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The utility of Reticulocyte Haemoglobin (Ret-He) for Iron Deficiency Screening at Canterbury Health Laboratory.

A thesis presented to the Massey University College of Health in partial fulfilment of the requirements for the Master of Health Science specializing in Medical Laboratory Research.

At Massey University, New Zealand

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2026

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to everyone who has supported me throughout this research journey.

First and foremost, I extend my sincere appreciation to my Massey University Student Research Advisor and Senior Lecturer (ret.) Chris Kendrick for his invaluable guidance, expertise, and patience for reaching me to the successful conclusion of my research. His experience, constructive feedback and encouragement have been instrumental in this study.

I am also grateful to Kit Norrish, whose insightful suggestions, continuous guidance and support have helped me shape this study to its finished form.

I extend my sincere gratitude to my clinical supervisor, senior medical officer, Consultant Haematologist Dr. Catherine Neal at Canterbury Health Labs in Health New Zealand Canterbury for her ongoing clinical guidance throughout the journey of my study. Her assistance has greatly improved the quality of my research.

I also extend my gratitude to Dr Nate Lucas for assisting me with statistical analysis of data and to Canterbury Health Labs and the Research Team at Health NZ Canterbury for providing the locality authorisation and necessary resources and support to conduct this research. Additionally, I am thankful to George Haremate at Health NZ Canterbury for providing Māori consultation for this study.

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April 2026

ABSTRACT:

Background: Iron deficiency (ID) is the most common nutritional deficiency worldwide and is the commonest cause of anaemia. Diagnosis of iron deficiency has traditionally been based on Biochemistry parameters such as Ferritin, total-iron binding capacity (TIBC), and Transferrin saturation. However, recent laboratory practices around the world have established that Reticulocyte Haemoglobin (Ret-He) is as a superior indicator of iron deficiency compared to the classical methods. Ret-He is a laboratory parameter that can be determined using automated haematology analysers. It is a measurement of haemoglobin of reticulocytes and provides a rapid and reliable measure of the functional iron available for the process of erythropoiesis. Thus, measuring Ret-He provides information about iron status change in the body much earlier compared to the measurement of ferritin. Ret-He is not affected by inflammation and is more reliable for iron studies compared to ferritin during inflammatory states of the body.

Aim: This study aimed to establish a normal reference interval for Ret-He and use sensitivity and specificity calculations to determine an optimum cut-off of Ret-He for determination of ID and IDA.

Methods: Reference intervals for Ret-He were calculated from 50 normal blood samples using +/-2SD from mean. 321 blood samples with existing Complete Blood Count (CBC) and iron studies tests were selected for retrospective Ret-He analysis in Sysmex XN-20 haematology analyser. Results of Ret-He, haemoglobin and ferritin were compared against each other. Sensitivities and specificities of Ret-He at different levels were calculated for ID and IDA using Chi square and 2x2 box plot. Receiver operating characteristic curve analysis, calculation of area under the curve with 95% confidence intervals and Youden’s Index calculations were performed to decide the optimum cut-off of Ret-He for ID and IDA.

Results: The reference interval for Ret-He was calculated to be 32.4 -37.5 pg. Based on the ROC curve analysis and Youden’s index calculation using the specificity and sensitivity values of Ret-He, an optimum value of 34 pg and 24 pg was calculated for diagnosis of ID and IDA respectively. Ret-He levels in IDA were decreased in comparison with non-IDA population of both sexes and the difference

was statistically significant. Ret-He levels were reduced in a number of patients with raised CRP who had normal or high ferritin results.

Conclusion: Ret-He is a cost-effective rapid response laboratory parameter that can be a valuable iron studies tool. Addition of Ret-He test to routine haematology tests such as CBC and Reticulocyte can add diagnostic value for screening and diagnosis of ID and IDA respectively. Ret-He is particularly valuable in inflammatory states of patients where ferritin results may not be reliable. Ret-He is not a substitution for existing iron studies tests like ferritin but is an additional tool with valuable clinical utility.

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LIST OF ABBREVIATIONS

AI	Anaemia of Inflammation
AUC	Area Under the Curve
BSH	British Society for Haematology
CBC	Complete Blood Count
CBCD	Complete Blood Count with Differentials
CHL	Canterbury Health Laboratories
CI	Confidence Interval
CRP	C-Reactive Protein
DE	Delphic Explorer
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
EQA	External Quality Assurance
F	Female
FID	Functional Iron Deficiency
FSC	Forward Scatter
HDEC	Health and Disability Ethics Committee
HFR	High Fluorescence
HIF	Hypoxia-inducible Factor
IANZ	International Accreditation New Zealand
IBM	International Business Machines Statistical Package for the Social
SPSS	Sciences
ID	Iron Deficiency
IDA	Iron Deficiency Anaemia
IL	Interleukin
IV	Intravenous
LFR	Low Fluorescence
LIS	Laboratory Information System
M	Male
MCH	Mean Cell Haemoglobin

MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Cell Volume
MFR	Mid Fluorescence
MPS	Mononuclear Phagocyte System
MUEC	Massey University Ethics Committee
NZ	New Zealand
PMID	PubMed Identifier
QQ	Quantile-Quantile
RACGP	Royal Australian College of General Practitioners
RBC	Red Blood Cell
RDW	Red-cell Distribution Width
RET	Reticulocyte
Ret-He	Reticulocyte Haemoglobin
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
RPI	Reticulocyte Production Index
SD	Standard Deviation
SF	Serum Ferritin
SFL	Side Fluorescence
SID	Sample Identity
SSC	Side Scatter
sTfR	Serum Transferrin Receptor
TIBC	Total Iron Binding Capacity
WBC	White Blood Cell
WHO	World Health Organisation
WQAP	Waikato Quality Assurance Program
ZPP	Zinc Protoporphyrin

1.0 INTRODUCTION

Iron deficiency (ID) is the most common nutritional deficiency worldwide and is the commonest cause of anaemia (Lopez et al., 2016). While bone marrow biopsy is the gold standard for measuring body's iron content storage, it is an invasive procedure. Diagnosis of iron deficiency has traditionally been based on venipuncture and measurement of biochemistry parameters such as ferritin, total iron-binding capacity (TIBC), and transferrin saturation (Brugnara et al., 2006). Various literature and recent laboratory practices around the world have established that reticulocyte haemoglobin (Ret-He) is a better indicator of ID compared to the classical methods (Rao et al., 2024Auerbach et al., 2020; Gelaw et al., 2019; Poffenroth et al., 2017; Rao & Mirji, 2024 and Wardhani & Oehadian 2021). Reticulocytes are younger red blood cells and Ret-He is a measurement of haemoglobin in these young cells. In an iron-deficient state, reticulocytes and red cells have a lower haemoglobin due to reduced bioavailability of iron. The measurement of haemoglobin in reticulocytes provides a direct quantification of iron supply for the process of erythropoiesis, thus detecting ID at an early stage (Wardhani & Oehadian, 2021).

Ferritin and transferrin, the primary parameters of iron status used for detecting ID offers limited value when a patient presents with co-morbidities such as Anaemia of Inflammation (Markovic et al., 2007). Ferritin and transferrin, being acute phase reactants, are elevated during infection, chronic disorders and other inflammatory states, thus reducing the reliability of these parameters for measuring iron levels. Ret-He, however, is not affected by acute phase response and has been demonstrated to be a better indicator of bone marrow iron status during these conditions (Markovic et al., 2007).

Canterbury Health Laboratory (CHL) provides Complete Blood Count (CBC), White Blood Cell (WBC) differentials and reticulocyte count (Ret-He) as part of the CBC test package. The Ret-He parameter is routinely available in Sysmex XN analysers as part of the reticulocyte count test. There will be significant cost advantage if a repeat blood test can be avoided to assess iron levels on patients who have CBC test requested. Ret-He test can prevent unwarranted iron studies test requests and can be a screening test for ID when paired with CBC test results (Aedh et al., 2023).

1.1 Aims

This study aims to establish a normal reference range for Ret-He and use sensitivity and specificity calculations to determine an optimum cut-off of Ret-He for determination of ID and IDA.

1.2 Objectives

i. To establish a reference range for Ret-He based on data collected from a normally distributed population.
ii. To evaluate the sensitivities and specificities of Ret-He at different cut-offs for diagnosis of ID and IDA.
iii. To calculate an optimum cut-off of Ret-He for ID and IDA based on ROC curve analysis and Youden's index.

We hypothesized that Ret-He would be an important tool in the overall iron level assessment for clinicians and at certain cut-off levels, Ret-He would be a useful indicator for diagnosis of ID and IDA. Moreover, we hypothesized that during inflammatory states when the body's C-reactive protein (CRP) levels are high, Ret-He would be a better alternative than serum ferritin for iron studies.

We focused this study at CHL to understand the clinical utility of Ret-He so that this test can be incorporated into the current haematology testing profile as an additional tool for clinicians wanting to study the iron levels of their patients.

1.3 Overview of Thesis

This thesis has been organised into seven chapters.

Chapter 1 is an introduction to the topic of this thesis- reticulocyte haemoglobin (Ret-He) and its use in detecting iron deficiency (ID). Chapter 2 includes a comprehensive literature review on current and past studies on laboratory parameters and their clinical utilities for iron level studies including Ret-He. Chapter 3 includes the methods and statistical analysis applied in this study. This chapter also includes the process of ethics approval for the study. Chapter 4 is a summary of the results of the study. The discussion and analysis of the results are included in Chapter 5. This

chapter also includes the limitations of the study and further recommendations and a conclusion of the study. The references used in the study are listed in chapter 6 and the last chapter includes appendices. A table of all the results of the study is part of the appendix as well.

2.0 LITERATURE REVIEW

2.1. Background

Iron is a vital mineral that is required primarily for the synthesis of the haemoglobin molecule which plays a key role in oxygen transportation in the human body. Iron deficiency (ID) is the most common nutritional deficiency worldwide and is the commonest cause of anaemia (Manish, 2024). Reduction of iron levels can lead to anaemia, fatigue, suppressed immune system and negatively alters physical and mental wellbeing (Hentze et al., 2010). Iron Deficiency Anaemia (IDA) is a state of depleted iron deposits in the body which is followed by reduction in haemoglobin levels. IDA affects both genders, though women are more frequently impacted, and studies have shown that IDA in the young is associated with cognitive function impairment (Aedh et al., 2023). The prevalence of anaemia rises with age and causes significant burden on health care systems (Nissenson et al., 2005). Thus, early detection of iron deficiency via laboratory methods provides clinicians valuable information that aids in the clinical management of the affected patients (Nissenson et al., 2005).

2.2. Iron and Haemoglobin

Iron is an essential mineral necessary for all eukaryotic cells due to its catalytic properties and chemical reactivity. Small molecules such as oxygen and nitrogen are easily activated when bound to iron. Iron plays an important role in oxygen binding and storage, cellular and mitochondrial respiration, protein synthesis and nucleic-acid synthesis and DNA replication (Khalimonchuk, 2024).

The protein responsible for transportation of oxygen from the lungs to the tissues and carbon dioxide from the tissue back to the lungs is haemoglobin. Haemoglobin is highly specialised and is composed of four hydrophobic globin chains that secure iron in its core in the reduced ferrous state and protect it from being oxidised (Figure 1). This erythrocyte protein makes up to a third of the volume of the human Red Blood Cells (RBC) in the bloodstream. Haemoglobin and iron content in the body is limited because mature RBCs do not have a nucleus or mitochondria, thus cannot produce new protein. Thus, the homeostasis of iron and haemoglobin is maintained

by the production of RBCs in the bone marrow and their destruction in the spleen (McKenzie & Williams, 2019).

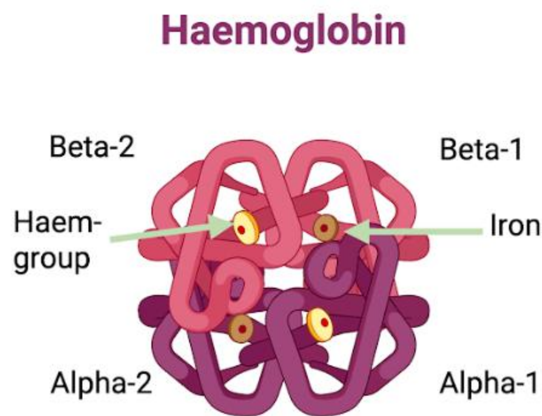

Figure 1: The molecular structure of the Haem complex and its coordinate system. The four hydrophobic globin chains (2 alpha and 2 beta) of the haemoglobin molecule protect iron in the core in its reduced ferrous state to prevent it from being oxidised. Image source: created in Biorender.

2.3. Iron Pathophysiology

Iron level homeostasis is maintained in the human body via a combination of dietary iron intake (10%) and recycling of existing iron in the body (90%). While the intake of iron is regulated by the increased and decreased absorption via intestines, the body does not have a regulated way to excrete iron. More than 90% of the total iron content is consumed by erythroblasts during the manufacture of the human RBCs and this iron content is recycled from damaged or aged erythrocytes by the macrophages (Hentze et al., 2010). Ferritin is the protein that stores iron in the body and is stored in macrophages of the mononuclear phagocyte system (MPS) and in the hepatocytes. Blood loss is the single most significant contributor to iron deficiency because RBCs contain a significant amount of the body's iron storage. In addition, insufficient iron intake, reduced iron absorption, and defects in the iron transfer mechanism from storage to availability may also lead to iron deficiency (Aedh et al., 2023).

2.4. Biochemistry Tests for Iron Measurement

While bone marrow biopsy is the gold standard for measuring body's iron storage, it is an invasive procedure. Diagnosis of ID has traditionally been based on venepuncture and measurement of biochemistry parameters (Brugnara et al., 2006). Laboratory testing to assess iron levels in the body consists of biochemical tests such *as* measurement of serum iron, total iron binding capacity (TIBC), calculation of percent saturation of transferrin, serum ferritin, and serum transferrin receptor (sTfR). Other laboratory tests such as zinc protoporphyrin (ZPP) are also an important indirect test to measure the availability of iron in the body.

Transferrin is a glycoprotein that mediates the transport of iron throughout the body. It can reversibly bind to ferric iron (Fe^{3+}) which makes iron soluble and aids in the delivery of iron to tissues such as the liver, spleen, and bone marrow. The assessment of the percent saturation of the transferrin glycoprotein with iron measures the body's ability to transport iron to tissues. Feedback mechanisms in the body cause changes in the total storage as the availability of iron in the serum and the iron binding capacity (TIBC) fluctuates (McKenzie & Williams, 2019).

Ferritin is a complex globular protein with high affinity to iron and can reversibly bind to iron. It plays key role in the binding and storage of iron in an intracellular level. In an extracellular level, serum ferritin levels are used as an indicator for the iron levels in the body. Ferritin's interaction with transferrin—the iron transporter of the body—enables the movement of iron to various cells and tissues of the body, particularly in the liver, spleen and immune system. Measured in serum, it is directly proportional to the stored iron levels of the body and is used to determine iron depletion or iron overload in the body. Decreased levels of serum ferritin signals depletion of iron levels and it is the most widely accepted laboratory parameter used for the first indication of developing IDA. While TIBC and serum iron is detected only after the body iron stores are depleted, ferritin levels are affected before the mobilizable iron stores run out. (McKenzie & Williams, 2019). Table 1 summarises how the classical parameters of iron studies—serum iron, transferrin saturation, and serum ferritin changes in the body during various clinical conditions.

Table 1: Interpretation of iron studies tests during various clinical conditions.

	Serum Iron	Transferrin Saturation	Serum Ferritin
Iron Deficiency	Decreased	Decreased	Decreased
Iron Deficiency + Acute Phase Response	Decreased	Normal or decreased	"Normal" <100 µg/L
Acute Phase Response	Decreased	Decreased	Increased
Iron overload	Increased	Increased	Increased

Source: RCPA.edu.au/manuals/RCPA-Manual/Pathology-Tests

Ferritin levels are regulated in the body by cellular iron status and are thus a good indicator of iron storage. Ferritin and transferrin, the primary parameters of iron status used for detecting ID offers limited value when the patient presents with co-morbidities such as AI (Markovic et al., 2007). Ferritin, being an acute phase reactant, is elevated during inflammation, infection and malignancy, and chronic disease thus reducing the predictive power of these parameters. In addition, in cases of functional IDA where iron storage is adequate but its availability to the RBCs is reduced due to enhanced formation of pro-inflammatory cytokines, ferritin levels do not necessarily correlate with the level of ID status in the body (Morceau et al., 2009). Moreover, ferritin levels fail to correlate with the body's stored iron levels once its levels are below 12 μg/L, primarily because the iron stores are completely depleted below that level (Knovich et al., 2008).

2.5. C-Reactive protein (CRP)

CRP is one of the markers of inflammation found in the human body and is the end-product of an inflammation process. It is produced in the liver in response to inflammation and has a characteristic pentagonal structure that allows it to transition between its two forms in response to inflammation (Singh et al., 2025). The pro-inflammatory property exhibited by this protein has helped medical researchers and clinicians understand the pathogenesis of various diseases and conditions. During an

immune response process, CRP can act both as an inflammation promotor or a suppressor of the existing inflammatory process (Melnikov et al., 2023).

CRP is an acute phase reactant and is induced by interlukin-6, one of the cytokines produced during the immune process. Plasma concentration of CRP can rapidly change in response to the triggers of the inflammation process, meaning the rise in concentration can happen acutely but also fall acutely when the inflammatory stimuli resolve. CRP is often found to be highly elevated in acute inflammatory conditions such as appendicitis, cholecystitis, pancreatitis and meningitis. Extended elevations of CRP happen during ongoing inflammatory conditions such as rheumatoid arthritis. (Sproston & Ashworth, 2018).

CRP is often paired with ferritin results while assessing iron levels of the body. Because ferritin and TIBC levels are unreliable for iron level monitoring during inflammatory states of the body, it is important to interpret ferritin and transferrin results with CRP results. To measure an accurate level of body's iron storage during inflammatory states, Urbanski et al., (2024) have proposed a laboratory parameter, serum ferritin/CRP ration that could be used as an 'effective biomarker for ID, even in the presence of systemic inflammation or comorbidities. This ratio would help clinicians determine if high ferritin is due to iron overload or inflammatory response.

2.6. Anaemia

The life span of a normal RBC is 120 days once it is released from the bone marrow to the blood stream. When there is loss of RBC due to acute haemorrhage or via increased destruction in the blood stream, the overall red cell mass of the body decreases. The bone marrow can respond to this decreased survival of RBCs by increasing the production of new RBCs to a level 5-10 times normal. If the raw materials for production of new cells are available, bone marrow can produce new RBCs and release them in the form of reticulocytes, earlier than normal, in the blood stream for compensation. However, if the survival of RBCs has decreased and bone marrow compensation mechanism is impaired or has already reached the maximum compensation capacity, the overall RBC mass in the body decreases and anaemia develops (McKenzie & Williams, 2019).

Anaemia is often diagnosed through laboratory testing by measuring haemoglobin concentration, typically as part of a CBC test. Functionally, anaemia is defined as the decreased ability of the body to transport oxygen from the lungs to the tissues which results in tissue hypoxia. Since anaemia itself is not a disease, but in fact a marker of a disorder, when the body enters an anaemic phase clinicians should be looking for the underlying cause of the anaemia and target the treatment towards the cause of anaemia. Treating anaemia without proper identification of the underlying cause is not only an ineffective approach, but also can further complicate the clinical condition (McKenzie & Williams, 2019).

2.7. Haematology laboratory analyses for anaemia

Clinicians rely on a variety of laboratory tests ranging from haematology and biochemistry to genetic analysis to screen for the presence of anaemia and to measure its severity. The degree of anaemia could be from mild to life threatening based on the haemoglobin concentration. The Complete Blood Count (CBC) is an important haematology test that has sub-tests such as haemoglobin, Mean Cell Volume (MCV), haematocrit, Mean Cell Haemoglobin (MCH), Red-cell Distribution Width (RDW) and Mean Cell Haemoglobin Concentration (MCHC) that provide important quantitative information on the degree of anaemia present in the blood sample. Haemoglobin is proportional to the oxygen carrying capacity in the body and defines anaemia. Haematocrit, expressed in percentage, measures the proportion of RBC volume compared to the total volume of blood. Both haemoglobin and haematocrit decrease in cases of anaemia related to acute and chronic blood loss but clinicians interpreting these results often follow it up with other laboratory tests as these values can depend on parameters such as age, sex, ethnicity and clinical details of the patient (McKenzie & Williams, 2019).

RBC indices such as MCV, MCH and MCHC are derived from RBC count, haemoglobin and haematocrit and depending upon the type of anaemia these indices may increase or decrease in comparison to their reference intervals and aid in the screening for anaemia. MCV is the average volume of all RBCs and is used to classify anaemia into normocytic (normal sized RBCs), microcytic (small sized RBCs), or macrocytic (large sized RBCs). MCV is an indicator of the anaemia and is

useful in the differential diagnosis and evaluation of pathophysiology, treatment and epidemiology of anaemia. MCH is derived from Haemoglobin and RBC count and indicates the average haemoglobin content (weight) in a single RBC. MCHC is derived from haemoglobin and haematocrit and is the concentration of haemoglobin in all RBCs. MCHC depicts whether the general RBC population is normochromic (normal concentration), hypochromic (reduced concentration) or hyperchromic (increased concentration). A decrease and increase in MCH is linked to microcytic and macrocytic anaemia respectively but interpretation of MCH needs to be in conjunction with other RBC indices such as MCV and MCHC. The RDW is another RBC index calculated using MCV and provides information on the size distribution of the RBCs. It reflects the degree of anisocytosis (unevenness in the size) of RBCs and complements in the differential diagnoses of anaemia. Increased RDW paired with microcytosis (decreased MCV) is related to iron deficiency anaemia (McKenzie & Williams, 2019).

Reticulocytes are immature erythrocytes in the blood stream that are one step close to full maturation. These cells do not have a nucleus but have organelles and ribosomal system for haemoglobin synthesis. After remaining in the bone marrow for 2-3 days, these cells are pushed out to the peripheral blood where they spend one more day and lose their ribonucleic acid (RNA) and mature fully to become a RBC. Reticulocytes are often called polychromasia in the blood smear as they appear with a bluish tinge on Romanosky-stained smears (McKenzie & Williams, 2019). The basophilic RNA of the ribosomes available in these immature cells account for the bluish tinge. Reticulocyte count also helps in classifying the pathophysiology of anaemia (McKenzie & Williams, 2019).

The production of RBCs in the bone marrow is regulated via erythropoietin (EPO) hormone. Anaemia stimulates reticulocyte production primarily through tissue hypoxia-mediated upregulation of EPO. When the haemoglobin concentration is decreased in the body, oxygen delivery to tissues is decreased as well. Organs like kidneys have specialised peritubular interstitial fibroblast-like cells that sense tissue hypoxia via activation of the hypoxia-inducible factor (HIF) pathway (Haase, 2010; Jelkmann, 2011). The end-product of this pathway is stabilization of transcription factor HIF-2α and eventual transcription and secretion of EPO into the circulation (Koury & Haase, 2015). In response to the circulating EPO, erythroid progenitor cells

in the bone marrow proliferate, differentiate and mature into reticulocytes (Jelkmann, 2011). As a result, reticulocytes are released prematurely into the peripheral blood as a compensatory response to anaemia, reflecting increased erythropoietic activity. The degree of reticulocytosis is also dependent on the body's iron availability, as decreased iron levels restrict erythropoiesis and causes low haemoglobin synthesis despite elevated EPO levels (Means, 2020; Piva et al., 2010).

The bone marrow responds to lower RBC count in peripheral smear by pushing out these immature RBCs into circulation. When the severity of anaemia increases, reticulocyte count in the peripheral smear increases proportionately. The reticulocyte count production is measured by a laboratory parameter—Reticulocyte Production Index (RPI). In an anaemic state, early release of reticulocytes into circulation can cause false elevations of reticulocyte percentage. The RPI is a corrected measure of erythropoietic activity that adjusts the raw reticulocyte count for both the degree of anaemia and the premature release of reticulocytes into circulation. The RPI is a ratio of corrected reticulocyte percentage and a maturation factor that reflects the extended lifespan of circulating reticulocytes in anaemia (Rodak et al., 2020). Hypo-proliferative anaemias such as IDA or anaemia of inflammation (AI) present with an RPI <2 and indicates inadequate bone marrow compensation. In conditions of haemolysis or acute blood loss, an appropriate or exaggerated erythropoietic response is seen with an RPI ≥2–3 (McKenzie & Williams, 2019). Thus, RPI remains a valuable, tool for the functional classification of anaemia and complements laboratory tests like reticulocyte count that assess iron-restricted erythropoiesis. While not routinely reported by laboratories as part of the CBC or the Reticulocyte test, RPI is an important indicator of whether the bone marrow is adequately responding to anaemia (McKenzie & Williams, 2019). Table 2 below lists the haematology parameters measured at Canterbury Health laboratory (CHL), New Zealand with their units of measurements and relationship of the indices to each other.

Table 2: The sub-tests of the CBC test package and their associated units and calculation at CHL

CBC parameters	Units	Calculation
RBC count	10^12/L	Direct measurement
Haemoglobin (Hgb)	g/L	Direct measurement
Haematocrit (Hct)	Ratio	Direct measurement
MCV	fL	(Hct/RBC) *10
MCH	pg	(Hgb/RBC) *10
MCHC	g/L	(Hgb/Hct)
RDW	%	(Std deviation of MCV/mean MCV) *100
Reticulocyte	10^9/L	Direct measurement

Source: Test Manager, CHL

A review of the blood smear is an important aspect of laboratory process for anaemia work-up. Film revision provides information on the morphology of the RBCs in relation to their shape, size, and colour. As ID develops progressively, the parameters of the CBC test and the morphology of RBCs change with the severity of the condition. Generally, ID presents with microcytic (MCV 55-74 fl), hypochromic (MCHC 22-31g/dl), MCH (14-23 pg) picture. Morphological features (Figure 2) in ID are dominated by microcytic and hypochromic RBCs with varying grades of anisocytosis and poikilocytosis, most notably of which are elliptocytes, tear drop cell and target cells. However, during cases of concurrent Vitamin B12 or folate deficiency, these typical morphological features may be masked. Reticulocyte count, both relative and absolute number, can be normal or slightly increased in IDA (McKenzie & Williams, 2019).

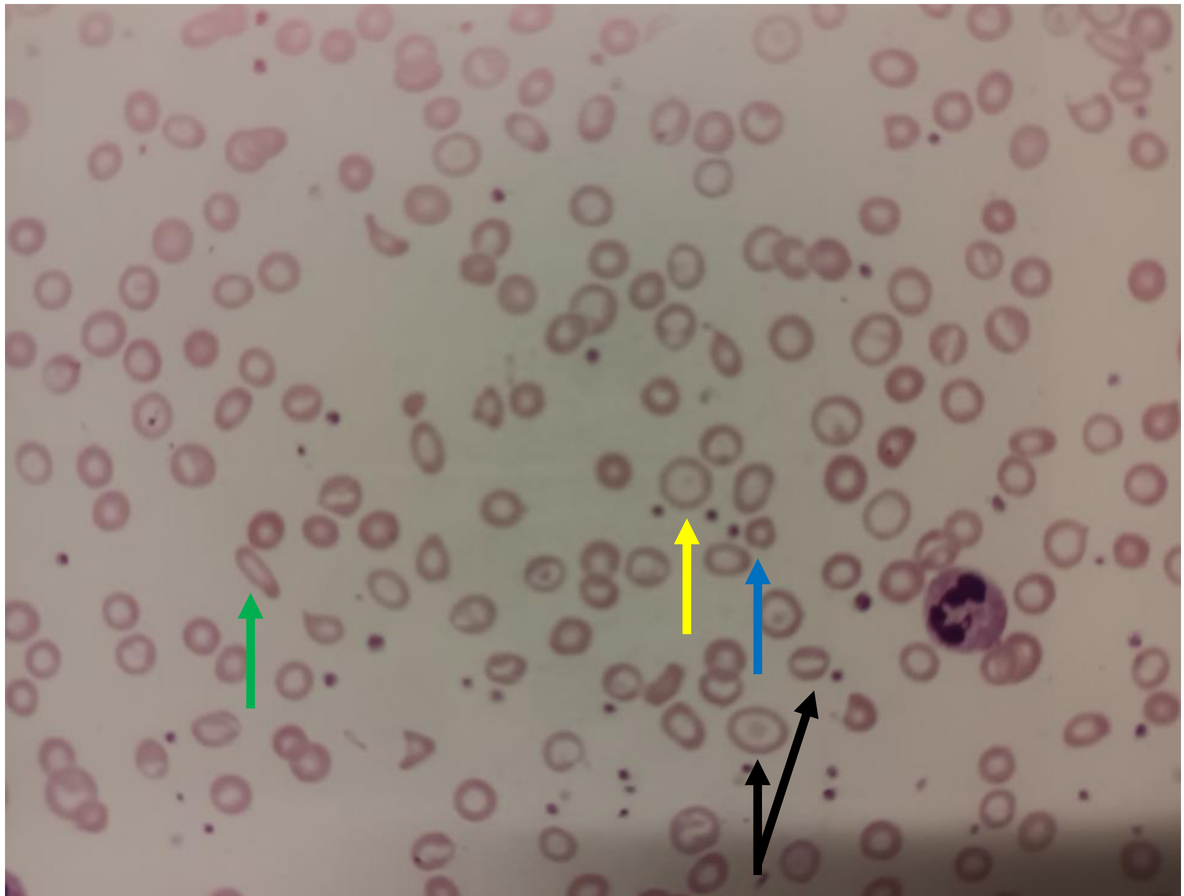

Figure 2: Image of a microscopic view of a peripheral blood smear stained with Wright stain under 100X magnification. The smear is from a patient with iron deficiency and its features are displayed in the smear such as microcytes (smaller than normal sized cells- blue arrow), hypochromic cells (increased central pallor- yellow arrow), anisocytosis (uneven size of the cells- black arrows), elliptocytes (elliptical in shape- green arrow). Image source: Prepared by the author of this Master's Thesis.

Anaemia may cause morphological aberrations of the RBCs and careful examination of these abnormalities can aid in diagnosis of type of anaemia present. Non-haematology tests such as bilirubin, haptoglobin, urinalysis and faecal analysis can be performed to test the presence of haemolysis (break-down of RBCs in circulation) and to find evidence of acute and chronic blood loss (McKenzie & Williams, 2019).

2.8. Ret-He and Iron levels

Reticulocytes are younger RBCs generated in bone marrow during erythropoiesis and released to the peripheral blood stream upon maturation. Peripheral blood samples contain approximately 1% reticulocytes, and Ret-He is a measurement of haemoglobin in reticulocytes. High precision automated analysers are used in laboratories to measure the reticulocyte count and their immature fractions. The immature fractions are transient populations that contain qualitative information regarding the changing iron status of the body. Measurement of the haemoglobin content of reticulocytes (Ret-He) can be used to assess not just the iron availability in the body but also to monitor the response of bone marrow to anaemia (Ucar et al., 2019). Brugnara et al., (2000) have shown that the Ret-He can reflect the iron levels accurately in an iron-deficient state because reticulocytes and red cells have a lower haemoglobin due to reduced bioavailability of iron. The measurement of haemoglobin in reticulocytes provides a direct quantification of iron availability for the process of erythropoiesis, thus detecting iron deficiency at an early stage (Wardhani & Oehadian, 2021). The clinical utility of Ret-He in detecting anaemia by providing expended information at cellular level and predicting bone marrow's iron stores has been demonstrated by Lourenco et al., (2019) whose team has looked into the utility of this parameter to differentiate between functional iron deficiency anaemia and true anaemia as well.

There have been reported cases of poor correlation between Ret-He and ferritin in cases with borderline ferritin levels and cases with presence of mixed anaemias (Choorapoikayil et al., 2025). Choorapoikayil's team have reported that the cause of anaemia other than iron deficiency can be chronic inflammation, chronic kidney disease, bone marrow disorders, acquired or inherited genetic conditions, vitamin deficiencies or other haematological malignancies. When these co-morbidities are present with anaemia, ID may not necessarily be the primary cause for anaemia and hence the correlation between Ret-He and ferritin is poor (Choorapoikayil et al., 2025).

2.9. Advantages of Ret-He over Ferritin

Various literature and recent laboratory practices around the world have established that Ret-He is a superior indicator of iron deficiency compared to the classical methods such as serum ferritin measurement (Auerbach et al., 2020; Gelaw et al., 2019; Poffenroth et al., 2017; Rao & Mirji, 2024 and Wardhani & Oehadian 2021). Additionally, Miwa et al., (2010) have shown that compared to ferritin, MCV and transferrin saturation, Ret-He is an earlier indicator of bone marrow's iron recovery status for patients who are iron deficient and are undergoing iron therapy.

Ferritin and C-Reactive Protein (CRP) are acute phase reactants, which increase during acute inflammation, infection or other stress conditions. This markedly reduces the reliability of ferritin for iron status monitoring during those physiological states. These markers can acutely elevate and their reliability for iron monitoring markedly reduces in those inflammatory states and chronic diseases. Ret-He, however, is not affected by acute phase response and has been demonstrated to be a superior indicator of bone marrow iron status during these conditions (Markovic et al., 2007). A combination of high inflammatory state and iron deficiency can result in a significant anaemia development which needs to be closely monitored and considered during management and therapy. A combined measurement of Ferritin, CRP and Ret-He can provide essential tools to help with that management of ID. (Jimenez et al., 2015).

2.10. Soluble Transferrin Receptor (sTfR)

During inflammatory states both CRP and ferritin are raised, and iron storage status of the body can be masked by the elevated ferritin. Soluble Transferrin Receptor (sTfR) test is often performed in laboratories to check the iron status of the body in anaemic patients who are in inflammatory states. This test is particularly often requested in CHL for patients who may have inflammation, infection of chronic conditions where ferritin concentration does not correlate with iron status. Conditions where ferritin results are unreliable are inflammation, infection, chronic disease, malignancy, pregnancy, neonates, competitive athletes, cystic fibrosis patients, transplant recipients and type-I diabetes with iron deficiency. In these conditions,

sTfR is used to evaluate suspected iron deficiency (Canterbury Health Laboratories, 2025). Immature RBCs adjust the number of transferrin receptors on their cell surface and is proportionately detected in the serum. In an iron deficient state, the receptor density increases and sTfR concentration rises subsequently (Braga et al., 2014). Immunoassays in laboratories can target sTfR in serum and detect small concentrations of it. sTfR is decreased when body iron levels are decreased and vice versa.

While sTfR has been shown to be a marker of erythropoietic activity and is not affected by inflammation (Ashiq et al., 2021), the clinical utility of sTfR is limited for ID screening in severe inflammation conditions. sTfR levels are not directly regulated by inflammatory cytokines and are generally regarded as independent of acute-phase responses, they can be indirectly influenced in severe inflammatory conditions due to suppression of erythropoietic activity. In disorders such as AI, malignancy, advanced chronic kidney disease, and critical illness, inflammatory cytokines promote hepcidin up-regulation and impair erythropoietin signalling, resulting in reduced proliferation of erythroid precursor cells (Means, 2020; Koury & Haase, 2015). As circulating sTfR reflects erythroid cell mass and cellular iron demand, diminished erythropoiesis may lead to normal or reduced sTfR values even in the presence of functional iron deficiency (Piva et al., 2010). Therefore, while sTfR can assist in differentiating iron deficiency anaemia from inflammation-associated anaemia in mild to moderate inflammatory states, its diagnostic utility is limited in severe inflammation where changes in erythropoiesis, rather than inflammation itself, predominantly influence sTfR concentrations. The British Society for Haematology recommends that routine use of sTfR is not recommended for investigating iron deficiency because of limited standardisation, assay variability and lack of robust studies (Fletcher et al., 2022). Moreover, poor turn-around-time and higher cost compared to Ret-He add to the downsides of sTfR. It takes at least 4 days for a sTfR result, compared to a few hours for Ret-He result and sTfR is close to 10 times the cost of a Ret-He test.

2.11. Ret-He: Clinical Utility during Iron therapy and EPO monitoring

2.11.1. Iron Therapy

Cancer cells have high metabolism and thus need more iron stores for survival. Tumour cells manage this by causing a disbalance in the hepcidin pathway to increase cellular iron uptake and decrease export (Vela & Vela-Gaxha, 2018). Management of iron levels in conditions such as Non-Hodgkin's lymphoma is an important therapeutic process as serum hepcidin levels have been found to be directly related to tumour activity (Tisi et al., 2014). Thus Ret-He could be an important laboratory parameter to quickly reflect the available iron levels during this therapeutic monitoring process.

2.11.2. EPO Monitoring

The erythropoietin (EPO) hormone regulates the productions of RBCs in the bone marrow. In an anaemic state, in response to low oxygen levels, feedback mechanisms in the body stimulate EPO production which aids in the subsequent release of immature red cells in the circulation (Jelkmann, 2011). EPO monitoring is an important part of therapeutic process in clinical conditions such as Renal Cell Carcinoma where EPO production is significantly increased and can result in polycythaemia (Kopel et al., 2019). Tumour activity in these conditions have been shown to be directly related to the EPO levels in bloodstream because the cancer cells are themselves involved in the production of the hormone. Peerschke et al., (2016) have shown that Ret-He is particularly useful in a cancer-care setting to evaluate iron deficiency via its clinical utility to monitor EPO levels. A reduced Ret-He level can indicate that the bone marrow lacks sufficient iron to effectively produce RBCs which in turn triggers the EPO stimulation. In cases of iron deficiency, despite a rise in EPO, haemoglobin and Ret-He levels remain low due to inadequate iron availability (Ervasti et al., 2008). This assists clinicians in optimising EPO dosing, reduce the risk of ineffective treatment, and avoid unnecessary transfusion, especially in patients with iron utilisation limitations due to inflammation or malignancy (Means, 2020).

2.12. Anaemia of Inflammation and Functional Iron Deficiency

Anaemia of inflammation (AI), previously known as anaemia of chronic disease, is a commonly encountered mild to moderately severe anaemia that develops from an inflammation due to impaired erythropoiesis paired with shortened RBC survival. AI can be seen in individuals with of chronic infection, immune activation, cytokine mediated suppression of bone marrow function and systemic inflammatory disorders. (Ganz, 2019). The inflammation in these patients is due to infections, auto-immune diseases, certain haematological malignancies and cancer. These patients present with low transferrin saturation, high ferritin, elevated CRP and normal transferrin receptor levels (National Institute of Diabetes and Digestive and Kidney Disease, 2025).

AI and IDA are the two most common anaemias in the world, and they often coexist in patients, of developing countries, with nutritional deficiencies and infections (Shaw et al 2011). As for patients with better access to health care and iron-rich nutrients, AI is linked with chronic systemic inflammatory disorders (Shaw et al 2011). AI is similar to IDA in terms of low serum ferritin levels, but in contrast to IDA where body iron stores are depleted, AI is primarily a result of impaired iron metabolism and distribution (Ganz, 2019). Inflammatory cytokines, especially IL-6 causes increased hepatic production of hepcidin in response to inflammation. Hepcidin, being the principal regulator of systemic iron balance, prevents iron export from macrophages and enterocytes and iron is trapped in storage sites rendering it unavailable for haemoglobin synthesis (Goodnough et al., 2010). Cytokines produced during inflammation also reduce bone marrow’s sensitivity to erythropoietin which contributes to the underproduction of RBCs and subsequently causes anaemia (Means, 2020).

Differential diagnosis of AI and IDA is challenging due to similar laboratory result patterns. Patients with AI show mild anaemia, a low reticulocyte count and slightly reduced MCV (Means, 2020). Iron studies in AI shows characteristic pattern of low serum iron and transferrin. Ferritin levels are not a reliable marker in AI due to co-existing inflammation. Soluble transferrin receptor (sTfR) tends to remain normal or only mildly elevated, providing some value when differentiating AI from IDA (Goodnough et al., 2010). However, Ret-He is an important laboratory test that aids

in differential diagnosis of AI and IDA because it is not affected by inflammation. Ret-He has been shown to be markedly decreased in IDA but normal in AI without iron deficiency (Thomas et al., 2013). In patients who underwent bone marrow assessment, CHr (equivalent of Ret-He) was found to be a more reliable indicator of iron deficiency compared with MCV, serum ferritin, or transferrin saturation (Mast et al., 2002). Canals et al. (2005) evaluated 504 patients with anaemia of chronic disease or other conditions causing iron restriction. Using a 25pg cut-off, Ret-He alone could differentiate between iron-deficient and iron-sufficient individuals with a sensitivity of 0.76. However, there was considerable overlap among patients with AI, mild iron deficiency anaemia, and reduced iron stores. Thus, incorporating Ret-He into diagnostic algorithms has been shown to improve the accuracy of anaemia assessment in chronic inflammatory conditions.

A common occurrence in patients with AI, chronic diseases, inflammation, and malignant diseases is an impaired supply of iron for RBC synthesis despite adequate iron stores in the body. This state of mismatch between iron availability and demand is called functional iron deficiency (FID) and this often leads to iron-restricted erythropoiesis and anaemia. In this disorder, the presence of adequate iron stores is demonstrated by stainable iron in the bone marrow and serum ferritin values are within normal limits. However, there is a partial block in the iron transport to bone marrow which results in iron unavailability for erythropoiesis (Goodnough et al., 2010). The high levels of hepcidin and IL-6 produced during inflammation in AI and chronic diseases further reduce the iron transfer to developing RBCs leading to FID. This makes it challenging for clinicians to predict the response to intravenous iron therapy in patients with AI and chronic diseases. Chuang et al. (2003) have shown that CHr compared favourably with other measures of iron status when predicting a response to intravenous iron. Traditional iron markers such as serum iron, ferritin, and transferrin saturation are less reliable to measure FID due to concurrent inflammation presence. Ret-He has been shown to be markedly reduced in absolute iron deficiency but only slightly reduced to normal in FID. Thus Ret-He has been shown to be a tool to differentiate absolute ID from FID which is otherwise challenging to differentiate by other iron studies parameters (Thomas et al., 2013).

2.13. Haemoglobinopathy and Thalassaemia:

Anaemia is also caused by alteration in the structure or synthesis of the haemoglobin molecule which is also known as haemoglobinopathy. The altered structure leads to change in biochemical properties such as the function, stability or solubility of the protein complex.

The quantitative disorder of the haemoglobin molecule is collectively known as thalassaemia. Thalassaemia constitutes a group of inherited disorders related to the mutation of one or more globin genes leading to the reduced production of haemoglobin (McKenzie & Williams, 2019). Disorders in the haemoglobin molecule can be caused due to mutations of the genetic sequence such as deletion, addition of promoter or a stop codon, nonsense, splice site etc. Theses mutations can lead to the decrease, increase or a complete absence of one or more of the globin chains of haemoglobin (McKenzie & Williams, 2019).

The decreased production of haemoglobin, ineffective production of RBCs and disproportionated production of unaffected globin chains are the hallmark features of thalassaemia (McKenzie & Williams, 2019). Symptoms of thalassaemia can range from asymptomatic to fatal depending on the type and amount of missing globin chains required for the synthesis of the haemoglobin molecule. Patients suffering from thalassaemia can present with anaemia, bone disorder, gall stones and can have higher likelihood of infection and inflammation (McKenzie & Williams, 2019). Laboratory tests of the peripheral blood can provide various clues to the type of thalassaemia a patient might have inherited. CBC tests including morphology of RBCs in blood samples of patients from thalassaemia show a characteristic microcytic, hypochromic anaemia often paired with decrease in MCV, MCH and MCHC. Sign, symptoms and the laboratory features of microcytic hypochromic anaemia (for e.g., IDA) are very similar and further tests would be required to find out the etiology of the anaemia and for differential diagnosis of the types of thalassaemia (McKenzie & Williams, 2019).

Ret-He is generally reduced in cases of congenital microcytic anaemia including thalassaemia and haemoglobinopathies. Because thalassaemia and haemoglobinopathies are related not only to iron deficiency but can also cause iron overload, a differential diagnosis between IDA and thalassaemia becomes important

(Perchard et al., 2023). Perchard's team studied Ret-he results of 145 patients of various age groups in the paediatrics population (<18 yrs) and concluded that Ret-He is more reliable in the non-thalassaemic patients with acute or chronic inflammation. While studies by Kadegasem et al. (2019) have recommended a Ret-He cut-off for IDA screening in thalassaemia trait patients, other studies have concluded that the reliability of Ret-He decreases during presence of thalassaemia trait (Buttarello et. al., 2016; Lian et al., 2019). This is because of the inability of current laboratory resources to determine the cause of reduced Ret-He which could be due to decreased ferritin levels or reduction of globin production. The reduction of globin production results to reduced alpha or beta globin chains of haemoglobin molecule seen in thalassaemia and haemoglobinopathies (Buttarello et. al., 2016). A study that included 443 samples from children and adults has concluded that while Ret-He alone is not a reliable test for iron levels in patients with haemoglobinopathy and thalassaemia; however, the researchers have shown that a combination of Ret-He, MCH, MCV, ferritin and RBC count is a powerful tool to identify thalassaemia and thalassaemic haemoglobin variants (Sudmann et al., 2012). Sudmann's team (2012) developed an algorithm that yields a higher specificity in separating beta-thalassaemia trait from non-haemoglobinopathy patients and displayed that Ret-He is an integral part of the algorithm.

2.14. Automation in Haematology Laboratories:

The use of automated analysers has progressively increased over the last 50 years and as a result automation is an integral part of current haematology laboratories. The evolution of the automated analyser due to advances in haematology instrumentation, current day analysers are known for their increasing precision and accuracy leading to their increasing reliability for hematological tests.

The Sysmex XN analysers use a combination of principles to produce results for the various aspects of the CBC and WBC differentiation tests. Blood cells are poor conductors of electricity and when they are passed through an electrical field, the increased resistance is measured using a principle called the impedance principle. This principle, also known as the Coulter principle, was founded in 1948 by Wallace H Coulter and is widely regarded as the most technological innovation in the field of

modern haematology (Robinson, 2013). The Coulter principle allows for enumeration of RBCs and platelets and their respective volumes. This electrical gating test is performed at very high speed and precision by modern day analysers yielding counting of up to 10,000 cells per second and a complete analysis of the CBC test in less than a minute. Histogram analysis of the accumulated resistance events and separation based on size is used to distinguish RBC and platelet counts (Figure 3).

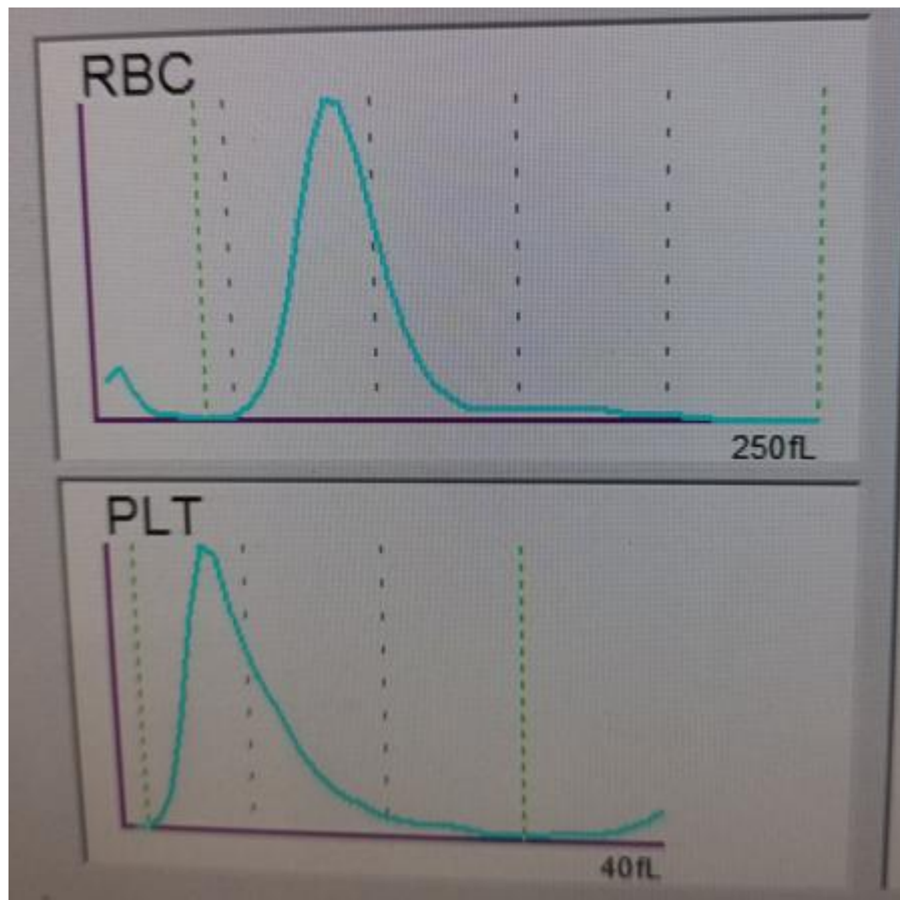

Figure 3: RBC and Platelet histogram the RBC channel of a Sysmex XN 9000 series analyser used for enumeration of RBC and Platelets. The X-axis is in units of volume (femtoliters) and the Y axis is the frequency of cells of that volume. Image source: Prepared by the author of this Master's Thesis.

After the enumeration of RBCs and platelets, the flow cytometry principle is then used to further analyse the cellular and intracellular components in blood. Enumeration of the nucleated cells such as WBCs and their differentiation is performed using a series of cell-lysis and flow-cytometry principles (McKenzie & Williams, 2019). A complete break-down of the RBCs allows for further analysis and differentiation of the WBCs from the same blood sample. Using a principle called

hydrodynamic focusing. Cells are pushed through a flow cell that allows measurement of cell volume, conductivity, and light scatter properties. Perforation of the cell membranes allows for tagging of the intracellular materials, mostly nucleic acids, using fluorescent markers and labels which results in separation of the WBC sub-population (Sysmex Europe, 2025). The intensity of the fluorescence signals is directly proportional to the amount of nucleic acid present in the sample. Florescence flow cytometry separates the cell population based on the light-scattering properties against the cell volumes yielding in a 3D scatterplot where cells with similar cytochemical and nuclear properties are gated in the same area in the scattergram. Measurement signals from forward scatter (FSC), side scatter (SSC) and side fluorescence (SFL) are used to produce scatterplots which are further analyzed by computer software algorithms (Sysmex Europe. (2025). An image of a scatterplot from a normal blood sample depicting the various subpopulations of WBCs in the WBC differentiation channel of a Sysmex XN-9000 series analyser is shown in Figure 4. Abnormal results are identified by the software by the usage of various rules and software generated flags or user-defined flags. Medical laboratory scientists and technicians in the haematology laboratory process the samples and analyse the scatterplots and other results produced by these automated analysers.

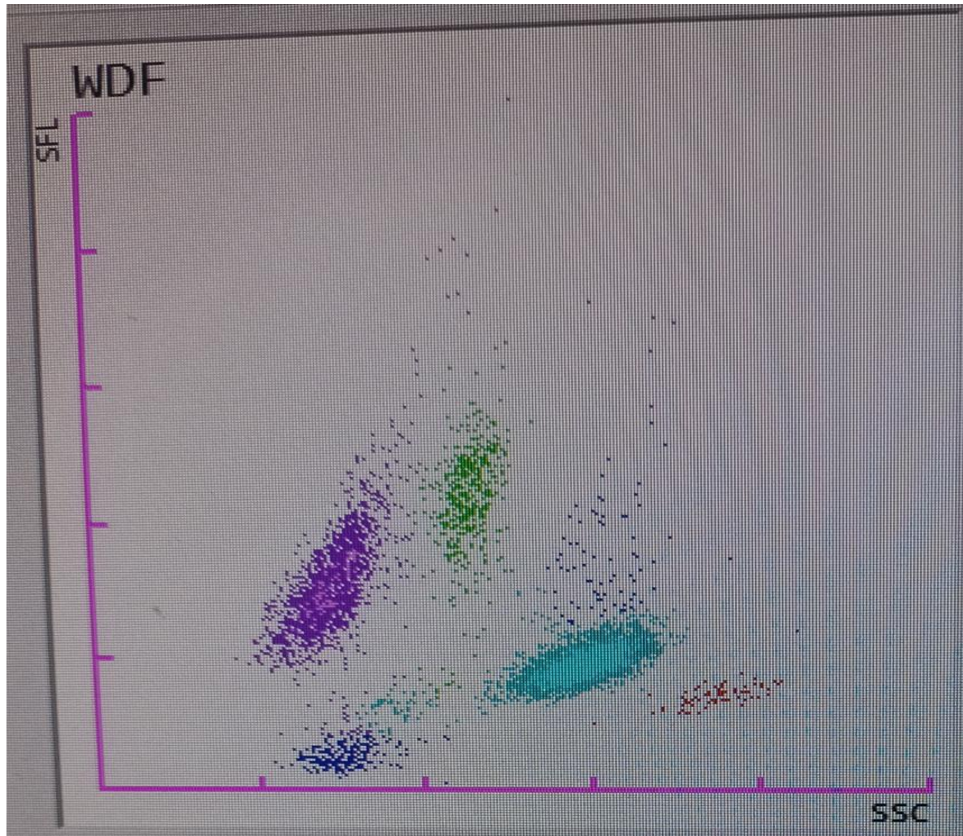

Figure 4: Scatterplot of a normal blood sample in the WBC channel of the Sysmex 9000 series analyser. Abbreviations: SFL= Side fluorescence, SSC= Side scatter, purple scatterplot cluster =Lymphocytes; green= Monocytes; aquamarine= Neutrophils + Basophil. Image source: Prepared by the author of this Master's Thesis.

2.15. Laboratory analysis of Ret-He:

Traditionally, supravital stain was used to stain the reticulocytes in the blood film smear and manual counting was performed via microscopic evaluation. The residual RNA in the immature red cells picks up the stain and reticulocytes appear blue, or blue green but mature red cells do not pick up the stain and appear pink or greenish blue. (McKenzie & Williams, 2019). Automated analysers such as Sysmex XN 9000 series use fluorescence markers in the RET channel to gate immature RBC populations based on their size and fluorescence (RNA content). Ret-He parameter is routinely available in these analysers as part of the Reticulocyte count test. In the RET channel, membranes of RBC, White Blood Cells, and Platelets are perforated by a surfactant reagent. Fluorescent markers in reagent dyes are used to tag nucleic

acids of cells including reticulocytes and cell populations are separated using forward scattered light and the fluorescence signal. Thus, the automated analyser uses a combination of laser excitation, fluorescent markers and flow cytometer counters to isolate reticulocyte cell cluster which is represented in a scatterplot (Roccafortea et al., 2021). As shown in Figure 5 below, cells can be categorized into the three stages of reticulocyte maturation—low fluorescence (LFR), mid fluorescence (MFR) and high fluorescence (HFR), with the degree of immaturity directly proportional to the degree of fluorescence.

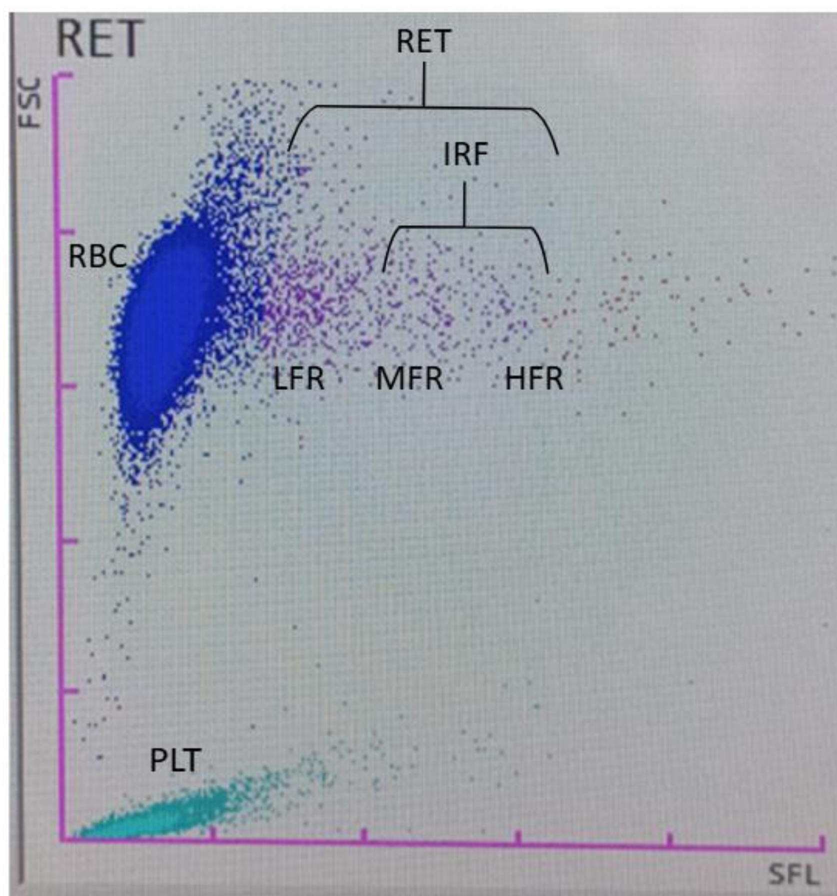

Figure 5: The RET channel of a Sysmex XN 9000 series analyser displaying a scatterplot of the subpopulations of reticulocytes. Abbreviations: RET= Reticulocyte; IRF= Immature Reticulocyte Fraction; LFR= Low Fluorescence; MFR= Medium Fluorescence; HFR= High Fluorescence. SFL = side fluorescence light intensity; FSC = forward light scatter; PLT= Platelets; RBC= Red Blood Cells. Image source: Prepared by the author of this Master's Thesis.

Scatterplots from the RET channel of a blood sample with normal results for Haemoglobin, Reticulocyte and Ret-He count is displayed in Figure 6A and one with reduced levels of those parameters is shown in Figure 6B.

Figure 6A Figure 6B

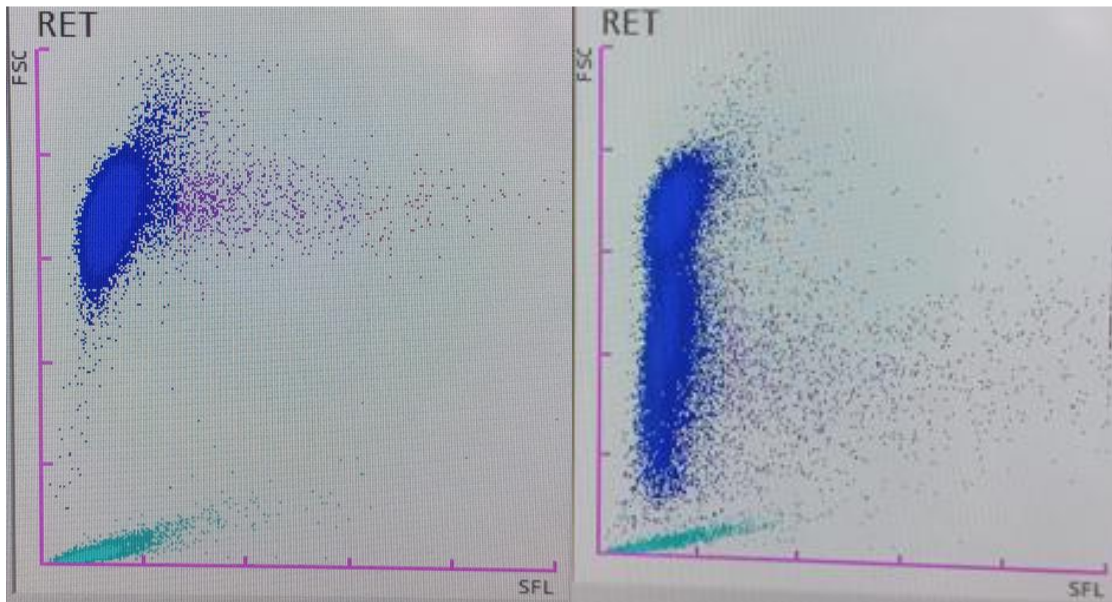

Figure 6: RET channel scatterplot of a blood sample with normal Ret-He count in figure L(left), and that of a sample with low Ret-He count in figure R (right). FSC denotes cell volume and SFL denotes RNA content. RBC population is displayed in dark blue; Reticulocyte population is displayed in purple; Platelets' cluster is displayed in aquamarine. Abbreviations: SFL = side fluorescence light intensity; FSC = forward light scatter. Image source: Prepared by the author of this Master's Thesis.

In an iron-deficient state (Figure 6B, above) the scatterplot of the reticulocyte population shifts down along the Y axis—indicating a reduction in the overall size of the red cells (decreased MCV) and towards the right along the Y axis—indicating an increase in the population of immature reticulocytes that have high RNA content (Schoorl, 2016).

2.16. Summary:

This literature review has described the importance of iron, haemoglobin and CRP in the human body and their dynamics during iron deficient and anaemic states. There are several laboratory tests that can be performed to assess the iron levels and availabilities but each tests have their limitations. Various literature has shown that Ret-He is one of the haematology tests that can be used as a tool to understand iron availability especially in cases of ID, IDA and during conditions of inflammation. However, many studies have reported that Ret-He has its own limitations as well during other comorbidities and haematological conditions like thalassaemia and hemoglobinopathies. There are limited studies that have included data from New Zealand population. There are various range of normal Ret-He levels published and the cut-offs of Ret-He for diagnosis of ID and IDA seem to vary as well.

3.0 METHODS:

3.1 Ethics approval

This study involved sample collection from humans, laboratory tests on human blood samples and use of human demographics. The Health and Disability Ethics Committee (HDEC) approval was not required for this study as the research was out of the scope for their review. Institutional ethics approval has been gained for this study from Massey University Human Ethics Committee (MUHEC) under ethics application ID OM1 24/51 in 8th of October 2024 (Appendix 7.3). A locality authorisation was gained from Health New Zealand before the data collection phase as the laboratory tests were performed in Canterbury Health Laboratories (CHL), Christchurch. *Health New Zealand Canterbury Research consultation with Māori* was completed before the data collection of this study.

3.2 Site of Testing:

This study was performed at Canterbury Health Laboratories (CHL), Christchurch, New Zealand. CHL is a Health New Zealand (Te Whatu Ora) pathology service provider and tertiary level reference and teaching laboratory in New Zealand. It is the largest laboratory in the South Island and one of the two reference labs in the country. CHL is accredited under International Accreditation New Zealand (IANZ) which is the accreditation body of the Testing Laboratory Registration Council in New Zealand. The haematology department at CHL has fully automated Sysmex XN-9000 series haematology analyser that processes over 1100 CBCD samples a day. This department offers CBC & WBC differentials (CBCD) and reticulocyte count (RET) testing as part of the 'CBCR' haematology test package. At the time of writing, the Ret-He test is currently a non-reportable research parameter at CHL, that is generated automatically as part of the RET testing. All tests, with the exception of Ret-He, were part of IANZ accredited test profiles of CHL. The tests performed for this study were conducted under standard laboratory conditions and were controlled by both internal and external quality control processes.

3.3 Patients and sample collection

This study comprised of two groups of blood samples: firstly 50 samples, (Male: Female = 1.0) for reference interval determination; secondly 353 samples (Male: Female = 1.66) for Ret-He versus ferritin, serum iron and CRP evaluation.

All samples were selected from patients who had previously presented to CHL for CBC and iron studies tests. Samples from pregnant (n=17) and paediatric (age <16 years, n=15) patients were also collected but test results of these cohorts were excluded from data analysis for a final sample size of 321 for Ret-He performance.

The following metadata was collected from the request form (refer to Appendix 7.2 for an example of request form) for each sample: Sample collection date, laboratory information system (LIS) episode number, location of sample collection, sex, test requests and clinical details. Data such as ethnicity were not collected as it was outside the scope of the proposal. Lab results (iron, transferrin, saturation, CRP, ferritin) were collected for each sample prior to addition of CBC and Ret-He tests.

Consent from patients for Ret-He test addition was not collected because the Massey University ethics committee granted a waiver of consent in accordance with section 7.49.b of National Ethical Standards of the National Ethics Advisory Committee of New Zealand. The ethics committee concluded that the results of this test would not affect patient management on the grounds that iron levels of the samples in this study's cohort were already being monitored by the requesting clinicians.

3.4 Laboratory analyses

The samples selected for Ret-He analysis were EDTA (Ethylenediaminetetraacetic acid) blood samples which had previously been collected from patients for Complete Blood Count with WBC Differentials (CBCD) tests and had been stored in refrigerated conditions for not longer than 6 hours from CBCD testing. Samples were collected in a tube containing EDTA anticoagulant. EDTA prevents the clotting of the whole blood by chelating calcium ions and preserves the morphology of the cells without significant alteration in size and shape of RBC, WBC and platelets. Moreover, anticoagulated blood samples remain stable for analysis in automated analysers for up-to 24 hours (McKenzie & Williams, 2019). Samples of patients with existing CBC,

Iron studies and CRP were used for addition of reticulocyte tests and analysed in an automated haematology analyser (Sysmex XN20). Ret-He is part of the reticulocyte test panel in the analyser. The existing iron studies were results of tests performed in the Biochemistry department of CHL. These tests were performed using Beckman Coulter DXI-800 analysers. Iron studies results including iron, transferrin, transferrin saturation, CRP and ferritin were extracted from Delphic Explorer (DE) which is the Laboratory Information System (LIS) in use at CHL.

3.5 Assessment of anaemia and iron deficiency:

The reference intervals used during this study are from CHL's LIS system and are listed in Table 3.

Table 3: Haematology Reference intervals utilised in Canterbury Health Laboratories.

Test	Reference interval	Units
Ret-He	n/a	pg
Haemoglobin- adult males	130-175	g/l
Haemoglobin- adult females	115-155	g/l
CRP	<5	mg/l
Serum iron (>18 yrs)	10-30	μmol/L
Transferrin Saturation	16-45	%
Ferritin- adult males (15-50 yrs)	20-350	μg/L
Ferritin- adult males (>50 yrs)	20-400	μg/L
Ferritin- adult females (15-50 yrs)	20-150	μg/L
Ferritin- adult females (>50 yrs)	20-300	μg/L

As per the ferritin and haemoglobin (Hb) intervals in Table 3, samples were further classified as shown inTable 4.

Table 4: Definition of ID, Anaemia and IDA in this study.

	Iron Deficiency	Anaemia	IDA
Male	Ferritin <20 μg/L	Hb <130 g/l	Presence of both iron deficiency and anaemia
Female	Ferritin <20 μg/L	Hb <115 g/l	

3.6 Study design and statistical analysis

This study utilised comparative non-experimental design and explored the relationship between Ret-he, ferritin and CRP levels on blood samples from human participants.

Data plotting was performed on Microsoft® 2010 Excel (Microsoft® 365 subscription). The data collected for reference interval calculation was analysed via various graphical and numerical tests to assess for normality. A normal distribution curve, often called a Gaussian curve, was plotted using IBM® SPSS® 20.0 software (year 2021) to analyse the shape of the histogram for the test of normality. This frequency histogram is an estimate of the probability distribution of a continuous variable and should be symmetric about the mean and bell-shaped if the distribution is normal. Furthermore, the normal and expected quantiles were plotted against each-other to form a Quantile-Quantile (Q-Q) plot. A normally distributed data yields a straight line on a Q-Q scatterplot (Mishra et al., 2019).

The Shapiro-Wilk and Kolmogorov–Smirnov tests were used to test for normality using IBM SPSS 20 software. These tests are widely used statistical methods for a goodness-of-fit between a sample data and a normal distribution. These tests measure the skewness or symmetry of the data and assume a null hypothesis that the same data is from a normally distributed population. The Shapiro-Wilk test is suitable for smaller sample sizes (<50) and the Kolmogorov–Smirnov is suitable for sample sizes larger than 50. In both the tests, if the significance level (p-value) is >0.05, the null hypothesis can be accepted, and data can be concluded to be normally distributed (Mishra et al., 2019).

The Chi-square test of independence is a non-parametric statistical tool that enables researchers to measure variables at a nominal level. It provides valuable information to determine if the association between the variables are significant or not. Using a significance level of 0.05 (p-value), this test calculates the likelihood of the observed data's occurrence by random chance. Null hypothesis of a chi-square test assumes that there is no association between the observed and expected frequencies, and when the p-value is ≤ 0.05, the null hypothesis can be rejected (McHugh ML., 2013). The chi-square calculations were performed in this study via an online calculator using Social Science Statistics website (Social Science Statistics, 2025). A 2x2 contingency table was setup prior to chi-square calculation. The results of this calculation were used to determine if the relationship between Ret-He values, haemoglobin and ferritin were statistically significant ($p \leq 0.05$).

The Chi-square results were further used to calculate the sensitivities and specificities of Ret-He for ID and IDA. Sensitivities and specificities are diagnostic test evaluation parameters that can demonstrate the likelihood of a presence or absence of a disease in a clinical diagnostic setting. Sensitivity is the proportion of true positives that are correctly identified by the test whereas specificity is the proportion of true negatives that are correctly identified by the test. A highly sensitive test is good for screening for a disease and specificity is a means to measure a test's ability to avoid false positives (Akobeng A., 2007). Sensitivity and specificity are calculated as follows:

Sensitivity= True Positive/ (True Positive + False Negative)

Specificity= True Negative/ (True Negative + False Positive)

Receiver Operating Characteristic (ROC) curve analysis is a graphical statistical tool widely used in clinical settings to assess the diagnostic performance of a test and to compare performance of multiple tests. This curve is obtained by plotting the sensitivity (y-axis) and specificity (x-axis) parameters of the concerned test. The curve is then used to calculate the Area Under the ROC Curve (AUC) to quantify the general accuracy of the test as a diagnostic marker (Nahm FS., 2022). 95% Confidence Interval (CI) range was calculated using a non-parametric approach according to Hanley & McNeil, 1982. An AUC of 0.5 generally does not indicate a diagnostic ability since it signifies that the test is only as good as chance at

distinguishing positive and negative cases. Since an AUC of 1.0 demonstrates a perfect cut-off for diagnosis and indicates perfect discrimination, an AUC above 0.8 are generally considered to have high clinical utility. A Youden’s index (calculated as Sensitivity+Specificity-1) is used to determine the optimum cut-off value with the best statistical balance of sensitivity and specificity (Çorbacıoğlu & Askel, 2023).

In this study, ROC curve analysis was performed using the STATA 18 software (year 2023). ROC curves were generated by plotting sensitivities (true positive rate) in Y axis against false positive rates (1-specificities) in X axis and AUC was calculated to quantify the over ability of Ret-He to discriminate between positive (for eg. iron deficient) and negative (for eg. non-iron deficient) cases. Youden’s index was calculated to determine a Ret-He cut-off with the best statistical balance of sensitivity and specificity.

4.0 RESULTS

4.1. Reference interval calculation

A total of 50 samples were used to check for normal distribution of Ret-He for the establishment of an adult reference interval. Table 5 provides the dataset used to determine the reference interval and the results.

Table 5:Results of the dataset used for reference interval calculation.

Sample no.	Ret-He (pg)	Sample no.	Ret-He (pg)	Sample no.	Ret-He (pg)	Sample no.	Ret-He (pg)
1	32.3	16	34.4	31	35.3	46	36.8
2	32.7	17	34.4	32	35.4	47	37.1
3	32.8	18	34.5	33	35.4	48	37.2
4	32.9	19	34.5	34	35.5	49	37.4
5	33.3	20	34.5	35	35.5	50	37.4
6	33.3	21	34.5	36	35.6		
7	33.4	22	34.5	37	35.7		
8	33.5	23	34.6	38	35.7		
9	33.5	24	34.9	39	35.8		
10	33.7	25	34.9	40	35.8		
11	33.8	26	34.9	41	36.3		
12	33.9	27	35	42	36.3		
13	34.2	28	35.2	43	36.4		
14	34.3	29	35.2	44	36.5		
15	34.3	30	35.3	45	36.5		
Mean +/- standard deviation			34.9 pg +/- 1.3				
Median of all results			34.2 pg				
Reference interval			32.4 - 37.5 pg				

Tests for normality of the distribution of reference interval sample data were performed and 95% confidence intervals were used to calculate a distribution curve which yielded an expected bell-shaped curve symmetrical about the mean (Figure 7). The bell-shaped curve and symmetry about the mean allows for assumption that the Ret-He data analysed is normally distributed.

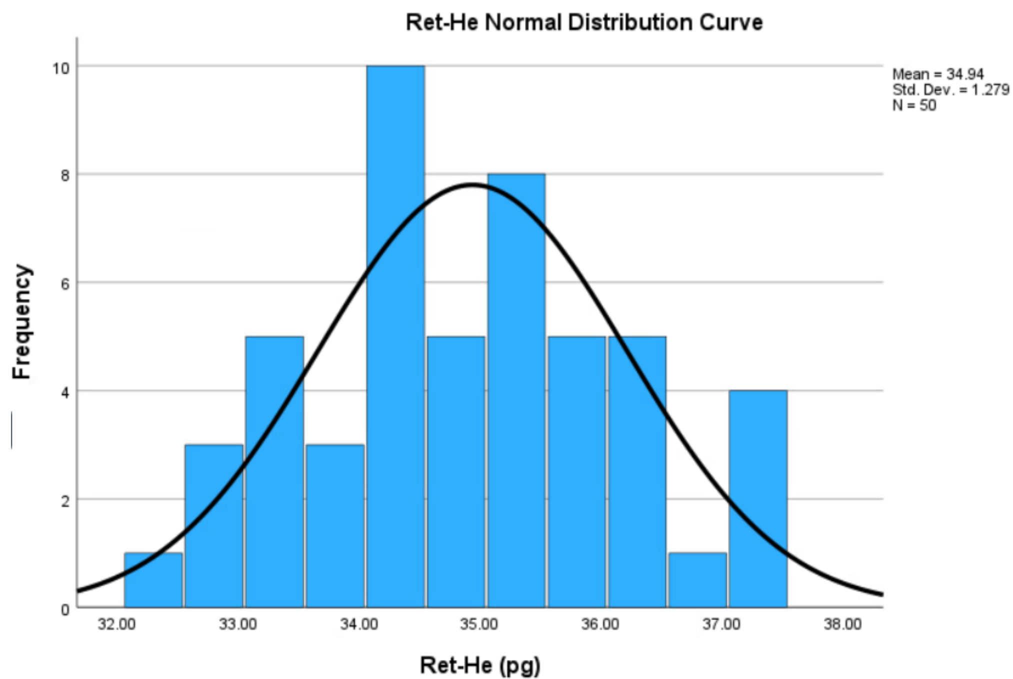

Figure 7: Frequency histogram of the Ret-He normal distribution curve generated using IBM SPSS 20.

A Q-Q scatterplot was generated to compare the distribution of the expected and the observed Ret-He data (Figure 8). A trendline of the points on this graphical plot formed a straight diagonal line, adding further confidence that the observed Ret-He data is statistically equal to the expected Ret-He data.

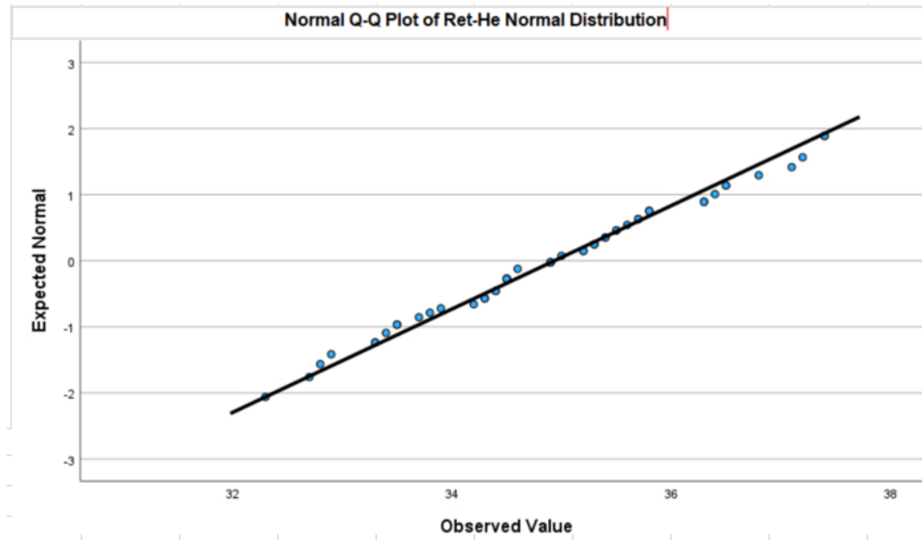

Figure 8: Q-Q plot results for assessment of normality of the data set used for calculation of Ret-He reference intervals. The points in this plot fell in a straight line demonstrating that the two data sets have similar distribution.

Both Kolmogorov-Smirnov and Shapiro-Wilk tests for normality confirmed the collected Ret-He data was normally distributed (Table 6). The calculated reference interval for Ret-He based on the normal distribution curve from these 50 samples was 32.4 - 37.5 pg. The mean of the data was 34.9 and Standard Deviation (SD) was 1.27. The reference interval was calculated as 2-SD from either side of the mean.

Table 6: The Kolmogorov-Smirnov and Shapiro-Wilk test results generated using IBM SPSS 20.

	Kolmogorov-Smirnov® Test		Shapiro-Wilk® Test	
	Statistic	Sig.	Statistic	Sig.
VAAR00001	0.073	0.200	0.982	0.650

The significance level (Sig.) or the p-value for both of these tests were above 0.05.

4.2. Performance of Ret-He participating samples

A table of the data collected from the 321 participant samples for this section of the study is presented in appendix 7.1. Tables of data of the excluded pregnant and paediatric populations are presented in Appendices 7.4 and 7.5 respectively.

The medians of Hemoglobin, ferritin and Ret-He results of the different categories are listed in Table 7.

Table 7: Medians of Haemoglobin, Ferritin and Ret-He,

	n	Haemoglobin (g/L)	Ferritin (μg/L)	Ret-He (pg)
Ret-He performance samples	321	129	64.5	34.0
ID	66	129	64.5	34.0
IDA	18	99.5	11	25.2
IDA male	7	103	10	22.9
IDA female	11	102	12	27.6

n= number of samples. Ret-He = reticulocytes haemoglobin equivalent

A box-whisker plot (Figure 9) displays the Ret-He values among the different population groups. It was found that Ret-He in ID and IDA were significantly decreased (p= 0.00001) in comparison with non-IDA population.

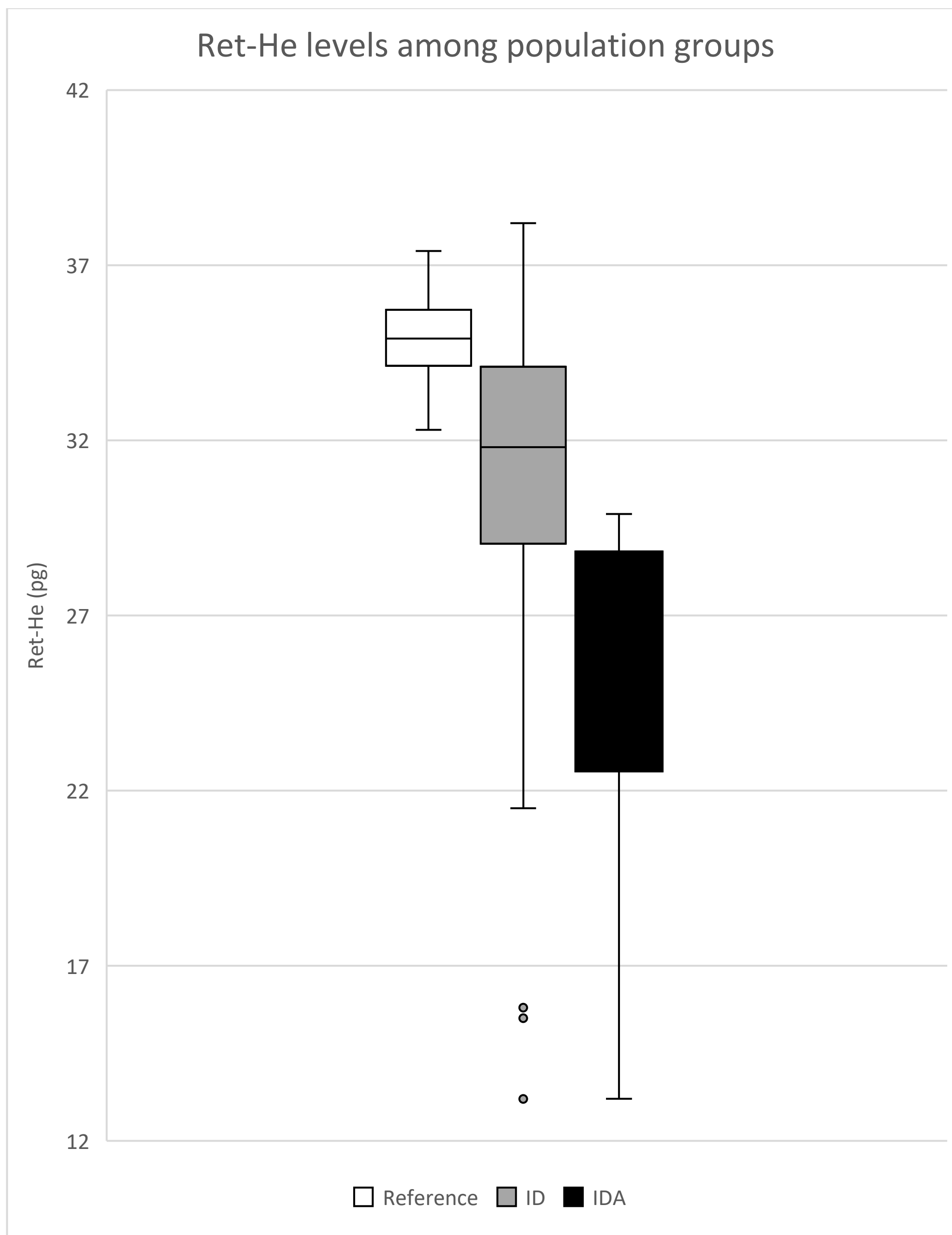

Figure 9: Box-and-Whisker plot displaying the Ret-He values among the different population groups. Abbreviations: Ret-He = reticulocytes haemoglobin equivalent; ID= Iron Deficiency; IDA= Iron deficiency Anaemia.

4.3. ID and Ret-He

A 2×2 contingency table comparing serum ferritin (<20 μg/L vs ≥20 μg/L) with Ret-He (<32 pg vs ≥32 pg) showed a statistically significant association ($\chi^2 = 28.4347$, $p < 0.00001$; Table 8). Ret-He results were compared against haemoglobin and ferritin results.

Table 8: 2x2 contingency table of Ferritin vs Ret-He with chi-square statistic and p-value

Iron Deficiency	Ret-He (pg)		Chi-square statistic	p-value
	<32	≥32	28.4347	<0.00001
Ferritin <20 μg/L	34	32		
Ferritin ≥20 μg/L	45	210		

Ret-He = reticulocytes haemoglobin equivalent

Sensitivities and specificities of Ret-He at various cut-offs for the detection of iron deficiency are presented in Table 9 below. Youden's index (J = sensitivity + specificity − 1) identified the optimal balance at a Ret-He cut-off of 34 pg (J = 0.307).

Table 9: Sensitivities, Specificities and Youden's indices of various Ret-He cut-offs for ID.

Ret-He (pg)	26	28	30	32	33	34	36
Sensitivity	0.152	0.182	0.364	0.561	0.636	0.742	0.955
Specificity	0.980	0.957	0.778	0.665	0.618	0.565	0.208
Youden's index (J)	0.132	0.139	0.142	0.226	0.254	0.307	0.163

Ret-He = reticulocytes haemoglobin equivalent.

A ROC curve analysis was performed to determine the optimum Ret-He cut-off for iron deficiency. The area under the curve (AUC) was 0.739 (95% CI 0.68–0.80) (Figure 10).

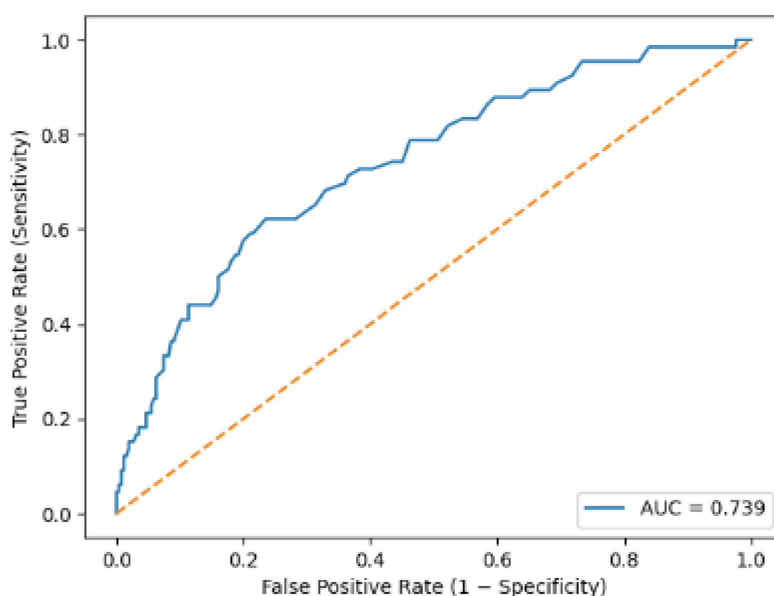

Figure 10: ROC curve for Ret-He in the diagnosis of iron deficiency. AUC = 0.739 (95% CI 0.68–0.80).

4.4. IDA and Ret-He

Ret-He levels in IDA were significantly decreased (p= 0.00001), in comparison with non-IDA population of both sexes (Tables 10 and 11). Sensitivities and specificities were calculated at various Ret-He cut-offs to determine the optimum value for determination of IDA (Table 10). Ret-He displayed the highest sensitivity at 24 pg and highest specificity at 30 pg with Youden's index *(J = sensitivity + specificity − 1)* identified the optimal balance at a Ret-He cut-off of 24 pg (J = 0.71) (Table 10).

Table 10: Sensitivities and Specificities of various Ret-He cut-offs for IDA.

IDA		Ret-He (pg)						
	32	30	28	26	24	23	22	20
Sensitivity	0.23	0.39	0.52	0.67	0.75	0.71	0.66	0.75
Specificity	1.00	1.00	0.98	0.97	0.96	0.96	0.96	0.95
Youden's index (J)	0.23	0.39	0.50	0.64	0.71	0.67	0.63	0.7

Ret-He = reticulocytes haemoglobin equivalent, IDA= iron deficiency anaemia.

A 2x2 contingency table evaluating the lower limit of the reference interval (Ret-He cut-off of 32 pg) for IDA is presented in Table 11 (χ^2 =45.1.50, p<0.00001).

Table 11: 2x2 Contingency Table of IDA versus Ret-He at a cut-off 32 pg with chi-square statistic and p-value.

Iron Deficiency	Ret-He (pg)		Chi-square statistic	p-value
	<32	>32	45.1059	<0.00001
IDA	18	0		
Non-IDA	61	242		

Ret-He = reticulocytes haemoglobin equivalent, IDA= iron deficiency anaemia.

4.5. CRP, Ferritin and Ret-He

The relationship between raised CRP, raised ferritin and reduced Ret-He is displayed in Figure 11. About 90% of samples with raised CRP levels also had normal or raised ferritin levels, but over a third of those samples had reduced Ret-He levels. Moreover, 42% of samples with raised CRP and markedly elevated ferritin levels (>400 µg/L) had reduced Ret-He levels.

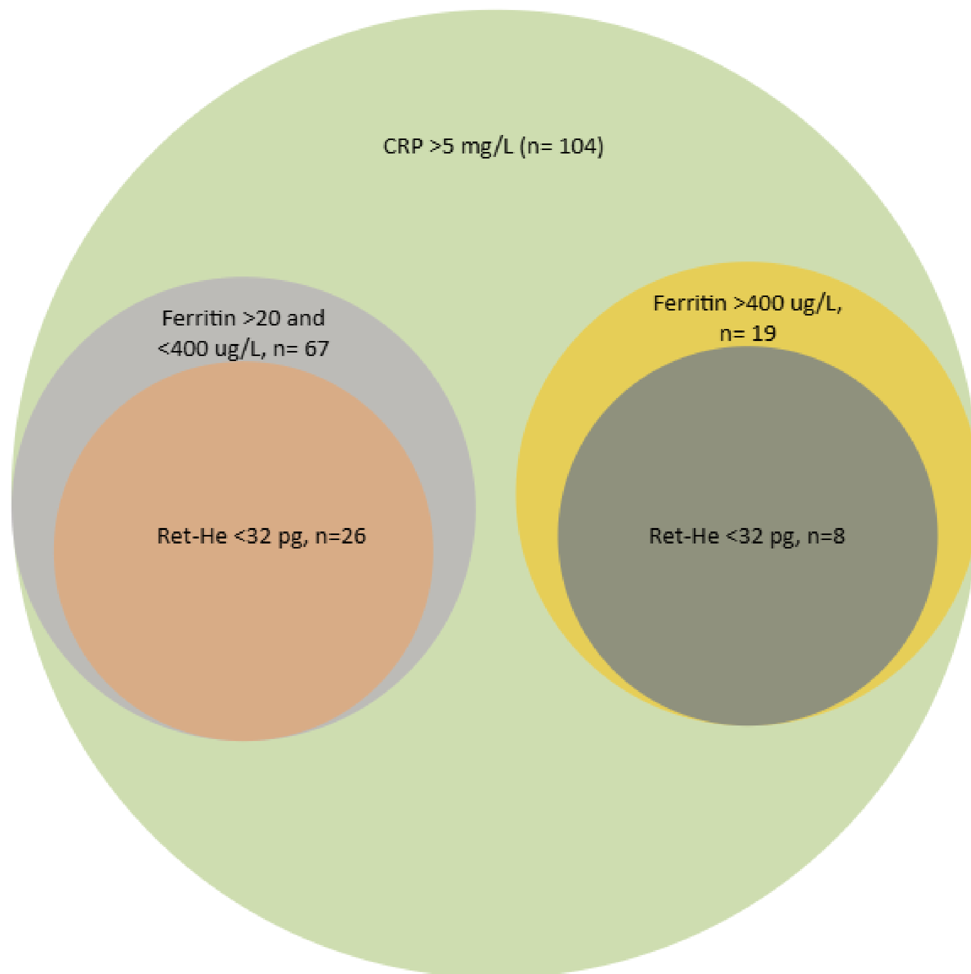

Figure 11: Venn Diagram of number of samples with CRP results >5 mg/L. CRP= mg/L, ferritin- μg/L, Ret-He=pg.

5.0 DISCUSSION:

This study was conducted with an aim to explore the clinical utility of Ret-He test for iron studies. Reference intervals for Ret-He calculated from 50 normal samples was 32.4 - 37.5 pg with a median result of 34.2 pg. Retrospective Ret-He analysis in 321 samples with existing Complete Blood Count (CBC) and iron studies tests and comparison of Ret-He results with ferritin was performed. There was a statistically significant difference in Ret-He values between iron deficient and non-iron deficient populations. Similarly, Ret-He was found to be significantly lower in Iron Deficiency Anaemia (IDA) population compared to non-IDA population. ROC curve was analysed and Area Under the Curve (AUC) was calculated with 95% CI and Youden's index was found to be highest at a Ret-He cut-off of 34 pg for ID. Ret-He displayed the highest sensitivity for IDA at 24 pg and highest specificity for IDA at 30 pg. Based on the comparison results, at cut-off levels of 34 pg and 26 pg, Ret-He could be useful for diagnosis of ID and IDA respectively. Ret-He was also found to be lower in 32.6% of the cohort with high CRP where ferritin was raised or normal, thus concluding that Ret-He can add clinical utility in assessing iron status when CRP is raised, and ferritin (which is an acute phase reactant) results are unreliable.

5.1. Exclusions

This study excluded test results from pregnant participants (n=17) and paediatric participants (n= 15, age<18 Years old). Pregnant participant samples were excluded because The pathophysiology of iron stores and metabolism of pregnant women is different compared to non-pregnant women due to maternal adaptation to pregnancy and increased iron demands to support fetoplacental development. Research has shown that iron requirements, iron absorption behaviour and iron recycling in the maternal body change during the various trimesters of pregnancy. As maternal RBC mass increase in adaptation to the increasing masses of foetus and placenta, iron demands rise in later stages of pregnancy (Bothwell, 2017). Thus, a separate study containing a larger sample size specific to pregnancy bloods is recommended to assess the relationship between iron and Ret-He levels during various stages of pregnancy.

Paediatric participant test results were excluded from data analysis in this study due to the inadequate sample size for confirmation of published age-related reference intervals. Ret-He parameter for ID screening has been found to be less effective in children due to high false positive rates (Hatuon et al., 2014). Hatuon's team have also found that it is more difficult in children to distinguish between ID and thalassaemia trait, so it is not recommended for use in paediatric population with high thalassaemia traits. Firdous et al., (2023) and Cheng et al., (2024) have excluded samples from paediatric patients with haematological malignancy and shown that Ret-He reference intervals varies with age groups. However, there are more other studies which cite the increasing use of Ret-He in paediatric population for screening of ID and IDA (Perchard et al., 2023). This illustrates that Ret-He in this age-group could have farther scope than current practice in laboratories and site-specific studies need to be performed before implementing in clinical practice. It is recommended that a separate study is recommended with a larger sample size for specific age-categories for the paediatric participants.

5.2. Reference intervals

The reference interval determined for this study was 32.4 - 37.5 pg with a median result of 34.2 pg. This range and median are higher compared to similar studies (Poffenroth et al., 2017; Chinudomwong et al., 2020). The sample size of Chinudomwong was much larger (n= 155) compared to this study (n=50). Poffenroth's team, a New Zealand based study, published a lower reference interval of 30.3 - 35.0 pg with a median of 32.7 pg using 66 normal samples. Similarly, the lower cut-off of reference interval of Ret-He in Health NZ Waikato Lab, a similar sized New Zealand laboratory in Waikato, is 28.9 pg (Health New Zealand Te Whatu Ora Waikato, 2025).

CHL participates in Waikato Quality Assurance Program (WQAP), a weekly external quality assurance program medical laboratories across New Zealand participate in to satisfy IANZ requirements. This external quality assurance program allows laboratories to compare results performed on a same batch of sample against other participating laboratories. WQAP includes reticulocyte count along with other haematology tests. This EQA program has 28 participants from New Zealand and

includes both smaller and larger sized laboratories comparted to CHL. Most of the participants use Sysmex XN series analysers. Ret-He parameter is also included as part of the reticulocyte count survey. Review of CHL's EQA performance history of the 14 participations (including the time the study samples were processed) revealed that Ret-He results are consistently above the mean of all the results (3.5% on average). This illustrates that an adjustment of the Ret-He channel of the XN analysers could be beneficial before this study's reference interval is implemented for clinical settings. It is recommended that a correction factor be applied to the reference intervals based on the RET channel adjustment. Alternatively, a recalculation of reference intervals based on a fresh set of data is recommended during a new-test verification/validation process of CHL. Moreover, to create a reference interval using a larger data set, a collection of all datasets used in New Zealand laboratories that currently report Ret-He could be performed.

5.3. Sex-based differences of Ret-He results:

Women naturally tend to maintain lower iron levels and storage (as measured by serum ferritin) in their body compared to men (Ryan et al., 2022) and hence are more prone to IDA. A nationwide study in the United States has shown that 11% of premenopausal women (16-49 yrs) maintain lower iron levels and experience iron deficiency compared to <1% men of the same age group. Moreover, up to 5% women suffer from IDA in this age group compared to <1% men (Looker et al., 1997). Our study's results reflect similar sex-based differences in ferritin levels as well. Among the 65 participants in this study who were classified as iron deficient, 80% were females. Participants (n=18) from that category had IDA of which 61% were females. Aedh et al., (2023) have discussed sex-based differences in laboratory parameters for tests such as iron studies, CBCD, reticulocyte count and Ret-He. Aedh's team analysed CBCD and Ret-He results from 108 outpatients (excluding people with known haematological malignancies) in Saudi Arabia. It was found that in a population with normal haemoglobin concentration and low ferritin levels the decrease in Ret-He levels was statistically significant only in the male population. While Ret-He level had a tendency to decrease in the female population of the study of Aedh's team, it was not statistically significant. Thus, the team concluded that Ret-

He is a good indicator of decreased iron levels only in men if the haemoglobin levels are normal. Our study's Ret-He results have reflected similar sex-based differences as well. The mean of the Ret-He results of iron deficient females was higher (26 pg) compared to that of males (21 pg) irrespective of the haemoglobin levels. In contrast to these sex-based differences, a small study (n=50) performed by Wardhani and Oehadian (2021), the difference between Ret-He levels was not found to be statistically significant between males and females. This discrepancy suggests that in order to obtain a statistically significant sex-based difference in Ret-He values, a study with a larger cohort of IDA is recommended.

5.4. Ret-He, ID and IDA

A comparison of Ret-He levels with ferritin levels was performed. A ROC curve analysis and Youden's index was calculated to identify a Ret-He cutoff with statistical balance between sensitivities and specificities. Based on our study results, AUC was 0.739 (95% CI) and highest Youden's index was observed at Ret-He cut-off of 34 pg for ID with the sensitivity of 74% and specificity of 56.5% for ID. However, this means that about a quarter of the iron deficient patients will not be picked up by this Ret-He cutoff. The British Society for Haematology (BSH) has published a guideline for the laboratory diagnosis of iron deficiency for adults and children (excludes pregnant population) with multiple recommendations (Fletcher et al., 2022). One of the recommendations is to look at the reticulocyte haemoglobin content if initial tests such as CBCD and ferritin results are inconclusive. The guideline has supported that Ret-He is less dependent on pre-analytical factors and under certain clinical settings Ret-He can predict iron response when ferritin levels are normal or raised. The guideline recommends a Ret-He level <29 pg as a cut-off for supporting iron-deficiency. While this cut-off is lower than the results of our study (cut off of 34 pg), our results are similar to a recent study published by Choorapoikayil et al., (2025), which includes a Ret-He and ferritin comparison on a significantly larger study (n= 2760, IDA= 412, ID= 487). Choorapoikayil's team have achieved a similar area under the curve (0.718) compared to ours (0.739) and have recommended a threshold of 34.6 pg for ID diagnosis in non-anaemic patients. In our study, data analysis to calculate a threshold of Ret-He for non-anaemic patients were not conducted.

Furthermore, a range of older and newer studies have concluded that Ret-He level is decreased in population of both sexes with ID (Auerbach et al. (2020), Gelaw et al. (2019), Poffenroth et al. (2017), Rao & Mirji (2024), and Wardhani & Oehadian (2021). Our study results mirrored this as we found out that average Ret-He value of the 65 ID participants was 30.6 pg, a value well below the Ret-He reference interval. This finding is statistically significant (p= 0.00001) and concludes that a Ret-He below the reference interval is likely to be associated with low ferritin levels.

Since the sensitivity of Ret-He for ID with the cut-off of 34 pg is 74%, it highlights the limitations of Ret-He specially on cases where ferritin is low, but Ret-He is normal. In our study, there were 25 participants where Ret-He failed to pick up a low ferritin result. Perchard et al. (2022) had similar results, especially on patients who have recently been on iron replacement therapy. Normal Ret-He and reduced ferritin was also seen in patients who had very recent depleted iron stores and the haemoglobinisation was yet to be affected. Our studies did not include a full clinical history search on patient's iron therapy status; thus, it was difficult to analyse the relationship between Ret-He levels and ferritins on patients with normal Ret-He but low ferritin results. However, it can be concluded that clinical history in known haematological patients is important in the interpretation of a Ret-He result.

The clinical utility of Ret-He in IDA population was explored via sensitivity and specificity parameters in this study. Our results demonstrated that Ret-He levels in IDA participants were significantly decreased (p= 0.00001) in comparison to non-IDA population of both sexes. This finding is consistent with other studies such as Auerbach et al. (2020), Gelaw et al. (2019), Poffenroth et al. (2017), Rao & Mirji (2024), and Wardhani & Oehadian (2021). We observed a Ret-He cut off of 24 pg to have the highest sensitivity (75%) and 98% specificity. However, this cut-off is lower in comparison to a similar study performed by Choorapoikayil et al., (2025). Choorapoikayil's team who reported a 69.4% sensitivity and 85.7% specificity at a cut-off value of 33.5 pg for Ret-He in the IDA group. Our results showed a poor sensitivity rate (23%) but excellent specificity rate (100%) at Ret-He cut-off of 32 pg. At Ret-He levels of 24 pg, the sensitivity and specificity were optimum, and Ret-He can be added as one of the quicker tools for clinicians assessing patient's iron level status.

Ret-He has demonstrated to have potential in adding value in assessing iron supply of the body in anaemic patients with normal ferritin results. In our study, 48 participants had normal iron levels (>20 μg/L) but Ret-He was lower than the reference range (32.4 pg). Of these, 18 females and 13 males were anaemic, demonstrating that this cohort potentially has iron levels disorder as shown by low Ret-He and anaemia status but ferritin alone is unable to demostrate the true iron status of the body. This cohort could include cases of functional iron deficieny where serum ferritin results appear normal or even elevated but is unavailable for erythropoesis leading to an iron-restricted erythropoesis. While the limitation of ferritin is highlighted in these cases, Ret-He in such scenarios could play an important role in adding value to the overall iron studies of the body.

Ret-He has a strong potential to add clinical value in non-anaemic patients. There are a number of studies that have looked into Ret-He in anaemic participants, but limited studies have been done to look at the utility of Ret-He in non-anaemic iron deficient patients. In our study, of the total 65 iron deficient participants, 37 of them were females and non-anaemic. This cohort had an average Ret-He count of 32.6 pg, which is near the lower end of the reference interval (32.4 pg). This illustrates that there could be a significant population of iron deficient samples who may appear as non-anaemic, but Ret-He can portray the true erythropoiesis status of the body compared to haemoglobin level. This is consistent with results of Ehram et al., (2020) whose results displayed that Ret-He can display real time assessment of the functional state of erythropoiesis and as it is 18-36 hours quicker to display true anaemia status of the body compared to haemoglobin.

5.5. Inflammatory states

During inflammatory states, both CRP and ferritin levels are raised, and iron storage status of the body can be masked by the elevated acute phase reactants. In our study, we found out that 90% of the samples with raised CRP levels (n=104) also had normal or raised ferritin levels, but over a third of those samples had reduced Ret-He levels. Because we did not collect age demographics from the participants, analysis of ferritin results above the age-specific reference range was not performed. We chose a ferritin upper cut-off of 400 μg to be classified as 'markedly raised'. In our

study, we found that 32.6% of samples with markedly raised ferritin (>400 μg) had reduced Ret-He levels (<32 pg). While gender and age-specific ferritin ranges are different, our results indicated that a significant proportion of this population may be iron deficient and ferritin levels alone are unreliable, but Ret-He has the potential to portray the iron deficient state of those samples. Our findings are consistent with Markovic et al., (2007) who have concluded that Ret-He can be an important tool to assess functional iron deficiency where Ret-He is reduced, and body's iron status is depleted but ferritin levels are unreliable due to the inflammation status of the body.

At CHL, Soluble Transferrin Receptor (sTfR) test is performed to check the iron status of the body in anaemic patients who are in inflammatory states. This study's results suggest that Ret-He can add a significant value because it is not affected by inflammation and the turn-around-time is the same as a CBC test. At CHL, the cost of performing Ret-He is about three folds less than that of sTfR (cost of CBCD= $15.5, sTfR= $45.78). The higher cost and increased turn-around-time becomes a barrier for laboratories to use sTfR routinely as part of iron deficiency screening tool. Ret-He could be a cheaper alternative. A further local study is recommended to draw correlation between sTfR and Ret-He to check the clinical utility of Ret-He in cases of markedly elevated ferritin and CRP levels.

5.6. Cost analysis

Ret-He has been shown to be advantageous in clinical practice due to its cost-effectiveness and non-invasive nature. There are recent overseas studies which have shown that ferritin and transfusion saturation cost more than the cost of a CBCD test (Tung et al., 2024; Neef et al., 2021). At CHL, there is no additional cost to produce a Ret-He result as long as a CBCD and Reticulocyte test is ordered. This is because Ret-He is performed in the same channel used by a reticulocyte count test. Ret-He results are readily available as one of the research parameters in the Sysmex XN-20 analysers when a reticulocyte test is performed. Table 13 below lists the current costs of these tests in the context of New Zealand by comparing costs between two laboratories- CHL and Labplus, Auckland. As shown in Table 12, the cost of performing a CBCD and Ferritin combined is more than the cost the CBCD

and Reticulocyte count. It is also worth noting that if a ferritin test is requested after the initial request, the cost will be significantly higher if a separate venepuncture is required to test the ferritin levels.

Table 12: Cost of tests in CHL and Labplus.

Tests	CHL Test Costs ($)	Labplus Test Costs ($)
CBCD	15.5	8.55
Ferritin	9.27	11.05
CBCD + Reticulocytes (Includes Ret-He)	15.81	17.67
CBCD + Ferritin	24.77	19.6

Sources: https://www.chl.co.nz/?s=cbcd, https://testguide.adhb.govt.nz/EGuide/?elv=1&name=LabPLUS%20Price%20List&pn=5057&mn=1478&sd=3&ts=12da0febddb.

Aedh et al. (2013) have shown that routine addition of RET testing request can prevent unwarranted iron studies test requests as Ret-He can be a screening test for ID when paired with other parameters of the CBC test package. Ret-He test can be particularly helpful for community general practitioners who are interested in a quicker and cheaper alternative for assessing patient's iron status. Clinicians who need to monitor patients' iron therapy of erythropoietin response can use Ret-He as a cheap alternate to gauze the body's iron levels on a regular basis.

5.7. Comparison with other automated analysers

Automated haematology analysers other than the Sysmex XN analysers are capable of calculating Ret-He equivalent in blood samples and studies have reported near-identical sensitivity and specificity for detecting iron deficiency. In a multi-centre study performed by Chung et al., (2022) comparison of RBCs and reticulocyte indices on a Sysmex XN-3000 analysers was performed against Advia 2021i, manufactured by Siemens. Advia 2021i can calculate Content of Haemoglobin in Reticulocytes (CHr) from EDTA samples which is equivalent to Ret-He in the XN analysers. Chung's team (2022) analysed 971 samples and reported that Ret-He from XN-3000 had a good linear correlation with CHr from Advia 2021i. Moreover, Urrechaga et al., (2011) have compared the reticulocyte parameters on an earlier model of Sysmex analyser

(XE 5000) and Beckman Coulter analyser- LH750. Red blood cell size factor (RSf) is a Ret-He equivalent parameter available in the LH750 analysers and is derived from the combined volume of RBCs and reticulocytes. In Urrechaga's study, comparison of Ret-He and RSf on samples from 417 patients yielded a good correlation with a correlation coefficient of 0.8184, thus concluding that both the parameters can add equal value in the diagnosis of inefficient erythropoiesis defined by iron levels.

5.8. Limitations

There were several limitations of this study. The exclusion of paediatric population and pregnant women due to low sample size was one of the significant limitations as the results obtained from this study cannot be applied to these population groups.

Lack of enough clinical information on the request forms was another limitation of this study. The only source of participants' demographics and clinical information were the blood test request forms. Poor correlation between Ret-He and ferritin would have been easier to explain if relevant clinical information were available in those cases. Moreover, the request forms lacked uniformity in ethnicity information for all participants. Thus, ethnicity information was not collected, and this parameter was not used to interpret data for this study.

Ret-He uses MCV (Mean Cell Volume) as part of the calculation and results can be affected by scenarios that affects RBC volume in conditions such as double RBC population, RBC agglutinations, and during hypo-natremia or hyper-natremia (Mast et al., 2002). This highlights the need to consider the RBC distribution Width (RDW) results when interpretating the Ret-He results. Moreover, during cases of anaplastic crisis where reticulocytes are decreased, clinical history need to be deeply considered while interpreting Ret-He results. The reliability of Ret-He to monitor iron status significantly decreases in these settings due to presence of macrocytosis or drug-induced-macrocytosis (Perchard et al., 2023). In this study, RDW or MCV data was collected but analysis was not performed to draw correlation with Ret-He. Thus, this remains as one of the limitations of the study. A separate study to study relationship between RDW and MCV would benefit clinicians to interpret the Ret-He results in cases that alters the RBCs size and distribution.

5.9. Further recommendations

The sample size for this study was initially 353 (including pregnancy and paediatric samples which were later excluded). It is recommended that a New-Zealand wide study with a larger sample size including all Health NZ districts be done to determine a national reference interval. Ret-He is a non-reportable parameter at CHL and IQC/EQA participation results for Ret-He are not being considered for regular calibration and sensitivity adjustment. It is recommended that a robust analysis of IQC/EQA results be performed, and the analyte's sensitivity parameters be adjusted accordingly. Comparison of our study's results with those of published literature suggests that a recalculation of reference interval studies would be beneficial after Ret-He sensitivity adjustment.

A CBC harmonisation project is currently in progress in New Zealand. Representatives of Haematology laboratories have met several times in-person and digitally from since mid-2024 and are working on a guideline for standardisation of various aspects of the CBC test. The scope of this project is reference intervals, sample integrity, auto validation, rerun/reflex rules, delta check rules, film making rules, critical ranges, and referral criteria to haematologists. The group started to work on CBC but is planning to expand to other parameters such as reticulocytes and coagulation. The only labs currently reporting Ret-He in New Zealand are Lab-plus in Auckland and Waikato Hospital Laboratory. Addition of Ret-He to the harmonisation project would be not only beneficial to those that are already reporting it, but also give confidence to those labs who are yet to add this test to their reporting profile. A nation-wide reference interval study as part of the harmonisation group would help to standardise and establish this test to other laboratories in the country.

There is a discrepancy in reference intervals for ferritin between CHL and World Health Organisation (WHO). While CHL defines iron deficiency as having ferritin levels less than 20 μg/L for adults above 18 years, the WHO cut-off is 15 μg/L for adults above 15 years (WHO, 2020). The WHO also recommends using a higher threshold for cases with concurrent inflammation or infections. In this study there were 23 samples from female participants with ferritin between 15-20 μg/L that would be considered normal as per WHO but have been classified as iron deficient in this study. Moreover, other widely used guidelines such as the Royal Australian College

of General Practitioners (RACGP) and British Society for Gastroenterology also recommend using a higher threshold (30 μg/L and suggest treating ferritin levels 15-30 μg/L for iron deficiency (Goddard et al., 2011; RACGP, 2023). Ferritin levels <15 μg/L has higher specificity but lower sensitivity for iron deficiency. It has been shown that diagnosis of iron deficiency at ferritin level <15 μg/L is easier compared to borderline ferritin levels of 15-30 pg (Wang et al., 2013). The value of Ret-He can be increased if Ret-He can add a diagnostic function with high specificity for patients with this borderline ferritin levels. In this study, a correlation of Ret-He values and ferritin values of samples (n=64) that had within the borderline ferritin levels (15-30 μg/L) yielded a poor correlation coefficient (0.133). However, due to limited clinical information available on the request forms and it was difficult to point out the root-cause of this poor correlation. A larger study to compare Ret-He and ferritin levels in samples with borderline ferritin levels (15-30 μg/L) be beneficial to further analyse the relationship in this cohort and calculate the sensitivity and specificity of Ret-He specific to this cohort.

The only source of clinical information related to participants for this study was the request-forms. Further investigations of participant's comorbidities and related clinical conditions were limited due to ethical restrictions. Clinical conditions such as pre-existing iron deficiency, anaemia of inflammation, thalassaemia, haemoglobinopathies and other haematological malignancies would have helped us categorise participants based on their clinical conditions and determine if the levels of Ret-He would be statistically different for each of the categories. A larger study with increased participants for separate categories is recommended to explore if Ret-He has unique clinical utilities for these clinical conditions. Similarly, a Ret-He study with specific participants who are being monitored for Erythropoietin (EPO) and Intravenous (IV) iron therapy would help determine the clinical utility of Ret-He in these cohorts. There are studies that have shown that the reliability of Ret-He decreases during presence of thalassaemia and haemoglobinopathies (Buttarello et al., 2016, Perchard et al., 2023). It is important for clinicians to treat anaemia not just based on the RBC indices but also based on iron levels due to the possibility of iron overload. Despite the known limitation, studies have recommended a Ret-He cut-off for IDA screening in thalassaemia trait patients. A study that incorporates larger participants for thalassaemia and haemoglobinopathies would be beneficial to draw

meaningful conclusion on the clinical utility of Ret-He for those cohorts. It is recommended that labs that report Ret-He draw clinicians' attention to its poor reliability when IDA presents with a concurrent thalassaemia or haemoglobinopathy. This can be done by developing an automated comment that gets reports with the Ret-He value.

This study was focussed on finding the correlation between Ret-He and ferritin in patients who have already been queried for iron studies tests. A reverse study would be beneficial to find the correlation between ferritin and Ret-He on samples with low Ret-He. This study would include addition of ferritin on those patients with Ret-He below the cut-off value. This study would look further into a cohort of patients with borderline ferritin results and enhance the clinical utility of Ret-He. Ultimately, it would aid to pick up early iron deficiency status based on Ret-He results, on those patients who do not have iron levels tested for. Whilst Ret-He is not a solitary ID screening tool, this reverse study can provide further insight to whether Ret-He can be a cheaper alternative to screen for potential iron deficiency in the community.

The soluble transferrin receptor (sTfR) test is a quantitation test performed at CHL to assess iron levels during inflammatory states where ferritin levels are unreliable. Ret-He can be a quicker and cheaper substitute of sTfR if adequate correlation studies are performed. It is recommended that and in-house comparison between sTfR and Ret-He be performed at CHL to portray the clinical utility of Ret-He in patients undergoing inflammatory states.

The sample acceptance criteria for performing a reticulocyte count at CHL is 'within 8 hours from the sample collection time'. In this study, we tightened that time limit by two hours, and Ret-He test was added to EDTA samples with 6 hours from the time of venepuncture. This assured that the results were not affected by degradation in sample quality due to extended time from collection. Most of the samples used for the study had already been stored in refrigerated conditions and were subsequently warmed in room temperature for 15 minutes before being tested for Ret-He. Brugnara et al., (2006) have demonstrated that Ret-He remains stable for up to 24 hours in room temperature and even longer (up to 72 hours) in refrigerated conditions of 4°C to 8°C. Thus, it is recommended that labs perform their in-house comparison of Ret-He performance for various time-lengths post collection in room

temperature or refrigerated conditions and implement their sample acceptance criteria for this test accordingly.

Our study results demonstrates that low Ret-He levels is linked to ID and IDA and the correlation is statistically significant. However, Ret-He is not a replacement for ferritin level monitoring. It is a cheaper and additional tool to assess early deficiency of iron levels. The final goal of monitoring/treatment of ID and IDA is restoration of iron stores and Ret-He can be a supplementary tool in understanding the iron stores of the body. Ret-He results should be reviewed in parallel with other serum iron studies tests to assess iron status. A combined measurement of ferritin, CRP, CBCD and Ret-He can provide essential tools to help with the detection and management of ID and IDA.

5.10. Conclusion:

Iron studies is one of the routine diagnostic tests in haematology and biochemistry laboratories. While bone marrow biopsy is the gold standard for measing body's iron levels, it is an invasive process. Ferritin is one of the most reliable laboratory tests used to measure body's iron storage. However, ferritin is an acute phase reactant and is often raised during inflammatory states which is marked by raised CRP levels. When CRP is raised ferritin's reliability to assess iron levels decreases. There are other laboratory parameters such as sTfR which can assess iron levels during inflammatory conditions, but the turn-around-time of these tests are higher, and the tests are more expensive. Ret-He measures the haemoglobin content of reticulocytes and reflects the body's iron status. It is a quick and inexpensive laboratory tool to assess iron stores of the body.

To explore the clinical utility of Ret-He, a reference interval was calculated from a normally distributed population. Ret-He results of the participating samples were compared against ferritin and haemoglobin results. Results concluded that there is a statistically significant difference in Ret-He values between iron deficient and non-iron deficient populations. Similarly, Ret-He was found to be significantly lower in iron deficiency anaemia population compared to non-anaemic population. Sensitivity and specificity tests were performed, and ROC curve was analysed to calculate an

optimum cut-off of Ret-He for both ID and IDA for diagnostic purposes. Using cut-off levels of 34 pg and 24 pg, Ret-He could be useful for diagnosis of ID and IDA respectively. Ret-He was also found to be lower in 32.6% of population with high CRP where ferritin was raised or normal, thus concluding that Ret-He can add clinical utility in assessing iron levels when CRP is raised, and ferritin results are unreliable.

Ret-He is an additional tool to assess body's iron status. It is not a complete substitute for the ferritin test where it's reliability decreases when there is concurrent congenital microcytic anaemia, thalassaemia or haemoglobinopathies. This study did not take MCV into account and did not categorise patients based on clinical details. It is recommended that laboratories reporting Ret-He results include adequate information regarding the limitations of this test to help clinicians interpret the results accordingly.

In conclusion, Ret-he is a simple and accessible test in the Haematology laboratory which can be performed with CBCD tests at a nominal cost. Measurement of haemoglobin content of reticulocytes by this test can provide early indication of ID and add diagnostic value in the screening of IDA.

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7.0 APPENDIX

7.1. Results

A table of the results included in the participation of Ret-He evaluation [excludes pregnant (in Appendix 7.4) and paediatric population (in Appendix 7.5)]

SID	Sex	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
1	m	2	3.3	2	50	5	43	1.69	45.8	13.2
2	f	53	1	100	7	96	86	4.14	127.9	31.1
3	m	53	1	100	7	96	83	2.54	70.1	31.8
4	m	2	2.1	4	136	129	158	1.11	57.2	34.7
5	m	22	2.3	38	L3	71	160	1.86	94.9	35.8
6	m	7	1.8	15	10	1153	79	1.47	39.5	32.8
7	f	9	3	12	3	24	104	1.14	40.9	33.6
8	m				3	62	152	1.61	79.4	34.9
9	f	16	2.1	30	12	270	90	1.56	44.3	29
10	m	11	2.3	1	5	162	142	1.42	6.2	34.9
12	f	20	1.8	44	3	873	131	1.38	55.1	37.4
13	m	14	2.6	21	3	119	152	1.89	96.2	33.8
14	m				3	107	151	1.39	71.4	34.3
15	m	12	2.1	23	3	39	122	2.12	87.1	33.8
16	f	10	2.9	14	3	82	127	1.46	72.3	28.9
17	m	16	1.9	34	3	375	103	1.5	48.9	34.9
18	f	9	3.9	9	7	129	148	2.58	138.8	31.2
19	m				5	20	139	1.48	67.2	34.6
20	m	28	2	56	3	130	153	1.46	72.3	36.3
21	m				3	146	162	1.26	66	36.4
22	f				3	58	125	1.12	46.7	34.5
23	f	16	2.6	24	3	117	125	1.54	60.2	36
24	f	14	2.4	23	9	18	124	1.71	71.5	33.7
25	f	5	3.3	6	3	6	124	0.89	39.6	29.5
26	f				6	156	138	1.21	55.3	33.9
27	m				7	82	112	1.87	65.8	34.7
28	f				3	21	140	0.7	30.4	36.1
29	m	20	1.8	44	3	93	141	1.22	59.9	33.3
30	m	7	1.2	23	3	286	85	4.62	110.9	35.4
31	f				3	3	114	1.55	59.5	31
32	f	22	2.7	32	3	49	140	1.31	57.2	36.5
33	f				8	52	141	1.08	55.4	32.9
34	f				5	590	126	2.52	94	40
35	f				3	40	130	1.09	54.6	29.9
38	f				3	187	141	1.54	72.5	34.5

SID	Sex	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
39	f				3	16	122	1.76	73	34.1
40	m	9	3.2	11	3	12	120	1.24	52.3	32
41	f	8	1.8	18	36	105	121	1	39.6	33.8
42	f	3	2.1	6	60	108	106	1.11	41.5	31.1
43	m	21	3.1	27	3	16	179	2.12	131	34.4
44	f	25	4.2	24	4	13	144	1.44	72.4	33.4
45	f				3	115	134	1.15	48.3	36.7
46	m	6	1.8	13	67	193	80	1	25.9	18.4
47	f				3	75	117	2.01	78.2	35.2
50	m	22	3.1	28	3	47	149	0.96	47.8	34.2
52	m				3	235	150	1.59	78.5	35.8
53	m				6	507	162	1.63	93.9	31.3
54	m				3	126	160	1.41	76.7	34
55	m				4	182	147	1.55	77.3	33.7
56	m	11	1.2	36	24	938	112	1.83	63.1	37.2
57	m				8	129	147	1.57	82.3	32.5
58	f	13	3	17	6	29	138	1.61	74.4	33.4
59	f				3	9	105	2.01	70.6	32.6
60	f	5	2.5	8	5	9	82	2.32	68	29.2
61	f				3	27	138	1.6	69.3	37.4
62	f	23	2.8	33	3	32	143	0.79	37	33.7
63	f	<2	2.6		65	36	81	1.6	42.2	29.6
64	m	19	2.3	33	3	439	163	3.3	171.9	36.7
65	m	17	2.6	26	3	68	150	1.58	83.3	33.5
66	f					9	97	2.45	86	28.9
67	f				3	46	126	0.62	27.5	32.3
68	f				7	27	109	3.16	108.1	36.5
69	f	7	3.6	8	3	187	75	5.03	199.7	26.4
71	m	14	2.3	24	3	282	154	1.92	100.2	35.1
72	m	3	1.3	9	146	792	89	1.24	44.8	24.5
73	f				3	36	145	1.21	55.2	36.4
74	m				3	47	149	1.3	64.7	34.6
75	m				41	190	137	1.17	56.7	32.9
76	m	9	0.9	40	12	160	162	1.6	75.7	40.6
77	f				4	30	135	1.7	78	34.2
78	f	8	3.2	10	5	12	116	0.82	37.8	28.6
79	f				3	184	130	1.27	54.9	34.5
80	f				3	84	139	2.03	92.8	34
81	m				3	124	130	1.08	44	36.3
82	f				88	86	128	0.77	32.6	34.3
83	f				6	313	132	2.03	88.3	35.3
84	m	3	2.5	5	12	11	106	0.87	40.3	22.9

SID	Sex	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
85	f				7	39	129	1.71	768	33.2
86	f				4	42	102	2.19	81.5	27.9
87	f				3	59	134	1.21	54.3	33.4
88	f	6	2.6	9	3	45	110	2.65	102.3	29.4
89	f	11	2.9	15	5	23	127	1.8	81.9	32.6
90	m	19	2.7	28	3	34	71	3.52	78.1	33.3
91	f	8	2	16	4	164	90	5.46	153.4	33.3
92	f	10	2	20	3	102	101	1.49	48	35.1
93	f				19	81	130	1.4	65.2	31.1
94	f	30	2.9	41	4	17	136	1.92	80.6	36.3
95	m				50	319	156	0.61	28.7	33.9
96	f	3	2.2	5	236	140	111	1.1	42.5	32.6
97	f				4	316	110	3	100.2	36.1
98	m	11	2.5	18	4	203	153	3.81	205	33.1
99	m	3	3.1	4	3	22	63	3.27	68.3	24
100	f	7	1.6	17	110	177	81	4.46	120.9	31.5
101	m	4	1	16	141	405	70	1.11	27.3	21.7
102	f		3		7	297	154	1.08	63.9	31.5
104	f				3	28	139	1.2	53.8	35.5
105	f	13	2.3	22	17	389	108	2.3	74.5	33.1
106	f	13	2.8	18	3	9	136	1.86	88.7	33.3
107	m				3	393	155	1.94	99.7	35.4
108	m				3	214	142	1.78	87.8	32.8
109	m	5	2	10	24	100	139	1	43.5	34.6
110	f				3	75	146	1.78	79	37.1
111	m	9	1.9	19	12	244	112	1.69	60.3	34.8
112	f				3	136	138	0.96	43.3	34.9
113	f	23	3	31	3	19	78	1.94	53	21.5
114	f				8	24	134	1.25	58.1	31.8
116	f				22	16	112	3.26	118	33.4
117	f	24	1.7	56	3	839	116	1.21	45.7	33.1
118	f	16	1.4	45	197	857	75	2.31	48.5	34.8
119	f				16	14	135	2.01	90.2	35.5
120	f	23	2.6	35	4	49	156	1.71	81.9	37.5
121	f				3	16	130	1.11	49.8	33.9
122	f				3	212	116	1.9	77.9	33.7
123	m	18	2.5	29	12	148	160	1.28	64.5	36.4
125	m	10	1.7	23	3	392	101	2.07	66.4	34
126	f				3	151	133	1.57	64.5	37.2
127	f				4	37	134	1.8	77.6	35.7
128	f	33	2	66	3	54	128	0.94	38.4	36.2
129	f	11	3.3	13	3	18	132	1.62	74.8	32.2

SID	Sex	Iron (µmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (µg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
130	f				14	108	135	2.3	98.9	33.7
131	f				4	4	117	1.02	45.7	29.2
132	f				51	188	142	1.05	48.7	30.2
133	f				5	10	116	1.43	56.8	30.7
134	m	12	1.3	37	125	242	117	0.98	38.8	31.4
135	f				10	27	85	1.16	36.8	27.2
136	f				3	64	129	1.41	62.5	35.5
138	f	21	2.8	30	3	35	133	1.81	79.5	34.9
139	f				7	45	150	1.62	82.3	34.2
140	m				7	229	128	2.63	103.1	36.7
141	m	10	2.4	17	3	242	119	1.07	44.4	33.5
142	f				3	18	120	2.36	94.6	32.4
143	f				5	66	126	2.41	103.4	33.5
145	m				5	149	135	1.1	47.3	35
146	f				3	23	132	1.7	79.1	32.2
147	f	8	2.1	15	3	132	113	0.49	20	32.2
149	m	15	2	30	3	208	143	1.25	60.1	35.2
150	m				42	186	128	0.72	28.9	34.9
151	f				3	51	125	1.34	52.3	35.6
153	m				3	134	144	0.83	37.8	35.9
154	m				3	70	155	1.58	73.6	36.9
155	f	17	3.3	21	4	25	142	1.62	82.5	32.4
156	m				3	158	147	1.4	64.7	36.5
157	f				3	40	127	1.3	55.3	33.2
158	f				4	31	97	1.4	49	30.5
159	f	14	1.3	43	64	232	123	0.77	31.3	31.2
161	m				5	232	141	1.03	50.9	32.6
162	f	5	3.1	6	3	11	86	2.62	82.5	25.7
163	f	26	4.1	25	3	18	124	2.94	120.8	35.6
164	m	12	1.7	28	94	779	81	1.16	29.9	33.5
165	m	16	2.8	23	3	15	137	1.13	45.2	38.2
166	m				3	129	146	1.01	48.9	34.7
167	f				3	23	115	1.33	50	35.3
168	f	15	2	30	3	82	136	1.61	71.8	35.9
169	f	8	1.7	19	15	589	113	2.65	99.4	31.6
171	f				3	48	123	1.7	66.5	35.7
172	f				3	65	138	2.2	97.5	35.3
174	f	6	3	8	5	13	108	1.33	56	27.6
176	f				6	7	138	1.3	57.5	34.7
177	f				6	7	128	2.04	92.8	31.6
178	f				3	51	119	1.01	41.1	33.1
179	m				41	476	139	1.21	53.6	34.2

SID	Sex	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
180	f	12	3	16	5	23	114	1.77	68.9	34.3
181	m	22	2.4	36	3	181	142	1.06	50.9	34.1
182	f	37	1.6	92	7	3200	116	0.3	11.3	41.3
183	f	7	2.2	13	3	32	102	3.21	108.5	32.8
184	f				3	13	135	1.24	52.7	35.1
185	f	6	1.8	13	64	71	85	2.5	69.8	34.7
186	f	17	2.2	31	3	96	129	1.63	64.4	35.8
187	m	<2	1.7	No result	165	191	82	2.09	53.9	27.6
188	f				3	21	136	1.51	69.2	33.9
189	m				3	57	152	2.76	313.9	36.8
190	f				9	23	110	0.87	34.7	30
191	f	17	2.6	26	11	81	142	1.77	84.1	33.1
192	f	15	2.7	22	5	34	113	1.6	61.4	34
193	f				3	91	132	1.79	72.9	36.7
194	m	23	2.8	33	12	729	88	2.9	95.7	38
195	f				3	32	139	1.11	50.1	35.5
197	f	14	1.9	29	3	88	127	2.19	91.8	34.6
198	f				3	184	125	1.15	49.4	33.3
199	f				3	141	128	1.34	56	34.5
200	f	23	2.3	40	4	265	107	1.58	52.3	35.3
201	m	8	1	No result	79	361	82	2.1	52.3	36.8
202	m				3	225	140	2.16	95	36.5
205	f				3	6	124	1.1	52	29.5
206	m				3	33	161	1.19	61.4	36.3
207	f				40	99	132	1.66	62.9	39.1
208	m	14	1.8	31	3	1176	109	1.56	65.4	31.1
209	m	13	1.6	32	15	635	98	1.03	39.7	28.7
210	m	8	2.9	11	3	8	121	1.00	40.9	34.1
211	f				3	72	130	1.57	65.8	36.5
212	m	15	2.5	24	3	72	161	1.17	60.8	36.2
213	f	17	2.2	31	3	72	137	1.09	50.9	34.3
214	m				3	80	140	1.03	51.9	31.8
215	m				3	93	151	0.96	49.4	34.1
216	m	14	2.6	21	5	97	143	2.71	123.0	36.8
217	f	2	1.1		49	319	88	1.96	75.7	30.6
218	m	14	2.8	20	3	33	156	2.08	108.8	34.3
219	f	24	2.6	37	3	35	127	1.31	59.5	32.9
220	f				3	36	144	1.18	55.6	35.9
221	m	13	2.6	20	3	39	135	1.88	82.7	34.6
222	f				3	39	130	1.47	60.0	37.6

SID	Sex	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
224	f	18	2.6	28	3	48	140	1.46	69.1	35.3
225	f				3	51	123	1.25	50.9	34.4
226	f	16	2.4	27	3	219	143	1.41	70.9	33.4
227	f				5	236	144	1.48	70.6	34.8
228	m				14	247	132	1.85	78.1	34.4
229	f				3	250	125	1.58	65.9	32.9
230	f	2	1.9		280	256	125	1.45	58.4	33.9
231	f	9	1.6	22	16	270	122	1.48	58.0	33.9
232	m	16	2.6	24	3	29	118	1.78	75.1	33.7
233	m	3	1.2	10	102	282	83	1.09	28.6	28.7
235	f	23	2.4	38	3	31	135	1.55	67.9	35.7
236	f				3	133	147	1.25	59.3	35.5
237	m	24	2.5	38	6	138	169	2.53	152.6	33.2
238	f				3	14	143	2.06	95.4	35.6
239	m	13	2.4	22	10	143	148	1.17	58.7	34.5
240	m	4	2.1	8	149	1488	144	1.80	85.0	33.4
241	f				6	17	125	1.25	62.0	30.1
242	m				5	164	129	1.89	78.6	36.5
243	f	17	2.2	31	3	19	144	1.46	70.7	33.5
244	f	9	1.7	21	47	202	139	1.65	78.5	32.8
245	f	8	2.3	14	10	218	84	4.89	141.3	30.1
247	f	7	2.4	12	3	19	129	1.52	68.2	33.2
248	f	25	2.4	41	3	22	144	1.30	60.2	35.8
249	m				3	26	120	1.35	57.8	32.8
250	m				3	65	151	1.81	88.7	35.4
251	f				57	585	89	2.22	56.2	37.4
253	f				4	44	145	2.06	101.4	33.0
254	f				3	60	129	1.20	49.0	35.2
255	f				5	106	140	1.51	70.5	34.6
257	m				3	93	147	1.68	81.6	35.5
258	m				3	131	153	1.50	79.0	33.9
259	f	26	2.5	41	3	986	79	3.04	80.3	33.5
260	m	9	2.8	13	3	579	156	1.33	68.1	36.2
261	m				3	249	150	1.49	70.8	36.8
262	m				3	55	127	1.43	57.3	35.8
263	f	9	1.9	19	11	1040	103	2.58	81.5	36.3
264	f				3	47	113	1.34	47.4	36.4
265	f				9	23	120	1.09	55.6	24.6
266	f				3	13	113	1.36	51.3	32.8
267	m	4	1.8	9	45	593	95	1.90	63.3	30.4
268	m	7	2	14	13	393	70	1.46	32.3	34.1
269	m				3	125	151	1.10	53.2	36.9

SID	Sex	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
270	f	13	2.7	19	3	43	120	1.23	47.4	34.9
271	f				185	447	89	1.49	51.4	27.9
272	f				3	19	139	1.78	79.6	35.4
273	m				3	76	113	1.37	49.0	35.6
274	m	6	1	24	252	1944	95	2.18	70.0	28.2
275	m	9	2	18	5	30	118	0.95	38.4	33.1
276	f	7	3.3	8	59	12	103	1.94	69.5	27.8
277	f	12	2.2	22	3	53	115	1.79	61.8	36.0
278	m				6	405	130	1.53	62.9	35.1
279	m	19	2.1	36	3	544	104	1.30	36.4	41.5
280	f				3	27	147	1.35	68.2	33.8
281	m				4	29	143	1.35	65.7	34.3
282	m				3	306	126	0.75	32.1	33.9
283	f				19	219	129	1.86	78.9	33.8
284	f				5	27	122	1.12	51.9	30.6
285	m				3	96	136	1.99	90.7	35.5
286	m	5	3.1	6	8	24	134	1.54	73.3	31.8
287	f				58	17	102	1.88	66.6	28.8
288	m				6	81	145	1.51	71.4	34.2
289	f				6	41	93	4.23	160.3	29.4
290	f				14	307	130	2.98	120.1	35.6
291	f	19	1.8	42	3	520	91	2.59	75.6	33.2
292	m	14	1.8	31	3	116	144	1.61	69.9	37.9
293	f				6	5	103	1.34	63.5	23.5
294	m				3	175	157	1.27	72.5	32.0
295	f				3	35	139	1.01	48.0	33.9
296	f	13	2.6	20	3	19	128	1.34	59.8	34.4
297	f				3	31	124	1.79	78.6	33.5
298	m				6	102	139	2.39	98.0	38.5
299	m				3	158	145	1.35	65.7	34.4
300	m	9	2.1	17	3	588	100	1.85	62.2	33.5
301	f	29	2.7	43	3	48	158	1.97	95.7	37.6
302	f				3	34	110	1.17	42.7	34.9
303	f	3	1.4	9	129	163	69	2.57	61.7	31.1
304	m				27	79	87	1.89	65.6	27.7
305	f				9	63	101	2.02	74.3	29.4
306	f				3	63	132	1.60	65.1	35.9
308	f	10	2.5	16	21	43	94	3.08	90.6	35.7
309	f				6	83	134	1.61	77.1	32.9
310	f				22	85	135	1.58	67.5	35.7
311	m	26	1.5	69	3	101	137	1.43	62.6	35.8
312	f				3	17	131	1.21	51.2	34.8

SID	Sex	Iron (µmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (µg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
313	f				3	13	126	1.72	69.7	34.7
314	f				3	24	134	1.03	44.6	37
315	f	26	2.5	41	3	24	125	1.15	48.2	32.7
316	f	7	2.6	11	12	17	119	1.36	61.9	31.7
317	f				3	25	107	0.65	23.3	34.5
318	m	5	3.9	5	15	7	33	4.27	72.6	15.5
320	f				5	20	132	1.37	57.1	33.5
321	f				3	23	131	1.59	67.9	34.4
323	m	6	3.7	6	14	14	60	4.27	108.5	15.8
325	f	4	2.9	5	7	12	124	1.01	44.2	29.4
326	f	15	3.2	19	3	14	134	1.67	73.6	34.5
327	f	8	3.1	10	3	20	108	1.57	60.6	29.6
328	f	13	3.5	15	3	8	125	1.11	49.3	32.5
329	f	8	3.1	10	3	11	129	1.58	65.6	32.8
330	f	20	3.1	26	3	12	141	2.01	95.3	33.6
331	f	12	2.4	20	3	23	126	1.14	47.2	35
332	m	9	2.9	12	3	25	153	1.84	86.7	37.6
333	m	4	3.1	5	3	7	103	1.74	71.7	24.6
334	f	11	3	15	6	14	117	1.51	66	30.4
335	f				3	13	112	1.51	52.7	36.3
336	m	25	2.7	37	3	23	135	1.48	63.8	35.6
337	f				3	23	144	10.4	50	36.7
338	f	11	2.8	16	3	16	134	1.35	64.9	31.8
339	f				3	22	144	1.65	73.3	36.7
340	f				3	10	131	1.39	70.5	30.2
341	f				3	12	125	1.57	65	31.7
342	f	19	2.3	33	3	24	131	1.41	59.8	35
343	m				3	15	129	0.99	45.7	29.9
345	m	18	3.1	23	3	8	150	1.08	55.8	34.1
346	f	5	3.2	6	5	11	103	0.27	12.9	24.2
347	m	6	3.3	7	6	10	122	0.95	41.5	28.3
348	f				3	20	119	1.04	40.9	36
349	f	4	3	5	3	18	83	24.1	82.2	24.1
350	m				9	16	143	2.29	116	31.5
352	f	16	3.3	19	3	12	134	1.61	78.6	29.9
353	f	4	3.1	5	5	19	112	1.69	63.7	29.2

7.2. Image of a request form used at CHL

Oct 2021 QF00050
Ref: 2402833

GENERAL REQUEST FORM

Surname | Given Names | Extra Copy to | Sample Collect DATE: DD-MM-YY | SAMPLES WHICH CANNOT BE SENT AT THE SAME TIME REQUIRE A SEPARATE FORM

D.O.B. | Sex | Hosp. | Patient No. | Charge to (HIC Code) | TIME: HH:MM | Requested by:

Ward | Consultant | TAKEN BY | Beep No. | MCRN

PROFILES - see pink pages pg 29

Urgent (circle tests required urgently) Phone Fax

LAB USE ONLY - ORDER OF DRAW - INDICATE No. SAMPLES TAKEN

B. CULTURE | CITRATE | PLAIN | LiHEP | EDTA | FLUORIDE | OTHER

Canterbury Health Laboratories will rely on the requestor to obtain informed consent for the requested tests, and any additional related tests, to be performed by the laboratory.

BIOCHEMISTRY ☐ Fasting

BIOCHEM

☐ Na, K ☐ Cl ☐ βHCG (pregnancy)
☐ Ca ☐ BIL
☐ CRN ☐ PO4 ☐ CBIL
☐ UREA ☐ Alb ☐ ALP
☐ URAT ☐ Mg ☐ AST
☐ GLU ☐ AMS ☐ GGT
☐ TPO ☐ CRP ☐ ALT
☐ LIPS ☐ FT4 ☐ CK
☐ Ferritin ☐ TSH ☐ TNI
☐ Iron studies ☐ PSA ☐ Digoxin (Dig)
☐ B12/Folate (VIT) ☐ AFP ☐ Lithium (Li)
☐ Carbamazepine ☐ Cortisol ☐ βHCG (tumour)
☐ Levertiracitam ☐ HbA1c
☐ Phenytoin ☐ Tobramycin
☐ Valproic Acid ☐ Vancomycin
☐ Gentamicin ☐ Lamotrigine

Dose
Dose time Dose date

HAEMATOLOGY

☐ CBCD (+DIFF) ☐ Retic

Patient on anticoagulants: Yes ☐ No ☐
☐ INR ☐ D-Dimer
☐ APTT ☐ Coag Screen
☐ DIC screen

BIOCHEMISTRY

BIOCHEM URINE ☐ Random ☐ 24 hour
☐ UNa, UK ☐ UCRN ☐ UOSM
☐ UPRO ☐ MALB

BLOOD GAS O2 therapy: %
☐ Arterial ☐ Venous
☐ Blood Gas ☐ Co-oximetry
☐ Blood Gas + Electrolytes

Dose duration (in mins.) if IV
Dose interval

MICROBIOLOGY

MICRO URINE **Specify Sample Type**
☐ Midstream ☐ Catheter
☐ Bladder puncture ☐ Bag ☐ Other

Test Req'd
☐ Micro/Culture

FAECES: Test(s) Req'd

☐ **CSF** ☐ **BLOOD CULTURE**
☐ **SPUTUM**
☐ **SWAB** Site:
☐ **ASPIRATE**
☐ **TISSUE** Test:

SEROLOGY*

Tests:

***Duration of illness in days**

IMMUNOLOGY

☐ ANA ☐ Coeliac Screen
☐ Tissue Abs ☐ ANCA Screen
☐ PR3/MPO ☐ ACCP
☐ DNA Abs ☐ Cardiolipin Abs
☐ ENA Abs ☐ IgE (total)
☐ Myositis Ab ☐ Skin Ab
☐ Neuronal (specify below)

PROTEINS

☐ Rheum Factor ☐ Ig's
☐ SFLC ☐ Myeloma Screen
☐ Complement

VIROLOGY

Specimen:
Test:

TOXICOLOGY

☐ Cyclosporin ☐ Tacrolimus
Dose
Dose time Dose date
Dose duration (in mins.) if IV
Dose interval

CLINICAL DETAILS
Recent Transfusions (Y/N)

Drug/Antibiotic Therapy:
Obstet: g ☐ p ☐ wk ☐

OTHER TESTS **Specify Sample Type**

7.3. Massey University Human Ethics Committee approval letter

26/11/2024

Dear: Ramesh Tiwari

Re: Ethics Application - OM1 24/51 - Research to study the effectiveness of Ret-He for Iron Deficiency screening at Canterbury Health Laboratory.

Thank you for the above application that was considered by the Massey University Human Ethics Committee:

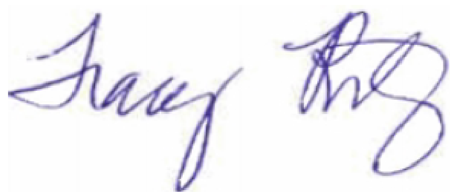
Ohu Matatika 1 at their meeting held on **Tuesday, 8 October 2024**

On behalf of the Committee I am pleased to advise you that the ethics of your application are approved.

Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely



Professor Tracy Riley,
Acting Chair, Research Ethics Chair's Committee

7.4. Excluded Results- Pregnant population

A table of results of pregnant participant samples

SID	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
11	3	3.7	3	3	6	113	1.29	59.9	26.5
37				5	14	144	2.89	135.8	34.4
48	5	3.9	5	3	29	111	1.89	68.8	27.9
49				4	18	113	3.08	107.5	35.8
51				7	55	118	3.29	130.6	33.7
70				3	36	110	1.89	67.1	35.3
103				3	21	115	1.87	78.4	28.9
115				15	10	110	2.12	85	31.1
124				6	8	119	2.28	96.7	32.1
144				3	68	131	1.35	59.3	33.7
160				4	11	115	1.56	60.8	33.4
170				3	82	96	3.4	108.5	32.6
175	30	4.2	28	4	552	111	6.53	237.7	36.8
196				7	29	122	2.1	91.8	30.3
204	20	2.7	29	3	35	108	1.36	48.4	35.5
246				3	27	122	1.80	75.2	34.2
344	9	4.2	9	7	10	122	2.37	115.4	30

7.5 Excluded Results- Pregnant population

A table of results of paediatric participant samples

SID	Iron (μmol/L)	Transferrin (g/L)	Saturation (%)	CRP (mg/L)	Ferritin (μg/L)	Haemoglobin (g/l)	Reticulocytes (%)	Reticulocytes (10^9/L)	Ret-He (pg)
36				5	15	145	1.67	85.3	33.3
137				3	17	120	1.14	48.9	32
148				3	43	147	1.19	62.8	32.8
152	12	3	16	3	34	129	1.19	66.8	27.5
173				3	32	124	2.1	82.3	35.5
203	32	2.2	58	3	71	148	1.71	77.6	37.2
223	37	2.3	64	3	47	138	1.78	81.9	34.4
234				3	31	136	1.17	53.7	35.0
252				3	36	133	2.30	104.7	34.6
256				3	40	140	0.90	43.6	33.1
307				3	37	131	1.72	69.5	37.0
319	2.4	27	3	14	127	0.92	43.9	31.5	
322	2.1	32	3	25	122	1.72	71.6	32.8	
324			3	19	118	1.05	48.3	30.8	78.7
351				3	10	138	1.61	78.7	34