THE ROLE OF SERUM FERRITIN IN BREAST TUMORIGENESIS:
IMPLICATIONS FOR CANCER-ASSOCIATED INFLAMMATION IN TUMOR
PROGRESSION AND THERAPY RESISTANCE

A Dissertation in
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ABSTRACT

Breast cancer is the most common malignancy amongst women with approximately 220,000 new cases every year in the United States alone. There has been increasing evidence to suggest a critical role for inflammation in breast cancer development, progression, and therapy resistance. However, the molecular mechanisms underlying this intriguing connection remain largely unresolved. The identification of therapeutic targets and predictive biomarkers within the context of inflammation would represent substantial steps in the fight against breast cancer.

Ferritin is the primary iron storage protein in cells. Although its intracellular iron storage function has been thoroughly studied, the significance of its extracellular function in human biology is poorly understood. Clinically, circulating ferritin is used as a biomarker of inflammation or as an indicator of the body’s iron stores. In the context of breast cancer, ferritin is elevated in tumor tissue as well as patients’ sera and this elevation correlates with poor clinical outcome and advanced histological grade. The goal of this dissertation is to identify a tumorigenic mechanism involving the classical iron storage protein ferritin in breast cancer. Moreover and in a broader clinical context, the project also aims at the development of serum ferritin as a prognostic and predictive biomarker in advanced cancer patients.

In support of this dissertation, I first performed a comprehensive investigation of the expression and localization of ferritin within breast tumors representing a spectrum of histological grades. As the tumor progressed from a ductal carcinoma in situ (DCIS) to an invasive ductal carcinoma, the ferritin content in cancer cells decreased while the tumor
infiltration by ferritin-rich macrophages increased. Surprisingly, ferritin also stained within the stromal fibers surrounding breast tumors suggesting local ferritin release within the breast. In order to examine potential sources for the secreted ferritin, I examined the ability of macrophages and breast cancer cells to secrete ferritin in vitro. Primary macrophages but not breast cancer cell lines were capable of ferritin secretion, and this secretion was potentiated in response to iron and inflammatory cytokines. The identification of macrophages as the primary site of ferritin expression and secretion within breast tumors introduces several diagnostic and therapeutic possibilities and advances our understanding of the molecular signature of tumor-associated macrophages.

In the second set of experiments, I utilized a breast cancer cell culture model to examine the ability of extracellular ferritin to stimulate cancer cells. Ferritin increased the proliferation of two epithelial breast cancer cell lines and was taken up by breast cancer cells indicating a direct functional interaction. These effects were seemingly independent of iron and were not due to iron delivery because: 1) iron-free ferritin had a similar effect to iron-rich ferritin, 2) the addition of iron to the culturing conditions did not increase the effects of ferritin, and 3) ferritin exposure did not increase intracellular iron levels. These novel findings propose that ferritin has an overlooked iron-independent functionality with tumorigenic significance.

Given the tumorigenic potential of extracellular ferritin, I then examined the clinical utility of serum ferritin in advanced cancer patients. Pre-treatment levels of serum ferritin were elevated in approximately half of a metastatic breast cancer cohort receiving trastuzumab-containing therapy (n=66). Elevation in serum ferritin was predictive of both progression-free and overall survival. When patients were stratified based on their serum
ferritin and C-reactive protein (CRP) levels, patients with elevation in both inflammatory biomarkers had the poorest response to therapy. To expand our findings to other cancer types, I examined the prognostic significance of serum ferritin and CRP in advanced pancreatic cancer patients from a phase III clinical trial serum bank (n=159). Serum ferritin levels were elevated in 88% of patients and they were predictive of shorter survival. Interestingly, only patients with elevation in both inflammatory biomarkers had a worse outcome than patients without detectable inflammation. Collectively, these findings provide strong support for the idea that cancer-associated inflammation represents a pro-tumorigenic phenomenon and may - directly or indirectly - impede response to cancer therapeutics.

In summary, I have identified a potential novel tumorigenic pathway by which tumor-associated macrophages stimulate breast tumors via the secretion of extracellular ferritin. I also identified a clinical predictive use for the inflammatory biomarkers serum ferritin and CRP which are elevated in a majority of breast and pancreatic cancer patients. Identifying the mechanism underlying the ferritin effect as well as elucidating the connection between serum ferritin and tumor-associated macrophages are important future research questions. Moreover, the clinical utility of anti-inflammatory drugs and life-style changes (such as exercise and diet) that may decrease inflammation should be explored as possible strategies to sensitize cancer patients to therapy.
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<th>Description</th>
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<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>AGP</td>
<td>α1-acid glycoprotein</td>
</tr>
<tr>
<td>ATCC</td>
<td>The American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5′-triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer-associated fibroblast</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>Colony-forming unit granulocyte-macrophages</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony stimulating factor 1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>CXC ligand 12</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC receptor 4</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DFO</td>
<td>Deferoxamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EpRE</td>
<td>Electrophile response element</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>FAC</td>
<td>Ferric ammonium citrate</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAS</td>
<td>Ferrous ammonium sulfate</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HK</td>
<td>High molecular weight kininogen</td>
</tr>
<tr>
<td>HKa</td>
<td>2-Chain high molecular weight kininogen</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>I</td>
<td>Iodine</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron response element</td>
</tr>
<tr>
<td>IRP</td>
<td>Iron regulatory protein</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PyMT</td>
<td>Polyoma middle T antigen</td>
</tr>
<tr>
<td>rHFR</td>
<td>Recombinant H-ferritin</td>
</tr>
<tr>
<td>rLFr</td>
<td>Recombinant L-ferritin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Scara5</td>
<td>Scavenger receptor class A member 5</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TfR1</td>
<td>Transferrin receptor 1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIM-2</td>
<td>Murine T cell immunoglobulin/mucin domains 2</td>
</tr>
<tr>
<td>TMHF</td>
<td>3,5,5-Trimethylhexanoyl-ferrocene</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
ACKNOWLEDGMENTS

I dedicate this dissertation to my parents, Awni and Latifa, whose unwavering support and encouragement has been the source and foundation of my drive as a student and scientist. Your sacrifice in accepting my passion and encouraging me to pursue it so freely is a debt that will take me a lifetime to repay. Also, I dedicate this dissertation to Khaldoon, Tariq, Majdoleen and Mohammed who always made an effort to make me feel at home even when oceans stood between us. Thank you for keeping me positive and optimistic. Finally, I dedicate this dissertation to the many friends who endured my friendship and blessed me with theirs.

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# CHAPTER 1

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I. Molecular properties of ferritin

Ferritin is the oldest known protein involved in iron metabolism. It was first described in 1894 by the German pharmacologist Oswald Schmiedeberg who noted an iron-rich component in horse livers (Laufberger 1937). However, it was not until 1937 that ferritin was purified from the horse spleen by the Czech biologist Vilém Laufberger who proposed that it “must be a substance which serves as a depot for iron in the organism” (Laufberger 1937; Crichton 1973). The early isolation of ferritin was facilitated by several distinct biochemical characteristics: its stability at high temperatures (>80°C), relative insolubility in ammonium sulfate solutions, and its crystallization with cadmium salts.

Ferritin is a 450,000 dalton hollow nano-cage (outside diameter 12-13 nm; inside diameter 8 nm) capable of incorporating up to 4500 iron atoms in a non-toxic but bioavailable form (Banyard et al. 1978; Harrison and Arosio 1996). In mammals, each ferritin complex is composed of 24 subunits that form a spherical symmetrical protein shell. Each ferritin subunit folds into a 4-helix bundle with a fifth short helix in close proximity to the C-terminus (Ford et al. 1984; Lawson et al. 1991). In its assembled form, the ferritin complex has six channels which serve as entry and exit ports for iron (Wardeska et al. 1986; Desideri et al. 1991; Levi et al. 1996). Iron is stored within the ferritin cavity as mineralized ferrihydrite (Fe$_3$O$_3$ · 9H$_2$O) with traces of phosphorus and nitrogen (Towe 1981).

Two functionally and genetically distinct ferritin subunits exist: L-ferritin and H-ferritin (also known as light chain and heavy chain ferritin). In humans, but not all species, their molecular masses are 19 and 21 kDa respectively (Arosio et al. 1978; Boyd
et al. 1985). Although the two subunits share approximately 55% of their sequence as well as their multi-helical three dimensional structures, they are functionally distinct (Boyd et al. 1985; Jain et al. 1985; Levi et al. 1988). The H subunit possesses enzymatic activity and can oxidize ferrous iron into ferric iron. The ferroxidase center in H-ferritin is composed of several residues (mainly glutamic acid) which are buried within the H-ferritin helical bundle and serve as metal ligands (Lawson et al. 1991; Cozzi et al. 2000). The ferroxidase activity of H-ferritin is not dependent on the assembly of the full ferritin complex and can be detected with the monomer form (Levi et al. 1993). The presence of a ferroxidase center within the ferritin subunit is essential and sufficient for efficient and rapid iron uptake (Levi et al. 1988; Wade et al. 1991; Cozzi et al. 2000). A mutant of H-ferritin generated by mutating two residues within the ferroxidase center (Glu62 and His65) was capable of forming stable ferritin complexes but lacked detectable ferroxidase activity (Lawson et al. 1989). Furthermore, introduction of several glutamic acid residues necessary for the ferroxidase center into L-ferritin was sufficient to increase its iron incorporation capacity to similar levels as H-ferritin (Levi et al. 1994).

L-ferritin lacks enzymatic activity and thus does not contribute to iron oxidization and uptake. However, it has a higher number of carboxy groups lining the ferritin cavity which serve as iron nucleation sites (Wade et al. 1991; Levi et al. 1992). In vitro experiments with recombinant L-ferritin homopolymers showed that it is capable of mineralizing iron faster than H-ferritin homopolymers (Levi et al. 1992). Moreover, the L-ferritin monomer contains a salt bridge within its helical fold which constitutes greater stability on the ferritin complex in acidic and reducing conditions (Santambrogio et al. 1992).
II. Intracellular Functions

II.A. Iron Storage

Ferritin is the primary iron storage protein in cells. Its high iron capacity and stable structure make it ideal for the long-term storage of iron. The two subunits composing the ferritin complex show differential stability and storage capacity and thus contribute to the storage of iron differently. For example, L-ferritin homopolymers are more stable than H-ferritin homopolymers and are more resistant to denaturants (Levi et al. 1989; Santambrogio et al. 1992). They also have a higher capacity to induce iron-core formation due to the multiple carboxy groups exposed within the ferritin cavity (Levi et al. 1992). On the other hand, H-ferritin with its ferroxidase center is far more efficient in iron uptake than L-ferritin (Levi et al. 1992). Overexpression of H-ferritin in several cell types led to the rapid sequestration of the labile iron pool and in some cases induced an iron-deficient phenotype (Picard et al. 1996; Cozzi et al. 2000). These effects were not seen with L-ferritin or the ferroxidase-deficient H-ferritin mutant.

H and L-ferritin have complementary roles in iron storage. H-ferritin is responsible for iron uptake (Santambrogio et al. 1993), whereas L-ferritin contributes to complex stability and iron core formation (Levi et al. 1994). Therefore ferritin heteropolymers have been noted to be more stable and have a higher storage capacity than homopolymers (Levi et al. 1992; Cozzi et al. 2000; Kim et al. 2003). In human tissue, ferritin is almost always found as a heteropolymer complex. Because the ratio of H to L within a single ferritin complex has important implications on the storage capacity of ferritin, the two subunits show differential tissue distribution. Generally, organs with high
Iron turnover (i.e. heart and brain) have been noted to have higher levels of H-ferritin, whereas organs involved in iron storage (i.e. liver and spleen) have higher levels of L-ferritin (Arosio et al. 1978; Dedman et al. 1992). For example, ferritin complexes extracted from the spleen may have a H2L22 distribution, whereas the ferritin extracted from the heart may have equal H and L subunits.

Iron release from ferritin occurs through two main pathways: ubiquitin-dependent proteasomal degradation or lysosomal degradation. In some cell types, ferritin degradation and the subsequent iron release was blocked with lysosomal or proteasomal inhibitors indicating the existence of two independent pathways functioning in a cell-specific manner (Radisky and Kaplan 1998; Konijn et al. 1999; Truty et al. 2001). Moreover, the regulation of ferritin degradation seems to be context-dependent and may be connected to intracellular iron levels. For example, overexpression of the iron exporter ferroportin led to a decrease in ferritin expression through a proteasome-dependent pathway (De Domenico et al. 2006). The kinetics of iron release from both pathways is yet to be understood. Interestingly, biochemical studies have demonstrated that several environmental conditions, such as iron chelation and exposure to reducing conditions (i.e. thioglycolic acid) can cause iron release from ferritin without grossly affecting ferritin protein structure (Joo et al. 1990; Chasteen and Harrison 1999). This release occurs through ferritin’s hydrophobic channels and may be associated with structural changes in the residues lining the channels (Liu et al. 2007). Whether or not this form of release occurs in the cytoplasm under physiological cellular conditions is still an open question.

II.B. Protection from iron-induced damage
As a consequence of its ability to sequester iron, ferritin plays a key protective role against oxidative stress. Unbound intracellular iron is capable of generating reactive oxygen species (ROS) through Fenton chemistry causing lipid peroxidation, DNA breaks and other forms of cellular damage (Halliwell and Gutteridge 1984; Stohs and Bagchi 1995; Harrison and Arosio 1996). Several over-expression and deletion studies have demonstrated the effect of ferritin, specifically H-ferritin, on survival under conditions of oxidative stress. In *C. elegans*, deletion of a mammalian H-ferritin homolog reduced lifespan under conditions of iron overload (Kim et al. 2004). In HeLa cells, the overexpression of H-ferritin was protective of oxidative stress and hydrogen peroxide-induced cytotoxicity (Cozzi et al. 2000). The expression of H-ferritin was also speculated to be a possible mechanism of resistance to chemotherapeutic agents that induce oxidative stress (Cermak et al. 1993).

The role of ferritin as an anti-oxidant is also evident in its protection against TNFα- and TGF-β1-induced oxidative stress. Overexpression of H-ferritin, but not L-ferritin, protected HeLa cells from TNFα-induced apoptosis (Cozzi et al. 2003). Furthermore, NFκB-medicated up-regulation of H-ferritin protected cells from the accumulation of ROS caused by TNFα exposure (Kwak et al. 1995; Pham et al. 2004). Remarkably, expression of H-ferritin in NFκB-null cells was sufficient to block TNFα-induced apoptosis. H-ferritin was also shown to be a downstream target for TGF-β1 (Zhang et al. 2009). In this study, TGF-β1 was shown to transiently increase the labile iron pool and thus increase the generation of reactive oxygen species. The induction of H-ferritin and the subsequent sequestration of free iron protected cells from the TGF-β1-induced spike in ROS.
In mice, deletions of H-ferritin are embryonic lethal signifying its protective role in the vulnerable early stages of development (Ferreira et al. 2000). Mice heterozygous for the H-ferritin deletion lacked any apparent abnormalities but had elevated levels of L-ferritin in serum and tissue (Ferreira et al. 2001). This elevation in L-ferritin is unlikely to be reflective of a compensatory role for L-ferritin in iron storage and is probably a consequence of increased intercellular free iron levels caused by the decrease in the iron storage capacity of H subunit-deficient ferritin complexes. Furthermore, a closer look at the brains of heterozygous mice revealed an increase in oxidative stress and apoptosis, indicating a possible connection between iron accumulation in excess of ferritin in the brain and neuronal diseases (Thompson et al. 2003). Using mice carrying LoxP-flanked H-ferritin gene, Darshan et al. silenced H-ferritin expression to below 5% of basal levels in the liver, spleen, and bone marrow (Darshan et al. 2009). These mice displayed increased iron levels in the serum and increased hepcidin expression in the liver. More importantly, iron-rich diets induced severe liver damage in these mice providing strong evidence for the central role played by H-ferritin in the detoxification of iron.

In 2001, a new ferritin gene was characterized (Levi et al. 2001). This gene is highly similar to H-ferritin and is termed mitochondrial ferritin because its product is eventually targeted to the mitochondria. The precursor protein includes a mitochondrial leader sequence which is cleaved upon entry into the mitochondria, and the mature protein is capable of assembly into full ferritin complexes with intact ferroxidase sites (Corsi et al. 2002). Mitochondrial ferritin serves a specialized role in sequestering free iron in the mitochondria and thus reducing oxidative stress in this iron-rich organelle.
II.C. Proliferation

In addition to iron storage, ferritin also possesses iron-independent functions that impact proliferation. In HeLa cells, transfections with L-ferritin cDNA or siRNA did not alter iron levels but modulated cellular proliferation (Cozzi et al. 2004). L-ferritin over-expression increased proliferation by approximately 50%, while L-ferritin downregulation decreased proliferation by approximately 50% (Cozzi et al. 2004). These effects were not observed with H-ferritin. Similarly, down regulation of L-ferritin in LM melanoma cells decreased proliferation and increased apoptosis in vitro and in vivo (Baldi et al. 2005). The underlying mechanism for these observations are yet to be elucidated but is believed to be iron-independent because the alterations of cellular L-ferritin levels did not affect the labile iron pool.

II.D. Modulation of cellular signaling

Aside from its role as an antioxidant, H-ferritin may have the ability to bind elements involved in signal transduction. Recently, H-ferritin has been reported as a negative intracellular regulator for the chemokine receptor CXCR4 (Li et al. 2006). CXCR4 is a co-receptor for T-tropic HIV (Feng et al. 1996) and is expressed on a wide range of human malignancies (Balkwill 2004; Kucia et al. 2004; Ehtesham et al. 2006). When bound to CXCR4, CXCL12 causes the activation of several signaling cascades including: MAPK/ERK, Jak/STAT and AKT (Ganju et al. 1998). H-ferritin over-expression in HeLa cells led to the inhibition of CXCL12-mediated signaling while the knockdown of H-ferritin reversed these effects and prolonged the activation of downstream pathways (Li et al. 2006). Moreover, H-ferritin was shown to interact
directly with the cytoplasmic domain of CXCR4 hindering the formation of signaling complexes. This study also generated a novel insight into nuclear translocation of ferritin. Exposure to CXCL12 was associated with a time-dependent phosphorylation and nuclear translocation of H-ferritin (Li et al. 2006).

II.E. Ferritin as a transcription factor

Ferritin has been reported to be unevenly distributed within the nucleoplasm of some cells raising the possibility of specific interactions with nuclear components (Cai et al. 1997; Surguladze et al. 2005). Nuclear fractionation studies in the astrocytoma cell line SW1088 demonstrated that the bulk of ferritin in the nucleus is within the soluble nuclear fraction, whereas intermediate levels of ferritin are associated with the nuclear matrix (Surguladze et al. 2005). In corneal epithelial cells, nuclear ferritin was reported to be found throughout the nucleus except for the nucleolus (Cai et al. 1997).

Several studies have argued that nuclear ferritin can serve as a transcriptional factor regulating the expression of the β-globin gene (Wu and Noguchi 1991; Pountney et al. 1999; Broyles et al. 2001; Surguladze et al. 2004). Incubation of nuclear extracts from K562 cells and oligonucleotide fragments representing the promoter region of the β-globin gene led to the formation of DNA-protein complexes (Broyles et al. 2001). The formation of these complexes could be blocked with ferritin antibodies. A closer look at the distal promoter region of the β-globin gene identified a highly conserved binding motif for ferritin (Broyles et al. 2001). The activity of a reporter gene driven by the β-globin promoter was significantly repressed when co-transfected with H-ferritin in CV-1 cells. Mutating the ferritin binding sequence abolished this effect.
III. Regulation of ferritin expression

The importance of iron in biological systems and its detrimental effects on cells when present in excess has led to the evolution of multiple layers of regulation to control the expression of the ferritin genes. Several lines of evidence have argued that the regulation of ferritin occurs on multiple levels and in response to a variety of stimuli and cellular conditions.

III.A. Labile iron pool

Probably the most prominent and best understood mode of ferritin regulation occurs at the mRNA level in response to the labile iron pool through the activity of iron regulatory proteins (IRPs). Zahringer et al. was the first to recognize that the increase in ferritin expression in the liver of iron-fed rats was not due to increased transcription of the ferritin gene, but increased association of the ferritin mRNA with ribosomes (Zahringer et al. 1976). In a hepatoma cell culture model, both H and L-ferritin protein levels increased in response to iron-loading and decreased with iron chelation indicating that chelatable cytoplasmic iron controls the expression of the ferritin proteins (Rogers and Munro 1987). Deletion of 157-nucleotide internal region in the 5’ untranslated region of the H-ferritin cDNA rendered ferritin insensitive to the addition or removal of iron (Hentze et al. 1987). Moreover, the cloning of this sequence into the cDNA of a reporter gene was sufficient to transfer iron-mediated regulation (Hentze et al. 1987). This regulatory cis-acting element in the ferritin mRNA is termed iron responsive element...

There are currently two proteins capable of binding to IREs – iron regulator protein 1 and 2 (IRP1 and IRP2). Both proteins have high affinity to the 5’ untranslated regions of ferritin and can repress translation in an iron-dependent manner (Leibold and Munro 1988; Rouault et al. 1988; Walden et al. 1988; Rouault et al. 1990). IRP1 contains an iron-sulfur cluster capable of reversible binding to iron (Rouault et al. 1992). When iron is depleted, IRP1 can bind to the IRE and suppress ferritin translation. But when iron is bound to the iron-sulfur cluster, IRP1 loses its mRNA binding capacity and acquires aconitase function (Rouault et al. 1992). On the other hand, IRP2 is degraded under iron-rich conditions through ubiquitination (Iwai et al. 1998). Although IRP1 and IRP2 have similar binding and functional kinetics (Kim et al. 1995), their expression ratios differ in various tissues indicating tissue-specific roles. For example, IRP2 knockout mice grew to adulthood without any observed alterations in their iron metabolism proteins, but displayed deregulated iron acquisition and storage in the intestinal mucosa and central nervous systems during adulthood (LaVaute et al. 2001).

In addition to ‘Iron-sensing’, protein modification can also regulate the function of the IRE-IRP system. In the leukemia cell line HL-60, treatment with the protein kinase K (PKC) activator phorbol 12-myristate 13-acetate (PMA) increased IRP1 and IRP2 phosphorylation and increased their RNA binding capacity (Eisenstein et al. 1993; Schalinske and Eisenstein 1996). Subsequently, PMA treatment decreased H and L-ferritin and increased transferrin receptor expression (Schalinske and Eisenstein 1996). In thyroid cells, stimulation with thyrotropin-releasing hormone or epidermal growth factor
led to increased phosphorylation of IRP1 and IRP2 via MAPK- and PKC-dependent pathways (Thomson et al. 2000). In several leukemia cell lines, exposure to erythropoietin, an activator of MAPK, increased the RNA binding capacity of IRPs and repressed ferritin expression (Weiss et al. 1997).

### III.B. Inflammatory cytokines

Another prominent mode of ferritin regulation occurs in response to inflammatory cytokines. Both TNFα and IL-1β increased H-ferritin mRNA levels in human and mouse cells (Torti et al. 1988; Rogers et al. 1990; Wei et al. 1990; Smirnov et al. 1999). In muscle cells, the effects of TNFα and IL-1β were additive and were seemingly independent (Wei et al. 1990). Interestingly, IL-1β also increased the expression of L-ferritin on a translational level by increasing the association of L-ferritin mRNA with ribosomes in human hepatoma cells (Rogers et al. 1990). Although this effect was blocked by iron chelation, it is still unclear if it was dependent on the increase in H-ferritin transcription and the possible saturation of the IRP-IRE system (Rogers et al. 1990). A similar observation in alveolar epithelial cells showed a fast induction of H-ferritin expression followed by an iron-dependent increase in both H and L-ferritin levels in response to inflammatory cytokines (Smirnov et al. 1999). Pinero et al demonstrated that the labile iron pool increases within 24 hours after exposure to inflammatory cytokines and that this correlates with increased aconitase activity for IRP1 indicating decreased mRNA binding (Pinero et al. 2000). Therefore, inflammatory cytokines can regulate the expression of ferritin on two levels: a transcriptional level (mainly H-ferritin) and a translational level (both H and L-ferritin).
The induction of H-ferritin transcription by TNFα and possibly IL-1β suggests that the ferritin expression is induced by transcription factors associated with inflammation. Deletion mapping of the H-ferritin promoter regions identified a cis-acting region 4.8kb upstream of the transcription start site (Kwak et al. 1995). This region contained two binding sites for members of the NFκB transcription factor family.

III.C. Hormonal regulation

Ferritin expression is also under hormonal regulation. The thyroid-stimulating hormone (TSH or thyrotropin) stimulates H-ferritin transcription in rat thyroid cells (Cox et al. 1988; Ursini and de Franciscis 1988). Thyrotropin plays an important role in thyroid development and functional activation by stimulating the transcription of genes involved in thyroid hormone synthesis (Van Heuverswyn et al. 1984; Gerard et al. 1988) and thyroid cell proliferation through the activation of c-myc (Dere et al. 1985) and c-ras (Dere et al. 1986). Many of the functions of thyrotropin are mediated by the second messenger cyclic adenosine monophosphate (cAMP). The treatment of thyroid cells with cAMP increased H-ferritin transcription to the same levels as thyrotropin (Chazenbalk et al. 1990; Chazenbalk et al. 1990). Moreover, the pancreatic hormone insulin and its homologous protein insulin-like growth factor 1 (IGF-1) stimulated the transcription of both H and L-ferritin in a dose-dependent manner (Chazenbalk et al. 1990; Yokomori et al. 1991). IGF-1 was approximately 10 times more potent than insulin, and its Kd was 10 times lower (Yokomori et al. 1991).

III.D. Oxidative stress
Cells respond to oxidative damage by increasing the transcription of genes that play a role in anti-oxidative protection. Due to its ferroxidase activity and iron storage capacity, ferritin may have beneficial roles during oxidative insult. Exposure to various oxidants increased the levels of H and L-ferritin mRNA in vitro (Cairo et al. 1995; Kennedy et al. 1997; Wasserman and Fahl 1997; Tsuji et al. 2000). Furthermore, chemical induction of oxidative stress in rat livers using phorones caused an elevation in H and L-ferritin mRNA (Cairo et al. 1995). A 75-bp segment located 4.1kb upstream of the transcription initiation site was identified as an electrophile response element (EpRE) and was shown to be essential for the ROS-induced ferritin transcription (Tsuji et al. 2000). In addition to the induction of ferritin transcription, oxidative stress modifies ferritin expression on a translational level through its effects on IRPs. Therefore, oxidative stress affects ferritin expression on both transcriptional and translational levels allowing cells to respond quickly to oxidative insult and thus minimizing ROS-induced cellular damage.

IV. Serum ferritin

In addition to its intracellular roles, ferritin is also an abundant protein in circulation. This form of ferritin, termed serum ferritin, was first detected in 1948 in animals experiencing hepatic cirrhosis or shock (Mazur and Shorr 1948). This original observation was later confirmed in humans with various forms of liver disease (Reissmann and Dietrich 1956). Serum ferritin showed similar immunologic reactivity, molecular size and isoelectrical focusing characteristics as ferritin extracted from the liver or spleen (Worwood et al. 1976; Arosio et al. 1977; Hazard et al. 1977;
Santambrogio et al. 1987). Furthermore, serum ferritin was surprisingly iron poor with approximately 4-20% of the iron content of liver or spleen ferritin (Worwood et al. 1976; Arosio et al. 1977). This relatively low iron content persisted even in patients with iron overload (Arosio et al. 1977). Based on its reactivity to antibodies raised specifically against L-ferritin and its ability to bind to concanavalin A, serum ferritin is believed to be composed primarily of a glycosylated form of L-ferritin (Santambrogio et al. 1987).

IV.A. Source of serum ferritin

The source of serum ferritin is still unclear. Several lines of evidence have demonstrated that hepatocytes, macrophages and microglia are capable of ferritin secretion in vitro (Tran et al. 1997; Ghosh et al. 2004; Zhang et al. 2006; De Domenico et al. 2011). This secretion was reflective of their intracellular iron levels and was responsive to iron loading and chelation (Tran et al. 1997; Zhang et al. 2006; De Domenico et al. 2011). Ferritin secretion was also induced by the inflammatory cytokines TNF-α and IL-1β through increased transcription of the ferritin genes in rat hepatoma cells (Tran et al. 1997). Although the secreted ferritin in those experiments contained both the H and L subunits, their ratios varied greatly between animal strains and cell types (Ghosh et al. 2004; Zhang et al. 2006; De Domenico et al. 2011).

Although different cell types are capable of ferritin release in vitro, serum ferritin seems to be primarily derived from macrophages in vivo (Ferring-Appel et al. 2009; Cohen et al. 2010). The macrophage-specific ablation of the iron response protein 2 (IRP2) – a negative regulator of ferritin expression – increased serum ferritin levels, whereas hepatocyte or intestinal epithelial-specific ablation did not affect serum ferritin
levels in mice (Ferring-Appel et al. 2009). Moreover, the size and immunological reactivity of serum ferritin in mice was similar to the ferritin found in organs with major macrophage populations such as the bone marrow and spleen (Cohen et al. 2010). Consistently, serum ferritin levels fell by 75% after splenectomy compared with sham operated mice (Cohen et al. 2010). Recently, studies utilizing a mouse model with macrophage-specific deletion in the iron exporter ferroportin showed a robust increase in serum ferritin levels (Zhang et al. 2011). Overall, the existing literature suggests that multiple cells are capable of ferritin secretion in response to various stimuli. However, macrophages seem to represent a primary systemic source for serum ferritin.

The existing literature on ferritin’s secretory pathway is conflicted, as evidence exists to suggest both classical and non-classical secretion. For example, a truncated and unglycosylated ferritin similar to ferritin found within lysosomes was detected in mouse serum and splenic macrophages suggesting macrophage-specific release of lysosomal ferritin (Cohen et al. 2010). In other studies, the secretion of ferritin by hepatocytes and macrophages was inhibited by brefildin A (BFA) which is a potent blocker of protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Ghosh et al. 2004; De Domenico et al. 2011). This study also demonstrated that the entry of the ferritin monomers into the ER system occurs under the relative absence of free cytosolic iron and that iron can induce ferritin cellular retention (De Domenico et al. 2011).

IV.B. Ferritin membrane receptors

Many normal and malignant cell lines specifically bind ferritin (Bretscher and Thomson 1983; Fargion et al. 1988; Fargion et al. 1991; Moss et al. 1994; Gelvan et al.
1996; Hulet et al. 2000). Moreover, studies with mouse brain membrane homogenates have also showed specific binding of ferritin in white matter tracts (Hulet et al. 1999; Hulet et al. 1999). Most of those studies focused on H-ferritin and demonstrated that it is internalized through receptor-mediated endocytosis with a binding association constant ranging from 5 to 30 nM (Mack et al. 1983; Fargion et al. 1988; Moss et al. 1994; Hulet et al. 2000).

The first ferritin receptor to be identified was the murine T cell immunoglobulin and mucin domains 2 (TIM-2) (Chen et al. 2005). TIM-2 shows specific affinity to H-ferritin but not L-ferritin and is expressed on splenic B cell, renal tubule cells, liver cells, and oligodendrocytes (Chen et al. 2005; Todorich et al. 2008). Moreover, it is capable of internalizing ferritin into endosomes and lysosomes (Moss et al. 1994; Chen et al. 2005), and its protein levels are responsive to iron loading and chelation (Todorich et al. 2008). TIM-2 does not have a human ortholog, and thus the question of the human H-ferritin receptor is still unresolved. Recently, Li et al. have argued that transferrin receptor 1 (TfR1) is responsible for most of the H-ferritin binding in human lymphocytes (Li et al. 2010). Using cell line and cDNA library screens, Li et al. identified TfR1 as a differentially expressed membrane protein in the cells capable of H-ferritin binding. However, whether or not transferrin receptor can account for the immunosuppressive effects observed with H-ferritin in lymphocyte cultures is yet to be determined (Fargion et al. 1991). Intriguingly, the extracellular domain of chemokine receptor CXCR4 seemed to directly interact with H-ferritin (Li et al. 2006). Further exploration of CXCR4 as a human H-ferritin receptor is warranted.
Recently, scavenger receptor class A member 5 (Scara5) was identified as the L-ferritin receptor (Li et al. 2009). Scara5 shows sequence and structural homology to class A scavenger receptors, but unlike other class A receptors it is unable to endocytose acetylated or oxidized low density lipoprotein (Jiang et al. 2006). Earlier expression profiling of Scara5 in mice has demonstrated that its expression is restricted to populations of epithelial cells primarily in the lung, trachea, testis, and bladder (Jiang et al. 2006). Scara5 was expressed in the murine developing kidney and was capable of delivering iron through ferritin endocytosis and thus replacing transferrin receptor as the major iron delivery pathway.

IV.C. Functions of extracellular ferritin

IV.C.1. Iron delivery

The existence of ferritin as a non-transferrin iron delivery system has been convincingly demonstrated by several groups. Injection of $^{59}$Fe-loaded H or L-ferritin into adult mice led to differential uptake by some organs. Radioactivity was detected in the liver, spleen, kidney, and lung (Fisher et al. 2007). More $^{59}$Fe was detected in the spleen, lung, and brain when loaded into H-ferritin than L-ferritin (Fisher et al. 2007). Moreover, mice embryos with total transferrin receptor deletions can still initiate organogenesis – a process that requires cellular iron accumulation- via ferritin endocytosis (Levy et al. 1999; Li et al. 2009).

Oligodendrocytes and myelination:

White matter tracts do not bind transferrin in rodents or humans and do not express transferrin receptor mRNA (Hulet et al. 1999; Han et al. 2003). However,
oligodendrocytes are the principle cell type that stains for iron in the brain (Dwork et al. 1988; Benkovic and Connor 1993). Todorich et al. have demonstrated that adding H-ferritin to oligodendrocyte progenitor cells cultured in transferrin-deprived chemically defined media can rescue cells and increase intracellular iron (Todorich et al. 2011). H-ferritin binding to oligodendrocytes is mediated by TIM-2 which, similar to transferrin receptor, is responsive to iron loading and chelation (Todorich et al. 2008). H-ferritin also increased expression of myelin-basic protein implicating the disruption of the TIM-2/ferritin system in demyelination diseases (Todorich et al. 2011). In a spinal cord mouse model, oligodendrocyte progenitor cells were able to uptake macrophage-derived ferritin suggesting that macrophages may serve as a local source of iron for oligodendrocytes during wound-healing and development (Schonberg et al. 2012).

Erythrocyte progenitor cells and erythropoiesis:

Ferritin can also deliver iron to erythrocyte progenitor cells for heme synthesis. Incubation of in vitro-differentiated erythrocyte progenitor cells with H-ferritin led to an increase in labile iron pool as indicated by decreased transferrin receptor expression, increased IRP activity and a decrease in the signal of the iron-sensitive tracer calcein AM (Gelvan et al. 1996; Meyron-Holtz et al. 1999; Leimberg et al. 2008). Moreover, the addition of $^{125}$I-tagged ferritin loaded with $^{59}$Fe to erythrocytes showed ferritin degradation and increased $^{59}$Fe incorporation into hemoglobin. Consistently, extracellular ferritin increased the proliferation of erythrocyte progenitor cells and their production of hemoglobin under transferrin-free conditions (Leimberg et al. 2003). These studies indicate that extracellular ferritin may serve as an iron delivery mechanism to ensure that sufficient iron is available for erythropoiesis.
Kidney Development:

Although mouse embryos with transferrin receptor deletions die at early embryonic stages, they can still initiate organogenesis of some organs such as the kidney (Levy et al. 1999; Li et al. 2009). Li et al. have demonstrated that the transferrin system is not essential for iron delivery in the capsule and stroma of the kidney (Li et al. 2009). Incubation of renal cells with L-ferritin-rich complexes showed increased proliferation with holo-ferritin (iron-rich) but not apo-ferritin (iron-deprived) indicating the ability of ferritin to deliver iron. Using gene expression arrays, the group identified Scara5 as the most up-regulated gene in kidney cells with transferrin receptor deletions (Li et al. 2009). Scara5 expression in Trvb cells, which do not express transferrin receptor (McGraw et al. 1987), was sufficient to bind and internalize L-ferritin. Moreover, L-ferritin exposure in these cells increased intracellular iron levels and rescued cells under serum-free conditions (Li et al. 2009).

IV.C.2. Immune-suppression

Extracellular ferritin exerts immunosuppressive effects on lymphocytes and myeloid-derived cells. These effects were first demonstrated in 1979 by Matzner et al. who showed that the addition of ferritin to lymphocyte cultures suppressed the mitogenic effects of phytohaemagglutinin (PHA) and concanavalin A (Con A) (Matzner et al. 1979). Using recombinant H and L-ferritin, Fargion et al. demonstrated saturable specific binding for H-ferritin but not L-ferritin on human peripheral lymphocytes (Fargion et al. 1991). The binding of H-ferritin increased markedly in PHA-stimulated lymphocytes and its presence in culture suppressed PHA-induced proliferation (Fargion et al. 1991).
Furthermore, immuno-screening of a cDNA library from the MM200 melanoma cell line identified H-ferritin as a secreted factor with immunosuppressive properties (Gray et al. 2001). Conditioned media from MM200 melanoma cells was able to suppress the proliferative responses to anti-CD3 antibodies in human lymphocytes (Gray et al. 2001). The effects of the conditioned media were replicated by recombinant H-ferritin but not L-ferritin. Interestingly, the addition of increasing amounts of L-ferritin with H-ferritin decreased the magnitude of the inhibition suggesting that the immunosuppressive effects are dependent on the monomer form of H-ferritin (Gray et al. 2001). Furthermore, the suppressive effects of H-ferritin were decreased by approximately 60% when incubated with anti-IL-10 antibodies indicating that the H-ferritin effect is partly mediated by increased IL-10 production (Gray et al. 2001). IL-10 is an immunosuppressive agent that inhibits the production of IL-2 and can downregulate class II MHCs in monocytes and thus reducing the activation of T cells (de Waal Malefyt et al. 1991; Itoh et al. 1994). Furthermore, H-ferritin is elevated in the sera of melanoma patients, and this elevation is associated with increased numbers of circulating CD4+ CD25+ regulator T cells (Gray et al. 2003). However, this small study did not observe a correlation between the elevation in H-ferritin and disease stage raising questions about the pathological significance of tumor-induced immunosuppression in melanoma patients. Overall, these studies indicate that cancer cells can secrete ferritin and this secretion might have direct immunosuppressive effects on lymphocytes.

H-ferritin has suppressive effects on myelopoiesis as well (Broxmeyer et al. 1981; Broxmeyer et al. 1989). Injecting into mice recombinant H-ferritin led to a decrease in hematopoietic progenitor cells in the bone marrow and spleen in vivo. The same group
also generated several H-ferritin mutants to gain more insight into its binding sites and mechanism of action. Interestingly, only one mutant lacked the immunosuppressive activity (Broxmeyer et al. 1991). This mutant had an inactive ferroxidase center and thus was incapable of efficient iron incorporation (Lawson et al. 1989). When colony-forming unit granulocyte-macrophage (CFU-GM) cultures were incubated with excess hemin as an iron source, the ferritin-mediated suppression on colony formation was blocked (Broxmeyer et al. 1991). Therefore, the immunosuppressive effects of ferritin on myelopoiesis seem to be iron-dependent and are specific to H-ferritin with an active ferroxidase center.

IV.C.3. Angiogenesis

Angiogenesis is a tightly-regulated process that requires the cooperation of many factors to ensure sufficient, but not excessive, vascularisation of new organs or damaged tissue. High molecular weight kininogen (HK) is a plasma glycoprotein that plays a role in the intrinsic coagulation pathway (Schiffman et al. 1980; Tait and Fujikawa 1987). It functions as a cysteine protease inhibitor and is a precursor to the vasodilator nanopeptide bradykinin (Henriques et al. 1969; Kerbiriou and Griffin 1979). Ferritin can bind HK and reduce its proteolytic cleavage and subsequent production of bradykinin (Torti and Torti 1998; Parthasarathy et al. 2002; Coffman et al. 2008). Ferritin can also bind the other HK cleavage product - the anti-angiogenic 2-chain high molecular weight kininogen (HKa) (Coffman et al. 2009). The binding of HKa to ferritin occurs at a Kd approximately 10 times lower than to HK indicating that the binding of ferritin to HKa may be more physiologically significant (Coffman et al. 2008; Coffman et al. 2009). The incubation of
endothelial cells with HKa and L subunit-rich ferritin protected them from HKa-induced apoptosis and allowed them to migrate and form vessel structures (Coffman et al. 2009). This protective effect was not dependent on the iron content of ferritin. Furthermore, deletion mapping of HKa revealed that ferritin specifically binds domain 5 in HKa which is necessary for its anti-angiogenic functionality (Coffman et al. 2009). In a tumor mouse model, the subcutaneous injection of a mixture of prostate cancer cells with HKa and ferritin induced normal vessel formation within the tumor. The protective effects of ferritin on endothelial cells are due to the restoration of survival and adhesion signaling, such AKT, ERK1/2, FAK and paxillin, which are blocked by the binding of HKa to UPAR (Tesfay et al. 2012). Interestingly, Ferritin, HKa and the macrophage marker CD68 seem to co-localize at sites of inflammation in mice challenged with chicken ovalbumin (Coffman et al. 2008). Thus, although ferritin on its own does not have a direct effect on signaling, its localized secretion by macrophages may indirectly enhance survival signaling in endothelial cells.

**IV.C.4. Ferritin as a growth factor**

A research team from the University of Tokyo has purified a growth factor from two different human cancer cell lines with immunological identity to ferritin (Kikyo et al. 1994; Kikyo et al. 1994). This growth factor stimulated the proliferation of the human leukemia cell line HL-60 and the melanoma cell line SK-28. The purified factor consisted of two fragments. The smaller band was reactive to L-ferritin antibodies, while the larger band showed sequence homology to H-ferritin. Antibodies against L-ferritin or H-ferritin were both effective in neutralizing the proliferative effects. However, it is still unclear
whether or not both subunits are required for the proliferative effects and what the underlying mechanism is.

**IV.C.5. Ferritin as a cytokine**

The increase in serum ferritin during inflammatory conditions has led Ruddell et al. to speculate a role for ferritin as a pro-inflammatory cytokine (Ruddell et al. 2009). This group has demonstrated that the addition of L subunit-rich ferritin complexes to primary rat hepatic stellate cells led to a time- and concentration-dependent increase in the phosphorylation of IKK α/β and the subsequent activation of NFκB transcription factor. Ferritin increased the expression of several NFκB-response genes including the pro-inflammatory cytokine IL-1β. The effects of ferritin were independent of iron as iron-rich and iron-depleted ferritins had similar effects. Although the ferritin receptor on hepatic cells is still unknown, it is believed to be capable of interacting with and activating phosphoinositide 3-kinase (PI3K) (Ruddell et al. 2009).

**IV.D  Clinical value of serum ferritin**

Serum ferritin is elevated during chronic and acute inflammation (Konijn and Hershko 1977; Feelders et al. 1998; Kalantar-Zadeh et al. 2004). Its rise correlates with the rise in other acute phase proteins such as C-reactive protein (CRP) and α1-acid glycoprotein (AGP) (Feelders et al. 1998; Beard et al. 2006). Consistently, chronic use of aspirin lowers serum ferritin and other parameters of inflammation in patients with various inflammatory diseases (Fleming et al. 2001). In cancer patients treated with TNFα and interferon gamma (IFN-γ), serum ferritin and other acute phase proteins
peaked 48 hours post perfusion but showed differential half-lives with the increase in serum ferritin persisting for 10 days post-transfusion (Feelders et al. 1998).

Serum ferritin is also a reliable indicator of the body’s iron stores (Addison et al. 1972; Jacobs et al. 1972; Walters et al. 1973). Its levels are significantly lower in individuals suffering from iron-deficiency anemia or undergoing phlebotomy (Jacobs et al. 1972; Walters et al. 1973). In contrast, serum ferritin levels are higher in patients with iron-overload disease and hemochromatosis (Jacobs et al. 1972; Allen et al. 2008). Generally, women tend to have lower levels of serum ferritin than men (Jacobs et al. 1972; Walters et al. 1973; O'Meara et al. 2011), possibly due to loss of hemoglobin during menstruation (Penckofer and Schwertz 2000).

Serum ferritin values in healthy individuals show some variability (O'Meara et al. 2011). In a study examining 620 healthy postmenopausal women, serum ferritin correlated positively with age, body mass index, iron supplement, and heme-iron intakes, but was inversely correlated with physical activity and aspirin use (Liu et al. 2003). Another study examining 1332 Danish men demonstrated that serum ferritin is positively correlated with body mass index, but not with the use of dietary iron supplement (Milman et al. 1999). Overall, the variability in serum ferritin observed in healthy individuals falls within the parameters of the established normal range.

V. Ferritin in tumor tissue

Ferritin is differentially over-expressed in tissues from multiple malignancies, including: hepatocellular carcinoma (Niitsu et al. 1975; Kew et al. 1978), Hodgkin’s lymphoma (Eshhar et al. 1974), breast (Marcus and Zinberg 1974; Weinstein et al. 1982;
Weinstein et al. 1989; Guner et al. 1992), and pancreatic cancer (Marcus and Zinberg 1974). Structural, immunological, and isoelectric analyses demonstrated that tumor ferritins differ in their subunit composition and are most likely composed of different ratios of the L and H subunits (Arosio et al. 1976). In breast tumors, the increase was specific to L-ferritin and was approximately six-fold higher compared to benign breast tissue (Weinstein et al. 1982; Weinstein et al. 1989; Jezequel et al. 2012). This increase was correlated with greater epithelial proliferation, histopathological dedifferentiation and mortality. Histological examination of ferritin distribution showed weak staining in ductal cells within benign breast tissue, moderate staining in breast cancer cells, and strong staining within the tumor stroma (Rossiello et al. 1984; Jezequel et al. 2012). It is still unclear what role this increase in ferritin has in tumor biology. It is important to note that breast cancer cells display an iron-deficient phenotype with increased expression of the iron importer transferrin receptor (Hogemann-Savellano et al. 2003) and decreased expression of the iron exporter ferroportin (Pinnix et al. 2010). These two observations suggest the increase in tumor ferritin might not be due to increased iron deposits within cancer cells.

VI.

Serum ferritin and cancer

VI.A. Serum ferritin and cancer risk

Serum ferritin is associated with a higher risk for some cancers. For example, an increase in serum ferritin but a decrease in transferrin was associated with a three-fold increase in hepatocellular carcinoma incidence and mortality in a Taiwanese cohort (Stevens et al. 1986). A similar trend in serum ferritin and transferrin levels was
associated with overall cancer incidence in women, but not men, participating in a large French study (Hercberg et al. 2005). Interestingly, increased dietary iron intake was not associated with increased cancer risk raising the possibility that the increase in serum ferritin is not due to increased iron stores (Hercberg et al. 2005). Moreover, consistent elevation in serum ferritin in patients with chronic liver disease is associated with a higher risk for the development of hepatocellular carcinoma than patients whose levels normalize after diagnosis (Hann et al. 1989). Conversely, the decrease in serum ferritin by phlebotomy in patients with peripheral arterial disease was associated with decreased cancer incidence and mortality (Zacharski et al. 2008). However, there was little evidence to suggest that serum ferritin is associated with a higher risk of colorectal cancer or recurrence of colorectal adenomas (Tseng et al. 2000; Chan et al. 2005; Cross et al. 2006), despite strong evidence suggesting that increased iron stores (as measured by iron parameters that excluded serum ferritin) is associated with a higher risk of colorectal cancer (Knekt et al. 1994).

Germline mutations in the high iron gene (HFE) are associated with increased overall iron stores (Jackson et al. 2001; Allen et al. 2008). There are two common loss-of-function mutations in HFE that can cause iron overload: a cystine to tyrosine substitution at residue 282 (C282Y), and a histidine to aspartic acid substitution at residue 63 (H63D) (Feder et al. 1996; Jackson et al. 2001; Allen et al. 2008). C282Y, but not H63D, homozygosity is associated with increased risk for breast cancer in women and colorectal cancer in men (Osborne et al. 2010). The C282Y allele is also more prevalent in breast cancer patients than healthy individuals (Kallianpur et al. 2004). Interestingly, individuals homozygous for the C282Y mutation have higher levels of serum ferritin than
patients with wild type or H63D alleles (Allen et al. 2008). It is unclear if the cancer risk associated with the C282Y polymorphism is connected to its effects on serum ferritin levels.

VI.B. Serum ferritin in breast cancer

The elevation in ferritin within breast tumor tissue encouraged further exploration of its levels in serum. The earlier studies utilized a radioimmunoassay for ferritin with antibodies that specifically recognized L-ferritin extracted from the human liver (Marcus and Zinberg 1975). When compared to the sera of 117 healthy women, serum ferritin was elevated in 40% of breast cancer patients before undergoing surgical resection, and in 67% of patients with metastatic breast disease (Marcus and Zinberg 1975). These initial findings were confirmed by a more focused study measuring serum ferritin in 250 healthy women and 229 women with early breast cancer (Stages I and II). Serum ferritin levels were significantly elevated in the cancer group, and the values observed in the top 10% of patients were predictive of tumor recurrence in the 5 years following diagnosis (Jacobs et al. 1976). This increase was also demonstrated to be primarily in L-ferritin (Jones et al. 1980). Nonetheless, H-ferritin was also detected, albeit in small quantities in contradiction to a previous report (Hazard and Drysdale 1977), in the sera of approximately 50% of advanced breast cancer patients compared to only 7% in healthy controls (Jones et al. 1980).

Although all of the studies examining serum ferritin in breast cancer patients showed statistically-significant increases in patients with both localized and metastatic diseases (Marcus and Zinberg 1975; Jacobs et al. 1976; Hazard and Drysdale 1977;
Tappin et al. 1979; Jones et al. 1980), one study showed an association between serum ferritin levels and metastatic breast cancer but not localized disease (Robertson et al. 1991). The elevation in serum ferritin seemed to be more pronounced in patients with metastases to the viscera (Robertson et al. 1991), more specifically the liver (Bezwoda et al. 1981), but was the lowest in patients with lung metastases (Williams et al. 1990). These reports, however, did not demonstrate a causative link and only speculated that liver metastases might be causing liver damage and thus ferritin release into circulation. This possibility has been rejected by studies that demonstrated that cancer-associated elevation in serum ferritin does not correlate with serum biomarkers of liver damage (Kew et al. 1978; Hann et al. 1980).

**VI.C. Serum ferritin in other cancers**

Serum ferritin is also elevated in patients with Hodgkin’s lymphoma (Bieber and Bieber 1973; Jacobs et al. 1976; Hann et al. 1990), hepatocellular carcinoma (Niitsu et al. 1975; Kew et al. 1978; Melia et al. 1983), neuroblastoma (Hann et al. 1980; Hann et al. 1985), glioblastoma (Sato et al. 1998), squamous cell carcinoma of the head and neck (Maxim and Veltri 1986), renal cell carcinoma (Partin et al. 1995), melanoma (Luger et al. 1983; Gray et al. 2003), non-small-cell lung cancer (Kukulj et al. 2010), and pancreatic cancer (Nitti et al. 1982; Plebani et al. 1988; Basso et al. 1991; Kalousova et al. 2012). This increase is often associated with more progressive disease. For example, the elevation in serum ferritin was more pronounced in patients with Stage III and IV tumors in neuroblastoma (Hann et al. 1985), head and neck carcinoma (Maxim and Veltri 1986), Hodgkin lymphoma (Jacobs et al. 1976) and melanoma (Luger et al. 1983). Serum
ferritin was also predictive of survival in neuroblastoma and pancreatic cancer patients (Hann et al. 1985; Kalousova et al. 2012). Moreover, serial measurement of serum ferritin in cancer patients receiving chemotherapy indicated that a return to normal level is associated with therapy response (Niitsu et al. 1975; Melia et al. 1983; Maxim and Veltri 1986).

A majority of those studies utilized antibodies capable of recognizing both H and L-ferritin and thus it is difficult to specify the identity of serum ferritin in cancer patients. However, several studies indicated that the increase can occur in either H or L-ferritin. For example, the increase in breast cancer patients sera is primarily in L-ferritin (Jones et al. 1980), whereas melanoma patients showed an increase exclusively in H-ferritin (Gray et al. 2003). These variations may represent molecular differences between malignancies and may be due to secretion by distinct sources.

VI.D. **Source of the cancer-associated elevation in serum ferritin**

The cancer-associated elevation in serum ferritin is most likely induced by an inflammatory state and is not due to liver damage or to alterations in the body’s iron stores. For example, the increase in serum ferritin observed in patients with Hodgkin’s disease was inversely correlated with serum iron, transferrin, transferrin saturation levels, and hemoglobin (Jacobs et al. 1976; Hann et al. 1990), but was positively correlated with a higher erythrocyte sedimentation rate (ESR) – a marker of inflammation (Hann et al. 1990). Moreover, serum ferritin levels in hepatocellular carcinoma and neuroblastoma patients did not correlate with the hepatic marker transaminase indicating that the rise in serum ferritin is not caused by liver damage (Kew et al. 1978; Hann et al. 1980). The iron
content of serum ferritin is below normal range in approximately 90% of non-small-cell lung cancer patients (Kukulj et al. 2010). The iron content of ferritin shows positive correlation with other iron parameters in cancer patients but negative correlations with serum ferritin levels and ESR (Kukulj et al. 2010). Overall, these findings support a role for inflammation-induced production of ferritin while arguing strongly against the involvement of body iron or liver damage.

The elevation in serum ferritin is partly due to localized release within the site of the tumor. In patients with elevated serum ferritin, surgical resection of tumors lowered serum ferritin levels by approximately 50% implicating a relationship between the tumor mass and the elevation in serum ferritin (Tappin et al. 1979). Moreover, analysis of intraductal fluid in breast cancer patients revealed a five-fold elevation in ferritin levels compared to healthy controls suggesting localized ferritin release within the breast tumor microenvironment (Mannello et al. 2010; Mannello et al. 2011). In patients with renal tumors, the concentration of serum ferritin measured from the renal vein was higher than the levels in inferior vena cava or the pre-operative systemic levels (Partin et al. 1995). Most convincingly, cerebrospinal fluid (CSF) ferritin increased approximately ten-fold in patients with glioblastoma compared to patients without neurological abnormalities or enteroviral meningitis (Sato et al. 1998). Although CSF contains little ferritin under normal conditions, almost 70% of the glioblastoma patients had CSF ferritin levels exceeding those in serum providing strong support for local synthesis and release (Sato et al. 1998).

Cancer cells from different malignancies have been reported to secrete ferritin or ferritin-like molecules in vitro (Sarcione et al. 1977; Hann et al. 1980; Kikyo et al. 1994;
Moreover, transplantation of human neuroblastoma or hepatocellular carcinoma cell lines into nude mice led to the detection of circulating human ferritin in the majority of tumor-bearing animals (Hann et al. 1984). All of the animals showed an increase in circulating levels of L-ferritin but few showed detectable levels of H-ferritin. However, it is still unclear whether or not tumor secretion alone could account for the high levels of serum ferritin seen in many cancer patients.

VII. The breast tumor microenvironment

It is now recognized that tumor cells do not grow in isolation and instead require a plethora of host cells to support their growth and invasion. Although some cell types, especially CD8 T cells, have anti-tumor capabilities and are a marker of favorable prognosis (DeNardo et al. 2011), many of the cell types within the tumor microenvironment are ‘re-programmed’ to contribute to tumor growth and spread. Remarkably, the activation of oncogenes in several in vitro and in vivo experimental models was not sufficient for transformation but required manipulations of the tumor microenvironment and tissue structure (Sieweke et al. 1990; Partanen et al. 2007; Daniluk et al. 2012). This section will address the current understanding of the tumor-promoting components of the tumor-microenvironment.

VII.A. Fibroblasts

Fibroblasts are mesenchymal cells that constitute the majority of the stroma of every organ. During development and organogenesis they play a key role in shaping organs through communicating with parenchymal cells and controlling their growth
In adulthood, they play a role in organ stability and repair through the deposition of extracellular matrix proteins (Kalluri and Zeisberg 2006; Egeblad et al. 2010). In tumors, the production of TGF-β by cancer cells or other infiltrating cells, such as macrophages, can activate and re-program fibroblasts (Elenbaas and Weinberg 2001). Cancer-associated fibroblasts (CAFs) contribute to tumor proliferation, angiogenesis, invasion and therapy resistance. The subcutaneous injection of breast cancer cells mixed with primary fibroblasts isolated from invasive breast tumors enhanced tumorigenesis, whereas normal fibroblasts or fibroblasts isolated from non-cancerous regions did not have an effect on tumor size (Orimo et al. 2005). CAFs can secrete the chemokine CXCL12 which leads to increased recruitment of endothelial progenitor cells to the site of the tumor (Orimo et al. 2005). Moreover, CAFs can secrete hepatocyte growth factor (HGF) which can induce therapy resistance and invasive growth in cancer cells directly (De Wever et al. 2004; Wang et al. 2009).

VII.B. Adipocytes

Growing evidence has been presented that adipocytes may not serve an exclusive role as fat depots but may also act as endocrine cells that secrete soluble factors, such as leptin, adiponectin, and HGF (Rajala and Scherer 2003). In the normal breast, adipocytes are a major constituent of mammary tissue. Recently, Dirat et al. have demonstrated that adipocytes in breast tumors show altered phenotype and decreased expression of adipocyte markers (Dirat et al. 2011). Through secretion of the pro-inflammatory cytokine IL-6, adipocytes can also increase the invasiveness of breast cancer cells in vitro and in vivo (Dirat et al. 2011).
VII.C. Neutrophils

Many innate immune cells show ‘bipolar’ roles in the tumor microenvironment in a context-dependent manner (de Visser et al. 2006; Qian and Pollard 2010; DeNardo et al. 2011; Gregory and Houghton 2011). For example, although neutrophils have been shown in several artificial models to have anti-tumor activity (Colombo et al. 1992; Gregory and Houghton 2011), their presence within tumors is predictive of poor prognosis. Neutrophils secrete the protease leukocyte elastase which is capable of gaining entry to cells via endocytosis (Houghton et al. 2010). Once inside the cell, elastases can cause the hyperactivation of PI3K and thus enhancing proliferation (Houghton et al. 2010). In addition to their effects on cancer cells directly, tumor-associated neutrophils can secrete MMP9 and MMP8 which can degrade the extracellular matrix within the tumor microenvironment and thus facilitating angiogenesis and invasion (Gregory and Houghton 2011).

VII.D. Macrophages

The most prominent innate immune cell type within the tumor microenvironment is the macrophage (Ruffell et al. 2012). In human breast tumors, tumor-associated macrophages (TAMs) were estimated to represent approximately 25% of infiltrating leukocytes in invasive ductal carcinomas second only to CD8 T cells (DeNardo et al. 2009; Ruffell et al. 2012). The extensive infiltration of macrophages into breast tumors are associated with decreased overall and recurrence-free survival (Leek et al. 1996; DeNardo et al. 2011). Strikingly, the prognostic value of macrophage
infiltration was most evident in tumors with high infiltration of CD4 T cells but low infiltration of CD8 T cells (DeNardo et al. 2011). CD4 T cells are capable of secreting cytokines that modulate the phenotype and effector functions of TAMs and thus contribute to tumorigenesis and metastasis indirectly (DeNardo et al. 2009; DeNardo et al. 2011).

Macrophages exhibit phenotypic plasticity allowing them to adapt to various physiological and pathological contexts (Mosser and Edwards 2008). The current paradigm holds that macrophages form distinct populations with specialized functions. The spectrum of activation and functional states for macrophages falls between two primary programs: pro-inflammatory (M1) and wound-healing (M2). The pro-inflammatory (M1) state is induced by exposure to IFN-γ or lipopolysaccharide (LPS) and is characterized by potent microbicidal and tumoricidal functions (Mosser and Edwards 2008; Herbst et al. 2011). On the other hand, the M2 program is induced by IL-4 and is characterized by pro-angiogenic, proteolytic and immunosuppressive functions (Kelly et al. 2007; Varin et al. 2010). The specific activation state of tumor-associated macrophages is still unknown with the existing literature pointing to an M2-like phenotype (Sica et al. 2006; DeNardo et al. 2009).

**VII.D. 1. Pro-tumorigenic Roles for TAMs:**

Several experimental approaches utilizing the well characterized MMTV-PyMT mammary tumor mouse model (see ref. (Guy et al. 1992)) revealed critical roles for TAMs in angiogenesis, metastasis, and therapy resistance. Lin et al. demonstrated that deletions of the macrophage recruitment factor colony stimulating factor 1 (CSF1)
decreased blood vessel density in tumors and delayed the onset of the angiogenic switch (Lin et al. 2006). The induction of early macrophage infiltration in the same mouse model led to earlier vascularisation of the benign tumor (Lin et al. 2006). In addition to the induction of angiogenesis during the early stages of tumor development, TAMs have a pronounced effect on tumor vasculature structure. For example, the presence of M2-like tumor-associated macrophages is associated with abnormal tumor vasculature and poor pericyte coverage (Rolny et al. 2011).

The presence of macrophages in breast tumors is also associated with increased metastasis (Lin et al. 2001; DeNardo et al. 2009). Although the increase in metastatic spread could be caused by the abnormal tumor vasculature (Lin and Pollard 2007; Rolny et al. 2011), macrophage-derived factor can enhance invasiveness directly in tumor cells via epidermal growth factor (DeNardo et al. 2009), NFkB (Hagemann et al. 2005), Wnt5a (Pukrop et al. 2006), and VCAM-1 (Chen et al. 2011) signaling.

In a broader clinical sense, macrophages also have a prominent role in modulating response to conventional therapies (i.e. chemo and radiotherapy). For example, cytotoxic therapies increased macrophage infiltration in human and mouse mammary tumors via increased expression of CSF1 in vitro and in vivo (Shiao and Coussens 2010; DeNardo et al. 2011; Ruffell et al. 2012). Blockage of CSF1 improved response to chemotherapy and reduced metastasis to the lungs in a mouse mammary tumor model (DeNardo et al. 2011). Another group utilizing the same mouse model demonstrated that inhibition of macrophage-derived cathepsins in vivo increased the efficacy of chemotherapeutic agents against primary and metastatic sites (Shree et al. 2011). Imaging studies have provided
further evidence showing that infiltration of myeloid cells into tumors impedes therapy response by decreasing drug tumor distribution (Nakasone et al. 2012).

VII.D. 2. Potential Anti-tumorigenic Roles for TAMs:

Although most of the existing literature suggests that the presence of TAMs in breast tumors is a marker of poor prognosis, few studies have indicated that the prognostic value is in fact due to the M2 polarization of TAMs (DeNardo et al. 2009; Ohri et al. 2009; DeNardo et al. 2011). For example, patient survival is improved in the few cases when there are more M1 macrophages than M2 macrophages within tumors (Ohri et al. 2009). The potential for the re-polarization of tumor-associated macrophages into a pro-inflammatory M1 program is currently being explored. Recently, studies using CD40 antibodies to activate macrophages have shown significant anti-tumor effects in pancreatic cancer animal models and pancreatic cancer patients (Beatty et al. 2011). Moreover, skewing TAM polarization away from M2 to a tumor-inhibiting M1 phenotype significantly improved tumor vascular structure and promoted anti-tumor immune responses (Rolny et al. 2011).

M1 macrophages can lead to tumor regression through two main effects:
1) Enhancing anti-tumor immunity: M1 macrophages produce low levels of the immunosuppressive cytokine IL-10 compared to M2 macrophages (Rolny et al. 2011). On the other hand, M1 macrophages can produce IL-6, IL-12, and IFN-β which can stimulate T cell proliferation (Trinchieri 2003; Mantovani et al. 2009; Mantovani and Sica 2010) . They can also produce chemokines such as CCL20, CXCL10, CXCL11 that would attract and activate natural killer (NK) cells and dendritic cells (DC) (Martinez et al. 2006; Martinez et al. 2008; Mosser and Edwards 2008).
2) Killing tumor cells directly: Macrophages can produce substances and factors that can kill tumor cells directly. For example, macrophages can produce nitric oxide (NO), TNFα and hydroxyl radical that can induce apoptosis or oxidative damage in some cell types (Keller et al. 1990; Herbst et al. 2011; Sindrilaru et al. 2011).

VIII. Macrophages in iron metabolism

Macrophages play a central role in systemic iron homeostasis. Their most prominent role is probably recycling iron from senescent erythrocytes to the bone marrow and thus ensuring undisturbed hemoglobin synthesis. Hemoglobin synthesis accounts for approximately 80% of the iron requirement in humans and demands far greater than the average iron dietary intake (Hentze et al. 2004). Therefore, the body depends on iron recycling through the endoreticular system to meet its daily iron demands. Earlier imaging studies have demonstrated that macrophages can engulf and ingest erythrocytes releasing their iron content which is later stored into ferritin complexes (Fedorko et al. 1973). Furthermore, staining for ferritin in multiple organs showed strong ferritin staining in resident macrophages in bone marrow, breast, spleen and liver (Mason and Taylor 1978).

This central role was recently highlighted in experiments utilizing transgenic mice with macrophage-specific deletions of the iron exporter ferroportin (Zhang et al. 2011). The inability to export iron from macrophages caused systemic anemia as indicated by a consistent decrease in serum iron and transferrin saturation levels and a transient decrease in some hematological parameters. However, iron levels and ferritin expression increased in macrophage-rich sites such as the bone marrow and spleen.
The iron and ferritin levels in macrophages are also linked to its role in innate immunity. Polarization of macrophages into pro-inflammatory (M1) or wound-healing (M2) programs showed distinct iron metabolism profiles (Corna et al. 2010; Recalcati et al. 2010). In those studies, unpolarized macrophages collected from healthy donors were cultured with either LPS or IL-4 in order to induce an M1 or M2 phenotype respectively. Expression analysis of multiple genes showed that M1 macrophages over-express H-ferritin but down-regulate transferrin receptor and ferroportin and thus have a higher intracellular iron levels. On the other hand, M2 macrophages over-expressed ferroportin and thus were able to release iron. These differences were suggested to be caused by changes in the RNA binding activity of IRPs, as well as increased transcription of some iron regulatory genes (Corna et al. 2010). Therefore, the activation of macrophages in response to various inflammatory stimuli is reflected by changes in key proteins involved in iron storage, import and export.

Iron can also have a direct effect on macrophage phenotype and functionality. Accumulation of iron in macrophages led to an increase in the production and secretion of several inflammatory cytokines (Fahmy and Young 1993; Weiss et al. 1997; Muoz and Olivares 2000; Zhang et al. 2011). For example, iron accumulation in ferroportin-deficient bone marrow macrophages led to increased secretion of TNFα and IL-6 (Zhang et al. 2011). Furthermore, iron loading of macrophage can cause unrestrained inflammation in vivo through increased cytokine production which may impair wound healing and the resolution of inflammation (Sindrilaru et al. 2011). Consistently, iron supplementation can exacerbate various infectious diseases such as tuberculosis (Murray et al. 1978; Gordeuk et al. 1996).
IX. Summary

The iron storage protein ferritin has been continuously studied for over 70 years and is now recognized as the primary iron storage protein in cells. Ferritin’s ability to sequester free iron protects cells from iron-induced oxidative damage. The expression of ferritin is tightly regulated and is influenced by various stimuli such as intracellular iron levels, inflammation, and oxidative stress. Although the intracellular functions of ferritin are for the most part well-characterized, the significance of its extracellular form (serum ferritin) in human biology is poorly understood. Several lines of evidence have suggested extracellular ferritin may have angiogenic, immunosuppressive, and iron delivery roles. However, most of those studies utilized iron rich H-ferritin, whereas serum ferritin is composed primarily of L-ferritin and is iron poor. Not all cell types are capable of ferritin secretion with the existing literature indicating that hepatocytes and macrophages are the main cell types with this capacity.

There is compelling evidence to suggest that ferritin plays a role in breast tumor development and progression. Ferritin is elevated in both tumor tissue and sera of patients with breast cancer and this elevation correlates with poor clinical outcome and advanced histological stage. Within breast tumors, ferritin is expressed primarily within the stromal compartments with the identity of the stromal ferritin-rich cells still unknown. Moreover, it is still unclear how the presence of ferritin within tumors could influence tumor growth and development. In light of the advances in our understanding of ferritin’s secretion and extracellular functions, I hypothesize that ferritin is localized primarily in tumor-associated macrophages within breast tumors and that once released this extracellular
ferritin stimulates breast cancer cells directly. I also hypothesize that serum ferritin is elevated in advanced cancer patients and that this elevation is predictive of poor clinical outcome. Chapter 2 will address the localization of ferritin in breast tumor tissue and provide support for the idea of local ferritin secretion. Chapter 3 will address the functional significance of extracellular ferritin in a breast cancer cell culture model and will explore the underlying mechanism for the observed effects. Chapter 4 and 5 will explore the clinical utility of serum ferritin (and other inflammatory biomarkers) in advanced breast and pancreatic cancer patients. The aims of this dissertation are depicted below.
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CHAPTER 2

Expression and Distribution of Ferritin in the Breast Tumor Microenvironment:
Evidence for Local ferritin Release

INTRODUCTION

Iron is an essential micronutrient in cells and is required for many critical cellular processes such as ATP and DNA production (Beard et al. 1993; Kauppi et al. 1996). Despite its importance, excess free iron within the cytoplasm can cause cellular damage and apoptosis (Dixon et al. 2012). Therefore, cells express the iron storage protein ferritin to manage their iron stores and to ensure a long-term supply of iron (Harrison and Arosio 1996). Ferritin is a 24-mer protein complex capable of sequestering free iron within its hollow core in a non-toxic but bio-available form. The expression of ferritin by mammalian cells is reflective of their iron status and is sensitive to changes in intracellular iron levels (Harrison and Arosio 1996).

The earlier studies on ferritin distribution in human tissue noted strong ferritin staining in liver parenchyma, stomach parietal cells, and bone marrow macrophages (Mason and Taylor 1978). In benign mammary tissue, ductal epithelial cells stained weakly while strong staining was observed in few dispersed resident macrophages (histiocytes) within the surrounding stroma (Weinstein et al. 1982; Rossiello et al. 1984). Interestingly, ferritin levels in malignant breast tissue lysates was six-fold higher than non-malignant tissue (Weinstein et al. 1982; Weinstein et al. 1989). This elevation had prognostic value as it correlated with patient survival and tumor histopathological grade (Weinstein et al. 1989). Further analysis of breast tumors revealed that ferritin seemed to
be primarily localized in the stromal compartments of the tumor while tumor cells stained moderately (Rossiello et al. 1984). The identity of those stromal cells has not been conclusively identified.

In addition to the impressive staining in the malignant breast tissue, serum ferritin is elevated in breast cancer patients, and this elevation correlates with advanced disease stage (Jacobs et al. 1976; Tappin et al. 1979; Jones et al. 1980; Robertson et al. 1991; Alkhateeb et al. 2012). The source of this elevation has not been addressed and has long been assumed to be due to inflammation-induced secretion by the liver. Recently, analysis of intraductal fluid from breast cancer patients revealed a five-fold increase in ferritin compared to healthy controls suggesting localized release within the breast (Mannello et al. 2011). Moreover, studies from other cancers have demonstrated local ferritin release in the tumor microenvironment (Partin et al. 1995; Sato et al. 1998). The source of secreted ferritin within tumors and its relative contribution to the systemic increase in serum ferritin are still open questions.

No study has examined alterations in the expression and localization of ferritin in breast tumors with disease progression. Furthermore, the identity of the ferritin-rich cells observed in the stroma of breast tumors has not been identified. In this study, I have comprehensively examined the distribution of ferritin in normal and malignant breast tissue at different stages in tumor development. In breast tumors, I observed decreased ferritin expression in cancer cells but increased infiltration of ferritin-rich CD68-positive macrophages with increased histological grade. In order to explore possible sources for secreted ferritin, we also examined the ability of macrophages and breast cancer cells to release ferritin in vitro. Macrophages, but not breast cancer cells, were capable of ferritin
secretion and this secretion was responsive to iron and inflammatory cytokines. Therefore, the elevation in the ferritin content of tumors is not due to increased ferritin expression in cancer cells but to increased infiltration of ferritin-rich macrophages.

METHODS AND MATERIALS

Tissue samples:

Normal and malignant breast tissue was obtained from the Department of Pathology at the Penn State Hershey Medical Center after approval by the institutional review board (IRB). The formalin-fixed, paraffin-embedded tissues were sectioned with a microtome into 5 μm sections. The diagnosis and classification of tumors was confirmed by a board-certified pathologist according to the World Health Organization (WHO) tumor classification criteria.

Immunohistochemistry:

Paraffin-embedded sections were deparaffinized and processed according to previously published standard laboratory protocol (Snyder et al. 2010). The slides were incubated for 45 minutes at room temperature with either polyclonal rabbit anti-L-ferritin (Abcam, Cambridge, MA) diluted 1:1500 in 10 mM phosphate buffered saline (PBS) or monoclonal mouse anti-CD68 (DAKO, Carpinteria, CA) diluted 1:200 in PBS. The slides were then washed in PBS and incubated for 1 hour with biotinylated anti-rabbit or anti-mouse secondary antibody (Vectastain Elite ABC Kit; Vector, Burlingame, CA) diluted 1:200 in PBS. After washing with PBS, sections were incubated with the avidin/horseradish peroxidase complex for 1 hour. The slides were then exposed to activated 3,3’-diaminobenzidine (DAB) for 2 minutes, counterstained with Gill-Modified
Hematoxylin (EMD, Darmstadt, Germany) for 45 seconds, washed, dehydrated, and coverslipped. For the pre-absorption studies, we pre-incubated the L-ferritin polyclonal antibody with the L-ferritin immunogen (Abcam, Cambridge, MA). Briefly, we added thirty-fold molar excess of L-ferritin immunogen to a PBS solution containing the L-ferritin antibody (1:1500) and incubated overnight at 4°C on a rocker. The staining was assessed and scored by Dr. Han Bing from the Department of Pathology at the Penn State Hershey Medical Center.

For confocal microscopy, sections were double-stained with ferritin and CD68 and then incubated with the appropriate fluorescent secondary antibodies (Molecular Probes, Invitrogen, Camarillo, CA) diluted 1:500 in PBS. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Invitrogen, Camarillo, CA).

**Cell Culture:**

Primary macrophages were collected from female adult Sprague Dawley rats by peritoneal washes using cold Hank’s-balanced solution (HBSS). Resuspended cells were centrifuged at 4°C for 10 minutes at 1620 g. Cells were then seeded into 6-well plates and cultured in DMEM growth media containing 10% fetal bovine serum (FBS) and 4.5 g/L glucose for 72 hours before proceeding with experiments. The human breast cancer cell lines MCF7, T47D, and SKBR3 were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were cultured in Minimum Essential Media (MEM) containing 10 mg/L insulin and 10% FBS. T47D cells were cultured in RPMI 1640 with 10% FBS. SKBR3 cells were cultured in DMEM with 10% FBS. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

**Secretion studies:**
Rat primary macrophages (1 x 10^7 cells/well in 6-well plates) cultured in 10% FBS media were treated with ferric ammonium citrate (FAC; 100 μM; Sigma, St. Louis, MO), recombinant human TNFα (10 ng/ml; Sigma, St. Louis, MO), IL-1β (10 ng/ml; Sigma, St. Louis, MO), IL-13 (10 ng/ml) or Deferoxamine (DFO; 100 μM; Sigma, St. Louis, MO) for 24 hours. The culturing media was replaced with serum-free media. The serum-free ‘conditioned media’ was collected after 24 hours and centrifuged at 720 g for 10 minutes to remove cellular debris. Ferritin content was measured using rat ferritin ELISA (Immunology Consultants Laboratory, Inc., Newberg, OR). Breast cancer cells (1 x 10^7 cells in 6-well plates) were cultured under normal and high-iron conditions (F.A.C.; 100 μM) for 24 hours in 10% FBS media. Media was replaced with serum-free media, collected after 24 hours, and centrifuged. Ferritin content was measured using human ferritin ELISA (AssayPro Corp., St. Charles, MO). Lactate dehydrogenase (LDH) activity was measured using a colorimetric method (Clontech, Mountain View, CA). Total protein levels were quantified using the bicinchoninic acid assay method (BCA; Thermo Fisher Scientific, Rockford, IL)

**Western blotting:**

Proteins were analyzed by SDS-PAGE according to standard conditions. Protein lysates (15-30 μg or 40 μL of conditioned media) were separated by electrophoreses on 4-20% polyacrylamide gels (Bio-Rad, Hercules, CA) at 150 V for 1.5 hours. The proteins were transferred to a nitrocellulose membrane with the Bio-Rad Trans-Blot apparatus at 100 V for 2 hours. The nitrocellulose membrane was blocked in 5% non-fat dry milk/TBS for 1 hour at room temperature. The membrane was incubated with the primary antibody (1:1000 L-ferritin, 1:1000 H-ferritin, or 1:5000 β-actin), diluted in 5% milk,
overnight, and washed extensively. The secondary antibody (1:5000, peroxidase conjugate, Sigma, St. Louis, MO) was added to milk and incubated with the membrane for 1 hour at room temperature. Visualization of the immunoreaction was achieved by using Lightning Plus-ECL (PerkinElmer, Santa Clara, CA) for 1 minute.

**Statistical analysis:**

Statistical analyses for the secretion studies were performed using one-way ANOVA with Dunnett’s post-hoc test (Compares each treatment to baseline condition) or Student’s t-test when comparing only two conditions. All quantitative data were generated from a minimum of three replicates. Statistical significance was defined as $p < 0.05$.

**RESULTS**

**Ferritin is weakly expressed in breast cancer cells and is primarily expressed within tumor-associated macrophages:**

To examine the distribution of ferritin in breast tumors, we performed immunostaining in normal and tumor tissue representing different histological grades using antibodies specific to L-ferritin (Figure 2.1). Previous reports have shown that L-ferritin is the primary subunit of ferritin detected in breast tumor lysates and patient sera (Jones et al. 1980; Jezequel et al. 2012). In normal tissue obtained from mammary reduction surgery, ferritin stained strongly in ductal epithelial cells, while only sparsely scattered cell staining was observed in the stroma (Figure 2.2 A). In ductal carcinoma in situ (DCIS), ferritin stained weakly to moderately in cancer cells, while the stroma contained a moderate density of ferritin-rich cells, particularly surrounding the in situ
tumor (Figure 2.2 B). Similarly, cancer cells from invasive ductal carcinomas showed weak to moderate ferritin staining, while the stroma showed high density of ferritin-rich cells (Figure 2.2 C).

Because macrophages extensively infiltrate breast tumors (DeNardo et al. 2009) and are often rich in ferritin (Simson and Spicer 1972; Cohen et al. 2010), we hypothesized that the ferritin-rich cells observed in the stroma of breast tumors were cells of the macrophage lineage. Therefore, we stained the sections for the macrophage marker CD68. In normal breast sections, we observed sparse staining for CD68 in the stroma probably representing resident histiocytes (Figure 2.2 D). DCIS tumor sections had moderate densities of CD68-positive cells with some cells infiltrating or circulating the in situ tumor (Figure 2.2 E). In invasive ductal carcinomas, there were moderate to high densities of CD68-positive cells with the highest densities within higher grade tumors (Figure 2.2 F). The results from the ferritin and CD68 staining are summarized in Table 2.1.

The staining of CD68 appeared to follow similar trends as the ferritin staining within the breast stroma (Table 2.1; Figure 2.2 B&E). In order to provide evidence that ferritin is localized in CD68-positive macrophages, we stained tumors for both ferritin and CD68 and visualized expression using fluorescence and confocal microscopy (Figure 2.3). All of the CD68-positive cells were ferritin positive and very few ferritin-rich cells were found that did not express CD68.

We also noted that ferritin staining could be detected within stromal fibers surrounding the invasive ductal carcinomas (Figure 2.4) suggesting ferritin could be
released from cells. This observation would support previous reports that speculated local release of ferritin within breast tumors (Tappin et al. 1979; Mannello et al. 2011).

**Macrophages are a source of extracellular ferritin in the tumor microenvironment:**

In order to address the source of ferritin in the tumor microenvironment, we first examined the ability of cancer cells to secrete ferritin in culture. The epithelial breast cancer cell lines MCF7, T47D and SKBR3 did not secrete detectable levels of ferritin even when intracellular ferritin levels increased following iron loading (Figure 2.5).

Because ferritin-rich macrophages are abundant in tumors, we next examined the ability of macrophages to secrete ferritin. Primary rat peritoneal macrophages secreted ferritin and this secretion was reflective of their iron status (Figure 2.6). In macrophages, iron loading had a stronger effect on ferritin expression and secretion than iron chelation by deferoxamine. Strikingly, when macrophages were cultured in 10% FBS (Conditioning media) they secreted little ferritin compared to the 0% FBS media (Conditioned media) and this secretion was not responsive to changes in iron levels (Figure 2.7).

Because serum ferritin levels increase during inflammation (Feelders et al. 1998), we examined the ability of inflammatory cytokines to induce ferritin expression and/or secretion. The inflammatory cytokines TNF-α and IL-1β increased ferritin secretion in macrophages, whereas the suppressive cytokine IL-13 had no effect on ferritin production (Figure 2.8 A). Exposure to TNF-α resulted in a modest increase (~30%) in intracellular ferritin levels but a robust, nearly 100%, increase in secreted ferritin, while IL-1β did not alter intracellular ferritin levels but doubled ferritin secretion. The drastic and selective increase in secreted, but not intracellular, ferritin following treatment with pro-inflammatory cytokines suggests that inflammation might represent a condition that
favors ferritin secretion in macrophages. On the other hand, pro-inflammatory cytokines did not increase ferritin secretion in MCF7 breast cancer cells suggesting that the effects of inflammatory cytokines on ferritin secretion are cell type-specific (Figure 2.8 B).

The release of ferritin from macrophages in response to iron or inflammatory cytokines was not due to cellular death or cytoplasmic leakage as lactate dehydrogenase (LDH) did not have differential activity in the conditioned media (Figure 2.9 A). Moreover, the different treatment did not affect total protein concentrations in macrophage lysates (Figure 2.9 B).

**Macrophages secrete L-ferritin:**

In order to examine the identity of the secreted ferritin, we used western blots and antibodies with specificity for against either L or H-ferritin. Both ferritin subunits were detected in macrophage lysates, but only L-ferritin was detected in conditioned media (Figure 2.10). Iron loading of macrophages or exposure to the inflammatory cytokine IL-1β led to a robust increase in the secreted form of L-ferritin. Although TNF-α exposure led to an increase in secreted ferritin as detected by the ELISA method (Figure 2.8 A), no increase in the secreted form of either L or H-ferritin was detected by western blots (Figure 2.10). This intriguing observation might suggest that a third ferritin subunit exists and is secreted by macrophages in a TNF-α-dependent manner.

**DISCUSSION**

**Ferritin localization within the breast tumor microenvironment:**

In this study, we have observed changes in the L-ferritin content of mammary epithelial cells and their surrounding stroma in association with transformation and tumor
progression (Table 2.1; Figure 2.2). The transformation of ductal epithelial cells to cancerous cells is associated with a detectable decrease in ferritin staining. This decrease might be indicative of low intracellular iron levels, which is consistent with the observations showing that breast cancer cells have high expression of the iron importer transferrin receptor (Hogemann-Savellano et al. 2003) and low expression of the iron exporter ferroportin (Pinnix et al. 2010). The high expression of ferritin in normal ductal epithelial cells contradicts the only published report examining ferritin in benign breast tissue which found it to stain only weakly in epithelial cells (Rossiello et al. 1984). This difference can be attributed to several possible factors: 1) the antibody used in the previous study was raised against ferritin complexes with both L and H subunits, whereas the antibodies used in the present study were raised specifically against L-ferritin, and 2) The tissue previously used represented benign neoplastic cases (fibroadenoma and fibrocystic disease), whereas we used normal tissue obtained from mammary reduction surgery.

We also observed increased infiltration of ferritin-rich CD68-positive macrophages with increased tumor histological grade. The progression from DCIS to invasive ductal carcinoma was associated with a marked increase in macrophage density. Therefore, there is a very strong association between increased macrophage infiltration and tumor invasion of the surrounding stroma. Although the increase in macrophage density could be a consequence of tumor invasion, several studies have argued that macrophage-derived factors can enhance invasion of breast cancer cells via increased angiogenesis or activation of tumorigenic signaling (Lin et al. 2006; DeNardo et al. 2009). Moreover, we observed a higher density of CD68-positive cells in close proximity
to the basal membrane of DCIS tumors (Figure 2.2 E; arrows) providing support for similar observations from a mouse mammary tumor model (Lin et al. 2002) and the idea that macrophage-derived proteases may create a “portal” through which tumor cells can invade the stroma (Pollard 2004).

Macrophages are the most prominent innate immune cell type present within breast tumors (Ruffell et al. 2012). There is mounting evidence demonstrating that macrophages play a critical role in tumor growth and progression through their effects on tumor angiogenesis (Lin et al. 2006), invasion (DeNardo et al. 2009), seeding at distant sites (Chen et al. 2011), and therapy resistance (DeNardo et al. 2011; Shree et al. 2011). For example, blocking monocyte/macrophage recruiting factor CSF-1 in a mouse mammary tumor model led to improved response to chemotherapy and reduced metastases in vivo (DeNardo et al. 2011). Moreover, using a similar mouse model lacking CSF-1, Lin et al. demonstrated a critical role for macrophage infiltration in initiating the ‘angiogenic switch’ (Lin et al. 2006).

**Ferritin secretion in the tumor microenvironment:**

Several lines of evidence have suggested that the cancer-associated increase in serum ferritin is due partly to local secretion within the tumor site. In breast cancer patients with elevated serum ferritin, surgical resection of tumors lowered serum ferritin levels by approximately 50% implicating a relationship between the tumor mass and the elevation in serum ferritin (Tappin et al. 1979). Analysis of intraductal fluid in breast cancer patients also revealed a five-fold elevation in ferritin levels compared to healthy controls suggesting localized ferritin release within the breast tumor microenvironment (Mannello et al. 2011). Moreover, cerebrospinal fluid (CSF) ferritin increased
approximately ten-fold in patients with glioblastoma compared to patients without neurological abnormalities or enteroviral meningitis (Sato et al. 1998). Although CSF contains little ferritin under normal conditions, almost 70% of the glioblastoma patients had CSF ferritin levels exceeding those in serum providing strong support for local synthesis and release (Sato et al. 1998). The staining of ferritin in acellular regions (Figure 2.4) within tumors provides further histological support for the concept of localized ferritin release. However, serum ferritin is commonly elevated in breast cancer patients so we cannot rule out that the detected extracellular ferritin by immunohistochemistry is extravasated from serum.

 Nonetheless, the source of serum ferritin elevation in cancer patients has not been directly addressed. Although, hepatoma cells are capable of ferritin secretion in vitro (Tran et al. 1997; Ghosh et al. 2004), several recent in vivo studies have argued that serum ferritin is primarily derived from macrophages and not hepatocytes (Ferring-Appel et al. 2009; Cohen et al. 2010). Moreover, the molecular size of murine serum ferritin was similar to ferritin extracted from macrophage-rich sites, such as the spleen and the bone marrow but not the liver (Cohen et al. 2010). In addition, we here demonstrate that macrophages specifically secrete L-ferritin — the predominant ferritin subunit in serum (Arosio et al. 1977). In light of our data showing increased infiltration of ferritin-rich macrophages with increased histological grade, it is tempting to speculate that the elevation in serum ferritin in breast cancer patients, which correlates with tumor stage (Jones et al. 1980; Robertson et al. 1991), may partly reflect an inflammatory state involving tumor-associated macrophages.
Interestingly, the secretion of ferritin by macrophages was suppressed by FBS-containing media (Figure 2.7). A similar observation has been noted from a study examining ferritin secretion by hepatoma cells *in vitro* (Ghosh et al. 2004). It is still unclear if a specific factor is serum is responsible for suppressing ferritin secretion, and whether or not this suppression is specific to ferritin. Nonetheless, the secretion of ferritin under serum-deprivation may have physiological relevance during conditions that may require macrophages to infiltrate poorly vascularized tissue such as wounds or tumors.

**The prognostic value of ferritin in the breast tumor microenvironment:**

It is still unclear why the elevation in ferritin levels in breast tumors, which we here show is primarily in L-ferritin within tumor-associated macrophages, is correlated with poor clinical outcome. Several lines of evidence have demonstrated that ferritin may have a multifunctional nature and may play a role in cellular proliferation (Cozzi et al. 2004), immunesuppression (Broxmeyer et al. 1989) and angiogenesis (Coffman et al. 2009). Further exploration of the value of extracellular ferritin in the breast tumor microenvironment is warranted to explore if the presence of extracellular ferritin may have direct tumorigenic influence on the growing tumor.

**CONCLUSIONS**

In summary, we have comprehensively analyzed the distribution of ferritin in benign and malignant breast tissue and noted increased infiltration of ferritin-rich macrophages into breast tumor with disease progression. Therefore, the elevation in the ferritin content of tumors is not due to increased ferritin expression in cancer cells but to increased infiltration of ferritin-rich macrophages. We have also provided evidence for
the presence of extracellular ferritin in the tumor microenvironment. Moreover, we have examined the regulation and specificity of ferritin secretion by macrophages. The identification of tumor-associated macrophages as the ferritin-rich cells within the breast tumor microenvironment raises the possibility for the therapeutic targeting of this cell type with anti-ferritin treatments. Moreover, the local release of ferritin can raise its levels in intraductal fluid which may be a useful diagnostic tool for the early detection of breast cancer.
Table 2.1. Ferritin and CD68 staining in normal and malignant breast tissue.

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<th>Ferritin</th>
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<td></td>
<td>Ductal/Tumor Cells</td>
<td>Stroma</td>
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<tr>
<td>Normal Breast Tissue (n=4)</td>
<td>Strong (4/4)</td>
<td>Weak (4/4)</td>
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<tr>
<td>Ductal Carcinoma In Situ (n=10)</td>
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<td>Weak (8/10); Moderate (2/10)</td>
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<tr>
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<td>Weak (4/5); Moderate (1/10)</td>
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<tr>
<td>High Grade</td>
<td>Weak (3/5), Moderate (2/5)</td>
<td>Weak (4/5); Moderate (1/10)</td>
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<tr>
<td>Invasive Ductal Carcinoma (n=15)</td>
<td>Weak (13/15); Moderate (2/15)</td>
<td>Moderate (12/15); Strong (3/15)</td>
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<tr>
<td>Low Grade</td>
<td>Weak (4/5); Moderate (1/5)</td>
<td>Moderate (5/5)</td>
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<tr>
<td>Intermediate Grade</td>
<td>Weak (4/5); Moderate (1/5)</td>
<td>Moderate (5/5)</td>
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<tr>
<td>High Grade</td>
<td>Weak (5/5)</td>
<td>Moderate (2/5); Strong (3/5)</td>
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FIGURES

Figure 2.1. The ferritin antibody specifically recognizes the L subunit. (A) Western blotting showing that the L-ferritin antibody used in immunohistochemistry recognized recombinant L-ferritin (rLFr) but not recombinant H-ferritin (rHFR). The L-subunit-rich human liver ferritin was used as a positive control. The appearance of the 17 kDa band in liver ferritin has been shown to be due to lysosomal cleavage of the L-ferritin protein (Cohen et al. 2010). Each lane was loaded with 0.25 μg of protein. (B) Ferritin staining in ductal carcinoma tissue is blocked by pre-absorption of the ferritin antibody with an L-ferritin fragment. The two images represent contiguous sections from the same tumor.
Figure 2.2. Ferritin and CD68 staining in normal and breast tumor tissue.

Contiguous sections from normal breast (A&D), ductal carcinoma in situ (B&E) and invasive ductal carcinoma (C&F) tissue stained for ferritin and CD68 respectively [magnification: 20x]. CD68-positive cells surround (arrows) and infiltrated (arrowheads) the in situ tumor in E. Brown pigment is the reaction product for DAB. Blue hematoxylin stain represents nuclei.
Figure 2.3. Ferritin in the breast tumor stroma is localized in CD68-positive cells.

(A) Double fluorescence staining for ferritin (green) and CD68 (red) in invasive ductal carcinoma tissue [magnification: 20x]. (B) Confocal microscopy images of different macrophages within breast tumors showing co-localization of ferritin (green) and CD68 (red) in invasive ductal carcinoma tissue. Bars: 10μm.
<table>
<thead>
<tr>
<th>DAPI</th>
<th>Ferritin</th>
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(B)
Figure 2.4. Ferritin stains in the stroma surrounding invasive ductal carcinomas. (A&B) ferritin stained stromal fibers surrounding invasive tumors. (C) Pre-absorbed ferritin antibodies did not stain the same regions as in B indicating that it is not due to non-specific reactivity of the antibodies or DAB [magnification: 10x].
**Figure 2.5. Breast cancer cells do not secrete ferritin.** Ferritin in lysates and conditioned media of breast cancer cell lines with or without exposure to 100 μM of ferric ammonium citrate as a source of iron. Data shown represent total ferritin in 1 x 10^5 cells and are expressed as mean +/- s.e.m. *p<0.05 between the indicated condition and the untreated (baseline) cells. Student’s t-test was used to compare treated and non-treated cells.
Figure 2.6. Macrophages secrete ferritin. Ferritin in lysates and conditioned media of primary rat macrophages following exposure to 100 μM of ferric ammonium citrate as a source of iron or 100 μM of the iron chelator deferoxamine (DFO). Data shown represent total ferritin in $1 \times 10^5$ cells and are expressed as mean +/- s.e.m. *$p<0.05$, **$p<0.01$ between the indicated condition and the untreated (baseline) cells. One-way ANOVA with Dunnett’s post-hoc test was used to compare treatments to the untreated cells.
Figure 2.7. FBS-containing media suppress ferritin secretion in macrophages.

Ferritin content in conditioning media (10% FBS) and conditioned media (0% FBS) from primary rat macrophages. Data shown represent total ferritin in $1 \times 10^5$ cells and are expressed as mean +/- s.e.m. *$p<0.05$, **$p<0.01$ between the condition and its respective untreated (baseline) control or as otherwise indicated. One-way ANOVA with Dunnett’s post-hoc test was used to compare treatments to the untreated (baseline) cells. Student’s t-test was used to compare the different treatments between the media conditions.
**Figure 2.8.** **inflammatory cytokines induce ferritin secretion by macrophages.** (A) Ferritin in lysates and conditioned media of primary rat macrophages following exposure to TNF-α, IL-1β, or IL-13. Pro-inflammatory (TNF-α and IL-1β) but not the suppressive cytokine IL-13 increased ferritin secretion while having a modest or no effect on intracellular ferritin levels. (B) Ferritin in conditioned media of MCF7 breast cancer cells following exposure to inflammatory cytokines. Data shown represent total ferritin in $10^5$ cells and are expressed as mean +/- s.e.m. *p<0.05, **p<0.01 between the indicated condition and the untreated (baseline) cells or as. One-way ANOVA was used to compare treatments to the untreated cells. N.S.: not significant.
Figure 2.9. Ferritin secretion by macrophages is not due to cellular damage. (A) LDH activity in conditioned media from primary rat macrophages demonstrating that the different treatments are not associated with compromised membranes that could leak intracellular ferritin. Macrophage protein lysates was used as a positive control. (B) Protein concentrations of macrophage lysates following the different treatments. One-way ANOVA with Dunnett’s post-hoc test was used to compare treatments to the baseline untreated cells.
Figure 2.10. Macrophages specifically secrete L-ferritin. Western blot analyses of cell lysates and conditioned media from primary rat macrophages with antibodies specific to H or L-ferritin. β-actin was used as an intracellular marker. Each lane was either loaded with 25 μg of total protein lysates or 35 μL of conditioned media.
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CHAPTER 3

Extracellular Ferritin Stimulates the Proliferation of Breast Cancer Cells Through an Iron-Independent Mechanism

INTRODUCTION

Although functions of ferritin are traditionally associated with intracellular iron storage, there is much more to this protein that is recently being discovered and investigated. For example, it has long been known that ferritin is an abundant protein in blood, but it is still unclear what role this form of ferritin plays in human biology. Serum ferritin is composed primarily of the L-subunit and is relatively iron poor (Arosio et al. 1977).

Several studies have explored a more expansive functional role for extracellular ferritin. These studies found extracellular ferritin to have angiogenic and immunosuppressive roles in endothelial and lymphocytes respectively (Broxmeyer et al. 1989; Coffman et al. 2009). Furthermore, injection of $^{59}$Fe-loaded ferritin into adult rats led to differential uptake of iron by some organs, suggesting an iron delivery role for this traditional iron storage protein (Fisher et al. 2007). Our laboratory also showed the ability of ferritin to deliver iron to developing oligodendrocytes through receptor-mediated endocytosis (Todorich et al. 2008; Todorich et al. 2011). Receptors with different affinities for the two subunits of ferritin have also been identified. TIM-2 has a high affinity for H-ferritin and is found on oligodendrocytes, lymphocytes, kidney and liver cells (Chen et al. 2005; Todorich et al. 2008), and scavenger receptor class A member 5
(Scara5) has been identified as the human L-ferritin receptor in the developing kidney (Li et al. 2009). Expression of Scara5 was sufficient to uptake ferritin, deliver iron, and rescue cells in serum free conditions (Li et al. 2009), while H-ferritin can replace the need for transferrin, once considered an obligate protein, in oligodendrocyte cultures (Todorich et al. 2011). Consistently, one study has argued that ferritin secreted by macrophages can be used to recycle iron for hematopoiesis (Leimberg et al. 2003; Leimberg et al. 2008). These studies are compelling evidence that ferritin is an iron delivery protein and that serum ferritin, through its ability to deliver iron, can be expected to impact cell proliferation and growth.

Serum ferritin is elevated in multiple human malignancies (Hazard and Drysdale 1977) including neuroblastoma (Hann et al. 1985), glioblastoma multiforme (Keir et al. 1993; Sato et al. 1998), melanoma (Luger et al. 1983; Gray et al. 2003), lymphoma (Eshhar et al. 1974; Sarcione et al. 1977), and breast cancer (Jacobs et al. 1976; Jones et al. 1980; Robertson et al. 1991; Alkhateeb et al. 2012). The functional significance of this elevation has been consistently ignored due to the prevailing paradigm on ferritin as a housekeeping iron storage protein and a non-specific acute-phase reactant. Intriguingly, the available data hints that this elevation correlates with disease stage in neuroblastoma and breast cancer patients (Jones et al. 1980; Hann et al. 1985; Robertson et al. 1991; Alkhateeb et al. 2012), and that it may be caused by localized release within the tumor microenvironment (Tappin et al. 1979; Mannello et al. 2011). The few studies examining the prognostic value of serum ferritin found it to be predictive of recurrence and disease progression in breast cancer patients (Jacobs et al. 1976; Alkhateeb et al. 2012). In one
study, serum ferritin showed independent prognostic value from other inflammatory biomarkers suggesting a functional significance (Alkhateeb et al. 2012).

Moreover, breast tumor lysates also show elevated levels of L-ferritin — the predominant subunit in serum — and this elevation correlates with advanced histological grade and shorter survival (Weinstein et al. 1982; Rossiello et al. 1984; Weinstein et al. 1989; Jezequel et al. 2012). Histological examination of ferritin distribution within the breast showed weak staining of ductal cells in benign breast tissue, moderate staining in breast cancer cells, and strong staining within the tumor stroma (Rossiello et al. 1984; Jezequel et al. 2012).

The aim of this study is to examine the functional significance of extracellular ferritin in tumorigenesis. We hypothesize that extracellular ferritin can impact the proliferation and growth of cancer cells through iron delivery and thus providing evidence that the elevation in serum ferritin is directly impacting tumorigenesis. In this study, ferritin increased the proliferation of two epithelial breast cancer cell lines and was taken up by breast cancer cells indicating a direct functional interaction. Moreover, these effects were seemingly independent of iron as iron was not essential for the ferritin effect.

MATERIALS AND METHODS

Cell culture:

The glioma cell lines U251, U-87, CCF-STTG1, T98G and LN-18 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in DMEM media containing 10% Fetal Bovine Serum (FBS; Gemini Bio-Products, West Sacramento, CA). The U251 culturing media was supplemented with 1%
of non-essential amino acids (v/v; Sigma, St. Louis, MO). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

The human breast cancer cell lines MCF7, T47D, MDA-MB-231, and SKBR3, and the normal mammary cell line MCF10A were obtained from ATCC. MCF7 cells were cultured in Eagles’s Minimum Essential Media (MEM) containing 10mg/L insulin and 10% FBS. T47D cells were cultured in RPMI 1640 with 10% FBS. MDA-MB-231 cells were cultured in Leibovitz media with 10% FBS. SKBR3 cells were cultured in DMEM with 10% FBS. The MCF10A normal mammary cells were cultured in DMEM/F12 media containing 10 mg/L insulin, 1% non-essential amino acids, and 1.8 g/L sodium bicarbonate. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂, except for MDA-MB-231 cells which were maintained in 0% CO₂.

The neuroblastoma cell line SH-SY5Y was obtained from ATCC and cultured in DMEM/F12 media containing 10% FBS, 1% non-essential amino acids, and 1.8 g/L sodium bicarbonate. The cell line was maintained at 37°C in a humidified atmosphere containing 5% CO₂. The melanoma cell lines A375M and UACC903 were obtained from ATCC and cultured in DMEM media containing 10% FBS. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

**Proliferation studies:**

Cancer cells (1 x 10⁵) were seeded in a 96-well plate under serum-free conditions and exposed to human liver ferritin (EMD, Darmstadt, Germany), horse spleen ferritin (Sigma, St. Louis, MO), horse spleen Apo-ferritin (Sigma, St. Louis, MO), or human holo-transferrin (Sigma, St. Louis, MO) for 48 hours. Proliferation was assessed by
CellTiter 96 AQueous cell proliferation assay kit (MTS; Promega, Madison, WI) and/or BrdU incorporation (EMD, Darmstadt, Germany) assays. The BrdU label was added in the last 24 hours of the experiment. Experiments were performed in quadruplicates and repeated at least three times. Molar concentrations for ferritin were calculated using the 450 kDa molecular weight of the entire complex. Some reagents (i.e. human liver ferritin) required extensive dialysis against phosphate buffered solution (PBS) to remove the sodium azide from solution.

**Uptake studies:**

Human liver ferritin was labeled with Alexa-fluor 488 labeling kit (Molecular Probes, Invitrogen, Camarillo, CA) according to manufacturer’s instructions. The labeled protein was run through a size exclusion column to remove excess free dye. The labeling efficiency was determined based on the equation suggested by the manufacturer:

\[
\text{Mole dyes per mole protein} = \frac{(\text{absorbance at 494nm x dilution factor})}{(71,000 \times \text{protein molar concentration})}.
\]

The minimum labeling efficiency in this experiment was 2 moles dye/mole of ferritin.

Breast cancer cells were grown on chambered slides, and were serum deprived for at least 12 hours before the addition of 25 nM fluorescence-conjugated ferritin for 1 hour at 37°C or 4°C. Cells were washed with Hank’s-balanced solution (HBSS; Life Technologies, Grand Island, NY) and fixed with 4% paraformaldehyde (w/v) before staining with α-tubulin (1:500, Abcam, Cambridge, MA) and 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Invitrogen, Camarillo, CA).

**Flow cytometry:**
MCF7 cells (5 x 10^6) were seeded in 100mm cell culture plates and grown to 80% confluency. Cells were washed with HBSS, and media was replaced with serum-free media. Fluorescence-conjugated ferritin (25 nM) was added to each plate for 1 hour at 37°C. Some cells were pre-incubated with five hundred-fold of unlabeled ferritin for 30 minutes. Cells were washed three times with cold PBS, and then collected with a cell scraper. Cells were centrifuged at 720 g for 5 minutes and resuspended in 2% paraformaldehyde. Cells were analyzed with FACScalibur at the Penn State Flow Cytometry Core Facility.

**Assessing the role of ferritin’s iron core on proliferation:**

MCF7 breast cancer cells (1 x 10^5) were seeded in a 96-well plate under serum-free conditions and exposed to horse spleen apo-ferritin with or without the addition of ferrous ammonium sulfate (FAS; Sigma, St. Louis, MO). Total cell number was measured using MTS assay (Promega, Madison, WI). The ability of apo-ferritin to take up iron was assessed *in vitro* by incubating 1µM of horse spleen apo-ferritin in 0.1 M HEPES (pH 7.4) with increasing concentrations of FAS on ice. Samples were run on PD-10 Size exclusion columns (GE Life Sciences, Pittsburgh, PA) to remove excess free iron. Perls Prussian blue (ferric ferrocyanide) staining was performed to visualize iron, and Coomassie (Sigma, St. Louis, MO) staining was performed to visualize total protein.

**Iron delivery studies:**

Changes in intracellular iron were measured by using the metal-sensitive dye calcein (Trevigen, Gaithersburg, MD). Cells were seeded in 10% FBS media for 24 hours, serum starved for 12 hours and then washed with HBSS. Serum-free media containing various concentrations of ferritin was added for 2 or 5 hours. Media was
removed and replaced with PBS containing 0.2 µM calcein. Fluorescent signal was measured 30 minutes later (excitation 490nm/emission 515nm). Cells were also treated with the membrane permeable iron complex 3,5,5-trimethylhexanoyl-ferrocene (TMHF) or transferrin as positive controls.

To assess the effect of ferritin on transferrin receptor levels, we incubated cells with 50 nM human liver ferritin for 24 hours under serum free conditions. Cells were washed and lysed with RIPA buffer (Sigma, St. Louis, MO) containing 1% protease inhibitor cocktail (v/v, Sigma, St. Louis, MO) and probed with western blotting for transferrin receptor (1:500, Sigma, St. Louis, MO). Densitometric quantification of signal intensity was performed using Multi-Gauge Software.

**Signaling studies:**

MCF7 breast cancer cells (3.5 x 10^5) were seeded in 30mm plates and allowed to adhere overnight. Cells were serum starved for 18-24 hours prior to the addition of human liver ferritin (25 nM), PBS (equal in volume to the ferritin treatment), or Insulin-like Growth Factor (IGF-1; 100 ng/ml; R&D systems, Minneapolis, MN) as a positive control. The cells were incubated at 37°C for several time points (0, 5, 10, 15, 30, 60 minutes, 24 hours), washed with HBSS and lysed using RIPA buffer (Sigma, St. Louis, MO) containing 1% protease and phosphatase inhibitor cocktails (v/v; Sigma, St. Louis, MO). The signaling analysis was performed using the 1) Pathscan Intracellular Signaling Array Kit (Cell Signaling, Danvers, MA), 2) Pathscan inflammation multi-target sandwich ELISA kit (Cell Signaling, Danvers, MA), and 3) western blotting. For the Pathscan inflammation ELISA, we used the lysates of Jurkat cells treated with 100 nM
calyculin A and 1 mM pervanadate to inhibit phosphotase as a positive control (Cell Signaling, Danvers, MA).

**Western blotting:**

Proteins were analyzed by SDS-PAGE according to standard conditions. Protein lysates (15-30 µg) were separated by electrophoresis on 4-20% polyacrylamide gels (Bio-Rad, Hercules, CA) at 150 V for 1.5 hours. The proteins were transferred to a nitrocellulose membrane with the Bio-Rad Trans-Blot apparatus at 100 V for 2 hours. The nitrocellulose membrane was blocked in 5% non-fat dry milk diluted in TBS for 1 hour at room temperature. The membrane was incubated with the primary antibody, which was diluted in 5% non-fat dry milk diluted in TBS-T, overnight and washed extensively. The secondary antibody (1:5000, peroxidase conjugate, Sigma, St. Louis, MO) was added to milk and incubated with the membrane for 1 hour at room temperature. Visualization of the immunoreaction was achieved by using Lightning Plus-ECL (PerkinElmer, Santa Clara, CA) for 1 minute.

**Antibodies:**

The following antibodies were used at 1:1000 concentration unless otherwise stated: total AKT (Cell Signaling, Danvers, MA), pS473 AKT (Cell Signaling, Danvers, MA), pS176/180 IKKα/β (Cell Signaling, Danvers, MA), total IKKβ (Cell Signaling, Danvers, MA), transferrin receptor (1:500; Sigma, St. Louis, MO), β-actin (1:5000; Abcam, Cambridge, MA), Scara5 (1:500; R&D systems, Minneapolis, MN).

**Statistical analysis:**

Statistical analyses for the proliferation studies were performed using one-way ANOVA with Dunnett’s post-hoc test (Compares each treatment to Control) or Student’s
t-test when appropriate. All quantitative data were generated from a minimum of three replicates. Statistical significance was defined as $p < 0.05$.

**RESULTS**

**Ferritin stimulates the proliferation of breast cancer cells:**

In order to investigate a functional role for extracellular ferritin in tumorigenesis, we first screened multiple cancer cell lines for ferritin response using MTS proliferation assay (Figure 3.1 A). The cell lines spanned multiple malignancies that have been associated with elevated serum ferritin levels. Of the 13 cell lines examined, only MCF7 and T47D breast cancer cells showed a consistent increase in cell number following exposure to human liver ferritin under serum free conditions. In order to validate this effect, we examined the effect of ferritin on DNA synthesis as measured by BrdU incorporation in the breast cancer cell lines (their characteristics are summarized in Table 3.1). The addition of ferritin to the culturing media led to an increase in BrdU incorporation in MCF7 and T47D cells, but not in SKBR3, MDA-MB-231 or MCF10A cells (Figure 3.1 B). Ferritin seemed to show a concentration-dependent effect on BrdU incorporation in MCF7 cells (Figure 3.1 C). In order to evaluate the contribution of the serum-free culturing conditions to the ferritin effect, we cultured MCF7 cells in varying concentrations of FBS-containing media and exposed them to ferritin. Ferritin increased MTS signal when cells were cultured under FBS-Free or FBS-Low conditions (Figure 3.1 D). The FBS lot used in this experiment had low levels of ferritin (Figure 3.1 E) suggesting that FBS might contain molecules other than ferritin that compensate for the ferritin effect.
**Ferritin is taken up by breast cancer cells:**

In order to examine whether or not the effect of ferritin in breast cancer cells is due to a direct interaction, we labeled human liver ferritin with Alexa-flour488 fluorescent probe and incubated it with the breast cancer cell lines MCF7 (responsive to ferritin) and MDA-MB-231 (unresponsive to ferritin). Immunofluorescence analysis showed that ferritin was taken up by the epithelial breast cancer cell line MCF7 but not the mesenchymal cell line MDA-MB-231 (Figure 3.2 A). Moreover, incubation of MCF7 cells with fluorescence-conjugated ferritin in 4°C blocked uptake suggesting a specific interaction (Figure 3.2 A). Flow cytometric analysis demonstrated that ferritin binds cells and that the majority of the binding signal can be blocked by excess unlabeled ferritin (Figure 3.2 B). Overall, these observations demonstrate that ferritin can be taken up by breast cancer cells.

**Ferritin does not deliver iron to breast cancer cells and may possess an iron-independent proliferative effect:**

One possible mechanism underlying the proliferative effects of ferritin is delivery of iron to cancer cells (Fisher et al. 2007; Li et al. 2009; Todorich et al. 2011). In order to evaluate the contribution of iron to the proliferative effects of ferritin, we exposed MCF7 breast cancer cells to holo (iron-rich) or apo (iron-free) forms of ferritin. Surprisingly, apo-ferritin had an effect at a lower concentration than holo-ferritin suggesting that iron is not essential for the proliferative effects of ferritin (Figure 3.3). Holo-ferritin had a significantly stronger effect than apo-ferritin at high concentrations suggesting ferritin may have both iron-dependent and iron-independent functionalities.
Because apo-ferritin still retains its ability to oxidize ferrous iron and store it within its core (Figure 3.4 A), it is hypothetically possible that apo-ferritin was capable of sequestering iron from the culture media and then delivering it to cells. To investigate this possibility, we added various concentrations of ferrous ammonium sulfate to MCF7 treated with a 25nM apo-ferritin. As expected, addition of ferrous ammonium sulfate (FAS) decreased cell number, as measured by MTS, possibly through the generation of free radicals (Figure 3.4 B,). Interestingly, the addition of apo-ferritin protected cells from FAS-induced cytotoxicity suggesting that it was capable of oxidizing and possibly storing ferrous iron (Figure 3.4 B,). However, the presence of increased concentration of iron in the culture media did not increase the proliferative effect of ferritin (Figure 3.4 B).

We also performed the reverse experiment by culturing cells with varying concentrations of apo-ferritin in the presence of a non-toxic concentration of FAS. The presence of iron did not significantly increase the magnitude of the apo-ferritin effect (Figure 3.4 C).

We examined iron delivery more directly by measuring changes in the intracellular labile iron pool using calcein-AM. Free intracellular iron is capable of binding calcein and quenching its fluorescent signal, and therefore establishing a direct correlation between intracellular iron levels and calcein signal (Breuer et al. 1995). Ferritin exposure did not change intracellular iron levels in MCF7 cells (Figure 3.5 A), whereas both the membrane-permeable iron complex 3,5,5-trimethylhexanoyl-ferrocene (TMHF) and the iron-delivery molecule transferrin quenched the calcein signal within the same timeframe (Figure 3.5 B & C).

We also examined the ability of ferritin to modulate the protein levels of transferrin receptor in MCF7 cells. Transferrin receptor has five Iron Response Elements
(IREs) in its 3’ UTR (Casey et al. 1988; Casey et al. 1989). The presence of iron in the cytoplasm inhibits the ability of iron regulatory proteins (IRPs) to bind IREs and thus decreases the stability of the mRNA and increases its degradation (Leibold and Guo 1992). Therefore, if ferritin is acting as an iron delivery molecule it would lead to a decrease in the protein levels of transferrin receptor. In order to test this prediction, we exposed MCF7 to human liver ferritin which is iron rich. Ferritin did not significantly decrease the expression levels of transferrin receptor suggesting that intracellular iron levels did not increase with ferritin exposure (Figure 3.6 A & B). As a positive control, we show that iron loading or chelation affect transferrin receptor levels in MCF7 within 24 hours (Figure 3.6 C).

The delivery of iron to MCF7 cells via transferrin can increase cell number as measured by MTS signal (Figure 3.7 A). If ferritin and transferrin were stimulating the proliferation of MCF7 via the same mechanism (i.e. iron delivery) then the simultaneous addition of both molecules would not confer an additive or synergistic advantage on proliferation. The addition of both ferritin and transferrin to MCF7 cells had additive effects providing further evidence for a novel iron-independent functionality for ferritin (Figure 3.7 B).

**The effects of ferritin on cellular signaling:**

Because ferritin does not seem to deliver iron to cells, we hypothesized that its proliferative effects on breast cancer cells are due to activation of cellular signaling. In order to address this hypothesis, we utilized two approaches: western blotting and ELISA-based arrays. The Pathscan Intracellular Signaling Array kit allows for the evaluation of 18 intracellular signaling targets using the ELISA principle (Targets and
array design are shown in Table 3.2). We first validated the sensitivity of this assay by exposing MCF7 cells to the potent AKT activator Insulin-like Growth Factor I (IGF-1). IGF-1 can visually increase the phosphorylation of AKT at serine 473, and two of its downstream targets: PRAS40 at threonine 246, and GSK-3β at Serine 9, within 15 minutes of exposure (Figure 3.8 A & B).

In order to comprehensively examine the effect of ferritin on cellular signaling, we exposed MCF7 to ferritin or vehicle for 5, 10, 15, or 60 minutes. The protein lysates were then examined using the Pathscan array. Ferritin did not show differential activation of any of the pathways examined (Figure 3.8 C & D). Furthermore, long-term exposure to ferritin (24 hours) did not activate any of the examined pathways (Figure 3.8 E & F).

A recent report has suggested that ferritin acts as an inflammatory cytokine and is capable of activating NFκB in hepatocytes independently of its iron content (Ruddell et al. 2009). Therefore, we examined the activation of both the transcription factor NFκB and its upstream regulator IKK. Ferritin exposure did not lead to the phosphorylation of IKK in MCF7 cells within the same time frame as previously reported (Figure 3.9 A). We also examined the ability of ferritin to activate NFκB using an ELISA assay. Ferritin did not activate NFκB transcription factor at any of the time points examined (Figure 3.9 B).

Because serum ferritin seemed to be predictive of trastuzumab response (Alkhateeb et al. 2012) and has been suggested to correlate with AKT activation in endothelial cells (Tesfay et al. 2012), we examined AKT activation more closely using western blotting. Neither apo- or holo-ferritin led to strong activation of AKT signaling in the same manner as IGF-1 (Figure 3.10 A). Interestingly, PBS-treated controls showed a weak time-dependent activation of AKT that seemed to subside at 60 minutes post
treatment. This weak activation was also observed with the different ferritin treatments. This activation may be due to the temperature-sensitivity of AKT (Oehler-Janne et al. 2008). To test this possibility, we examined the effects of placing MCF7 cells at room temperature for 2 minutes without adding any reagents and then placing them back in their 37°C incubators. This slight alteration in culturing conditions led to weak AKT activation within the same time frame observed with PBS and ferritin (Figure 3.10 B). Therefore, the weak activation of AKT we observed with the ferritin treatments is probably an unavoidable experimental artifact.

**Scara5 expression is not sufficient for mediating the ferritin effect:**

The ability of ferritin to directly stimulate some cancer cells but not others (Figure 3.1 A & B, Figure 3.2 A) suggests that some cancer cells express a ferritin receptor. Scara5 is the putative L-ferritin receptor (Li et al. 2009). Therefore, we examined Scara5 expression in the breast cancer cell lines. Both responsive (i.e. MCF7) and unresponsive (i.e. MDA-MB-231) cell lines expressed Scara5 suggesting that Scara5 is not sufficient for ferritin uptake (Figure 3.11 A & B). Interestingly, the normal mammary epithelial cell line MCF10A showed the lowest expression suggesting that this protein is up-regulated in breast cancer cells. We also examined Scara5 expression in breast and glioma tumor lysates (Figure 3.11 C). Scara5 was expressed in 6/6 of the breast tumors and 5/6 of glioma tumors. In breast tumors, all tumors primarily expressed a 60 kDa band, whereas some glioma tumors expressed two bands – 53 and 60 kDa.

**DISCUSSION**
In this study we have examined the functional significance of extracellular ferritin in cancer cells. First, we have demonstrated that ferritin stimulates the proliferation of epithelial breast cancer cells. Second, we demonstrated that ferritin can interact directly with breast cancer cells. Third, we have provided several lines of evidence to suggest that the proliferative effects of ferritin are caused by an iron-independent mechanism as i) apo-ferritin stimulated proliferation similarly to holo-ferritin, ii) the increase in the iron content of ferritin did not enhance its proliferative effects, and iii) ferritin exposure did not lead to an increase in intracellular iron levels. Collectively, these data demonstrate that extracellular ferritin interacts directly with breast cancer cells and stimulate their growth via a novel iron-independent mechanism. Therefore, the elevation in serum ferritin observed in many breast cancer patients may be reflective of pro-tumorigenic phenomenon.

**A novel proliferative role for ferritin in tumorigenesis:**

Ferritin has long been exclusively viewed as a house keeping iron storage protein, and this view consistently ignored a functional component to extracellular ferritin. Several lines of evidence have suggested that extracellular ferritin has a multi-functional nature in human biology. It has been implicated in proliferation (Broxmeyer et al. 1989; Cozzi et al. 2004), angiogenesis (Coffman et al. 2009), and iron delivery (Fisher et al. 2007; Li et al. 2009; Todorich et al. 2011). The iron delivery functionality of ferritin is associated primarily with H subunit, while the L subunit is associated with proliferation and angiogenesis. For example, overexpression of L-ferritin, but not H-ferritin, increased cellular proliferation in HeLa cells (Cozzi et al. 2004). Also, Coffman et al. have demonstrated that ferritin rich with the L subunit can bind to high molecular weight.
kininogen and block its anti-angiogenic effects on endothelial cells (Coffman et al. 2009). The mechanism by which ferritin stimulates the growth of HeLa cells or endothelial cells are still undiscovered and are believed to be independent of iron. In our study, we provided evidence that ferritin complexes, rich with the L subunit, can directly interact with breast cancer cells and stimulate their proliferation. Serum ferritin contains relatively little iron and is composed primarily of the L subunit (Arosio et al. 1977). Our results demonstrating a direct proliferative effect of apo-ferritin provides support to the idea that serum ferritin possesses an overlooked non-canonical function. The ability of ferritin to stimulate cellular proliferation may have implications during pathologic conditions that cause an elevation in serum ferritin, such as inflammation and cancer.

Serum ferritin is elevated in breast cancer patients and this elevation correlates with disease stage (Jones et al. 1980; Robertson et al. 1991). One study has found that high levels of pre-operative serum ferritin are predicative of recurrence in early breast cancer patients (Jacobs et al. 1976). Another study examining the predictive value of serum ferritin in advanced breast cancer patients receiving trastuzumab-containing therapy found it to have independent prognostic value from other inflammatory biomarkers (Alkhateeb et al. 2012). These observations support a possible direct role for ferritin in tumorigenesis.

Although the mechanism by which ferritin stimulates breast cancer cells is still unknown, we have provided evidence showing that it is not due to iron delivery and is not dependent on the iron content of ferritin. Future studies examining the transcriptomes of ferritin-treated cells might provide insights to the mechanism underlying the ferritin effect.
We also provided evidence showing that the putative L-ferritin receptor Scara5 is not sufficient for the proliferative effects, as originally speculated (Li et al. 2009). Scara5 shows sequence and structural homology to Class A scavenger receptors but is unable to endocytose acetylated or oxidized low density lipoprotein (Jiang et al. 2006). Earlier expression profiling of Scara5 in mice have demonstrated that its expression is restricted to populations of epithelial cells, primarily in the lung, trachea, testis, and bladder (Jiang et al. 2006). However, it is still unclear what drives its expression, what role it may be playing in the breast, and how it is connected to innate immunity. Moreover, the insufficiency of scara5 to uptake ferritin in breast cancer cells raises the possibility of the involvement of another L-ferritin receptor with possible implications in breast cancer development and progression.

Interestingly, scara5 was differentially expressed in breast cancer cells line but not normal mammary cells. Moreover, it was detected by western blotting in glioma and breast tumors. The significance of scara5 expression in tumor biology is still unclear. The few published reports have speculated a role in: 1) the modulation of intracellular signaling, such as focal adhesion kinase (Huang et al. 2010), and 2) induction of cellular survival through increased cellular adhesion (Ojala et al. 2012). It is still unclear if those functions are connected to the ability of Scara5 to directly interact with ferritin (Li et al. 2009).

Ferritin in epithelial-stromal communication within the tumor microenvironment:

Earlier studies on the role of iron in tumorigenesis have indicated that iron accumulated in the stroma, but not malignant cells, within iron-induced tumors (Singh et al. 1994; Diwan et al. 1997). The involvement of the stroma suggests that iron-dependent
tumorigenic effects may be mediated by non-malignant cells with high iron storage capacity – such as macrophages. Moreover, high expression of ferritin in the stroma of breast tumors was predictive of poor clinical outcome in breast cancer patients with early disease (Jezequel et al. 2012). Another independent group has proposed the inclusion of the ferritin receptor, Scara5, in a prognostic gene expression panel for breast cancer (Miller et al. 2011). Therefore, our study provides a molecular mechanism through which the presence and release of ferritin in the tumor microenvironment directly stimulates tumorigenesis.

**Ferritin as an inflammatory effector mechanism:**

It is still unclear what molecular mechanisms underlie the association between the use of anti-inflammatory drugs and the decrease in cancer incidence and mortality (Cotterchio et al. 2001; Johnson et al. 2002). Serum ferritin, often overlooked as a bystander protein, is elevated during acute and chronic inflammation (Konijn and Hershko 1977; Kalantar-Zadeh et al. 2004) possibly through the activation of the NF-κB transcription factor (Pham et al. 2004). Moreover, chronic use of anti-inflammatory drugs significantly reduces serum ferritin levels in patients with inflammatory diseases (Milman et al. 1999; Fleming et al. 2001). Intriguingly, elevated levels of serum ferritin had prognostic and predictive value in advanced breast cancer patients independently of the inflammatory biomarker C-reactive protein (CPR) suggesting that ferritin may possess a functional role (Alkhateeb et al. 2012). Therefore, it is logical to argue that the ability of extracellular (serum) ferritin to stimulate cancer cells represents an inflammatory effector mechanism through which inflammation induces proliferation in the growing tumor. The
cancer-associated elevation in serum ferritin may represent a functional link connecting inflammation and tumorigenesis.

**CONCLUSIONS**

The ability of ferritin to stimulate the proliferation of breast cancer cells represents a novel role for this classical iron storage protein. Ferritin may have strong implications in breast cancer as the elevation in serum ferritin, which correlates with disease stage (Jones et al. 1980; Robertson et al. 1991), may have tumorigenic influences on the growing tumor. In light of the great number of breast cancer patients with elevation in serum ferritin, the development of therapies targeting ferritin functionality or its secretion represents an attractive therapeutic strategy. Our study also raises questions about the influence of diet and life style changes, which may alter serum ferritin levels, on tumorigenesis and disease progression. Identifying the mechanism underlying the ferritin effects as well as elucidating the connection between serum ferritin and tumor-associated macrophages are important future research questions.
Table 3.1. Characteristics of breast cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Estrogen Receptor</th>
<th>HER2/neu</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Breast Adenocarcinoma</td>
<td>+</td>
<td>-</td>
<td>Epithelial</td>
</tr>
<tr>
<td>T47D</td>
<td>Breast Invasive Ductal Carcinoma</td>
<td>+</td>
<td>-</td>
<td>Epithelial</td>
</tr>
<tr>
<td>SKBR3</td>
<td>Breast Invasive Ductal Carcinoma</td>
<td>-</td>
<td>+</td>
<td>Epithelial</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast Adenocarcinoma</td>
<td>-</td>
<td>-</td>
<td>Mesenchymal</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>Normal Breast</td>
<td>-</td>
<td>-</td>
<td>Epithelial</td>
</tr>
</tbody>
</table>
Table 3.2. The intracellular signaling molecules examined by Pathscan intracellular signaling array kit. Every block represents duplicate spots, except for positive (1 spot) and negative (3 spots) controls.

<table>
<thead>
<tr>
<th>Positive Control</th>
<th>ERK1/2 (p-T202/Y204)</th>
<th>Stat1 (p-Y701)</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat3 (p-705)</td>
<td></td>
<td>AKT (p-T308)</td>
<td>AKT (p-S473)</td>
</tr>
<tr>
<td>AMPKα (p-T172)</td>
<td>S6 Ribosomal Protein (p-S235/236)</td>
<td></td>
<td>mTOR (p-S2448)</td>
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<tr>
<td>HSP27 (p-S78)</td>
<td>Bad (p-S112)</td>
<td></td>
<td>p70 S6 Kinase (p-T389)</td>
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<tr>
<td>PRAS40 (p-T246)</td>
<td>p53 (p-S15)</td>
<td></td>
<td>p38 (p-T180/Y182)</td>
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<tr>
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<td>PARP (D214)</td>
<td></td>
<td>Caspase-3 (D175)</td>
</tr>
<tr>
<td>Positive Control</td>
<td>GSK-3β (p-S9)</td>
<td></td>
<td>Negative Control</td>
</tr>
</tbody>
</table>
FIGURES

Figure 3.1. Extracellular ferritin stimulates the proliferation of breast cancer cells.

(A) MTS signal in a cancer cell line panel following exposure to varying concentration of human liver ferritin for 48 hours under serum-free conditions. Note that the y-axis starts at %80. (B) BrdU incorporation in breast cancer cell lines following exposure to human liver ferritin for 48 hours under serum-free conditions. (C) Ferritin has a concentration-dependent effect on BrdU incorporation in MCF7 cells. (D) The contribution of fetal bovine serum (FBS) to ferritin’s proliferative effects on MCF7 cells. Each treatment group was compared to its respective untreated control. (E) FBS contains low levels of ferritin as measured by a human ferritin ELISA. All error bars, s.e.m. All quantitative data were generated from a minimum of three replicates. n.s.: not significant. *p<0.05, **p<0.01 between the indicated conditions and untreated controls. One-way ANOVA with Dunnett’s post-hoc test as used to compare treated and control cells. Student’s t-test was used to compare conditions in E.

(A)
(B) Ferritin (nM)

% Control (BrdU)

MCF7  T47D  SKBR3  MDA-MB-231  MCF10A

(C) Ferritin (nM)

% Control (BrdU)

0  5  10  25  50  100  200

(D) Fetal Bovine Serum (FBS)

% Control (MTS)

10%  15%  20%  25%  30%  35%  40%

(E) Ferritin (nM)

Serum-free Media  FBS
Figure 3.2. Extracellular ferritin is taken up by breast cancer cells. (A) Ferritin was conjugated to AlexaFluor488 (green) and added to MCF7 and MDA-MB-231 cells for 1 hour in 37°C. The incubation of MCF7 cells in 4°C inhibited this uptake. Cells were stained for α-tubulin (red) and nuclei (Blue; DAPI). Bars: 50 μm [magnification: 20x]. (B) Flow Cytometry analysis of ferritin uptake in MCF7. Incubation with unlabeled ferritin (five hundred-fold) decreased the strength of the signal suggesting specific uptake.
Figure 3.3. Ferritin has an iron-independent effect on breast cancer cells. Both Apo- and holo-ferritin stimulated BrdU incorporation in MCF7 cells under serum free conditions after 48 hours suggesting an iron-independent functionality. All quantitative data were generated from a minimum of three replicates. *p<0.05, **p<0.01 between the indicated conditions and untreated controls. One-way ANOVA with Dunnett’s post-hoc test was used to compare treated and control cells.
**Figure 3.4. The iron-content of ferritin does not influence its proliferative effects on breast cancer cells.** (A) Perls Prussian blue and Coomassie staining of apo-ferritin following incubation with varying concentrations of ferrous ammonium sulfate (FAS). (B) MTS signal in MCF7 cells following incubation with varying concentrations of ferrous ammonium sulfate with or without a fixed concentration of apo-ferritin (C) MTS signal in MCF7 cells following incubation with varying concentrations of apo-ferritin with or without a non-toxic concentration of ferrous ammonium sulfate (FAS). All error bars, s.e.m. *p<0.05, **p<0.01 between the indicated conditions and untreated controls. One-way ANOVA with Dunnett’s post-hoc test or Student’s t-test were used to compare the the parited conditions in B and C.
Figure 3.5. Ferritin does not increase intracellular iron levels in breast cancer cells.

(A) Calcein AM-quenching assay showing no significant change in intracellular iron levels following ferritin exposure in MCF7 cells after 2 and 5 hours. (B) The membrane-permeable iron complex 3,5,5-trimethylhexanoyl-ferrocene (TMHF) and (C) the iron delivery molecule, transferrin, quench the calcein signal after 2 and 5 hours respectively demonstrating iron delivery. All error bars, s.e.m. *p<0.05, **p<0.01 between the indicated conditions and untreated controls. One-way ANOVA with Dunnett’s post-hoc test was used to compare treated and control cells.
Figure 3.6. Ferritin does not decrease the expression levels of transferrin receptor.

(A&B) Western blotting of transferrin receptor in MCF7 cells following exposure to 50 nM of human liver ferritin for 24 hours. Expression levels were normalized to β-actin levels. (C) The iron chelator deferoxamine (50 μM; DFO) increases TfR levels while ferric ammonium citrate (100 μM; FAC) decreases it in MCF7 cells within 24 hours. All error bars, s.e.m.; (n.s.) not significant according to Student’s t-test.
Figure 3.7. Ferritin and the iron delivery molecule transferrin have additive effects. 

(A) MTS signal in MCF7 following exposure to human holo-transferrin for 48 hours under serum free conditions. (B) MTS signal in MCF7 cells treated with 50nM of both human liver ferritin and transferrin. All error bars, s.e.m. All quantitative data were generated from a minimum of three replicates. *p<0.05, **p<0.01 between the indicated conditions. One-way ANOVA with Dunnett’s post-hoc test or Student’s t-test were used when appropriate.
**Figure 3.8. Ferritin does not activate the major proliferation pathways.** (A&B) The effects of IGF-1 (100 ng/ml) on intracellular signaling following 15 minutes of exposure as detected by Pathscan intracellular signaling array. (C&D) The effects of short term exposure to 25 nM of human liver ferritin or PBS on the activation of proliferation/survival pathways in MCF7 cells. (E&F) 24-hour exposure to ferritin did not activate proliferation/survival pathways in MCF7 cells.
**Figure 3.9. Ferritin does not activate NFκB signaling.** (A) Western blot analysis showing no phosphorylation of IKKα/β following exposure to 25 nM ferritin extracted from the human liver. (B) ELISA quantification of total NFκB p65 protein levels and its activated form (S536) in MCF7 cells following short term exposure to PBS or ferritin. As a positive control, we used Jurkat cells treated with 100 nM calyculin A and 1 mM pervanadate to inhibit multiple serine/threonine and tyrosine protein phosphatases.
**Figure 3.10. Ferritin does not activate AKT signaling.** (A) Western blotting showing no significant activation of AKT (S473) following treatment with 25 nM liver ferritin or apo-ferritin. (B) Simple alteration in culturing conditions of MCF7 cells led to weak activation of AKT signaling. Cells were taken out of their 37°C humidified incubators, placed at room temperature for 2 minutes without addition of any reagents and then placed back in the incubator.
Figure 3.11. The L-ferritin receptor Scara5 is expressed in multiple cancer cell lines and tumors. (A) Recombinant human Scara5 is detected by the Scara5 antibody. (B) Western blotting showing Scara5 expression in breast cancer cell lines. Both cells responsive (i.e. MCF7) and unresponsive (i.e. MDA-MB-231) to ferritin expressed Scara5. (C) Scara5 expression in glioma and breast tumor lysates. Based on amino acid sequence, the expected size of Scara5 is 53 kDa.
REFERENCES


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**Int J Cancer** **131**(2): 426-37.


CHAPTER 4

Elevation in the Inflammatory Serum Biomarkers Serum Ferritin and C-Reactive Protein Predicts Response to Trastuzumab-Containing Therapy

INTRODUCTION

Inflammation plays a critical role in breast cancer development and progression (Coussens and Werb 2002; Joyce and Pollard 2009). Epidemiological studies have consistently demonstrated that the chronic use of anti-inflammatory drugs is associated with reduced breast cancer incidence and mortality (Cotterchio et al. 2001; Johnson et al. 2002; Holmes et al. 2010). Moreover, inflammatory serum biomarkers, such as C-reactive protein (CRP) and serum ferritin, are elevated in breast cancer patients and correlate with advanced tumor stage and poor clinical outcome (Jacobs et al. 1976; Jones et al. 1980; Robertson et al. 1991; Pierce et al. 2009; Allin et al. 2011).

The role of the inflammatory microenvironment in modulating response to cancer therapy has only been recently appreciated (DeNardo et al. 2011; Shree et al. 2011; Nakasone et al. 2012). For example, blockage of monocyte/macrophage recruitment factors can improve response to chemotherapy and reduce metastasis to the lungs in a mouse mammary tumor model (DeNardo et al. 2011). Also, inhibition of macrophage-derived cathepsins in vivo increases the efficacy of chemotherapeutic agents against primary and metastatic sites (Shree et al. 2011). Imaging studies have provided further evidence showing that infiltration of myeloid cells into tumors impedes therapy response (Nakasone et al. 2012). Taken together, these studies suggest that drug distribution within
the tumor increases with vascular permeability, which can be negatively influenced by macrophage-derived factors (Priceman et al. 2010; DeNardo et al. 2011; Nakasone et al. 2012).

Trastuzumab is a humanized monoclonal antibody targeting the HER2/neu growth factor receptor. When administered as a single agent or in combination with chemotherapy, trastuzumab impedes tumor progression and increases survival of HER2/neu-overexpressing breast cancer patients (Slamon et al. 2001). However, approximately half of all HER2/neu-overexpressing breast cancer patients do not respond to trastuzumab-containing therapy (Slamon et al. 2001), and only 25% of patients respond when trastuzumab is given as a first-line mono-therapy (Vogel et al. 2002). In addition, trastuzumab therapy is associated with severe and possibly life-threatening cardiac dysfunction which occurs in 10-20% of treated patients (Slamon et al. 2001). Therefore, there remains an urgent and unmet clinical need to develop predictive biomarkers for trastuzumab response to spare them from the needless financial and physical burden.

Few studies have provided insights into the mechanism of trastuzumab resistance. Yu et al. have demonstrated that PTEN activation is an early molecular event involved in the anti-tumor activity of trastuzumab, and its loss was predictive of trastuzumab response in patients (Nagata et al. 2004). Moreover, Insulin-like growth factor I (IGF-I) signaling was implicated as a possible resistance mechanism through rapid activation of AKT, and its receptor (IGF-IR) was shown to bind and phosphorylate HER2 (Lu et al. 2001; Nahta et al. 2005). Consistently, HER2 heterodimerization was shown to predict trastuzumab resistance through its activation of the PI3K/AKT pathway (Ghosh et al.)
Overall, the existing literature supports the concept that trastuzumab resistance is induced by the hyper-activation of the PI3K/AKT signaling pathway.

Because inflammation within the tumor might be decreasing the efficacy of cancer therapeutics, we hypothesize that the elevation in inflammatory biomarkers is associated with a decrease in therapy response. The aim of this study is to evaluate the clinical utility of the inflammatory biomarkers serum ferritin and CRP in predicting response to trastuzumab-containing therapy in advanced breast cancer patients.

MATERIALS AND METHODS

Ethics statement:

Signed informed consent to participate in the present study was obtained from all patients before sample collection. This study was reviewed and approved by the institutional review boards at the Pennsylvania State University Hershey Medical Center and the University of Vienna.

Patients:

A comprehensive description of the eligibility criteria for this patient series was previously reported (Kostler et al. 2004). The patient characteristics are summarized in Table 4.1. Briefly, eligible patients had HER2/neu- overexpressing (immunohistochemistry 2+ or 3+ as determined by the HercepTest; DAKO Diagnostics, Austria) metastatic breast cancer and were scheduled to receive trastuzumab (Herceptin; Roche Pharmaceuticals, Vienna, Austria) +/- chemotherapy at the discretion of the treating physician. Trastuzumab (4 mg/kg of body weight i.v. loading dose for 90 min followed by a weekly 2 mg/kg maintenance dose for 30 min.) administered until evidence
of disease progression, consent withdrawal, or toxicity prompting cessation of treatment. Blood was drawn into native tubes immediately before each infusion of trastuzumab. Serial serum was also collected from some patients during follow-up visits when possible.

Complete response was defined as a complete disappearance of any tumor-related symptoms and all lesions in imaging studies, without appearance of any new lesions lasting for at least 4 weeks. Partial response was defined as a 50% decrease in diameter of all measurable lesions. Progression was defined as a 25% increase in the products of all measurable lesions, an unequivocal increase of nonmeasurable disease, or the appearance of new lesions. The median clinical follow-up duration was approximately 5.5 years.

In order to compare our findings to other disease types, we also examined serum ferritin in prostate cancer patients with castration resistant advanced tumors. This patient cohort has been previously described (Oh et al.).

**Ferritin and CRP measurements:**

Ferritin and CRP levels were determined using a human ferritin or CRP ELISA (AssayPro Corp., St. Charles, MO).

**Statistical analysis:**

Statistics for the pretreatment serum biomarkers were analyzed as dichotomous (median cutoff point) groups. Univariate analysis for progression-free survival (PFS) and overall survival (OS) was performed using the Cox proportional Hazards model. Multivariate analysis for PFS and OS was analyzed using Cox modeling. The correlation between serum ferritin and CRP was examined using Pearson’s correlation coefficient. For all analyses, a $p < 0.05$ was considered statistically significant.
RESULTS

In order to examine the predictive value of inflammatory biomarkers in breast cancer patients, we measured serum ferritin and CRP in HER2/neu-overexpressing patients before undergoing trastuzumab-containing therapy (Patients characteristics are described Table 4.1). We examined overall and progression-free survival to assess therapy responsiveness.

Of the 66 patients, 33 (50%) had elevated pre-treatment serum ferritin levels which were defined as being greater than the median, which at 250 ng/ml is near the upper limit of the normal range reported for serum ferritin (Liu et al. 2003; O’Meara et al. 2011) (Table 4.2, Figure 4.1 A). We next examined the value of serum ferritin in predicting overall survival (OS) and progression-free survival (PFS) in the trastuzumab-treated series. When analyzed as dichotomous categorical groups using the median pretreatment serum ferritin level (250 ng/ml) as a cutoff point, the patients with elevated serum ferritin had a significantly reduced OS (Figure 4.2 A, \( p < 0.0001 \), median OS 12.73 vs. 69.57 months) and PFS (Figure 4.2 B, \( p = 0.004 \), median 8.30 vs. 23.90 months) compared to patients with serum ferritin values below the median.

CRP also showed a wide distribution of values in HER2/neu-overexpressing patients (Table 4.2, Figure 4.1 B; Range: 0.26-114.88 mg/l). When analyzed as dichotomous groups with the median CRP level (7.25 mg/l) as a cutoff point, higher CRP predicted shorter OS (Figure 4.2 C, \( p = 0.009 \), median OS 17.3 vs. 69.5 months) and reduced PFS (Figure 4.2 D, \( p = 0.002 \), median PFS 8.3 vs. 16.1 months). Therefore, both of the inflammatory biomarkers serum ferritin and CRP have strong prognostic and
predictive value in advanced breast cancer patients receiving trastuzumab-containing therapy. CRP displayed a moderate linear correlation with serum ferritin (Pearson’s correlation coefficient, \( p<0.0001, r^2 = 0.4436; \) Figure 4.1 C).

In order examine the independent prognostic value of both serum ferritin and CRP, we stratified patients in the trastuzumab-treated series by serum ferritin and CRP levels using the median values as the cutoff points. We created four groups: Low CRP/Low ferritin, High CRP/Low Ferritin, Low CRP/ High ferritin, and High CRP/High ferritin. It is important to note that the median value of CRP in this cohort is more than three-fold higher than previously reported (Pierce et al. 2009; Allin et al. 2011), while the median value for serum ferritin is relatively close to the upper limit of the reported normal range (Liu et al. 2003; O’Meara et al. 2011). Patients with high serum ferritin and high CRP had the poorest response to trastuzumab-containing therapy as assessed by both overall survival (Figure 4.3 A) and progression-free survival (Figure 4.3 B). It is noteworthy that we observed that the increase in mortality and progression in patients with elevated levels of inflammatory biomarkers occurs primarily in the first year of treatment. Almost 75% of patients with elevated levels of both serum ferritin and CRP died or progressed in the first year compared to only 25% in the other patients groups.

It is unlikely that the elevation in serum ferritin and CRP is merely reflective of more advanced or terminal disease. Multivariate analysis of this patient group (Table 4.3) have shown that the serum biomarker CA 15-3 (a marker of breast tumor burden) is not predictive of progression free survival (HR 1.00027, \( p =0.25 \)) and is barely significant in predicting overall survival (HR 1.00048, \( p =0.046 \)). Moreover, serum ferritin was elevated in approximately 60% of patients with metastatic prostate cancer but this
elevation was not predictive of shorter survival (Figure 4.4 A & B). The lack of
prognostic value for serum ferritin in prostate cancer patients indicates that its elevation
is not merely a biomarker for terminal disease. This also indicates that its prognostic
value in breast cancer patients may be specific to this disease or the chosen therapeutic
intervention.

Interestingly, patients with high serum ferritin/low CRP had a significantly poorer
outcome compared to patients with low serum ferritin/low CRP (Figure 4.3 A, \( p = 0.02 \)),
while the low serum ferritin/high CRP patient group was not statistically different (Figure
4.3 A, \( p=0.186 \)). Furthermore, multivariate analysis using estrogen receptor status, age,
treatment modality, and CA 15-3 in addition to serum ferritin and CRP as covariates
showed a stronger prognostic value for serum ferritin than CRP. Serum ferritin remained
an independent factor for progression-free survival (HR 2.22, \( p=0.023 \)) and overall
survival (HR 3.43, \( p=0.002 \)), while CRP was only an independent factor for progression-
free survival (HR 2.6, \( P=0.006 \)) but not overall survival (\( P=0.072 \)). These observations
raise the possibility that serum ferritin is an independent prognostic factor for breast
cancer patients and its prognostic value is not dependent on the elevation in other
inflammatory biomarkers.

In order to examine the changes in serum ferritin and CRP that might occur
following treatment, serial serum was collected from ten patients (15% of total patients
participating) in the subsequent visits after the first injection. Because pre-treatment
serum ferritin in those patients showed more variability than CRP, we divided patients
into three groups based on their pre-treatment serum ferritin levels: 1) within the normal
range (\(< 200 \text{ ng/ml, Figure 4.5}\), 2) moderately elevated (200-1000 ng/ml, Figure 4.6),
and 3) highly elevated (>1000 ng/ml, Figure 4.7). All of the patients with low pre-treatment ferritin (n=4) showed modest fluctuations in their serum ferritin levels during the course of the treatment, and no value exceeded 500 ng/ml (Figure 4.5). On the other hand, CRP values showed drastic fluctuations in some patients (patients 35 and 82) that were not similar to the trends seen with serum ferritin. In the second group (Figure 4.6), two patients (patient 11 and 41) showed a declining trend with the progression of treatment, whereas one patient (patient 45) showed a consistent increase following treatment. When compared to CRP, almost all of the patients had low pre-treatment CRP levels (<10 mg/L). Moreover, CRP levels did not replicate the same trends observed in serum ferritin. In the third group (Figure 4.7), two patients (patients 21 and 71) showed a strong and consistent decline in serum ferritin levels following treatment. In one patient (patient 21), the levels of serum ferritin returned to normal levels (~200 ng/ml) after the 5th visit. When compared to CRP, only patient 21 showed a strong and consistent decrease in CRP mirroring the decrease in serum ferritin. The other two patients seemed to have CRP levels within the normal range.

**DISCUSSION**

In this report, we have demonstrated that both serum ferritin and CRP are strong prognostic factors in advanced breast cancer patients and can predict response to trastuzumab-containing therapy. Patients with high levels of both inflammatory biomarkers had the poorest clinical outcome suggesting that cancer-associated inflammation may have clinical utility in predicting response to trastuzumab-containing therapy.
Elevation in inflammatory biomarkers is a common phenomenon amongst patients with advanced cancers (Heikkila et al. 2007). However, most of those studies utilized only one inflammatory biomarker which might have increased error as the circulating levels of many inflammatory biomarkers are often influenced by environmental (diet, exercise, body mass, etc.) and genetic factors (Tchernof et al. 2002; Jehn et al. 2004; Crawford et al. 2006; Ma et al. 2006; Allen et al. 2008). In our study, we have observed that: 1) some advanced cancer patients have normal levels of both CRP and serum ferritin, 2) some patients have elevation in only one of the two biomarkers which might be caused by non-inflammatory factors or sub-threshold inflammation, 3) some patients have elevation in both biomarkers indicating a robust inflammatory state. Strikingly, patients with elevation in both biomarkers had the poorest response to therapy providing evidence that inflammation might be the underlying phenomenon for the decrease in therapy response.

It is still unclear what causes the elevation in some inflammatory biomarkers but not others in patients with comparable disease. In our study, we have observed that almost one third of the patients studied had elevation in either serum ferritin alone or CRP alone. As mentioned, there was also a moderate linear correlation ($r^2 = 0.4436$) between serum ferritin and CRP levels. These observations suggest that serum ferritin and CRP might not be produced by the same mechanism or cell type. CRP is believed to be produced exclusively by the liver (Zhang et al. 1995; Streetz et al. 2001). On the other hand, the source of serum ferritin in physiological or pathological conditions is still unclear. Although immortalized hepatocytes have been shown to secrete ferritin in vitro (Tran et al. 1997; Ghosh et al. 2004), several in vivo studies have argued that ferritin is
secreted primarily by macrophages and that hepatocytes do not contribute significantly to
the secretion of ferritin into circulation (Ferring-Appel et al. 2009; Cohen et al. 2010;
Zhang et al. 2011). Therefore, the elevation in serum ferritin might reflect an
inflammatory state involving macrophages –systemically from macrophage-rich sites (i.e.
spleen and bone marrow), and locally from within the tumor microenvironment. The
relative contribution of tumor-associated macrophages to the systemic increase in serum
ferritin requires further investigation.

Patients with low serum ferritin or low CRP had a median survival approximately
six times longer than patients with high serum ferritin or CRP (Figure 4.2 A & C). More
importantly, patients with high levels of serum ferritin or CRP had a shorter progression-
free survival indicating that that trastuzumab-containing therapy was not effective (Figure
4.2 B & D). Therefore, our data suggests that cancer-associated inflammation, as assessed
by serum ferritin and CRP, is either 1) inducing resistance to cancer therapies
(trastuzumab alone, chemotherapy alone, or the combination of both) either directly
through activation of molecular pathways or indirectly through affecting the structure and
density of tumor vasculature and thus decreasing drug distribution, or 2) enhancing
tumorigenesis through various mechanisms that counteract and compensate for the anti-
tumorigenic effects of this therapy.

It is possible that the elevation in inflammatory biomarkers represents a subset of
breast cancer patients with more aggressive disease that may or may not be inducing
resistance to therapy. However, several recent studies have demonstrated that the
inflammatory tumor microenvironment may contribute directly to therapy resistance. For
example, infiltration of myeloid cells into tumors can impede therapy response through
the production of proteases (i.e. Cathepsins, MMP9) or angiogenic factors (i.e. VEGF) (Priceman et al. 2010; DeNardo et al. 2011; Shree et al. 2011; Nakasone et al. 2012). In one study, cathepsin proteases seemed to increase therapy resistance in cancer cells directly (Shree et al. 2011). Aside from their ability to induce therapy resistance in cancer cells, the presence and activation of inflammatory cells within the tumor can affect vascular structure and permeability, and thus drug distribution within the tumor (Priceman et al. 2010; Rolny et al. 2011; Nakasone et al. 2012). Those studies indicate that inflammatory cells, such as macrophages, can impede drug response on multiple levels. They can induce therapy resistance in cancer cells directly or decrease drug distribution within the tumor.

The continuous monitoring of inflammatory biomarkers, specifically serum ferritin, in patients receiving trastuzumab-containing therapy may be useful in identifying responsive patients. Serum ferritin levels did not fluctuate drastically as much as CRP possibly indicating a longer serum half life as previously reported (Feelders et al. 1998). Several patients (patients 21, 71, 11, and 41) showed a consistent decrease in serum ferritin levels following treatment with some cases returning to normal levels. Although it is hard to draw strong conclusions from these limited observations, the decrease in inflammatory biomarkers might suggest a decrease in tumor size and subsequently the activation of immune cells in the tumor microenvironment. Therefore, trastuzumab, chemotherapy, or the combination of both might be directly or indirectly affecting the causes of the elevation in inflammatory biomarkers. Further investigation of the predictive value of serum ferritin and other inflammatory biomarker to monitor response in patients is warranted.
Based on our data showing an independent prognostic value for serum ferritin (Figure 4.3 A), it is tempting to speculate that serum ferritin may be tumorigenic. Recently, the ferritin receptor, Scara5, has been included in a prognostic gene panel for breast cancer indicating that the capability of cancer cells to uptake ferritin may confer a survival or proliferative advantage (Miller et al. 2011). Moreover, several lines of evidence have suggested that extracellular ferritin may have angiogenic, immunosuppressive and iron delivery roles (Broxmeyer et al. 1989; Coffman et al. 2009; Todorich et al. 2011). However, these studies did not address the role of serum ferritin in cancer patients or whether ferritin can interact directly with cancer cells.

CONCLUSIONS

In summary, this study demonstrates that elevation in the inflammatory biomarkers serum ferritin and CRP is a common phenomenon in advanced breast cancer patients and is predictive of response to trastuzumab-containing therapy. Further investigation of the mechanisms underlying the strong association between the elevation in inflammatory biomarkers and therapy responsiveness is warranted. Moreover, the clinical utility of anti-inflammatory drugs and life-style changes that may decrease levels of inflammatory biomarkers should be explored as possible strategies to sensitize patients to trastuzumab-containing therapy.
**Table 4.1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Total number of Patients</th>
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<tbody>
<tr>
<td>Mean Age</td>
<td>53.6 years</td>
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<tr>
<td>Std. Deviation</td>
<td>11.4 years</td>
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<tr>
<td>Treatment</td>
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<tr>
<td>Trastuzumab + Chemotherapy</td>
<td>58 (88%)</td>
</tr>
<tr>
<td>Trastuzumab alone</td>
<td>8 (12%)</td>
</tr>
<tr>
<td>Line of Chemotherapy</td>
<td></td>
</tr>
<tr>
<td>First-line</td>
<td>53 (80%)</td>
</tr>
<tr>
<td>Second-line</td>
<td>9 (13.5%)</td>
</tr>
<tr>
<td>Third-line</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (1.5%)</td>
</tr>
<tr>
<td>Estrogen Receptor Status</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>33 (50%)</td>
</tr>
<tr>
<td>Positive</td>
<td>33 (50%)</td>
</tr>
<tr>
<td>Mean Follow-up</td>
<td>3.1 years</td>
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</tbody>
</table>
Table 4.2. Serum ferritin and CRP levels in HER2/neu-overexpressing breast cancer patients (n=66).

<table>
<thead>
<tr>
<th></th>
<th>Serum Ferritin (ng/ml)</th>
<th>CRP (mg/l)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Minimum</td>
</tr>
<tr>
<td></td>
<td>11.55</td>
<td>0.26</td>
</tr>
<tr>
<td>25&lt;sup&gt;th&lt;/sup&gt; percentile</td>
<td>98.45</td>
<td>2.23</td>
</tr>
<tr>
<td>Median</td>
<td>249.99</td>
<td>7.26</td>
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<tr>
<td>75&lt;sup&gt;th&lt;/sup&gt; percentile</td>
<td>490.79</td>
<td>13.18</td>
</tr>
<tr>
<td>Maximum</td>
<td>3270</td>
<td>114.88</td>
</tr>
<tr>
<td>Mean</td>
<td>443.26</td>
<td>13.70</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>624.50</td>
<td>21.12</td>
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</table>
Table 4.3. Multivariate analysis of patients receiving trastuzumab-containing therapy with all of the available data.

<table>
<thead>
<tr>
<th></th>
<th>Overall Survival</th>
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<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>p value</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>3.435</td>
<td>1.54-7.63</td>
<td>0.002</td>
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<tr>
<td>CRP</td>
<td>2.001</td>
<td>0.94-4.26</td>
<td>0.072</td>
</tr>
<tr>
<td>Age</td>
<td>0.945</td>
<td>0.91-0.98</td>
<td>0.002</td>
</tr>
<tr>
<td>CA 15-3</td>
<td>1.000487</td>
<td>1.00-1.00</td>
<td>0.046</td>
</tr>
<tr>
<td>Therapy Modality</td>
<td>1.983</td>
<td>0.64-6.11</td>
<td>0.233</td>
</tr>
<tr>
<td>Estrogen Receptor</td>
<td>0.467</td>
<td>0.21-1.02</td>
<td>0.057</td>
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<table>
<thead>
<tr>
<th></th>
<th>Progression-free Survival</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>p value</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>2.219</td>
<td>1.11-4.12</td>
<td>0.023</td>
</tr>
<tr>
<td>CRP</td>
<td>2.604</td>
<td>1.32-5.12</td>
<td>0.006</td>
</tr>
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<td>Age</td>
<td>0.969</td>
<td>0.93-0.99</td>
<td>0.047</td>
</tr>
<tr>
<td>CA 15-3</td>
<td>1.00027</td>
<td>0.99-1.00</td>
<td>0.25</td>
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<tr>
<td>Therapy Modality</td>
<td>0.413</td>
<td>0.18-0.94</td>
<td>0.035</td>
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<tr>
<td>Estrogen Receptor</td>
<td>0.703</td>
<td>0.37-1.34</td>
<td>0.285</td>
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</table>

HR: Hazard Ratio. CI: Confidence Interval.
Serum ferritin (low vs. high; median cutoff)
CRP (low vs. high; median cutoff)
Age (continuous variable)
CA 15-3 (continuous variable)
Therapy modality (trastuzumab monotherapy vs. combinational therapy)
Estrogen receptor (positive vs. negative)
FIGURES

Figure 4.1. Serum ferritin (A) and CRP (B) levels in HER2/neu-overexpressing patients before undergoing trastuzumab-containing therapy (n=66). The pretreatment values of both serum biomarkers were moderately correlated (C).

(A)  
(B)  
(C)
Figure 4.2. Serum ferritin and CRP predict response to trastuzumab-containing therapy. Pre-treatment serum ferritin and CRP predict overall survival (A&C respectively) and progression-free survival (PFS; B&D respectively) in patients receiving trastuzumab-containing therapy. Low serum ferritin or CRP (<250 ng/ml and <7.25 mg/l respectively) had an overall better clinical outcome than high ferritin or CRP. Difference in OS and PFS were analyzed by Kaplan-Meier survival model with the median serum ferritin or CRP value as a cutoff point.
Figure 4.3. Patients with elevation in both serum ferritin and CRP have the poorest response to trastuzumab-containing therapy. Kaplan-Meier analysis of overall survival (A) and progression-free survival (B) in trastuzumab-treated patients stratified by their CRP and serum ferritin levels. High CRP (>7.25 mg/l); High ferritin (>250 ng/ml).

(A)
Figure 4.4. Serum ferritin is elevated in metastatic prostate cancer patients but is not predictive of overall survival. (A) Serum ferritin levels in patients with metastatic castration-resistant prostate cancer (n=60). (B) Kaplan-Meier survival analysis showing that elevation in serum ferritin levels is not predictive of overall survival. Low ferritin: <175 ng/ml; Mid Ferritin: 175-528 ng/ml; High ferritin: > 528 ng/ml. No significant associations were detected when patients were stratified into three groups (shown below) or dichotomous groups using the media value (528 ng/ml; Data not shown) as a cutoff point.
Figure 4.5. Serum ferritin (A) and CRP (B) in advanced breast cancer patients with pre-treatment serum ferritin levels below the normal range (<200 ng/ml). Patients received trastuzumab-containing therapy on their first visit. The serum samples from the first visit were taken immediately before drug infusion.
Figure 4.6. Serum ferritin (A) and CRP (B) in advanced breast cancer patients with moderate (200-1000 ng/ml) pre-treatment serum ferritin levels. Patients received trastuzumab-containing therapy on their first visit. The serum samples from the first visit were taken immediately before drug infusion.
Figure 4.7. Serum ferritin (A) and CRP (B) in advanced breast cancer patients with high (>1000 ng/ml) pre-treatment serum ferritin levels. Patients received trastuzumab-containing therapy on their first visit. The serum samples from the first visit were taken immediately before drug infusion.

(A) Serum ferritin levels over visits.

(B) CRP levels over visits.
REFERENCES


heterodimerization contributes to trastuzumab resistance of breast cancer cells."

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CHAPTER 5

Elevation in Multiple Serum Inflammatory Biomarkers Predicts Survival of Pancreatic Cancer Patients with Inoperable Disease

INTRODUCTION

Pancreatic cancer is the fourth-leading cause of cancer death in the United States and has the lowest 5-year survival rate of any cancer (Parker et al. 1996; Siegel et al. 2012). Therefore, there is an urgent and unmet clinical need for the development of reliable biomarkers that can aid in the early detection, monitoring, and treatment of pancreatic cancer patients. Currently, only CA 19-9 has been recommended as a biomarker to predict outcome and response to therapy in pancreatic cancer patients (Steinberg 1990; Rocha Lima et al. 2002; Locker et al. 2006). However, more sensitive and clinically meaningful biomarkers are needed to aid in the stratification of patients, and to uncover the molecular mechanisms underlying the pathogenesis of this aggressive disease.

Inflammation plays a critical role in pancreatic cancer development and progression. Early epidemiological reports have demonstrated that chronic pancreatitis is associated with a higher risk for pancreatic cancer (Lowenfels et al. 1993). On a molecular level, the onset of inflammation in the pancreas can amplify oncogene activity and inhibit cellular senescence all of which drives the transformation of pre-cancerous lesions (Fukuda et al. 2011; Guerra et al. 2011; Daniluk et al. 2012). Moreover, inflammation is associated with increased epithelial-to-mesenchymal transition (EMT)
and tumor dissemination even before the development of detectable tumors (Rhim et al. 2012). These studies provide a strong argument that inflammation may contribute not only to the development of pancreatic cancer, but also to its aggressive, highly-metastatic behavior.

Inflammatory serum biomarkers are elevated in a majority of pancreatic cancer patients (Falconer et al. 1995; Jamieson et al. 2005; Tingstedt et al. 2007; Douglas et al. 2011). Most of those studies focused primarily on the inflammatory biomarker C-reactive protein (CRP) and found it to have prognostic value in patients with inoperable pancreatic tumors as well as after undergoing surgical resection. However, elevated CRP levels lacked diagnostic value as they failed to distinguish pancreatic cancer patients in case-control studies (Douglas et al. 2011). Because CRP is influenced by various genetic and environmental factors (Tchernof et al. 2002; Crawford et al. 2006; Ma et al. 2006; Timpson et al. 2011), a more specific inflammatory signature is needed to improve the clinical and prognostic utility of cancer-associated inflammation in pancreatic cancer patients.

Serum ferritin is elevated in multiple human malignancies including pancreatic cancer (Hann et al. 1985; Basso et al. 1991; Robertson et al. 1991). In breast cancer patients, elevation in serum ferritin was a strong predictor of response to trastuzumab-containing therapy and displayed independent prognostic value from other inflammatory biomarkers (Alkhateeb et al. 2012). In neuroblastoma, the elevation in serum ferritin correlated with advanced disease and was predictive of survival (Hann et al. 1985).

Recent evidence has suggested that serum ferritin is derived primarily from macrophages and not solely the liver as previously believed (Ferring-Appel et al. 2009;
Cohen et al. 2010; De Domenico et al. 2011). Our understanding of the functional significance of serum ferritin has also improved as several studies have demonstrated that extracellular ferritin has immunosuppressive, angiogenic, and iron delivery roles (Broxmeyer et al. 1989; Coffman et al. 2009; Todorich et al. 2011). These studies raise the possibility that the elevation in serum ferritin observed in cancer patients may represent a specific inflammatory state involving macrophages and may have a pro-tumorigenic role.

In light of the recent advances in our understanding of the source and role of serum ferritin, it is logical to examine its prognostic value in pancreatic cancer patients. The aim of this study is to evaluate serum ferritin as a prognostic biomarker for patients with advanced unresectable pancreatic tumors and to expand our understanding of the role of cancer-associated inflammation by comparing the prognostic value of multiple inflammatory biomarkers.

**PATIENTS AND METHODS**

**Ethics statement:** Signed informed consent to participate in the present study was obtained from all patients before sample collection. This study was reviewed and approved by the institutional review board at the Pennsylvania State University Hershey Medical Center.

**Patients:** The Novartis phase 3 trial of octreotide (SMS 201-995 pa LAR (SMS PA LAR) and continuous infusion (CI) 5-FU in unresectable advanced pancreatic cancer has been reported (Roy et al. 1998). Briefly, this was a randomized, double-blind, placebo-controlled, multi-center phase III clinical trial of octreotide [somatostatin (somatotrophin-
release inhibiting factor) analogue, plasma half life of 2 hours) and 5-FU vs. placebo and 5-FU in advance pancreatic cancer. Patient inclusion criteria included: 1) at least 18 years of age, 2) measurable or evaluable, Stage III or IV, pathologically, histologically, or cytologically confirmed unresectable adenocarcinoma of the exocrine pancreas, and 3) no prior chemotherapy, radiation, or hormonal therapy. The trial was powered to achieve 330 evaluable patients (165 per arm) with planned randomization of 412 patients (206 per arm) to detect an increase in the one-year survival rate from 10% in the 5-FU + placebo group to 20% in the 5-FU + octreotide group with a power of 0.85. The primary objective of this trial was overall survival; secondary objectives were progression-free survival (PFS), clinical benefit, tolerability of octreotide, and objective response rate. Treatment after randomization was octreotide (160 mg intramuscular initiation, then every 2 weeks for 4 injections, then every 4 weeks until disease progression) vs. placebo. 5-FU was given immediately following the first octreotide injection, by continuous intravenous infusion at a total daily dose of 225 mg/m2, for 8 weeks, followed by a 7 day rest period, then 5-FU repeated until diseases progression. The trial was stratified for gender, Karnofsky performance status (70–100 % vs. 50–60 %), and stage of disease (III vs. IV).

The interim efficacy analysis of this phase III clinical trial (284 patients) reported one complete (Stage II SMS arm) and two partial remissions (Both Stage IV; 1 SMS: 1 placebo). There was no significant difference in overall survival between treatment arms (P=0.649). The median survival of octreotide-treated patients was 22.6 weeks (95% CI 18.1,27.7) vs 21.6 weeks in the placebo arm (95% CI 17.9,28.3)(p= 0.649) (Roy et al. 1998). Therefore this trial is optimal for biomarker discovery and analysis.
**Ferritin and CRP measurements:** Ferritin and CRP levels were determined using a human ferritin or CRP ELISA (AssayPro Corp., St. Charles, MO).

**Cell lines:** The human pancreatic cancer cell lines PNC-1, Capan-2 and BxPC-3 were obtained from The American Type Culture Collection (ATCC, Manassas, VA) and cultured per manufacturer’s recommendations in 37°C and 5% CO₂.

**Proliferation Studies:** Pancreatic cancer cells (1 x 10⁵ cells in 96-well plate) were seeded in serum-free media, starved for 24 hours, and then exposed to horse spleen Apo-ferritin (Sigma, St. Louis, MO) for 48 hours. Proliferation was assessed by BrdU cell proliferation assay (EMD, Darmstadt, Germany) according to manufacturer’s instructions. The BrdU label was added in the last 24 hours of the experiment before cells were fixed and probed for BrdU incorporation.

**Statistical analysis:** Statistics for the pretreatment serum biomarkers were analyzed both as continuous and dichotomous variables. Univariate analysis for overall survival (OS) was performed using the Cox proportional Hazards model. Multivariate analysis for OS was analyzed using Cox modeling. The correlation between serum and CRP was determined by Pearson’s correlation coefficient. For all analyses, a \( p < 0.05 \) was considered statistically significant.

**RESULTS**

We measured ferritin in pretreatment sera from 159 patients with unresectable pancreatic adenocarcinoma. Serum ferritin was elevated (>300 ng/ml) in 83% of pancreatic cancer patients (Table 1, Figure 5.1 A). Kaplan-Meier analysis of serum ferritin showed the strongest and most significant predictive value when patients were
analyzed as dichotomous categorical groups using the median serum ferritin (840 ng/ml) as a cutoff point (Figure 5.2 A). Median survival for patients with high ferritin was 121 days, whereas patients with low ferritin had a median survival of 182 days (HR 1.64, 95% CI 1.14 to 2.35; \( p = 0.007 \)).

In order to compare the elevation in serum ferritin to other inflammatory biomarkers, we measured the hepatic inflammatory biomarker CRP in the same patients (Table 1, Figure 5.1 B). Kaplan-Meier analysis of CRP showed the strongest and most significant predictive value when patients were analyzed as dichotomous groups using the 25% percentile (7.3 mg/L) as a cutoff point which is comparable to the upper limit of the normal range (Figure 5.2 B). Median survival for patients with high CRP was 132 days, whereas patients with low CRP had a median survival of 229 days (HR 1.81, 95% CI 1.17 to 2.78; \( p = 0.007 \)).

Serum ferritin and CRP demonstrated a weak linear correlation (Figure 5.1 C; \( p = 0.0002 \), \( r^2 = 0.087 \)) suggesting that ferritin and CRP might represent distinct pathological or inflammatory states. Moreover, both serum ferritin and CRP showed independent prognostic value in a multivariate model (\( p = 0.015 \) and \( p = 0.018 \)).

In order to directly examine the independent prognostic significance of both serum ferritin and CRP, we stratified the 159 patients using their serum ferritin and CRP levels, with 840 ng/ml and 7.3 mg/L as cutoff points respectively (Figure 5.3). We created four groups: Low CRP/ Low Ferritin (baseline), Low CRP/ High ferritin, High CRP/Low Ferritin, and High CRP/High ferritin. Patients with only high CRP or high ferritin were not statistically different from the baseline group (\( p = 0.29 \) and \( p = 0.924 \) respectively). On the other hand, patients with elevation in both inflammatory biomarkers
had the worse clinical outcome. Patients with elevated levels of both serum ferritin and CRP had a median survival of 117 days compared to 261 days in the baseline group (HR 2.37, 95% CI 1.4 to 4.0; \( p=0.001 \)). Although patients with High CRP/Low Ferritin did not have a shorter survival than the baseline group, they were statistically different from patients with elevation in both serum ferritin and CRP \( (p=0.007) \). Therefore, the elevation in CRP alone is not predictive of survival in advanced pancreatic cancer patients.

In order to examine the functional significance of extracellular ferritin in pancreatic cancer more directly, we exposed pancreatic cancer cell lines to ferritin, similar in subunit composition to ferritin detected in the serum (Figure 5.4). Exposure to ferritin did not stimulate proliferation of pancreatic cancer cell lines as previously demonstrated in several cell lines (Alkhateeb et al. 2013).

**DISCUSSION**

Most of the studies examining inflammatory biomarkers in pancreatic cancer patients have utilized only one serum biomarker (Basso et al. 1991; Falconer et al. 1995; Jamieson et al. 2005; Tingstedt et al. 2007; Haas et al. 2010). This is potentially problematic as inflammatory biomarkers (such as CRP) are influenced by multiple factors such as genetics, body mass and diet (Tchernof et al. 2002; Crawford et al. 2006; Ma et al. 2006; Timpson et al. 2011). Therefore, the elevation of one inflammatory biomarker might not be sufficiently specific to provide a clinical description of the disease. In this study, we have demonstrated that CRP and serum ferritin are weakly correlated, are independent predictors of survival, and concomitant elevation of both biomarkers identifies patients with the poorest clinical outcome. These findings suggest
that cancer-associated inflammation may be the underlying phenomenon for the prog nostic value for both serum ferritin and CPR.

CRP is a hepatic inflammatory biomarker that is almost exclusively produced by the liver in response to IL-6 via the activation of the stat3 transcription factor (Streetz et al. 2001; Arnaud et al. 2005). On the other hand, serum ferritin could be produced by macrophages (Cohen et al. 2010; De Domenico et al. 2011), and possibly hepatocytes (Tran et al. 1997), in response to inflammatory cytokines activating the NF-κB pathway such as TNFα and IL-1β (Pham et al. 2004). Therefore, serum ferritin and CRP might be produced by different cell types and in response to the activation of different signaling pathways. In pancreatic cancer, stat3 is critical in K-Ras-driven tumor development and dissemination (Fukuda et al. 2011). Furthermore, NF-κB activation amplifies the pathological activity of the K-Ras oncogene in pancreatic tumors (Daniluk et al. 2012). Therefore, the elevation in CRP and serum ferritin in pancreatic cancer patients may be reflective of a robust inflammatory state that activates multiple inflammatory pathways that contributes to increased tumorigenicity in pancreatic tumors.

The widespread elevation in serum ferritin (~83%) in patients with inoperable pancreatic tumors provides further support to the role of inflammation in pancreatic cancer and raises many questions about the predictive value of serum ferritin in a clinical setting. Although CRP’s prognostic utility has been explored, serum ferritin may be more reliable because its elevated levels can persist for a longer time. Studies in animals have shown that elevation in serum ferritin can persist for 10 days while CRP levels drop sharply after 48 hours (Feelders et al. 1998). Therefore, serum ferritin may be a more persistent and accurate indicator of inflammation in cancer patients than CRP.
Although the data do not directly suggest that serum ferritin has functional significance on its own, the size of the patient group with Low CRP/High ferritin might have been too small to make credible conclusions (n=14). However, the patient group with High CRP/Low ferritin (n=53) did not have prognostic value and was statistically different from patients with high levels of both CRP and serum ferritin. Nonetheless and in light of the recent advances in our understanding of serum ferritin, the elevation and independent prognostic value of serum ferritin suggests that ferritin might have functional significance in pancreatic cancer patients. Serum ferritin possess a multifunctional nature that may contribute to tumorigenesis on several levels: 1) extracellular ferritin is capable of binding high molecular weight kininogen inhibiting its anti-angiogenic effects (Coffman et al. 2009), 2) extracellular ferritin has immunosuppressive properties and can inhibit the proliferation of hematopoietic progenitor cells and lymphocytes (Broxmeyer et al. 1989; Fargion et al. 1991), 3) Ferritin can act as an iron delivery molecule in some cell types through receptor-mediated endocytosis (Li et al. 2009; Todorich et al. 2011), and 4) extracellular ferritin is capable of binding and stimulating the proliferation of cancer cells through an iron-independent mechanism (Alkhateeb et al. 2013).

CONCLUSIONS

In this study, we have demonstrated that serum ferritin is an independent prognostic factor in pancreatic cancer patients with inoperable tumors. We also demonstrate that stratification of patients based on multiple inflammatory biomarkers identifies patients with the worst clinical outcome, whereas the elevation in only one of
the two biomarkers does not have prognostic significance. These observations provide support to the concept that there is a range of inflammatory states with distinct clinical presentation. Based on our findings, we propose that further exploration of the prognostic and therapeutic significance of inflammation in pancreatic cancer patients should examine serum ferritin in addition to CRP.
Table 5.1. CRP and Serum ferritin values in pancreatic cancer patients with inoperable disease (n=159).

<table>
<thead>
<tr>
<th>Serum Ferritin (ng/ml)</th>
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<tr>
<td>Minimum</td>
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<tr>
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<tr>
<td>Median</td>
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<tr>
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<table>
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<tbody>
<tr>
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<td></td>
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<tr>
<td>Median</td>
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</tr>
<tr>
<td>Mean</td>
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<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>58.73</td>
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</tbody>
</table>
FIGURES

**Figure 5.1.** Serum ferritin (A) and CRP (B) levels in patients with metastatic pancreatic cancer with inoperable disease. The serum ferritin and CRP values were weakly correlated (C).

(A) 

(B) 

(C) 

P=0.0002  
$r^2 = 0.087$
Figure 5.2. Kaplan-Meier survival analysis of 159 patients with inoperable pancreatic cancer according to serum ferritin (A, cutoff 840ng/ml) and CRP (B, cutoff 7.3 mg/L).
Figure 5.3. Kaplan-Meier survival analysis of 159 patients with inoperable pancreatic cancer stratified according to both serum ferritin and CRP levels. Patients with elevation in only serum ferritin or CRP were not statistically different from the baseline group ($P=0.924$ and $P=0.290$ respectively). Patients with elevation in both inflammatory biomarkers were significantly different from the baseline group ($P=0.001$).
Figure 5.4. Ferritin does not stimulate the proliferation of pancreatic cancer cell lines. Ferritin exposure did not increase BrdU incorporation in BxPC-3, Capan-2 and PNC-1 after 48 hours in serum free conditions. All error bars, s.e.m. Statistical analysis was performed using one-way ANOVA analysis.
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"Secretion of ferritin by rat hepatoma cells and its regulation by inflammatory cytokines and iron." Blood 90(12): 4979-86.
CHAPTER 6

Summary and Future Directions

The main objective of this dissertation project was to elucidate a novel tumorigenic mechanism involving the classical iron storage protein ferritin in breast cancer. Moreover and in a broader clinical context, the project also aimed at the development of serum ferritin as a prognostic and predictive biomarker in advanced cancer patients. The primary novel finding of my thesis is the identification of serum ferritin as a tumorigenic mechanism in breast cancer with clinical value as a prognostic and predictive serum biomarker. I have also identified macrophages as the major cell type expressing ferritin within the breast tumor microenvironment.

In ‘Chapter 2’, I observed changes in the expression and localization of ferritin in breast tumors. As the tumor progresses from a ductal carcinoma in situ to an invasive ductal carcinoma, the ferritin content in cancer cells decreases while tumor infiltration by ferritin-rich macrophages increases. Therefore, I am proposing from my work that the increase in ferritin that has long been noted in breast tumors is not due to increased expression by cancer cells but increased recruitment of macrophages as part of the body’s response to tumor development. Interestingly, we also observed ferritin staining in acellular stromal regions surrounding tumors suggesting local release within the breast. To support this notion we showed that macrophages, but not breast cancer cells, can secrete ferritin, specifically the L subunit, and that this secretion increases with exposure to iron and pro-inflammatory cytokines.
In ‘Chapter 3’, I examined the functional significance of extracellular L ferritin in a breast cancer cell culture model. Ferritin stimulated the proliferation of breast cancer cell lines as assessed by MTS and BrdU incorporation assays which measure total cell number and DNA production respectively. These effects were seemingly independent of iron and were not due to iron delivery because: 1) iron-free ferritin had a similar effect to iron-rich ferritin, 2) the addition of iron did not increase the effects of ferritin, and 3) ferritin exposure did not increase intracellular iron levels. Furthermore, the effects of ferritin on breast cancer cells did not activate NFκB signaling or were mediated by the ferritin receptor Scara5 as previously speculated (Li et al. 2009; Ruddell et al. 2009). Collectively, these findings provide support to the novel idea that ferritin secretion by tumor-associated macrophages directly stimulates breast tumorigenesis. Moreover, they provide basis for a possible molecular connection between inflammation and tumorigenesis.

Another major novel finding from my dissertation was the demonstration that serum ferritin is elevated in advanced breast and pancreatic cancer patients (Chapters 4 & 5). This increase was weakly-to-moderately correlated with the increase in the hepatic inflammatory biomarker C-reactive protein (CRP). When patients were stratified based on their serum ferritin and CRP values, patients with elevation in both biomarkers had the worst clinical outcome. However, serum ferritin showed independent prognostic value when compared to CRP and the elevation in CRP alone was not predictive of shorter survival. Those findings also provided support to the novel concept that cancer-associated inflammation negatively modulates response to cancer therapeutics, such as the
monoclonal antibody trastuzumab. The major findings of my dissertation are summarized in Figure 6.1

**Figure 6.1** Graphic representation of the functional and predictive value of ferritin in breast cancer patients.

The results summarized in Figure 6.1 demonstrate that the findings in this dissertation have made several important and novel contributions to our basic and clinical understanding of breast cancer and cancer-associated inflammation. However, there are many important questions that stem from this new understanding that should be the subject of further research. Some of the questions are listed below.
What is the clinical potential of ferritin-rich tumor-associated macrophages?

The identification of tumor-associated macrophages (TAMs) as the major site of ferritin expression and release within breast tumors as well as the tumorigenic effects of extracellular ferritin introduces several novel therapeutic and diagnostic possibilities:

1) Down-regulation of ferritin as an anti-TAM therapeutic strategy:

Iron is a pro-oxidant and has been recently proposed to cause non-apoptotic cell death (termed ‘ferroptosis’) when not safely stored within ferritin complexes (Dixon et al. 2012). Consequently, the down-regulation of ferritin has been shown to increase sensitivity to iron-induced oxidative damage in multiple cell types (Cozzi et al. 2000; Kim et al. 2004; Liu et al. 2011). Therefore, it is reasonable to expect that the down-regulation of ferritin within tumor-associated macrophages would impair their ability to safely maintain their iron stores which can lead to cell death. The ablation of tumor-associated macrophages has been shown to sensitize breast tumor to chemotherapy and impair their vascularisation (DeNardo et al. 2011; Shree et al. 2011).

In addition to the resulting cytotoxic effects from unbound intracellular iron, the down-regulation of ferritin and the subsequent increase in free iron may induce an unrestrained pro-inflammatory (M1) state in macrophages. When loaded with iron, macrophages have been shown to secrete more pro-inflammatory cytokines (TNFα and IL-6) and hydroxyl radicals which damage and induce apoptosis in cancer cells (Sindrilaru et al. 2011; Zhang et al. 2011). Therefore, down-regulation of ferritin in macrophages and the subsequent decrease in their iron storage capacity could lead to the reprogramming of tumor-associated macrophages from a pro-tumorigenic wound-healing (M2) to an anti-tumor pro-inflammatory (M1) program. Moreover, the reprogramming of tumor-
associated macrophages may improve the structure of tumor vascular and thus improve drug delivery (Rolny et al. 2011). The expected effects of ferritin down-regulation in macrophages are depicted in the following diagram.

In addition to the direct effects that the down-regulation of ferritin would have on tumor-associated macrophages, the overabundance of free iron within the tumor microenvironment may have cytotoxic effects on cancer cells as well. For example, in ‘Chapter 3’ we have shown that the addition of soluble ferrous iron to the culturing conditions is cytotoxic to breast cancer cells \textit{in vitro} (Figure 3.4 B). Therefore, the down-regulation of ferritin within TAMs may turn them into ‘iron bombs’ capable of killing tumor cells and other cells within the tumor microenvironment (endothelial cells, cancer-associated fibroblasts, etc).

2) Neutralizing ferritin’s pro-tumorigenic functionality or secretion:

Because extracellular ferritin can bind and stimulate cancer cells, neutralizing ferritin directly, blocking its binding site, or decreasing its release from macrophages may represent attractive therapeutic strategies for breast cancer patients. Although humanized monoclonal antibodies for ferritin or its receptor can be developed and tested, blocking
ferritin secretion might be a more straightforward therapeutic approach. For example in this study, we have demonstrated that the FDA-approved iron chelator deferoxamine can decrease ferritin secretion by macrophages. Moreover, we have noted that fetal bovine serum (FBS) suppressed macrophage ferritin secretion raising the possibility of the existence of a suppressive factor in serum that may be used clinically to suppress macrophage secretion of ferritin or other factors. In order to deliver the iron chelators or other factors to macrophages specifically, our laboratory has developed LPS-conjugated nanovesicles that target the TL4 receptors on activated microglia/macrophages (Wiley et al. under revision). These nanovesicles have not been tested in a cancer model yet, but would be a logical extension from my data. Further exploration of the specific activation state of tumor-associated macrophages may allow us to identify receptors that are differentially expressed on TAMs but not resident or circulating monocytes/macrophages and therefore increase specificity of the nanovesicles.

3) Local secretion of ferritin as a diagnostic tool:

From a diagnostic perspective, the local release of ferritin in breast tumors has the potential of being used as a molecular diagnostic tool for the early detection of breast cancer. Studies examining the effects of metals on breast tumorigenesis have noted that ferritin, which the group used as a surrogate for tumor iron levels, is detected at higher levels in the intraductal fluid (nipple aspirate) of breast cancer patients compared to controls (Mannello et al. 2010; Mannello et al. 2011). Because the work in this dissertation demonstrates that ferritin is primarily localized and secreted by TAMs, the elevation in ferritin within intraductal fluid may serve as a possible marker for the recruitment and activation of macrophages which has been proposed as a critical step in
mammary transformation into malignancy and invasiveness (Lin et al. 2001; Lin et al. 2006; DeNardo et al. 2009). Measuring ferritin levels in serum and intraductal fluid from both breasts may allow clinicians to make diagnostic decisions with a high degree of confidence. Although the use of intraductal fluid as a method for the early detection of breast cancer has long been proposed, most of the research utilizing this approach was almost exclusively focused on cytology and has thus lacked the specificity and sensitivity needed for clinical practice (Dua et al. 2006). The screening of molecules, such as ferritin, instead of cells may present a more sensitive and specific approach to cancer detection. Interestingly, macrophages have been found in the majority of intraductal fluid samples obtained from patients with in situ or invasive breast tumors (Krishnamurthy et al. 2003).

**Can the elevation in serum ferritin be used as a marker for tumor-associated macrophages?**

Several lines of evidence from animal studies have demonstrated that serum ferritin is primarily derived from macrophages and not hepatocytes or enterocytes (Ferring-Appel et al. 2009; Cohen et al. 2010; Zhang et al. 2011). Therefore, the elevation in serum ferritin observed in cancer patients may represent a specific inflammatory state involving the activation of macrophages. Although it is unlikely that tumor-associated macrophages are solely responsible for the increase in serum ferritin, they are undoubtedly contributing to this increase. The inflammatory cytokines causing the systemic elevation in serum ferritin are produced locally within the tumor and therefore will stimulate tumor-associated macrophages before any other site. Therefore,
the question is not whether or not tumor-associated macrophages contribute to the systemic increase in serum ferritin but what their relative contribution to this increase is. A tumor mouse model treated with drugs that block macrophage recruitment into breast tumors, as previously demonstrated (DeNardo et al. 2011), could be utilized to answer this question. Based on those studies, a signature of inflammatory biomarkers (for example, low CRP and high ferritin) may identify patients whose ferritin elevation is due to local secretion and not part of a systemic response.

Several drugs targeting tumor-associated macrophages are currently in development or in clinical trials (DeNardo et al. 2011; Shree et al. 2011). Therefore, the development of markers for tumor-associated macrophages to identify patients more likely to benefit from those drugs is an unmet clinical need. So far, only one group has developed a method to detect TAMs in vivo (Daldrup-Link et al. 2011). This method utilizes magnetic resonance imaging (MRI) to detect the specific uptake of FDA-approved iron oxide particles by TAMs (Daldrup-Link et al. 2011). However, the use of iron-oxide particles may have unintended consequences as it may increase the production and release of ferritin which we here show has tumorigenic potential (Chapter 3). The development of a safe and objective biomarker for TAM recruitment and activation would satisfy this clinical need.

**Is ferritin an inflammatory effector mechanism?**

A connection between inflammation and cancer was perhaps first hypothesized by Rudolf Virchow in the 1860s and has recently been receiving increasing attention for its role in tumor promotion and progression (Balkwill and Mantovani 2001; Coussens and
Werb 2002). In the context of breast cancer, epidemiological studies have demonstrated that chronic use of non-steroidal anti-inflammatory drugs (NSAID) is associated with reduced breast cancer risk and mortality (Cotterchio et al. 2001; Johnson et al. 2002; Holmes et al. 2010). Moreover, elevation in inflammatory biomarkers is associated with poor clinical outcome regardless of tumor stage (Robertson et al. 1991; Pierce et al. 2009; Allin et al. 2011). However, the molecular mechanisms underlying this intriguing association remain largely unresolved.

Serum ferritin, often overlooked as a bystander protein, is elevated during acute and chronic inflammation (Konijn and Hershko 1977; Feelders et al. 1998). Consistently, chronic use of NSAIDs has been shown to significantly reduce serum ferritin levels in patients with inflammatory or infectious diseases (Fleming et al. 2001). However, the function of serum ferritin during inflammation has not been thoroughly addressed. The only function speculated for serum ferritin thus far has been to deny infectious pathogens access to free iron and thus blunting their growth (LeGrand and Alcock 2012). Although this idea seems reasonable and may be part of the overall function of ferritin during inflammation, it is unlikely to be its primary function. For example, serum ferritin is composed primarily of L ferritin which is not efficient in taking up iron (Arosio et al. 1977). Moreover, the elevation in serum ferritin occurs not only during pathogenic infections but as part of a nonspecific inflammatory response.

The novel findings of this dissertation challenge the classical outlook on serum ferritin as a bystander protein and propose that it may have functional significance in some pathological conditions such as cancer. The increase in serum ferritin may be part of a tumor-promoting inflammatory state. Targeting serum ferritin or understanding its
pathological context may allow for the development of effective preventive and therapeutic strategies to cancer regardless of tumor molecular subtype.

The proliferative potential of ferritin may also play a role in other pathological states. For example, there is a strong association between obesity and cancer (Basen-Engquist and Chang 2011). However, it is still unclear what molecular mechanisms underlie this association. The widespread elevation in inflammatory biomarkers observed in obese people as well as the extensive infiltration and activation of macrophages in their adipose tissue led to speculation that inflammation may represent the missing link between obesity and cancer (Milman et al. 1999; Liu et al. 2003; Weisberg et al. 2003; Ye et al. 2007; Lecube et al. 2008). Interestingly, the elevation in serum ferritin - but not iron - has been demonstrated by several studies to be associated with body mass index and onset of type II diabetes (Ford and Cogswell 1999; Milman et al. 1999; Liu et al. 2003; Chen et al. 2006; Lecube et al. 2008). In light of our findings showing that ferritin may be tumorigenic, it is tempting to speculate a possible role for ferritin in inducing tumorigenesis as part of an obesity-induced inflammatory state. Overall, obesity can lead to the development of hypoxic regions within adipose tissue which triggers the recruitment and activation of macrophages (Weisberg et al. 2003; Ye et al. 2007; Pasarica et al. 2009). Macrophages can then secrete ferritin locally which can stimulate some cells to divide and thus increasing the chances for tumor development.

**Ferritin as a wound-healing cytokine: mechanistic considerations.**

The ability of extracellular L ferritin to interact directly with breast cancer cells and stimulate their proliferation independently of iron represents a novel mechanism for
this protein in cellular biology. The existing literature views extracellular ferritin as a multi-functional molecule with direct or indirect roles in angiogenesis, immune-suppression, and proliferation. The immunosuppressive functions are H ferritin-specific and were dependent on its ability to oxidize iron (Broxmeyer et al. 1989; Broxmeyer et al. 1991). On the hand, the proliferative and angiogenic effects are associated with L ferritin and are independent of ferritin’s iron content (Cozzi et al. 2004; Coffman et al. 2008; Coffman et al. 2009). In normal individuals, serum ferritin is primarily composed of L ferritin and is relatively iron poor (Arosio et al. 1977). In breast cancer patients, the elevation is primarily in L ferritin but an increase in H ferritin is also detected (Jones et al. 1980). Therefore, it is possible that the cancer-associated elevation in serum ferritin is stimulating the proliferation of breast cancer cells, inducing angiogenesis, and suppressing immune cells simultaneously. Interestingly, all of those functions are characteristics of a wound-healing program. For example, many of the immune cell types that can suppress the immune response can also induce angiogenesis when appropriately polarized (Motz and Coukos 2011). Moreover, several molecules, such as VEGF and prostaglandin E2 (PGE2), have a multifunctional nature with angiogenic and immunosuppressive properties (Baratelli et al. 2005; Della Porta et al. 2005; Alfranca et al. 2008; Ellis and Hicklin 2008). Therefore, ferritin could be viewed as a factor with a multifunctional role during wound healing. For example, a single ferritin complex can include both H and L subunits and therefore could have functional significance as a proliferative/angiogenic or suppressive factor. Furthermore, the H:L ratio within a single complex can modulate its functional potential in a context-dependent manner. Therefore, extracellular ferritin may represent a multifunctional molecule that simultaneously
contributes to the many aspects of wound healing. The view of ferritin as a cytokine was recently corroborated as it was demonstrated that extracellular ferritin can directly activate NFκB inflammatory signaling pathway in hepatocytes (Ruddell et al. 2009).

In light of the possibility that ferritin may act as a cytokine or an inflammatory mediator (Ruddell et al. 2009), it is tempting to hypothesize that ferritin is acting as a proliferative cytokine and thus activating pathways connected to wound healing such as Stat3 (Dauer et al. 2005), JNK (Bosch et al. 2005), and Smad3 (Ashcroft et al. 1999). Aside from probing the activation of specific signaling pathways, examining the transcriptomes of ferritin-treated cells may provide clues to which pathway ferritin activates as well as the downstream effector pathways that are directly responsible for increased proliferation.

**If not Scara5, then what is the ferritin receptor?**

Scavenger receptor class A member 5 (Scara5) was identified in 2006 by screening DNA sequences with homology to other scavenger receptors (Jiang et al. 2006). Although the three dimensional structure has not been resolved, the amino acid sequence was predicted to encode a multidomain type II transmembrane protein with an organization characteristic of the class A scavenger receptors (Jiang et al. 2006). The highest amino acid sequence homology was observed in the cysteine-rich and collagenous domains which represent the majority of the extracellular domain of Scara5 (Jiang et al. 2006). However, unlike all of the other class A scavenger receptors, Scara5 was not capable of binding acetylated or oxidized low density lipoprotein (Jiang et al. 2006).
Therefore, the classification of Scara5 as a scavenger receptor is based solely on partial sequence and structural homology and not functional similarities.

The function of Scara5 is still unclear with the existing few reports indicating contradictory roles in cell biology. In hepatocellular carcinoma, Scara5 expression was shown to inhibit proliferation and invasiveness of cancer cells by inhibiting focal adhesion kinase (FAK) signaling (Huang et al. 2010; Yan et al. 2012). Consistently downregulation of Scara5 increased proliferation and invasion in vitro and in vivo (Huang et al. 2010). Interestingly, this report noted a direct, albeit inhibitory, interaction between Scara5 and FAK. Contradictory to this report, Scara5 expression in cancer cells was shown to promote survival and proliferation via increased cellular adhesion (Ojala et al. 2012). Scara5 was also identified as the human L ferritin receptor in the developing murine kidney (Li et al. 2009). Its artificial expression in a human cell line increased survival in serum free conditions following treatment with iron-rich ferritin (Li et al. 2009). However, the function of Scara5 as a human ferritin receptor is yet to be demonstrated in vivo. Overall, Scara5 may represent an adhesion molecule with the capacity to interact and uptake ferritin.

In my thesis work, I found expression of Scara5 in almost every breast cancer cell line and tumor lysate examined. Moreover, Scara5 seemed to be overexpressed in cancer cells compared to the normal mammary cell line MCF10A (Figure 3.10). Therefore, I argue that the proliferative effects of ferritin, observed in some but not all breast cancer cell lines, may not be mediated by Scara5 alone. It is important to note that the size of the Scara5 band detected in cancer cell lines was 53 kDa which is the expected size based on amino acid sequence. On the other hand, we detected primarily a 60 kDa band in tumor
lysates indicating possible post-translational modifications (i.e. glycosylation). It is possible that these modifications are essential for membrane localization and are required for the iron delivery function. Nonetheless, our *in vitro* observations from breast cancer cell lines indicate the existence of another ferritin receptor whose proliferative effects are iron-independent.

The human ferritin receptors for either the H or L subunit are still unanswered questions. Several lines of evidence have indicated the possibility for the presence of multiple ferritin receptors with preferential or non-preferential binding to the ferritin subunits (Bretscher and Thomson 1983; Fargion et al. 1988; Fargion et al. 1991; Moss et al. 1994; Gelvan et al. 1996; Hulet et al. 2000). Thus far, transferrin receptor 1 (TfR1) and Scara5 are the only proposed human ferritin receptors – TfR1 for H ferritin and Scara5 for L ferritin (Li et al. 2009; Li et al. 2010). However, both are ubiquitously expressed in breast cancer cells and do not show differential expression and thus cannot account for the effects observed in this project (Figure 3.10, (Eckard et al. 2010)). One potential receptor for ferritin in human cells is the chemokine receptor CXCR4. Although this idea has not been explored in detail, some experimental data suggests an interaction between the extracellular domain of CXCR4 and ferritin (Li et al. 2006). However, it is unclear whether or not this interaction is subunit-specific and what functional significance it has in cell or tumor biology.

*Is ferritin a molecular sensing mechanism that links iron availability and cellular division?*
Iron is an essential micronutrient and is required for normal cellular functioning. It is found in the mitochondria as part of iron-sulfur clusters and thus is required for efficient ATP production. Iron is also a cofactor for ribonucleotide reductase and is needed for DNA production during S phase (Nordlund and Eklund 1993; Nyholm et al. 1993; Fu and Richardson 2007). In order to maintain optimal performance for these two critical cellular functions, cells need to maintain sufficient iron deposits. Because iron is shared between the daughter cells, if cells initiate mitosis without sufficient iron stores both cells may not maintain optimal functionality or even survive the division. This scenario may be of particular importance during the early and vulnerable stages of embryonic development when iron is not systemically stored or shared by the organism. Therefore, it is logical to speculate that a molecular sensing mechanism would exist to help cells trigger division when iron is abundant. In light of our data showing that ferritin can stimulate proliferation and that ferritin secretion by macrophages increases with iron loading, the ability of ferritin to stimulate proliferation may represent an autocrine or paracrine mechanism by which cells stimulate division in their surrounding environment in response to iron availability.

This possible mechanism might come into play during the early stages of breast tumor development. For example, there is compelling evidence for a relationship between iron and breast tumorigenesis. A large cohort study involving 24,000 women born in Australia, New Zealand, United Kingdom, and Ireland demonstrated an association between the polymorphism in the High Iron (HFE) gene (promoting enhanced iron uptake) with breast cancer risk; even after all other risk factors were considered (Osborne et al. 2010). In this study, patients homozygous for the C282Y HFE polymorphism were
at a higher risk for breast cancer development. Interestingly, the other common loss-of-function HFE polymorphism, H63D, was not associated with cancer risk. This observation raised the possibility that iron overload alone was not responsible for the increased cancer risk. One possible distinguishing factor between these polymorphisms is serum ferritin which is more frequently elevated in the presence of the C282Y gene variant than H63D (Allen et al. 2008; Gurrin et al. 2008). Several cohort studies have supported this notion by showing higher serum ferritin to be associated with cancer risk (Stevens et al. 1986; Zacharski et al. 2008).

Moreover, the incidence of chemically-induced mammary tumors is increased when animals are fed high iron diets or injected with an iron solution subcutaneously (Thompson et al. 1991; Singh et al. 1994; Diwan et al. 1997). Interestingly, histological examination of iron-induced tumors showed that iron was primarily present in the stroma and not the malignant cells (Singh et al. 1994; Diwan et al. 1997). In addition, not only did the tumor incidence increase with iron administration, but in one study the tumors were larger (Diwan et al. 1997). Therefore, it is possible that the effects of iron on tumor development and growth are due to a paracrine molecular mechanism that is influenced by iron availability through extracellular ferritin

**Does cancer-associated inflammation induce tumorigenesis and therapy resistance?**

In the context of cancer, inflammation has long been considered to be an anti-tumor phenomenon and its promotion has been considered to be a therapeutic strategy. Although this is true for some tumors, it does not seem to be the rule in the majority of cancer patients. For example, the presence of CD8 cytotoxic T cells in breast tumors is
indeed correlated with favorable prognosis, while the presence of CD4 T cells and macrophages is associated with poor clinical outcome and therapy resistance (DeNardo et al. 2009; DeNardo et al. 2011). Therefore, immune cells seem to have a bipolar functionality within the tumor microenvironment and can either destroy or promote tumors.

In this dissertation, I have demonstrated that a majority of breast and pancreatic cancer patients have elevation in serum inflammatory biomarkers and that this elevation is predictive of poor clinical outcome (Chapter 4 and 5). This cancer-associated elevation in inflammatory biomarkers is believed to be due to the production of inflammatory cytokines, such as IL-6 and TNFα, within the tumor microenvironment (Tran et al. 1997; Feelders et al. 1998; Arnaud et al. 2005). Locally, these cytokines have been shown to stimulate tumorigenesis and metastatic behavior through the activation of the inflammatory mediators Stat3 and NFκB signaling within cancer cells (Fukuda et al. 2011; Marotta et al. 2011; Daniluk et al. 2012). Also, we herein propose that ferritin secreted by macrophages in response to inflammatory cytokines acts as a pro-tumorigenic factor. Therefore, cancer-associated inflammation, as assessed by serum inflammatory biomarkers, may be reflective of a local pro-tumorigenic inflammatory state with direct stimulatory effects on cancer cells.

Moreover, our data suggest that the elevation in inflammatory biomarkers may be inducing resistance to cancer therapy. Based on our findings, it is difficult to determine whether or not cancer-associated inflammation is inducing resistance to therapies directly or if it represents an aggressive tumorigenic state that negates any benefits from those therapies. Further examination of the predictive value of inflammatory biomarkers in
patients treated with placebo, chemotherapy alone, and combinational therapy is needed to further understand this phenomenon. Mechanistically, there are several possibilities of how inflammation could be inducing therapy resistance:

1) Cancer-associated inflammation could be reflective of an immuno-suppressed tumor microenvironment. For example, the presence of CD4 T cells and M2 macrophages could be the source of many suppressive cytokines such as IL-4 and IL-10 (Maeda et al. 1995; DeNardo et al. 2009). Moreover, ferritin which we here show is secreted in response to inflammatory cytokines has been shown to have immunosuppressive effects on lymphocytes via inducing the production of IL-10 (Gray et al. 2001). In the case of monoclonal antibodies such as trastuzumab, one of the major mechanisms of action is antibody-dependent cell-mediated cytotoxicity (Park et al. 2010). Therefore, in an immuno-suppressed microenvironment, monoclonal antibodies cannot lead to the efficient clearance of tumor cells.

2) One interesting possibility through which inflammation may be inducing therapy resistance is the effects of immune cells on tumor vasculature. Recently, imaging studies examining drug penetration into tumors have noted decreased drug diffusion in correlation with the presence of myeloid-derived cells (Nakasone et al. 2012). Moreover, the production of angiogenic factors by tumor-associated macrophages has been demonstrated to be responsible for the ‘abnormality’ of tumor vasculature – which is characterized by collapsed lumen, irregular flow, and inadequate pericyte coverage (Ruoslahti 2002; Rolny et al. 2011).

3) Finally, cancer-associated inflammation could be producing factors that induce resistance in cancer cells directly. For example, macrophage-derived cathepsins have
been shown to interact directly with cancer cells and increase their resistance to chemotherapy (Shree et al. 2011). Furthermore, the tumorigenic effects of ferritin, as demonstrated herein, could also be inducing resistance to cancer therapy.

In light of these possibilities, anti-inflammatory drugs or lifestyle changes that may lower inflammation (such as diet and exercise) could be viewed as possible strategies to sensitize patients to cancer therapy. Once the pathways connecting inflammation and cancer are elucidated, targeted anti-inflammatory strategies may represent an attractive strategy to sensitize patients to many of the existing therapies.
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