Understanding the Complexities of Anemia in Chronic Inflammatory Diseases from Diagnosis to Treatment

Naomi Rae Flindt
Brigham Young University

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Understanding the Complexities of Anemia in Chronic Inflammatory Diseases from Diagnosis to Treatment

Naomi Rae Flindt

A dissertation submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Richard K. Watt, Chair
Roger G. Harrison
John C. Price
David L. Kooyman

Department of Chemistry and Biochemistry
Brigham Young University

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ABSTRACT

Understanding the Complexities of Anemia in Chronic Inflammatory Diseases from Diagnosis to Treatment

Naomi Rae Flindt
Department of Chemistry and Biochemistry, BYU
Doctor of Philosophy

Iron is an essential nutrient for energy and DNA replication. Its homeostasis is commonly perturbed by chronic inflammatory mechanisms. Chronic inflammation upregulates a cytokine, hepcidin, that degrades the iron export protein ferroportin. Without a way to export iron into the bloodstream iron availability in blood becomes depleted. Iron depletion in the blood stream hinders erythropoiesis and is termed anemia. Herein I investigate and inhibit the mechanism of hepcidin activation. Inhibition of hepcidin activation has released iron from tissues and alleviated anemic conditions in a cancer model. I have laid the foundation to investigate this pathway in a 3D spheroid model. The results show that hepcidin 25 inhibition is a promising treatment for anemia of cancer. More work needs to be done to confirm efficacy in an in vivo model. In addition to anemia of cancer I have also worked with diabetic rats and investigated their anemic state using common anemia diagnostic methods. I found that in this high fat high sugar diet Wistar rat model anemia was not induced. In addition to my studies on anemia I have investigated the use of portable x-ray fluorescence (pXRF) as an accessible and affordable elemental analysis technique for lateral flow immunoassays and biological samples such as cell lysates and animal tissue. While pXRF shows promising results more work needs to be done to increase its sensitivity and pixel size.

Keywords: Ferroportin, Hepcidin, Anemia, Cancer, Furin, X-ray Fluorescence, Lateral Flow Immunoassay
ACKNOWLEDGEMENTS

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ABBREVIATIONS

ACD—anemia of chronic disease

ADAM-17—ADAM metallopeptidase domain 17

ARE—antioxidant response element

BACH-1—BTB and CNC homology 1

BMP-6—bone morphogenic protein-6

Cat—catalase

CKD—chronic kidney disease

CREBHcyclic adenosine monophosphate(CAMP)-responsive element-binding protein H

DMT-1—divalent metal transferase-1

ELISA—enzyme linked immunoassay

EPO—erythropoietin

ERFE—erythroferrone

ESA—erythropoietin stimulating agents

FPN—ferroportin

GPx—glutathione peroxidase-1

Ft—ferritin

Hb—hemoglobin

HGF—hepatocyte growth factor

HIF-1α—hypoxia inducible factor-1

HIV—human immunodeficiency virus

HO-1—heme oxygenase-1

IFN-γ—interferon gamma

IRE—iron response elements
IRP—iron response proteins
IV—intravenous
Jak—janus tyrosine kinase
JNK—c-jun N-terminal kinase
IL-6—interleukin-6
IGFR-1—insulin-like growth factor-1
LIP—labile iron pool
LPI—labile plasma iron
MTP-1—metal transcription factor-1
MT1-MMP—membrane-type 1 metalloproteinase
NF-κB—nuclear factor kappa B
Nrf2—nuclear factor erythroid 2-related factor 2
NTBI—non-transferrin bound iron
PC—proprotein convertase
PDGF—platelet derived growth factor
Prx—Peroxisiredoxin
sHJV—soluble hemojuvelin
SMAD—small mothers against decapentaplegic-1/5/8
SOD—super oxide dismutase
SREBP-1—sterol regulatory element binding protein-1
STAT-3—signal transducer and activator of transcription-3
TACE—tumor necrosis factor-alpha converting enzyme
TFN—transferrin
TGF-β—transforming growth factor β
TfR-1—transferrin receptor-1
TNF—tumor necrosis factor
VEGF—vascular endothelial growth factor
1. Introduction

1.1 An Introduction to Iron Pathology

Iron, the fourth most abundant element on the earth, is an essential nutrient for cell growth and proliferation.\(^2-4\) Adult bodies contain anywhere from 3-5 g of iron.\(^5,6\) The chemistry that iron can perform is what makes it a unique and critical nutrient. Iron shuttles electrons as it changes oxidation states between ferrous (Fe\(^{+2}\)) to ferric (Fe\(^{+3}\)) iron. Although iron is found in other oxidation states, the ferrous and ferric oxidation states are most biologically relevant. Ferrous and ferric iron have different binding properties, solubility’s, preferred ligand preferences, coordination geometries, affinities etc. The ability of iron to change oxidation states by 1-electron chemistry leads to radical chemistry that can be damaging to biological systems. To protect against radical damage, the body tightly regulates iron. Iron is often shuttled, bound, or chaperoned by other proteins.

Bodily Iron Stores

The most abundant example of iron and its unique properties in the body is found in hemoglobin (Hb). As shown in Figure 1.1 roughly 70% of iron in the body is bound in Hb and stored in erythrocytes.\(^5-7\) The body generates 2 million erythrocytes every second in the bone marrow through a process called erythropoiesis. Erythropoiesis is stimulated by erythropoietin (EPO) a hormone released from peritubular cells in the kidney. EPO is stimulated by a lack of oxygen.\(^8\) As the body senses low oxygen levels EPO stimulates erythroferrone (ERFE) which has been shown to inhibit a protein hepcidin to allow more iron to be released for erythrocytes
As developing erythroid progenitors mature, they gradually accumulate iron from macrophages. Iron sits in the center of the porphyrin ring in the ferrous iron (II) state where it can bind to oxygen. The Fe-O₂ interaction is not too strong thus allowing O₂ to be released under acidic conditions acting as an important oxygen shuttle throughout the body. Oxygen is an essential nutrient for glucose and energy metabolism. There are many metabolic enzymes and proteins that are dependent on oxygen as well as iron to produce glucose which is a building block of porphyrin. This chemistry is critical for the survival of life.
In the body ferrous iron is the most relevant form of iron. Our bodies are only able to directly absorb ferrous iron through our diet. In enterocytes divalent metal transferase-1 (DMT-1) absorbs primarily ferrous iron since ferric iron (Fe³⁺) is insoluble and precipitates under biological conditions.¹³ The acidic conditions of the gut favor the Fe⁺² oxidation state.⁷ Other indirect iron absorption are in the form of heme or ferritin (Ft). These are taken up by separate absorption pathways.⁴

The next major iron reservoir in our body is the liver (Figure 1.1). About 25% of our bodily iron is stored in the liver.⁶, ⁷ In liver hepatocytes, iron is stored in Ft, an iron storage protein that stores up to 4,500 atoms of iron in the ferric state.¹³, ¹⁵ Generally, most of the stored iron in the liver can be mobilized and used if bodily iron needs are not met. About 10% of our bodily iron is stored as myoglobin in muscles and found in macrophages and other organs as metalloproteins (i.e. Fe-S clusters). Macrophages play an important role in recycling senescent erythrocytes through erythrophagocytosis. Thus, releasing iron back into the transferrin pool.¹⁶ Metalloproteins play important roles in oxygen sensing, transcription, metabolism, electron transfer, and DNA synthesis.⁶

Lastly, roughly 0.1% of bodily iron is trafficked through the bloodstream in its ferric state by a protein transferrin (TFN).⁵, ⁷ Under healthy physiological conditions about one third of circulating TFN is saturated with iron. Iron absorbed from the intestinal enterocytes or recycled from splenic macrophages performing erythrocyte turnover is exported into the bloodstream by ferroportin (FPN). Once in the bloodstream TFN shuttles iron to budding erythrocytes or to hepatocytes for storage. About one half of the circulating iron is lost daily to
When iron is unchaperoned it can readily participate in redox chemistry to produce reactive oxygen species (ROS) via Fenton-like chemistry shown in Figure 1.2. ROS can damage lipids, proteins, DNA, and RNA among others. Chronic levels of inflammation or excess iron
can lead to higher levels of ROS as well as ferric iron. These become a major driving force of ROS and therefore inflammatory diseases. Under chronic circumstances an abundance of ROS and iron will deplete the levels of protective antioxidants and other chaperones in the body.\textsuperscript{18} When speaking about unchaperoned iron we often refer to the cytosolic labile iron pool (LIP) inside the cells and the non-transferrin bound iron (NTBI) or Labile Plasma Iron (LPI).\textsuperscript{2,5} The increase in unprotected iron has no specific location. The excess unchaperoned iron in the LIP contributes to the buildup of more ROS as shown in Figure 1.2. It is difficult to measure the presence of labile ROS but as we see in the Fenton reaction their presence is often associated with the presence of free unbound iron. Therefore, iron levels and the ratio of free ferric to ferrous iron levels themselves can be a sign of oxidative stress levels.\textsuperscript{19}

Due to the deleterious side effects of ROS, our bodies have mechanisms in place to protect themselves from high levels of free iron and ROS. Excess iron is often stored away in Ft. Any iron that is transferred extracellularly is chaperoned by transferrin. There are also known iron response elements (IRE) in the mRNA of iron regulatory proteins that are shielded from binding when iron is present. When iron levels become depleted this allows iron response proteins (IRPs) to bind to the IREs in the mRNA. Depending on where the IRE is on the mRNA it can prevent the initiation of translation (5′IRE bound to IRP) or protect the mRNA from degradation (3′IRE bound to IRP). Ft, mAconitase and FPN mRNAs contain a 5′IRE while DMT1 and transferrin receptor-1 (TfR1) transcripts have a 3′IRE.\textsuperscript{11} In addition to iron regulatory proteins, antioxidants are also upregulated in response to free iron levels via nuclear factor erythroid 2-related factor 2 (Nrf2). These iron regulatory responses are helpful in maintaining iron homeostasis throughout the body and protecting from oxidative stress.
Iron can mediate autophagy, also known as ferroptosis via iron specific ROS generation. Ferroptosis mechanisms block transcription factor Nrf2 from binding to its antioxidant response element (ARE) leading to a depletion of antioxidants (i.e. heme oxygenase-1 (HO-1), glutathione reductase, Cat, and GPx) and an abundance of iron mediated ROS thus leading to cell death via iron generated ROS overload. In the study of Chen et al. the presence of iron chelators prevented ferroptosis response implying that free iron is indeed an essential amplicant for ferroptosis mediated cell death.

Iron Homeostasis Response to Acute Inflammation

Because iron is an essential resource for life and is found abundantly in the body it is also useful for pathogens. Microbes can use ferroproteins to extract iron from their hosts. These microbes also often secrete iron chelating proteins termed “siderophores”. The small amount of iron in the bloodstream bound to transferrin is the biggest target for microbes. Microbes also seek out NTBI or free iron that has not yet bound to TFN. Our bodies have developed responses to fight off these types of microbes and infection which are protective under acute circumstances. Mechanisms such as the interleukin-6 (IL-6) inflammatory response prove to be protective to the host as shown in Figure 1. IL-6 signals through the janus tyrosine kinase (JAK) signal transducer and activator of transcription-3 (STAT-3) pathways. JAK phosphorylates STAT-3 which then homodimerizes and binds to a HAMP promoter region in the nucleus. HAMP is the...
name of the gene that encodes hepcidin. Once HAMP gene expression is initiated, the mRNA is made and translated into pre-prohepcidin by the ribosome. The pre-region targets hepcidin for secretion. Once preprohepcidin enters the secretory pathway, the pre region of pre-prohepcidin is cleaved from prohepcidin and prohepcidin moves into the golgi where it can be further cleaved by the protease furin as shown in Figure 1.3.\textsuperscript{24} Furin cleaves the pro region resulting in hepcidin-25 excretion from the cell as shown in Figure 1.4. Once excreted, hepcidin-25 binds to FPN where the hepcidin/FPN complex is then ubiquitinated for proteasome degradation.\textsuperscript{4,25,26} When FPN, the only iron export protein, is degraded, iron begins to accumulate in the cell. This leads to depletion of iron in the bloodstream. Iron accumulated in the cell can be stored in Ft or used in metabolic processes. The hepcidin mechanism is protective in acute scenarios of infection or injury as it starves the microbe of iron, by storing up iron inside cells where microbes cannot easily access it. This is a healthy response of cells to a bacterial infection.\textsuperscript{13}

In response to iron overload, hepcidin is upregulated via the bone morphogenic protein-6 (BMP-6) pathway. BMP-6 signals through the suppressor of mothers against decapentaplegic 1/5/8 (SMAD 1/5/8) pathway. The BMP-6 receptor and other cellular mediators phosphorylate SMAD 1/5/8 which then binds to SMAD-4. This complex then moves to the HAMP promoter region in the nucleus. High levels of iron can cause increased inflammation and drive disease progression in many diseases such as anemia, diabetes, fibrosis, arthritis, cancer, and neurodegenerative diseases. Iron dysregulation can have severe consequences and often promotes disease progression. Iron mediated oxidative stress can create a cycle of disease
progression as disease is brought on by chronic inflammation that leads to prolonged dysregulation of iron leading to increased inflammation to further disease progression.

These common inflammatory stimuli for hepcidin, IL-6 and BMP-6 are shown in Figure 1.3. These two pathways work together synergistically to upregulate the gene expression of HAMP.\textsuperscript{9,27,28} However, as shown in Figure 1.3, other inflammatory stimuli are also known to upregulate hepcidin. In response to iron overload, hepcidin is upregulated via the bone

\textbf{Figure 1.3. Hepcidin/Ferroportin Axis}

The hepcidin/ferroportin axis is regulated by the IL-6/STAT-3 pathway in response to the immune system and by BMP-6/SMAD-1/5/8 in response to endogenous iron levels. Both pathways upregulate HAMP expression leading to increased levels of preprohepcidin.
morphogenic protein-6 (BMP-6) pathway. BMP-6 signals through the suppressor of mothers against decapentaplegic 1/5/8 (SMAD 1/5/8) pathway. The BMP-6 receptor and other cellular mediators phosphorylate SMAD 1/5/8 which then binds to SMAD-4. This complex then moves to the HAMP promoter region in the DNA. High levels of iron can cause increased inflammation and drive disease progression in many diseases such as anemia, diabetes, fibrosis, arthritis, cancer, and neurodegenerative diseases. Iron dysregulation can have severe consequences and often promotes disease progression. This oxidative stress can create a cycle of disease progression as disease is brought on by chronic inflammation that leads to prolonged dysregulation of iron leading to increased inflammation to further disease progression.

1.2 Anemia of Chronic Inflammation Overview

Upregulated Inflammatory Markers & Hepcidin

Iron is known to accumulate in cells experiencing chronic inflammatory environments common in diseases such as diabetes, chronic kidney disease (CKD) and cancer. This iron accumulation in cells has a direct effect in upregulating a gene called HAMP as mentioned earlier in Figure 1.3. The production of hepcidin is influenced mostly by circulating and tissue iron levels (BMP-6) secondly it is influenced by infection/injury (IL-6), erythropoiesis/hypoxia (ERFE), protein misfolding (CREBH), among other inflammatory signals. Other common inflammatory events or mechanisms that have an indirect effect on hepcidin regulation are included in Table 1.1 below. This list shows the inter-relatedness of different inflammatory
pathways that can affect iron regulation via hepcidin response. It is apparent that hepcidin and therefore iron plays a significant role in inflammatory diseases.

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Associated Diseases &amp; Disorders</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNK</td>
<td>Arthritis, Cancer</td>
<td>(Kanamori et al, 2018).</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Fibrotic diseases, Cancer</td>
<td>(Peng et al, 2022).</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Multiple Sclerosis, Arthritis</td>
<td>(Pollard et al. 2013)</td>
</tr>
<tr>
<td>TNF</td>
<td>Cancer, Irritable Bowel Disease (IBD), Diabetes, Non-Alcoholic Fatty Liver Disease</td>
<td>(Kunnumakkara et al, 2018), (Chen &amp; Ma, 2019)</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>IDB, Multiple Sclerosis (MS), Arthritis</td>
<td>(Park &amp; Hong, 2016)</td>
</tr>
<tr>
<td>UV-radiation</td>
<td>Cancer</td>
<td>30</td>
</tr>
</tbody>
</table>
Hepcidin Regulation via Furin During Chronic Inflammation

In order to understand further how to regulate hepcidin as a treatment for inflammatory disease we need to further understand how furin, the hepcidin activator, is regulated under chronic inflammation. Pro-hepcidin has been shown to be found in the golgi and nucleus where it autoregulates its own expression by binding to the HAMP promoter inhibiting its upregulation. Pro-hepcidin in the golgi is cleaved by furin. Prohepcidin can also be excreted from the cell but at a lower rate than that of hepcidin-25. Figure 1.4 shows the furin consensus sequence on

**Figure 1.4 Hepcidin Maturation Process**
Hepcidin is a pre-proprotein. The prehepcidin is cleaved once it enters the secretory pathay. The prohepcidin portion is cleaved next in the golgi activating the mature hepcidin-25 which is the primary signalling portion of hepcidin. Figure modified from Poli et al.
hepcidin. Furin is a proprotein convertase (PC) in the family of prohormone convertases that activate hormones by cleaving the inactive pro region.

Hepcidin is inhibited under hypoxic conditions through furin cleaving soluble hemojuvelin (sHJV) a co-receptor to the BMP-6 receptor. Matriptase is another protease shown to cleave HJV in a similar spot however yields a different product than that of furin cleavage.32 sHJV levels block BMP-2 and BMP-4 from upregulating furin and hepcidin.1, 32, 33 Although furin activity attenuates hepcidin expression with sHJV activation, furin also contributes to hepcidin activation. Under hypoxic conditions furin gene expression is also promoted via hypoxia inducible factor-1α (HIF-1α) which is stabilized by iron deficiency.32 As one of the most important PCs furin activates many inflammatory cytokines and receptors as shown in Figure 1.5 such as transforming growth factor β (TGF-β), insulin-like growth factor-1(IGFR-1), VEGF-C, platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), membrane-type 1 metalloproteinase (MT1-MMP), and tumor necrosis factor-alpha converting enzyme (TACE/ADAM17), sterol regulatory element binding protein-1c (SREBP-1), and BMP-4.34, 35 Some of these activated pathways such as NF-κβ, TGF-β, SMAD-2, SMAD-4, and PDGF cytokines also promote increased gene expression of fur the gene encoding for furin.35-38 Furin activation creates a cycle as its activity promotes an increase in its gene expression leading via maturation of inflammatory signals that can then exponentially increase inflammatory signaling.39, 40 Elevated furin levels have been associated with arthritis, obesity, type 2 diabetes, and anxiety.35 Furin is important in processing viral targets like COVID-19 and HIV.35
Figure 1.5 Furin Mediated Inflammation Pathways
Furin is an important pro-protein convertase that has been shown to play a crucial role in many cell related pathways from matrix remodeling to signaling. It is also crucial in activating viruses (not shown in figure above).

Chronic Inflammation & Ferroportin

While hepcidin has shown both systemic and cellular regulation of FPN, other inflammatory pathways have been shown to contribute to FPN regulation outside of the hepcidin/FPN axis. FPN transcription is upregulated by Nrf2 and downregulated by BTB and CNC homology 1 (BACH1).\textsuperscript{22,41} BACH1 is known to enhance ROS. BACH1 is commonly found upregulated with age and diseases such as arthritis.\textsuperscript{22} On the translational level FPN mRNA is silenced via iron response proteins and elements (IRPs and IREs) in response to iron deficiency. FPN has been shown to be regulated on a cellular level by heme levels as well as zinc.
and cadmium levels through metal transcription factor-1 (MTP-1). Low FPN levels lead to iron sequestration in cells whether that is tissues or erythrocytes. This accumulation of iron over chronic periods can deplete protective proteins and chaperones leading to increased oxidative damage that leads to a decrease in erythrocytes.

**Inflammation & Erythropoiesis**

Erythropoiesis depends on four things: proliferation of erythroid progenitor reserve, potency of stimuli for erythropoiesis, nutrient disposability, and erythrocyte survival. As mentioned earlier inflammation and disease upregulate many cytokines including IL-1, IL-6, IL-10, and TNF. These inflammatory markers negatively regulate EPO by impairing proliferation and differentiation of erythroid progenitors while stimulating erythrophagocytosis. These inflammation mediated impairments result in a lower number of erythrocytes. This can lead to anemia with a reduced amount of iron available to make new red blood cells (hypoferremia) and normal Ft levels. Ineffective erythropoiesis leads to fewer erythrocytes and increased hypoxia. Hypoxia leads to and upregulation the of HIFs which are transcription factors that downregulate hepcidin transcription. Essential nutrients for heme synthesis include glucose and iron. Under hypoxic conditions glucose metabolism is slowed along with nutrient availability.

Shortened erythrocyte survival occurs under inflammation as more macrophages undergo macrophage polarization. Inflammation is also involved in suppressed erythropoiesis in bone marrow. Alterations in iron metabolism, and oxygen use result in iron-restricted erythropoiesis leading to the induction of hepcidin. The combination of interrupted iron delivery to the bone marrow and inflammation induced inhibition of erythropoiesis cannot keep up with the rate of
Erythrocyte destruction. Erythrocyte destruction is increased from a higher amount of oxidative stress and macrophage polarization. Patients with chronic inflammatory diseases such as cancer or diabetes show decreased EPO synthesis via hypoxic stimuli and are inadequately too low to keep up with the demands for Hb. As the energy metabolism and the nutritional status of patients under inflammatory stress decline these same inflammatory signals perpetuate the inflammation cycle.

**Systemic Effects of Hepcidin**

In the body, systemic inflammatory signaling occurs primarily from macrophages in the blood. Although hepcidin is primarily produced in the liver it has a systemic influence as a cytokine. Other organs that have been shown to respond to and sometimes secrete hepcidin include brain, liver, spleen, intestine, kidneys, lungs, prostate, periodontal tissues, bone marrow, and heart. Hepcidin plays a role in many different organs and is important in metabolism and nutritional uptake. The way hepcidin regulates metals is different depending on the organ. For example, hepcidin has been shown to upregulate Fe, Mn, and Mo content in the spleen whereas it only upregulates iron in the liver. Additionally, hypoxia has shown to decrease hepcidin levels in the liver and increase cardiac hepcidin levels. These findings imply that inflammation in one organ can cause systemic signaling that can impact other organs as well. This is why understanding the effects of hepcidin on metal sequestration on a systemic level is an important step in understanding the effects of inflammation on a systemic level.
1.3 **Hepcidin as a Biomarker in Disease**

Chronic diseases resulting in increased systemic inflammation may be associated with increased hepcidin production, leading to the “anemia of chronic disease”. Hepcidin is a biomarker for liver fibrosis and cirrhosis. Elevated Pediatric levels of hepcidin have been correlated with juvenile idiopathic arthritis. Elevated hepcidin has also been shown to be involved with an increased risk of cardiovascular events. Hepcidin-25 has been shown to be a biomarker for inflammation status. Hepcidin has become a biomarker of interest in predicting cancer severity and outcome. In a recent study serum samples from a breast cancer clinical trial in the early 1990’s were surveyed for hepcidin-25 levels using an ELISA. Results showed a significant correlation of cancer resurgence in obese patients with high hepcidin levels. The hepcidin results also significantly correlated well with iron indices. However, no trend was observed in the overall cohort. While this data shows promise that hepcidin may be a useful biomarker, some variables in this study could be causing different trends. One variable is that the serum samples for this study were taken post-surgery and therefore could easily have elevated IL-6 signaling from surgery alone leading to increased hepcidin levels systemically. Alternatively, elevated hepcidin could be due to obesity alone thus fueling the higher hepcidin levels.

In acute liver failure serum hepcidin levels have been shown to correlate with survival. The lower the serum levels of hepcidin the lower the survival rate of the patient. Iron deficiency anemia vs anemia of chronic inflammation could be better diagnosed if hepcidin levels were measured. Hyperferritinemia in metabolic syndrome is easier to diagnose with hepcidin level information. Hepcidin-25 has been shown to strongly correlate with Ft levels in CKD and can aid in differentiating what type of therapy may be best. There have been mixed
results of associating Hepcidin-25 to inflammation markers like C-reactive protein (CRP) or IL-6. These paradoxical outcomes may be due to the difficulty in probing Hepcidin-25. ESA therapies have shown to be more effective when Hepcidin-25 levels are high in the patient suggesting that hepcidin-25 markers would be useful in deciding on a treatment strategy. Atherosclerosis has been correlated with Hepcidin-25 levels in rheumatoid arthritis, non-alcoholic fatty liver disease, and menopausal women. Hepcidin has been correlated with low energy availability in athletes and is being investigated as a biomarker. Hepcidin has been shown to be a biomarker useful in diagnosing and treating anemia of inflammation. Despite the many studies that have shown interesting correlations there is still no FDA approved hepcidin test.

1.4 Anemia

Anemia in chronic disease (ACD) is the second most common type of anemia in the world. Studies have shown that Hb is inversely correlated with inflammatory markers such as hepcidin, EPO, Ft, and ROS species. This correlation shows that anemia can be brought about by a variety of inflammatory avenues. Many studies show that anemia has a multifaceted mechanism that is affected by nutrition, energy metabolism, and oxidative stress due to limited oxygen transportation. The cardiovascular and renal systems are most affected as they attempt to compensate for anemic conditions. Vasodilation and reduced vascular tone are associated with increased cardiac output leading to symptoms such as paleness and vertigo. Lack of oxygen to essential organs is what causes other symptoms such as headache, lethargy,
depression, limited functional capacity, and fatigue.\textsuperscript{12} Immunodepression is another significant side effect of anemia making anemic patients more susceptible to infection.\textsuperscript{12}

**Diagnosis of Anemia**

Anemia of chronic disease is commonly diagnosed via a complete blood count (CBC) among other blood parameters as shown in Table 1.2. In anemia of chronic inflammation include Hb levels under 9 g/dL, significantly lower serum iron, increased Ft, and decreased serum EPO, and low reticulocyte count. While iron deficiency anemia patients experience normal hemoglobin levels with a decrease in serum iron and mean cell volume (MCV) or mean cell height (MCH) while experiencing an increase in reticulocyte counts and EPO. Distinguishing between these two scenarios of anemia is critical for understanding the underlying driving force of the anemia.

**Table 1.2 Diagnostic Biomarkers for Iron Deficiency**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Iron Deficiency Anemia</th>
<th>Anemia of Chronic Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of Other Chronic Diseases</td>
<td>rarely</td>
<td>often</td>
</tr>
<tr>
<td>Average Baseline Hb Concentration</td>
<td>≥ 9 g/dL</td>
<td>≤ 9 g/dL</td>
</tr>
<tr>
<td>Serum Iron Concentrations</td>
<td>decreased</td>
<td>significantly decreased</td>
</tr>
<tr>
<td>MCV/MCH</td>
<td>decreased</td>
<td>normal</td>
</tr>
<tr>
<td>Serum Ferritin Concentration</td>
<td>low</td>
<td>increased</td>
</tr>
<tr>
<td>Serum Hepcidin Concentration</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>% of Reticulocytes in the Blood Serum</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Serum Folic Acid Concentration</td>
<td>normal</td>
<td>decreased</td>
</tr>
<tr>
<td>Serum Vitamin B\textsubscript{12} Concentration</td>
<td>normal</td>
<td>decreased</td>
</tr>
<tr>
<td>Serum Creatine Concentration</td>
<td>normal</td>
<td>increased</td>
</tr>
<tr>
<td>Serum EPO</td>
<td>increased</td>
<td>decreased</td>
</tr>
</tbody>
</table>

Reformatted from Abbaspour et al.\textsuperscript{7}
**Treatment Options**

It is important when treating anemia of chronic inflammation to understand how to silence the inflammatory signaling of the chronic disease in order to sufficiently get nutrients absorbed by the patient. Current treatment options include oral, intravenous (IV), or intramuscular iron supplementation. Oral treatments have a high percentage of mild side effects and when a patient has inflammation their ability to absorb iron from the diet is severely hindered. Additional supplementation with the combination of other treatments such as erythropoietin stimulating agents (ESAs) has shown to sometimes be effective in aiding iron uptake and ultimately increasing Hb levels. ESAs do come with the risk of negative cardiovascular events and are increasingly more expensive. Other issues that make oral iron supplementation complex are effects of diet on iron absorption. There are many plant derived compounds that inhibit iron absorption as well as other medications. Ascorbic acid and meats can help to increase iron absorption. IV iron supplementation is associated with more risks as free labile iron introduced into an inflammatory environment with excess ROS can be a catalyst for more oxidative stress. IV iron has also been associated with a higher risk of infection. Blood transfusions work well in severe cases however they are not a long-term solution as they come with serious risks such as iron overload, infectious disease, immunosuppression, etc.
Treatment of Anemia of Chronic Diseases

• Tumor removal
• Chemotherapy/radiotherapy
• Treatment of autoimmune diseases, including RA (methotrexate, tocilizumab)
• Treatment CKD
• Recombinant erythropoietin analogues and derivatives.

Supportive Treatments

• Monitoring drug side effects
• Pain control
• Fall prevention
• Vitamin C

Supplementing Deficiencies

• Iron rich diet, folic acid, vit. B12
• Oral/intramuscular/intravenous iron supplements
• Oral folic acid treatment
• Oral/intramuscular administration of vit. B12

When ACD is prevalent in patients iron supplementation may not be the best treatment as the mechanisms and conditions described earlier result in iron overload for iron stores and cells while the bloodstream remains iron deficient. Increasing iron supplementation will aid in increasing the iron overload and thus perpetuating the inflammation and disease cycle. Other treatments have been explored to mitigate hepcidin levels directly or indirectly to ultimately release iron from iron stores and alleviate anemia.1, 8, 9, 12, 66-69, 70 Treatment strategies that target the hepcidin/FPN axis to alleviate anemia are shown below in Figure 1.7. Mechanisms of action that have been explored include inhibition of hepcidin production, protection of FPN activity from hepcidin suppression, neutralization of circulating hepcidin, and suppression of hepcidin-inducing signals, such as the inflammatory pathway mediated by the cytokine IL-6.55 A potential promising therapeutic approach to modulate hepcidin has emerged from the development of hypoxia-inducible factor prolyl hydroxylase inhibitors (HIF-PHIs). These agents stabilize hypoxia-inducible factors and subsequently improve anemia by stimulating endogenous EPO
production and iron release from macrophages and enterocytes. Alternative strategies have aimed at pharmacological modulation of hepcidin function by targeting different regulatory steps, such as the BMP/HJV/SMAD pathway. The BMP/HJV/SMAD pathway is the primary regulator of hepcidin expression in response to systemic iron levels. Other therapeutic approaches include hepcidin sequestering agents (antibodies, anticalins, and RNA aptamers), FPN stabilizers, inhibitors of BMP/SMAD, IL6/STAT3, or hepcidin transduction (siRNA/shRNA) pathways.1

These emerging strategies to reduce or antagonize hepcidin can facilitate release of endogenous iron from the reticuloendothelial system (RES) to the circulation which may reduce ESA dose requirements and, consequently, reduce adverse cardiovascular events observed in clinical trials.72, 73, 74

1.5 Inhibition of the Bottleneck Furin

Many angles to hepcidin inhibition and FPN stabilization have been explored in chronic inflammatory models.25 From upstream antibodies and inflammatory inhibitors to direct inhibitors of hepcidin, none of these treatments have proven sufficient to inhibit hepcidin in humans in a pharmaceutically acceptable manner. Inhibition of inflammatory pathways using drugs or antibodies that block the Jak/STAT3 pathway or the BMP/SMAD pathway have not been successful due to the simultaneous activation of multiple inflammatory pathways (Figure 1.7). This is further complicated by additional systemic inflammation that occurs through many convoluted signaling pathways that are not yet fully understood. Other inflammatory pathways (Figure 1.7). This is further complicated by additional systemic inflammation that occurs
through many convoluted signaling pathways that are not yet fully understood. Other inflammatory pathways are beginning to emerge as potential hepcidin regulators such as GDF-
This makes it difficult for drugs that target one specific inflammatory cytokine or pathway to work effectively. The attempts to bind and clear hepcidin have proven difficult by the quantity of antibodies or other binding molecules required to clear hepcidin and by the fact that hepcidin rebounds rapidly after clearance due to continued inflammation.

An alternative route, that has been investigated with some promising results, is to target the hepcidin activating PC furin. Even though furin may be expressed at high levels under inflammatory states, its enzymatic protease activity may be altered by other factors in the diet. It has been shown that curcumin can inhibit the activity of furin leading to better disease outcome for COVID-19. Other groups have investigated furin silencing in cancer decreased tumor progression as well as the inflammatory response. Other groups have inhibited cancer metastasis via bone targeted furin inhibition to prevent inflammatory mechanisms and breast cancer bone metastasis. There are known furin inhibitors like Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK). In cancer however, furin ablation in the liver has shown to be an ineffective treatment in treating liver carcinoma in mice. This may be due to the fact that SREBP-1 was inhibited which led to the mislocalization of lipids. This in turn shows that completely knocking out furin may be too extreme.

We propose the use of HIV protease inhibitors, a class of drugs that have been extensively reviewed and are already FDA approved. HIV protease inhibitors have been used for many years in HIV treatment. They have also been found to have anti-tumor effects which have been reviewed. Nelfinavir has been shown to have the best antitumor effects in comparison to other HIV protease inhibitors. In previous studies the effect of these protease inhibitors on hepcidin and therefore anemia has never been explored. We propose the use of Nelfinavir to inhibit Hepcidin-25 activation via furin inhibition. By inhibiting the activation of
hepcidin through furin inhibition, we can attenuate hepcidin regulation regardless of the source of inflammation. This will aid in stabilizing FPN, restoring iron to the serum and eventually to the bone marrow. Ultimately, we will be able to treat the anemic conditions via FPN stabilization while simultaneously mitigating the cancer progression. This is a unique approach to anemia as well as inflammatory diseases such as cancer and diabetes.
2. HIV-Protease Inhibitor, Nelfinavir, Stabilizes Ferroportin to Alleviate Anemia in Cancer

Naomi Flindt, Steve Christiansen, Grant Flindt, Alexis Gardner, Paulina Medellin, Annie Taylor, Jacob Parker, Roger Woolley, Ellis Truman, Clayton Grundvig, Phillip Smit, Richard Watt

2.1 Abstract

Anemia of Cancer is commonly found in 50-65% of breast cancer patients upon diagnosis. Anemic breast cancer patients have a lower chance of survival and prognoses. It has been found the patients with high FPN levels have a 90% chance of survival. Many groups have targeted the hepcidin/FPN axis to stabilize FPN however, there is still no FDA approved treatment. We have employed the use of an FDA approved HIV protease inhibitor, Nelfinavir, to stabilize FPN and alleviate anemic symptoms. In this study we investigated the effect of Nelfinavir on FPN stabilization using 2D and 3D tumorigenic MCF-7 cell culture models. In our 2D results we were able to observe that Nelfinavir significantly inhibits furin activity. Inhibition of furin was correlated with a significant decrease in the furin mediated Hepcidin-25 activation. Hepcidin-25 inhibition led to a significant increase in FPN levels. We observed trends showing that iron was released when treated with Nelfinavir. These preliminary results show that Nelfinavir has potential to treat of anemia in cancer. More work needs to be done to understand the pleiotropic effects of Nelfinavir on anemia as well as anticancer pathways.

2.2 Introduction

Anemia of Cancer

Anemic conditions are found in about 45-60% of breast cancer patients and increase to about 90% post chemotherapy. Anemia of cancer has a significant impact on patient outcomes.
Anemia can affect quality of life by decreasing energy levels and cognitive function. Side effects common in anemia of cancer include fatigue, lethargy, depression, patient morale, immunodepression, and reduced treatment efficacy.\textsuperscript{12} Iron is an essential nutrient for cell growth and proliferation.\textsuperscript{2, 4, 29} Sequestered iron intensifies malignant phenotypes of several cancers, including breast cancer to drive cancer growth through metabolic processes.\textsuperscript{6, 12, 14, 30, 43, 51, 66, 85, 87, 88, 89} Breast cancer cells exhibit a perturbed iron metabolism, giving them a metabolic advantage over neighboring cells to accumulate iron.

Breast cancer cells accumulate iron through the hypoxia induced upregulation of the iron uptake protein transferrin receptor-1 (TFR-1).\textsuperscript{90} Elevated TFR-1 allows the breast cancer cells to accumulate iron. Additionally, cancer cells were shown to have elevated levels of the hormone hepcidin.\textsuperscript{29, 50, 91} High levels of hepcidin are associated with the degradation of the only iron export protein ferroportin (FPN).\textsuperscript{4, 8, 13, 70, 85, 92} Without a way for iron to exit the cell, iron accumulates in the cancer cells and iron is depleted from the bloodstream. The combination of elevated TFR-1 to accumulate iron and the secretion of hepcidin to prevent iron release through FPN degradation, allows cancer cells to accumulate iron for metabolic purposes. This occurrence leads to the development of anemia of cancer, a common symptom of aggressive cancers.\textsuperscript{12, 87}

The mechanism of tumor aggressiveness ties back to inflammation. In a study of 133 breast cancer patients, it was found that 35% of cancer patients had anemia upon diagnosis. The more severe the prognosis correlated with a higher incidence of anemia as well as inflammatory markers (hepcidin, IL-6, Ft, and ROS).\textsuperscript{87} While hepcidin is primarily expressed and secreted from the liver, it has been shown to also be produced at lower levels in other organs, macrophages and epithelial breast cells.\textsuperscript{8, 12, 29, 75, 85} Hepcidin is upregulated by various
inflammatory pathways an anemia focused set of inflammatory pathways are shown in Figure 1.7. Common injury related inflammatory pathways such as those activated by interleukin 6 (IL-6) and bone morphogenic protein (BMP-6) increase hepcidin. IL-6 and BMP-6 synergistically upregulate the gene expression of HAMP via the JAK/STAT-3 and SMAD 1/5 pathways respectively. HAMP is translated into a pre-proprotein that is subsequently activated through proteolytic cleavage of the pre and pro regions. Pro-hepcidin is the inactive precursor of hepcidin and prohepcidin cleavage by the protease, furin, produces active hepcidin-25. Active hepcidin-25 is secreted into the bloodstream where it binds to and degrades membrane bound FPN, the only iron export protein in the cell. This regulatory axis of FPN via hepcidin-25 is heavily dependent on the post-translational modification of prohepcidin via the protease furin.

**The Role of Inflammation in Tumor Progression**

Although the inflammatory response is healthy and protective in acute scenarios, it can become a breeding ground for cancers under chronic conditions. Chronic inflammation can initiate cancer related DNA mutations from ROS induced DNA damage. These same chronic inflammatory pathways also increase matrix remodeling proteins to enhance cancer metastasis. Up to 20% of cancer deaths are linked to inflammation or chronic infection. As shown in Table 2.1 many inflammatory events diseases are linked with the potential outcome for associated cancers. This can include inflammation from chronic irritation. One example includes chronic bronchitis from smoking as a predisposition for lung cancer. Another is someone with IBD has an increased risk for bowel cancer. Obesity and arthritis are also linked with lymphoma development.
<table>
<thead>
<tr>
<th>Inflammation/ Infection</th>
<th>Related Cancer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulcerative colitis, Autoimmune Diseases</td>
<td>Colorectal</td>
<td>94</td>
</tr>
<tr>
<td>Emphysema, Tobacco, tuberculosis, Macular Degeneration</td>
<td>Lung</td>
<td>95</td>
</tr>
<tr>
<td>Hemochromatosis, hepatitis Band C, Alcohol, obesity</td>
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<td>79</td>
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<tr>
<td>Gastritis/helicobacter pylori</td>
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<td>Alcohol, pancreatitis</td>
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<td>Cervicitis/papilloma virus</td>
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<tr>
<td>Prostatitis</td>
<td>Prostate</td>
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<tr>
<td>Cystitis and Schistosoma haematobium</td>
<td>Bladder</td>
<td>100</td>
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<tr>
<td>Sunburn/ultraviolet light</td>
<td>Melanoma</td>
<td>101</td>
</tr>
<tr>
<td>Esophagitis/Gastric acid, alcohol, tobacco</td>
<td>Esophageal</td>
<td>102</td>
</tr>
<tr>
<td>AIDS</td>
<td>Non-Hodgkin’s lymphoma, Kaposi’s sarcoma</td>
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</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Non-Hodgkin’s lymphoma, Hodgkin lymphoma</td>
<td>104</td>
</tr>
<tr>
<td>Epstein-Barr virus</td>
<td>Nasopharyngeal carcinoma, Burkitt lymphoma</td>
<td>105</td>
</tr>
<tr>
<td>Multiple Sclerosis, Obesity</td>
<td>Breast</td>
<td>106</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>Oral</td>
<td>107</td>
</tr>
</tbody>
</table>
In breast cancer, both the tumor and immune cells express inflammatory cytokines.\textsuperscript{12, 108} These inflammatory cytokines include IL-6, HIF1, VEGF, TNF, mTOR, hepcidin, etc. lead to the polarization of M1 macrophages. These polarized macrophages can retain more iron further aggravating anemic conditions.\textsuperscript{12, 109} In response to inflammation and reduced production of EPO, EPO receptor are increased in cancer and correlated with poor \textit{erythrocyte maturation}.\textsuperscript{110} When nutrient pools like iron are reduced in the cell, mTOR activation inhibits Hb synthesis.\textsuperscript{12} Low Hb synthesis will in turn lead to low oxygen (O\textsubscript{2}) levels (hypoxia) which has been correlated with a negative effect on the efficacy of antineoplastic treatment.\textsuperscript{12, 16, 34, 35, 39, 87} Although many inflammatory pathways can have an effect on hepcidin and tumorigenicity, we are focusing on a narrow scope of inflammation that has shown to directly interact with iron regulatory proteins. The scope of our inflammatory model Figure 1.7 focuses on IL-6 and BMP-6 pathways and their effect on hepcidin.

\textbf{Role of the Tumor Microenvironment}

Due to the role that cancer tissues and immune cells play in inflammatory signaling it is important to understand the role of the tumor microenvironment on hepcidin signaling. Recent work has shown that inflammatory signaling and HAMP gene expression are significantly increased when MCF-7 tumorigenic breast epithelial cells are grown in 3D cell culture as opposed to 2D cell culture as shown in Figure 2.1.\textsuperscript{75} Pro-hepcidin levels have been shown to be
significantly increased in 3D spheroid breast cancer cell models when compared to 2D cell culture. This shows that hepcidin signaling pathways vary depending on the model used. As shown in BMP-6 signaling is significantly increased in the 3D model. This group also found that hypoxia induce GDF-15 plays a role in upregulating hepcidin in 3D culture models that is not present in 2D cultures. They found that 3D spheroids co-cultured with tumor fibroblasts allowed for the IL-6 signaling to increase pro-hepcidin expression further. Recent reviews suggest the

![Figure 2.1 2D v 3D Signaling Models in Breast Cancer](image)

2D culture models have simplified signalling pathways in comparison to that of 3D tumor models. The increased signalling and increased inflammatory pathways present in a 3D model have been shown to increase hepcidin levels. This increase in hepcidin may lead to an increased in iron dysregulation and therefore could be a more realistic tumor environment. Understanding of the effects of treatment on a 3D spheroid may helpful in understanding treatment efficacy.
role that tumor assisted macrophages can also play on hepcidin regulation in the tumor microenvironment. These findings stress the importance of using more realistic tumor models in cancer research and drug discovery.

The Role of FPN in Tumor Progression

Pinnix et al. investigated FPN levels in various breast cancer cells lines including: HME, R5, SUM19, SUM102, MCF-7, and MCF-10a. They compared the tumorigenic epithelial cell lines and their corresponding non-tumorigenic controls. The results show that tumorigenic cells have significantly lower FPN expression and higher pro-hepcidin expression than that of the non-tumorigenic cell lines. This group went on to investigate ferroportin stabilization as a therapeutic method for tumorigenicity. They found that when mice were given knock-in FPN tumor xenografts they had significantly smaller tumors than mice that were given control xenografts.

In this same study, breast cancer patients with high FPN mRNA levels were found to have a 90% chance of cancer survival after 10 years. In contrast, cancer patients with low FPN mRNA levels, have only a 65% chance of survival after 10 years. Thus, finding a treatment to stabilize FPN, in other words prevent FPN from being degraded by hepcidin-25 induced degradation, is imperative to increasing cancer patient survival rates. Herein, we investigate the role that the hepcidin plays on FPN stabilization in anemia of cancer.

As mentioned in the introduction, treatments to inhibit hepcidin have focused on inhibiting the inflammatory signaling pathways of Jak/STAT or BMP/SMAD. Figure 1.7 depicts a few of the signaling pathways that have been detailed in attempts to understand
hepcidin inhibition and FPN stabilization. Small molecule inhibitors of these pathways or expensive antibodies that target these upstream inflammatory signaling pathways to inhibit hepcidin have been attempted (Figure 1.7). Upstream inhibition of hepcidin has proven to be problematic because in many cases, multiple inflammatory signaling pathways are activated at the same time so inhibiting only one pathway is insufficient to stop the production of hepcidin. As mentioned earlier there are many known inflammatory cytokines that participate in hepcidin activation. Because hepcidin depletion treatments do not appear effective, the ability to stabilize FPN has also been attempted. FPN agonist strategies include antibody stabilizers but currently no treatments have made it to market. Despite the prevalence of anemia in breast cancer, the only treatment options include ESAs when chemotherapy induces anemia. Other targeted treatments for anemia have yet to be approved.

The Role of Furin in Cancer

A new mechanism we are proposing is to target the protease Furin to inhibit Hepcidin-25 activation to thus stabilize FPN. Furin is the most studied PC in cancer and is elevated in all human cancer types including breast cancer. Furin leads to increased tumor activation, migration and inflammation signaling. Inhibition of furin in breast cancer cells decreased cancer tumorigenicity and proliferation. Loss of furin in T-cells represses mammary tumorigenesis in triple negative breast cancer. Furin inhibition via Sunitib treatment showed a decreased migration and activation of MCF-7 cells via decreased interaction between furin and substrates pro-VEGF-C and pro-MT1-MMP. Furin targeting in Ewing’s Sarcoma, colorectal cancer also showed promising results. However, furin KO in liver carcinoma has...
shown to be an ineffective treatment in mice. This may be because SREBP-1 was inhibited which led to the mislocalization of lipids. This in turn shows that completely knocking out furin may be too extreme. These findings suggest that furin is a viable target for aiding in cancer treatment. Additionally, furin inhibition has the potential to aid in stabilizing FPN to alleviate anemic conditions. Although furin inhibition has been actively studied no groups have made the connection between furin inhibition to FPN stabilization.

Our goal is to inhibit hepcidin and stabilize FPN in a way that is independent of any inflammatory pathway. To be independent of inflammatory pathways, the inhibition must be downstream of translation. Preprohepcidin is synthesized followed by two cleavage steps that accompany targeting to the secretion pathway and activation by furin. We identified Furin as a potential site of inhibition because without the cleavage of prohepcidin to hepcidin, there is no biological activity that degrades FPN. Through a series of computer docking, enzyme assays, tissue culture studies and animal studies we discovered that some of the HIV protease inhibitors have favorable binding affinities to inhibit furin. We suggest that HIV Protease inhibitors, which have been extensively reviewed and are already FDA approved, can be used for FPN stabilization to alleviate anemia of cancer.

**Nelfinavir Treatment**

HIV protease inhibitors have been used since 1995. It is also well documented that this class of protease inhibitor also exhibits strong anti-tumor effects. Although many other anti-cancer properties have been detailed, Furin inhibition and subsequent FPN stabilization has never been documented before our lab made this discovery. The HIV protease inhibitor
Nelfinavir has been involved in several cancer related clinical trials (NCT01728779, NCT00589056, NCT02066311, NCT00589056, NCT01065844) as a chemosensitizer and has been shown to have significant anti-tumor effects and is the drug investigated herein. We hypothesize that the use of HIV protease inhibitors will inhibit Hepcidin-25 activation via Furin inhibition. Inhibition of hepcidin activation should lower active hepcidin levels, regardless of the source of inflammation. In turn, decreased active hepcidin will stabilize FPN concentration and ultimately should treat the anemic conditions and mitigate cancer progression. This is a unique approach to treat anemia of cancer as well as cancer aggression in breast cancer cells.

In this study we evaluate the efficacy of inhibiting IL-6 and BMP-6 stimulated hepcidin activation via furin inhibition using the HIV protease inhibitor Nelfinavir. The objective of this study is to show that hepcidin inhibition via Nelfinavir will restore FPN levels in cancer cells and prevent cancer cells from accumulating iron, subsequently resulting in less cancer proliferation and tumor aggression. We aim to evaluate hepcidin activation, FPN expression, and iron homeostasis in breast cancer cell models. Using these models, we will use Nelfinavir as a furin inhibitor to block the activation of prohepcidin to hepcidin to lower hepcidin-25 levels and increase levels of FPN. We propose that furin inhibition with Nelfinavir will alter cancer cell viability, growth rates, migration rates, intracellular iron levels, and levels of other iron regulatory proteins that relate to cancer aggressiveness. Due to the narrow inflammatory scope of our model we do not claim that this strategy will be anti-inflammatory however, it may aid in the restoration of iron homeostasis alleviating anemia and iron mediated inflammation.
2.3 Methods

**Cell Culture:** The 2D and 3D cell cultures of MCF-7 breast cancer cells were cultured using a modified method from Blanchette-Farra et al\textsuperscript{5}. Briefly, DMEM/F-12 (Corning 10-092-CV) with 10% FBS, 1% Pen-Strep-Amphotericin (Lonza 17-745E), 10 μg/mL bovine pancreas insulin (Sigma 1882) and 5% CO\textsubscript{2} at 37C. The MCF-10a were cultured using DMEM/F-12 with 5% donor horse serum (R&D Systems S12150), 0.5 μg/mL hydrocortisone ((Sigma H-0888,), 20 ng/mL hEGF (Peprotech AF-100-15), 100 ng/mL cholera toxin (Sigma C-8052), 10 μg/mL insulin, and 1% Pen-Strep-Amphotericin. Spheroid cell culture has 0.024% methylcellulose (Sigma m-7027). Spheroids are cultured in a 2% agarose gel 3D spheroid plate made with a mold that was a generous gift from the Van Ry lab at Brigham Young University.

**Western Blotting:** Cells were rinsed with PBS and scraped while on ice for lysis. Cells were lysed with RIPA buffer, frozen in liquid nitrogen, thawed, and then stored at -20\textdegree C for long-term storage. Protein quantifications are carried out with the Lowry protein assay. Protein samples are prepared by adding 2.5-5 μL of 5x SDS sample loading buffer with β-mercaptoethanol and samples were boiling for 5 minutes. Approximately 25 μg of protein are electrophoresed by SDS-PAGE and transferred to a nitrocellulose membrane. Following transfer, the nitrocellulose was rinsed in room temperature Tris Buffered Saline (TBS), dried for 10 minutes, and rehydrated with Milli-Q water. The membranes were blocked with 5 mL of TBS, containing 5% BSA and 0.1% Tween, for 1 hour at room temperature. The membranes were then incubated overnight at 4\textdegree C with primary antibodies. Rabbit monoclonal antibodies, pSTAT3 (Y705, 9145S) and pSMAD1/5 (s463/465, 9516S), purchased from Cell Signaling Technology were used in this assay. Transferrin Receptor-1 (TFNR-1) was from Thermo Fisher Scientific (Rabbit pAb, PA5-
β-Actin (8H10D10, 3700S), a mouse monoclonal antibody, was also purchased from Cell Signaling Technology for use as a loading control. Following overnight incubation, membranes were rinsed 3 times for 5 minutes with 5 mL of TBS with 0.1% Tween. The membranes were blocked with 5 mL of TBS with 5% BSA (pSMAD1/5, TFNR-1) or milk (pSTAT3) with 0.1% Tween and 1:5000 dilution of secondary antibodies for 45 minutes at room temperature. LICOR IRDye secondary antibodies, 680 RD Donkey Anti-Mouse (926-60872) and 800 CW Donkey Anti-Rabbit (926-32213), were used in this assay. The membranes were rinsed 3 times for 5 minutes with 5mL TBS and then imaged on LI-COR Odyssey® CLx imaging software. The images were quantified using ImageStudio™ Lite from LI-COR.

**Viability Assay:** In vitro EC50 was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which evaluates cytotoxicity. Nelfinavir was tested with a 12-dose EC50 regime starting at 1-5 μM and increasing to 75-100 μM at regular intervals. DMSO was used as the MTT solvent. The resulting % Survival (relative to untreated controls) was plotted in GraphPad Prism. EC50 values were determined using a sigmoidal dose-response (variable slope) curve fit, with a bottom constraint of 0.

**Flow Cytometry:** Cells were rinsed 2x with PBS followed by an incubation in accutase. Cells were collected and rinsed 3x in PBS. Following this treatment, cells were fixed in 2% methanol free PFA (define PFA) for 15 minutes at 4C. Following this, cells were rinsed 3x in PBS and permeabilized in 0.1% saponin for 20 min at room temperature. Then blocked with Fc block for 15 min followed by an incubation in the FPN (Novus 21502PE) antibody for 20 min at room
temperature then rinsed 3x with PBS and analyzed on the Cytoflex. All treatments had 6 replicates with 20,000 events per sample.

**Confocal Microscopy:** Cells were seeded on coverslips and two days later were fixed with methanol free 4% PFA in PBS for 10 min. Cells were permeabilized with 0.1% Triton X-100 (in PBS). Cells were blocked using Seablock Blocking Buffer (ThermoFisher Scientific) at room temperature for 1 h. Hepcidin primary antibodies (70R-6236 Fitzgerald) were diluted 1:300 in blocking buffer (10% Seablock Blocking Buffer in PBS with 0.1% Tween) overnight at 4 °C. Coverslips were washed 3 times with PBS, and then incubated with FITC secondary antibodies at a 1:1000 dilution in blocking buffer for 1 h at room temperature. Cells were then washed 3 times with PBS and incubated with 1:300 PE conjugated FPN/SLC40A1 conjugated (NBP1-21502PE) in blocking buffer overnight (PE). Cells were then washed 1 time with PBS followed by a 5 min 4’,6-diamidino-2-phenylindole (DAPI) 0.1 μg/mL stain in blocking buffer. Coverslips were then washed with PBS-T once followed by 2 washes with PBS. Coverslips were mounted on slides using ProLong Diamond Antifade Mountant (ThermoFisher Scientific) and cured for 24 h before imaging. Imaging was performed using Leica TCS SP8 DMi8 confocal laser microscope using the 40x oil immersion lens with the Leica Application Suite X software version 3.1.1.15751 for collection of images and analysis. After the acquisition, images were processed using Huygens Deconvolution and 3D analysis software 19.04.0p664b for quantitation and colocalization analysis. Image channels were acquired sequentially using appropriate settings for DAPI, PE, and FITC (Invitrogen, A-21126, 1:500).
**Furin Assay:** MCF-7 and MCF-10a 96 well plates were seeded at 10,000 cells per well. Cells were induced with various treatments for 24 hours. Method based on work of\textsuperscript{117}. The media is removed and cells are washed three times with PBS followed by a resuspension in 100 µL of 5x lysis buffer (500 mM HEPES, pH 7.0, 2.5% Triton X-100, 5 mM calcium chloride, 5 mM β-mercaptoethanol). After incubating for 15 minutes in lysis buffer 20 µL was added to the fluorescence compatible Perkin Elmer OptiPlate\textsuperscript{TM}-96F (6005270) along with 10 mM furin substrate (Enzo Life Sciences Cat# ALX-260-040-M005) and milli-Q water for a total volume of 100 µL. Fluorescence was measured before addition of substrate and for 60 minutes after adding substrate every 5 minutes on a Perkin Elmer Victor Nivo Multimode Plate using an excitation of 355/40nm and the emission of 460/30nm and the D400 dichroic mirror. A BCA was run for total protein concentration on remaining lysis buffer.

**Ferrozine Assay:** Whole cells from a T-75 cell culture flask (Starstedt, Inc.) were placed on ice, rinsed with PBS, and scraped into 15 mL conical vials (Starstedt, Inc.). Cells were centrifuged, excess supernatant was removed, and approximately 50 µL PBS was added to each vial. Cells were frozen in LN2 and stored at -80ºC. Cells were thawed and 260 µL of PBS was added to each vial. Cells were lysed with 155 µL of 50mM Trace Metal Grade NaOH on a shaker in a humidified atmosphere for 2 hours. The glassware used to make the 50mM NaOH and other solutions was rinsed in Trace Metal Grade 12M HNO3 and Mili-Q water and dried in the oven at 50ºC. Cell lysate was stored at -20ºC. After thawing, 155 µL of an acid solution containing 0.6M Trace Metal Grade HCl and 0.6M Ascorbic Acid solution were added to each vial. The vials were heated at 60ºC in an oil bath in a fume hood for 2 hours. During this time, a standard curve was made with a 2:1:1 ratio of stock Fe solution, 50mM NaOH and HCl/Ascorbic Acid solution.
Stock Fe solution was made using Mohr’s Salt and Milli-Q water. The standard curve covered 0-1.4 ppm in 0.2 ppm increments and 0-14 ppm in 2 ppm increments. Samples were cooled to room temperature and centrifuged to gather all liquid. The cell lysate samples, and standard curve samples were then brought to a pH of 4-5 using NaOH as visualized using pH paper. 210 µL of each cell lysate and standard curve sample were added to a 96-well plate. Cell lysate samples were done in triplicate while standard curve samples were done in duplicate. 70 µL of the Iron-Chelating Reagent (6.5mM Ferrozine, 13.1mM Neocuproine, 5M Ammonium Acetate, 2M Ascorbic Acid) were added to each well. The plate was covered with parafilm and foil and put on a shaker for 30 minutes at room temperature. The plate was read by a plate reader (Synergy H4 Hybrid Reader, BioTek) for absorbance at 562 nm.

**Real-time qPCR:** RNA was isolated using a Micro Kit (cat# 74004) following the manufacturer’s instructions. Samples were then quantified via fragment analysis for quantity and purity. RNA samples were diluted to 10 ng/ml and 2.2 µL of diluted RNA was used with the iTaq Universal SYBR Green One-Step Kit (Bio-Rad cat# 172-5151) with 320 nm primers total volume was 18 µL. Three biological replicates were run in duplicate for each treatment. To make standard curves, serial dilutions of RNA were added to the RT reaction. Samples were analyzed on the Bio-Rad CFX96 RealTime PCR Detection System.

**ELISA:** Media was collected and frozen immediately to -80C. Cell lysates were scraped and rinsed followed by three freeze thaw cycles from -80C to RT. Followed by centrifugation at 1500 RPM for 15 minutes. Media samples were also centrifuged at 1000 RPM for 15 minutes and diluted 50:50 with sample diluent. BCA was performed on lysate samples. Hepcidin-25 ELISA
kit (LSBio cat#LS-F40093) was used and the procedure was executed according to manufacturer’s instructions.

2.4 Results

Iron Sequestration in Cancer Cells

To investigate the mechanism used by tumorigenic cells to accumulate iron, we began by investigating how cancer cells acquire iron. We measured TFR-1 levels as shown in Figure 2.2. TFR-1 is present at much higher levels in the MCF-7 tumorigenic cell line when compared to the non-tumorigenic MCF-10a cells. Secondly, we see that regardless of increased inflammatory cytokine treatment or drug treatment the iron import protein is present at significantly higher levels in the tumorigenic MCF-7 cell line. Elevated TFR-1 is consistent with the accumulation of iron in cancer cells.

Since tumorigenic cells have elevated levels of the iron import protein TFNR-1, we investigated whether they had more internalized iron via total iron content measurements using the ferrozine assay. Results are shown in Figure 2.3. We see that the total levels of iron are significantly higher in MCF-7 cells.
We postulated that the tumor cells accumulated iron for multiple reasons. The first is caused by the elevated levels of TFR-1 that imports iron. However, tumor cells exist in elevated inflammatory conditions where hepcidin is present, and we postulated that FPN might exist at lower levels where iron export from cells would be inhibited. As mentioned earlier, the only iron export protein and thus a key regulator in the iron export process is FPN. The goal of this work is to determine if we can modulate FPN levels to regulate iron levels in cancer cells.

Figure 2.2 Transferrin Receptor-1 Levels in MCF-7 and MCF-10a Cell Lines
A) Western blot using a 12% gel. Data comparing MCF-7 and MCF-10a lines with various treatments. B) Lanes of MCF-7 lysates are quantified in graph below from left to right: 1) normal growth conditions, 2) 10 ng/mL IL-6, 3) 20 ng/mL IL-6, 4) 10 ng/mL IL-6 + 10 μM Nelfinavir 5) 20 ng/mL IL-6 + 10 μM Nelfinavir, 6) 10 μM Nelfinavir. All cells were induced for 24 hours.
We first measured FPN levels with confocal microscopy. Figure 2.4 shows the results of fluorescence imaging of FPN in the tumorigenic cells (MCF-7). MCF-7 cells were grown using several conditions including normal media, the addition of inflammatory cytokines (10 ng/mL IL-6 and 10 ng/mL BMP-9), 10 μM Nelfinavir as a control to evaluate Nelfinavir’s effect on FPN and finally cells treated with a combination of inflammatory cytokines IL-6 and BMP-9 and Nelfinavir. Our lab has previously demonstrated that Nelfinavir can inhibit hepcidin. We postulated that Nelfinavir treatment would increase FPN on the surface of MCF-7 and MCF-10A cells.

Figure 2.4 shows that FPN is present in MCF-7 cells in normal media. Treatment with IL-6 and BMP-9 decreases FPN levels in these cells. 10 μM Nelfinavir treatment in normal media shows FPN at intensities similar to cells grown in normal media. It is interesting to note that Nelfinavir treatment appears to concentrate FPN on the membrane of the cells (Figure 2.4 third row). When MCF-7 cells treated with IL-6 and BMP-9 are also treated with 10 μM
Nelfinavir, the FPN levels are similar or higher than in non-inflamed MCF-7 cells. This indicates that Nelfinavir can maintain FPN on the surface of the cells.

We also compared FPN levels in MCF-7 cells and MCF-10A cells, the non-tumorigenic control cells in these studies. Analysis of this data showed that FPN levels were logarithmically lower than the non-tumorigenic cell lines (MCF-10A).
Stabilizing FPN with Nelfinavir Treatment

To this point, our data has shown that MCF-7 cells have elevated iron levels compared to MCF-10A cells. The elevated levels of TFR-1 and decreased levels of FPN account for increased uptake of iron and decreased iron export. Nelfinavir treatment also showed stabilization of FPN in MCF-7 cells. Our overall goals are to treat the tumor cells with Nelfinavir to ultimately stabilize FPN and release iron sequestered in cells. To fully understand the effect of Nelfinavir on the cells we need to understand viability of the different cell lines with Nelfinavir treatment.

We investigated the IC50 of Nelfinavir in MCF-7 and MCF-10a cell lines. We performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability experiment to determine the IC50 and or toxicity of Nelfinavir in each cell line. As we see in Figure 2.5 the tumorigenic MCF-7 cells are more sensitive to Nelfinavir treatment. The IC50 in the MCF-7 tumorigenic cells was 9 μM and 22 μM in the MCF-10A non-tumorigenic cells. Although our goal is to lower hepcidin, the fact that the tumor cells are less viable with nelfinavir treatment is an important side benefit that may slow cancer growth. This shows that we can use a lower dose of Nelfinavir that can target the tumor cells while mitigating damage to healthy cells.

As mentioned above, Figure 2.4 shows FPN in images of inflamed, inflamed treated, and treated control samples. It is important to note that we were measuring prohepcidin levels. The active form Hepcidin-25 is more rapidly excreted from the cell and would not be present in sufficient levels inside the cell. We see that Nelfinavir treatment alone is correlated with a decrease in prohepcidin and that Nelfinavir with inflammation increases prohepcidin levels. Based on our model we would expect prohepcidin levels to build up when furin was inactivated.
We see increased FPN levels in red when treated with 10 μM Nelfinavir in the presence of inflammation. These images are the result of one biological replicate. More imaging experiments are underway to confirm with statistics.

In complement to this experiment, we ran flow cytometry experiments to quantify the amount of FPN on each cell type (Figure 2.6). We verified that FPN was indeed sensitive to hepcidin regulation by including a hepcidin induced control. In treated MCF-10a cells FPN decreases in response to elevated hepcidin-25 levels. This however was not the case for the MCF-7 cell line. They showed an insignificant response to hepcidin-25 treatment. We also have included an isotype control for another measure of FPN antibody specificity. It seems that the antibody bound more strongly to FPN on the MCF-7 cell lines than the MCF-10a cell lines. We
draw this conclusion from comparing the isotype control levels. This makes it difficult for us to compare FPN levels from different cell lines. It seems that Nelfinavir treatment significantly increases the amount of FPN in the cancer cells.

Figure 2.6 Flow Cytometry Ferroportin Levels
Cells were treated for 24 hours with the indicated treatments. Cells were fixed with 2% PFA permeabilized with 0.01% saponin and blocked with human Fc block prior to incubation with FPN antibody. P values were calculated using an ordinary one-way anova. n=6 replicates for each treatment. P<0.04=*, P<0.01=**, P=0.006=***, P<0.0001=****.
Our next question was to validate that increased hepcidin-25 activation by furin was the cause for FPN protein levels being lower. It is well established that hepcidin-25 binds to FPN and degrades it. Based on that assumption we hypothesize that levels of hepcidin-25 should be

**Figure 2.7 Hepcidin-25 ELISA**

MCF-7 cells were treated with the indicated treatment for 24 hours with various treatments. Media from the cells (n=3) was tested for Hepcidin by ELISA. P values were calculated using an ordinary one-way anova. n=3 replicates for each treatment. P<0.03=*, P=0.0001=***, P<0.0001=****.

**Nelfinavir Treatment and Hepcidin Levels**

Our next question was to validate that increased hepcidin-25 activation by furin was the cause for FPN protein levels being lower. It is well established that hepcidin-25 binds to FPN and degrades it. Based on that assumption we hypothesize that levels of hepcidin-25 should be
higher in the tumorigenic cells (MCF-7) and lower when treated with Nelfinavir. We grew MCF-7 cells and treated them with different concentrations of Nelfinavir and with inflammatory cytokines with and without Nelfinavir. We investigated total levels of Hepcidin-25 secreted into the media using an ELISA. MCF-10A cells were also treated identically and Hepcidin-25 measured, and these cells responded in a similar fashion as the MCF-7 cells (data not shown). Results show a dose response of decreasing hepcidin-25 in the media with increasing Nelfinavir concentration treatment in cells grown in normal media and in cells treated with inflammatory cytokines (Figure 2.7).

**Figure 2.8 Furin Assay Procedure.**
Cells were grown in a 96 well plate and induced with various treatments for 24 hours. Following this they were lysed and a furin consensus substrate was added. When the substrate is cleaved by furin it then allows for its probe 7-amino-4-methylcoumarin to fluoresce. The more fluorescence the more active the furin. The fluorescence is measured over time to observe the rate of furin’s activity.
Nelfinavir Treatment and Furin Activity

Next, we investigated the potential mode of action for Nelfinavir stabilizing FPN through furin activity inhibition. Our lab has previously demonstrated that Nelfinavir is a furin inhibitor\textsuperscript{118}. These docking studies showed promising binding activities however these had not been tested in vitro or in vivo. We were able to use the furin assay to evaluate the efficacy of Nelfinavir for inhibiting furin. As shown in Figure 2.8 the furin assay is a fluorogenic assay where a peptide with the furin cut sequence is attached to a fluorescent marker. Upon cleavage of the peptide by furin, the fluorophore is released and becomes fluorescent.

We studied furin enzyme activity in tumorigenic (MCF-7) and non-tumorigenic (MCF-10a) cell lysates from cells grown under a variety of inflammatory and Nelfinavir treated conditions. Our results in Figure 2.9 shows that 15 $\mu$M Nelfinavir has similar inhibition properties against Furin as 10 $\mu$M CMK, a known furin inhibitor. Interestingly cells that are inflamed seem to have lower levels of furin activity which was unexpected especially as furin is typically elevated with inflammation\textsuperscript{118}. This data show that furin plays a significant role in regulating the FPN/hepcidin axis and that Nelfinavir treatment can inhibit the activation of hepcidin-25.

Genetic Regulatory Effects of Nelfinavir

Next we were interested to see if it may be due to some genetic regulation. We performed qPCR analysis on various treatment groups as shown in Figure 2.10. We see that Nelfinavir treatment is shown to significantly increase furin expression in MCF-7 cell line. It is important to note that all other differences are statically insignificant.
Figure 2.9 Furin Activity of MCF-7 and MCF-10a Cell Lines
Cells were treated with the indicated treatment for 24 hours. After the 24-hour treatment, the cells were lysed, and cell lysates were tested for furin enzyme activity. In A and B, we see the effect of Nelfinavir treatments on cells grown under normal conditions (controls). In C and D, the furin enzyme activity is shown for inflamed cells treated with 10 ng/mL IL-6 and BMP-6 and 10 μM Nelfinavir. E and F show the comparisons of the slopes of the kinetic graphs in A-D. P values were calculated using an ordinary one-way anova. n=3 replicates for each treatment. P<0.03=*, P=0.0001=***, P<0.0001=****.
Using MCF-10a and MCF-7 cell lines we were able to produce viable 3D spheroids according to methods outlined. Images shown in Figure 2.11 are taken at the same magnification.

Figure 2.10 qPCR Results for 2D Cell Culture Treatments.
qPCR was performed on n=3 replicates. An unpaired t-test was used to produce listed p value. All other differences were statistically insignificant.

Establishing 2D & 3D Anemia of Cancer Tissue Culture Models

Using MCF-10a and MCF-7 cell lines we were able to produce viable 3D spheroids according to methods outlined. Images shown in Figure 2.11 are taken at the same magnification.
to show the difference in structure. The use of such models has shown to have increased signalling pathways that can better mimic the tumor or tissue microenvironment.\textsuperscript{75, 89}

We investigated the inflammatory signalling and iron regulatory differences in the tumorigenic and non-tumorigenic cell lines. We see elevated levels of iron in the cells in the 3D culture as shown in Figure 2.12. We also see that with Nelfinavir treatment, has a trend of lowering iron content in the cells, however changes are not statistically significant. We propose that the release of iron is due to FPN stabilization.

We were able to investigate the differences in mRNA levels of hepcidin mRNA, FPN mRNA and Furin mRNA between 2D and 3D cell cultures of MCF-7 and MCF-10A. In each comparison of cells, the mRNA levels were lower in the 3DF cultures than the 2D cultures. It seems that furin mRNA (Figure 2.13) is much higher in the MCF-10A cells than in the MCF-7 cancer cells. More research needs to be done to investigate the role of furin, Hepcdin-25, and FPN in 3D cells. It may be that these signalling pathways are exacerbated like the iron findings.
2.5 Discussion

Nelfinavir IC$_{50}$ values range from 8-17 µM in various leukaemia cell lines.$^{119}$ In our studies we found the IC$_{50}$ to be at 9 µM which would be on the lower end of the range making it an option in breast cancer treatment. Nelfinavir has shown to have several anti-cancer effects such as the inhibition of E-cadherin and MMPs that are critical for metastasis and tumor growth.$^{84, 116}$ It has also been shown to inhibit drug metabolism pathways. Studies have investigated using Nelfinavir as a chemosensitizer in combination with other chemotherapeutics like Doxorubicin and Tamoxifen.$^{81, 116}$

![Figure 2.12 Total Iron in 2D vs 3D Cell Culture](image)

**Figure 2.12 Total Iron in 2D vs 3D Cell Culture**
All cells were treated for 24 hours followed by iron quantification via ferrozine analysis. In A we see differences between iron levels in 2D and 3D as well as non-tumorigenic and tumorigenic cells. In B and C results show differences between treatment groups. n≥5 for each treatment group.
Nelfinavir was able to significantly stabilize FPN levels as shown in Figure 2.4 and Figure 2.6 through the mitigation of hepcidin-25 activation as shown in Figure 2.7. Hepcidin-25 levels decreased in a dose dependent manner when treated with Nelfinavir. We also saw that Nelfinavir was able to help in rescuing inflamed cells. FPN has been a particularly difficult protein to quantify. Our lab has spent significant resources in implementing new quantification techniques for FPN. Flow cytometry has been the gold standard for our lab, but this technique makes it difficult to compare protein levels between cell lines due to different antibody binding interactions. In our study we used an isotype control to illustrate the difference of this binding between the two cell lines. Ideally western blotting or mass spectrometry would be a more definitive way to quantify total protein between both cell lines. Hepcidin-25 is another difficult to probe protein due to its small size and presence in inactive precursors as shown in Figure 1.4. Antibody selectivity is critical when probing for hepcidin-25. In our imaging studies we chose to probe for prohepcidin as it is known to be excreted at a slower rate than hepcidin-25.24 We also

Figure 2.13 Differences in mRNA Levels Between 2D and 3D cells
Control lysates from each type were compared with n=3 replicates. There was no significant difference between cell types.
hypothesize that pro-hepcidin will increase in concentration when furin activity is inhibited because it will remain uncut. We see this occurring in Figure 2.4 where nelfinavir treatment increases the prohepcidin signal. We see that a majority of this prohepcidin seems to localize at or near the nucleus. It is known the prohepcidin binds in the nucleus to regulate its own mRNA expression.\textsuperscript{31}

It seems that the effect of Nelfinavir on FPN does show an emerging trend of lower iron however iron levels were not to a statistically significant level when FPN was stabilized with Nelfinavir. This may be due to the fact that fluctuations in iron levels are very small over the course of a 24-hour period. Longer induction times should be investigated to see if the stabilization of FPN can induce a significant decrease in iron stores. Nelfinavir is known to induce autophagy and apoptosis. It is possible that iron is being used to promote a process similar to ferroptosis. More investigations need to be done to understand what role iron is playing in Nelfinavir treatment. It seems that if this were the case it would make Nelfinavir treatment even more selective as it would be more deadly for tumors known to sequester iron than healthy tissues deficient in iron.

Nelfinavir was shown via docking studies to be a suitable target for furin inhibition.\textsuperscript{118} Our findings support this hypothesis as we see in Figure 2.9A and B that Furin activity is significantly lowered with the treatment of Nelfinavir. It is particularly interesting that the inhibition of furin is as effective as the known furin inhibitor-1 CMK. Currently there are no FDA approved Furin inhibitors. Nelfinavir has been FDA approved for more than two decades with manageable side effects. The repurposing of Nelfinavir as a Furin inhibitor is intriguing because furin has many targets other than hepcidin.
We see some interesting findings in Figure 2.9C where inflammation seems to lower furin activity. It has been shown that IL-6 can inhibit MCF-7 cell proliferation when expressed in high levels (~100 nM). In our study we induced with IL-6 levels near 0.3 nM. This could have played some role in furin inhibition. Previous studies have found that IL-6 downregulates MMP-2/9 mRNA levels which are also known furin targets. More studies should be done to understand the interplay between furin and the many cytokines that influence its regulation including IL-6.

IL-6 and BMP-6 were endogenously added to mimic a more realistic tumor microenvironment based on the work of Blanchette. Our 3D Models mimic these signalling pathways in a more realistic way. Ideally, in the future we would prepare the 3D cultures with fibroblasts and other cell types to enhance the tumor microenvironment. Although 3D cells models provide a much better model for drug evaluation, they are difficult to test. We ran into issues with cell differentiation in flow cytometry thus preventing us from being able to clearly define cell populations. 3D imaging is a difficult skill and was not able to be achieved in the time frame we had to work with. This resulted in only being able to produce total iron level data. We see that indeed the 3D cells sequester much more iron than the 2D cells. We have some supplementary data that illustrates the increased signalling pathways of BMP-6 and IL-6 in the 3D model. Future work would include a full evaluation of the iron regulatory pathways involved in iron sequestration and regulation.

Our qPCR data in Figure 2.10 showed insignificant differences in HAMP and slc40a1 but a significant increase in Furin. This may be due to promoter binding proteins that require furin activity for maturation as mentioned in the introduction. The insignificant differences in gene expression with treatment further support the hypothesis that Nelfinavir inhibits furin activity post-translationally. In Figure 2.13 showed an insignificant difference between 3D and 2D
mRNA levels of HAMP, slc40a, and fur. Previously Blanchette et al.\textsuperscript{75} found HAMP levels in 3D cell lysates to have significantly higher levels of HAMP mRNA. This difference could be due to different 3D growth procedures, primer efficiencies, or other experimental conditions.

### 2.6 Conclusion

More work needs to be done to understand the role of furin inhibition on anemia of cancer. Nelfinavir is a selective and anti-tumorigenic option. Our studies indicate that Nelfinavir does significantly inhibit Hepcidin-25 activation and stabilize FPN through Nelfinavir inhibition of furin. These novel findings connecting furin to key iron regulatory proteins in the hepcidin/FPN axis suggest the need for further studies. Understanding the nuances of furin may lead to understanding the nuances of anemia in chronic diseases such as cancer.
Appendix A. Investigation of Anemia in a High Fat High Sugar Diet in Wistar Rats
Naomi Flindt, Steve Christiansen, Brandon Rose, Roger Woolley, Alexis Gardner, Annie Taylor, Paulina Medellin, Jacob Parker, Grant Flindt, Ellis Truman, Cade Nordhagen, Kirsten Flindt, Grant Balls, David Kooymen, Richard Watt

A.1 Abstract

The western diet has been shown to have many deleterious effects on health as the inflammatory stimulus can often perpetuate and sometimes initiate disease. It has been shown that a high fat high sugar diet can induce diabetes mellitus and osteoarthritis. These inflammatory induced diseases are often accompanied by anemic conditions. In this study we investigate the effects of a protease inhibitor Nelfinavir on the inhibition of arthritis and anemia pathways. Nelfinavir has been shown to inhibit furin is a protease known to activate matrix remodeling proteins (MMPs) and facilitate cartilage build up. Furin is also known to inhibit other iron sensing pathways and can potentially treat anemia which is commonly prevalent in diabetic patients. We investigated the effects of 30 days of Nelfinavir treatment on Wistar rats. We were able to collect complete blood counts (CBC), total iron levels by organ, glucose testing, and arthritis scoring. It seems that the high fat high sugar rats did not develop significant anemic symptoms in comparison to the control rats. Based on our results Nelfinavir seemed to develop a more severe anemic conditions as well as intensify arthritic conditions.

A.2 Introduction

Diet induced inflammation has been shown to upregulate inflammation causing a host of chronic disease comorbidities including: arthritis, diabetes mellitus, fatty liver disease, CKD and Fibrosis.\textsuperscript{67, 121, 122} Diets high in fats and sugars have been associated with causing inflammation.
These inflammatory diseases are often found to have higher levels of furin.\textsuperscript{123} Furin is potentially upregulated via the IL-6 and BMP-6 same pathways outlined in Chapter 1. High furin expressing pancreatic cells have low insulin but fast growth while low expressing cell lines have slow growth and larger pools of insulin.\textsuperscript{124} SREBP-1c transduces the insulin signal and induces expression of glucose utilization and fatty acid synthesis.\textsuperscript{125} In addition to increased metabolic utilization, inflammatory diseases are often correlated with a 25-30\% increase in the rate of lipogenesis and oxidative stress in the liver.\textsuperscript{125} Increased matrix remodeling via furin mediated MMP activation is another oxidative stress driver prevalent in arthritis.\textsuperscript{78, 96, 101, 126} Increased oxidative stress, when chronic, can deplete protective antioxidants leading to an increase in unchaperoned iron and deleterious effects of radical oxygen species (ROS). ROS lead to anemia through damaging erythrocytes and decreasing available iron for erythropoiesis by upregulating hepcidin thus perpetuating ROS and disease progression.\textsuperscript{122}

Treatment options for anemia of chronic disease are nuanced as discussed in chapter one. It has been shown that many standard therapies such as ESA are correlated with adverse cardiovascular events.\textsuperscript{74} In another study they used anti-hepcidin treatments to address the inflammation first by decreasing hepcidin levels. This treatment led to reduced iron in macrophages and a decrease in foam cell formation and atherosclerosis.\textsuperscript{60} Thus, implying a strategy that can mitigate hepcidin-25 activation while also stabilizing FPN has potential to treat anemia of chronic inflammation. Nelfinavir an FDA approved pro-protein convertase has been shown to inhibit hepcidin levels in HepG2 cells as well as a previous LPS inflamed Lewis rat study.\textsuperscript{118} In addition to Nelfinavir mitigating Hepcdin-25 activation it has potent anti-inflammatory effects as it inhibits furin. Since furin is an integrated inflammatory cytokine
processor, targeting it can potentially mitigate MMP activation among other inflammatory mechanisms that drive disease progression.

In this study we seek to inhibit arthritis progression via Nelfinavir induced furin inhibition while also alleviating anemia of chronic disease. We plan to investigate changes in metal localization with the upregulation of related metalloproteins and their potential protective effects on chronic inflammation. These aims collectively will aid in understanding how hepcidin regulates the localization of various metals during inflammation in an in vivo model. This is an important step in understanding the role that metals play in the pathogenesis of inflammation and related diseases such as diabetes, and arthritis.

A.3 Methods

Animals: were maintained in a humane manner following IACUC guidelines and protocols. Animals were obtained from Jackson Labs. We used a high-fat, high-sugar (HFHS) diet comprising TD.08811 (Envigo) and 25% fructose in double-distilled sterilized drinking water and allowed to consume these diets ad libitum for 4 weeks; the control groups received a standard rat chow (SC) comprising LabDiet 5001 (LabDiet) and distilled drinking water. Our choice of this regimen was based on a proprietary diet used in a previous publication. Lewis Rats were raised on a HFHS sugar diet for the last 16 weeks of their life. The other 16 rats were raised on a control diet for the entire study. 8 male HFHS rats, 8 male control, 8 female HFHS, and 8 female controls. Four rats in each group were treated and the other four received a vehicle control treatment. Except for 3 females in the nelfinavir treated HFHS and 3 males in the nelfinavir treated HFHS males. Treatment began at 49 weeks. The HFHS rats had been on a
HFHS diet for 16 weeks prior to the beginning of treatment. One of the nelfinavir treated female HFHS rats had a tumour and data has been omitted. Another nelfinavir treated HFHS male rat was severely immunocompromised and died shortly after treatment began. Treatment was administered via intraperitoneal (IP) injection twice daily for 30 days. Rats were dosed at 18 mg/kg. Females received a 1.8 mL injection, males received a 3.2 mL injection of a 4 mg/mL solution of Nelfinavir in 5% DMSO 5% PEG-400 and 5% Tween-80 with sodium chloride. Nelfinavir was extracted in DMSO from crushed 625 mg Viracept® pills (Agouron Pharmaceuticals ndc 63010-027-70) with ~95% efficiency. Nelfinavir was quantified using HPLC prior to dilution for injection based on the methods of Charbe et al.127 Vehicle control rats were injected with equal volumes of a 5% DMSO 5%PEG-400 and 5%Tween 80 with sodium chloride based on the methods of Gassart et al.128 Dissection was performed under anaesthesia via cardiac exsanguination.

Pain assessment in rats employed the Brigham Young University rodent pain assessment Standard Operating Procedure (SOP) developed by the University Veterinarian, based upon American Veterinary Medical Association (AVMA) guidelines and approved by the University IACUC.

**Blood Tests:** Blood was taken the day before the first day of treatment and at day 14 and at dissection. 1-1.5 mL of blood per draw via tail vein. Blood was collected in lithium heparin and EDTA tubes following the order of draw. Blood was kept on ice. EDTA tubes were used for CBC analysis at Utah Valley Hospital and blood smears for a differential, reticulocyte count, and abnormal morphology scoring.
**Morphology Scoring:** Blood smears were fixed with methanol and stained with Wright’s Stain (Electron Microscopy Sciences Cat# 26060) following manufacturer instructions. This allows visibility of white blood cells for differentiation. It also allows a clear view of red blood cell morphology which can be indicative of anemia status. Red Blood Cell Morphology was graded on a scale of 1-3 dependent on the number of event type found in the sample. 10 replicates per sample were counted.

**Insulin Tolerance Test:** Four days prior to euthanasia, were performed on day 27 of treatment to determine the effect of the HFHS diet and/or Nelfinavir on insulin responsiveness. Animals were fasted for six hours’ time (6:00 A.M.-12:00 P.M.), receiving only distilled water during that span. Blood glucose was measured using a Contour 7151H Glucometer and 7097C test strips (Bayer) immediately before injection of insulin (fasting blood glucose) as well as at time points 15, 30, 60, 90, and 120 minutes after insulin injection. Novolin R (Novo Nordisk Medical) was diluted in 1X PBS (Fisher Scientific BP399-500) to a concentration of 0.75 IU/mL and administered IP at a dosage of 0.75 IU/kg. Following the cessation of ITT, animals resumed assigned diets and drinking fluids.

**Flow Cytometry Erythropoiesis:** was monitored using Bone marrow cells were filtered then blocked using Rat Fc Block from BD followed by staining with CD45 and HIS49 for 20 minutes on ice. Cells were then washed 3x prior to analysis. CD44 conjugated to FITC and HIS49 conjugated to APC. Gating strategy included gating for healthy cells, single cells, HIS49 positive cell, and finally a scatter plot of SSC vs CD44.
**Flow Cytometry Ferroportin/Macrophage:** Tissues were collected in PBS, digested, and red blood cell lysed using RBC lysis buffer for two minutes. This was followed by 3 rinses in PBS and an accutase digestion for 1 hr at 37C. Following this samples were filtered and washed 3x. Then cells were fixed in 1% methanol free PFA for 15 min on ice followed by permeabilization in 0.1% saponin for 20 min on ice. Following this samples were blocked for 15 min on ice and then stained and washed 3x prior to analysis. Single and unstained controls were also accounted for. Samples stained with CD45 were blocked and stained prior to fixation/permeabilization then the other stains were used. FPN NBP1-21502 from Novus conjugated to PE, CD45 conjugated to APC-A750, CD68 conjugated to PerCP, CD163 conjugated to Pacific Blue.

**ELISA:** Hepcidin-25 ELISA was acquired from LS-Bio (Cat # LS-F56097). Aliquoted serum samples were quantified for protein content. Samples were then normalized and ran according to manufacturer instructions.

**Microscopy:** Tissue was immediately immersed in 4% PFA for 24 hours at 4C followed by sucrose cryopreservation using a 15% sucrose solution for 24 hours followed by a 30% sucrose solution for 24 hours before being trimmed and cryo-embedded into tissue Tek OCT.

**Histology:** Tissue was immediately immersed in 4% PFA for 24 hours at 4C followed by sucrose cryopreservation using a 15% sucrose solution for 24 hours followed by a 30% sucrose solution for 24 hours before being trimmed and cryo-embedded into tissue Tek OCT.

**qPCR:** Tissue was snap frozen and stored at -80C until ready for RNA extraction.
**ICP-MS:** Tissue was snap frozen and stored at -80°C until ready for nitric acid digestion. Frozen samples were crushed, weighed, and suspended in trace metal grade HNO₃ at 100 mg/mL. Samples were sealed and digested at 65°C overnight. Following this samples were centrifuged and 50μL of digested sample were diluted to 5 mL in Milli-Q water. Chromium was added as an internal standard. Samples were filtered using a 0.45 μm PES filter prior to analysis.

**A.4 Results & Discussion**

**CBC Data**

Complete Blood Count (CBC) data is an important diagnostic tool used in assessing anemia. We were able to run CBC samples prior to starting treatment. Two weeks into treatment and at 4 weeks of treatment. The two tables provided show interesting comparisons between different treatment groups. In Table A.1 the top table shows that the high fat high sugar female rats did not have a significant decrease in haematocrit when on the HFHS diet. However, males on the HFHS diet had a significant decrease Hb, haematocrit, red blood cell count and white blood cell counts. It also seemed that the male rats receiving Nelfinavir treatment had a decrease in iron parameters leading to an increased anemic phenotype. These changes in CBC parameters seem to suggest a very slight decrease that may not be clinically apparent. Additionally, it is interesting to see a decrease of all blood cell types (white and red). This is typically not the case for anemic patients. Table A.2 lists additional comparisons for the HFHS diet rats. Table A.3
lists the additional comparisons for control diet rats. These additional comparisons help to aid as controls.

**Erythropoiesis**

Bone marrow samples were stained for erythrocyte progenitors. This experiment was critical in understanding the influence of Nelfinavir on erythropoiesis. The graphs in Figure A.1 show the changes in erythrocyte maturation. We see that with Nelfinavir treatment we have an increased amount of Reticulocytes however not an increased amount of total red blood cells. This data suggests that Nelfinavir may be inhibiting erythropoiesis. This would also explain the decrease observed in all blood cell types form the CBC data.

**Arthritis Scoring**

Mankin scoring was performed using a stain and scoring system. It seems that all the rats were in the arthritic region and Nelfinavir treatment seemed to increase the severity of arthritis score however this increase was not statistically significant. Results are shown in Figure A.2. Because Nelfinavir has been shown to inhibit matrix remodeling proteins these results were surprising. More work needs to be done to understand the specific activity of furin on MMPs as well as what affect Nelfinavir may have on their activity.

**Glucose Intolerance**
Glucose intolerance seemed to be more prevalent in the high fat high sugar rats. Results are shown in Figure A.3. It has been shown that glucose intolerance can be induced by a HFHS diet\textsuperscript{121}. It seems that our rats had similar glucose profiles regardless of diet and Nelfinavir treatment.

**Hepcidin-25 Expression Levels in Serum**

Hepcidin-25 levels in serum after four weeks of treatment were measured using an ELISA kit. The results did not seem to correlate as expected as shown in Figure A.4. We see that the Male rats on both diets as well as female control rats seemed to have a slight increase in hepcidin-25 with Nelfinavir treatment however, this increase is statistically insignificant. The female HFHS rats did show a decrease in hepcidin levels with Nelfinavir treatment.

**Iron localization by Organ**

Iron levels were measured in the liver, spleen, and lungs. Iron levels were significantly lowered in the liver in response to Nelfinavir treatment as shown in Figure A.5. These results suggest that Nelfinavir is potent at releasing iron stored up in the liver back into the bloodstream. Interestingly, Nelfinavir seemed to have the opposite effect on the liver and spleen. When rats were treated with Nelfinavir spleen and lung concentrations increased. This increase was not significant.

**Ferroportin Expression in Activated Macrophages**
FPN levels on activated macrophages were measured using flow cytometry. The results in Figure A.6 show an increase in FPN with Nelfinavir treatment. This increase was significantly higher in the female rats as shown in. This data confirms that activated macrophages key in erythrocyte recycling were able to release iron back into plasma when treated with Nelfinavir. This is an important step in alleviating anemia.

A.5 Conclusions

This study It seems that there were several important parameters such as arthritis scoring, hepcidin-25 levels and anemia data form the CBCs that were unexpected. It seems that this model of inducing anemia did not occur. This may be due to the short amount of treatment. Additionally, Nelfinavir had some interesting effect on erythropoiesis. More studies need to be done to confirm whether Nelfinavir is inhibiting erythropoiesis. Some positive effects of Nelfinavir include its ability to release iron from key bodily iron stores like the liver and macrophages. This is an important in treating anemia of chronic inflammation. We are looking into testing combination treatments of ESAs with Nelfinavir to induce erythropoiesis while also releasing iron to facilitate erythropoiesis. Additionally, other protease inhibitors may be more suitable targets that can continue to release iron from liver and macrophages while not inhibiting erythropoiesis. We are looking into new ways to screen our protease inhibitors for the inhibition of furin while not inhibiting erythropoiesis targets. Other PCs and concentrations should be investigated for their concentrations.
Table A.1 CBC Results for Control vs. HFHS Diet Comparisons

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<td><strong>Control</strong></td>
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</tr>
<tr>
<td><strong>% RSD</strong></td>
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<td><strong>HFHS</strong></td>
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### Table A.2 CBC Results for HFHS Diet

#### Nelfinavir Treated HFHS Diet Females: 0 wk vs. 4 wk Treatment

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<th>Units</th>
<th>WBC</th>
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<th>HGB</th>
<th>HCT</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
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<th>RDW-CV</th>
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<th>MPV</th>
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<tr>
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<td>%</td>
<td>fl</td>
<td>pg</td>
<td>fl</td>
<td>%</td>
<td>10^3/μL</td>
<td>fl</td>
<td></td>
<td></td>
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<td>0 wk</td>
<td>6.7</td>
<td>8.2</td>
<td>14.9</td>
<td>43.3</td>
<td>52.8</td>
<td>18.2</td>
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<td>24.7</td>
<td>18.5</td>
<td>778.3</td>
<td>7.6</td>
</tr>
<tr>
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<td>1%</td>
<td>3%</td>
<td>2%</td>
<td>2%</td>
<td>3%</td>
<td>1%</td>
<td>5%</td>
<td>6%</td>
<td>5%</td>
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<tr>
<td>4 wk</td>
<td>2.7</td>
<td>7.1</td>
<td>12.5</td>
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#### Nelfinavir Treated HFHS Diet Males: 0 wk vs. 4 wk Treatment

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<tr>
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<th>WBC</th>
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<tr>
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<td>8.6</td>
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#### Vehicle Treated HFHS Diet Females: 0 wk vs. 4 wk Treatment

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<td>fl</td>
<td>%</td>
<td>10^3/μL</td>
<td>fl</td>
<td></td>
<td></td>
</tr>
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<td>6.7</td>
<td>8.2</td>
<td>14.9</td>
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<td>2%</td>
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<td>3%</td>
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<td>5%</td>
<td>6%</td>
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#### Vehicle Treated HFHS Diet Males: 0 wk vs. 4 wk Treatment

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<td>fl</td>
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### Nelfinavir Treated Control Diet Males: 0 wk vs. 4 wk Treatment

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<td>54.1</td>
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<td>4%</td>
<td>4%</td>
<td>3%</td>
<td>3%</td>
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Figure A.1 Erythropoiesis Maturation in Bone Marrow
Erythrocyte maturation was monitored by using a series of cell markers. FSC-A stands for forward scatter area. Cells scattered farther to the right on the FSC-A axis are more granular. FITC-A is the probe for CD-44 antibody. CD-44 is a marker for Erythrocyte maturation. Cells expressing lower levels of CD-44 are more mature. Categories of erythrocyte progenitors displayed in graphs are 1-Proerythroblast, 2-basophilic erythroblast, 3-polychromatic erythroblast, 4-orthochromatic erythroblast, 5-polychromatic erythrocyte, 6-erythrocyte. The more red the higher the density of cells. In the top left graph we are looking at the scattering of erythrocyte progenitors in a representative replicate for female control rat receiving vehicle. In the top right we see the Nelfinavir treated female control rat. In the bottom left vehicle control male rat. In the bottom right the Nelfinavir treated male rat.
Figure A.2 Mankin Scores
Mankin scores are indicative of arthritis. A score of 5 or above is considered arthritic and above 8 is considered osteoarthritic.

Figure A.3 Insulin Tolerance
Test rats were injected with insulin and glucose concentrations were monitored over the course of 120 minutes. Some rats were severely affected and we released them early due to severe hypoglycemic shock symptoms.
Figure A.4 Hepcidin-25 Levels in Serum After 30 Days of Treatment
Figure A.6 ICP-MS Data for the Liver, Spleen, and Lungs

Figure A.5 FPN Expression on M1 Macrophages in the Bone Marrow
Appendix B. pXRF Quantification of Lateral Flow Immunoassays
Naomi Flindt, Annie Armitstead, Lara Grether, M. Jill Clapperton, Bruce Kaiser, Richard Watt

B.1 Abstract

New solutions for rapid and sensitive point of care testing have been required since the beginning of the COVID-19 pandemic. The pandemic caused broad challenges for rapid diagnostic testing that include issues from supply chain and limited material availability to technology limitations such as the inability to detect and notify individuals of infection status at the earliest times of infection. We developed and evaluated the use of cost-effective printer paper Lateral Flow Immunoassay (LFI) tests that can be constructed when supply lines cannot provide materials to construct LFI tests. We also evaluated portable x-ray fluorimeter (pXRF) as a reader to quantify LFI results. To maximize LFI quantitation using pXRF, we created background clearing chase buffers and wash steps to increase visualization of the test and control lines. When using pXRF limits of detection were increased by 100-fold for common hCG LFI tests.

B.2 Introduction

The Covid-19 pandemic infused the rapid diagnostic field with motivation to create rapid Covid-19 tests for early detection of the SARS-CoV-2 virus. The goal of virus testing is to detect viruses like SARS-CoV-2 at the earliest stages of infection. A positive test result informs an infected individual to quarantine to prevent spreading Covid-19. Rapid testing coupled with efficient trace contacting, has the potential to inform contagious individuals to quarantine and slow the progress of an epidemic or pandemic. To successfully battle a viral pandemic, tests need to
detect the virus at low viral counts and as early in the infection as possible. With proper tests, contagious individuals can quarantine, and the spread of a virus can be limited.

During the early stages of the Covid-19 pandemic, Polymerase Chain Reaction (PCR) testing was the gold standard because PCR testing identified SARS-CoV-2 mRNA at very low levels and early in the infection. Several drawbacks hindered PCR testing during the early stages of the Covid-19 pandemic. These challenges included problems with obtaining PCR supplies, faulty tests and extended delays for patients receiving results of the tests. These delays in informing infected individuals that they were contagious allowed the virus to spread.

Antigen tests or lateral flow immunoassays (LFIs) do not have the low detection limit of PCR but are cheaper to make and distribute and give results in less than 30 minutes. Since LFI tests directly measure elevations in the viral load, virus detection by LFI tests correlates with the contagious stage of viral infection. In the work of Mina et al. and Paltiel et al. the authors modeled test sensitivity, selectivity and cost. Both groups conclude that frequent testing with lower sensitivity LFI tests and strict behavioral interventions provides the most effective and economical strategy for controlling viral outbreaks.

Another study by Larremore et al. was able to show that the more sensitive PCR tests given frequently showed a marginal benefit in controlling the spread of the virus, but the cost and delayed reporting might not justify the benefit. From these studies we learn that an ideal test will have a low detection limit like PCR and give rapid results at low cost that allow increased frequency of testing, like the LFI tests.

An additional challenge faced during the Covid-19 pandemic were disrupted supply lines caused when countries shut down during the pandemic. Additionally, a surge to produce LFI tests for the Covid-19 pandemic, depleted the sources of nitrocellulose, absorbance, conjugate, and
sample pads as well as other required supplies for LFI tests. To address this challenge, our group developed methods to create simplified LFI tests using printer paper. These simplified tests did not require any of the normal absorbance, conjugate or sample pads associated with LFI tests. These simplified tests can allow the preparation of LFI tests for pandemic testing if supply lines were slow or inaccessible. We have named the in house tests the simple empowering LFI or seLFI\textsuperscript{132}.

Most at home LFI tests use a visible qualitative readout typically visualized using gold nanoparticles (AuNP) conjugated to an antibody that binds specifically to the biomarker of interest. If the biomarker is present, it will bind to the antibody and as it migrates through the test it will bind to the test line antibody bound to the paper. The resulting sandwich of the biomarker between two antibodies with the attached AuNP gives a red line at the test line (Figure B.1). A second control line is made by attaching an antibody that recognizes the Fc domain of the AuNP conjugated gold nanoparticle. This is known as the control line and indicates that the test ran properly. The intensity of the red color of the test line is dependent on the amount of antigen present. If low amounts of antigen are present in the sample, the test line may be too faint to see, leading to a false negative. This has been a common issue during the COVID-19 pandemic when tests are taken early in the infection stage when the viral load is not sufficient to give a positive test line\textsuperscript{133, 134}.

When the intensity of the test and control lines is below the visible detection limit, LFI readers can be used to determine if the antigen is present. LFI readers commonly quantify fluorescently tagged antibodies on the test strip. Fluorescence detection allows for quantitative detection, and some fluorescence tags provide a degree of signal enhancement. Using LFI readers
increases the overall cost and would require testing to be done at testing sites like those used during the Covid-19 pandemic.

However, even with fluorescent tags and readers, the detection limit of many biomarkers can fall below the detection limit of fluorescence detection in LFI tests. This study evaluates the hypothesis that a portable x-ray fluorescence (pXRF) instrument could detect the AuNP conjugated antibodies bound to the test line at a detection limit lower than visualized tests or fluorescent tests. pXRF is an analytical instrument that is used for elemental analysis of archeological artifacts, minerals, plants, and various materials. It has also been used more
recently in the biological field to measure animal and peripheral blood specimens\textsuperscript{136, 137}. This convenient technique can be used under ambient conditions to quantify elements from Sodium to Uranium. We propose pXRF can be used on LFI tests to detect and quantitate if any non-visible gold is bound to the test and control lines of LFI tests.

The goal of this paper is to determine if pXRF can detect gold nanoparticles bound to test and control lines on LFI tests at a lower detection limit than existing LFI tests. If this works, pXRF detected LFI tests will combine the speed and cost of LFI tests with the lower detection limits of PCR.

Herein, we use pXRF measurements to quantify AuNPs bound to the background, the test line, and the control line in a pregnancy test. We used purified human chorionic gonadotropin (hCG) as the positive biomarker in these LFI test strips. We used pXRF to measure elemental levels of gold and also visual methods to evaluate seLFI strips and compared the results of these in house seLFI strips to that of commercially available LFI strips. Our findings suggest the pXRF is a useful tool for LFI development as well as for increasing sensitivity.

\textbf{B.3 Methods}

\textit{Lateral flow Immunoassays:} The method used to run seLFI tests is illustrated in Figure B.0.2. The seLFI tests were run in a vertical flow format with 90 µL of blocking buffer (Tris Buffered Saline with 1% Tween and 0.1% dry milk), 10 µL of pre-conjugated AuNP anti-hCG antibody from Fitzgerald Industries, and
various concentrations of hCG (0 -5.38 µg/mL). The seLFI strip was placed into the solution and the sample flowed up the seLFI test by capillary action. Once the sample had saturated the top control line, the strip was moved into a new tube containing 100 µL of blocking buffer and allowed to run for 1 hr. After the samples had run for 1-hour they were transferred into washing containers. Each strip was shaken in 5 mL of blocking buffer for 5 min. The buffer was emptied, and the blocking buffer wash procedure was repeated two more times. After washing the test strip was laid flat to dry. After the test strips had dried, they were quantified with the pXRF.

**Figure B.2 seLFI Wash Procedure**

The seLFI test strips were evaluated by A) preparing 100 µL samples that were prepared with various concentrations of hCG in blocking buffer containing 10 µL of pre-conjugated anti-hCG antibody bound to AuNP’s. B) The LFI strip is placed into sample. C) The anti-hCG-AuNP-conjugate binds the hCG and migrates up the LFI strip. The AuNP-conjugate binds at the control or test lines. D) While the test is running 100 µL of blocking buffer was prepared. When the sample had saturated the top control line the seLFI strip is moved to E) as a washing step. This helped to clear the pink non-specifically bound AuNPs from the background as blocking buffer washed any non-specific bound AuNP in the background to the top of the test strip. F) The resulting sample showed reduced background signal. G) The resulting strip had reduced background signal but still had a visibly pale pink background. H) The residual gold background signal was further reduced by performing three five-minute washes in blocking buffer. I) The resulting samples had significantly improved clearance in comparison to the unwashed samples.
Commercially Available hCG LFI tests: A commercially available hCG pregnancy LFI strip from ClinicalGuard® (Item # THCG25ZZ) was used as a reference for comparison to our seLFI strip tests. ClinicalGuard® test strips were placed in a solution of 90 μL blocking buffer (TBS with 1% tween and 0.1% dry milk) and 10 μL of pre-conjugated AuNP anti-hCG antibody (Fitzgerald 62-H25C), and then spiked with varying amounts of hCG. After the initial run, the test strip was placed in the 100 μL of blocking buffer to wash the test of background AuNPs. Strips were left in the blocking buffer for 1 hour then laid flat to dry. After the test strips had dried, they were quantified with the pXRF. The washing procedure was less stringent for the commercial test because it had an absorbance pad that efficiently pulled the gold nanoparticle-antibody conjugate through the test strip.

pXRF Imaging: Schematic showing flow of running tests to quantifying gold is shown in Figure B.3. Quantification of test and control lines was performed by testing the background (a location on the test that did not correspond to a test or control line), the test line, and the control line. Quantification was performed using a Bruker Tracer 5i handheld XRF with an 8 mm collimator and a copper, titanium, and aluminum filter with the respective metal widths: 75 μm, 25 μm, and 200 μm. All measurements were taken using an incident x-ray energy of 42 keV and 67 currents for 180 seconds ambient conditions. Using Artax software we quantified the amount of AuNPs using the Lβ1 band for gold at 11.4430 keV representing the electronic transition of L2-M4. Placement of test strips was standardized with a self-made strip holder. The 3D holder was employed to hold the LFIs in consistent locations that analyzed only the location of the test or
control lines or a location that corresponded with the background of the test (Figure B.6). The three samples were measured three different times in each of the three positions.

**Figure B.3 Experimental Schematic for pXRF Quantification of LFIs**

LFI samples are run in a buffer with AuNP antibody conjugates. The sample and AuNP conjugates wick up the strip. Once sample has reached the top of the strip, strips are then laid flat to dry. Following this the test and control lines are quantified using the pXRF to measure the amount of gold conjugated to the antibody of interest. The XRF spectrum on the right shows the incident emission spectrum. We quantified gold levels using the Lβ₁ electron transition energy to its Gaussian like distribution and low interference with other elemental transitions.

**B.4 Results**

The original seLFI half strip, shown in Figure B.4A, shows a purple background signal throughout the test. We were able to decrease the purple background by doubling the length of the test where the longer strip acted as an absorbance pad (Figure B.4B). The background is lighter, and the test and control lines show more distinctive lines and the excess AuNP was cleared and is observed at the top of the test. Even with the longer seLFI strip, the background still appeared pink due to non-specific binding of AuNPs. In Figure B.3C we introduced the chase buffer and washing steps mentioned in . The chase buffer and washes provided a decrease in pink color from AuNP nonspecifically bound to the paper and the residual gold signal in the
background was further reduced by performing three five-minute washes in the blocking buffer. 

The resulting test strips had significantly reduced background signal in comparison to the unwashed sample resulting in increased visual sensitivity. We proceeded with a quantitative assessment on the amount gold bound to the control and test lines as well as the background in the washed test strips using pXRF.

We evaluated the best energy transition peak to quantify gold in the XRF spectrum as shown in Figure B.5. The ideal pXRF peak for elemental analysis quantitation will have a Gaussian distribution. Other elements like zinc appear in the spectrum and overlap with the more intense peaks for gold making the gold peak appear lopsided or non-Gaussian. We found that although the Lβ transition for the L2-M4 fluorescence emission is not the most intense peak for gold, it did have less interference of other elements\textsuperscript{138}. There are other energy level transitions that could be used but the Lβ had the least amount of background for other elements present in our sample matrix. Therefore, we chose this peak for quantitative analysis of gold bound at the

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**Figure B.4 seLFI Strip Modifications to Enhance Sensitivity**

All strips show two lines. The bottom line is the test line and the top line is the control line. A) Original seLFI strip had high background signal from nonspecifically bound AuNPs. B) Elongated seLFI strip shows a reduction in background signal because the longer paper acted as an absorbance pad. C) Elongated and washed seLFI strip showed the most significant reduction in background signal. These samples were used for pXRF quantitation. D) Amazon LFI for an example of gold standard clearance from an absorbance pad and visual sensitivity. All samples were run at 25 μg/mL hCG.
test and control lines. We compared the intensity of the Lβ₁ peak when evaluating differences between AuNP levels in the background, test line, or control lines.
The pXRF results for the background, control lines and test lines of the washed seLFI tests are shown in Figure B.6. We see that samples consistently have reduced background signal after wash steps. The resulting S/N ratio of 76 was calculated for the test line on the washed strips shown in Figure B.6. This S/N is more than five standards deviations above the background signal for gold. We did not introduce quantitative measurements on the seLFI strips before we introduced the washing steps.

The washed seLFI strips gave proof of concept data showing quantitation of the test and control lines can be done with pXRF on LFI tests but the seLFI strips have poor reproducibility in terms of test and control line distance, width, and linearity. We used an 8 mm collimator to

![Figure B.6 seLFI Strips & pXRF Quantifications](image)

Three identical seLFI samples, photo to the right, were run with a 0.484 ng/mL concentration of hCG. Each sample was analyzed by the XRF in triplicate. P values were calculated using an ordinary one-way ANOVA. P<0.0001=**** using n=3 measurements per sample.

The washed seLFI strips gave proof of concept data showing quantitation of the test and control lines can be done with pXRF on LFI tests but the seLFI strips have poor reproducibility in terms of test and control line distance, width, and linearity. We used an 8 mm collimator to
collimate the excitation energy. This means that we evaluated samples with an 8 mm circular area. The placement of the lines was not exact from strip to strip, and this variation led to averaging with more background leading to decreased reproducibility in the results. Due to this variability in the striping method and strip construction of our seLFI strips we decided to evaluate the use of pXRF on a commercially available LFI test. The commercial LFI test strips have more reproducible test and control lines.

The pXRF analysis using the commercially available ClinicalGuard® LFI test strips are shown in Figure B.7. As the hCG concentration increases the visibility of the test line increases (Figure B.7A) and the quantity of AuNPs bound at the test line is quantitated by pXRF (Figure B.7C). When high concentrations of hCG were added (2.7 and 5.38 μg/mL) the intensity of the control line begins to decrease (Figure B.7A and Figure B.7B). This shows that when the hCG concentration is very high, most of the gold binds to the test line and less gold is available for binding at the control line. The pXRF scans show this trend quantitatively in Figure B.7. Our results show that the pXRF has a limit of detection of approximately 0.025 μg/mL on the ClinicalGuard® LFI test.

### B.5 Discussion

The goal of this work was to evaluate the usefulness of printer paper LFI tests and to develop methods to improve the limit of detection of AuNPs bound at the test and control lines. We observed that the short half-strip seLFI tests had high AuNP background compared to longer seLFI tests or commercial tests with an absorbance pad (Figure B.4). At higher levels of biomarker, all tests showed positive results but background from AuNPs would prevent
visualization of weak positive test lines in the seLFI half strip because the signal would be lost in the background. We determined that longer seLFI strips gave improved AuNP clearance that will improve the ability to visualize positive results.

To properly evaluate the pXRF for quantitative analysis of the LFI tests, we needed to lower the AuNP signal in the background of the tests. To decrease the AuNP background signal we elongated the seLFI test strips and introduced a chase buffer and washing steps (Figure B.2, Figure B.4). The improved clearance of AuNP from the background allowed the evaluation of pXRF as a quantitative reader of LFI tests.

The introduction of wash steps introduces more opportunities for user error or variability. In the seLFI strips we did not use a sample or absorbance pad, both of which are known to help in clearing
the background levels of AuNPs\textsuperscript{139}. Another alternative to minimize background signal from AuNPs employs bovine serum albumin (BSA) in the running buffer\textsuperscript{140}. BSA (68 kDa) is a protein that is smaller than antibodies (~250 kDa) and will wick up the test strip faster than the antibody-AuNP conjugate blocking the test strip and mitigating non-specific binding of AuNPs. We chose not to use BSA because we were investigating low resource options in this simple LF1 and used the minimal buffer components to keep the testing conditions simple. Additionally, elongating the test strip also increased run time to 1 hour. The combination of a longer run time and introducing more steps increases the time it takes to run and analyze a test to about 2.5 hours. This would no longer provide a “rapid” test option meaning results in <30 minutes\textsuperscript{133, 141}.

Since one of our goals was a proof-of-concept study to evaluate pXRF as a detection method for the seLFI test, we proceeded with the longer washed seLFI tests to evaluate pXRF. Figure B.5 shows the spectrum of the test and control lines. Some intense gold peaks are observed but several of the gold peaks are close to zinc peaks that could interfere with properly quantifying the gold. We chose the L\textsubscript{\beta\textsubscript{1}} XRF band for gold at 11.4430 keV which is the electronic transition of L\textsubscript{2}-M\textsubscript{4} because as it has no zinc interference. We recognize that choosing a more intense gold peak will allow a lower limit of detection to be obtained but might require the use of metal free buffers or chelators to eliminate interfering metals.

In this study, only one size of AuNPs were used. Using a larger sized AuNP will provide increased the pXRF signal and lower the limit of detection. Additionally, there are many other types of nanoparticles to explore. Strategically choosing metal nanoparticles that have intense absorbance peaks in the pXRF spectrum is an option to increase sensitivity of this method. Other metals may also have peaks in the pXRF spectrum that do not have overlap from other metals.
Additionally, exploring simplified running buffers or metal free buffers could also aid in improving the pXRF analysis.

We tested the pXRF on commercially available LFI strips with much more accurate deposition of antibodies with carefully deposited test and control lines (Figure B.7). In a series of tests evaluated at different biomarker concentrations, the commercial test performed well. The test and control lines could be evaluated quantitatively using pXRF (Figure B.7B and C). Interestingly, in the ClinicalGuard test in Figure B.7A we see visually that as hCG concentrations increase more gold binds to the test line and less gold binds to the control line. This data supports the idea that there is a finite amount of gold in the test and that quantity is sufficient to obtain a positive or negative answer with the test.

The results of the ClinicalGuard® LFI test strips from Figure B.7 are helpful to understand common limits of detection when building a strip. If the goal is to target a positive test for lower or specified concentration ranges, the concentration of test line antibodies and conjugate antibodies need to be increased or amplified in order to visually see the test line. The limit of detection for the ClinicalGuard tests is advertised as 25 mIU/ml which is equivalent to approximately 2.5 μg/mL using a conversion of 0.1 IU=1mg. Our results suggest that the pXRF is able to lower that detection limit to 0.025 μg/mL which is a 100-fold increase in detection with the current pXRF settings.

The results from pXRF quantitation also give insight into the amount of AuNP-antibody conjugate needed for a test. Supplying the correct quantity of AuNP-conjugate antibody for the specific levels of antigen required for a positive test is critical. For a pregnancy test, where a positive or negative answer is required, an excess amount of AuNP-conjugated antibody is required to see both a test and control line. In other words, if a sample had too much hCG it
could potentially bind all of the AuNP antibody conjugate to the test line resulting in nothing binding to the control line. However, for a quantitative LFI test, it is important to understand the AuNP-conjugated antibody quantity to know how to interpret the result. For example, a known total concentration of a limited quantity of AuNP-conjugated antibody could allow quantitation by measuring total AuNP captured and determining the difference in AuNP bound at each line. Other tests might be designed so that the test and control lines can be completely saturated to validate a quantitative number. More research here is needed to develop these quantitative analysis methods.

### B.6 Conclusion

The pXRF is a viable quantitative companion tool for evaluating sensitivity in the development of LFI techniques. The pXRF quantification helped us to increase the detection limit by a factor of 100 in commercially available strips. The pXRF can quantitatively define differences in gold nanoparticle concentrations. New method combinations of sensitization and quantification should be explored to allow for the detection of low-level biomarkers. Functionalized gold surfaces can provide quick chemistry to bind more visible compounds like larger nanoparticles with higher extinction coefficients. Investigations on other electronic transition peaks in the XRF spectrum could provide a peak with higher sensitivity for LFI evaluation. It is important to keep in mind that some added steps to improve sensitivity in quantification may compromise the user-friendliness of LFI tests as well as the time to run the tests. Aside from introducing a reader and wash steps, other methods can be employed to increase the sensitivity of LFIs. Including some listed in recent reviews.\textsuperscript{139, 142}
Appendix C. Elemental Mapping of biological samples using portable XRF
Naomi Flindt, Jill Clapperton, Bruce Kaiser, Richard Watt

C.1 Abstract

Iron and other metals mechanisms of transport throughout the body are not fully understood in part because we don’t have accessible tools that can sensitively investigate metal localization. I explored the limitations and possibilities of using portable x-ray fluorescence (pXRF) to investigate metal localization in biological samples including cell lysates and animal tissues. While the pXRF has potential its current limitations include sensitivity and pixel size.

C.2 Elemental Mapping: A Potential Diagnostic Tool

A hallmark of AD is the accumulation of Fe, Zn, and Cu deposits in the brain. Diabetes, arthritis, macular degeneration, and other chronic inflammatory diseases that are precursor diseases to AD all show disruptions in metal homeostasis. The role that metals play during chronic inflammation is not well understood. Most research tends to focus solely on iron however we are finding that iron and other metals are often found localized together. FPN, Ft, DMT1 and other iron regulatory proteins have shown to transport and store metals other than iron. Understanding the interplay between iron and other metals is key to understanding inflammatory related mechanisms. For example Zinc metallothionein (MT) releases Zn$^{+2}$ with concomitant oxidation of cysteine residues as an antioxidant mechanism to quench iron induced ROS in the KEAP1-Nrf2 pathway. Copper is found in hephaestin (HEPH) and ceruloplasmin (Cp), proteins that bind to the iron transporter (FPN). These copper proteins are ferroxidase enzymes that oxidize iron as it leaves the cell to facilitate Fe$^{+3}$ loading onto TFN. How hepcidin
affects these metals and metalloproteins and their respective metabolic pathways is not fully understood. Other metalloproteins and peptides that are known to have crossover with iron and other metals include super-oxide dismutase (Cu, Zn), catalase (Mn), and xanthine oxidase (Mo).

Hepcidin is present systemically in the bloodstream and comes primarily from the liver. It has been shown that hepcidin alters the content of iron in the liver and other organs. Other organs such as the spleen, kidneys, heart, and brain also respond to the hepcidin/FPN axis as cited earlier. In the work of Cavey et al. molybdenum and manganese have different trends in the spleen and liver in response to hepcidin. This data shows that organs respond differently to hepcidin. Understanding hepcidin’s influence on each organ is key to characterizing which organs are most susceptible to metal accumulation in response to inflammation. Multi-elemental analysis is a challenging task especially in biological samples.

Previous models such as the work of Milanino et al. have studied the effects of acute inflammation and arthritis on copper and zinc localization in the kidneys, liver, spleen, and blood. These studies have been limited to a small subset of metals and organs. Ma et al. investigated the distribution of 18 elements across kidney, liver, brain, and heart in 26 different mammalian species. This study broadened the scope on the number of metals and made hypotheses to their correlation with metalloprotein and ROS levels but never quantified or correlated them.

Previous metal quantitation studies were performed via colorimetric or ICP-MS assays alone. While inductively coupled mass spectrometry (ICPMS) and colorimetric methods are popular, they have limitations due to laborious sample preparation, cost of equipment, and sample destruction. Mass spectrometry can be limited by the ionization efficiency of the
sample. This limitation often occurs in complex sample matrices, like biological samples, and can alter ICP-MS results.

There have been several recent advancements in XRF instrumentation particularly, the Bruker Tracer 5i. The Bruker Tracer 5i is a handheld laboratory grade XRF that can detect elements from Mg to Ur. The Tracer has a 10-40 keV energy range and a silicon diode detector. Detector advancements, collimators, and filters allow for more precise measurements and improved resolution. Additionally, the matrix effects that are a limitation due to ionization efficiency in ICPMS are not an issue with XRF. These improvements in instrumentation make biological analysis on portable XRF a viable method for developing a quicker and easier method for multi-elemental analysis in biological tissues. I explored the limitations and possibilities of using portable XRF to investigate metal localization in biological samples. When appropriate I used ICP-MS as a comparative technique.

C.3 Methods

samples were from a previous rat study. Lewis rats were treated with an lipopolysaccharide (LPS) injection to mimic bacterial infection. Half the rats were treated with Nelfinavir which significantly lowered hepcidin and iron levels in different organs. Using these known controls, we reinvestigated the iron levels in the soleus as well as other metal levels using pXRF. Mouse placenta samples were a gift from the Ganz Lab at UCLA. They were paraffin embedded and sectioned onto Chemplex Etnom® film and shipped to us on dry ice. Mouse healthy control liver and kidney samples were a gift from the VanRy Lab at BYU.
**Digested Tissue Quantification:** Chicken liver sampling Tyson brand Chicken livers were prepped in three different ways and analyzed on the XRF. Whole livers were analyzed first. Livers were then sliced into section with a scalpel and analyzed. Following these measurements livers were homogenized in RIPA buffer and analyzed. For analysis of homogenate 1 mL samples were placed over the aperture on top of a Chemplex Etnom® film (Chemplex cat# 095) secured with a sample cup (Chemplex cat#3115). Samples were analyzed on the XRF in triplicate.

**ICPMS:** Homogenates were weighed and suspended in trace metal grade HNO₃ at 100 mg/mL. Samples were sealed and digested at 65°C overnight. Following this samples were centrifuged and 50 μL of digested sample were diluted to 5 mL in Milli-Q water. Chromium was added as an internal standard. Samples were filtered using a 0.45 μm PES filter prior to analysis. and standards were diluted in 3% nitric acid, sonicated, and filtered. Samples were run on the Agilent and the samples analyzed by ICPMS.

**Whole Cell Lysates:** HepG2 cells were grown and investigated with XRF and ICPMS. It seems that matrix effects could be playing a major role in the ICPMS samples. HepG2 cells were grown to 80% confluency in a T-175 plate. Cells were starved of iron for 24 hours prior to induction and then treated with 0, 10, and 25 μM iron citrate for 24 hours. After 24 hours, media was collected and cells were detached from the plates and pelleted in the centrifuge. Cell pellets were rinsed three times with PBS and lysed in 500 μL of RIPA buffer. For analysis a 100 μL droplet of media or lysate sample was placed over the aperture on top of a Chemplex Etnom® film. Samples were analyzed on the XRF in triplicate.
**Whole Tissue Imaging:** Mouse livers from healthy control mice were cryo-embedded with OCT and sectioned in 50 μm sections onto Chemplex Etnom® film slides for XRF metal localization analysis. Mouse kidneys from healthy control mice were sectioned onto glass slides (Thermo Scientific) in 50 μm sections. One set of kidneys were stained with a Perl’s Prussian Blue Stain kit (Polysciences cat#24199-1).

**pXRF Imaging:** Measurements were taken using the 0.7 mm collimator under helium with various incident x-ray energies, currents, integration times, and filters. Each image will specify the instrument settings. All measurement and quantification were made using Artax software. Imaging samples were mounted to a motorized stage to raster over image.

**C.4 Results**

First, I investigated the limit of detection for iron using the pXRF in a simple aqueous matrix. Results are shown in Figure C.1 below. I compared the limit of detection to that of the ICP optical emission spectrum (OES). For the settings on the pXRF the limit of detection was lower about 50 times higher than that of the ICP-OES. Considering that the pXRF is significantly less expensive than the ICP-OES it can still aid in investigating biological samples if we have a large enough sample size. I investigated whether an increased integration time may aid in lowering the detection limit as shown in Figure C.2 below. Sixty seconds of integration led to a
higher detection limit to 59 μM detection limit as opposed to 45 μM when integrating for 30 seconds. One issue with iron is that it is very abundant in water and dust. Contamination can
become an issue. Further studies should be done using iron free water and acid cleaned containers.

I investigated the detection limits of copper using a simple aqueous matrix. I investigated the difference in the detection of copper at two different settings as shown below in Figure C.3. The 5010 represents an incident energy of 50 eV and 10 currents while 4267 represents the settings used previously of 42 eV and 67 currents. Both use the red filter made from a copper, titanium, aluminum alloy. The use of the filter helps us to selectively probe a lower range of electronic transitions. This helps to prevent our spectrum from becoming oversaturated by electronic transitions that are not in our scope of interest. We see that the LOD for the 4267 was 0.26 µM and 1.073 µM in the 5010 settings. The settings at 4267 are more than four times as sensitive. We decided to continue probing for both iron and copper with these settings.

![Graphs showing copper signal at 50 eV and 10 currents vs. copper signal at 42 eV and 67 currents]

**Figure C.3 Copper LOD Investigation**
We investigated the use of different incident energies, currents, and filters. One the left we used an incident energy of 50 eV and 10 currents. On the right results for the typical incident energy of 42 eV and 67 currents. Both used a 30 second integration time, helium, and the red filter

Next I was curious to know if these LODs were still in range for biological samples. We also wanted to introduce more complex sample matrices, we wanted to see if that would hinder
our measurements. In order to amplify our signal, we decided to begin with an iron overload induction. In Figure C.4 below we see a difference between iron levels in the HepG2 cells between the control cells grown without added iron and the iron overloaded cells treated with 25 μM iron. We also experimented with integration time and we see that sample variation decreased when integrating for 60 seconds. This led to more significant differences between treatments. It seems that RIPA buffer had a similar iron content to that of water. This implies that iron quantification via pXRF may not be as susceptible to matrix effects often found in ICPMS.

**Figure C.4 pXRF Iron Quantification in HepG2 Cell Lysates**

We used the incident energy of 42 eV and 67 currents with the red filter and helium. Samples were induced for 24 hours with various treatments of iron citrate added to media. Lysates were digested and analyzed in RIPA buffer.
Next we were excited to understand whether iron content could also be quantified in the media and how this content compared with iron quantification results in ICPMS. We induced with iron overload as well as TGF-β to induce fibrosis. Our hypothesis is that iron is sequestered in fibrotic cells. The pXRF results in Figure C.5 show that iron increases in both TGF-β induced cells as well as iron overloaded cells. It is interesting to note that iron also increases in the media when induced with TGF-β. This could be due to some cells dying off prior to collection. This data would be more meaningful if it was normalized to protein content, confluency, or total number of cells digested. On the other hand, the ICPMS results were normalized to protein content and reveal a different trend. It seems the control sample had the highest amount of iron while the iron overloaded sample had the lowest amount of iron. This could be due to issues with matrix effects in ICPMS and the sample preparation process.

Figure C.5 Iron Quantification in Fibrotic and Iron Overload HepG2 Cells
Samples were quantified using an incident energy of 42 eV and 67 currents with the red filter under helium with a 60 second integration time.
Iron Levels in Digested Tissues

In order to conserve samples, we used purchased Tyson chicken liver for use in our experimental parameter investigation. Shown in Figure C.6 below digesting or looking at whole liver intact yielded similar results for our biological replicates. It is interesting to note that the intensities were much higher in the whole liver than in the homogenate. This is most likely due to a higher sample concentration for the whole liver, where the homogenate has a dilution effect from the buffer. This may work for the liver but more investigations need to be done on other tissue types. Organs that are more heterogeneous may pose more of a sampling issue.

Figure C.6 Sample Method Comparison for pXRF on Liver
Two sample preparations were compared. Whole liver measurements and homogenized samples in RIPA buffer. We also compared these XRF results to that of the ICPMS.

Seeing that whole liver gave the same trend in iron as that of homogenate on the XRF. It seems that sample preparation does not make a big difference assuming thickness of sample does
not vary significantly. We decided to continue to investigate trends of whole tissue iron in other organs such as muscle. We used Lewis rat soleus muscle from a previous animal study. The setup for this animal study is shown in Figure C.7. Although no significant differences were found we were excited about seeing a potential trend. It would have been better to have investigated iron levels with an increased integration time as this has been shown to decrease sample variation. Additionally these samples were not normalized in anyway. Normalizing them by thickness may provide more definitive results. Using sectioned tissue samples may provide a more uniform way of analyzing tissues.

**Figure C.7 Comparison of Iron in Soleus Muscle in Lewis Rats**
Rats were inflamed via LPS injection and treated with Nelfinavir. We evaluated levels of iron in the soleus tissue to see if treatment groups had altered levels of iron. XRF measurements were taken with an incident energy of 42 eV and 67 currents with the red filter under helium with a 30 second integration time.
Iron localization in Mouse Tissue

In addition to whole tissue probing, we have been exploring the use of the handheld XRF to scan over tissue slides to define the localization of metals within an organ. This imaging technique provides a unique perspective as to where the metals are localizing within the organ and can provide information on the mechanism of metal localization during inflammation and disease. To begin our investigation, we used paraffin embedded mouse placenta from the Ganz...
lab at UCLA. Figure C.8 shows a picture of the placenta tissue mounted on the film. The spectra on the right show the localization of iron, potassium and calcium. It seems that potassium has the best outline for where the tissue is and may be in the paraffin wax. Iron looks slightly more localized. We employed the use of two other setting but found the 25 eV and 90 currents with the yellow filter worked the best for seeing iron while mitigating background levels from paraffin. The general localization of elements is present although resolution is poor. It may also be that the

**Figure C.9 Mouse Liver Elemental Imaging**
Mouse livers were sectioned at 50 μm onto glass slides. The top two rows show elemental analysis of the plain sample. The bottom two rows show the localization for the Prussian blue stained sample. Images were taken using an incident every of 25 eV and 90 currents with no filter using the 0.7 mm collimator and 30 seconds of integration.
sample was too thin to produce a strong enough signal. Much of the signal is quenched by ambient conditions and could be strengthened if run under nitrogen or argon as opposed to helium or air.

In addition to mouse placenta we also investigated livers and kidneys. We started with the livers on glass. We stained one with Perl’s Prussian Blue stain and the other was not stained. We wanted to investigate whether we could use a more accessible sample platform such as glass and secondly whether samples being investigated for other things could be simultaneously investigated for iron analysis. It seems there were not as many background matrix effects in the OCT embedding compound as there were in the paraffin as shown in Figure C.9.

Perl’s Prussian blue staining seemed to enhance the signal for iron in the liver. Further studies need to be done to investigate whether or not this iron is introduced from the stain or is amplified by the stain. It is also important to note that background iron levels in glass could have also played a role in the low sensitivity of the liver on glass. Further studies can and should be done to improve the sensitivity of this measurement.

Following the analysis in livers we investigated the appropriate settings to analyze several elements simultaneously in the kidney. The kidney samples were mounted onto Etnom® film which had much better sensitivity for elements like iron. Images are shown in Figure C.10 below. We see that the lower energy was useful for smaller elements such as sulfur, calcium, and potassium. While, the higher energy was better at producing a signal for larger elements such as copper and zinc.
Figure C.10 Elemental Distribution in Kidney
Cryo-embedded kidneys were sectioned at 50 μm onto Etnom® film. Two different settings were used. The left two columns used an incident energy of 25 eV and 90 currents with no filter. The right two columns show an increased excitation energy of 35 eV and 90 currents with the use of a yellow filter. A 30 second integration time and 0.7 mm collimator was used.
C.5 Discussion
Understanding metal localization can be a useful tool in understanding the roles that these metals play in disease. Limited accessibility to imaging platforms due to cost and availability can be a factor in advancing imaging techniques. The use of pXRF as a cost effective alternative has shown potential in evaluating biological samples such as tissues and cell lysates. We see some exciting preliminary results in Figure C.0.4 and Figure C.0.5 where differences in HepG2 lysate and media were observed. Evaluating cell lysates in ICP-MS has been a particularly challenging task due to matrix effects. It seems that those matrix effects are not as prevalent in the pXRF results as water and RIPA show similar results unlike in ICPMS. A benefit to using pXRF is the potential for simpler sample preparation techniques. As we see in Figure C.6 and Figure C.7 where whole tissue was analyzed and trends in iron levels were revealed. It is interesting to note that these did not compare well with ICP-MS results. ICP-MS is considered the gold standard for elemental analysis however, laborious sample preparation and matrix effects may be playing a role in nuanced results. Iron is a particularly difficult to probe element due to issues with contamination. It is possible that pXRF may not be subject to matrix effects as in ICP-MS. Normalization techniques for pXRF sample need to be explored. In the work of Cavey et al.47 they employ the use of certified biological reference standards.

Metal imaging provides a new dimension to elemental analysis allowing for metal co-localization analyses. It is interesting that sulfur is consistently an element found localized in the tissue. This helps to understand where the tissue is. In Figure C.9 we see the localization of iron and calcium increase after staining with Perl’s Prussian Blue. This is most likely due to element abundant in the stain. A similar metal bound antibody may be used to target the localization of different proteins in conjunction with localized metals. This can be particularly useful in
investigated metal transport proteins like zinc transporters (ZIPs) They have been shown to transport other metals aside from zinc.

Among the limitations in pXRF the resolution needs to be improved if cellular localization is to be explored. This would also benefit the organ and tissue imaging as well. In our images we see some localization to where the tissue is present. Increased resolution could be provided with the use of a smaller collimator and imaging pixel size. This would require increased imaging time. Another improvement to increase sensitivity would be to use other environments such as argon or nitrogen to probe samples. Ideally ambient pressure is critical to preserve sample integrity however vacuum conditions could also help to improve sensitivity.

We have shown that pXRF can be useful in determining the amount of copper in biological samples with a reasonable limit of detection. It seems that depending on the sample type and platform excitation energies and filters must be optimized. As we see in Figure C.10 copper was much more prevalent in the higher excitation images. This is particularly interesting as the elements with full d-block configurations like copper and zinc require higher excitation energies to emit their tightly held electrons. This is exciting as zinc and copper are notoriously difficult to probe via ICPMS and OES due to poor ionization efficiency and stability.

**C.6 Conclusion**

pXRF has the potential to be a useful technique. This was a preliminary investigation and further work is required to understand its limitations. Elemental imaging is already being used in the synchrotron and ICP forms which yields much better resolution however it is difficult and expensive to access these instruments. Implementing a smaller aperture and gases to prevent
incident x-ray quenching may improve this methods sensitivity and resolution. Creating more accessible imaging techniques more available will aid in advancing knowledge in the role that metals play in disease.
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