)
3. **Results for specific aim 3**

Macrophage FPN1 was positively associated with tumor epithelial FPN1 (cases, \(r=0.50; p=0.005\)) but was not significantly associated with normal epithelial FPN1 (controls, \(r=0.28; p=0.15\)). In cases plasma hepcidin was not associated with the tumor epithelial FPN1 score (\(r=-0.16, p=0.39\)) or the macrophage FPN1 score (\(r=-0.14, p=0.43\)) (Table IV-IX). However, sTFR was weakly negatively associated with FPN1 in epithelial cells among controls (\(r=-.33; p=0.08\)). CRP was negatively associated with the FPN1 score in adipose tissue (\(r=-45; p=0.009\)) in cases but not in controls (\(r=0.03; p=0.9\)). The association among cases remained after adjusting for BMI (\(r=-44; p=0.01\)). Among the cases, bigger adipocytes were associated with reduced tissue iron accumulation (iron score as continuous variable) (\(r=-0.35; p=0.05\)).

**Table VI-IX: Correlations of plasma hepcidin with iron and FPN1 content in overall participants, cases and controls**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall (n=64)</th>
<th>Cases (n=34)</th>
<th>Controls (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron Content</td>
<td>0.09 (p=0.49)</td>
<td>0.05 (p=0.80)</td>
<td>0.04 (p=0.84)</td>
</tr>
<tr>
<td>FPN1 in epithelium</td>
<td>-0.11 (p=0.41)</td>
<td>-0.16 (p=0.39)</td>
<td>0.08 (p=0.67)</td>
</tr>
<tr>
<td>FPN1 in macrophages</td>
<td>-0.15 (p=0.25)</td>
<td>-0.14 (p=0.43)</td>
<td>0.07 (p=0.70)</td>
</tr>
<tr>
<td>FPN1 in adipose tissue</td>
<td>-0.09 (p=0.46)</td>
<td>-0.17 (p=0.35)</td>
<td>0.01 (p=0.94)</td>
</tr>
</tbody>
</table>
VII. DISCUSSION

1. Discussion for specific aim 1

**Hepcidin and sTFR levels in healthy postmenopausal women**

We have demonstrated that obesity-associated hepcidin elevation previously reported in children [18], adolescent [19] and premenopausal women (17) is also present in healthy postmenopausal women. Iron insufficiency (i.e. increased sTFR) occurred in 20% of healthy obese (BMI= 35± 8) postmenopausal women, which is much lower than the 45% rate observed in our earlier study of premenopausal morbidly obese women (BMI = 50 ± 11) seeking bariatric surgery (17). This difference likely reflects the lower prevalence of iron deficiency following the cessation of the menstrual cycle [137] and/or differences in the level of obesity between the two studies. Lecube et al. [90] reported a significant association between sTFR and BMI in similarly aged postmenopausal women as those in our study; however, they did not measure hepcidin or any marker of inflammation. In our controls sTFR was significantly positively associated with inflammation (CRP), but not with BMI. This lack of association may be due to the wider BMI range (19–60 kg/m² vs. 19-42 kg/m²) of participants in Lecube’s study compared to ours. The positive correlation between CRP and sTFR indicates the presence of greater iron insufficiency associated with low-grade inflammation, similar to earlier findings of obesity-associated iron deficiency via elevated inflammation [22, 23, and 17]. The hepcidin levels in our obese postmenopausal controls were significantly higher than what was reported in our earlier study of healthy premenopausal women post-bariatric surgery at similar BMI (113 ng/ml, IQR 46 vs 31.3 ng/ml, IQR 38). These age-related elevations in hepcidin in the present study are similar to those reported by Galesloot et al. in healthy women over 55 years; however they did not account for any measure of adiposity [138]. Overall these findings indicate the obesity-induced elevation in hepcidin among postmenopausal women do not induce changes in iron status. The increase in overall inflammation that accompanies aging [139] and cessation of menstrual cycle may also stimulate hepcidin production and represents an age-related factor (menopausal status) contributing to increased hepcidin levels.
**Hepcidin and sTFR levels in postmenopausal women with breast cancer**

There was no significant difference between obese and non-obese cases for plasma hepcidin or sTFR. We speculate cancer-induced inflammation exceeded the pro-inflammatory influence of obesity, masking its effect. It is likely this also diminished plasma level differences for estrogen between obese and non-obese cases (Table IV.IV) that were present in controls. Post-hoc power analysis based on the mean and standard deviation for hepcidin levels indicated a sample size of 109/group would be needed to detect a significant difference between obese and non-obese cases. The insignificant influence of obesity on hepcidin levels may have occurred due to the inclusion of overweight women in the non-obese group. Restricting our comparison to obese vs. lean women with the current sample size may have resulted in a greater difference between groups in plasma hepcidin. This approach was considered, however the original cohort had limited lean Caucasian postmenopausal participants recruited at the time the current study was initiated (N=16 cases and 24 controls), impeding this restriction. Differences between obese and non-obese may also have been reduced by our use of BMI as a measure of obesity. More accurate assessment for adiposity such as waist circumference or body composition assessment using Dual-energy X-ray absorptiometry (DXA) may have improved our ability to detect the association between obesity and hepcidin in cases. Future studies with larger sample sizes or greater differences for BMI between groups or more precise measures of adiposity are needed to evaluate the influence of obesity on hepcidin levels in women with postmenopausal breast cancer.

**Hepcidin and breast cancer association**

To our knowledge this is the first study to demonstrate elevated hepcidin levels associated with breast cancer risk when controlling for other established risk factors. Obese postmenopausal women have almost 2.5 times higher risk for breast cancer compared to their lean counterparts [2]. The obese healthy controls had significantly higher hepcidin then their non-obese counterparts (Table IV.IV) supporting the hypothesis that the obesity-associated elevated risk for postmenopausal breast cancer in part may be due to its effect on plasma hepcidin. Hepcidin levels are affected by the interplay of various simultaneous stimulatory (i.e. iron overload and inflammation) and inhibitory pathways (i.e. hypoxia and erythropoiesis) (18). The relative strength of these signals, rather than any hierarchy, determines hepcidin production [140, 141]. Our participants’ hepcidin levels were associated with iron status and inflammation among controls and cases with double positive tumors (ER+PR+, 73% of cases); these
tumor types are generally Luminal A subtype [142]. Miller et al. found Iron regulatory gene signature significantly identified high-risk patients in women with Luminal A breast cancer [113]. A recent meta-analysis showed a 5-unit increase in BMI was associated with a 33% (95% CI= 20 to 48%) increased risk of double positive tumors in postmenopausal women [143]. Our results suggest obesity may increase the risk of double-positive tumors via its influence on hepcidin levels. As Luminal A tumors account for 66.5% of postmenopausal breast cancer in Caucasians [144] these findings have important public health implications.

The significant association between elevated systemic hepcidin and breast cancer in our findings is similar to and extends those reported by Orlandi et al. [117]; however, their crude OR for hepcidin-breast cancer association (OR=1.42; 1.18-1.70) was much higher than ours (OR=1.01; 1.00-1.02). This likely reflects differences in the participation criteria for each study. Orlandi et al. recruited pre- and postmenopausal women and healthy controls; our study was restricted to postmenopausal women, with controls having benign non-proliferative lesions. Additionally, the Orlandi study did not control for the influence of BMI or age while we matched our cases and controls on these two variables. Zhang et al. [108] found elevated plasma hepcidin (p=0.006) among cases (n=91) compared to healthy controls (n=51). We found hepcidin levels trended (insignificantly) higher in cases than controls (p=0.1). The failure to detect significant differences in the present study may be due to our smaller sample size compared to Zhang’s study (n= 44 cases and 44 controls vs. n= 91 cases and 51 controls respectively). The discrepancy in the hepcidin comparison results between these studies may reflect differences in participants’ mean age and race. We included Caucasian postmenopausal women, and Zhang’s study had Chinese women, both pre-and postmenopausal who were much younger than ours (mean age 47.25 ± 0.74 vs. 60.9 ± 7 years respectively). Additionally our cases and controls were matched on BMI, which is an important factor affecting both hepcidin levels and breast cancer risk. However, the study by Zhang did not account for any measure of obesity. In our study, final conditional logistic model predicting disease status was derived after considering all the assessed variables that are established significant risk factors for breast cancer. Postmenopausal women with elevated hepcidin (>152 ng/ml) were found to have four times higher risk for breast cancer compared to those with lower levels (<152 ng/ml) when controlled for other variables. These findings indicate plasma hepcidin has a good capacity to predict breast cancer and can be further explored as a novel plasma marker for breast cancer detection and screening.
**Adiponectin and breast cancer association**

We found BMI-adjusted adiponectin levels were higher among newly diagnosed cases than controls. Similar findings were established by Awadhi et al. [145], although others have reported the opposite association [146-149] or no correlation between these parameters [81, 150]. Similar to our design, Gross et al. included only postmenopausal Caucasian women matched for age and found lower adiponectin in cases than controls (44).

The contrasting results from this study may be due to their cases having significantly higher BMI (p=0.02) compared to controls, whereas our cases and controls were matched on BMI. In vitro studies indicate adiponectin enhances apoptosis and inhibits the growth in ER/PR negative breast cancer cell lines [151, 152]. It has been reported that low level of adiponectin is associated with poor outcome in ER/PR negative tumors, whereas ER/PR positive tumors had no association [153]. Another study found adiponectin to be inversely associated with breast cancer only in low estrogen environments [154]. Thus, the lack of a negative association between adiponectin and breast cancer in the present study may reflect the majority of our cases having ER+, PR+ (73%) tumors. Llanos et al. [7] evaluated plasma and breast tissue adiponectin levels and found the strongest positive correlation between the two in their obese participants (r=0.31, p= 0.04). They hypothesized the inconsistent epidemiological results for the adipokine-breast cancer relations were due to the influence of participants’ background characteristics.

Breast tissue adiponectin is positively associated with postmenopausal breast cancer [155]. With our elevated levels of adiponectin among the cases compared to BMI-matched controls, we speculate the breast tumors might be producing more adiponectin. Therefore more studies are warranted to evaluate the effect of adiponectin on breast cancer considering tissue, as well as systemic levels.

2. **Discussion for specific aim 2 and 3**

**Breast tissue iron content**

We have demonstrated breast tumors have higher iron accumulation compared to benign breast tissues in postmenopausal women. These results are consistent with our previous findings and those of others in patients with colorectal cancer comparing tumors to normal colon [156, 157]. Most of the iron was accumulated within the macrophages, inside and around the tumor, indicating iron is trapped within these storage sites. Similar findings were reported using Perls’ staining in murine mammary tumors [158] and ferritin staining in human breast tumors.
carcinoma [159, 160]. In our analysis, tumor tissue macrophage iron accumulation varied by obesity status. However, contrary to what we hypothesized higher tissue iron was found in the non-obese cases compared to their obese counterparts. We speculate this reflects differences in polarization of the macrophages within these individuals. It is known that M1 macrophages store more iron and express lower FPN1 compared to M2 macrophages [161, 162]. This difference in iron handling enables M2 to release more iron spontaneously, even at low concentrations [162, 163]. Tumor promotion is an established function of M2 macrophages [161], and tumor-associated macrophages are more like M2 than M1 [164]. Thus, low iron staining in the obese than the non-obese cases supports the hypothesis for greater proportions of the M2 phenotype in the breast tissue that enables efficient provision of iron to the surrounding tumor in these individuals, and may represent an additional obesity-associated pathway for poor prognosis. Further studies evaluating the difference in distribution of M1 and M2 macrophages surrounding tumors of obese and lean subjects are needed to understand this link better.

Macrophages play a critical role in tumor angiogenesis [135] and are associated with tumor progression [160]. Cell culture studies have shown that inflammation increases ferritin secretion by macrophages, independent of iron [160]. Similar to previous investigators [31, 32, 165] we found obesity was significantly associated with large tumors (>2cm). Additionally, we reported these big tumors trended to have lower iron in their macrophages compared to small tumors (<2cm). The neoplastic progression of breast tissue from normal to non-invasive carcinoma (DCIS) to invasive carcinoma (IDC) is positively associated with its iron needs as indicated by increasing expression of TFR1 [12]. We speculate decreased iron in macrophages surrounding large tumors reflect their adaptation for increased iron acquisition, which may facilitate tumor growth. Iron staining was lower in obese cases than controls, possibly reflecting their high prevalence (70% of obese cases) of large tumors. We speculate these large tumors may have the ability to acquire and utilize the iron stored in surrounding macrophages for their growth. The increased macrophage iron accumulation around small tumors in the non-obese cases when compared to their obese counterparts may also represent a local defense mechanism to minimize iron availability. This type of iron sequestration accompanies acute trauma/inflammation, which limit its availability to invading microbes [166]. Thus, we speculate small tumors in non-obese cases may be able to withhold iron in surrounding macrophages while the large tumors in obese women cannot, possibly due to increased tumor iron requirements of larger tumors or alterations in defense mechanism of obese.
**Breast tissue FPN1 content**

Women with breast tumors expressing low FPN1 have a poorer prognosis than those expressing high FPN1 [15]. Studies on other cancers (colorectal carcinoma [157] and hepatocarcinoma [167]) have also demonstrated association between tumor's low FPN1 expression and disease progression. A study using similar methods for FPN1 staining reported lower FPN1 in the tumors compared to healthy breast tissues [15]. Tumor-epithelial FPN1 in our cases were insignificantly lower than normal-epithelial FPN1 in controls (p=0.15). The lack of significance may be due to our controls having benign non-proliferative lesions, rather than healthy breast tissues examined by Pinnix et al. [15] and/or due to different scoring methods used in the two studies. We scored ferroportin on epithelium, macrophages and adipose tissue separately using H-score, whereas Pinnix’s study collectively gave a single score from 0 to 2. Based on the mean and standard deviation for FPN1 score for our cases and controls, post-hoc power analysis indicated a sample size of 59/group would be needed to detect a significant difference between the groups. Sample size estimation for this study was based on systemic hepcidin levels. Thus, we may not have had adequate power to detect the ferroportin differences between tumor and benign breast tissue epithelium using IHC. Analyzing this difference with a larger sample size may extend the earlier findings by Pinnix’s study (compared tumor vs. normal breast tissue for ferroportin using IHC). Our findings of lower FPN1 at tumor epithelium coupled with decreased FPN1 at surrounding macrophages when compared with normal breast tissue epithelium and macrophages respectively suggests alternation in iron homeostasis in the tumor microenvironment. We speculate the reduction in iron export at tumor epithelium increases the labile iron pool for cancer growth while its reduction in macrophages sequester iron to minimize tumor access to iron. Additional investigations are needed to clarify the role of FPN1 at different cell types that are important components of the tumor microenvironment.

**Breast tissue adipocyte size**

Breast tumors grow in close proximity with adipose tissue, and recent studies have emphasized the role of surrounding adipose tissue in altering the local microenvironment. Our histological examination revealed the presence of smaller and more rounded adipocytes on the invasive front of the tumor, which resulted in lower average adipocyte cell size in cases than controls when adjusted for BMI. Wang et al. [168] also reported smaller adipocytes near the tumor compared to distant breast adipocyte; however, they did not measure the exact cell
size. They termed these adipose tissues “cancer-associated adipocytes” (CAA) which over-expressed Matrix metalloproteinase-11 (MMP-11), Plasminogen activator inhibitor (PAI-1) and IL-6 when compared to adipocytes from normal breast [66]. We found average breast adipocyte size was significantly associated with BMI and was higher in the obese compared to their non-obese counterparts in both cases and controls. Similar findings were reported by Morris et al. [4] in breast cancer patients. They also reported larger breast adipocytes to be positively associated with CLS; IL-6 and aromatase (enzyme required for estrogen synthesis) in breast tissue [4]. Both in-vivo and ex-vivo experiments demonstrated estrogen up-regulates leptin [167]. Leptin is synthesized and secreted by adipocytes and associated with increased breast cancer risk [88, 169]. Adipocytes influence the tumor microenvironment via their production of estrogen, pro-inflammatory adipokines and other pro-angiogenic factors [8]. The inverse association between adipocyte size and iron accumulation in our cases may have been driven by the “cancer associated adipocytes” influence on iron homeostasis locally, although more studies are warranted in this area.

**Plasma hepcidin and breast tissue parameters**

Hepcidin is primarily produced by hepatocytes and its release in circulation affects FPN1 expression at the hepatocytes, enterocytes and macrophages [21]. Studies in other cancers (renal and colon) have shown no correlation between circulating and tumor hepcidin mRNA levels [170]. There is limited information on systemic hepcidin in breast cancer and its relation with tumor expression of FPN1 or iron. We recently reported elevated systemic hepcidin in colon cancer patients with iron-positive tumors compared to those with iron-negative tumors [15]. This association was not found in the current study. The insignificant relation may be due to differences in the participants’ gender (the colon cancer study was restricted to males) and/or tumor- specific variation in iron accumulation (colon vs. breast). In the present study BMI-adjusted elevated hepcidin levels in cases were accompanied by higher tissue iron acquisition and decreased macrophage FPN1 compared to controls. Overall hepcidin levels in postmenopausal women were positively associated with systemic inflammation (CRP, IL-6) and negatively associated with iron-insufficiency (sTFR) as expected. Although, we did not find any association between systemic hepcidin and iron or FPN1 score, directionally inverse relations for circulating hepcidin with FPN1 score at macrophages, and tumor-epithelial cells were found. This insignificant correlation may be due to our small sample size or failure of plasma hepcidin to influence FPN1 expression in the breast tissues. We
speculate local factors (i.e. tissue hypoxia, inflammation and tumor iron requirement [21]), other than systemic hepcidin—predominantly governs the hepcidin-FPN1 axis in the tumor microenvironment. Our semi-quantitative method for iron and FPN1 detection rather than a more precise measure, such as tissue mRNA expression and protein content of hepcidin and FPN1, limits our interpretation for the effect of plasma hepcidin. Future studies with these measures are needed to expand our understanding on the role of systemic hepcidin in breast cancer and its potential therapeutic role in treating breast cancer.

We hypothesized higher plasma hepcidin among cases would negatively influence FPN1 causing more iron accumulation in the breast tissues compared to controls and this effect would vary by obesity status. As hypothesized cases had elevated plasma hepcidin, lower breast tissue macrophage FPN1 and higher breast tissue iron accumulation than controls. Stratifying by obesity status showed no significant difference in plasma hepcidin or breast tissue iron, but lower macrophage FPN1 at the tissue level in obese cases compared to obese controls. Other plasma variables (i.e. CRP, IL-6 and estrogen) indicated obese cases and controls were metabolically similar. We speculate the development of tumors in obese women locally influences FPN1 at the iron storage site (macrophages) due to factors that were not assessed in our study (e.g. breast tumor hepcidin production stimulated by tumor IL-6 and/or leptin synthesis). Among non-obese, cases had higher plasma inflammation and hepcidin, and breast tissue iron content than controls with no significant difference in tissue ferroportin scores. These findings suggest the onset of cancer in non-obese influences inflammation-induced hepcidin production and tumor tissue iron accumulation. The mechanistic link of how elevated hepcidin production influences breast cancer risk coupled with other obesity-associated pathways in lean and obese women remains unknown.
3. **Study’s strengths and limitation**

**Strengths**

There are several strengths of our study that enhance the ability to interpret our findings. First we used a balanced study design with an equal number of cases and controls and obese and non-obese participants, which facilitated the exploration of two exposure variables (breast cancer and obesity). Next, several steps were taken to improve the homogeneity of our sample to increase the power to detect differences between cases/controls and obese/non-obese participants. Other recent studies examining the influence of plasma hepcidin on breast cancer did not account for obesity or any other established breast cancer risk factors and included both pre- and postmenopausal women. Specifically, we restricted our participants to Caucasian postmenopausal women with newly diagnosed IDC who were not taking any cancer medication or therapy at the time of their blood draw. Third, we matched cases and controls on age and BMI. Finally we assessed other established breast cancer risk factors including patient’s baseline characteristics, plasma inflammatory markers, adipokines and estrogen levels and included them in our regression analysis to further control for their confounding for the hepcidin-breast cancer association.

**Limitations**

There are some limitations in the present study that need to be addressed. First, limiting the sample to Caucasian postmenopausal women improved our power; however it restricts generalizability to this ethnicity. Second, only indirect assessment of adiposity of participants was used (i.e. BMI) and self-reported height was used to calculate BMI as the original cohort study did not include direct measurements. Additional anthropometric measures of obesity (e.g. waist circumference) and precise measures of adiposity (e.g. DXA scans) could have resulted in more accurate categorization of obesity status, improving our ability to detect its influence. Third, serum iron could not be assessed because plasma collection in the cohort was done in EDTA-containing tubes, which hinders the assessment of iron and limited our assessment of iron status to sTFR. Plasma sTFR is one of the most sensitive serum markers of iron status that is minimally influenced by inflammation [171], thus we do not believe this constraint influenced our overall findings and interpretation of the results. Fourth, subjects were not fasting at the time of the blood draw and insulin resistance could not be assessed. This information would have expanded our understanding of the obesity/inflammation/hepcidin interactions. Fifth, due to restricted availability of the tissue
samples, FPN1 and iron analysis was only completed on 73% of study participants. This smaller sample size limited our power to detect significant relationships where they may have existed. Sixth, iron content was assessed with a semi-quantitative method (Perls’ staining) which is not a sensitive technique for iron detection in cancer cells, however it does detect iron in the storage sites where iron is present in high concentration (i.e. macrophages). Future studies with more precise methods of measuring iron (e.g. laser ablation inductively coupled plasma-mass spectrometry) are required. In the present study due to unavailability of frozen samples we could not measure mRNA or protein expression of IL-6, hepcidin and ferroportin among our participants. Extending analysis using such measures would have added precision to our exploration of the obesity-iron-breast cancer link.

All the women recruited in the cohort study were evaluated for breast biopsy following an abnormal mammography or physical examination. For our nested case-controls study, we compared women with breast cancer (cases) to those with benign non-proliferative lesions (controls). This strengthened our case-control design, as at recruitment both the study staff and participants were unaware of their disease status, fundamentally limiting the potential of selection bias and recall bias which typically plague case-control studies. Unfortunately, the control group had non-proliferative breast abnormality which may have resulted in greater inflammation and reduced our power to detect the differences in some parameters between the groups. Finally, assessment of a causal relation is not possible with this nested case-control study design.
VIII. CONCLUSIONS AND FUTURE DIRECTIONS

In summary, we found healthy postmenopausal women exhibit obesity-associated increase in plasma hepcidin, and this effect is masked in the presence of breast cancer. Alterations in iron homeostasis in postmenopausal women with breast cancer is characterized by elevated plasma hepcidin, decreased breast tissue macrophage ferroportin and increased iron accumulation when compared to their healthy counterparts. The association between elevated plasma hepcidin and breast cancer supports further investigations to determine if hepcidin have a potential value in breast cancer screening. The higher hepcidin levels in newly diagnosed cancer patients may also increase their risk of developing cancer-related anemia. Hepcidin degrades ferroportin in macrophages and enterocytes, reducing iron availability for hemoglobin synthesis [21]. Dietary and supplemental iron absorption is also diminished with elevated plasma hepcidin, limiting the effectiveness of oral treatment for iron deficiency. Thus, our findings support the assessment of serum hepcidin and a complete blood count in postmenopausal women with newly diagnosed breast cancer prior to iron supplementation.

The carcinogenic nature of excess iron makes it an important player in the tumor microenvironment. Further tissue level studies examining more precise measures of iron regulatory proteins such as FPN1, hepcidin, IRP-2 and sTFR1, along with plasma iron and hepcidin, are needed to clarify the mechanistic link of how obesity-induced altered iron homeostasis in postmenopausal women may enhance breast cancer risk. To this end these parameters will be assessed in the future project including 12 postmenopausal women with breast cancer scheduled for surgical treatments and compared to 12 healthy postmenopausal women scheduled for breast reduction mammoplasties at UIC. To detect the influence of obesity 50% (6/12) of cases and controls will be obese, and 50% (6/12) will be lean.

We also found obesity status altered iron homeostasis in the tumor. Obesity is an established risk factor for breast cancer and is associated with poor prognosis. Our obese cases had significantly bigger breast adipocytes compared to their non-obese counterparts. Differences in handling of breast tissue iron by obese and non-obese cases suggest a potential role of adiposity-induced iron dysregulation of the tumor microenvironment in causing
poor prognosis among obese. Although Pinnix et al. [15] reported the tumor tissue’s iron metabolism was associated with poor prognosis of breast cancer, they did not account for obesity. Additional cohort investigations focusing on the effect of obesity-associated altered iron metabolism and breast cancer outcomes are needed to establish the obesity-iron-breast cancer prognosis link.

Most of the iron accumulation in the breast tissue was found in the macrophages (iron storage sites). We suggest low iron content in the obese women’s larger tumors may indicate more M2 type macrophages in the tumor microenvironment. In contrast, iron sequestration occurred in non-obese cases, which may reflect a defense mechanism characterized by more M1 type macrophages that can store iron. These hypotheses can be assessed by M1 and M2 specific staining in breast tumor tissues of obese and non-obese women. Recent studies have focused on the critical role of macrophages and their polarization in the tumor microenvironment. Future investigations for therapeutic strategies targeting differently polarized macrophages and their iron storing capacity may provide promising results for improving breast cancer risk and poor prognosis.
IX. APPENDICES
APPENDIX - A

Baseline characteristics and tumor characteristics in a subsample of cases and controls included for tissue analysis (n=64)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases (n=34)</th>
<th>Controls (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>62.3 ± 7</td>
<td>60.5 ± 7.6</td>
<td>0.32 ~</td>
</tr>
<tr>
<td>BMI, Median (IQR)</td>
<td>30.47 (9.7)</td>
<td>26.5 (8.8)</td>
<td>0.52 ^</td>
</tr>
<tr>
<td>Age of menarche n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12 years</td>
<td>6 (18)</td>
<td>7 (23)</td>
<td></td>
</tr>
<tr>
<td>12-13 years</td>
<td>17 (50)</td>
<td>12 (40)</td>
<td></td>
</tr>
<tr>
<td>&gt;=14 years</td>
<td>6 (18)</td>
<td>6 (20)</td>
<td></td>
</tr>
<tr>
<td>Don't remember/ missing</td>
<td>5 (14)</td>
<td>5 (17)</td>
<td></td>
</tr>
<tr>
<td>Age of first live birth n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25 years</td>
<td>22 (65)</td>
<td>15 (52)</td>
<td></td>
</tr>
<tr>
<td>25-29</td>
<td>8 (23)</td>
<td>9 (31)</td>
<td></td>
</tr>
<tr>
<td>30 +</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td>0.25</td>
</tr>
<tr>
<td>Never gave birth</td>
<td>4 (12)</td>
<td>3 (10)</td>
<td></td>
</tr>
<tr>
<td>Missing (n=1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth control pills n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>19 (56)</td>
<td>18 (62)</td>
<td>0.60</td>
</tr>
<tr>
<td>Missing (n=1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT (ever used) n (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15 (44)</td>
<td>13 (45)</td>
<td>0.95</td>
</tr>
<tr>
<td>Missing (n=1)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Family history of breast cancer n (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15 (44)</td>
<td>19 (63)</td>
<td>0.03</td>
</tr>
<tr>
<td>No</td>
<td>17 (50)</td>
<td>6 (20)</td>
<td></td>
</tr>
<tr>
<td>Don’t know/missing</td>
<td>2 (10)</td>
<td>5 (17)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (&gt;2cm) n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17 (50)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nodal status n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18 (53)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Metastasis (M)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (3)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Double positive tumors (ER, PR +) n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>25 (73)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note: Except for age and BMI all other characteristics were compared using chi-square test among cases and controls. ~ T-test comparing the means between two groups, ^Wilcoxon rank test comparing the means
APPENDIX - B

Plasma levels in a subsample of cases and controls included for tissue analysis (n=64)

<table>
<thead>
<tr>
<th>Plasma Variables</th>
<th>Cases (n=34)</th>
<th>Controls (n=30)</th>
<th>P value¹</th>
<th>P value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTFR (nmol/l)</td>
<td>23.3 ± 8.9</td>
<td>23.2 (9.0)</td>
<td>0.74</td>
<td>0.68</td>
</tr>
<tr>
<td>Hepcidin (ng/ml)</td>
<td>123.4 (93.4)</td>
<td>95.2 (49.5)</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>CRP(µg/ml)</td>
<td>2.6 (3.5)</td>
<td>1.8 (3.0)</td>
<td>0.10^</td>
<td>0.16</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.8 (1.6)</td>
<td>1.7 (1.8)</td>
<td>0.26</td>
<td>0.30</td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>5.8 (7.9)</td>
<td>5.5 (9.3)</td>
<td>0.42^</td>
<td>0.45</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>13.3 (15.1)</td>
<td>10.4 (17.1)</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>Adiponectin (µg /ml)</td>
<td>10.5 (8.4)</td>
<td>9.6 (9.5)</td>
<td>0.23</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Note: All the values are median (interquartile range (IQR)) or frequency; Variables compared using Student T-test; ^ compared using Wilcoxon Rank test. ¹ unadjusted p values; ² adjusted for BMI
### APPENDIX - C

Plasma level comparisons in obese and non-obese cases and controls included for tissue analysis (n=64)

<table>
<thead>
<tr>
<th>Plasma Variables</th>
<th>Cases (n=34)</th>
<th>Controls (n=30)</th>
<th>P Value b</th>
<th>Obese Case / Control</th>
<th>Non-obese Cases/ Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obese (n=19)</td>
<td>Non-obese (n=15)</td>
<td>P value a</td>
<td>Obese (n=13)</td>
<td>Non-obese (n=17)</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>33.0 (5.2)</td>
<td>24.6 (2.4)</td>
<td>&lt;.0001^</td>
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</tr>
<tr>
<td>sTfR (nmol/l)</td>
<td>22.1 (8.4)</td>
<td>25.7 (8.4)</td>
<td>0.06</td>
<td></td>
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<tr>
<td>Hepcidin (ng/ml)</td>
<td>151.5 (87.2)</td>
<td>104.9 (81.6)</td>
<td>0.26</td>
<td>106.70 ± 1.42</td>
<td>79.04 ± 1.68</td>
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</tr>
<tr>
<td>CRP (µg/ml)</td>
<td>3.38 (4.2)</td>
<td>1.25 (2.0)</td>
<td>.0016^</td>
<td>2.66 (3.8)</td>
<td>0.76 (1.2)</td>
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</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>2.4 (1.8)</td>
<td>1.4 (0.7)</td>
<td>.04^</td>
<td>2.3 (2.3)</td>
<td>0.9 (0.8)</td>
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</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>6.4 (7.8)</td>
<td>5.21 (9.9)</td>
<td>0.53^</td>
<td>8.4 (6.2)</td>
<td>3.1 (6.4)</td>
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</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>21.9 (9.8)</td>
<td>7.6 (4.0)</td>
<td>&lt;.0001</td>
<td>23.8 (10.9)</td>
<td>6.8 (5.6)</td>
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</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>9.4 (7.2)</td>
<td>14.0 (8.8)</td>
<td>0.04</td>
<td>8.2 (8.6)</td>
<td>11.8 (6.5)</td>
</tr>
</tbody>
</table>

Note: All the values are median (IQR) or frequency (n, %); a Compared using Student T test; ^ compared using Wilcoxon Rank test.
X. CITED LITERATURE


Curriculum Vitae

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EDUCATION:

- B.Sc., Home Science (Honors), University of Delhi, India 2001
- M.Sc., Home Science (Food and Nutrition), University of Delhi, India 2003
- Post Graduate diploma in Business Administration (Human Resource Development), Symbiosis Centre for Distance Learning, Pune, India 2006
- Ph.D., Human Nutrition, University of Illinois at Chicago, Chicago, Illinois, 2014

TEACHING EXPERIENCE:

Teaching Assistant, Department of Kinesiology and Nutrition, Jan 2008 – May 2009 and Aug 2010 - Dec 2010

- Nutrition
- Nutrition Science I
- Nutrition Assessment
- Clinical Nutrition I

RESEARCH EXPERIENCE:

Research Assistant, Department of Kinesiology and Nutrition, University of Illinois, Chicago, Aug 2009 – May 2010 and Jan 2013 to May 2014. Supervisor: Dr. Carol Braunschweig, Ph.D., RD
COUNSELING EXPERIENCE:

- Diabetes Educator – Sitaram Bhartia Institute of Science and Research, New Delhi, June 2003 – Nov 2004. Supervisor: Dr. A.S. Lata, Chief Endocrinologist

LAB EXPERIENCE:

- Analysis of various parameters in plasma using ELISA. Project: Dissertation project
- DNA extraction and 8-OHdG analysis using HPLC. Project: Quinn study and Prostate cancer study

FIELD EXPERIENCE:

- Collection and analysis of dietary information using dietary assessment tools (Block Food Frequency Questionnaire and 24-hour diet recall) and NDSR. Project: Health Empowerment Zone (HEZ) project; Happy Healthy Kids study; Chicago Colorectal Cancer Consortium (CCCC) at UIC; Eating behavior in African-American women study
- Public Health Counseling: At Non-government organization Prayas and Mobile crèches and a government program “Anganwadi” (Integrated Child Development Services, India)

PUBLICATIONS:

CONFERENCE PRESENTATIONS AND PUBLISHED ABSTRACTS:

  - Could not go due to visa issue, Dr. Lisa Tussing-Humphreys presented the talk


PROFESSIONAL MEMBERSHIPS:

- American Society of Clinical Oncology