ASSESSMENT OF CARDIOVASCULAR ADAPTATION, ORGAN PERFUSION, AND TISSUE HYPOXIA IN AN ANTIBODY MEDIATED MODEL OF MODERATE ANEMIA

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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ABSTRACT
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ASSESSMENT OF CARDIOVASCULAR ADAPTATION, ORGAN PERFUSION, AND TISSUE HYPOXIA IN AN ANTIBODY MEDIATED MODEL OF MODERATE ANEMIA

Anemia is associated with increased brain and kidney injury, and mortality in surgical patients by undefined mechanisms. We hypothesize that anemia-induced tissue hypoxia is a potential unifying mechanism. A transgenic mouse model (HIF-ODD Luciferase) was utilized to assess the impact of antibody-mediated anemia on adaptive cardiovascular responses, tissue PO₂(PtO₂), and hypoxia-inducible factor (HIF)-expression. Red blood cell (RBC)-specific antibody (TER119) induced anemia via intravascular hemolysis, and splenic and hepatic RBC sequestration. Anemia-induced cardiovascular adaptations included increased: 1) peripheral arterial oxygen saturation, 2) cardiac output, 3) internal carotid blood flow, and 4) cerebrovascular reactivity. These mechanisms contributed to maintain brain P_tO₂. By contrast, renal perfusion was not increased, leading to a reduced renal P_tO₂. Also, increased HIF-1α expression was observed in renal, hepatic, and gut regions. These findings demonstrate that anemia-induced tissue hypoxia occurs in an organ specific manner. These data support the hypothesis that renal and splanchnic hypoxia may contribute to increased organ injury and mortality in anemic patients.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2,3-DPG</td>
<td>2,3-Diphosphoglycerate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>C_{O_2}</td>
<td>Arterial Oxygen Content</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic Helix-Loop-Helix</td>
</tr>
<tr>
<td>P_{O_2}</td>
<td>Brain Tissue Oxygen Tension</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac Output</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CVR</td>
<td>Cerebrovascular Reactivity</td>
</tr>
<tr>
<td>DO_{O_2}</td>
<td>Delivery Of O_{2}</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HbA</td>
<td>Hemoglobin A</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin Concentration</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxic Response Element</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobin M</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neural Nitric Oxide Synthase</td>
</tr>
<tr>
<td>O_{2} max</td>
<td>O_{2} Consumption</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>O_{2}</td>
<td>Oxygen</td>
</tr>
<tr>
<td>ODD</td>
<td>Oxygen Dependent Domain</td>
</tr>
<tr>
<td>S_{O_2}</td>
<td>Oxygen Saturation Of Arterial Blood</td>
</tr>
<tr>
<td>P_{CO_2}</td>
<td>Partial Pressure Of Arterial Carbon Dioxide</td>
</tr>
<tr>
<td>P_{O_2}</td>
<td>Partial Pressure Of Oxygen</td>
</tr>
<tr>
<td>P_{O_2}</td>
<td>Partial Pressure Of Oxygen In Arterial Blood</td>
</tr>
<tr>
<td>PAS</td>
<td>Per-Arnt-Sim</td>
</tr>
<tr>
<td>P_{i}</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PHD</td>
<td>Proline Hydroxylase</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>RR</td>
<td>Relative Risk</td>
</tr>
<tr>
<td>SCA</td>
<td>Sickle Cell Anemia</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation Domain</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel Lindau Protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
1. **STATEMENT OF HYPOTHESIS AND AIMS**

The following series of experiments utilizes a RBC-specific antibody mediated model of anemia to test the following hypotheses:

1.1. **GENERAL HYPOTHESIS**

Anemia-induced tissue hypoxia is a unifying mechanism for organ injury and mortality.

1.2. **SPECIFIC HYPOTHESIS**

i. Moderate anemia causes tissue hypoxia, disrupts oxygen homeostasis and depletes cellular energetics in an organ specific manner

ii. Moderate anemia triggers adaptive cardiovascular responses which do not fully compensate for a reduction in blood oxygen content and tissue oxygen delivery resulting in tissue hypoxia.

1.3. **OBJECTIVES AND SPECIFIC AIMS**

The following specific aims are proposed:

Aim 1. To characterize the mechanism of moderate anemia produced by an RBC binding antibody and its effects on circulating hemoglobin levels.

Aim 2. To identify the degree of adaptive cardiovascular responses associated with anti-RBC mediated anemia.

Aim 3. To determine if organ specific tissue hypoxia (brain, kidney) occurs during anti-RBC mediated anemia.

Aim 4. To determine if increased stabilization of tissue specific HIF-1α is occurs during anti-RBC mediated anemia.

Aim 5. To assess the impact of anti-RBC mediated anemia on cerebrovascular CO₂ reactivity as a surrogate of microvascular reserve.
2. INTRODUCTION

ANEMIA IS A WORLDWIDE HEALTH PROBLEM

WHAT IS THE IMPACT OF ANEMIA?

Anemia is defined as a reduction in circulating red blood cells (RBC), resulting in a decreased hemoglobin concentration (Hb) and impairment in oxygen (O\textsubscript{2}) delivery. A report assessing the global burden of disease predicted that anemia affected 32.9% of the global population as of 2010.\textsuperscript{1} Due to variations in baseline Hb caused by ethnicity, sex, and age, the World Health Organization (WHO) has defined anemia as a Hb threshold value of $<130$g/L for men and $<120$g/L for women\textsuperscript{2} These definitions have been validated to be two standard deviation from the mean of a normally distributed healthy population. In 1989, the WHO updated its definition to establish criteria for describing the severity of anemia, where Hb values $>80\%$ of the cutoff are classified as mild anemia ($Hb \approx >100$g/L), values between 80% and 60% of the cutoff are classified as moderate anemia ($Hb \approx 80$-100g/L), and values $>60\%$ of the cutoff are severe anemia ($Hb \approx <80$g/L) to identify populations at greatest risk.\textsuperscript{3} The purpose of this thesis is to assess an RBC-antibody mediated model of anemia which produced a stable Hb within the moderate anemia range, as this Hb range has been associated with increased organ injury and morbidity.

POPULATIONS AT HIGHEST RISK OF DEVELOPING ANEMIA

Geographically, anemia is most prevalent in developing nations. South Asia accounts for 37.5% of global anemia burden, and Sub-Saharan Africa accounts for 23.9% of the global anemia burden.\textsuperscript{1} These areas are associated with malaria and parasitic diseases which are a primary cause of anemia. By contrast, the lowest anemia prevalence rates are in higher-income regions such as North America and Western Europe.\textsuperscript{1} It has been suggested that developed nations have better access to resources to treat and prevent anemia. This is supported by the
finding that poorer populations in high-income nations have been demonstrated to have an increased risk of developing anemia. In a study assessing patients in Minneapolis, Minnesota; children in households with very low food security (limited access to nutritionally adequate foods) are twice as likely to have iron deficiency anemia compared to children from families with higher food security.\(^4\) Therefore, anemia is a problem specific to geographic regions, as well as economic prosperity.

Anemia also targets patients with specific age demographics. For example, post neonatal children and children younger than 4 years old represent age groups with a very high prevalence and severity of anemia.\(^1\) The impact of anemia in children and young adults has been characterized by the Global Burden of Disease Pediatrics Collaboration, who listed anemia as one of the leading causes of years lived with disability.\(^5\) In addition, school children with anemia associated with malaria have been shown to have deficiencies in academic performance, which are corrected by malaria treatments.\(^6\) Developing children have been identified as the most vulnerable patient populations subject to adverse outcomes associated with the disease, and as such, has been granted the highest priority for treatment from the WHO.\(^7\) Children with sickle cell anemia (SCA) provide an example. These children experience an increased incidence of stroke at an early age, likely secondary to the imbalance in cerebral oxygen delivery as a result of its complex disease process.\(^8\) These examples provide some of the rationale for our assessment of the impact of anemia on tissue oxygen delivery in animal models.
CAUSES OF ANEMIA

Experimental studies within this thesis specifically assess physiological adaptations in an acute model of anemia. However, for the context of this review, a cross section of specific types of acute and chronic anemia will be referenced for their associations with major clinical outcomes. The focus of this review will be to assess types and degrees of acute and chronic anemia that have been clearly been associated with organ dysfunction, organ injury and increased mortality.

NUTRITIONAL DEFICIENCIES

Nutritional deficiencies are the leading etiology of anemia.¹ The deficiency can be due to inadequate consumption or malabsorption of the required nutrient. The major nutritional deficiencies inducing anemia are attributed to inadequate iron metabolism, vitamin B12 levels, and folate levels; all which are co-factors required for the formation a porphyrin ring for heme synthesis.⁹ Any irregularities in the absorption of the micronutrient, or in the regulation of its transport to the bone marrow for erythropoiesis, can result in the inadequate production of hemoglobin; thus, reducing the production of RBC and leading to anemia.⁹

In addition to inadequate absorption, dysfunctional iron metabolism can also be caused by increased iron sequestration leading to iron-restrictive anemia. Major causes for iron-restrictive anemia are systemic inflammatory diseases, where pro-inflammatory cytokine interleukin-6 (Il-6) promotes production of hepcidin.¹⁰ Hepcidin is an inhibitor for ferroportin, a transmembrane protein allowing stored iron from the cells to the bloodstream. Therefore, increased hepcidin may restrict iron transport to the bone marrow for erythropoiesis, reducing circulating RBC levels.¹⁰ Iron deficiency and iron sequestration anemia affect up to 50% of patients in acute care settings, identifying this as a major health issue.¹¹
GENETIC DISORDERS CAUSING ANEMIA

Hemoglobin is a major component of the RBC and is composed of a heme group and globin tetramer. In adults, approximately 98% of hemoglobin isoform expressed is hemoglobin A (HbA), which consists of 2α- and 2β-globin subunits, each tightly associated with a heme group facilitating oxygen binding in a cooperative fashion. Hemoglobinopathies arise from genetic mutations of the genes encoding the structure of hemoglobin, leading to irregular globin protein shape (sickle cell anemia), or reduced globin protein production (thalassemia). Patients with sickle cell anemia most commonly have a missense mutation within their β-globin gene, in which a single nucleotide mutation (adenosine to thymine) results in a change in a single amino acid where glutamic acid is replaced with a valine residue. This single amino acid substitution results in a complex series of pathophysiological changes, including: 1) a change in hemoglobin protein confirmation after Hb deoxygenation, causing the RBC to sickle and become rigid and fragile, and subject to lysis, 2) an increase in inflammatory mediators, 3) macrovascular and microvascular vasculopathy. These changes lead to reduced organ perfusion and sickle cell crisis. Sickle cell anemia is of specific interest because it is associated with increased incidence of stroke in children.

Mutations to glycolytic enzymes, crucial for defense reactive oxygen species can also cause anemia. For example, glucose-6-phosphate dehydrogenase (G6PD) catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate, which facilitates the reduction of glutathione. Reduced glutathione is utilized as a buffer in the RBC, neutralizing hydrogen peroxide to produce water. The G6PD gene is located on the X-chromosome, and many mutations can lead to G6PD deficiency. Patients with genetic mutations for G6PD have a reduced anti-oxidant capacity of the RBC, which may contribute to membrane damage and hemolysis. This type of anemia is associated with splenomegaly thought to be due to filtration of
damaged RBC and RBC fragments by the reticuloendothelial system and spleen.\textsuperscript{16} This is a pattern seen by our currently utilized model of anemia.\textsuperscript{17}

**AUTOIMMUNE ANEMIA**

Autoimmune hemolytic anemia is a condition affecting 1-3 patients per 100,000 per year.\textsuperscript{18} It is caused by the production of antibodies specific to antigens on the RBC surface, leading to RBC clearance primarily through intravascular hemolysis or sequestration into the reticuloendothelial system. The major causes for autoimmune hemolytic anemia include: drug induced autoantibody production, a preexisting autoimmune disorder, allogeneic RBC exposure, and idiopathic onset of the disease.\textsuperscript{19} Clinical cases of autoimmune hemolytic anemia are categorized by the temperature at which they are most reactive. During warm autoimmune hemolytic anemia, antibodies are most reactive around 37°C, and it is typically Immunoglobulin G (IgG) mediated. Therefore, RBC are cleared in warm autoimmune hemolytic anemia primarily via sequestration in the reticuloendothelial system.\textsuperscript{19} In cases of cold autoimmune hemolytic anemia, antibodies are most reactive around 0-4°C, and it is typically Immunoglobulin M (IgM) mediated. Therefore, RBCs are cleared in cold autoimmune hemolytic anemia primarily via compliment mediated intravascular hemolysis.\textsuperscript{19} The presence of warm and cold autoantibodies is not mutually exclusive, as there are clinical cases where patients present with a combination of both autoantibodies.\textsuperscript{19} This mechanism of anemia is of particular interest as we have utilized an RBC-binding antibody to make rodents anemic in order to study the effects of anemia on organ perfusion and tissue hypoxia.
CLINICAL EVIDENCE OF ANEMIA-INDUCED MORBIDITY AND MORTALITY

ANEMIA IMPACTS OVERALL QUALITY OF LIFE

It has been demonstrated in many patient populations that anemia, regardless of its etiology, is associated with a reduced quality of life. In elderly and orthopedic surgery patients, iron deficiency anemia is associated with a decreased quality of life, and reduced ability to perform activities of daily living.\textsuperscript{9} Quality of life surveys in young renal transplant patients (mean age = 39.2±11.5 years) demonstrate that anemia is associated with poor mental health status, that is proportional to the decreasing Hb concentration.\textsuperscript{20} In addition, children with sickle cell anemia and preexisting silent cerebral infarction have low quality of life scores. Interestingly, these scores are improved with chronic blood transfusion therapy.\textsuperscript{21} This suggests that strategies to correct anemia have the potential to improve patient quality of life in many patient populations.

ANEMIA IS ASSOCIATED WITH A DECREASE IN EXERCISE CAPACITY

During exercise, the rate of O\textsubscript{2} consumption can increase from a resting rate of \textasciitilde 250ml/min, up to 6000ml/min.\textsuperscript{22} This increase in O\textsubscript{2} requirement is provided by increases in respiratory gas exchange, cardiac output, skeletal muscle blood flow, and O\textsubscript{2} extraction. Anemia can significantly impair the capacity of high performance endurance athletes. A reduction in Hb concentrations reduces skeletal muscle O\textsubscript{2} delivery, and insufficiently supplying skeletal muscle O\textsubscript{2} below its metabolic requirements. For example, healthy adults exposed to acute hemodilutional anemia have increased fatigue levels.\textsuperscript{23} Another highly publicized example occurred during the London 2012 Olympics, where Canadian triathlete Paula Findlay struggled to complete her event due to an undiagnosed and/or undertreated iron deficiency anemia. Conversely, it has been demonstrated that augmentation of Hb concentrations, by blood doping, can significantly increase the storage capacity and delivery of O\textsubscript{2}; as evidenced by 7-time Tour
de France winner, Lance Armstrong. Clinically, in diseases such as heart failure, anemia is also associated with a reduced exercise capacity. In the RELAX trial, exercise capacity was measured in heart failure patients. In these patients, anemia was associated with a reduction in peak $O_2$ consumption (VO$_2$ max). The level of exercise capacity was reduced in proportion to the degree of anemia, suggesting a link between Hb, blood oxygen content, tissue $O_2$ delivery, and exercise capacity. Treatment of patients with chronic heart failure by administration of erythropoietin (EPO) was associated with increased Hb concentrations, higher peak $O_2$ consumption, and increased distance travelled in 6 minutes. These findings have also been demonstrated in children with sickle cell anemia, where patients on chronic blood transfusion therapy and on hydroxyurea, increasing Hb concentrations, have improved 6-minute walk test scores relative to untreated patients. This suggests that during anemia, patients have a reduced capacity for exercise, and restoration of RBC allows for optimization of $O_2$ delivery to reverse impairments to exercise function.

ANEMIA IMPACTS COGNITIVE FUNCTION

Clinical trials assessing patients with acute and chronic anemia have demonstrated evidence of neurological dysfunction. For example, children with sickle cell anemia have impaired cognitive function as evidenced by reduced school scores, cognitive decline, and learning disabilities. In addition, in a randomized controlled trial assessing the impact of malaria in Kenyan school children demonstrated lower than expected academic scores. Interestingly, when these children received treatment for malaria, their Hb concentrations increased and their test scores were improved relative to the placebo group. Furthermore, healthy adults exposed to severe acute hemodilutional anemia (Hb <60g/L) experienced impaired cognitive function (reduced response time and error rate) and neurological latency. These effects reversed by reinfusion of autologous blood or $O_2$ administration. These findings suggest
anemia causes cognitive deficits in a spectrum of healthy individuals and systemic disease. Although a direct link to inadequacy of cerebral oxygen delivery has not been made, we hypothesize that this lack of cognitive ability may be attributable to a lack of oxygen delivery to the brain during anemia for the following reasons: 1) the increase in metabolic requirement for oxygen associated with mental processing places an increased demand on neuronal function; 2) animal models of anemia have demonstrated an acute reduction in brain partial pressure of oxygen (PO$_2$) at hemoglobin levels associated with reduced cognitive function in humans; and 3) cognitive deficits are improved by autologous or allogenic RBC transfusion. Therefore, evidence of reduced cognitive abilities in anemic patients may be an early sign of inadequate oxygen delivery to the brain.

ANEMIA INCREASES THE RISK OF ORGAN INJURY

Anemia has been independently associated with increased risk of organ injury and mortality across many patient populations. Although no randomized controlled clinical trials have been conducted to formally test the causality between anemia and organ injury, many retrospective observational trials have compared the outcomes associated with anemia versus age matched control patients. One of the largest retrospective trials to date assessing the risk of anemia in surgical patients was conducted by Musallam and colleagues. Musallam et al. analyzed data from 227,425 patients undergoing major non-cardiac surgery to determine the impact of mild and moderate to severe anemia on developing a 30 day composite morbidity (development of two or more major morbidities). It was found that among all risk-factors analyzed, including systemic sepsis, central nervous system (CNS) disease, renal disease, and cardiac disease, the presence of anemia with the risk-factor significantly increased the risk of developing a composite morbidity. The association of anemia and organ-specific injury will be discussed in this section.
Risk of stroke in perioperative anemic patients

As previously discussed, the reduction in $O_2$ content due to anemia induces neurological dysfunction, and clinical data supports anemia as a factor linking brain dysfunction with brain injury. In both cardiac and non-cardiac surgery patients, preoperative anemia has been associated with increased risk of stroke.\textsuperscript{49, 41, 50} Preoperative anemia increases the risk of intraoperative anemia,\textsuperscript{51} which has also been associated with increased risk of stroke in cardiac surgery patients. The incidence of stroke has been found to increase with lowest nadir hematocrit (Hct) during cardiopulmonary bypass.\textsuperscript{40} These findings suggest anemia-induced tissue hypoxia may be a contributory mechanism for stroke in surgical patients.

Changes in cerebrovascular reactivity may predict adverse cerebral outcomes

Cerebrovascular reactivity is a measure of change in cerebral blood flow in response to a vasodilatory physiological stimulus, such as carbon dioxide (CO$_2$). The mechanisms of CO$_2$ mediated vasodilation are not fully understood, and are thought induce vasodilation via changes in extracellular pH. Davies et al. has measured increased activation of cerebral pH sensitive extravascular potassium channels following CO$_2$ exposure. Activation of these channels mediates an efflux of potassium, leading to hyperpolarization of vascular smooth muscle and ultimately vasodilation.\textsuperscript{52} Changes in cerebrovascular reactivity may predict adverse cerebral outcomes. A reduction in cerebrovascular reactivity is associated with increased risk of developing cerebral injury in patients with internal carotid stenosis\textsuperscript{53} and Moyamoya disease,\textsuperscript{54} which will be further discussed in subsequent sections.
Risk of stroke in sickle cell anemic patients

It is predicted that up to 11% of children with sickle cell anemia have had at least one overt stroke, and up to 37% having at least one silent cerebral infarct by 14 years of age. Although a clear mechanistic link to tissue hypoxia has not been made, there is evidence supporting this hypothesis. In experimental models, sickle cell anemia is associated with cerebral tissue hypoxia. Presumably, cerebral tissue hypoxia signals for an increase in cardiac output and cerebral blood flow. Patients with sickle cell anemia have in increase in cardiac output, and marked index in cerebral blood flow. The increase in cerebral blood flow in anemic patients is proportional to the decrease in Hb concentrations, suggesting that this is a compensatory adaptation to optimize cerebral oxygen delivery. (Kosinski et al., Eur J Hematol, In Press) In addition, in patients with sickle cell anemia, increased cerebral blood flow is proportional to stroke risk. Increased cerebral blood flow is associated with a reduction in cerebrovascular reactivity in animals and humans. (Kosinski et al., Eur J Hematol, In Press) Stroke occurs in macrovascular and microvascular patterns; thus the overall picture for stroke is an inadequate degree of brain perfusion and low brain tissue oxygen tension ($P_{O_2}$). This is may act as a factor contributing to morbidities associated with sickle cell disease, including: increased RBC sickling, altered RBC metabolism, increased inflammation, and vasculitis.

Transfusion of RBC reduces the risk of stroke associated with sickle cell anemia

Transfusion of RBC has been demonstrated to reduce cerebral blood flow velocity, and the incidence of both silent cerebral ischemia and stroke in these patients. Also, treatment of sickle cell anemia with hydroxyurea, increasing fetal hemoglobin content, has been shown to reduce cerebral blood flow velocity, and reduces the risk of recurrent stroke. This evidence
suggests that inadequacy of global tissue O$_2$ delivery contributes to cerebral injury, and correction of anemia restores blood oxygen delivery and is sufficient to prevent adverse outcomes associated with sickle cell anemia.

**Risk of Renal Injury in Anemic Patients**

Anemia is independently associated with increased risk of kidney dysfunction, leading to injury.$^{50,62,63}$ The renal medulla has a high metabolic demand and low perfusion to maintain its ionic gradient for solute reabsorption, causing the kidneys to function in a borderline hypoxic state. Therefore, during anemia, reduced oxygen content induces tissue hypoxia in the kidney, potentially contributing to kidney pathophysiology.$^{64}$ Many clinical trials have demonstrated perioperative anemia leading to increased renal dysfunction,$^{62}$ and acute kidney injury at hemoglobin concentrations as high as 120g/L.$^{41,50,63}$ Acute kidney injury is an independent risk factor for mortality in surgical patients.$^{65}$ In addition, anemia has been associated with increased risk for renal replacement therapy.$^{66}$ These findings suggest that impaired oxygen delivery, in conjunction with ongoing tissue hypoxia, may be a mechanism for anemia-induced renal injury during anemia.

**Risk of developing cardiac events associated with anemia**

Clinical trials have demonstrated the association of anemia, ranging from mild to severe, with increased risk of developing postoperative cardiac events in many patient populations.$^{42,67-71}$ In cardiac surgery patients, one factor contributing to anemia increasing the risk of developing cardiac events is the patient’s baseline status. Patients selected for surgery are not healthy and are often at their cardiovascular capacity. The onset of perioperative anemia may progress preexisting myocardial lesions, and could lead to the onset of myocardial infarction. In addition, anemia and blood loss have been associated with an increase in troponin levels, suggesting
damage to the myocardium. Recently, subgroup analysis of data from the TRACS trial, assessing transfusion thresholds in cardiac surgery patients undergoing cardiopulmonary bypass, has shown that elderly patients on a restrictive transfusion strategy were at increased risk of developing cardiogenic shock as opposed to patients on a liberal strategy. Cardiogenic shock may be due to inadequate perfusion; acting as another mechanism increasing global tissue hypoxia and ultimately leading to anemia-induced organ injury.

_Gut Ischemia_

Gut ischemia, leading to enterocolitis, is a significant factor causing mortality, predominantly in infants. The development of neonatal enterocolitis was originally attributed to RBC transfusion; but recent evidence suggests anemia increases the risk. Anemia has been shown to be an independent predictor of developing necrotizing enterocolitis in very low birth weight infants in a given week (Odds Ratio (OR): 5.99 [2.00-18.0], p<0.001). In addition, evidence of gut ischemia is also consistent with acute hemodilutional anemia. Clinical evidence presented by Mathru et al. has demonstrated splanchnic hypoperfusion during acute hemodilution, where a reduction in gut perfusion and an increase in gut O2 extraction was measured during anemia. This evidence suggests that splanchnic hypoperfusion potentially contributes to anemia-induced tissue hypoxia, which may act as a mechanism for gut injury. Splanchnic hypoperfusion has also been experimentally demonstrated by Van Bommel et al., who observed decreased gut perfusion in a pig, and reduced intestinal microvascular PO2 during hemodilution. Data from a recent randomized controlled transfusion trial for cardiac surgery patients, TITRe2, has demonstrated that patients randomized to the restrictive arm (patients receiving transfusions if their Hb <75 g/L) had a non-significant increase incidence of gut infarction, as compared to patients in the liberal arm. Therefore, it is suggested that anemia-
induced gut hypoxia may be a factor leading to increased enterocolitis and gut ischemia in anemic patients.

**Acute and Chronic Anemia Increases the Risk of Mortality**

Many clinical trials have associated both acute and chronic anemia with increased risk of death amongst many patient populations. Anemia is associated with increased mortality in cardiac and noncardiac surgery patients. In cardiac surgery patients, preoperative anemia⁴¹,⁵⁰ and intraoperative anemia⁷² are associated with increased mortality. In non-cardiac surgical patients, Beattie et al. were the first to report that anemia was associated with an increase in mortality.⁷¹ In a later study, Musallam et al. found that among all risk factors analyzed, the presence of anemia in conjunction with the risk-factor had significantly increased the risk of 30 day mortality (OR for mortality in anemic patients with: CNS disease – 1.48 [1.31-1.67]; Renal disease – 2.52 [2.23-2.85]; Cardiac disease – 1.58 [1.41-1.76]).⁴²

In addition, in a meta-analysis assessing iron deficiency anemia and mortality in nearly 12,000 African children between 28 days and 12 years, it was found that the risk of mortality decreases by 24% for every 10g/L increase in Hb. From these values, it was estimated that approximately 1.8 million deaths could be prevented annually if Hb values increase by 10g/L in this subset of African children.⁷⁶ Iron deficiency anemia is preventable, and through the implementation of strategies to optimize Hb concentrations there is a potential to prevent mortality within a very large patient population; however, further study is required to better understand the mechanism of anemia-induced mortality.
Transfusion of RBC reduces the risk of mortality associated with anemia

Randomized controlled trials assessing the safety of transfusion thresholds in surgical patients have measured an increased risk of death in patients on a restrictive transfusion strategy compared to patients on a liberal strategy.\textsuperscript{47-80} Patients on a restrictive strategy are transfused at a lower Hb threshold versus patients on a liberal strategy, often resulting in restrictive patients having lower perioperative Hb concentrations and receiving fewer transfusions. This suggests that the differences in mortality rates between restrictive and liberal patients are associated with reduced O\textsubscript{2} content during anemia. In the TITRe2 randomized controlled trial, transfusion thresholds in patients undergoing cardiac surgery were assessed, and a doubled risk for mortality was measured in patients assigned to the restrictive arm, relative to the liberal arm.\textsuperscript{47} Increased mortality in the restrictive arm of transfusion trials has also been measured in the TRICC trial (Critical care patients; ischemic heart disease subgroup)\textsuperscript{78}, TRACS trial (Cardiac surgery patients)\textsuperscript{77}, MINT trial (Cardiac surgery patients)\textsuperscript{79}, and in elderly hip fracture patients\textsuperscript{80}; supporting that inadequate O\textsubscript{2} delivery during anemia may be a risk factor for mortality.
CARDIOVASCULAR AND CELLULAR ADAPTATIONS TO ANEMIA

DIFFERENCE BETWEEN ANEMIA AND HYPOXIA

In order to understand the cardiovascular adaptations to anemia, we must first define the fundamental differences anemia and hypoxia have on $O_2$ gradients as outlined in Figure 2-1. During both anemia and hypoxia, there is a decrease in arterial oxygen content ($C_aO_2$), which is calculated from the equation: $C_aO_2 = ([Hb] \times 1.39) \times (%S_aO_2/100) + (0.003 \times PaO_2)$, where $S_aO_2$ is the saturation of $O_2$ bound to Hb, and $PaO_2$ is the partial pressure of oxygen in arterial blood. During anemia, a reduced $C_aO_2$ is caused from a decrease in Hb, while $PaO_2$ and $S_aO_2$ are preserved. Assuming the inhalation of room air (21% oxygen), preservation of high $PaO_2$ (~100mmHg $O_2$) and $S_aO_2$ (~100%) allows for the maintenance of an oxygen gradient from arterial blood to the tissue (~60mmHg $O_2$). This allows for oxygen to diffuse over a large gradient (~40mmHg $O_2$) to tissues of high metabolic demand. However, during hypoxia, hemoglobin concentrations are preserved, and a reduction in $C_aO_2$ is caused by reduced $PaO_2$ and $S_aO_2$. Assuming the inhalation of 15% $O_2$, $PaO_2$ is lowered to ~60mmHg $O_2$, and $S_aO_2$ to ~75%, reducing the oxygen gradient considerably to all tissues by near ~20mmHg $O_2$. This will reduce the diffusion of $O_2$ to the tissue, and result in systemic tissue hypoxia.

Therefore, anemia and hypoxia are different, and induce differential cardiovascular and cellular adaptations, as highlighted in Table 2-1. Cardiovascular and cellular differences between anemia and hypoxia have been characterized in a neural nitric oxide synthase (nNOS) deficient mouse model, where cardiovascular and molecular adaptations (hypoxia inducible factor (HIF) stabilization) during anemia were found to be nNOS dependent. In addition, during anemia, nNOS deficient mice had increased mortality at higher hemoglobin concentrations relative to control mice, suggesting a protective role of nNOS during anemia. Conversely, during acute hypoxia (5% $O_2$) nNOS deficient mice had increased survival relative to control mice, indicating a maladaptive role of nNOS during hypoxia. This thesis will discuss cardiovascular and cellular adaptations during anemia in further detail.
Figure 2-1: Difference in oxygen gradients during anemia and hypoxia. During anemia, a reduction in oxygen content is due to reduced hemoglobin concentrations, whilst arterial blood oxygen tension and oxygen saturation remain preserved. This results in maintenance of oxygen gradient during anemia, where oxygen can freely diffuse to tissues to satisfy metabolic requirements. However, during hypoxia, hemoglobin concentrations are maintained, and a lower oxygen content is due to a reduced fraction of inspired oxygen. Correspondingly, there is a decrease in arterial oxygen tension and arterial oxygen saturation, reducing the overall oxygen diffusion gradient. This reduced gradient is experienced by all tissues, resulting in systemic hypoxia and a reduction in tissue oxygen delivery. (Modified from Hare GM et al., Best Pract Res Clin Anaesthesiol 2013)
### Table 2-1: Summary of outcomes in response to low oxygen content due to anemia and hypoxia in wild type and nNOS deficient mice.

A reduction in oxygen content was observed in anemia via reduced hemoglobin concentrations, and in hypoxia via reduced $P_aO_2$. nNOS deficient mice produced differential cardiovascular and cellular responses to anemia and hypoxia. During anemia, nNOS deficient mice had decreased survival, which was associated with nNOS dependent HIF expression, suggesting an adaptive role of nNOS during anemia. In contrast, nNOS deficient mice had increased survival during hypoxia, suggesting an adaptive role of nNOS during anemia (Data in table from Tsui AKT et al., PNAS 2011)

<table>
<thead>
<tr>
<th>Response to Low Oxygen Content</th>
<th>Anemia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen Content</td>
<td>Decreased ↓</td>
<td>Decreased ↓</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>Decreased ↓</td>
<td>No Change ↔</td>
</tr>
<tr>
<td>$P_aO_2$</td>
<td>Increased ↑</td>
<td>Decreased ↓</td>
</tr>
<tr>
<td>Cardiac Output</td>
<td>Increased ↑</td>
<td>Increased ↑ ↔</td>
</tr>
<tr>
<td>Systemic Vascular Resistance</td>
<td>Decreased ↓</td>
<td>Decreased ↓</td>
</tr>
<tr>
<td>Mean Arterial Pressure</td>
<td>No Change ↔</td>
<td>Decreased ↓</td>
</tr>
<tr>
<td>HIF Expression</td>
<td>Increased ↑</td>
<td>Increased ↑</td>
</tr>
<tr>
<td>Survival</td>
<td>Decreased ↓</td>
<td>Increased ↑</td>
</tr>
<tr>
<td>nNOS Dependent HIF Expression</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>
**TISSUE HYPOXIA IS SENSED**

The main role of the red blood cell is to facilitate and maintain O$_2$ delivery to peripheral tissues to satisfy its metabolic requirement and prevent tissue hypoxia. Over time, many redundant mechanisms have evolved to sense low oxygen and induce adaptations to ensure adequate O$_2$ delivery. The full scope of this topic is beyond the scope of the thesis, thus specific examples of tissue hypoxia sensing will be discussed.

*Examples of cellular hypoxia sensing:*

*Biochemical Oxygen Sensing of tissue hypoxia*

At the cellular level, the mitochondria, being the ultimate site of oxygen consumption, senses oxygen through regulation of metabolism.\(^8^2\) Oxidative phosphorylation drives the energy state of the cell, ([ATP]/[ADP]/[Pi]) at an equilibrium value near 2x10$^6$ per mole of metabolite.\(^8^3\) The mitochondria can regulate the energy ratio close to 1x10$^5$ per mole of metabolite, indicating the extremely high level of sensitivity and precision of O$_2$ regulation of this sensor.\(^8^3\)

*Transcriptional regulation by tissue hypoxia*

Hypoxia inducible factor-1α (HIF-1α), which is expressed in all metazoan cell types, enables for all cells to have oxygen sensing capabilities.\(^8^4\) HIF-1α undergoes oxygen-dependent degradation in normoxic conditions. During hypoxia, stabilization of HIF-1α enables for transcription of hypoxia related genes to support oxygen homeostasis.\(^8^5\) HIF biology will be described in further detail in subsequent sections.
Examples of hypoxia sensing at the organelle level

At the level of the organelle, peripheral chemoreceptors within the aortic arch and the carotid bodies can sense low $P_aO_2$, increased partial pressure of arterial carbon dioxide ($P_aCO_2$), and decreased pH.\textsuperscript{86} Activation of peripheral chemoreceptors stimulates activation of respiratory centers within the medulla to increase ventilation to restore $P_aO_2$ and prevent anemia-induced tissue hypoxia.\textsuperscript{87-89} Experimental models denervating aortic chemoreceptors have demonstrated an attenuation of the cardiac output response during acute anemia.\textsuperscript{90,91}

Examples of hypoxia sensing at the organ level

The kidney and brain are hypoxia sensitive organs which can induce adaptations to low oxygen through hormonal mechanisms and neuronal activation.\textsuperscript{81} Erythropoietin (EPO) is primarily produced by fibroblasts within the renal medulla, and induces production of RBC in response to anemia. In addition, it is suggested that afferent nervous signals from the kidney, as well as central chemoreceptor activity, induce sympathetic activity through adrenergic nervous system activation.\textsuperscript{81} As a result, cardiovascular adaptations are activated in response to anemia, increasing cardiac output to increase blood flow and optimize $O_2$ delivery, as a potential mechanism to prevent anemia-induced tissue hypoxia.

Physiological Adaptations to Maintain Tissue Oxygen Delivery

Respiratory Adaptation to Anemia

An increase in respiration is observed during acute anemia, thus increasing minute ventilation, SpO$_2$, and overall $C_aO_2$.\textsuperscript{92} Increased SpO$_2$ is caused by nitric oxide (NO)-mediated mechanisms optimizing ventilation perfusion matching, functioning to maximize $O_2$ delivery during anemia.\textsuperscript{92,93} This respiratory adaptation is characteristic of acute anemia, and results in an increase in $C_aO_2$; ultimately optimizing the delivery of $O_2$ during anemia.
CARDIOVASCULAR RESPONSES TO ANEMIA

Anemia Increases Cardiac Output

An increase in cardiac output is one of the most characteristic and well defined examples of physiological adaptation to anemia. The delivery of O\(_2\) (DO\(_2\)) can be characterized by the equation: DO\(_2\) = CO x C\(_a\)O\(_2\), where CO is cardiac output and C\(_a\)O\(_2\) is arterial O\(_2\) content. Anemic patients have an inherently low C\(_a\)O\(_2\) due to a reduced Hb compared to healthy patients, ultimately restricting systematic O\(_2\) delivery. As previously described, low C\(_a\)O\(_2\) is sensed at various levels and induces an increase in cardiac output via redundant mechanisms, primarily believed to be sympathetic nervous mediated.\(^8^6\) This has been demonstrated in experimental models of hemodilutional anemia, where increased aortic chemoreceptor activation has been measured with decreasing hemoglobin concentrations,\(^8^9\) and aortic chemoreceptor denervation has been shown to attenuate the cardiac output response.\(^9^0,9^1\) Therefore, during anemia, it is suggested that cardiac output increases in order to maintain adequate O\(_2\) delivery to prevent anemia-induced tissue hypoxia.

Many animal and human studies have demonstrated progressive increases in cardiac output as Hb reduces.\(^3^1,3^4,5^7,9^4,9^5\) In a study by Wieskopf et al., healthy volunteers underwent severe normovolemic hemodilution to determine the effects of severe anemia on cardiovascular responses.\(^3^1\) A progressive increase in cardiac output was measured during anemia, with heart rate contributing near 75% and stroke volume contributing near 25% of the increased cardiac output, indicating a proportional sensing and adaptation to reduced blood oxygen content during anemia.\(^3^1\) In addition, in an experimental model of chronic anemia, mice with sickle cell anemia had increased cardiac output relative to healthy controls.\(^5^7\) These findings suggest that cardiac output is increased during anemia in order to compensate for the reduction in blood oxygen content and to maintain adequate oxygen delivery to maintain tissue oxygen homeostasis.
Effects of Reduced Viscosity on Microvasculature during Anemia

The main factors affecting blood viscosity are hematocrit, RBC mechanical properties, and plasma viscosity. Therefore, during anemia blood viscosity is primarily decreased due to a reduction in hematocrit.\(^9\) This is a potential mechanism contributing to increased cardiac output, where decreased viscosity increases venous return to the heart and thus ventricular preload. Also, blood viscosity is an integral component of maintaining peripheral vascular resistance by providing shear stress on the vessel surface.\(^9\) Interestingly, it has been demonstrated in animal models that decreased viscosity lowers shear stress and reduces endothelial mediated NO vasodilation; ultimately decreasing microscopic tissue perfusion by lowering functional capillary density.\(^9\)\(^,\)\(^9\) Conversely, hyperviscosity has been shown to increase functional capillary density, increasing tissue perfusion.\(^9\) It has been demonstrated in a model of hemodilution with high viscosity plasma that there is increased vital organ functional capillary density and endothelial nitric oxide synthase (eNOS) expression during anemia.\(^9\) In addition, during anemia regional microvascular blood flow to the brain is increased, and regional microvascular blood flow to the kidney is reduced.\(^1\)\(^0\) Increased functional capillary density of organs of higher importance (brain) suggests the activation of mechanisms contributing to increased oxygen extraction during anemia, relative to organs of lower importance (kidney).

Increased Organ-Specific Blood Flow during Anemia

The distribution of increased cardiac output during anemia is heterogeneous, whereby \(O_2\) delivery is matched to organs of higher metabolic demand. During anemia, differential organ blood flow is observed, where flow through the heart (cardiac output) is increased.\(^3\)\(^1\)\(^,\)\(^9\)\(^5\) A higher proportion of the cardiac output is directed to the brain, as evidenced by increased brain blood flow, or ‘preferential perfusion’ in experimental animal and clinical data through nNOS mediated vasodilation.\(^3\)\(^4\),\(^3\)\(^5\),\(^5\)\(^7\) This has also been observed in an experimental model of sickle cell
anemia, whereby sickle cell anemic mice had increased cardiac output and carotid artery diameter, which was associated with an increase in brain (carotid artery) blood flow. These findings suggest that cardiovascular adaptations function to preserve O₂ delivery to organs of higher metabolic demand. However, blood flow to less vital organs, such as the kidney, liver, and intestines remains unchanged. Tsui et al. has measured increased brain blood flow during hemodilutional anemia, but no change in flow to the renal artery or abdominal aorta as hemoglobin concentrations decrease. In addition, Van Bommel has measured increased brain blood flow during anemia, but a decrease in intestinal flow during anemia. These findings support the role of cardiovascular adaptations during anemia functioning to preserve blood flow to organs of higher importance (brain, heart), by reducing blood delivery to organs of less importance (kidney, gut), which may act as a mechanism contributing to anemia-induced tissue hypoxia.

RBC ADAPTATION TO ANEMIA

As previously described, the primary role of hemoglobin within the RBC is to facilitate gas exchange, predominantly delivering O₂ to the tissue. Hemoglobin oxygen delivery is characterized by the oxygen-hemoglobin dissociation curve, outlining the efficient loading of O₂ at high PO₂ (lungs), and efficient unloading in low PO₂ (tissue). By definition, a P₅₀ of <60mmHg O₂ has been defined as hypoxemia, in part due to the transition in the shape of the oxygen-hemoglobin dissociation curve, which declines sharply below this PO₂. Another point of interest on the oxygen-hemoglobin dissociation curve is the venous oxygen saturation of 75%, which is associated with a mixed venous PO₂ of 40mmHg O₂. Another important point on the curve is the p50, which refers to the PO₂ level at which hemoglobin saturation is 50%. Different physiological states can shift the p50 of the hemoglobin dissociation curve, where anemia
induces a right shift, causing a decrease in hemoglobin-O$_2$ affinity, favoring offloading of O$_2$ at the level of the tissue.$^{102}$

Anemia increases oxygen offloading at the tissue

Clinical studies have demonstrated that anemic patients have increased concentration of 2,3-diphosphoglycerate (2,3-DPG) within their RBCs.$^{103}$ This has the impact of reducing the affinity of O$_2$ to hemoglobin, causing a right shift in the hemoglobin dissociation curve.$^{103}$ Increased concentrations of cellular 2,3-DPG provides evidence of metabolic adaptations of the RBC during anemia. 2,3-DPG is a metabolite produced during glycolytic ATP production in the RBC through the Rapoport-Luebering shunt within the Embden-Meyerhof Pathway.$^{104}$ 2,3-DPG allosterically binds to deoxygenated hemoglobin, maintaining the deoxyhemoglobin confirmation.$^{105}$ In a rat model, it has been demonstrated that modification of RBC 2,3-DPG levels impacts metabolic activity cerebral ischemia. In ischemic rat brain, transfusion of 2,3-DPG-enriched RBC maintains cerebral production of ATP, suggesting that O$_2$ delivery was adequate to support aerobic metabolism. Conversely, transfusion with 2,3-DPG-subnormal (deficient) RBC resulted in a reduction in measured ATP levels, suggesting that oxygen delivery was inadequate to maintain aerobic metabolism. This provides an example by which allosteric modification of Hb by 2,3-DPG enhances O$_2$ delivery to the tissue.$^{106}$ While clinical studies have demonstrated anemia increases RBC 2,3-DPG concentrations in humans, the impact of this shift on tissue O$_2$ delivery has not yet been clearly demonstrated.
TISSUE BLOOD FLOW IS PROPORTIONAL TO OXYGEN DELIVERY

Traditional cardiovascular physiologists have stated that both cardiac output and tissue blood flow are primarily driven by the cellular requirement for oxygen to produce ATP under aerobic conditions.\textsuperscript{107,108} As previously described, experimental studies assessing organ specific blood flow during anemia have demonstrated that organs with the highest metabolic consumption for O\textsubscript{2} receive the greatest proportion of cardiac output associated with anemia. As such, the heart receives a greater increase in blood flow compared to the brain, which in turn has a higher relative blood flow compared to other organs.\textsuperscript{109-111} Van Bommel et al. have clearly demonstrated these findings in experimental studies in a porcine model of hemodilution, where brain perfusion is increased relative to gut perfusion during anemia.\textsuperscript{94} In addition, Ragoonanan et al., demonstrated increased cerebral blood flow during anemia relative to the kidney, which was associated with maintaining brain P\textsubscript{t}O\textsubscript{2} during severe anemia.\textsuperscript{111} These findings suggest that during anemia, cardiovascular adaptations function to optimize tissue oxygen delivery to organs of higher metabolic demand as a potential mechanism to prevent anemia-induced tissue hypoxia.

Increased oxygen extraction during anemia

Characterization of the pathway of O\textsubscript{2}, as it offloads from Hb to the mitochondria, has been traditionally difficult to assess quantitatively. Utilizing sophisticated O\textsubscript{2} imaging methodologies, Vinogradov et al. has demonstrated a clear but shallow gradient of PO\textsubscript{2} as O\textsubscript{2} transitions from the red cell toward the vascular wall and within the perivascular tissue.\textsuperscript{112} In addition, Vinogradov et al. has demonstrated that the gradient to cross the cell membrane is very low (1-2mmHg O\textsubscript{2}).\textsuperscript{112} These studies have also demonstrated that the PO\textsubscript{2} of mitochondria was much higher than traditionally believed, (in the range of 15-20mmHg).\textsuperscript{107} Knowing the intracellular and mitochondrial PO\textsubscript{2} is maintained in the range of 15-20 mmHg enables for the mitochondria to function as an O\textsubscript{2} sensor for its ability to regulate ATP to ADP ratio, as
previously described. The background is of importance, as it dictates that mitochondrial consumption of O$_2$ creates a PO$_2$ sink which drives O$_2$ from the RBC to the tissue.

The capacity or proportion of O$_2$ extracted from Hb is organ specific. For example, it is estimated that the heart extracts $\sim$75% of delivered O$_2$.

This indicates that the coronary sinus venous blood is highly desaturated, and any increases in cardiac O$_2$ extraction are reliant on increased blood flow. Conversely, the brain has a high metabolic requirement, but only extracts $\sim$30% O$_2$ delivered, resulting in jugular venous oxygen saturation of $\sim$70%. In contrast to organs of high oxygen extraction, other tissues in body extract O$_2$ at lower percent (Gut: $\sim$20%; Kidney: $\sim$15%).

The overall effect of global O$_2$ delivery of all tissue results in a global mixed venous oxygen saturation of $\sim$75%.

During times of reduced cardiac output, inadequate tissue O$_2$ delivery is associated with increased O$_2$ extraction, and a reduction in tissue O$_2$ tension. Similarly, during anemia, cardiac output and organ specific blood flow are increased. There is also an associated decrease in mixed venous O$_2$ saturation. This suggests that despite cardiovascular adaptation and increased blood flow, there is overall inadequacy in O$_2$ delivery. This is associated with a decrease in tissue PO$_2$, which is the driving force for an increase in O$_2$ extraction. Direct evidence has been provided by Van Bommel et al., who demonstrated an increase in brain O$_2$ extraction during anemia, decreasing jugular venous O$_2$ saturation from 70%–50%. In addition, our laboratory has demonstrated the effect of anemia on global mixed venous desaturation during normovolemic hemodilutional anemia. This effect is much more profound during inadequate volume resuscitation, where anemia in conjunction with a decreased cardiac output augmented O$_2$ extraction to a much higher degree. (Kei et al., Intensive Care Med Exp, Under Review)
Evidence of anemia-induced morbidity and mortality at hemoglobin thresholds higher than the critical hemoglobin level

Human and animal studies have demonstrated that global oxygen consumption remains stable until relative low Hb thresholds (~50 g/L). However, as Hb drop to even lower to values (~30 g/L), O2 metabolism is reduced. This point is described as the critical Hb level at which organism metabolism supply dependent. The critical O2 delivery threshold has been established in rats, dogs, and humans. It has also been shown that the rate of metabolic O2 consumption of the brain does not change to very low hemoglobin concentrations. Evidence of anemia induced tissue hypoxia, organ dysfunction, organ injury, and mortality have been demonstrated to occur at Hb thresholds as high as 90 g/L. The onset of anemia-induced morbidities and mortality at Hb thresholds much higher than than the critical Hb level raises criticism regarding clinical relevance of the critical hemoglobin level.

Evidence of inadequate tissue oxygen delivery during anemia

Despite these described adaptive cardiovascular mechanisms to maintain blood O2 delivery, experimental models have demonstrated organ-specific reductions in tissue O2 tension (P(O2)) during anemia. Therefore cardiovascular adaptations functioning to increase tissue O2 supply are inadequate to meet O2 demand, resulting in the onset of organ-specific tissue hypoxia. The onset of tissue hypoxia is graded, where cardiovascular adaptations maintain P(O2) of organs of more importance (brain, heart) at lower hemoglobin thresholds compared to organs of less importance (kidney, gut, liver). In a hemodilutional model of anemia, Van Bommel et al. measured a low tolerance of anemia in the kidney, reducing microvascular P(O2) at a hematocrit threshold as high as 35.5%, followed by the intestine at 17.5%, and the heart at 8.7%. In addition, graded tissue hypoxia has been measured in our laboratory where cardiovascular adaptations are sufficient to maintain brain P(O2) following a 50% reduction in
blood O\textsubscript{2} content. However, under the same conditions, a significant reduction in kidney P\textsubscript{1}O\textsubscript{2} was measured.\textsuperscript{111} Anemia induced tissue hypoxia is sensed and induces cellular adaptations as evidenced by increased stabilization of HIF-1\textalpha protein. Following acute hemodilution to different hemoglobin thresholds, Tsui et al. demonstrated increased kidney and liver HIF-1\textalpha stabilization as anemia becomes more severe, with the highest levels of HIF-1\textalpha stabilization measured in hepatic tissue.\textsuperscript{35} Kidney HIF-1\textalpha expression has been localized over the glomelular and tubular regions, suggesting renal cortical and medullary cellular adaptations during anemia. In addition, stabilization of renal HIF induced transcription of hypoxia related genes governing glucose transport, glucose metabolism, and erythropoiesis; functioning to increase oxygen delivery in response to reduced blood oxygen content during anemia.\textsuperscript{35}

Cardiovascular adaptations are vital to preserve brain P\textsubscript{1}O\textsubscript{2} during anemia; however as anemia becomes more severe, O\textsubscript{2} delivery is unable to meet the metabolic demands of brain tissue resulting in hypoxia. This has been demonstrated by our laboratory, where brain tissue hypoxia has been measured during severe hemodilutional anemia.\textsuperscript{34,111,118} In addition, abolishment of anemia-induced cardiovascular adaptations optimizing flow to the brain (cardiac output, cerebral vasodilation) with \textbeta\textsubscript{1} and \textbeta\textsubscript{2} adrenergic receptor antagonists result in a reduction of brain P\textsubscript{1}O\textsubscript{2}.\textsuperscript{111,118} This provides evidence that active regulation of increased cardiac output and cerebral vasodilation are required to optimize tissue O\textsubscript{2} delivery during anemia. Cerebral tissue hypoxia is sensed at the cellular level through nNOS mediated stabilization of HIF-1\textalpha. During acute hemodilution, increased brain HIF-1\textalpha is measured as hemoglobin concentrations decrease, which is associated with increased transcription of hypoxia related genes.\textsuperscript{34} nNOS has been shown to regulate brain cardiovascular adaptations, and HIF-1\textalpha stabilization and gene activation; as nNOS deficient mice lack these cardiovascular and cellular adaptations.\textsuperscript{34} Paradoxically, at a moderate anemic hemoglobin threshold (Hb = 90g/L), there is a reduction in HIF-1\textalpha stabilization
suggesting cardiovascular adaptations during moderate anemia is optimized to preserve brain \( O_2 \) tissue levels.\(^{35}\) However, despite reduced brain HIF-1\( \alpha \) stabilization during moderate anemia, there is an associated increase in transcription of hypoxia related genes governing glucose metabolism and erythropoiesis.\(^{35}\) This indicates that despite a preferential perfusion of the brain, cellular adaptations to tissue hypoxia are still activated during moderate anemia, as a potential protective mechanism to prevent anemia-induced tissue hypoxia.

Interestingly, in a chronic model of sickle cell anemia (Hb ~ 80g/L), a 46\% decrease in brain \( P_tO_2 \) was measured relative to control mice. This hypoxia is sensed at the molecular level, increasing expression of HIF-1\( \alpha \) in perivascular regions in the brain.\(^{57}\) These findings suggest that the cardiovascular adaptations optimizing oxygen delivery are insufficient to satisfy the metabolic oxygen requirement of the tissue in chronic models of anemia.

**CELLULAR RESPONSES**

**HYPOXIA INDUCIBLE FACTOR SIGNALING**

Hypoxia inducible factor (HIF) protein is a marker of tissue hypoxia, composed of \( \alpha \) and \( \beta \) subunits which dimerize to induce transcription of genes associated with the hypoxic response element (HRE).\(^{119}\) HIF-1\( \alpha \) is differentially spliced to produce isoforms HIF-1, -2, and -3\( \alpha \). HIF-1\( \alpha \) is ubiquitously expressed in all cell types, whereas HIF-2\( \alpha \) expression is restricted to endothelium, and cell types within small intestine, lung, kidney, and heart. HIF-3\( \alpha \) is the least understood, and has been suggested it binds and inhibits HIF-1\( \alpha \) transcriptional activity.\(^{120}\) HIF-1\( \alpha \) and HIF-2\( \alpha \) proteins contain a basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) motif which facilitate dimerization with HIF-1\( \beta \) and binding with DNA. HIF-1\( \alpha \) and HIF-2\( \alpha \) subunits contain an oxygen dependent domain (ODD) which regulating protein stability, and a
transactivation domain (TAD) which binds transcriptional activator protein CBP/p300 to promote HRE transcription.\textsuperscript{120}

HIF-1\(\alpha\) is regulated by post-translational modifications at the protein level, where it is rapidly degraded in normoxia. Prolyl hydroxylation of amino acid residues Pro402 and Pro564 by proline hydroxylase (PHD) within the HIF-1\(\alpha\) ODD region induce its interaction with the tumor suppressor von Hippel Lindau protein (pVHL), resulting in proteosomal degradation.\textsuperscript{119} The enzymatic reaction mediated by PHD is \(O_2\) dependent, as molecular oxygen is necessary to form the hydroxyl group. Other post-translational modifications, including lysine acetylation and transactivation domain hydroxylation promote proteosomal degradation.\textsuperscript{119} In addition, HIF-1\(\alpha\) undergoes hydroxylation of its N803 residue, inhibiting the recruitment of the CBP/p300. HIF-1\(\alpha\) is constitutively produced, and undergoes rapid degradation during normoxia.\textsuperscript{119}

In hypoxic conditions, HIF-1\(\alpha\) does not undergo prolyl hydroxylation, resulting in no pVHL interaction and subsequent degradation. The stabilized HIF-1\(\alpha\) subunit binds to HIF-1\(\beta\) and translocates into the nucleus, where it associates with the HRE.\textsuperscript{119} NO has also been shown to stabilize HIF-\(\alpha\). During acute anemia, it has been demonstrated that nNOS derived NO is required for stabilization of HIF-1\(\alpha\). HRE activation, with the recruitment of CBP/p300, facilitates activation of gene transcription of factors promoting: erythropoiesis (EPO), angiogenesis (vascular endothelial growth factor; VEGF), hypoxia sensing (HIF-\(\alpha\)), nitric oxide synthesis (nNOS, iNOS), iron metabolism, glucose metabolism, and cell proliferation.\textsuperscript{121} It remains yet to be defined whether HIF activation is an adaptive or maladaptive response to anemia.
TISSUE HYPOXIA AS UNIFYING MECHANISM FOR ORGAN INJURY AND MORTALITY

As previously described, moderate anemia is associated with increased risk of organ injury (stroke, acute kidney injury, gut infarction, myocardial infarction), and mortality. Experimental models have demonstrated inadequate cardiovascular adaptations during moderate anemia leading to anemia-induced tissue hypoxia. However, the causality between anemia-induced tissue hypoxia and organ injury, and subsequent mortality, remains yet to be defined.

To test our hypothesis of anemia-induced tissue hypoxia as a mechanism for organ injury and mortality, we utilized an antibody mediated model of anemia to assess its corresponding cardiovascular and cellular adaptations. The RBC-specific antibody utilized was TER119, which is specific to the glycoporphin-A complex on RBC. RBC-specific antibody has been demonstrated to reduce RBC-counts following administration; however, the effects of RBC-specific antibody on hemoglobin concentrations remains unknown. In addition, the mechanism of RBC-clearance induced by RBC-specific antibody is believed to function independent of complement activation and macrophage activation; however, characterization of the mechanism remains beyond the scope of this thesis. We aim to compare the physiological adaptations induced by RBC-specific antibody to other models of hemodilutional and sickle cell anemia with the goal of defining the mechanisms associated with anemia-induced morbidity and mortality.
GENERAL HYPOTHESIS

Anemia-induced tissue hypoxia is a unifying mechanism for organ injury and mortality.

SPECIFIC HYPOTHESIS

i. Moderate anemia causes tissue hypoxia, disrupts oxygen homeostasis and depletes cellular energetics in an organ specific manner.

ii. Moderate anemia triggers adaptive cardiovascular responses which do not fully compensate for a reduction in blood oxygen content and tissue oxygen delivery resulting in tissue hypoxia.

OBJECTIVES AND SPECIFIC AIMS

The following specific aims are proposed:

Aim 1. To characterize the mechanism of moderate anemia produced by an RBC binding antibody and its effects on circulating hemoglobin levels.

Aim 2. To identify the degree of adaptive cardiovascular responses associated with anti-RBC mediated anemia.

Aim 3. To determine if organ specific tissue hypoxia (brain, kidney) occurs during anti-RBC mediated anemia.

Aim 4. To determine if increased stabilization of tissue specific HIF-1α is occurs during anti-RBC mediated anemia.

Aim 5. To assess the impact of anti-RBC mediated anemia on cerebrovascular CO₂ reactivity as a surrogate of microvascular reserve.
3. MATERIALS AND METHODS

All procedures on mice were approved and performed in accordance to the Animal Care Committee guidelines at St. Michael's Hospital and the Toronto Centre for Phenogenomics following the Canadian Council on Animal Care standards.

3.1. ANIMAL MODEL:

HIF-ODD mice were purchased (Jackson Laboratory, Bar Harbor, ME, USA), and bred for colony maintenance. This model is of FVB background and has firefly luciferase fused to the ODD region of HIF-1α, and has no impairment on its ability to undergo proteosomal degradation. Genotyping of mice was outsourced to an automated genotyping facility (Transetyx, Cordova, TN, USA), where tail clippings were sent and analyzed for the following probes: 1) LUC (indicating the presence of the ODD-Luc Allele); and 2) ROSA WT (indicating the presence of the WT allele). Heterozygous male HIF-ODD mice between the ages of 8-12 weeks were used for all experiments. Animals had access to food and water ad libitium and were housed in a facility with a 12h light-dark cycle. Unless otherwise noted, spontaneously breathing mice were anesthetized with 2% isoflurane at 21% O₂ and core body temperature was maintained between 36-37°C. To assess hemoglobin levels, 10μl of blood was collected via tail nick and analyzed with a Hemocue Hb201+ analyzer (Radiometer, Angelholm, Sweden).

i. ANTIBODY-INDUCED ANEMIA:

Antibody specific to RBC, TER119, or isotype control, Rat IgG2b, were administered to mice induce anemia (BioXCell, West Lebanon, NH, USA). As previously described, TER119 is a monoclonal antibody specific to the glycoporphin-A complex on the surface of RBC; however, the precise mechanism of RBC clearance induced by TER119 remains incompletely defined. Stock aliquots of TER119 or Rat IgG2b were stored at -80°C. Thawed antibody was diluted with
filter-sterilized saline to a concentration of 0.2μg/μl. Using a 30G needle, anesthetized mice were injected with antibody at a dose of 1μg/g body weight via tail vein.

3.2. **Degree of Anemia and Mechanism of RBC Clearance by RBC-Specific Antibody**

i. **Degree of Anemia Induced by RBC-Specific Antibody:**

Mice were administered control antibody (n=16) or TER119 (n=23), and hemoglobin values were assessed daily until recovery. In a subgroup of mice (n=6/group), mice were re-injected with a second dose of antibody at the day 4 time point, and hemoglobin values were measured until recovery (Day 5, 6, 7, 10, and 14).

ii. **Assessment of Plasma Hemoglobin, and Spleen and Liver Weights:**

Following administration of control or anemia-inducing antibody administration, blood was collected from the abdominal aorta of anesthetized mice at different time points (n=6-8 at each time point), and measured with a blood gas analyzer (Radiometer, ABL800Flex). Blood was centrifuged at 1400G for 10 min at 4°C to separate and obtain plasma. Plasma hemoglobin content was analyzed (co-oximetry, Radiometer ABL800Flex). Mouse spleen and liver were extracted and weighed following blood extraction.

3.3. **Peripheral Oxygen Saturation:**

Pulse oximetry was used to assess peripheral arterial blood saturation in 6 control mice (mean weight = 23.1±1.6 g) and 6 anemic mice (mean weight = 24.0±1.7 g) (MouseOx, Starr Life Sciences Corp). Peripheral oxygen saturation measures were recorded every 4 seconds and averaged over a 10 minute time period.
3.4. **Effect of Moderate Anemia on Tissue Oxygen Tension**

i. **Tissue Oxygen Tension:**

Tissue oxygen tension was measured with a G4 Oxyphor probe, which utilizes a mechanism of phosphorescence quenching of oxygen to measure focal tissue oxygen tension. As previously described, a burst of light at an excitation wavelength (637 nm) induces G4 oxyphor to undergo phosphorescence, emitting light at a wavelength readily absorbed by oxygen (813 nm). The duration, or lifetime, of phosphorescence is inversely proportional to the PO$_2$ within the tissue. This relationship can be defined by the Stern-Volmer equation: \( \frac{1}{\tau} = \frac{1}{\tau_0} + \left( k_q \right)(PO_2) \), where \( \tau \) is the lifetime of G4 oxyphor, \( \tau_0 \) is the lifetime of G4 oxyphor in the absence of oxygen, and \( K_q \) is the quenching constant, which temperature dependent.

Brain P$_{tO2}$ was measured in 12 control mice (mean weight = 25.3±3.0 g) and 13 anemic mice (mean weight = 25.0±2.6 g). Different groups of control and anemic mice were measured at baseline, day 3, and 4 time points. To access brain tissue, mice were placed in prone position, and the skull was exposed. A burr hole of 1-2 mm in diameter was created to allow for microsensor placement. Kidney P$_{tO2}$ was measured in 10 control mice (25.4±2.8 g) and 14 anemic mice (25.7±2.4 g). The kidney was exposed via abdominal incision in mice placed in supine position. A 20G needle was used as a guide when placing the microsensor into the brain or kidney tissue. After probe insertion and stabilization, PO$_2$ values were recorded with a PMOD 5000 (Oxygen Enterprises, Philadelphia, PA, USA) every 10 seconds, and averaged over a 10 minute period. The PMOD 5000 device is calibrated to measure PO$_2$ at 37°C.
Organ-specific temperature was measured in a separate subset of mice (n=7/group), to measure discrepancies in tissue temperature from 37°C. These experiments were not performed on the same animals due to the impact of tissue trauma on PO$_2$. Brain and kidney tissue was exposed as previously described and a temperature probe was inserted into the tissue. Data was collected with a PowerLab system and LabChart Software (ADInstrument, Dunedin, New Zealand). After probe insertion, tissue temperature was recorded every 10 seconds, and averaged over a 40 minute period. Tissue-specific average temperatures for control and anemic mice were used to calibrate quenching constant ($K_q$) values for each respective tissue for PO$_2$ calculation.

3.5. ASSESSMENT OF CARDIOVASCULAR RESPONSES TO MODERATE ANEMIA

i. ULTRASOUND BIOMICROSCPY:

A Vevo 2100 high frequency ultrasound biomicroscope (VisualSonics, Toronto, ON) was used to assess blood flow through the aortic orifice, common carotid artery, internal carotid artery, and renal artery in control (n=6; mean weight = 22.6±2.8 g) and anemic (n=8; mean weight = 24.1±2.3 g) mice at the baseline and day 4 time point. For ultrasound imaging, anesthesia was maintained with 1.5% isoflurane. Mice were placed in supine position, and abdominal and neck hair was removed with Nair (Church & Dwight, Ewing, NJ, USA). Using a 30-MHz transducer, M-mode traces and Doppler measures were conducted. To calculate blood flow through the aortic orifice, a 2-dimensional image and Doppler measure of the aortic annulus at peak systole was measured. For all other vessels, M-mode traces were recorded perpendicular to the cross-sectional center of each respective vessel, and all Doppler recordings were measured at the smallest intercept angle to vessel flow (<60 degrees) (Figure 3-1). Vessel area was calculated from diameter measures obtained from M-mode images. The velocity-time-integral (VTI) was measured from the average intensity weighted mean velocity of the Doppler spectrum.
All measurements were averaged over three cardiac cycles. Stroke volume was calculated from the product of vessel area and VTI. Blood flow measures were calculated from the product of Stroke volume and Doppler measured heart rate.

Acquisition and analysis of all images were conducted in a blinded randomized method. Mice were randomized in blocks of 4 or 6, and individual mouse assignments were blinded from the ultrasound associate during data acquisition. Once all data was collected, the ultrasound associate blinded the data analyzer by renaming all the data files. Once the data analyzer completed the analysis, the ultrasound associate and data analyzer unblinded each other to reveal mouse assignments.
Figure 3-1: Transducer orientations utilized during ultrasound biomicroscopy data collection. M-Mode traces were recorded perpendicular to the cross sectional center of each respective vessel, enabling for the acquisition of data relating to vessel diameter used to calculate vessel area. Doppler spectrum were recorded at the smallest intercept angle to vessel flow (<60°) to minimize error when measuring blood velocity values. Vessel area and blood velocity values were used to calculate blood flow within each respective vessel.
3.6. **Effect of Moderate Anemia on Cerebrovascular Reactivity**

i. **Response of Common and Internal Carotid Artery to Hypercapnia:**

Cerebrovascular reactivity was assessed in 6 control mice (mean weight = 25.4±1.3 g), and 6 anemic mice (mean weight = 25.8±1.2 g), at baseline and at the day 4 time point (Figure 3-2). Following an induction of anesthesia of 2% isoflurane in 21% O₂ for 20 minutes, mice underwent endotracheal intubation with a 22G catheter. Mice were placed on a TOPO small animal ventilator set at 135 cycles per minute (Kent scientific, Torrington, CT, USA). Blood flow parameters of the common carotid and internal carotid artery were assessed and analyzed following the ultrasound biomicroscopy methodology (outlined in protocol 2). Under 1.5% isoflurane, mice were subjected to a cycling of inhalation of room air (normocapnia: 21% O₂, 79% N₂), and a carbon dioxide challenge (hypercapnia: 5% CO₂, 30% O₂, 65% N₂). Mice underwent 2 carbon dioxide challenges during each timepoint. A period of 5 minutes between measurements was allotted for mice to return to normocapnia following each carbon dioxide challenge. As previously described, acquisition and analysis of all images was conducted in a blinded randomized method.
Figure 3-2: Experimental Protocol Timeline for Cerebrovascular Reactivity Assessment
Measurement of common carotid artery and internal carotid artery blood flow during normocapnic and hypercapnic conditions.
3.7. **ASSESSMENT OF MOLECULAR ADAPTATIONS TO MODERATE ANEMIA**

i. **REAL-TIME IN VIVO ASSESSMENT OF HIF-LUCIFERASE:**

HIF-Luciferase bioluminescence was assessed in 11 control mice (mean weight = 25.6±2.3 g) and 12 anemic mice (mean weight = 27.1±3.4 g) with repeated measurements at the baseline, day 3 and day 4 time points. At each measurement time point, mice were administered an intraperitoneal injection of D-luciferin at a dose of 50μg/g body weight (Promega, Madison, WI, USA). After 10 minutes, mice were anesthetized (1.5% isoflurane) and placed into an IVIS Lumina II imaging system (Perkin Elmer, Waltham, MA, USA). Bioluminescence images of the dorsal side, followed by the ventral side, were measured over a 10 second exposure time. Prior to analysis, all images were blinded and randomized to prevent any potential measurement bias. Images were analyzed with Living Image software (Caliper, Hopkinton, MA, USA).

ii. **ANALYSIS STRATEGY TO QUANTIFY HIF-LUCIFERASE BIOLUMINESCENCE**

3.7.ii.a. **REGION OF INTEREST DEFINITIONS AND RATIONALE**

In order to conduct a regional analysis of HIF Luciferase radiance, multiple regions of interest (ROIs) on the dorsal and ventral sides were measured.

Previous qualitative analysis of dorsal HIF-luciferase radiance images of HIF-ODD mice have consistently demonstrated increased radiance produced from the right dorsal region compared to the left dorsal region (Safran PNAS 2006; Tsui PNAS 2011; Tsui AJP 2014). To assess the anatomical explanation for these differences, we measured serial cross sectional magnetic resonance images (n=3 mice). From cross sectional analysis of the sagittal plane centered over the right kidney compared to the left kidney, we observed more liver tissue on the right side relative to the left side. (Figure 3-1A) In addition, assessment of the transverse plane centered over the left and right kidneys show a greater proportion of the left kidney obstructed by paraspinal muscles compared to the right kidney (Figure 3-1B). Previously, our laboratory has
found that HIF-1α levels within the liver are increased during hemodilutional anemia, and these data strongly suggest the source HIF-luciferase radiance produced on the right dorsal side is produced from a combination of the kidney and liver. Also, a greater degree of left kidney obstruction from paraspinal muscles suggests an attenuation of HIF-luciferase radiance produced by the left side. Therefore the combination of liver-induced amplification of right dorsal HIF-luciferase radiance and attenuation of the left side may contribute to the increased HIF-luciferase observed over the right dorsal region relative to the left dorsal region.

On the dorsal side, ROIs assessing: the total dorsal region, total kidney region, left kidney region, and right kidney and liver region were constructed (Figure 3A). On the ventral side, ROIs assessing: the total ventral region, total abdomen region, liver region, and gut region were constructed (Figure 3-2B). Sizes of all respective ROIs were consistent throughout. Brain HIF-Luciferase radiance was unable to be quantified due to shielding of bioluminescence from the skull.
Figure 3-3: MRI cross-sectional analysis assessing kidney (red arrow), liver (blue arrow), and gut (green arrow) anatomical placement. Cross-sectional images were analyzed in n=3 mice. A) Analysis of the sagittal plane centered over the left and right kidneys shows increased liver tissue over the right side of the mouse. B) Analysis of the transverse plane centered over the left and right kidneys shows a greater proportion of the left kidney blocked dorsally by paraspinal muscles relative to the right kidney, potentially obstructing a greater amount of HIF-Luciferase radiance produced by the left kidney.
Figure 3-4: Regions of interest measured for HIF-luciferase assessment. Respective sizes were maintained during analysis over the A) dorsal regions of interest and B) ventral regions of interest.
3.7.ii.b. RATIONALE FOR MEASURING TOTAL HIF-LUCIFERASE RADIANCE

Previously, our laboratory has measured average radiance when assessing ROIs. Contrary to average radiance, total radiance (total flux) was measured because it provided an absolute radiance value for each ROI, not influenced by any ‘dead space’. In a separate analysis, (Figure 3-5), two ROIs of the same height but different widths were analyzed (n=4 mice). Size differences in these ROIs resulted in varying amounts of ‘dead space’, where the larger ROI had more dead space than the smaller ROI. After taking radiance measurements, we observed no change in ‘total flux’ values when comparing the different ROIs (Rank sum test, p=0.686). However, ‘average radiance’ measures were greatly influenced by the amount of ‘dead space’ within the image, resulting in a -49.5% change in radiance values (Rank sum test, p=0.029). Due to differences in mouse size and placement within each image, the amount of ‘dead space’ within each ROI varies; thus, affecting the ‘average radiance’ value. Therefore, a total flux value provides a more accurate measure of tissue luciferase when comparing mice with standardized ROIs.
Figure 3-5: Region of interest analysis comparing measures of Total flux and Average Radiance. Total Flux and Average Radiance was measured to assess the effect of varying dead space in bioluminescence images (n=4). A small region of interest (ROI 1) and large region of interest (ROI 2) were constructed to quantify radiance of areas with varying dead space. Total Flux values were not affected by varying dead space (p=0.686). By contrast, a significant decrease in Average Radiance was observed as dead space area increased (#p=0.029 relative to ROI 1). (Rank Sum Test)

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<tr>
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<th>Total Flux (p/s)</th>
<th>Average Radiance (p/s/cm²/sr)</th>
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<tbody>
<tr>
<td></td>
<td>ROI 1</td>
<td>ROI 2</td>
</tr>
<tr>
<td>Average Radiance</td>
<td>1.10±0.13 x10⁸</td>
<td>1.11±0.13 x10⁸</td>
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<tr>
<td>Percent Change</td>
<td></td>
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<tr>
<td>from ROI 1</td>
<td>0.6 %</td>
<td>- 49.5 %</td>
</tr>
<tr>
<td>ROI 1</td>
<td>8.00±0.94 x10⁵</td>
<td>4.04±0.48 x10⁵</td>
</tr>
<tr>
<td>ROI 2</td>
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3.8. **Statistical Analysis**

Statistical analysis was conducted using SigmaPlot 11.0 software (Systat Software Inc., San Jose, CA, USA). Box boundaries outline the 75th and 25th percentiles of each data spread. Whiskers mark the 90th and 10th percentiles and are calculated and displayed with more than 8 data points available. Comparisons of two groups were assessed by Rank Sum tests. A Two Way ANOVA test, with repeated measures when applicable, was used to assess group, time, and interaction effects between sets of data for the following experiments comparing: hemoglobin concentrations, spleen and liver weights, peripheral arterial oxygen saturation, vessel specific blood flow, and HIF-Luciferase radiance. Cerebrovascular reactivity to anemia was assessed by a Three Way ANOVA for group, time, treatment, and interaction effects. For Two and Three Way ANOVA tests, when a significant effect was observed, a Tukey post hoc multiple comparison test was performed. All data is presented as mean ± SD. A value of p<0.05 was taken to be significant.
3.9. **Contributions to Thesis Data**

All data collection, data analysis and statistical analysis were performed by the author (Nikhil Mistry) except during the following instances:

**Dr. Yu-Qing Zhou:** Ultrasound blood flow and cerebrovascular reactivity measurements were performed by Dr. Zhou as outlined in sections 3.5 and 3.6. Dr. Zhou also provided guidance in the analysis of the ultrasound images, which was completed by the author (Nikhil Mistry). All data and statistical analysis was performed by the author.

**Dr. Lindsay Cahill:** Intubation for cerebrovascular reactivity measurements was performed by Dr. Cahill as outlined in section 3.6. Dr. Cahill also provided guidance in the analysis of the ultrasound images, which was completed by the author (Nikhil Mistry). In addition, Dr. Cahill provided the MRI cross sectional analysis reference images as outlined in section 3.7. All data and statistical analysis was performed by the author.

**Mr. Max Solish:** Assistance in hemoglobin measurements, and measurement of liver and spleen weights, as outlined in section 3.2 were performed by Mr. Solish under the supervision of the author (Nikhil Mistry). All data and statistical analysis was performed by the author.

**Mr. Alexander Hare:** Independently performed the double injection hemoglobin assessment experiment in a subset of mice, as outlined in section 3.2. All data and statistical analysis was performed by the author (Nikhil Mistry).
4. RESULTS

4.1. DEGREE OF ANEMIA AND MECHANISM OF RBC CLEARANCE INDUCED BY RBC-SPECIFIC ANTIBODY (TER119)

Hemoglobin changes after a single and double injection of RBC-specific antibody:

A significant group effect (p<0.001), time effect (p<0.001), and group-time interaction (p<0.001) was measured when assessing hemoglobin concentrations after injection with experimental antibodies. Baseline hemoglobin values were not different between groups (Control = 143±7 g/L, Anemia = 144±9 g/L; p=0.293) (Figure 4-1). Control mice demonstrated a slight reduction in hemoglobin concentrations over time, reaching a minimum value at 14 days (134±7 g/L; p<0.001). A single injection of RBC-specific antibody produced a moderate level of anemia, with a nadir hemoglobin concentration of 94±11 g/L at day 4 (p<0.006 vs baseline, p<0.001 vs control). Hemoglobin values for mice receiving a single injection returned to control values by 14 days following injection (Anemia = 135±6 g/L; p=0.994). Double-injected mice received their second injection at the day 4 time point, when their hemoglobin concentrations are at a nadir from the first injection. Hemoglobin concentration decreased to a value of 51±7 g/L, at 1 day following the second injection (6 days following baseline injection). Similar to single-injected mice, hemoglobin values for double-injected mice returned to control values by 14 days following injection (141±5 g/L; p=0.287).

Assessment of the rate of hemoglobin reduction following RBC-specific antibody injection:

There was no significant group effect for the rate of hemoglobin clearance (p=0.134). However, there was a significant time effect (p<0.001) and group-time interaction (p<0.001). After a single antibody injection, the rate of hemoglobin decrease was -11±12 g/L/day during the first day (Figure 4-2), and accelerates to a fastest rate of -27±17 g/L/day during the second day.
following antibody injection (p=0.003 vs. previous day). During the third day following antibody injection, single-injected mice experience a reduction in hemoglobin concentration of -11±12 g/L/day (p=0.003 vs. previous day). Following a double-injection with RBC-specific antibody, hemoglobin is reduced at a faster rate of -46±6 g/L/day during the first day (p<0.001 vs. single-injection), and the rate of hemoglobin reduction is attenuated to -4±9 g/L/day during the second day (p<0.001 vs. previous day; p<0.001 vs. single-injection). During the third day, contrasting single-injected mice, double-injected begin a recovery where hemoglobin concentrations increase at a rate of 12±11 g/L/day (p<0.001 vs. previous day; p<0.001 vs. single-injection).

**Plasma hemoglobin and spleen and liver weight after injection of RBC-specific antibody:**

Plasma hemoglobin values, and spleen and liver weights were measured to assess the degree of intravascular RBC hemolysis and extravascular RBC clearance following injection with RBC-specific antibody (Figure 4-3). When analyzing plasma hemoglobin values, a significant group effect (p=0.050), time effect (p=0.046), and group-time interaction (p=0.003) was observed. Plasma hemoglobin concentrations, spleen weight, and liver weight did not change over time in control mice. Plasma hemoglobin levels increased after 1 hour following RBC-specific antibody administration (Figure 4-3A), reaching a peak value of 1.15±0.32 g/L by 6 hours following injection, significantly higher than baseline and control values (Baseline = 0.52±0.41 g/L, p<0.001; Control = 0.33±0.35 g/L). No gross hematuria was observed; all urine samples analyzed for hemoglobin content yielded a null result.
When analyzing spleen weight, a significant group effect (p<0.001), time effect (p<0.001), and group-time interaction (p<0.001) was detected. Spleen weight increased to a maximum of 0.21±0.04 g by day 4 after injection (Figure 4-3B), >2 fold higher than baseline and control measures (Baseline = 0.09±0.01 g, p<0.001; Control = 0.09±0.01 g/L, p<0.001). Spleen weights returned to 0.11±0.01 g by 14 days, which was not different from baseline (p<0.05) or control values (0.10±0.01 g/L, p=0.062)

A group interaction was measured where livers from anemic mice were heavier than livers of control mice (p=0.010) (Figure 4-3C). In addition, a time effect was detected (p=0.006); however, a Tukey post-hoc test yielded no significant differences between groups. No interaction effect was measured for liver weights (p=0.202).
Figure 4-1: Degree of anemia induced by RBC-specific antibody (TER119). A single injection of RBC-specific antibody induced a moderate level of anemia, with a nadir hemoglobin concentration observed at day 4 (Hb: control = 143±7 g/L, n=23; anemia = 94±11 g/L, n=22). Anemic mice re-injected with RBC-specific antibody developed severe anemia at 5 days following baseline injection (Hb: Control = 139±10 g/L, n=23; Single-injection = 95±11 g/L, n=22; Double-injection = 54±5 g/L). All mice returned to control hemoglobin values by 14 days following baseline injection *p<0.006 relative to baseline; #p<0.001 anemia relative to control; $p<0.001 double injection relative to single injection. (Two Way RM ANOVA)
Figure 4-2: Daily rate of hemoglobin reduction following injection with RBC-specific antibody. A differential rate of hemoglobin reduction is observed following a single and double injection of RBC-specific antibody, where the rate of hemoglobin reduction is greatest during the second day following the first injection, and greatest during the first day following the second injection (Severe anemia) *p<0.003 relative to previous day; p<0.001 relative to single-injection. (Two Way RM ANOVA)
Figure 4-3: Assessment of plasma hemoglobin concentration and spleen and liver weights following injection with RBC-specific antibody (TER119). Plasma hemoglobin, and spleen and liver weights were measured to assess the degree of intravascular hemolysis and extravascular RBC clearance respectively (n=6-8 per group) Plasma free hemoglobin levels, spleen weight, and liver weight was unchanged in control mice. **Panel A:** Plasma hemoglobin increased to a peak value at 6-hours following RBC-specific antibody injection, and returned to baseline values by day 1 (*p<0.001 relative to baseline; #p<0.006 relative to control.) **Panel B:** Spleen weights increased at day 2, reaching a peak value at day 4, and returned back to baseline by day 14 (*p<0.002 relative to baseline; #p<0.001 relative to control.) **Panel C:** No clear pattern in liver weight was observed during anemia, however a group and time interaction was detected where livers of anemic mice were heavier than control (p=0.006 ANOVA group interaction). (Two Way ANOVA)
4.2. **Peripheral oxygen saturation following anemia:**

These experiments were performed to assess whether RBC-specific antibody contributes to systemic hypoxia within our model. There was no significant group effect for peripheral arterial oxygen saturation (p=0.365). However, there was a significant time effect (p=0.033) and group-time interaction (p=0.003).

No change in peripheral arterial oxygen saturation was observed in control mice. Peripheral arterial oxygen saturation remained at 97% following administration of RBC-specific antibody (Figure 4-4). During anemia, a reduction in hemoglobin concentration resulted in increased peripheral oxygen saturation at day 3 (Anemia = 98.1±0.4 % vs. Baseline = 97.6±0.1 %, p=0.002; vs. Control = 97.4±0.4 %, p<0.018) and at day 4 (Anemia = 97.8±0.3 % vs. Control = 97.0±0.8 %, p<0.018).
Figure 4-4: Peripheral arterial oxygen saturation during anemia. Peripheral arterial oxygen saturation was assessed (n=6 control, n=6 anemia) and remained at around 97%, until anemia where peripheral arterial oxygen saturation increased at nadir hemoglobin concentrations *p=0.002 relative to baseline; #p<0.018 relative to control. (Two Way RM ANOVA)
4.3. **Effect of Anemia on Tissue Oxygen Tension**

Brain and kidney tissue temperatures were not significantly different between control and anemic animals (Table 4-1). Average tissue temperatures were used to calculate a quenching constant (Kq) for each respective organ for PO\(_2\) calculation. Tissue-specific Kq values are outlined in Table 5-2. We detected no change in difference in rectal, brain, and kidney temperature between anemic and control mice. Anemic mice had a reduced difference in rectal and brain temperature relative to control mice (Anemia = 0.66±0.46 °C vs. Control = 1.28±0.55 °C, p=0.026).

During anemia, focal measures of brain and kidney tissue oxygen tension were assessed (Figure 4-5). We did not detect any change in brain tissue oxygen tension during anemia (Anemia = 22.7±5.2 mmHg PO\(_2\) vs. Control = 23.4±9.8 mmHg PO\(_2\)). By contrast, a significant reduction in kidney tissue oxygen tension was measured, where kidney tissue oxygen levels reduced to 13.1±4.3 mmHg PO\(_2\) relative to a control value of 20.8±3.7 mmHg PO\(_2\) (p<0.001).
Table 4-1: Brain and kidney tissue temperature measures during anemia

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<th>Rectal Temperature</th>
<th>Tissue Temperature</th>
<th>Difference from Rectal Temperature</th>
<th>Adjusted Quenching Constant (Kq)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain, °C</td>
<td>Kidney, °C</td>
<td>Brain, °C</td>
<td>Kidney, °C</td>
</tr>
<tr>
<td>Control</td>
<td>36.09 ± 0.80</td>
<td>34.82 ± 1.30</td>
<td>34.82 ± 0.83</td>
<td>33.67 ± 1.50</td>
</tr>
<tr>
<td>Anemia</td>
<td>35.95 ± 0.46</td>
<td>34.30 ± 0.46</td>
<td>35.29 ± 0.66</td>
<td>33.87 ± 0.52</td>
</tr>
</tbody>
</table>

#p=0.026 relative to control (Rank Sum Test)
Figure 4-5: Changes to brain and kidney tissue oxygen tension during anemia. Brain tissue oxygen tension did not change during anemia (n=12 control; n=13 anemia). A significant reduction in kidney oxygen tension was observed during anemia (n=10 control; n=14 anemia) #p<0.001 relative to control. (Rank Sum Test)
4.4. ASSESSMENT OF CARDIOVASCULAR RESPONSES TO ANEMIA

Measurement of cardiac output and blood flow using ultrasound biomicroscopy:

Cardiac output, common carotid blood flow, internal carotid blood flow, and renal artery blood flow did not change in control mice. During anemia, cardiac output significantly increased from a baseline value of 10.21±2.41 ml/min to 12.38±1.98 ml/min (p=0.011, Figure 4-6A). No change in common carotid artery flow was detected during anemia (Group effect: p=0.713, Time effect: p=0.489, Interaction effect: p=0.146). Internal carotid artery flow significantly increased from baseline value of 0.35±0.09 ml/min to 0.60±0.13 ml/min during anemia (p<0.001) (Figure 5-5C). The absolute change in internal carotid artery flow at the day 4 time point from baseline is greater in anemic mice versus control mice (Anemia = 0.26±0.13 ml/min vs. Control = 0.04±0.10 ml/min, p<0.003, Figure 4-7). The increase in internal carotid artery flow during anemia occurred in association with increased heart rate from baseline (Anemia = 520±48 bpm vs. Baseline = 468±40 bpm, p=0.018), increased velocity from baseline (Anemia = 18.4±2.7 mm vs. Baseline = 14.2±2.1 mm, p=0.001), and increased diameter from baseline (Anemia = 0.28±0.02 mm vs. Baseline = 0.26±0.02 mm, p=0.015, Table 5-1). Baseline internal carotid artery diameter of anemic mice was smaller than control mice (Anemia = 0.28±0.03 mm vs. Control = 0.26±0.02 mm, p<0.05). Renal artery flow did not change during anemia (Group effect: p=0.084, Time effect: p=0.088, Interaction effect: p=0.239, Figure 4-6D). A reduction in renal artery diameter was measured relative to baseline during anemia (Anemia = 0.41±0.05 mm vs. Baseline = 0.44±0.03 mm, p=0.034, Table 4-2).
Figure 4-6: Hemodynamic changes during anemia. Cardiac output, brain blood flow, and kidney blood flow was assessed during anemia (n=6 control; n=8 anemia). Cardiac output, common carotid, internal carotid, and renal artery blood flow did not change in control mice. **Panel A:** Cardiac output increased during anemia (*p=0.011 relative to baseline.) **Panel B:** Common carotid blood flow did not change during anemia. **Panel C:** A significant increase in blood flow was observed through the internal carotid artery (*p<0.001 relative to baseline.) **Panel D:** We did not detect any change in blood flow to the kidney during anemia. (Two Way RM ANOVA)
Figure 4-7: Change in blood flow from baseline during anemia. Cardiac output, common carotid artery, and renal artery flow did not change during anemia (n=6 control; n=8 anemia). An increase in flow through the internal carotid artery flow was observed \(^{#}p=0.003\) relative to control. (Rank Sum Test)
Table 4-2. Ultrasound blood flow parameters during anemia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aortic Orifice</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Flow, ml/min</td>
<td>10.58 ± 2.39</td>
<td>10.36 ± 3.74</td>
</tr>
<tr>
<td>Heart Rate, beats/min</td>
<td>460 ± 74</td>
<td>540 ± 55</td>
</tr>
<tr>
<td>VTI, mm</td>
<td>27.4 ± 3.4</td>
<td>22.8 ± 6.1</td>
</tr>
<tr>
<td>AO Diameter, mm</td>
<td>1.03 ± 0.06</td>
<td>1.03 ± 0.05</td>
</tr>
<tr>
<td><strong>Common Carotid Artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Flow, ml/min</td>
<td>0.89 ± 0.15</td>
<td>0.83 ± 0.27</td>
</tr>
<tr>
<td>Heart Rate, beats/min</td>
<td>507 ± 60</td>
<td>531 ± 50</td>
</tr>
<tr>
<td>VTI, mm</td>
<td>13.4 ± 1.3</td>
<td>10.6 ± 2.6</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>0.41 ± 0.03</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td><strong>Internal Carotid Artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Flow, ml/min</td>
<td>0.47 ± 0.13</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>Heart Rate, beats/min</td>
<td>513 ± 72</td>
<td>535 ± 55</td>
</tr>
<tr>
<td>VTI, mm</td>
<td>14.4 ± 3.8</td>
<td>13.7 ± 1.9</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>0.28 ± 0.03</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td><strong>Renal Artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Flow, ml/min</td>
<td>0.73 ± 0.22</td>
<td>0.85 ± 0.11</td>
</tr>
<tr>
<td>Heart Rate, beats/min</td>
<td>476 ± 82</td>
<td>469 ± 71</td>
</tr>
<tr>
<td>VTI, mm</td>
<td>12 ± 1.7</td>
<td>13.2 ± 2.1</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>0.41 ± 0.06</td>
<td>0.42 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SD. AO, aortic orifice; VTI, velocity-time integral. *p<0.05 vs. baseline; #p<0.05 vs. control (Two Way ANOVA, Tukey post hoc test)
4.5. **Effect of Anemia on Cerebrovascular Reactivity**

A three way ANOVA statistical analysis was performed to measure the impact of three independent variables on vascular reactivity of the common carotid and internal carotid arteries to CO$_2$: 1) a group effect was assessed to compare flow in control versus anemic mice; 2) a time effect was assessed to compare values measured at baseline versus day 4; and 3) a treatment effect was assessed to compare blood flow during normocapnia versus hypercapnia.

With respect to common carotid blood flow, we observed no group effect (p=0.136) or time effect (p=0.060, Figure 4-8A); however, a treatment effect was observed (p<0.001). No interaction effect was observed between group and time (p=0.658); or group and treatment (p=0.739). A time and treatment interaction was detected (p=0.017). The interaction between group, time, and treatment was not significant (p=0.860).

Within the internal carotid artery, a group effect was observed (p=0.028), but no time effect was observed (p=0.100, Figure 4-8B). A treatment effect was detected (p<0.001). No interaction effect was observed between group and time (p=0.081); or group and treatment (p=0.206). A time and treatment interaction was detected (p=0.024). The interaction between group, time, and treatment was not significant (p=0.685).

*Common carotid and internal carotid response to hypercapnia during anemia:*

The impact of CO$_2$ (treatment) increasing cerebral blood flow was observed universally (Figure 4-8A/B). Exposure to CO$_2$ (hypercapnia) increased flow in the common carotid and internal carotid arteries within both anemic and control animals at baseline and day 4 (p<0.001, 3 Way ANOVA, treatment effect: hypercapnia vs. normocapnia blood flow).
Interestingly, a second exposure to CO\(_2\) increased cerebral blood flow to a greater magnitude in all mice. A repeat exposure to CO\(_2\) at day 4, following an initial exposure at baseline, resulted in increased cerebral blood flow during hypercapnia within both the common carotid and internal carotid arteries in control and anemic mice (p<0.024, 3 Way ANOVA, time and treatment interaction).

*Common carotid artery-specific responses to hypercapnia during anemia:*

The pattern of common carotid blood flow reactivity to CO\(_2\) was comparable in both anemic and control mice over time (Figure 4-8A). During the day 4 time point, there were no differences in blood flow between anemic and control mice (p=0.172, 3 Way ANOVA, group and time comparison: day 4 blood flow in anemic vs. control mice). No change in CO\(_2\) reactivity was observed when comparing anemic and control mice (p=0.197, 3 Way ANOVA, group and treatment comparison: hypercapnic blood flow in anemic vs. control mice).

*Internal carotid artery-specific responses to hypercapnia during anemia:*

By contrast, basal internal carotid artery blood flow demonstrated a different pattern following CO\(_2\) exposure in anemic mice relative to control mice over time (Figure 4-9). At the day 4 time point, blood flow was significantly increased in anemic mice relative to control mice (p=0.006, 3 Way ANOVA, group and time comparison: day 4 blood flow in anemic vs. control mice). The increase in internal carotid artery basal flow observed during anemia contributed to an elevated reactivity to CO\(_2\) in anemic mice. Following exposure to CO\(_2\), anemic mice had significantly increased blood flow relative to control mice (p=0.016, 3 Way ANOVA, group and treatment comparison: hypercapnic blood flow in anemic vs. control mice). These findings demonstrate an augmentation to cerebrovascular reactivity of the internal carotid artery during anemia to adequately maintain brain perfusion to maintain brain tissue oxygen tension.
Figure 4-8: Cerebrovascular reactivity of the common carotid artery and internal carotid artery during anemia. Panel A/B: CO₂ exposure increased common and internal carotid blood flow in all mice (n=6 control; n=6 anemia, treatment effect: p<0.001). At day 4, common carotid and internal carotid blood flow increased during hypercapnia in both anemic and control mice (*p=0.003 relative to baseline hypercapnia; $p<0.001$ relative to day 4 normocapnia). (Three Way ANOVA)
Figure 4-9: Internal Carotid Artery-Specific Vascular Reactivity During Anemia. During the day 4 time point, anemic mice had increased internal carotid artery flow relative to control mice (Anemia= red bars vs. Control = green bars, p=0.006). This elevated flow caused anemic mice had to have an increased internal carotid reactivity to CO₂ relative to control (p=0.016). (Three Way ANOVA)
4.6. **Assessment of Molecular Adaptations to Anemia in HIF-ODD Luciferase Mice**

*Assessment of dorsal radiance in kidney and liver regions:*

Qualitative analysis of HIF-luciferase radiance over the dorsal side suggests no change in radiance in control mice (Figure 4-10A), and increased radiance over the ‘Right Kidney and Liver Region’ during anemia at the day 4 time point (Figure 4-10B, white arrow).

When conducting our regional analysis, we did not detect any change in HIF-luciferase radiance within any region in control mice. Baseline HIF-luciferase radiance was not different between groups for all regions of analysis. We did not observe any change in HIF-luciferase radiance over the ‘Total Dorsal Side’ during anemia (Group effect: p=0.494, Time effect: p=0.413, Interaction effect: p=0.085, Figure 4-10C).

Similarly, no group effect (p=0.855) or time effect (p=0.493) was detected over the ‘Total Dorsal Kidney and Liver Region’; however, a group time interaction was observed (p=0.042, Figure 4-10D). A post-hoc Tukey test yielded no significant differences between groups.

No changes in HIF-Luciferase radiance were observed over the ‘Left Kidney Region’ (Group effect: p=0.089, Time effect: p=0.282, Interaction effect: p=0.310, Figure 4-10E).

Over the ‘Right Kidney and Liver Region’, no group effect (0.425) or time effect (p=0.382) was detected. A significant time group interaction was measured (p=0.009). No change in HIF-luciferase radiance was observed at day 3 (p=0.205 vs. baseline; p=0.298 vs. control); but at day 4 HIF-Luciferase radiance significantly increased from a baseline value of $5.30 \times 10^7 \pm 1.23 \times 10^7$ p/s to $6.72 \times 10^7 \pm 1.63 \times 10^7$ p/s (p=0.006), and was significantly higher than control values (Control = $5.48 \times 10^7 \pm 1.27 \times 10^7$ p/s, p=0.034; Figure 4-10E).
Assessment of ventral radiance in liver and gut regions:

Qualitative analysis of HIF-luciferase radiance over the ventral side suggests no change in radiance in control mice (Figure 4-11A), and increased radiance over the ‘Liver’ and ‘Gut’ Regions during anemia at the day 4 time point (Figure 4-11B, white arrow).

When conducting our regional analysis, we did not detect any change in HIF-luciferase radiance within any region in control mice. Baseline HIF-luciferase radiance was not different between groups for all regions of analysis. We did not observe any change in HIF-luciferase radiance over the ‘Total Ventral Side’ during anemia (Group effect: p=0.485, Time effect: p=0.189, Interaction effect: p=0.111, Figure 4-11C).

Similarly, no group effect (p=0.517) or time effect (p=0.165) was detected over the ‘Abdomen Region’; however, a group time interaction was observed (p=0.049, Figure 4-11D). No change in HIF-luciferase radiance was observed at day 3 (p=0.969 vs. baseline; p=0.929 vs. control). At day 4 HIF-Luciferase radiance increased to $6.51 \times 10^8 \pm 4.34 \times 10^8$ p/s, which was not significantly different from a baseline value of $4.49 \times 10^8 \pm 1.72 \times 10^8$ p/s (p=0.060), but significantly higher than a control value of $4.37 \times 10^8 \pm 1.70 \times 10^8$ p/s (p=0.029).

No changes in HIF-Luciferase radiance were observed over the ‘Liver Region’ (Group effect: p=0.653, Time effect: p=0.415, Interaction effect: p=0.544, Figure 4-11E).

Over the ‘Gut Region’, no group effect (p=0.365) or time effect (p=0.188) was detected. A significant time group interaction was measured (p=0.043). No change in HIF-luciferase radiance was observed at day 3 (p=0.964 vs. baseline; p=0.782 vs. control). At day 4 HIF-Luciferase radiance increased to $4.38 \times 10^8 \pm 4.00 \times 10^8$ p/s, which was not significantly different from a baseline value of $2.70 \times 10^8 \pm 1.24 \times 10^8$ p/s (p=0.073), but significantly higher than a control value of $2.36 \times 10^8 \pm 0.93 \times 10^8$ p/s (p=0.017).
Figure 4-10: Dorsal HIF-luciferase bioluminescence during anemia. Panel A/B: Observational analysis of characteristic dorsal images of control (n=11) and anemic (n=12) mice demonstrate increased HIF-luciferase radiance over the ‘right kidney and liver region’ during anemia. Dorsal regional HIF-luciferase radiance does not change in control animals. Panel C: No change was observed in total dorsal HIF-luciferase bioluminescence during anemia. Panel D: HIF-luciferase radiance did not change over the total kidney and liver region during anemia. Panel E: No change in HIF-luciferase radiance was detected over the left kidney region during anemia. Panel F: A significant increase in HIF-luciferase radiance was observed during anemia at day 4 over the right kidney and liver region *p=0.006 relative to baseline; #p=0.034 relative to control. (Two Way RM ANOVA)
Figure 4-11: Ventral HIF-Luciferase bioluminescence during anemia. **Panel A/B:** Observational analysis of characteristic ventral images of control (n=11) and anemic (n=12) mice demonstrate increased HIF-luciferase radiance over the gut region during anemia. Ventral regional HIF-Luciferase radiance does not change in control animals. **Panel C:** No change was observed in total ventral HIF-Luciferase bioluminescence during anemia. **Panel D:** Radiance over the total abdomen region significantly increased during anemia at day 4 #p=0.029 relative to control. **Panel E:** Liver HIF-Luciferase radiance did not change during anemia. **Panel F:** Gut HIF-Luciferase significantly increased during anemia at day 4 #p=0.017 relative to control. (Two Way RM ANOVA).
5. **DISCUSSION**

**SUMMARY OF FINDINGS**

The current study measured physiological and hypoxic cellular responses to antibody-mediated anemia, to assess if these adaptations were similar to other models of hemodilutional anemia and sickle cell anemia; and to test the hypothesis that anemia-induced tissue hypoxia is a unifying mechanism for organ injury and mortality.

*Cardiovascular and cerebrovascular responses to moderate anemia are sufficient to prevent brain tissue hypoxia*

Cardiovascular and cerebrovascular responses to moderate anemia were characterized by an expected increase in peripheral arterial O$_2$ saturation, cardiac output, cerebral blood flow, and cerebrovascular reactivity. These responses increased brain blood flow and O$_2$ delivery to compensate for decreased blood O$_2$ content. This was manifest in a specific increase in internal carotid blood flow and cerebrovascular reactivity. The observed increased in cerebrovascular reactivity to CO$_2$ has not been previously described in anemia, and may represent an adaptive mechanism to promote cerebral perfusion (i.e a heightened reactivity to vasodilatory stimuli). These changes were associated with a maintained brain P$_t$O$_2$ suggesting that oxygen delivery to the brain was maintained. Collectively, these data support the finding that augmentation of cerebral blood flow, oxygen delivery, and vascular responsiveness acted to maintain O$_2$ homeostasis within the brain.

*Cardiovascular adaptations to moderate anemia were insufficient to prevent renal and splanchnic tissue hypoxia*

In contrast to the brain, perfusion of the kidney was not augmented during anemia. We observed no increase in renal blood flow and an associated drop in renal P$_t$O$_2$. This was associated with an increase in HIF-luciferase signal over the right kidney region, suggesting the
activation of adaptive cellular responses to tissue hypoxia. Increased HIF-luciferase was observed over the dorsal right kidney region, but not over the left kidney region. We suggest that this discrepancy occurred for a number of reasons: 1) MRI analysis demonstrated that the right kidney is more superficial compared to the left kidney; 2) the right kidney is adjacent to a superficial segment of the liver which also expresses HIF-luciferase during anemia, thus augmenting the dorsal HIF signal in the region of the right kidney.

In addition, a second region of increased HIF-luciferase signaling was measured over the ventral abdominal or gut region. This provides evidence of splanchnic tissue hypoxia in response to moderate anemia. Further investigation is required to determine whether this increased HIF expression is adaptive or maladaptive, and whether a decrease in kidney $P_{O_2}$ contributes to increased morbidity and mortality.

*Model of anemia induced by RBC-specific antibody*

This RBC-specific antibody induced moderate anemia (94±11 g/L) without excessive intravascular hemolysis, overt organ failure, or hematuria; and with 100% survival. Although there is evidence of early (Day 1) intravascular hemolysis, splenic and hepatic sequestration appears to be the primary mechanism for RBC clearance. The greatest increase in spleen size and weight occurred around day 4, the time point at which the nadir hemoglobin concentration occurred. The hemoglobin threshold which was achieved by our RBC-specific antibody model of moderate anemia is of clinical importance as the achieved nadir hemoglobin concentration has been associated with renal, gut, and cerebral morbidity and mortality in surgical patients. In addition, antibody-induced anemia produces cardiovascular and cellular adaptations, characteristic of other models of anemia, making it a valuable tool to assess the mechanisms associated with anemia-induced morbidity and mortality.
CARDIOVASCULAR ADAPTATIONS TO MODERATE ANEMIA MAINTAIN CEREBRAL TISSUE

OXYGEN DELIVERY AND HOMEOSTASIS

MECHANISMS THAT OPTIMIZE OXYGEN DELIVERY DURING MODERATE ANEMIA

Peripheral arterial oxygen saturation is increased during antibody-induced moderate anemia

A well-established characteristic response to anemia of all etiologies is an increase in arterial partial pressure of O\textsubscript{2} (P\textsubscript{a}O\textsubscript{2}). This effect has been demonstrated by a measured increase in arterial O\textsubscript{2} in experimental\textsuperscript{33,34,124} and clinical studies.\textsuperscript{103} In our model of anemia, we elected to measure peripheral arterial O\textsubscript{2} saturation (S\textsubscript{p}O\textsubscript{2}) instead of P\textsubscript{a}O\textsubscript{2} for technical reasons; notably the small blood volume of mice which limited the availability for an adequate volume for blood sample analysis. S\textsubscript{p}O\textsubscript{2} is a surrogate of PaO\textsubscript{2}, as measured via pulse oximetry. The relationship between PaO\textsubscript{2} and S\textsubscript{p}O\textsubscript{2} is characterized by the Hb-O\textsubscript{2} dissociation curve. As reported, we observed a late increase in SpO\textsubscript{2} by ~2% at day 3 and 4 in anemic mice. This change reflects the characteristic increase in P\textsubscript{a}O\textsubscript{2} observed in all forms of anemia. This increase in S\textsubscript{p}O\textsubscript{2} occurred during the time at which there were maximum reductions in Hb concentrations, indicating that activation of physiological adaptations within the lungs did not occur until the nadir Hb value was reached. Deem et al. has characterized this response by showing that anemic mice have an increase in ventilation and perfusion matching as a compensatory mechanism to optimize O\textsubscript{2} exchange during anemia, and proposed NO as a mechanism for mediating pulmonary vasodilation.\textsuperscript{93} Other factors, such as the increase in respiratory rate (tachypnea) may also act to improve ventilation perfusion matching.\textsuperscript{125} This finding of increased arterial O\textsubscript{2} saturation during anemia ruled out the possibility that our injected antibody had additional adverse effects, such as interaction with the lung which might have caused decreased lung function and arterial hypoxemia. The increase in arterial O\textsubscript{2} saturation to a value of 98.1±0.4% ensured that arterial O\textsubscript{2} content was optimized. Absence of blood gas analysis did not allow for us to determine if there
was a right shift in the O₂- Hb dissociation curve. This effect is present in more chronic models of anemia, as a result of increased cellular 2,3-DPG within the RBC. Further investigation will be needed to determine whether if such a shift occurs in this model.

*Antibody mediated moderate anemia is associated with an increase cardiac output*

In our model of anemia, we observed a moderate increase in cardiac output (24%). This increase is consistent with our observations in other experimental models of acute anemia (ie. hemodilution). The increase in cardiac output is in proportion to the reduction in Hb concentrations, as observed in most mammalian models studied, as well as in humans.

Anemia results in tissue hypoxia, which is known to activate a number of afferent signals to the central nervous system and brain. For example, Hatcher et al. have demonstrated an increase in aortic autonomic afferent nervous activity in response to chemoreceptor activation in proportion to reductions in Hb concentrations. This afferent signal to the brain results in an efferent sympathetic output to the heart via known autonomic cardiac sympathetic innervation leading to a corresponding increase in cardiac output. Denervation of aortic chemoreceptors results in an attenuation of the cardiac output response to hemodilution, supporting this mechanism by which anemia augments cardiac output. In addition, inhibition of the cardiac sympathetic responsiveness to anemia by administration of systemic β adrenergic antagonists prevents the characteristic increase in cardiac output resulting in more profound tissue hypoxia. Thus, adrenergic signaling from chemoreceptors, and subsequent sympathetic output, contribute to increasing cardiac output during moderate anemia.

With respect to our traditional understanding of aortic and carotid body chemoreceptor sensing; anemia presents a paradoxical situation. These chemoreceptors are located in organelles within the large conduit arteries and are known to respond to hypoxia. However, as we have
demonstrated, the blood in these conduit vessels has a high $P_aO_2$ and increased $S_pO_2$. This evidence is puzzling and paradoxical because, as just described, there is an increase in $S_pO_2$ and presumably $PaO_2$ during anemia.\textsuperscript{103} Thus, either the chemoreceptors are able to detect a decrease in arterial blood oxygen content ($C_aO_2$), denervation affects other efferent (or afferent) signaling pathways besides $O_2$-sensing chemoreceptors, or the traditional chemoreceptors are situated within tissue that experiences local tissue hypoxia as a result of a decrease in the $O_2$ supply demand ratio. Our laboratory’s previous findings of increased HIF expression in the perivascular or endothelial region of conduit arterioles suggests that such a sensing mechanism may be in place in all vessels.\textsuperscript{34,111} It has yet to be established how this specific decrease in $C_aO_2$ is sensed at the tissue level by traditional chemoreceptors which detect hypoxia.

Experimental studies have demonstrated that acute anemia causes organ specific tissue hypoxia, in the kidney, gut, brain, and heart.\textsuperscript{33-35,94,109,111,118} Presumably, this occurrence of tissue hypoxia can act as an afferent signal to nervous system; however, the mechanism by which signaling occurs is as of yet not fully defined. It is likely that multiple efferent signals from many sources contribute to signal that results in increase in cardiac output during anemia. The increase in cardiac output is a compensatory mechanism to optimize or maintain global organism $O_2$ homeostasis in response to reduction in $C_aO_2$ during anemia. Interruption of this nervous response, by antagonism of $\beta$-adrenergic receptors, has been demonstrated to prevent the cardiac output response and increase tissue hypoxia in response to anemia.\textsuperscript{111} This effect is largely thought to be a $\beta$1 mediated effect, as demonstrated by consistent results by $\beta$1 antagonists.\textsuperscript{111,118} The increase in cardiac output is critical to ensure adequacy of $O_2$ delivery during anemia.

While changes in viscosity have been argued to contribute to the increase in cardiac output, these changes are likely secondary and less important than the mechanisms described above. The concept that reduced viscosity contributes to maintain cardiac output originated from
Guyton et al in 1961. In that study, Guyton performed spinal anesthesia and ganglionic blockade to dogs, followed by hemodilution to low Hb concentrations. He observed a maintenance of cardiac output in response to anemia, and therefore concluded that removal of active sympathetic and ganglionic responses are not required for the anemia-induced increases in CO; and suggested that passive features in circulation, such as reduced viscosity are primarily responsible for increasing cardiac output during anemia. While at the time this conclusion may have been valid, it was made with the lack of an understanding of the redundant mechanisms of hypoxia sensing in the body. These characterized hypoxia signaling mechanisms including nNOS and HIF, as well as mitochondrial sensing mechanisms, may enable active regulation of cardiac output despite sympathetic nervous system block. The idea that reduced viscosity improves perfusion has been staunchly defended by a number of investigators in the past. However, the strongest data investigating microvascular responses during anemia have reported by Tsai; who has demonstrated that increased rather than decreased blood viscosity improves tissue perfusion by increased functional capillary density.

Evidence that cerebral perfusion is optimized by antibody induced moderate anemia

A number of experimental and clinical observations have demonstrated that during anemia, there is preferential perfusion of brain, relative to other less vital organs. For example, utilizing a hemodilution model in pigs, Van Bommel et al. have demonstrated that a much higher proportion of cardiac output is directed to the brain during anemia, relative to the gut, which receives a smaller proportion of the increased cardiac output. This resulted in the maintenance of brain PO$_2$ at much lower Hb concentrations than were tolerated by the gut, in which early tissue hypoxia was observed. In addition, similar comparative assessments were performed for cerebral and kidney perfusion in a rat model of acute hemodilutional anemia; again evidence of relatively enhanced or preferential perfusion of the brain was observed relative to the kidney.
where brain \( P_{tO_2} \) was maintained at Hb concentrations associated with kidney tissue hypoxia.\(^{34,111}\) In addition, Van Bommel demonstrated that brain PO\(_2\) is preserved during anemia to a much lower Hb concentration relative to tissue PO\(_2\) in the heart, kidney, and gut; all of which demonstrate drops in PO\(_2\) in these tissues at Hb concentrations higher than that of the brain.\(^{109}\)

This preferential increase in cerebral perfusion was also observed in our antibody mediated model of anemia. Specifically, we observed the greatest percent increase in flow through the internal carotid artery, specifically functioning to perfuse the brain. This was clearly illustrated where a 24% increase in cardiac output caused no significant increase in flow through the common carotid artery (20%), but an 80% increase in flow through the internal carotid artery; while renal flow did not change. This clearly demonstrates a preferential redistribution of blood flow to brain as a mechanism for maintaining cerebral O\(_2\) delivery. Evidence that brain perfusion was maintained is also provided by the finding that brain \( P_{tO_2} \) does not decrease relative to control mice; providing direct evidence that brain \( P_{tO_2} \) is during moderate anemia (~80-90 g/L). Other experimental studies support this; Hare et al demonstrated that severe hemodilution to 50 g/L resulted in a small reduction brain \( P_{tO_2} \).\(^{33}\) Tsui et al. demonstrated that moderate anemia (90g/L) did not increase expression of HIF.\(^{35}\)

Our experimental data also support these findings, as brain \( P_{tO_2} \) is preserved during moderate anemia, but kidney \( P_{tO_2} \) is reduced. Thus the synthesis of blood flow and PO\(_2\) data demonstrate that preferential perfusion of brain functions to maintain brain O\(_2\) homeostasis at physiological levels during anemia. We did not measure tissue HIF levels, as previous studies of HIF at this level have shown to decrease.\(^{35}\) As opposed to assessing cellular levels of HIF, we chose another novel approach; we assessed cerebrovascular reactivity to CO\(_2\) as an additional surrogate measure of cerebrovascular adaptation to anemia. We chose this outcome because
previous clinical and experimental studies have demonstrated that a reduced cerebrovascular reactivity (CVR) is associated with increased risk of cerebral injury (transient ischemic attacks, stroke) in different patient populations. Evidence of decreased cerebrovascular responsiveness to CO₂ has been associated with increased stroke incidence in three different clinical conditions: 1) sickle cell anemia, 2) Atherosclerotic carotid disease and 3) Moyamoya disease. In children with sickle cell anemia, anemia in association with increased basal cerebral blood flow has been associated with an increase in the incidence of stroke.³⁶ Interestingly, transfusion of red blood cells reduced the incidence of stroke by ~90%, suggesting that augmentation of blood oxygen content may have alleviated the stroke risk. Additional evidence from a randomized clinical trial demonstrated that red blood cell transfusion also reduced the incidence of silent cerebral ischemia as detected by MRI, suggesting that cerebral injury was prevented at the microvascular level.⁵⁹ Furthermore, the evidence that sickle cell anemia is associated with increased basal cerebral blood flow and reduced CVR suggested that maximal basal cerebral vasodilation prevented further vascular reactivity to CO₂. These observations were made in both an animal model of sickle cell anemia and in human measurements.⁵⁷,⁶⁰ In the animal model these changes were associated with a reduction in basal brain tissue PO₂ relative to controls. Thus, we hypothesize that anemia leads to brain tissue hypoxia, and an adaptive increase in basal cerebral blood flow to compensate; these changes detract from the cerebral vasodilatory reserve and make the brain more susceptible to cerebral injury. If these hypotheses are correct; then transfusion should reverse these changes by reducing basal cerebral blood flow and restoring cerebrovascular reactivity to CO₂. Indeed, this is what was found in a recently published clinical study by Kassner et al. CVR was reduced with decreasing Hb concentrations; with transfusions increasing Hb concentrations restoring CVR in these patients. (Kosinski et al., Eur J Hematol, In Press) This suggests a reduced CₐO₂ from anemia is a primary factor reducing CVR, and may
predispose sickle cell anemic patients to anemia-induced tissue hypoxia leading to stroke. A second example of increased stroke risk with reduced cerebrovascular reactivity occurs in in patients with carotid artery stenosis. In these patients and decrease of 20% in cerebral blood flow reactivity was associated with increased risk of developing stroke or transient ischemic attack. In addition, it has been demonstrated that patients with Moyamoya disease undergoing surgical intervention, a >10% reduction in preoperative CVR is associated with increased risk of developing postoperative cerebral infarct. Thus, reduced cerebrovascular reactivity is a predictor of stroke and this predictor can be modified by treatments such as blood transfusion to reduce the risk of stroke (sickle cell anemia). Furthermore, a reduction in CVR may increase the risk of mortality of sickle cell anemic patients in response to systemic hypoxemia. Crawford et al. demonstrated increased mortality of sickle cell anemic mice relative to wild type mice exposed to hypoxia (6% O2). In addition, sickle cell anemic mice administered an hemoglobin-based oxygen carrier which restored C4O2 and potentially CVR, had increased survival, again further providing evidence that an anemia-induced reduction in CVR may be a factor increasing the risk of mortality in sickle cell anemic patients.

From these studies, we hypothesize that during conditions of elevated cerebral blood flow there may be a reduction in CVR; and therefore a reduced microvascular response to CO2. Clinical studies have elegantly characterized this by inducing step-wise hypercapnia with very high precision by targeting end tidal PCO2. Based on our measured increase in basal cerebral blood flow during moderate anemia, one should expect a comparable reduction in CVR. Our CVR studies were performed in a blinded randomized fashion to prevent any bias. Paradoxically, our results demonstrated the opposite, and there was an increase in CVR in anemic mice. This result was accentuated following repeat exposures with CO2. In addition, increased CVR during anemia was specific to the internal carotid artery;
suggesting that this may be a component of a mechanism designed to improve brain perfusion during anemia.

The mechanism of cerebrovascular reactivity increase remains yet to be determined. Due to the Fahraeus-Lindqvist effect, it is suggested that decreased Hct during anemia may influence the apparent blood viscosity in the microvasculature. It has been demonstrated experimentally and mathematically that decreases in Hct induce an increase in capillary wall sheer stress in the perfusion sub-network, inducing an overall increase in blood flow rate.\textsuperscript{129} As previously described, Tsai et al. has demonstrated that increased capillary wall shear stress improves tissue perfusion by increasing functional capillary density via NO mediated vasodilation.\textsuperscript{97} Potentially, during anemia, increased wall shear stress in the perfusion sub-network may increase functional capillary density, increasing perfusion, blood flow rate, and overall cerebrovascular reactivity. In addition, another mechanism which may contribute to an increased cerebrovascular reactivity during anemia may be due to a reduction of NO scavenging. Hemoglobin is a potent scavenger for NO.\textsuperscript{130} During anemia, reductions in hemoglobin concentrations may lead to reduced hemoglobin-induced NO uptake. This may contribute to cerebral microvasculature vasodilation, increasing blood flow, and overall cerebrovascular reactivity. These two factors may contribute to the observed increase in cerebrovascular reactivity measured during moderate anemia.

There is little evidence in the literature assessing the effects of an increased CVR on cerebral outcomes. It remains yet to be defined whether an anemia-induced increase in cerebrovascular reactivity is an adaptive or maladaptive response. An increase in CVR may suggest the presence of a stimulated vascular reserve as a mechanism that may maintain and benefit cerebral perfusion during anemia. This stimulated vascular reserve may induce adaptive increases in basal cerebral blood flow to compensate for reduced $\text{C}_3\text{O}_2$ during anemia. It could be proposed that this anemia-induced vascular reserve does not detract from the cerebral
vasodilatory reserve in response to hypercapnia. Conversely, these adaptations may potentially supplement the cerebral vasodilatory reserve to hypoxia. This suggests a potential synergistic effect of cerebrovascular adaptations to anemia and acute hypoxia, functioning to enhance brain perfusion to adequately deliver O₂ to meet the metabolic demands of the brain tissue, protecting against brain injury and stroke. Conversely, increased cerebrovascular reactivity has been measured in patients during sustained hypoxemia. This evidence suggests that an increase in CVR may be a sign of slight brain tissue hypoxia, as a potential predictor for cerebral injury. Further studies are required to determine whether this observed response is adaptive or maladaptive.

*Experimental and clinical evidence of reduced CVR leading to injury and mortality*

A reduced CVR is associated with an increased risk of developing cerebrovascular accidents in many patient populations, as previously described. The mechanism surrounding a reduction in CVR increasing the risk of cerebral injury is not yet fully defined; however, it is suggested that a reduced CVR impairs the ability for cardiovascular adaptations to adequately deliver O₂ in times of increased O₂ demand. Therefore, according to this hypothesis, cardiovascular adaptations are unable deliver O₂ to the brain above its critical threshold of metabolic consumption, leading to tissue hypoxia and potentially cerebral injury. This progression of events has been demonstrated in an experimental model of sickle cell anemia by Cahill et al., and is summarized in Table 5-1. In sickle cell anemic mice, similar to our model, there is an increase in CO, with a higher proportion of blood flow to the brain. However, in contrast to our model, Cahill et al. measured a reduced CVR in sickle cell anemic mice, which was associated with 46% reduction in brain P₉O₂. In addition, it was found that these mice spontaneously developed strokes, as assessed by MRI (Cahill et al., unpublished data). Therefore, these data provide evidence that a reduction in CVR may limit brain O₂ delivery.
during times of increased demand, acting as a mechanism for anemia-induced stroke. In addition, as previously described, Crawford et al. has demonstrated increased mortality of sickle cell anemic mice relative to wild type mice exposed to hypoxia, suggesting a reduction in C$_3$O$_2$, and potentially CVR, prevents adequate brain O$_2$ delivery, leading mortality.\textsuperscript{128} Contrasting the model of sickle cell anemia, in antibody-mediated anemia, we measured an anemia-induced increase in CVR. This may be an adaptive mechanism protecting brain P$_t$O$_2$ and enabling for cardiovascular adaptations to increase brain O$_2$ delivery in times of need. As a result, this augmented vasodilatory reserve prevents brain tissue hypoxia, suggesting no indication of anemia-induced stroke or mortality. The mechanisms governing the vasodilatory reserve to anemia, and whether they are separate from the vasodilatory reserve to hypoxia are unknown, and remain yet to be defined. To our knowledge, this is the only model demonstrating an anemia-induced increase in CVR. Therefore, further investigation is required to determine and better understand the adaptations to cerebrovascular vessels during anemia, which may potentially augment brain O$_2$ delivery to maintain O$_2$ homeostasis.
<table>
<thead>
<tr>
<th>Response to Anemia</th>
<th>Antibody-Mediated Anemia</th>
<th>Sickle Cell Anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin Concentration</td>
<td>94 ± 11 g/L</td>
<td>83 ± 8 g/L</td>
</tr>
<tr>
<td>Cardiac Output</td>
<td>Increased↑</td>
<td>Increased$^{57}$↑</td>
</tr>
<tr>
<td>Internal Carotid Artery Blood Flow</td>
<td>Increased↑↑</td>
<td>Increased$^{57}$↑↑</td>
</tr>
<tr>
<td>Cerebrovascular Reactivity</td>
<td>Increased↑</td>
<td>Decreased$^{57}$↓</td>
</tr>
<tr>
<td>Brain Tissue Oxygen</td>
<td>No Change ↔</td>
<td>Decreased$^{57}$↓</td>
</tr>
<tr>
<td>Evidence of Stroke</td>
<td>??</td>
<td>YES</td>
</tr>
<tr>
<td>Hypoxia Exposure</td>
<td>??</td>
<td>↑MORTALITY$^{128}$</td>
</tr>
</tbody>
</table>

Table 5-1: Comparison of physiological adaptations during antibody-induced anemia and sickle cell anemia. Both antibody-induced anemia and sickle cell anemia induce an increase in cardiac output, with a higher proportion of blood directed through the internal carotid artery. Antibody mediated anemic mice have augmented cerebrovascular reactivity, which contributes to maintaining brain tissue oxygen levels. Sickle cell anemic mice have a reduced cerebrovascular reactivity, which is associated with brain tissue hypoxia, increased incidence of stroke, and increased rates of mortality.
CARDIOVASCULAR ADAPTATIONS TO MODERATE ANEMIA ARE INSUFFICIENT TO PREVENT RENAL TISSUE HYPOXIA

RENAL PERFUSION IS JEOPARDIZED DURING ANEMIA

As outlined before, during anemia there is an increase in cardiac output; and this increase is disproportionately directed through the internal carotid artery to the brain.\textsuperscript{35,57} By contrast, we observed no increase in renal blood flow, consistent with models of acute hemodilutional anemia.\textsuperscript{35} In the setting of anemia, and reduced blood O\textsubscript{2} content, this represents a net reduction in O\textsubscript{2} delivery to kidney. The mechanism of reduced renal O\textsubscript{2} delivery may be due to macrovascular and microvascular responses. We measured a reduction in renal artery diameter during moderate anemia. Despite an increase in cardiac output, this macrovascular response of renal artery constriction may have contributed to the no change in renal blood flow detected, potentially reducing renal O\textsubscript{2} delivery. In addition, our laboratory and others have consistently demonstrated that reductions in O\textsubscript{2} delivery during anemia result in a reduction in kidney P\textsubscript{T}O\textsubscript{2}, suggesting an inadequacy in kidney tissue perfusion.\textsuperscript{64,111,132} During anemia, the combination of renal artery constriction and reduced renal tissue perfusion may contribute to anemia induced kidney hypoxia as a mechanism contributing to acute kidney injury.

We utilized a real time HIF-Luciferase mouse model to assess whether this measured reduction in renal P\textsubscript{T}O\textsubscript{2} translated to increased expression of hypoxia signaling molecules such as HIF. In a blinded analysis, we demonstrated a clear increase in HIF-luciferase expression on day 4 in the region of the right kidney (dorsal). Paradoxically, there is no increase in left renal HIF expression. This finding is consistent with Safran et al., who developed this model of real time HIF-luciferase expression.\textsuperscript{122} To better understand this observation, we analyzed serial cross-sectional images to determine if there was an anatomical explanation for the difference in right versus left renal HIF response. Interestingly, our analysis demonstrated that the right kidney is...
more superficial, and less shielded by paraspinal structures; providing an explanation for why decreased HIF-signal is detected over the left side. An additional anatomic finding was that there is a greater proportion of superficial liver tissue on the right side relative to the left. Tsui et al. has demonstrated a profound increase in liver HIF expression during anemia. Therefore, we propose that the combination of the superficial anatomical placement of the right kidney, and the contribution of HIF-luciferase radiance produced by the liver, induced a greater HIF-Luciferase signal over the dorsal right side.

We did not observe acute renal failure in any animals; therefore, the effect of decreased kidney $\text{PtO}_2$ and increased HIF requires interpretation. On one hand the kidney is a hypoxia sensing organ which is typically hypoxic during normoxia as a part of its normal function (to produce systemic EPO). By contrast, there is evidence that anemia is associated with acute kidney injury in cardiac and noncardiac surgery patients. In addition, low intraoperative Hb during cardiopulmonary bypass is associated with renal failure. Evidence that anemia may lead to renal tissue hypoxia as a mechanism for renal dysfunction and increased mortality is provided by studies assessing patients undergoing cardiac surgery under cardiopulmonary bypass. Preoperative anemia is associated with postoperative renal dysfunction, and predisposes cardiac surgery patients to a lower intraoperative Hb concentration when on bypass. Low Hct on cardiopulmonary bypass is associated with postoperative acute kidney injury. Finally, lowest Hct on bypass associated with increased post-operative mortality. We do not know whether low kidney $\text{PtO}_2$ contributes to this pattern, as the causality has not yet been established. However, it is proposed that hypoperfusion during cardiopulmonary bypass exacerbates anemia-induced renal tissue hypoxia, contributing to the development of renal morbidities and mortality in these patients. Due to the heterogeneity of blood flow and oxygen consumption of the kidney, it is suggested that the renal medulla faces a higher degree of
hypoxia as compared to the cortex during moderate anemia. Therefore it is suggested that the medulla has an increased risk for anemia-induced injury. Relative to the renal cortex, the medulla is relatively less perfused\textsuperscript{132} and has a higher metabolic demand in order to maintain its ion gradient for solute and water reabsorption. In a model of hemodilutional anemia, an immediate drop in renal cortical and medullary microvascular PO\textsubscript{2} has been measured, with the medulla reaching lower microvascular PO\textsubscript{2} levels compared to the cortex.\textsuperscript{64} These findings further support that cardiovascular adaptations to moderate anemia are insufficient to prevent kidney hypoxia, increasing the risk hypoxic injury of the renal medulla relative to the cortex in our model. However, further assessment of markers associated with acute kidney injury (creatinine, estimated glomerular filtration rate, urine output) should also be assessed to determine the effects of anemia on kidney function and potentially kidney injury.\textsuperscript{64,132}

**MODERATE ANEMIA-INDUCED TISSUE HYPOXIA AS A POTENTIAL MECHANISM FOR SPLANCHNIC INJURY AND MORTALITY**

**CARDIOVASCULAR ADAPTATIONS TO MODERATE ANEMIA ARE INSUFFICIENT TO PREVENT SPLANCHNIC HYPOXIA**

We assessed splanchnic perfusion in our HIF-luciferase mouse model in the context of whole organism anemia during which there is an increase in cardiac output, with a greater proportion of blood flow directed through the internal carotid artery, and no increase in flow through the renal artery. Interestingly, we observed a significant increase in HIF-Luciferase radiance over the ‘ventral gut region’ during moderate anemia; suggesting that splanchnic visceral perfusion was reduced. This is consistent with other experimental models, which have demonstrated that the gut is particularly vulnerable to a reduction in PO\textsubscript{2} during anemia.\textsuperscript{35,109,134} Also, in human studies, Mathru et al. demonstrated a reduction in splanchnic and gut perfusion in hemodilutional
anemia. Presumably, this was a result of inadequate blood flow in the context of reduced $C_aO_2$, however, tissue $PO_2$ values were not measured.

Clinically, these findings have also been demonstrated in premature neonates developing enterocolitis. There is an increased relative risk of developing necrotizing enterocolitis in anemic patients (CI: 5.99 [2.00-18.00]; $p<0.001$). We hypothesize that during anemia, cardiovascular adaptations are insufficient to prevent gut tissue hypoxia; leading to the breakdown of bowel wall tissue integrity. This may potentially lead to the translocation of abdominal bacteria as a mechanism leading to increased inflammation, and potentially mortality. An additional example of anemia-induced gut hypoxia leading to mortality was demonstrated by the TITRe2 transfusion trial, comparing the effects of a liberal and restrictive transfusion protocol in cardiac surgery patients. A non-significantly higher incidence of gut infarction was observed in the restrictive arm in which Hb levels were allowed to drop to the region of 80 g/L; however, further studies are required to assess the causality between anemia and gut tissue hypoxia as a mechanism for gut injury.

**Model of Anemia Induced by RBC-Specific Antibody**

**Clinically Relevant Level of Moderate Anemia Induced by RBC-Specific Antibody**

Antibody specific to RBC induces a nadir Hb concentration of 94±11 g/L, which is precisely within the scope moderate anemia, as defined by the WHO. Many clinical trials have associated moderate anemia with increased risk of morbidity and mortality in a variety of patient populations. Musallam et al. conducted a large retrospective trial, assessing 227 425 non-cardiac surgery patients, and associated moderate preoperative anemia with increased risk of developing postoperative renal injury (OR: 1.38 [1.18-1.62]; $p<0.05$), and mortality across all morbidities assessed (OR: 1.44[1.29-1.60]; $p<0.05$). Also, in cardiac surgery patients, preoperative and intraoperative moderate anemia associated with increased risk of developing cardiac events,
stroke, renal injury, and mortality. In addition, Hb thresholds near 90g/L are being assessed by many transfusion trials to determine whether the safety and risks associated with transfusion outweigh the risks associated with moderate anemia. Therefore, the level of moderate anemia induced by antibody specific to RBC has significant clinical relevance, and this model can be used as a tool to assess the mechanisms for morbidities and mortality at this Hb concentration.

MODEL OF ANTIBODY MEDIATED ANEMIA

The mechanism of anemia induction includes a small degree of intravascular hemolysis, as determined by a slight increase in plasma Hb, within the first 24 hours following antibody injection. This rise in plasma Hb was relatively low, and was not primarily responsible for the producing the nadir Hb concentration by day 4. The degree of free Hb did not cause observable morbidities, as mice were always healthy in appearance, maintained their weight, did not express gross hematuria, and had 100% survival. It is suggested that the primary mechanism for RBC removal in our model is sequestration into the spleen, as spleen weights increased by >2 fold between day 2 and 4, and this time was associated with the fastest reductions in Hb concentrations. Similar to our findings, Chen et al. measured a nadir RBC count at 4 days with maximal splenomegaly at the day 4 time point. Conversely, this group observed significant hematuria at the day 1 time point, contrasting our observations. Their group utilized a higher dose of RBC-specific antibody within a different mouse background strain, suggesting a potential dose and/or strain dependent effect of RBC clearance induced by this antibody; however, further study is required to characterize these differences.

COMPARISON TO OTHER MODELS OF ANEMIA

Anemia induced by RBC-specific antibody produces cardiovascular and cellular responses characteristic of other models of anemia. There is a measured increase in cardiac
output, with a higher proportion of blood directed to the brain via increased internal carotid artery flow, across all experimental models of anemia studied.\textsuperscript{35,57} However, we measured differences in cardiovascular adaptations when assessing CVR, whereby we observed an increase in CVR in antibody induced anemia, which we predict as a mechanism against brain tissue hypoxia and injury; and a reduced CVR in experimental models of sickle cell anemia,\textsuperscript{57} which we predict is associated with increased brain tissue hypoxia leading to stroke and mortality. In order to better understand the effects of anemia on CVR, experiments assessing CVR in response to hemodilutional anemia should be conducted to determine if cerebrovascular vessels have an adaptive or maladaptive response during acute moderate anemia. Ultimately, in all models of anemia, cardiovascular adaptations are insufficient to prevent tissue hypoxia, which induces increased stabilization of HIF-1\textalpha.\textsuperscript{34,35,57} The causality between anemia-induced tissue hypoxia and organ injury, and subsequent mortality remains yet to be defined, and this model of antibody mediated-anemia can be utilized as a valuable tool to assess the mechanisms leading to anemia-induced organ injury and mortality.

LIMITATIONS OF MODEL

All models of anemia have limitations. One limitation of ours is that there is no direct clinical translational disease profile which fits our model of antibody-induced anemia. It can be suggested that this model of antibody-induced anemia mimics warm autoimmune hemolytic anemia; which is also primarily IgG mediated, and does induce splenic RBC sequestration resulting in splenomegaly.\textsuperscript{19} However, the etiology of warm autoimmune hemolytic anemia is often drug induced, or the result of a preexisting autoimmune disorder, and results in chronic anemia that does not spontaneously correct, from the constant production autoimmune RBC antibodies.\textsuperscript{19} In our model of antibody mediated anemia, the nadir Hb concentration is sustained only between days 3 to 5, and Hb concentrations gradually return to baseline (14 days). This Hb
profile follows closer to that of a surgical patient, who faces an acute reduction of Hb concentration, followed by a gradual recovery to baseline; rather than that of a patient with chronic autoimmune disorder.

In addition, mice receiving two injections of RBC-specific antibody developed severe anemia, but these mice lacked a healthy appearance and were lethargic. These findings suggested severe anemia induced by 2 doses of RBC-specific antibody may have associated morbidities; and thus, this model was not explored.

Although we observed no evidence of macroscopic hematuria, further assessment is required to determine whether RBC-specific antibody induces any pathology associated with the small measured increase of plasma free Hb. Free Hb has been associated with increased oxidative damage of the kidney, which may confound our assessment of anemia-induced tissue hypoxia as a mechanism for kidney injury. However, within our model it is suspected that the small rise in free Hb does not contribute to renal pathology as we did not measure any: 1) gross hematuria; 2) overt kidney failure; and 3) difference in HIF-luciferase expression over the left kidney region, suggesting no change in hypoxic signaling, which we propose as a potential marker for hypoxia-induced injury. However, further assessment of kidney injury markers, such measurement of creatinine levels, or other renal specific biomarkers, estimated glomerular filtration rate, and urine output should be conducted in both our model of antibody mediated anemia, and other models of anemia, to determine the model-specific impacts of anemia on kidney injury.

Overall, this model of antibody-induced moderate anemia easily and effectively allows for the assessment of physiological adaptations during moderate anemia, making it a valuable tool for assessing the mechanism for morbidities and mortality associated with moderate anemia.
6. CONCLUSIONS

1. RBC-specific antibody induces a moderate level of anemia with no overt hematuria, organ dysfunction; and with 100% survival
   a. Mechanism of RBC clearance includes early intravascular hemolysis followed by a rapid clearance of RBC associated with sequestration to the spleen.
   b. No evidence of systemic hypoxia by antibody-lung interaction, as evidenced by an increase in $S_pO_2$ during anemia

2. Cardiovascular adaptations during anemia included an increase in $S_pO_2$, and a 24% increase in cardiac output, optimizing global oxygen delivery.

3. Cardiovascular adaptations function to increase flow through blood vessels specifically supplying oxygen to the brain, and are sufficient to maintain brain $P_tO_2$. This was manifest in two experiments:
   a. Blood flow through the common carotid artery did not increase during anemia; however, we measured an 80% increase in flow through the internal carotid artery.
   b. No change in common carotid artery cerebrovascular reactivity was detected; however, we measured an increase in cerebrovascular reactivity through the internal carotid artery during anemia. This novel finding suggests cardiovascular adaptations supplement the vasodilatory reserve to hypoxia during anemia as a potential protective mechanism to maintain brain oxygen homeostasis.

4. No change in renal blood flow was measured during moderate anemia. Due to the reduction in blood oxygen content during anemia, this reduced kidney oxygen delivery by 33%. This insufficient cardiovascular adaptation was associated with a drop in kidney $P_tO_2$. 
5. Increased stabilization of HIF-1α over the dorsal ‘kidney and liver’ region, and ventral ‘gut’ region suggest moderate anemia induces renal, hepatic, and splanchnic tissue hypoxia. This anemia-induced tissue hypoxia may act as a mechanism for organ injury (acute kidney injury, necrotizing enterocolitis) and mortality.
7. LIMITATIONS AND FUTURE DIRECTIONS

The current study did not assess the causality of anemia-induced tissue hypoxia as a mechanism for organ injury and mortality. We plan to further characterize cardiovascular adaptations during anemia to investigate the mechanism of anemia induced organ-injury and mortality by:

1. Assessing cerebrovascular reactivity in moderately anemic mice only at the day 4 time point (one exposure to CO₂). This experiment will help isolate the effect of a repeat exposure of hypoxia on CVR, to help us better understand effects of anemia on CVR.

2. Measuring organ-specific (brain, kidney, liver, gut) HIF-Luciferase radiance values, and mRNA expression of HRE related genes affecting erythropoiesis (EPO), angiogenesis (VEGF), and glycolytic enzymes (PDK1). These experiments will help us quantify organ-specific increases in HIF-luciferase radiance to better understand organs of highest risk of anemia-induced hypoxic injury.

3. Assessing markers for kidney injury, such as serum creatinine and urine output, during moderate anemia to determine if anemia-induced tissue hypoxia causes any impairment in renal function; potentially leading to renal injury.

In addition, further experiments will be conducted with an iron-deficiency model of anemia to determine if physiological adaptations to chronic anemia are similar to other types of anemia (acute: hemodilutional, moderate: antibody-mediated, chronic: sickle cell anemia).
8. **SUMMARY OF THESIS**

The purpose of this study was to assess whether cardiovascular adaptations in a model of anemia induced by a RBC-specific antibody caused a disruption in tissue oxygen homeostasis, leading to tissue hypoxia. We measured that RBC-specific antibody induced a moderate level of anemia with no associated toxicity (no overt organ failure or dysfunction, no hematuria) or mortality. The primary mechanism of RBC-clearance is suggested to be splenic sequestration, as it was associated with the fastest rates of hemoglobin reduction. Anemia induced characteristic cardiovascular adaptations including: an increase in SpO₂, a 26% increase in cardiac output, and an 80% increase in internal carotid artery flow; suggesting cardiovascular adaptations during anemia function to maintain brain oxygen delivery. In addition, we measured an increase cerebrovascular reactivity of the internal carotid artery during anemia, suggesting cardiovascular adaptations during anemia supplement the vasodilatory reserve to hypoxia as a protective mechanism preserving brain oxygen delivery. These cardiovascular adaptations preserved brain PtO₂ during anemia. Conversely, no change in renal blood flow was detected, which was associated with a reduction in renal oxygen delivery renal tissue hypoxia. Increased HIF-luciferase radiance over the dorsal ‘kidney liver’ and ventral ‘gut’ region suggest cardiovascular adaptations during moderate anemia are insufficient to prevent renal, hepatic, and splanchnic tissue hypoxia. Anemia-induced renal, hepatic, and splanchnic hypoxia may act as a mechanism for anemia-induced organ injury and mortality. In conclusion, cardiovascular adaptions during moderate anemia maintain brain PtO₂, but are insufficient to prevent visceral (renal, hepatic, and splanchnic) tissue hypoxia. This model may enable us to further assess the mechanisms of anemia-induced morbidity and mortality in surgical patients.


9. **REFERENCES**


50 Karkouti, K., Wijeysundera, D. N., Beattie, W. S. & Reducing Bleeding in Cardiac Surgery, I. Risk associated with preoperative anemia in cardiac surgery: a multicenter


Lieberman, J. A. *et al.* Critical oxygen delivery in conscious humans is less than 7.3 ml O2 x kg(-1) x min(-1). *Anesthesiology* **92**, 407-413 (2000).


Schaer, D. J., Buehler, P. W., Alayash, A. I., Belcher, J. D. & Vercellotti, G. M.