Anemia in Pediatric Intestinal Failure: Prevalence, Types, Etiologies and Predictors

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A Research Proposal for Dissertation
for the degree of Master of Science in Clinical Epidemiology
Submitted to the Department of Community Health Sciences
University of Calgary
Fall Semester 2021

Outline

1. Introduction

Outcomes in children with intestinal failure (IF) have improved considerably over the last decade and the majority of children are now surviving through childhood.[1] However, with improved survival, new burdens and complications are becoming more apparent. Anemia is an important complication in the pediatric IF population with a reported prevalence greater than 90%.[2-4] Anemia has considerable negative long-term consequences including associated impairments in growth, neurocognitive development, immune function and quality of life.[5-8] Children with IF are at high risk for iron deficiency anemia (IDA), anemia of inflammation (AI) and nutritional anemia and there are limited data on the prevalence of the varied types of anemia in pediatric IF as well as a paucity of data on the underlying etiologies of these anemias. This knowledge gap limits our ability to effectively treat and prevent anemia in these children. Thus, we propose a cross-sectional, multicenter study to 1) determine the prevalence of IDA, AI, mixed IDA/AI and nutritional anemia in the children with IF in Western Canada; 2) identify associated factors of anemia in children with IF including hematologic indices, vitamin and micronutrient deficiencies, inflammatory markers, underlying IF etiology, gastrointestinal anatomy, surgical history, liver disease, renal disease, small intestinal bacterial overgrowth (SIBO), degree of parenteral nutrition (PN) dependence and endoscopic findings; and 3) evaluate the diagnostic utility of soluble transferrin receptor and soluble transferrin receptor/log ferritin index in discriminating IDA, AI and mixed IDA/AI in children with IF. This study will provide novel information on the prevalence of the various types of anemia and their underlying causes in children with IF and will be an important first step towards developing a rationalized approach to evaluation and management of anemia in children with IF.

2. Literature Review

2.1. Pediatric Intestinal Failure (IF)

Pediatric IF is a devastating condition characterized by the inability of the gastrointestinal tract to absorb adequate macronutrients and water to sustain normal growth, development and health due to a reduction of functional intestinal mass.[9] The causes of pediatric IF can broadly be defined as disorders of intestinal function, which includes motility disorders (*e.g*. hirschsprung disease, chronic intestinal pseudo-obstruction) and mucosal enteropathies (*e.g*. congenital diarrhea disorders, tufting enteropathy), or disorders of insufficient bowel length. Short bowel syndrome (SBS), defined as bowel length < 25% predicted, is the leading cause of IF in children and is most often secondary to necrotizing enterocolitis, gastroschisis, congenital intestinal atresias or volvulus.[9] A Canadian population-based study in 2004 estimated the incidence of neonatal SBS to be 24.5 per 100 000 live births.[10] The resulting intestinal anatomy and bowel length in SBS predisposes individuals to specific micronutrient and vitamin deficiencies, many of which play key roles in gastrointestinal structure, function and adaptation.[11] Initially, all children with IF are dependent on parenteral nutrition, (PN), nutrition received directly into a vein, for survival. While up to 50% will achieve adequate enteral autonomy and come off of PN, this can take months to years to achieve.[1, 10] Due to advances in care, including multidisciplinary intestinal rehabilitation programs, alternative PN formulations and innovative bowel lengthening procedures, the majority of children with IF are now surviving through childhood and thus, new long-term complications and comorbidities are

being identified.[12] Anemia is a frequent, yet poorly described complication in pediatric IF with considerable implications for long-term outcomes.

2.2. Anemia

Anemia is defined as a reduction in the total number of red blood cells (RBCs) or hemoglobin concentration in the blood.[13] Acute signs and symptoms of anemia can include pallor, fatigue, dizziness, weakness, exercise intolerance and arrhythmias. The causes of anemia are varied and include inherited defects in hemoglobin or RBCs (*e.g.* sickle cell disease, thalassemia), infection, chronic diseases or inflammatory disorders (*e.g.* leukemia, renal disease, inflammatory bowel disease), nutritional deficiencies (*e.g.* iron, vitamin B12, folate), acute or chronic blood loss, or medication side effects which can cause increased RBC destruction (hemolysis) or decreased RBC production (myelosuppression).[14] Accurate classification and diagnosis of the underlying cause of anemia is important for effective management. Categorizing anemia by the mean corpuscular volume (MCV), a measure of RBC size, is a common means used to guide an organized approach to investigations to determine the underlying cause of anemia.[14] Microcytic, normocytic and macrocytic anemias are defined by a low, normal and high MCV respectively. Initial classification and diagnosis of the specific causes of anemia is dependent on the assessment of a number of hematologic indices, as well as additional investigations specific to the underlying etiology, as seen in Figure 1.[14]

The global burden of anemia is large, affecting approximately 1/3 of the world population.[15] However, the estimated prevalence of anemia in Canada is < 5% in school-aged children.[16] Iron deficiency anemia (IDA) is the most common cause of anemia and is characterized by a reduction of total body iron stores, also referred to as absolute iron deficiency.[17] Children are at an increased risk of anemia as their increased growth can rapidly deplete iron stores.[18] Anemia of inflammation is considered the second most prevalent anemia and is due to immune system activation resulting in (i) the inability to use iron stored in the body (functional iron deficiency), (ii) decreased production of RBCs (myelosuppression), and (iii) decreased RBC lifespan.[19, 20] In AI, inflammatory signals stimulate the synthesis of hepcidin, a peptide hormone produced by the liver. Hepcidin inhibits the export of iron from cells into the bloodstream by blocking the iron transporter ferroportin. The trapped iron is not accessible for new RBC production, thus resulting in a functional iron deficiency anemia, as opposed to an absolute iron deficiency, as seen in IDA. Nutritional anemias are another, less common form of anemia due to deficiencies in vitamin B12 and/or folate which result in ineffective red blood cell production, also known as erythropoiesis.[21]

Iron deficiency is the first step in the development of IDA and is defined by low iron stores.[22] The gold standard for detection of iron deficiency is the absence of stainable iron on bone marrow aspirate.[23] However, this is impractical for routine use as it is invasive and expensive. Thus, iron deficiency is usually assessed using laboratory markers in peripheral blood. Serum iron is often assessed as part of a panel of "iron studies" and is typically low in IDA. However, serum iron is not a measure of iron stores as it simply reflects the movement of iron in and out of the plasma pool. Serum ferritin is the primary storage form of iron. It has been shown to be a reliable marker of bone marrow stores and is the most effective test to detect iron deficiency.[24] Ferritin < 12-15 μg/L is confirmatory for iron deficiency, however, a cut-off of ferritin < 30 μg/L is more frequently used as it increases the sensitivity to detect iron deficiency from 25% to 92% with a specificity of 98% .[25]

[26] A major diagnostic limitation of ferritin is that it is also an acute phase reactant and thus, its accuracy in detecting iron deficiency is diminished in the presence of acute or chronic inflammation. Transferrin saturation (TSAT), a measure of the availability of utilizable iron, is another marker of iron status which is readily available. A TSAT < 16% is typically used in screening for iron deficiency, but is reduced to < 20% in the presence of inflammation.[19, 27] A threshold of TSAT< 20% has been demonstrated to correlate with iron deficiency detected in bone marrow in chronic inflammatory conditions, including heart failure and chronic kidney disease.[28-31] TSAT alone has a relatively high sensitivity for detecting iron deficiency (up to 90%), but lower specificity (63% to 84%).[28-31] However, one study in patients with kidney disease demonstrated that the specificity improved to 98% when TSAT <20% was combined with ferritin < 100 μg/L.[32] One limitation of TSAT is that serum iron levels are required for the calculation of TSAT and serum iron levels show diurnal fluctuation and can be influenced by iron in the diet and oral iron supplementation.[33] Thus, current practice for diagnosis of iron deficiency is to use serum ferritin < 30 μg/L in the absence of an inflammatory condition/state and serum ferritin < 100 μg/L or TSAT < 20% in inflammatory conditions. In cases where serum ferritin is 100-300 μg/L, a TSAT < 20% is required to confirm iron deficiency.[19]

Anemia of inflammation usually presents as normocytic anemia in the presence of an underlying inflammatory condition, but it may also present as a microcytic anemia, similar to IDA. In addition to low hemoglobin, both IDA and AI typically have low serum iron and TSAT. Ferritin is a useful measure to distinguish the two, as ferritin is low in IDA and elevated (typically > 100 ug/L) in AI.[20] However, IDA and AI can occur together (mixed IDA and AI), and as a result, ferritin may be low, normal or elevated and MCV may be low or normal in mixed IDA/AI. There is no single test which can be used in isolation to classify anemia as IDA, AI, or mixed, and instead, a pattern of numerous key hematologic and biochemical indices, including hemoglobin, MCV, ferritin, total iron binding capacity (TIBC), TSAT, and CRP or other markers of inflammation, is used for classification (Table 1). [19]

Additional hematologic markers to determine iron status and differentiate between a functional iron deficiency as seen in AI and absolute iron deficiency in IDA have been available for several years. Soluble transferrin receptor (sTfR) is a test which has shown promise in research settings but is not yet widely available as a routine standard of care test. sTfR is a highly sensitive and specific measure of iron deficiency which is not impacted by inflammation or hepatic disease.[34] Soluble transferrin receptor is a truncated form of the transferrin receptor and is present in every cell. The serum concentration of sTfR is proportional to the total number of transferrin receptors expressed on cell membranes, thus reflecting cellular iron requirements (an increase in transferrin receptors indicates increased iron needs).[35, 36] The sTfR concentration increases in IDA and remains normal in AI. Thus, an increase in sTfR in the presence of AI suggests the presence of concomitant iron deficiency (mixed IDA/AI). [37] The soluble transferrin receptor/log ferritin index (sTfR-F index) may further increase diagnostic accuracy as it is based on the relationship between transferrin (an indicator of iron availability for erythropoiesis) and ferritin (a measure of iron stores). It is calculated by dividing the sTfR in mg/L by the log value of ferritin in μg/L. Both sTfR and the sTfR-F index have been demonstrated to effectively discriminate between IDA, AI and mixed IDA /AI in a variety of populations, including rheumatoid arthritis, pediatric inflammatory bowel disease and young children hospitalized or at risk for various chronic infections or inflammatory states.[38-41] The

use of ferritin, sTfR and sTfR-F index more than doubled the detection of iron deficiency from 41% (with ferritin alone) to 92%.[42] The ability to distinguish IDA from mixed IDA/AI and AI using sTfR and sTfR-F index would be particularly advantageous in the pediatric IF population, as it may 1) identify the presence of AI in the absence of elevated markers of systemic inflammation, indicating the need for further investigation into potential causes of AI requiring treatment, such as intestinal inflammation or liver or renal disease, and 2) detect iron deficiency before it progresses to IDA, thus triggering further investigations into the cause of iron deficiency, resulting in earlier treatment. This is important as a recent study indicated that parents of children with IF prefer pre-emptive treatment of iron deficiency as opposed to a reactionary approach to IDA which may result in the need for invasive iv iron therapies or blood transfusions for management.[43]

2.3. Anemia in Pediatric Intestinal Failure

2.3.1. Prevalence and Classification

Anemia is a frequent complication in children with IF with the reported prevalence ranging from 40% in a single-center cross-sectional study to as high as 90 to 97% in three, single-center, retrospective studies. [2-4, 44] The higher prevalence in the retrospective studies reflects the ability to assess multiple blood samples over several years as opposed to a single blood sample in the cross-sectional study. Unfortunately, the primary aim of each of these studies was to assess micronutrient deficiencies, not anemia, and thus, the underlying causes of anemia were not evaluated.

Children with IF are at risk for both IDA and AI. We are aware of only 1 study to date which has evaluated the prevalence of IDA and AI in the IF population.[45] This single center, retrospective study included 54 adult and pediatric IF patients who had been on PN for more than 5 years. Using serum levels of ferritin with TSAT, the prevalence of AI was 36%, IDA 38% and anemia with indeterminate iron status was 21%. They attempted to identify possible causes of AI, specifically renal and liver disease, and found that 23% of patients met criteria for chronic kidney disease (CKD) stage 2 or 3. However, the retrospective study design was a considerable limitation of the study, as the necessary investigations to thoroughly assess the potential causes of AI, such as intestinal failure associated liver disease (IFALD), renal disease and intestinal inflammation, as well as the underlying causes of IDA, were absent, thus highlighting an important gap in the research.

Differentiating between IDA, AI and mixed IDA/AI is critical to ensuring appropriate identification and treatment of the underlying cause of anemia. Iron is a micronutrient which is essential to the survival of nearly all living cells, including microbes.[46] The sequestration of iron seen in AI is believed to be an evolutionary strategy termed “nutritional immunity” to make iron less accessible to circulating pathogens, therefore inhibiting the ability of potential pathogens to survive, replicate and invade host cells.[47, 48] Indeed, conditions associated with iron overload, such as hereditary hemochromatosis and thalassemia are associated with increased risk of infection.[49] Indiscriminate iron supplementation can lead to increased morbidity and mortality, including enteric infections due to bacterial utilization of excess iron, which is of particular concern in the pediatric IF population, as these children are at high risk for enteric infections and sepsis, specifically in the presence of a central venous catheter for PN.[20] Iron overload from aggressive iron supplementation can also contribute to liver fibrosis.[50] On

the other hand, given that iron deficiency in infancy and childhood has been shown to have negative effects on growth, immune function, intestinal adaptation, motor and neurocognitive development, and school achievement, failure to identify and treat concomitant iron deficiency in a child with AI can contribute to long-term morbidity.[5-8] Thus, an improved understanding of the types and underlying causes of anemia in pediatric IF is essential to ensure effective management and improve long-term outcomes.

2.3.2. Potential Etiologies of Iron Deficiency Anemia in Pediatric Intestinal Failure

The potential underlying causes of IDA in children with IF are varied and can be broadly classified as inadequate iron intake, inadequate iron absorption and increased gastrointestinal (GI) losses.

2.3.2.1. Inadequate Iron Intake

Iron is an essential micronutrient obtained through the diet. There are 2 types of iron; heme iron, which is found in animal meat (e.g. beef, chicken, fish) and non-heme iron, which is found in plant products (e.g. spinach, lentils, tofu). Infants and children require 7-15 mg per day of iron from their diet depending on their age and sex.[18, 51] Children who have limited enteral intake due to feed intolerance or malabsorption secondary to IF are dependent upon iron supplementation in order to meet their iron requirements. Iron supplementation can be achieved by oral/enteral administration of iron fortified formulas/cereals or elemental iron supplements or via intravenous iron infusions. In cases of severe iron-deficiency anemia, blood transfusions are required. Adherence to oral/enteral supplementation is frequently low in children due to oral aversion, poor palatability, or gastrointestinal side effects such as abdominal pain and diarrhea and concerns regarding anaphylaxis and incompatibility prevents direct supplementation of iron in PN mixtures in many centres, including those that manage pediatric IF in Western Canada.[43, 52, 53] Thus, achieving adequate iron supplementation can be challenging. Persistent IDA in the presence of adequate supplementation and adherence is an indication for further investigations into the underlying cause, including intestinal inflammation or occult GI bleeding.

2.3.2.2. Inadequate Iron Absorption Secondary to Intestinal Inflammation

Intestinal inflammation inhibits the absorption of Iron, which occurs in the duodenum and proximal jejunum, as well as other related nutritents.[54] Evidence of acute and chronic intestinal inflammation diagnosed by endoscopy/colonoscopy has been reported in several studies in children with IF.[55-58] Endoscopy is not routinely performed without specific clinical indications, such as worsening malabsorption or frank GI bleeding and thus, there is a paucity of information regarding the true prevalence of intestinal inflammation in pediatric IF. A single retrospective study describing surveillance endoscopy found histological intestinal inflammation in 54% of “asymptomatic” children with IF.[57] Unfortunately, the underlying cause for this inflammation was unknown and anemia was not assessed. Known contributors to intestinal inflammation and anemia include small intestinal bacterial overgrowth (SIBO) and eosinophilic/allergy related inflammation.[59-62]

SIBO is characterized by qualitative and quantitative changes in endogenous bacteria in the small bowel and is estimated to occur in 34-75% of pediatric SBS patients.[60, 63] Children

with IF are at increased risk of SIBO due to alterations in intestinal motility and anatomy, including loss of the ileocecal valve (ICV) and small bowel dilatation due to intestinal adaptation, as well as increased exposure to antibiotics and gastric acid suppressing medications.[64] SIBO contributes to IDA through intestinal inflammation and mucosal injury, as evidenced by endoscopic ulcerations and villous blunting on histology, resulting in impaired absorption of micro and macronutrients, including iron. In addition, SIBO may cause a macrocytic anemia secondary to bacterial utilization of vitamin B12.[65] An accurate and non-invasive diagnostic test for SIBO in pediatric IF does not currently exist. Hydrogen breath tests are frequently used to diagnose SIBO, however, their accuracy in children with altered intestinal anatomy and function is not known and thus, in the pediatric IF population, SIBO is a clinical diagnosis based on increased stool outputs, abdominal distention, gas or D-lactic acidosis which are not otherwise explained and which improve with empiric antibiotic treatment.[63, 66] Interestingly, fecal calprotectin (FC), a stool marker specific for intestinal inflammation, was previously found to be more elevated in children with SBS with confirmed SIBO compared to children with SBS without SIBO.[60] The potential role of FC as a non-invasive marker of SIBO or intestinal inflammation in general has not been evaluated in the pediatric IF population and may be useful in identifying the causes of anemia in pediatric IF.

Eosinophilic gastroenteritis is an inflammatory condition which can affect the upper or lower GI tract and is characterized by infiltration of the tissue by eosinophils, a type of white blood cell frequently associated with allergic conditions.[61] Following several case reports of eosinophilic inflammation in IF, a large, retrospective study of children with IF who had undergone endoscopy and/or colonoscopy reported an overall prevalence of eosinophilic intestinal inflammation diagnosed by histology of 37%.[55, 56, 67] While the presence of anemia was not evaluated in this cohort, anemia has been found to be associated with eosinophilic gastroenteritis in otherwise healthy children and thus, may be an important contributor to anemia in pediatric IF.[62]

2.3.2.3. Gastrointestinal Blood Loss

Anastomotic ulceration (AU) is a known cause of anemia in short bowel syndrome.[68, 69] The etiology of AU is unknown, but ischemia, SIBO, excessive acid, bile salt exposure and allergy have been proposed.[68] AU may require surgical intervention or treatment with anti-inflammatory medications such as steroids, sulfasalazine or infliximab.[68, 70] AU is associated with occult GI blood loss and thus, diagnosis of AU may be delayed several years, resulting in prolonged oral or intravenous iron supplementation for IDA while failing to identify the underlying cause.[70] In the course of evaluation and management of chronic anemia in the IF population at Alberta Children's Hospital, we have detected a high incidence of intestinal ulceration; ulcers have been identified in 10 of 32 patients in our current cohort (unpublished data). This is strikingly higher than the reported 30-year AU prevalence of 0.3% in the pediatric IF population in France.[68] Interestingly, ulcers in our patients occur not only at anastomosis sites and staple lines from bowel lengthening procedures, but also at other sites within the GI tract. The occurrence of non-anastomotic ulceration has not been described in detail the pediatric IF literature and thus, characterization of the prevalence of

GI ulcers may help to elucidate their role in anemia and indicate a need for routine endoscopy in evaluation of anemia.

2.3.3. Potential Etiologies of Anemia of Inflammation in Pediatric Intestinal Failure

With only 1 study to date examining the prevalence of AI in pediatric IF, the underlying causes remain largely unknown.[45] Potential contributors include chronic intestinal inflammation, as described above, renal disease and liver disease. Anemia is a well described complication of chronic kidney disease (CKD) with multiple etiologies, including inflammation and a decrease in erythropoietin (EPO), a renal hormone which stimulates erythropoiesis.[71] Glomerular filtration rate (GFR) is a measure of renal function and is used in diagnosis and disease staging, with disease progression defined by decreasing GFR. There is growing evidence of renal dysfunction and development of CKD in children with IF. [71-73] Studies in both adults and children on long-term PN have demonstrated a gradual decline in GFR and abnormalities on renal ultrasound, including increased echogenicity and nephrocalcinosis. Increased echogenicity is a finding which can be due to inflammation, tubular disorders or fibrosis, while nephrocalcinosis is caused by precipitation of minerals within the renal parenchyma. The causes of CKD in long-term PN remains largely unexplained.[74] However, recurrent episodes of sepsis, dehydration, electrolyte imbalances, nephrotoxic medications and impaired intestinal absorption are proposed mechanisms which exist in IF patients.[73] A retrospective study of 54 pediatric IF patients found sonographic renal abnormalities in 41% of patients while another study reported a decrease in GFR in 29%.[71, 72] In both studies, increased PN duration and shorter remaining bowel length were associated with impaired renal function. Anemia was not assessed in these studies, however, Namjoshi *et al* (2020) found evidence of CKD in 23% of their patient cohort, which suggests that CKD may be an important contributor to AI in this population.

Intestinal failure associated liver disease (IFALD) is a major source of morbidity and mortality in pediatric IF. IFALD is characterized by a persistent elevation in bilirubin and eventual progression to cirrhosis and liver failure.[75] The role of chronic liver disease in AI is poorly understood, however, Namjoshi *et al* (2020) did find an association between elevated bilirubin and AI, suggesting a potential role for IFALD in AI in children with IF. This association requires further investigation, including correlation with ultrasound findings.

2.3.4. Nutritional Anemia and the Role of Micronutrient and Vitamin Deficiencies in Anemia in Pediatric Intestinal Failure

Nutrients are absorbed differentially throughout the GI tract and thus, the resultant length and anatomy of the GIT following intestinal resection in SBS will predispose to specific micronutrient and vitamin deficiencies.[76] For example, children with resection of the duodenum and proximal jejunum are at risk for iron deficiency while resection of the ileum is a risk for B12 deficiency and fat soluble vitamin deficiency (vitamins A, D, E and K) secondary to bile malabsorption.[12] Micronutrients and vitamins play a critical role in GI structure and function and deficiencies may inhibit the intestinal adaptation necessary to achieve enteral autonomy.[11] Several small cross-sectional studies ($n \leq 10$) and larger single center, retrospective studies ranging in size from 30 to 178 study participants, have demonstrated a high prevalence of micronutrient and vitamin deficiencies in children with IF on PN and after transition to full enteral nutrition.[2, 4, 77-80] Using

regression analysis, Ubesie et al, (2013) and Namjoshi et al, (2018) found that longer PN duration was associated with micronutrient deficiencies, while Yang et al (2011) found the presence of the ileocecal valve (ICV) was protective for micronutrient deficiencies. The prevalence of deficiencies was particularly high for zinc (20-52%), copper (29-56%) and vitamin D (20-38%).[2, 4, 78] These vitamins and micronutrients are of particular relevance in anemia as zinc and copper have roles in iron metabolism and hemoglobin synthesis and have been demonstrated to perpetuate IDA, while there is growing evidence of a relationship between vitamin D deficiency and AI .[4, 81-83]

2.3.4.1. Role of Zinc in Anemia

Zinc is an essential micronutrient which serves as a cofactor in several enzymes and metabolic processes including iron metabolism and erythropoiesis and thus, plays as role in iron-deficiency and anemia.[84, 85] Zinc is absorbed mainly in the duodenum and small intestine and has been demonstrated to be a strong predictor of hemoglobin concentration in healthy school age children and adults.[86, 87] There is no storage form of zinc in the human body and thus, zinc status is determined by daily oral intake (chicken, meat, lentils, nuts) and supplementation of PN.[88] Zinc may also contribute to AI as deficiency has been demonstrated to induce pro-inflammatory cytokines and aggravate mouse models of colitis and has also been demonstrated to correlate with increased serum levels of pro-inflammatory markers including IL-6, IL-8, TNF-α, CRP and fecal calprotectin in patients with rheumatoid arthritis and inflammatory bowel disease.[89-92] Plasma zinc is a reliable means of assessing zinc status, however, albumin is the main carrier protein of zinc and zinc is also a negative acute phase reactant and thus, zinc deficiency must be interpreted with caution in the presence of hypoalbuminemia and inflammation .[93]

2.3.4.2. Role of copper in anemia

Similar to zinc, copper serves as a cofactor for a variety of important enzymes and metabolic processes including hemoglobin synthesis and iron oxidation.[94] Deficiency can present with anemia and neutropenia.[95] Low levels of copper can result from inadequate dietary intake or PN supplementation, inadequate absorption in the GI tract which occurs mainly in the stomach and proximal duodenum but also in the jejunum, or increase losses due to malabsorption and diarrhea.[96] Copper shares some common transporters with both zinc and iron and thus, these micronutrients may compete for absorption.[97] Excess zinc supplementation can cause a copper deficiency anemia which may be microcytic, normocytic or macrocytic.[95] Copper deficiency can be detected by serum copper levels and copper increases in response to inflammation. Ceruloplasmin, a major copper-carrying protein in the blood which also plays a role in iron metabolism will be low in serum only in the presence of severe copper deficiency.[98]

2.3.4.3. Role of Vitamin D in Anemia

An association between Vitamin D deficiency and AI has been described in various populations including children with chronic kidney disease (CKD) and inflammatory bowel disease (IBD).[99, 100] *In vitro* studies have demonstrated the role of vitamin D in decreasing hepcidin induced pro-inflammatory cytokines levels via downregulation of hepcidin expression, and vitamin D supplementation has resulted in decreased serum hepcidin levels and decreased need for erythropoietin stimulating agents in patients with CKD.[101-105]

2.3.4.4. Role of Vitamin B12 and Folate in Anemia

Vitamin B12 and folate both play critical roles in DNA and RNA synthesis and deficiencies in either of these vitamins results in ineffective erythropoiesis characterized by production of fewer, but larger abnormal red blood cells and premature cell death.[21] Nutritional anemias due to B12 and/or folate deficiency are macrocytic and are associated with anisocytosis, poikilocytosis and hypersegmented neutrophils on blood smear.[106] Vitamin B12 is found in animal products (meat, eggs, dairy) while folate can be obtained from ingestion of leafy greens and fortified cereals.[107] It requires adequate gastric acid to release it from dietary proteins and it is absorbed in the terminal ileum. Children with IF are at risk for vitamin B12 deficiency if they have short bowel involving surgical resection of >15 cm of the ileum, chronic gastric acid suppression (*e.g.* proton-pump inhibitor therapy), or SIBO, as enteric bacteria compete with the host bowel for vitamin B12.[108, 109] The risk of folate deficiency is considered low in children with SBS and those at risk for SIBO, as folate is synthesized by enteric flora.[110] Indeed, several retrospective and small case studies have assessed vitamin B12 and folate status in children with IF and folate deficiency was not present in any of the children, while vitamin B12 was found to be deficient in 6.9% to 22% of patients.[2, 78, 80] Vitamin B12 and folate status are evaluated via serum levels, however, deficiencies in associated vitamin B12 binding proteins can result in normal B12 levels in individuals with functional vitamin B12 deficiencies.[111, 112] Therefore, simultaneous assessment of vitamin B12 and methylmalonic acid (MMA), a metabolic product which requires vitamin B12 for metabolism, is recommended.[113] Levels of MMA are elevated in >98% of patients with a clinical B12 deficiency and is considered to be a highly sensitive and specific test diagnosing B12 deficiency and levels of MMA decrease with B12 supplementation.[106, 113-115]

Patterns of micronutrient and vitamin deficiencies may help to identify the underlying causes of anemia. For example, vitamin B12 deficiency with elevated methylmalonic acid (MMA) and normal folate is observed in SIBO, while deficiencies in fat soluble vitamins (A, D and E) and elevated bilirubin are indications of liver disease.[116, 117] Thus, evaluating the associations of micronutrient and vitamin deficiencies with AI and IDA may identify predictors useful in diagnosis and monitoring of anemia in pediatric IF.

Many micronutrients and vitamins are acute phase reactants and should be interpreted cautiously in the presence of a systemic inflammatory response. A systematic review evaluating the magnitude of changes of serum vitamin and micronutrient levels in the presence of a systemic inflammatory response, as measured using CRP, found that the levels of zinc, copper and vitamins A, D, E and B12 were within the normal reference range in the presence of mild inflammation (CRP < 10 mg/L).[93] In the presence of major inflammation (CRP >80 mg/L) the concentrations of vitamin B12, vitamin E and copper changed by approximately 10%, while the concentrations of zinc and vitamins A and D decreased by 25 to 55%. Correction of plasma micronutrients using CRP and albumin have been examined for zinc, vitamin A and vitamin D and may be a means to more accurately assess micronutrient/vitamin status in patients with chronic inflammation.[118, 119]

2.3.5. Medications and Additional Causes of Anemia

Medications are an important cause of anemia in all children and should not be overlooked in children with IF. Medications can contribute to anemia through various mechanisms, including hemolysis (*e.g.* septra), myelosuppression (*e.g.* 5-ASA, anticonvulsants, immunomodulators) or inhibition of absorption of vitamins and micronutrients (*e.g.* proton-pump-inhibitors, cholestyramine).[120] Some of these classes of medications are used frequently in children with IF to manage IF specific complications, such as the use of proton-pump-inhibitors to manage gastric acid hypersecretion or broad-spectrum antibiotics for SIBO, while other classes of medications may be used to manage other comorbidities, such as anticonvulsants for epilepsy. Therefore, a detailed inventory of patient medications is necessary when evaluating causes of anemia. Similarly, it is also important to asses for additional causes of anemia not specific to pediatric IF, such as thalassemia, hemoglobinopathies or thyroid disorders.[121] These disorders are generally easily identified through additional blood tests once anemia has been diagnosed, as indicated in the approach to anemia algorithm in Figure 1.[14]

3. Research Aims and Objectives

3.1. Aim: To describe the contributions of the various causes of anemia in the pediatric intestinal failure population in Western Canada and identify predictors associated with these causes.

3.2. Objectives

Primary Objective: To determine the prevalence of iron deficiency anemia, anemia of inflammation, mixed iron deficiency anemia/anemia of inflammation and nutritional anemia in children with intestinal failure in Western Canada.

Secondary Objective: To identify factors associated with iron deficiency anemia, anemia of inflammation and nutritional anemia in the pediatric IF population, including hematologic indices, micronutrient deficiencies, inflammatory markers, underlying IF etiology, gastrointestinal anatomy, surgical history, liver disease, renal disease, small intestinal bacterial overgrowth, degree of PN dependence and endoscopic and histologic findings.

Tertiary Objective: To examine the diagnostic utility of soluble transferrin receptor and the soluble transferrin receptor/log ferritin index in discriminating iron deficiency anemia, anemia of inflammation and mixed iron deficiency anemia/anemia of inflammation in children with intestinal failure.

4. Methods

4.1. Study Design

This is a multicenter, cross-sectional study with retrospective chart review of pediatric IF patients managed by the Western Canadian Children's Intestinal Rehabilitation Program Network (CHIRP-Net), during the enrolment period (November 1, 2021 – August 1, 2022). The Western Canadian CHIRP-Net includes pediatric patients with IF who receive care and are followed by intestinal rehabilitation teams at Alberta Children's Hospital (Calgary, AB), BC Children's Hospital, (Vancouver, BC), Stollery Children's Hospital (Edmonton, AB) and Children's Hospital of Winnipeg (Winnipeg, MB). Together, these hospitals include a catchment area covering British Columbia, Alberta, Saskatchewan, Manitoba, Northwest Ontario, Nunavut and Yukon and the Northwest Territories. Following approval at the University of Calgary, this protocol will be submitted for ethics approval at the Universities of Alberta, British Columbia, and Manitoba to include patients

managed at those sites. The pediatric IF population is a small and heterogeneous population with ≤50 patients at each site in Western Canada and thus, a multi-center design is necessary to increase sample size and overall study power. A cross-sectional study design will ensure all relevant investigations are performed, mitigating the risk of missing data in retrospective studies, which are frequently used in this patient population. The retrospective chart review will encompass a 10-year time span dating back from the time of enrolment.

The retrospective chart review component of this study will also include children with IF managed by the Group for the Improvement of Intestinal Function and Treatment (GIFT) at the Hospital for Sick Children (Toronto, Ontario). The GIFT program at Toronto Sick Kids Hospital services a much larger population than the programs in Western Canada and routinely performs most of the investigations proposed. Thus, for purposes of study feasibility, available retrospective data on an estimated 150 patients managed by the GIFT program will be collected and utilized for analysis, as opposed to cross-sectional data collection.

4.1.1. Assumptions

We anticipate high participation rate (>95%), based on this study design and previous multi-site studies performed by the Western Canadian CHIRP-Net (unpublished), resulting in high external validity. Laboratories used in each province are accredited and monitored by quality assurance programs, ensuring high internal validity in test results. All children with IF require care by intestinal rehabilitation teams at tertiary centers for survival. Thus, while the catchment area for the centers involved in this study is large, the population is well known to these teams, and we can assume that we will be able to reliably capture the entire pediatric IF population in Western Canada.

4.2. Population

4.2.1. *Inclusion and Exclusion Criteria*

Inclusion Criteria: All children > 6 months and < 18 years of age with IF, defined as the need for PN > 60 days due to intestinal disease or dysfunction, managed by the Western Canadian CHIRP-Net during the surveillance period are eligible.[9] Participants do not need to be on PN at time of evaluation, but had to have been on PN at some point within the 2 years prior to the time of enrolment. Written informed consent and/or assent where appropriate is required for all participants given that we will be investigating 1 lab test (sTfR) which is not considered routine standard of care.

Exclusion Criteria: Children with liver or intestinal transplants will be excluded, as the potential risk factors and causes of anemia are different in children with organ transplants and using transplant related immune-suppressing medications.

4.2.2. *Recruitment*

Patients eligible for participation will be identified by the local CHIRP physician and co-investigator who will directly obtain informed consent/assent or obtain permission for the research coordinator to contact the patient/family to obtain informed consent/assent remotely or in person. As per current research ethics board guidelines, assent will be obtained from the child and consent from the parent for children < 14 years, or those ≥14 years without decision

making capacity. The primary IF physician will assess decision making capacity in patients ≥14 years; if deemed to have capacity, consent will be obtained from the child instead of the parent. Enrollment will occur over 1 year (Sept 2021-August 2022; dates pending ethics approval and program timeline) and identification of eligible participants and status of recent investigations will ideally be determined prior to a patient's routine medical visit to ensure completeness of investigations ordered/performed at each visit.

4.2.3. *Sample Size and Study Power*

There are currently an estimated 110 pediatric IF patients in the in Western Canadian CHIRP-Net catchment area, 5 of whom have had liver/intestinal transplant. Assuming 95% participation, we anticipate a study size of 100 participants, allowing us to capture nearly the entire eligible pediatric IF population in Western Canada. Assuming a conservative prevalence of anemia of 50%, and a sample size of 100, a 95% confidence interval around the proportion would result in a confidence interval with a standard error of less than 10%.

Calculations:

Confidence Interval (CI) = $\hat{p} \pm z \sqrt{\frac{\hat{p}\,(1-\hat{p}\,)}{n}}$

= $0.50 \pm 1.96 \sqrt{\frac{0.50\,(1-0.50)}{100}}$ = 0.50 ± 0.098

Based on an estimated prevalence of anemia of 50% with a sample size of 100, the 95% confidence interval = 40.2-59.8%.

A composite prevalence of anemia based on chart review of participants in Western Canada (n=100) along with retrospective data from 150 pediatric IF patients followed by the IF team at Toronto Sick Kids Hospital will also be determined. Assuming a conservative prevalence of anemia of 50%, with a sample size of 250, the 95% confidence interval around the proportion would result in a confidence interval with a standard error of less than 7%.

Calculations:

Confidence Interval (CI) = $\hat{p} \pm z \sqrt{\frac{\hat{p}\,(1-\hat{p}\,)}{n}}$

= $0.50 \pm 1.96 \sqrt{\frac{0.50\,(1-0.50)}{250}}$ = 0.50 ± 0.062

95% confidence interval = 43.8-56.2%.

4.3. Definitions

<u>Outcome Variables</u>:

Anemia: Serum hemoglobin level ≥ 2 standard deviations below normal for age and sex (Table 2).

Iron Deficiency Anemia (IDA): Ferritin < 30 μg/L **or** TSAT < 16% in the presence of microcytic anemia (Table 3-A).

*** IDA classification using soluble transferrin receptor (sTfR):** Ferritin < 30 μg/L **and** either sTfR > normal for age (>1.6 mg/L for 1-12 years, >1.5 mg/L for 12-19 years) **or** sTfR-F index > 1.5 in the presence of microcytic anemia (Table 3-B).

****Note:** A sub-analysis using sTfR values will be performed to assess its utility in distinguishing IDA vs AI vs mixed IDA/AI.

Anemia of Inflammation (AI): Normocytic anemia with ferritin > 100 μg /L and TSAT <20% in the presence of evidence of inflammation (Table 3-A). Evidence of inflammation includes elevated serum CRP, ESR, fecal calprotectin or evidence of intestinal inflammation on histology. Normal reference values are reported in Table 2.
***Anemia of Inflammation classification using soluble transferrin receptor (sTfR):** Ferritin > 100μg/L **and** sTfR normal for age (1-11 years: 0.8-1.6 mg/L, 12-19 years: 0.7-1.5 mg/L) **and** sTfR-F index < 1.0 (Table 3-B).

Mixed IDA and AI: Normocytic or microcytic anemia with ferritin >30 μg /L and TSAT < 20% in the presence of evidence of inflammation (elevated serum CRP, ESR, fecal calprotectin or evidence of intestinal inflammation on histology) (Table 3-A).
***Mixed IDA and AI classification using soluble transferrin receptor (sTfR):** Ferritin > 30 μg /L **and** sTfR > normal for age **and** sTfR-F index > 2.0 (Table 3-B).

Nutritional anemia: Macrocytic anemia in the presence of folate deficiency (folate < 12.2 nmol/L) or vitamin B12 deficiency (B12 < 155 pmol/L or MMA > 0.4 μmol/L).[122]

Exposure Variables:
Iron Deficiency: Serum ferritin < 30 μg /L or TSAT < 16%. In presence of inflammation (systemic or intestinal – described below), serum ferritin < 100 μg /L or serum ferritin >100 μg /L and TSAT < 20%.[26]

Vitamin/Micronutrient Deficiency: Defined by serum micronutrient level below the lower limit of normal specific to that micronutrient. Normal reference values for vitamins A, D and E, copper, ceruloplasmin, zinc, folate, vitamin B12 and MMA are found in Table 2. Levels of Vitamins A, B12, D and E as well as copper and zinc may be falsely elevated or depressed in the presence of CRP > 10 mg/L and thus, will need to be repeated when CRP is < 10 to be included in the analysis.[93]

Small Intestinal Bacterial Overgrowth (SIBO): a diagnosis of SIBO can be made based on clinical symptoms, including an increase in the following above baseline that is not otherwise explained and responds to antibiotic therapy: increased abdominal distention, flatulence, increased stool/ostomy outputs, watery stools, foul-smelling stools, or d-lactic acidosis. A positive hydrogen breath test (increase in hydrogen $\geq$ 20 ppm above baseline within 90 mins of ingestion of 75g glucose or 10g lactulose) or positive small bowel aspirate and culture (> 10^3 CFU/mL of bacteria) are also diagnostic of SIBO, if these procedures have been performed by the primary physician.[123]

Intestinal Inflammation: Fecal calprotectin > 150 ug/g or histologic evidence of active or chronic inflammation on intestinal biopsy, as reported by the pathologist.[124]

Systemic Inflammation: Investigations supportive of the presence of systemic inflammation included elevated CRP, ESR, WBC or platelets above normal reference range for age (Table 2) which is not otherwise explained by acute injury, infection, hematologic disorder or medication side effect.

Chronic Kidney Disease: GFR < 60 mL/min/1.73m^2 or persistent proteinuria $\geq$ 3 months. GFR will be estimated using bedside Schwartz equation; GFR (mL/min/1.73 m²) = (0.41 × Height in cm) / Creatinine in mg/dL.[125] Staging of CKD is per the Kidney Disease: Improving Global Outcomes (KIDGO) organization guidelines (Table 4).[126]

Intestinal Failure Associated Liver Disease (IFALD): Persistent elevation of direct bilirubin > 34 μmol/L (2 mg/dL) for minimum of 2-4 weeks in the context of intestinal failure, or evidence of cirrhosis/fibrosis, inflammation or portal hypertension (as determined by liver doppler and/or low platelets +/- low albumin and splenomegaly) or chronic cholestasis on ultrasound, liver elastography or liver biopsy.[75, 127]

4.4. Data collection

Primary CHIRP physicians and nurses at each study site will identify eligible participants and obtain consent/assent for participation in the study. Data collection will involve a combination of new investigations and chart review. An electronic data abstraction tool (excel spreadsheet) will be used by data abstractors at each research site. Data collected from chart review will include (i) patient demographics and characteristics, such as age, sex, primary etiology of IF and intestinal anatomy and length, (ii) nutritional intake (current and at time of lowest hemoglobin), including oral/enteral intake [mode (oral, nasogastric, g-tube), dietary restrictions (vegan, lacto-ovo-vegetarian, exclusive formula), percent caloric intake via oral/enteral nutrition], PN (hours per week, volume, calories, supplementation with copper, zinc and iron) and oral vitamin/micronutrient supplementation (vitamin D, vitamin B12, folate, iron, zinc), (iii) treatment of iron deficiency and/or anemia during the study period (oral or iv iron, blood transfusion), (iv) previous diagnoses/history of intestinal ulceration, liver disease (diagnosed via liver enzymes, liver imaging (elastography) or liver biopsy) or renal disease (diagnosed via GFR, imaging or biopsy) (v) history of small intestinal bacterial overgrowth and management, (vi) history of histologic inflammation or endoscopic abnormalities (ulcers, erosions, edema, erythema) on endoscopy/colonoscopy, (vii) medications, (viii) clinical data/features at time of enrolment and at lowest hemoglobin during study period including height and weight, as well as signs of GI bleeding, malabsorption/SIBO and liver disease (hepatomegaly, splenomegaly).

Chart review will be performed mainly by the primary student investigator, who has conducted previous chart reviews with this same population and is familiar with site specific records. The ability to obtain remote access to electronic charts has been confirmed for patients followed at BC Children's Hospital (Vancouver, BC) and Stollery Children's Hospital (Edmonton, AB) and is under review for Children's Hospital of Winnipeg (Winnipeg, MB). Remote access is not available to obtain the retrospective data on patients followed at Toronto Sick Kids. Funding for the student investigator to travel to Toronto to collect the data on a research elective is being investigated, as is the possibility of identifying a student researcher to serve as a data abstractor. A training session via telehealth at all sites prior to initiation of the study will be held to discuss recruitment, investigations and documentation of clinical data to ensure reliability and validity of data documentation and abstraction. The chart review will extend back 10 years from date of enrolment.

Investigations to be collected at time of enrolment are summarized in Table 5. Serum studies include: (i) hematologic indices, (ii) micronutrient and vitamin levels, (iii) inflammatory markers (serum and stool – fecal calprotectin) and (iv) markers of liver and renal disease (serum and urine) along with renal/abdominal ultrasound with liver elastography. All investigations are routine standard of care in pediatric IF patients, except for sTfR. Serum studies are often spread out over several months to avoid large volume blood draws in young children. sTfR will be performed at Toronto Sick Kids Hospital and will require 2.5 mL of blood. These samples will be frozen and stored locally to shipped in a batch from each site to limit shipping costs. Vitamin and micronutrient levels performed within 3 months and ultrasounds performed within 6 months of enrolment do not need to be repeated unless there is new or worsening anemia, SIBO or GI bleeding. An investigation checklist will be used to ensure no investigations are missed. Additional investigations to rule out other causes of anemia not specific to IF, such as inherited hemoglobinopathies or thyroid disease, will be performed by the IF physician at each site if indicated following the algorithm depicted in Figure 1. Pediatric Gastroenterologists are well versed in the use of this algorithm and the judicious use of investigations for anemia.

Evaluation of anemia status will not be assessed using labs obtained within 4 weeks of a major surgery, acute infection or illness, including neonatal or pediatric intensive care (NICU/PICU) stay; investigations collected outside the time of these conditions will be used for analysis. Similarly, vitamin and micronutrient levels collected in the presence of evidence of acute systemic inflammation with CRP > 10 mg/L will be re-evaluated at a time when inflammation is not present in order to ensure accurate interpretation. Endoscopy/colonoscopy will not be a required investigation, as it is currently not standard of care in all IF patients and is resource intensive, requiring operating room time, an anesthetist, procedural nurses and pathologists for analysis of biopsies. Furthermore, due to the impact of the COVID-19 pandemic on healthcare resources over the last year, operating room time is limited to clinically indicated cases. Endoscopy/colonoscopy and histology available on retrospective chart review will be included in the chart review component of the study.

4.5. Confidentiality

Each patient will be assigned a unique subject code for use on the data collection tool; there will be no personal identifying information on the data collection sheets. A spreadsheet with the unique subject code and the patients' personal identifying information (name, date of birth, personal health number) will be kept separate from the data collection sheets and stored on a password-protected computer at each research site. Paper consent forms will be stored in a locked cabinet in a locked office. The electronic data collection tool will be stored on a secure, electronic shared drive (Onedrive).

4.6. Statistical analysis

All descriptive statistics (e.g. age, etiology of IF, bowel length, PN dependence) will be described as means with standard deviations if the data is normally distributed, or medians with interquartile ranges if the data is not normally distributed. The prevalence of anemia, its subtypes (IDA, AI, mixed IDA/AI or nutritional), as well as iron and micronutrient deficiencies will be reported as proportions, as will other categorical variables (e.g. presence/absence of intestinal ulcers, SIBO,

supplements). Possible relationships between the presence of anemia and associated micronutrient deficiencies and disease related data will be analyzed using Fisher's exact test and the Chi square test of independence where appropriate for categorical data and reported as odds ratios. Fisher's will be used preferentially, particularly in instances where expected frequencies in cells are <5, as the Chi square test is not reliable in these conditions.[128] Given that some subjects will be on treatment for anemia (e.g. current or recent oral iron, folate or B12 supplementation, iv iron or red blood cell transfusions) and may not meet diagnostic criteria for anemia at time of evaluation, a composite measure of anemia prevalence will be determined to include these subjects found with a history of anemia on chart review, which will extend back 10 years from date of inclusion in the study.

There are 5 categorical outcome variables: 1) no anemia, 2) iron deficiency anemia (IDA), 3) anemia of inflammation (AI), 4) mixed IDA/AI and 5) nutritional anemia (Table 3-C). Multinomial logistic regression will be used which allows for more than 2 categories of the outcome variable. Univariate to multivariable regression modelling will be performed to identify potential predictors for anemia and its subtypes (IDA, AI, mixed IDA/AI, nutritional), with the strength of association and level of uncertainty expressed as odds ratios with 95% confidence intervals; the coefficients for all univariable models will be reported. Decision to include exposure variables in the multivariable model for each type of anemia (outcome variable) will require a p-value of 0.20 or less in the bivariable model and an alpha <0.05 will be considered statistically significant. Given the large number of variables being assessed, forward stepwise regression modelling will be used, which will aid in selecting variables which provide the best model fit for each anemia subtype. Comparisons between models will be carried out, with the model for the "no anemia" group serving as a control to compare the other models (IDA, AI, mixed IDA/AI, nutritional) against separately. Additional comparisons between the models for IDA and AI will also be performed to identify key predictors distinguishing these models/outcomes. Model comparisons will be assessed using the Area Under the Curve (AUC)

Based on previous studies, we anticipate that deficiencies in zinc and iron, % PN dependence and intestinal ulcers will be the strongest predictors for iron deficiency anemia, while IFALD and renal disease will be predictors of AI and mixed IDA/AI.[4, 45]

Given that endoscopy/colonoscopy results will not be available for all study subjects, a sub-analysis will be performed on subjects with this data available. Given that the main indication for endoscopy/colonoscopy in this patient population is occult GI bleeding and/or persistent IDA, we predict that this data will be heavily skewed to those with IDA. However, we also anticipate finding evidence of intestinal inflammation, which may correlate with AI or mixed IDA/AI as well.

A sub-analysis to assess the utility of using the sTfR and sTfR-F index in distinguishing IDA, AI and mixed IDA/AI will be performed. Correlations between sTfR and hematologic parameters used to classify anemia will first be examined by scatter plots to identify and evaluate the shape and direction of any relationships and Spearman's rank correlation coefficient will be calculated if a monotonic relationship is present. The Kruskal-Wallis test will be used to compare differences between these types/groups of anemia. Receiver Operating Characteristic (ROC) curves will be used to evaluate a series of cutoffs and the Area Under the Curve (AUC) and 95% confidence intervals will be determined to evaluate the sensitivity and specificity of sTfR and the sTfR-F index.

All statistical analysis will be performed using STATA® version 16.1. (STATACorp LLC). Variable descriptions and analyses are summarized in Table 6.

5. Strengths and Limitations

Small sample size and a heterogeneous patient population are frequent limitations in research in pediatric IF. The multicenter, cross-sectional design is a key strength of this study; a larger population (Western Canada and Toronto Sick Kids vs single site) will result in increased precision of confidence intervals and study power. A cross-sectional study enables us to ensure collection of data on all variables of interest and limits the risk of missing data seen with retrospective studies, while remaining feasible in comparison to the higher costs/time associated with prospective studies. All bloodwork is routine and performed yearly at the very minimum with some investigations performed as frequent as every 3 months depending on the patient/clinical scenario. Any missed labs will be performed with next routine bloodwork to avoid additional pokes. The costs of all investigations, apart from soluble transferrin receptor (nation-wide) and fecal calprotectin (in British Columbia) are covered by provincial health care as they part of routine care ordered by physicians for patients with IF. Additional funding has been obtained through the Canadian Association of Gastroenterology to enable testing of fecal calprotectin and soluble transferrin receptor on all patients in Western Canada. Endoscopy/colonoscopy is not currently performed in all IF patients without indication (e.g. anemia, bleeding). Due to the burden of an anesthetic required to perform this, as well as limited OR time due to the COVID-19 pandemic, this investigation will not be performed unless clinically indicated or combined with another procedure requiring anesthesia; endoscopy/colonoscopy will otherwise only be assessed on chart review and thus, this data will not be available for all participants. The large number of variables being assessed is a risk for model overfitting and type 1 error. Univariate analysis, as well as previous studies, will help to mitigate this by identifying key variables to include in the final model. The small population size is a risk for type 2 error, which is inherent in all studies with this population. However, the sample size in this study is larger than similar studies in this patient population in the literature.[44, 45] A Biostatistician will be consulted to aid modeling and ensure adequate power. Finally, while a cross-sectional design prevents the ability to prove causation, identification of associations will result in hypothesis generation for future studies.

6. Significance, Impact and Knowledge Translation

With the majority of children with IF now surviving through childhood, clinical care and research focused on optimizing development and long-term outcomes is increasingly important. This study will provide novel information on the prevalence and predictors of the various causes of IDA and AI in the pediatric IF population and is an important first step in the development of evidence-based guidelines for screening, diagnosis, and management of anemia in this complex patient population. In addition, this study can serve as the baseline for a longitudinal study to follow the evolution, management, and outcomes for this patient cohort, which will provide valuable information regarding the natural history of pediatric IF.

The pediatric IF community is small and clinicians and researchers rely on shared experiences to guide management. Dissemination of our study findings through publications in key surgical, gastrointestinal and nutritional journals (e.g. Journal of Pediatric Gastroenterology and Nutrition,

Journal of Parenteral and Enteral Nutrition), and at the two major IF conferences held biannually (Pediatric Intestinal Failure and Rehabilitation Symposium (PIFRS) and the Congress of the Intestinal Rehabilitation and Transplant Association (CIRTA)). These forums may lead to changes in the clinical, surgical, and nutritional management provided by IF multi-disciplinary care teams worldwide. While the physicians, surgeons, dietitians, and social workers who make up the intestinal rehabilitation teams are stakeholders in this research, it is ultimately the children and their families who will benefit from improved outcomes and quality life as a result of this study. An initiative is currently underway with several patient partners to establish an internet-based information resource for our IF families, and this will be a key tool for sharing our findings with our patients and informing them about anemia.

7. Budget

The budget can be found in Table 7. All proposed investigations are considered routine in this patient population and associated costs fall under the participants' provincial health care plans. Fecal calprotectin testing for children with IF is covered by provincial health plans in Alberta and Manitoba. Thus, expenses for fecal calprotectin testing in participants in British Columbia will be covered by funding received for this study. The soluble transferrin receptor test is not currently available in provincial labs in Western Canada and thus will need to be shipped to and performed at the laboratory at Toronto Sick Kids Hospital. These expenses are described in Table 7. The majority of data collection will be performed by the student investigator through remote access to electronic medical records at the participating sites. In the event that remote data access is not available, data collection will be performed by the co-investigator and/or a student at that site through in-kind hours.

8. Timeline

Study enrolment at each site will begin following ethics approval at that site (currently anticipated in November 2021 for UofC) and will occur over a 9-month period. However, given delays in obtaining ethics approval at all sites, we will attempt to expedite enrolment of all subjects by June 1, 2022. Identification of eligible participants prior to their routine visits will enable timely and efficient data collection. The surveillance period for the cross-sectional data will include investigations performed up to 6 months prior to enrolment, with retrospective chart data including data dating back to January 1, 2019. Data collection will extend to August 2022. Patients are seen in clinic a minimum of once per year and as often as every 2 weeks, allowing ample opportunity to complete all investigations. While this study is limited by the 2-year requirement of a master's program, the frequency of clinic visits and combined use of cross-sectional and retrospective data will help to complete nearly all the data collection in under 8 months, allowing for sufficient time (2 months) to complete the final data analysis.

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10. Appendices

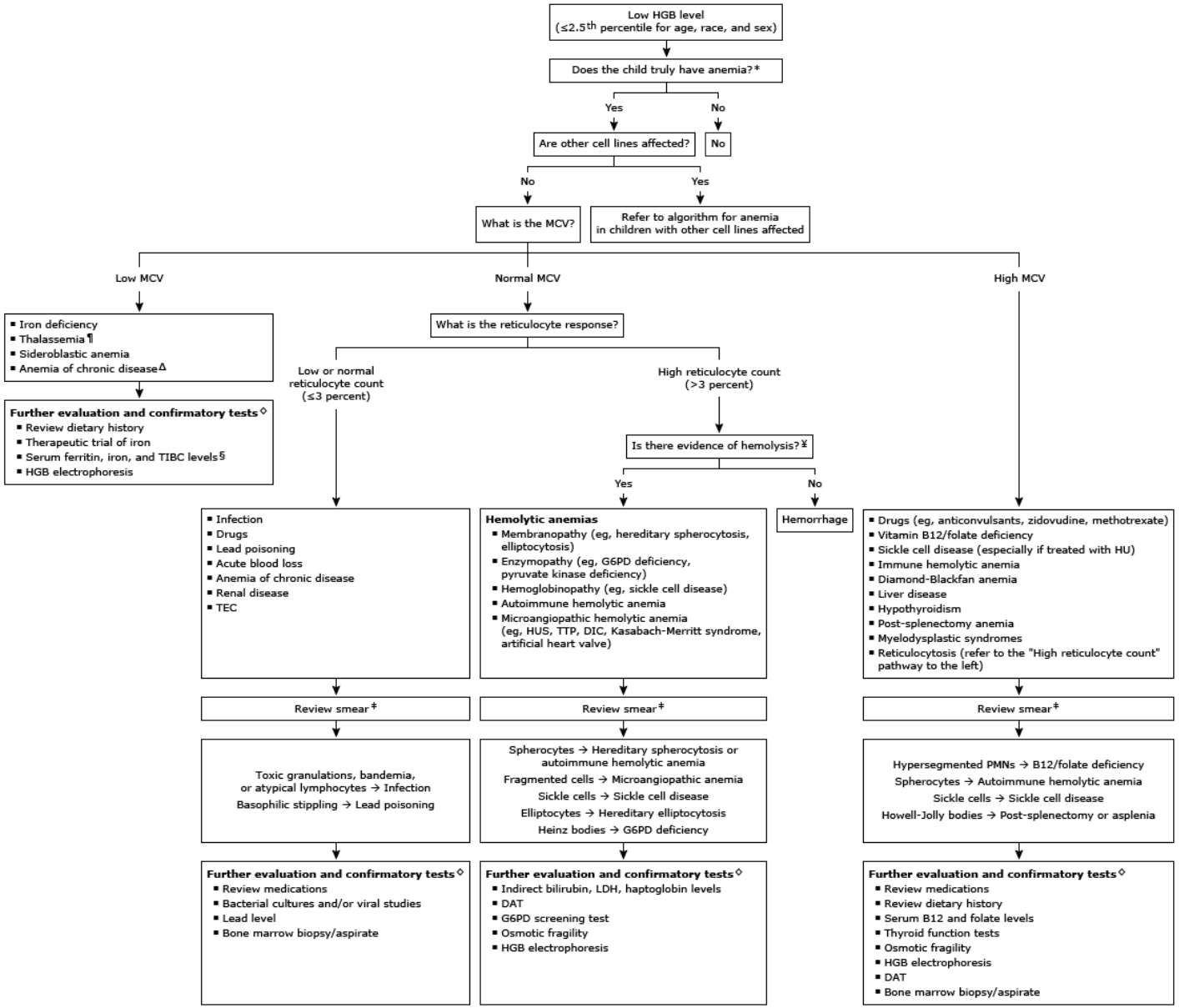
Low HGB level
(≤2.5th percentile for age, race, and sex)
Does the child truly have anemia?*
Yes
No
Are other cell lines affected?
No
No
Yes
What is the MCV?
Refer to algorithm for anemia in children with other cell lines affected
Low MCV
Normal MCV
High MCV
Iron deficiency
Thalassemia¶
Sideroblastic anemia
Anemia of chronic disease△
Further evaluation and confirmatory tests◇
Review dietary history
Therapeutic trial of iron
Serum ferritin, iron, and TIBC levels§
HGB electrophoresis
What is the reticulocyte response?
Low or normal reticulocyte count (≤3 percent)
High reticulocyte count (>3 percent)
Is there evidence of hemolysis?¥
Yes
No
Infection
Drugs
Lead poisoning
Acute blood loss
Anemia of chronic disease
Renal disease
TEC
Hemolytic anemias
Membranopathy (eg, hereditary spherocytosis, elliptocytosis)
Enzymopathy (eg, G6PD deficiency, pyruvate kinase deficiency)
Hemoglobinopathy (eg, sickle cell disease)
Autoimmune hemolytic anemia
Microangiopathic hemolytic anemia (eg, HUS, TTP, DIC, Kasabach-Merritt syndrome, artificial heart valve)
Hemorrhage
Drugs (eg, anticonvulsants, zidovudine, methotrexate)
Vitamin B12/folate deficiency
Sickle cell disease (especially if treated with HU)
Immune hemolytic anemia
Diamond-Blackfan anemia
Liver disease
Hypothyroidism
Post-splenectomy anemia
Myelodysplastic syndromes
Reticulocytosis (refer to the "High reticulocyte count" pathway to the left)
Review smear‡
Review smear‡
Review smear‡
Toxic granulations, bandemia, or atypical lymphocytes → Infection
Basophilic stippling → Lead poisoning
Spherocytes → Hereditary spherocytosis or autoimmune hemolytic anemia
Fragmented cells → Microangiopathic anemia
Sickle cells → Sickle cell disease
Elliptocytes → Hereditary elliptocytosis
Heinz bodies → G6PD deficiency
Hypersegmented PMNs → B12/folate deficiency
Spherocytes → Autoimmune hemolytic anemia
Sickle cells → Sickle cell disease
Howell-Jolly bodies → Post-splenectomy or asplenia
Further evaluation and confirmatory tests◇
Review medications
Bacterial cultures and/or viral studies
Lead level
Bone marrow biopsy/aspirate
Further evaluation and confirmatory tests◇
Indirect bilirubin, LDH, haptoglobin levels
DAT
G6PD screening test
Osmotic fragility
HGB electrophoresis
Further evaluation and confirmatory tests◇
Review medications
Review dietary history
Serum B12 and folate levels
Thyroid function tests
Osmotic fragility
HGB electrophoresis
DAT
Bone marrow biopsy/aspirate

Figure 1. Diagnostic approach to isolated anemia in children: morphologic classification.
(from Powers and Sandoval; https://www.uptodate.com/contents/approach-to-the-child-with-anemia2021)

HGB: hemoglobin; MCV: mean corpuscular volume; TIBC: total iron binding capacity; TEC: transient erythroblastopenia of childhood; G6PD: glucose-6-phosphate dehydrogenase; HUS: hemolytic uremic syndrome; TTP: thrombotic thrombocytopenic purpura; DIC: disseminated intravascular coagulation; LDH: lactate dehydrogenase; DAT: direct antiglobulin test; HU: hydroxyurea; PMNs: polymorphonuclear cells; RDW: red cell distribution width.

* Hemoglobin levels vary considerably by age, race, and sex; when diagnosing anemia, hemoglobin values should be compared with age-, race-, and sex-adjusted norms. Mild anemia occurring at six to nine weeks of life is consistent with "physiologic anemia" and is not pathologic. Falsely elevated hemoglobin values may occur when measured using capillary samples (eg, finger or heel "sticks"), particularly when using microhematocrit measurements. Spurious results may also occur with automated counters in the presence of lipemia, hemolysis, leukocytosis, or high immunoglobulin levels.

¶ The RDW can be helpful in differentiating thalassemia from iron deficiency. High RDW is typical of iron deficiency, whereas the RDW is usually normal in patients with thalassemia (though elevated RDW can occur).

Δ Anemia of chronic disease typically presents as a normocytic anemia, but can have low MCV.

◊ Selected testing is based upon review of the patient's history and examination of the peripheral blood smear.

§ In children with mild microcytic anemia and dietary history that is suggestive of iron deficiency, serum iron studies (ie, ferritin, iron, and TIBC levels) are generally not necessary. In these children, a therapeutic trial of iron can be used to confirm the diagnosis.

¥ Evidence of hemolysis includes jaundice, indirect hyperbilirubinemia, elevated lactate dehydrogenase, and/or decreased haptoglobin.

‡ Findings on blood smear may suggest an underlying etiology of anemia but they are generally not diagnostic. Further confirmatory testing should be carried out to confirm the diagnosis

Table 1. Pattern of test results used to differentiate iron deficiency anemia (IDA), anemia of inflammation (AI) and mixed IDA/AI.

	IDA	AI	Mixed IDA + AI
Hemoglobin	↓	↓	↓
MCV	↓	↓/normal	↓/normal
Ferritin	↓	↑	↑
Iron (unreliable)	↓	↓	↓
Transferrin saturation (TSAT)	↓	↓/normal	↓
Total iron binding capacity (TIBC)	↑	↓/normal	↓/normal
CRP	Normal	↑	↑

Adapted from Weiss *et al* (2019) and Goyal *et al* (2020).
MCV=mean corpuscular volume; CRP=C-reactive protein.

Table 2: Normal reference ranges for variables being investigated in serum and stool

Variable	Normal Reference Range
Hematologic indices	
Hemoglobin	
Age 6 months – 2 years	106-145 g/L
Age 3 – 11 years	110-157 g/L
Male 12-14 years	125-170 g/L
Female $\geq$ 12 years	120-160 g/L
Males 15 $\geq$ years	137-180 g/L
Mean corpuscular volume (MCV)	
Age 6 months – 2 years	71-90 fL
Age 3 – 11 years	75-91 fL
Age $\geq$ 12 years	82-100 fL
Mean Corpuscular Hemoglobin Concentration (MCHC)	
Age 6 months – 2 years	310-350 g/L
Age 3 – 11 years	315-360 g/L
Age $\geq$ 12 years	320-360 g/L
Red cell distribution width (RDW)	11-16 %
Platelets	150-400 x10^9/L
White blood cell count (WBC)	
Age 6 months – 2 years	6.0-16.0 x10^9/L
Age 3 – 11 years	4.0-14.0 x10^9/L
Age $\geq$ 12 years	4.0-11.0 x10^9/L
Reticulocyte	0.2-2.0 %
Iron	8-35 μmol/L
Ferritin	
Age 6 months – 15 years	15-100 μg/L
Female > 15 years	20-300 μg/L
Male > 15 years	30-500 μg/L
Transferrin Saturation (TSAT)	0.15-0.50 %
Total iron binding capacity (TIBC)	50-85 μmol/L

Soluble transferrin receptor (sTfR) (reference values for Toronto Sick Kids Hospital Laboratory)	
1 - < 12 years	0.8-1.6 mg/L
12 - < 19 years	0.7-1.5 mg/L
sTfR-Ferritin index[129]	
1-11 years	0.49-1.46
12-18 years	0.51-1.44
Micronutrients and vitamins	
Vitamin A	0.9-1.7 μmol/L
Vitamin D[130]	50-125 nmol/L
Vitamin E	10-21 μmol/L
Copper	11.0-28.0 μmol/L
Ceruloplasmin	0.16-0.45 g/L (female) 0.15-0.30 g/L (male)
Zinc	8-20 μmol/L
Vitamin B12	155-700 pmol/L
Folate	> 12.1 nmol/L
Methylmalonic acid (MMA)	0-0.4 μmol/L
Markers of inflammation	
Albumin	
< 1 year	22-45 g/L
$\geq$ 1 year	30-45 g/L
C-reactive protein (CRP)	< 8.0 mg/L
Erythrocyte sedimentation rate (ESR)	
0-12 years	0-10 mm/hr
> 12 years	0-20 mm/hr (female) 0-15 mm/hr (male)
Fecal calprotectin (stool test)[124]	< 150 mg/Kg
Markers of liver disease	
Bilirubin – direct (age 15 days-150 years)	0-7 μmol/L
ALT	< 35 U/L

AST	
< 1 year	10-65 U/L
1-3 years	10-55 U/L
4-10 years	10-45 U/L
≥ 11 years	8-32 U/L (female) 8-40 U/L (male)
ALP	
< 1 year	130-500 U/L
1-12 years	130-430 U/L
13-14 years	60-225 U/L (female) 130-500 U/L (male)
15-17 years	50-140 U/L (female) 60-250 U/L (male)
≥ 18 years	40-120 U/L
GGT	
< 1 year	< 100 U/L
1-17 years	< 27 U/L
≥ 18 years	< 50 U/L (female) < 80 U/L (male)
INR	0.9-1.1
Markers of renal function/disease	
Creatinine	
< 2 years	10-40 μmol/L
2-5 years	20-45 μmol/L
6-12 years	20-75 μmol/L
13-14 years	30-85 μmol/L
15-150 years	40-100 μmol/L 50-120 μmol/L (male)

All reference values from Alberta Precision Laboratories (https://www.albertaprecisionlabs.ca/tc/Page13850.aspx), unless otherwise indicated.[131]

Table 3-A. Classification of Iron deficiency anemia (IDA), anemia of inflammation (AI) and mixed iron deficiency anemia/anemia of inflammation based on routine iron studies

Anemia	MCV (fL)*	Ferritin (μg /L)	Transferrin Saturation (TSAT) (%)	Evidence of Inflammation**
Iron deficiency anemia	Low	< 30	< 15%	No
Anemia of inflammation	Low or normal	> 100	Low or normal	Yes
Mixed iron deficiency anemia and anemia of inflammation	Low or normal	> 30	< 20%	Yes

***** MCV normal values for age listed in Table 2 ******ESR, CRP, Fecal calprotectin
Adapted from Capellini *et al* (2017).[19]

Table 3-B. Classification of Iron deficiency anemia (IDA),, anemia of inflammation (AI) and mixed iron deficiency anemia/anemia of inflammation based on soluble transferrin receptor

Anemia	Ferritin (μg /L)	Soluble transferrin receptor (sTfR) (mg/L)	Soluble transferrin receptor index (sTfR-F index)	Evidence of Inflammation**
Iron deficiency anemia	< 30	> 1.9 (1-11 years) > 2.0 (12-18 years)	> 1.5	No
Anemia of inflammation	> 100	Normal for age	< 1.0	Yes
Mixed iron deficiency anemia and anemia of inflammation	30-100	> 1.9 (1-11 years) > 2.0 (12-18 years)	> 2.0	Yes

******elevated ESR, CRP or Fecal calprotectin
Adapted from Vázquez-López *et al* (2015)[132] and Vázquez-López *et al* (2016).[129]

Table 3-C. Classification of outcome variables of anemia

	Outcome Variables				
	No anemia	**Iron deficiency anemia (IDA)**	**Anemia of inflammation (AI)**	**Mixed IDA and AI**	**Nutritional anemia**
Hemoglobin (g/L)	normal	↓	↓	↓	↓
MCV (fL)	normal	↓	↓/normal	↓/normal	↑
Ferritin (μg /L)	↔	**< 30**	**> 100**	**30-100**	↔
TSAT (%)	↔	**< 16%**	**↓/normal**	**< 20%**	↔
Evidence of inflammation*	↔	↔	**Yes**	**Yes**	↔
Vitamin B12 (pmol/L)	↔	↔	↔	↔	**↓/normal**
MMA (μmol/L)	↔	↔	↔	↔	↑
Folate (nmol/L)	↔	↔	↔	↔	↓

MCV: mean corpuscular volume. TSAT: transferrin saturation. MMA: methylmalonic acid.

↑: above normal reference value for age. ↓: below normal reference value for age. ↔: equivocal – no direct relationship to type of anemia.

Table 4. Chronic Kidney Disease Classification[126]

Stage	Description
1	Kidney damage with a normal or increased GFR (> 90 mL/min/1.73m^2)
2	Mild reduction in the GFR (60-80 mL/min/1.73m^2)
3	Moderate reduction in the GFR (30-59 mL/min/1.73m^2)
4	Severe reduction in the GFR (15-29 mL/min/1.73m^2)
5	Kidney failure GFR (< 15 mL/min/1.73m^2)

Table 5. Investigations for evaluation of the causes of anemia and potential predictors

Category of Investigation	Specific Investigations	Rationale/utility for Investigations
Hematologic	• CBC + differential, MCV, MCHC, RDW, reticulocyte count • Iron, ferritin, Tsat, TIBC • sTfR, sTfR-F index (to be performed at Toronto Sick Kids Hospital)	• Identification and classification of anemia
Micronutrients and Vitamins	• Vitamin A, D, E • Copper, ceruloplasmin, zinc • Vitamin B12, folate, MMA	• Associated contributors to anemia • Nutritional anemia
Inflammation	• Albumin, WBC, platelets • CRP, ESR • Fecal calprotectin	• Identify evidence of inflammation for anemia of inflammation • Differentiate systemic from intestinal inflammation
Liver and Renal Disease	• ALT, AST, ALP, GGT, direct bilirubin, INR, albumin • Creatinine (for eGFR) • Urinalysis (for proteinuria) • Renal ultrasound • Liver ultrasound with elastography	Evidence of liver and renal disease
Endoscopy and Histology**	Upper endoscopy and colonoscopy (historic on chart review)	• Identify presence of intestinal ulcers/bleeding • Histologic evidence of intestinal inflammation
Additional investigations to identify underlying causes of anemia not specifically related to IF (as per Figure 1 algorithm)	• Blood smear – hemoglobinopathies, lead poisoning, hemolysis • G6PD screening test • Lead level – lead poisoning • Haptoglobin, LDH, indirect bilirubin, DAT – hemolytic anemia • Hemoglobin electrophoresis - thalassemia • Osmotic fragility – hemoglobinopathies • Thyroid hormones – thyroid disease	• Rule out other causes of anemia

** Endoscopy/colonoscopy will only be done on patients for whom it is deemed clinically relevant. Information from previous endoscopy/colonoscopy will be collected via chart review.

CBC=complete blood count; MCV=mean corpuscular volume; MCHC=mean corpuscular hemoglobin concentration (MCHC); RDW=red cell distribution width; TSAT= transferrin saturation; TIBC=total iron binding capacity; MMA= methylmalonic acid; CRP=C-reactive protein; ESR=erythrocyte sedimentation rate; ALT=alanine transferase; AST=aspartate transferase; ALP=alkaline phosphatase; GGT=gamma glutamyl transpeptidase; INR=international normalized ratio; G6PD=glucose-6-phosphate dehydrogenase.

Table 6. Variables for statistical analysis

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
Outcome variable – Anemia				
Iron deficiency anemia (IDA)	Categorical (present/not present)	• Low serum hemoglobin with, • low MCV and, • low ferritin and/or, • TSAT < 16%	Proportions	Primary outcome
Anemia of inflammation (AI)	Categorical (present/not present)	• Low serum hemoglobin with, • low or normal MCV and, • elevated ferritin (> 100 μg/L) and, • TSAT <20% or normal	Proportions	
Mixed IDA/AI	Categorical (present/not present)	• Low serum hemoglobin with, • low or normal MCV • ferritin > 30 μg/L, **and** • TSAT < 20%, **and** • Evidence of inflammation*	Proportions	
Nutritional Anemia	Categorical (present/not present)	• Low serum hemoglobin with, elevated MCV and: • low vitamin B12, or • high MMA, or • low folate	Proportions	
No Anemia	Categorical (present/not present)	• normal serum hemoglobin for age	Proportions	

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
Exposure Variables - Demographic				
Age	Continuous	Subject age in months	Median and Interquartile Range	Younger children at increased risk for anemia due to increased demands[18]
Sex	Binary/Categorical (male, female)	Subject's biologic sex	Proportions Chi square/Fisher's exact test	Male sex is a risk factor for iron deficiency in young children[18]
Etiology of IF	Categorical (7 categories)	Primary cause of IF		Underlying etiology will impact risk for types/causes of anemia (e.g. anastomotic ulcers in SBS, SIBO in motility disorders)
History of bowel lengthening procedure	Categorical (yes/no)	History of surgical bowel lengthening procedure		Potential increased risk of ulcers at staple lines[69, 133]
Presence of Ileocecal valve	Categorical (yes/no)	Intact ileocecal valve		Absence of ICV is a risk factor for SIBO[134]
SBS type I, II or III	Categorical (3 categories)	Intestinal tract anatomy • SBS type I = enterostomy • SBS type II = jejuno-colic anastomosis • SBS type III = jejuno-ileocolic anastomosis		Resulting GI anatomy is a risk factor for malabsorption and various micronutrient/vitamin deficiencies (increased risk with type I > II > III[76]
Bowel length/% predicted	Continuous	Remaining bowel length at last surgical procedure or predicted bowel length for gestational age	Median and IQR Chi square/Fisher's exact text	Short bowel a risk factor for malabsorption[76]
Comorbid conditions/ previous diagnoses	Categorical (10 categories)	Comorbid conditions which could impact risk of anemia or previous diagnoses of	Proportions Chi square/Fisher's exact text	Comorbid conditions, such as IBD, will impact risk for the various types of anemia or predispose to anemia unrelated to IF (e.g. hemoglobinopathy)

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
		intestinal ulcers, liver disease or renal disease		
Exposure Variables – Hematologic investigations[14]				
Hemoglobin	Continuous	Oxygen carrying protein in RBC	Mean and SD or median and IQR	Low hemoglobin is defining criteria for anemia
MCV	Continuous	Average size of RBC		MCV is used to describe and categorize types of anemia
Ferritin	Continuous	Measure of iron stores in the body. Also an acute phase reactant.		Used in diagnosis of iron deficiency (ferritin <30 μg/L). Levels with increase in the presence of inflammation
TSAT	Continuous	Measure of iron content in circulating transferrin (iron transport protein) Reflects availability of utilizable iron		Required for diagnosis of iron deficiency anemia. Low in iron deficiency anemia (<16%). Cut-off for iron deficiency is <20% in presence of inflammation.
TIBC	Continuous	Amount of iron needed to saturate plasma transferrin		Used to help classify iron status. Elevated in iron deficiency.
MCHC	Continuous	Average concentration of hemoglobin in RBC		Used to describe and categorize types of anemia
RDW	Continuous	Measure of variability of RBC size		Used to describe and categorize types of anemia
Reticulocyte count	Continuous	Measure of bone marrow response to anemia		Used to help identify underlying cause of anemia
WBC	Continuous	Measure of number of WBCs in serum		Marker of inflammation or infection (high WBC)

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
Platelet	Continuous	Measure of number of platelets in serum		Marker of inflammation (high) or liver disease (low)
Blood smear	Categorical	Physical characteristics of blood cells by microscopy	Proportions	Used to help identify underlying cause of anemia
Soluble transferrin receptor (sTfR)	Continuous	Measure of number of available transferrin receptors in blood as marker of iron load in cells	Mean and SD or median and IQR Spearman's correlation coefficient Kruskall-Wallis Test ROC and AUC	Used to discriminate IDA from AI and mixed IDA/AI, as well as identify iron deficiency before development of anemia in various patient populations[38-42]
Soluble transferrin receptor/log ferritin index (sTfR-F index)	Continuous	Determined by dividing sTfR by log ferritin index. Marker of iron supply available for erythropoiesis		
Exposure Variables – Vitamin and micronutrient levels				
Iron deficiency	Categorical (deficient/not deficient)	Low level of stored iron in cells • Ferritin < 30 μg/L or TSAT < 16% or TSAT < 20% in presence of inflammation*	Proportions Chi square/Fisher's exact test Univariate/multivariate odds ratio	First stage of iron deficiency anemia[18]
Vitamin A deficiency	Categorical (deficient/not deficient)	Low serum vitamin A level (< 0.9 μmol/L)		Indicative of intestinal malabsorption[12]
Vitamin D deficiency	Categorical (deficient/not deficient)	Low vitamin D level (< 80 nmol/L)		Vitamin D deficiency is associated with both IDA and AI[82, 83, 101]
Vitamin E deficiency	Categorical (deficient/not deficient)	Low vitamin E level (<10 μmol/L)		Indicative of intestinal malabsorption[12]

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
Vitamin B12 deficiency	Categorical (deficient/not deficient)	Low serum vitamin B12 (< 155 pmol/L) or elevated MMA (> 0.4 μmol/L)		Defining criteria for nutritional anemia[106]
Folate deficiency	Categorical (deficient/not deficient)	Low serum folate level (< 12.1 nmol/L)		Defining criteria for nutritional anemia[106]
Copper deficiency	Categorical (deficient/not deficient)	Low serum copper level (11 μmol/L)		Important cofactor in several enzymes and metabolic processes involved in hemoglobin synthesis and iron oxidation. Copper competes with iron and zinc for absorption[94, 95]
Zinc deficiency	Categorical (deficient/not deficient)	Low serum zinc level (< 8 μmol/L)		Important cofactor in several enzymes and metabolic processes involving iron metabolism and erythropoiesis. Zinc competes with iron and copper for absorption[84-86]
Exposure Variables – Biomarkers of inflammation				
CRP	Continuous	Serum marker frequently elevated in systemic inflammation	Mean and SD or median and IQR Univariate/multivariate odds ratio	Marker of systemic inflammation. Frequently elevated in cases of anemia of inflammation[62, 135]
ESR	Continuous	Serum marker frequently elevated for age in systemic inflammation		Marker of systemic inflammation. May be elevated in cases anemia of inflammation[135]
Fecal Calprotectin	Continuous	Biomarker of inflammation specific to the intestine found in stool		Marker of intestinal inflammation.[136] Evidence of elevated levels in various patient population with SIBO, including short bowel syndrome.[60, 137] May also be elevated in systemic inflammation[138, 139]
Exposure Variables – Liver disease[75, 127, 140, 141]				

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
IF associated liver disease (IFALD)	Categorical (present/not present)	• Direct bilirubin > 34 μmol/L for minimum of 2-4 weeks, or • evidence of fibrosis or cirrhosis or portal hypertension on ultrasound +/- doppler or liver elastography, or • Histologic evidence of fibrosis or cirrhosis or inflammation or chronic cholestasis on liver biopsy, not due to another process	Proportions Chi square/Fisher's exact test Univariate/multivariate odds ratio	Intestinal failure associated disease may be a cause of anemia of inflammation[45]
Direct bilirubin	Continuous	Serum marker of cholestasis used in defining IFALD	Mean and SD or median and IQR Univariate/multivariate odds ratio	Used in diagnosing IFALD
ALT	Continuous	Serum liver enzyme		Elevated levels seen in hepatic inflammation and are a nonspecific indicator of liver disease
AST	Continuous	Serum liver enzyme		
ALP	Continuous	Serum liver enzyme		Elevated levels in combination with elevated GGT are suggestive of cholestasis and are indicative of liver disease
GGT	Continuous	Serum liver enzyme		Elevated levels in the context of elevated bilirubin are indicative of liver disease
Albumin	Continuous	Serum protein synthesized in the liver		Low levels may be indicative of systemic inflammation, intestinal inflammation or liver disease
INR	Continuous	Serum measure of clotting time of blood		Low levels may be indicative of liver disease or vitamin K deficiency

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
Liver ultrasound – parenchyma	Categorical (3 categories)	Test used in the assessment of the liver parenchyma	Proportions Chi square/Fisher's exact test Univariate/multivariate odds ratio	Used to identify evidence of inflammation (steatosis) and cirrhosis, which are indicative of liver disease
Liver ultrasound - doppler	Categorical (2 categories)	Test used in the assessment of portal hypertension		Used to identify evidence of portal hypertension, which is indicative
Liver elastography	Continuous	Measure of liver stiffness	Mean and SD or median and IQR Univariate/multivariate odds ratio	High measures of liver stiffness are indicative of liver fibrosis[142]
Exposure Variables – Renal disease				
Estimated GFR (eGFR)	Continuous	Measure of kidney function • Calculated via bedside Schwartz equation[125]	Mean and SD or median and IQR Univariate/multivariate odds ratio	Used in diagnosis and staging of chronic kidney disease[126]
Creatinine	Continuous	Protein metabolism by-product found in serum		Elevated levels are indicative of reduced renal function and used to calculate estimated GFR used to stage chronic kidney disease[126]
Proteinuria	Categorical (present/not present)	Presence of protein in urinalysis	Proportions Chi square/Fisher's exact test Univariate/multivariate odds ratio	Presence is suggestive of renal disease and requires further investigation[126]
Renal ultrasound findings	Categorical (4 categories)	Imaging of the kidneys by ultrasound		Findings such as the presence of nephrocalcinosis, nephrolithiasis and increased echogenicity in the renal parenchyma may be early signs of renal disease in children with IF[71-73]
Exposure Variables - Nutrition				
PN duration	Continuous	Months on parenteral nutrition	Mean and SD or median and IQR	Longer PN duration previously shown to be associated with risk of micronutrient deficiencies and risk of renal and liver disease[4, 45, 71, 72, 78]

Variable	Variable Type	Variable Definition	Variable Analysis	Variable Justification
Percent PN dependence	Continuous	Measure of nutritional/caloric dependence on PN determined by the ratio of non-protein energy intake (NPEI) in PN over subject's calculated resting energy expenditure (REE)[143]	Univariate/multivariate odds ratio	Higher PN dependence may be at higher risk of iron deficiency due to poor GI absorption and difficulty in providing iron in PN, as well as increased risk of IFALD[75, 127]
Oral/enteral dietary restrictions	Categorical (4 categories)	Restrictions in sources of nutrition (e.g. meat, all animal products) taken in via the intestinal trat	Proportions Chi square/Fisher's exact test Univariate/multivariate odds ratio	Absence of good sources of dietary iron and/or B12 (e.g. meat) may increase risk for iron deficiency anemia and nutritional anemia[18, 51, 107]
Exposure Variables – Medications				
Current Medications	Categorical (6 categories/drug classes)	Current medications belonging to specific identified classes of medications	Proportions Chi square/Fisher's exact test Univariate/multivariate odds ratio	Identification of medications belonging to classes of drugs known to contribute to anemia[120]
Exposure Variables – Intestinal findings on endoscopy and histology (where available)				
Endoscopic evidence of intestinal ulcers	Categorical (4 categories)	Endoscopic visualization of intestinal ulcers	Proportions Chi square/Fisher's exact test Univariate/multivariate odds ratio	Potential cause of iron deficiency anemia[68, 69]
Histologic intestinal inflammation	Categorical	Intestinal biopsies with histologic evidence of inflammation		Potential cause of anemia of inflammation and/or iron deficiency anemia[134]

MCV=mean corpuscular volume; TSAT= transferrin saturation; TIBC=total iron binding capacity; MMA= methylmalonic acid; MCHC=mean corpuscular hemoglobin concentration; RDW=red cell distribution width; WBC=white blood count; CRP=C-reactive protein; ESR=erythrocyte sedimentation rate; ALT=alanine transferase; AST=aspartate transferase; ALP=alkaline phosphatase; GGT=gamma glutamyl transpeptidase; INR=international normalized ratio; PN=parenteral nutrition; SD=standard deviation; IQR=interquartile range.

Table 7. Budget

Budget Summary

A. Research Procedure Costs			
Procedures/Services	**Cost per item**	**Projected Number of Participants**	**Total**
Soluble transferrin receptor testing (Toronto Sick Kid's Hospital)	$19.50	100	$1,950.00
Fecal Calprotectin (Dynacare)	$59.00	20	$1,180.00
Shipping (soluble transferrin receptor)	$40.00	100	$4,000.00
Shipping (fecal calprotectin)	$50.00	20	$1,000.00
Sum			**$8,130.00**
B. Personnel Costs			
Data Collection	**Rate**	**Hours**	**Total**
in kind hours	$0.00	40	$0.00
Sum			**$0**
C. Additional Costs			
Printing/photocopying	**Cost per page**	**Total Number of pages**	**Total**
Consent forms	$0.10	2000	$200.00
Sum			**$200.00**
		Total Costs	**$8,330.00**