The laboratory assessment of haemoglobin, Ret-He, IRF, plasma ferritin, serum vitamin B12 and folate deficiency in anaemic and non-anaemic women during pregnancy: Can laboratory screening predict outcomes?

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The laboratory assessment of haemoglobin, Ret-He, IRF, plasma ferritin, serum vitamin B12 and folate deficiency in anaemic and non-anaemic women during pregnancy: Can laboratory screening predict outcomes?

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Declaration

With the exception of any statements to the contrary, all the data presented in this report are the results of my own efforts and have not previously been submitted in candidature for any other degree or diploma. In addition, no parts of this report have been copied from other sources. I understand that any evidence of plagiarism and/or the use of unacknowledged third party data will be dealt with as a very serious matter.

Signed:

Dated: 28.07.2022

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List of abbreviations

4PLC	Four Parameter Logistic Curve
ANOVA	Analysis of Variance
ALAS	δ-Aminolevulinic Acid Synthase
CHr	Reticulocyte Haemoglobin Content
CI	Confidence Interval
CRP	C-Reactive Protein
CV	Coefficient of Variation
dUMP	deoxyuridine monophosphate
DMT1	Divalent Metal Transporter 1
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELBW	Exceptionally Low Birth Weight
FBC	Full Blood Count
Fe^{2+}	Ferrous iron
$Fe3^{+}$	Ferric iron
Hb	Haemoglobin
HCP1	Haem Carrier Protein 13
HCRW	NHS Health Research Authority and Health and Care Research Wales
HFR	High-fluorescence reticulocytes
HO1	Haemoxygenase 1
ID	Iron Deficiency
IDA	Iron Deficiency Anaemia
IF	Intrinsic Factor
IL6	Interleukin 6
IMD	Index of Multiple Depravation

IMDD	Index of Multiple Depravation Decile
IDWA	Iron Deficiency Without Anaemia
IQR	Inter Quartile Range
KSD	Kolmogorov-Smirnov D statistic
LFR	Low-fluorescence reticulocytes
IRE	Iron Responsive Elements
IRF	Immature Reticulocyte Fraction
LBW	Low Birth Weight
LIMS	Laboratory Information Management System
MCH	Mean Cell Haemoglobin
MCV	Mean Cell Volume
MFR	Medium-fluorescence reticulocytes
MTHF	Methyltetrahydrofolate
NICE	National Institute for Health and Care Excellence
OLS	Ordinary Least Squares
RBC	Red blood Cell
Ret-He	Reticulocyte Haemoglobin Equivalent
RNA	Ribose Nucleic Acid
RT	Room Temperature
SLS	Sodium Lauryl Sulphate
THF	Tetrahydrofolate
TFR1	Transferrin Receptor
TNF-α	Tissue Necrosis Factor Alpha
TSAT	Transferrin Saturation
VB12	Vitamin B12
VLBW	Very Low Birth Weight

WHO	World Health Organisation

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Abstract

Anaemia in pregnancy affects women in both developed and developing countries. In the UK a high incidence of iron deficiency has been reported, although the incidence of women with iron deficiency in the absence of anaemia is unknown. High incidence has been attributed to socioeconomic background. Iron deficiency is thought to be the main contributor of anaemia during pregnancy, although vitamin B12 and folate deficiency may also be associated. The aim of the study was to evaluate the haematinic variables as predictors of three birth outcomes (gestational delivery age, birth weight and pregnancy loss) in concurrent 1st and 2nd trimester pregnancies for women both with or without anaemia (IDWA) and across socioeconomic groups.

Methods: This was a prospective observational longitudinal study in the East Riding of Yorkshire examining n= 545 pregnant women presenting in the 1st and 2nd trimesters of pregnancy. Haemoglobin (Hb), reticulocytes and associated variables (reticulocyte haemoglobin equivalent (Ret-He), immature reticulocyte fraction (IRF)), plasma ferritin, C-Reactive protein (CRP), vitamin B12 and folate were measured using univariate and multivariate analysis to establish independent predictors of birth outcomes.

Results: At univariate level, associations with birth outcomes were seen for reticulocyte count, Ret-He, IRF, ferritin, delta Hb, delta ferritin, delta Ret-He and delta IRF. There was a significant difference between live birth and pregnancy loss for Ret-He, IRF and vitamin B12. At multivariate level, Hb, reticulocyte count, Ret-He, IRF, delta Hb, delta IRF and IMDD were shown to be independent predictors of birth outcomes.

Conclusion: The use of reticulocyte counts together with extended variables (Ret-He and IRF) provides an excellent opportunity to screen women during pregnancy, for iron deficiency,

using the existing FBC sample taken providing a low cost alternative to ferritin. These can be used to not only predict birth outcomes but also identify those most at risk and provide earlier intervention.

1.0. INTRODUCTION

1.1. ANAEMIA

Anaemia is described by the World Health Organization (WHO) as "a condition in which the number of red cells (and consequently their oxygen-carrying capacity) is insufficient to meet the body's physiological needs**"** (WHO, 2011a). It is defined as a low haemoglobin concentration level lower than the limit of the current reference interval; two standard deviations below a healthy population mean (Beutler and Waalen, 2006). There is strong evidence suggesting anaemia can significantly contribute to morbidity and mortality (Davis and Littlewood, 2012). There are three main mechanisms of anaemia, which are ineffective erythropoiesis, haemolysis and blood loss. These can be categorised by the features of the red cells into microcytic (small red cells), normocytic (normal size red cells) and macrocytic (large red cells). Microcytic anaemia with iron deficiency is the most common cause worldwide (figure 1.1).

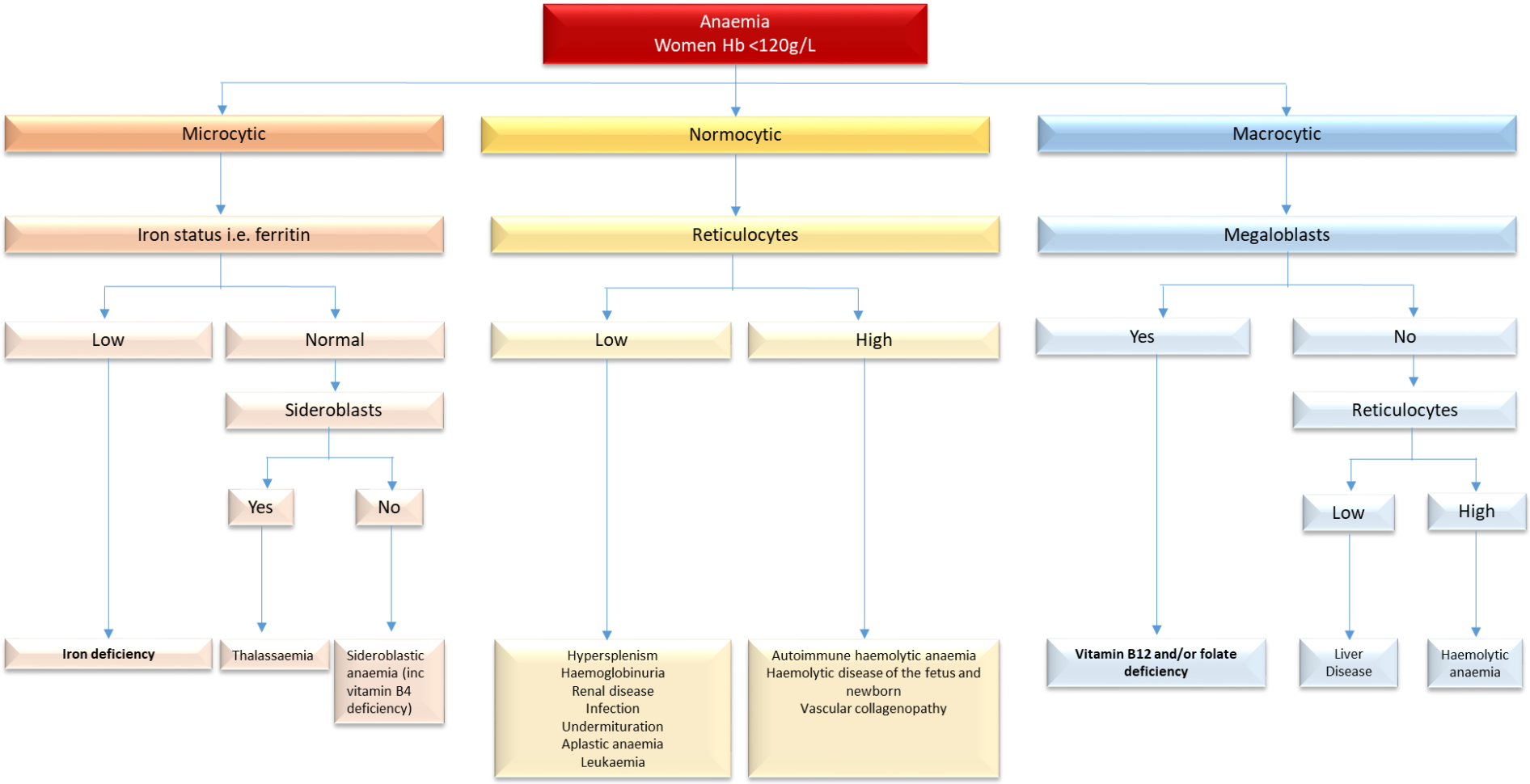


Figure 1.1. Diagram detailing types of anaemia based on red cell size, diagnostic features and differential diagnoses. Diagram adapted from World Health Organisation, Nutritional anaemias: tools for effective prevention and control (WHO, 2017).

1.2. PREVALENCE

Anaemia can influence all age groups affecting approximately 33% of the world population (Lopez, et al., 2016). There is a prevalence of iron deficiency anaemia in the population of between 0 and 6% according to age and sex, and higher rates recognised in certain demographic groups; very young children, teenage girls, menstruating and pregnant women, with adults over 85 years of age having an increased risk (Nutrition, S.A.C.o., 2010). Deficiency in vitamin B12/cobalamin and folate may also contribute to anaemia via different mechanisms. Vitamin B12 deficiency affects between 1.5% and 15% of the population (National Institutes of Health, 2021) with the true prevalence of folate deficiency being unknown (McLean, 2008). In some cases, iron, vitamin B12 and folate may be found in combination confounding diagnosis.

1.3. CLINICAL FEATURES

The clinical features of anaemia can be variable and is influenced by the severity of the anaemia. There are many signs and symptoms of anaemia (table 1.1) which include general fatigue, pallor, dyspnoea, tachycardia, poor concentration, and headaches. In severe cases koilonychias (spooning nails) and /or pica, glossitis and pagophagia may be observed but are rarely seen in developed countries (Cook, 2005; Killip, et al., 2007).

Table 1.1. Signs and symptoms of anaemia (Lopez, et al., 2016; Parker, 2012)

	Symptoms
Common	• Pallor of skin, conjunctivae and nail beds • Fatigue • Dizziness • Dyspnoea • Headache
Frequent	• Diffuse and moderate alopecia • Atrophic glossitis • Dry and rough skin • Dry and damaged hair • Restless leg syndrome • Cardiac Murmur • Tachycardia • Palpitations • Chest pain • Neurocognitive dysfunction • Tinnitus • Muscular weakness • Angina pectoris • Vertigo • Low temperature • Depression • Infection • Lactation failure
Rare	• Koilonychia • Haemdynamic instability • Syncope • Plummer-Vinson Syndrome

1.4. SYNTHESIS OF HAEM AND FORMATION OF HAEMOGLOBIN

The synthesis and formation of haemoglobin has long been established. Haem is a complex molecule synthesized in the cytoplasm of red cells and mitochondria of erythrocytes developing in the bone marrow (Moore, Knight, and Blann, 2016). The enzymatic reactions leading to the synthesis of haem require various macronutrients which are derived from the diet and include iron, vitamin B12, vitamin B6 and folate along with many others (Moore, Knight, and Blann, 2016). The first step in haem formation is the synthesis of the protoporphyrin ring initiated by the condensation of one glycine and one succinylCoA by pyridoxal phosphate containing the enzyme δ-aminolevulinic acid synthase (ALAS) (figure 1.2). This is a highly regulated reaction with defects in the ALAS gene leading to deficiencies in iron-containing cells (Harmening, 2002). The final reaction takes place in the mitochondria by the insertion of the iron atom into the ring system catalysing a reaction known as ferrochelatase. Iron is held in place in the centre of the porphyrin ring by four atoms of nitrogen (Moore, Knight, and Blann, 2016).

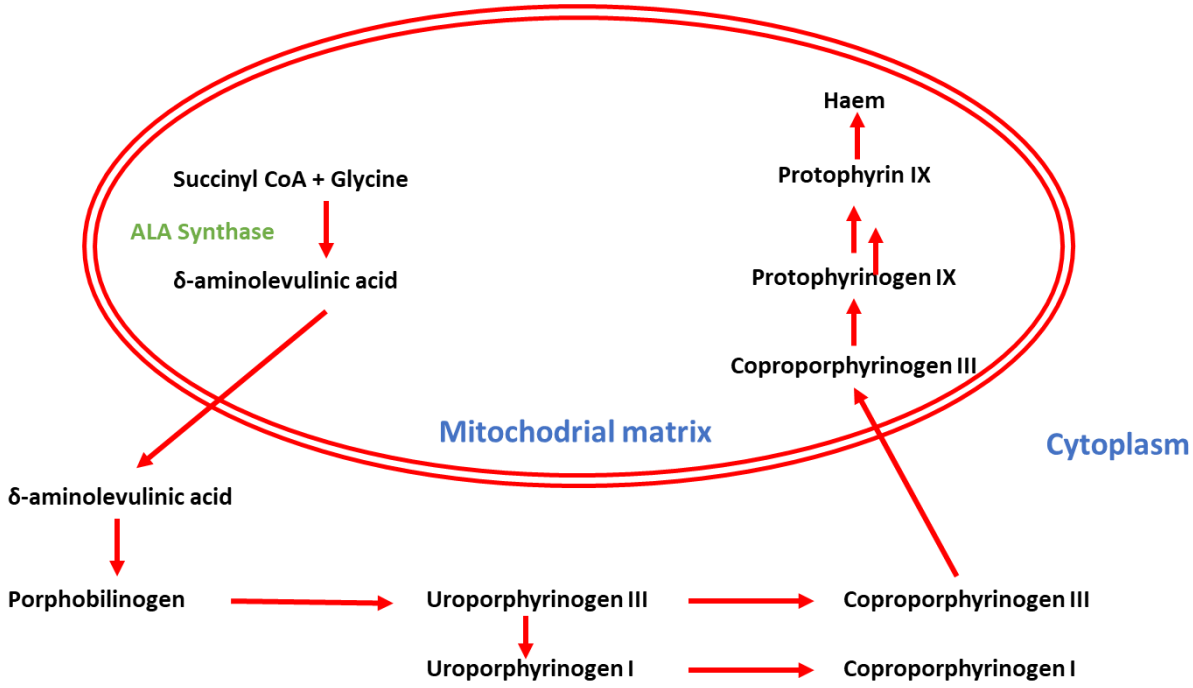


Figure 1.2. Formation of haem in the protoporphyrin ring. Diagram adapted from The Medical Biochemistry Page (1996-2021)

There are approximately 640 haemoglobin molecules contained in each red cell (Hoffbrand and Moss, 2012). A normal mature adult haemoglobin molecule is comprised of four individual polypeptide chains, referred to as a tetramer and composed of two alpha chains and two beta globin chains. Each chain is a molecule of haem and contains a ferrous iron (Fe^{2+}) and protoporphyrin with iron combining reversibly with CO^{2+} at its core (figure 1.3) (Howard and Hamilton, 2013). Haemoglobin has a high affinity for oxygen in the lungs but low affinity in tissues (Howard and Hamilton, 2013).

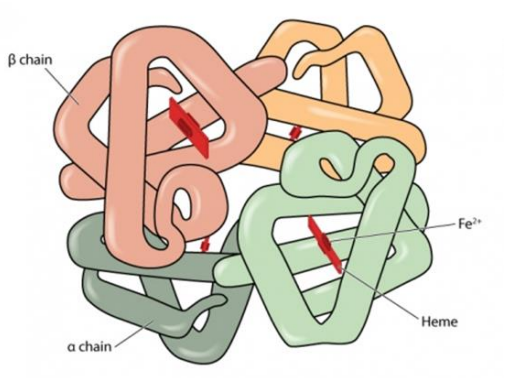


Figure 1.3. Formation of haemoglobin tetramer, 2 alpha and 2 beta globin chains with ferrous iron at the core of each molecule. Image from Public Health England, (2018) Understanding haemoglobinopathies.

Iron together with protoporphyrin forms the haem molecule. Haem then combines with the globin chain synthesis to form the complete haemoglobin structure (figure 1.4). The synthesis of the haemoglobin molecules in cytoplasm of erythrocyte precursors occurs in parallel with synthesis of haem and together form haemoglobin (Moore, Knight, and Blann, 2016).

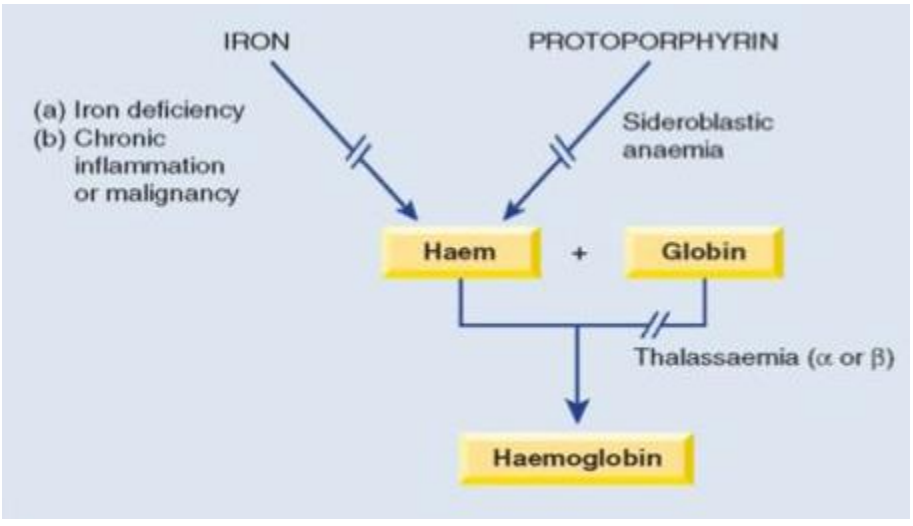


Figure 1.4. Formation of haemoglobin. Image from Hoffband and Moss, (2012)

1.5. HAEMATINIC HOMEOSTASIS

An adequate balance of vitamin and minerals including iron, vitamin B12 and folate is important for every individual for general well-being and quality of life. They play an important role in growth (proliferation and microbial), normal functioning of cells (immune), as well as being co-factors for biochemical and enzyme reactions in energy metabolism, for synthesis of DNA and microbial growth (Gasche, et al., 2004; Milman, 2008). During pregnancy a higher rate of DNA synthesis is required for both mother and fetus (Karabulut, et al., 2015). Vitamin B12 and folate are essential for fetal neurodevelopment as well as supporting erythropoiesis throughout pregnancy (Carretti, et al., 1994; Black, 2008; Dror and Allen, 2008). Iron, vitamin B12 and folate are especially important for the normal functioning of red cells and general cell renewal causing morphological variations when deficient.

1.6. IRON HOMEOSTASIS

Iron homeostasis is a complex process and has been extensively reviewed with thousands of publications (Guo, et al., 2016). Ferritin and transferrin, the proteins involved in iron homeostasis have been known for many decades, with more understanding of molecular mechanisms coming in the last few decades (Anderson and Frazier, 2017). The proteins, which contain iron, are essential for a wide range of bodily functions including energy metabolism, nucleotide synthesis as well as signalling pathways and host defence (Ganz, 2013; Guo, et al., 2016). As well as being a trace element, iron is a redox active metal which needs to be tightly regulated as in excess is toxic, can cause formation of reactive oxygen species and lead to tissue fibrosis and organ dysfunction (Guo, et al., 2016). When required iron can be mobilised both from bodily stores and absorbed from the diet and down regulated when stores are replete.

1.6.1. Pathophysiology of iron

In normal physiological conditions, iron concentrations are generally stable with regulation of iron tightly controlled. Deviations in iron balance can lead to iron deficiency or iron overload. The average adult human has approximately 3-4g of iron, most of which is found in erythrocyte haemoglobin (2-3g). The remainder found in tissues (3-5mg) and other stores including the liver and spleen where iron is stored in the storage protein ferritin, in hepatocytes and macrophages respectively. Iron contained in muscles is predominantly myoglobin, an oxygen storage protein (Ganz, 2013). Only 2-4mg of iron bound to transferrin (transport protein) circulates in blood plasma with around 20-25mg of iron a day moved around the system, turning over every few hours. The lifespan of a human erythrocyte is

approximately 120 days with 15-25mg of red cell iron being recycled each day Figure 1.5. (Ganz, 2013).

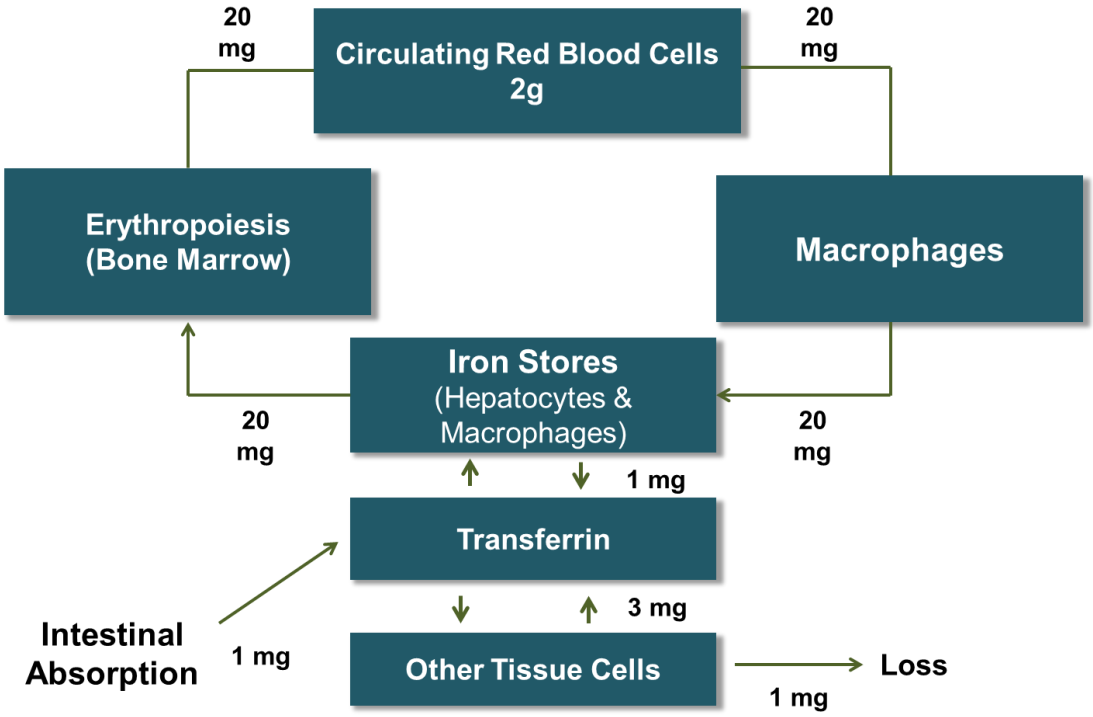


Figure 1.5. Daily iron balance in humans.

The body loses 1-2mg of iron per day mainly from desquamated epithelial cells (Camaschella and Strati, 2010). Via the reticuloendothelial system (RES), macrophages recycle iron from senescent red cells demonstrating the efficiency of the reutilisation of systemic iron (Scientific Advisory Committee on Nutrition, 2010). The equilibrium is maintained with dietary absorption and iron stores are generally stable in most humans consuming an iron-adequate diet (Ganz, 2013). Good quality data on the efficiency of iron uptake from the diet is lacking (Scientific Advisory Committee on Nutrition, 2010). However, the efficiency of iron uptake from food in influenced by systemic requirements. In a state of iron deficiency, more iron is absorbed and less when iron replete. The absorption of haem iron from the diet is more efficient than from non-haem sources (i.e. medications) with meat and fish being more readily absorbed (20-30%) than

non-haem- plant based diets (5-15%) (Martinez-Torres and Layrisse, 1971; FAO, 1988). Haem iron from the diet is 2-6 times more available for absorption that non-haem iron (Scientific Advisory Committee on Nutrition, 2010). Imbalance in absorption is often due to the variation in differing diets, vegetarian versus mixed diets (Hurrell and Elgi, 2010; Ganz, 2013). Other variations can occur when there is extra demand due to growth or blood loss. Insufficient iron supply can have adverse consequences resulting from impaired synthesis of proteins, which are required for normal cellular physiology (Anderson and Frazier, 2017).

1.6.2. Iron absorption

The iron absorption mechanisms has been extensively studied. Iron enters the human body via the diet, except when therapeutic administration is required via exogenous sources (intravenous iron or blood transfusion). Dietary iron comes from either non-heme or heme sources. Non-heme is of animal and plant origin with absorption being affected by a range of other dietary constituents and luminal factors such as stomach pH, citric and ascorbic acid (enhances absorption), plant derived phylates, tannins, and polyphenols (impedes absorption) (Anderson and Frazier, 2017). Non heme iron is converted from $Fe3^{+}$ to $Fe2^{+}$ and transported across the apical membrane of intestinal enterocyte by DMT-1, exported into circulation via ferroportoin (figure 1.6) (Camaschella and Strati; 2010, Anderson and Frazier, 2017). Most of the heme iron in the diet is from myoglobin and haemoglobin and is more efficiently absorbed and less dependent on diet although the absorption mechanism is still poorly understood (Anderson and Frazier, 2017). Heme iron is transported across the cell membrane via the Haem Carrier Protein 13 (HCP1). When demands in the body for iron are high, intracellular iron is rapidly transferred across the basolateral membrane by ferroportin.

In times when demand is low, it is stored as ferritin (an intracellular iron storage protein complex) (Gulec, et al., 2014).

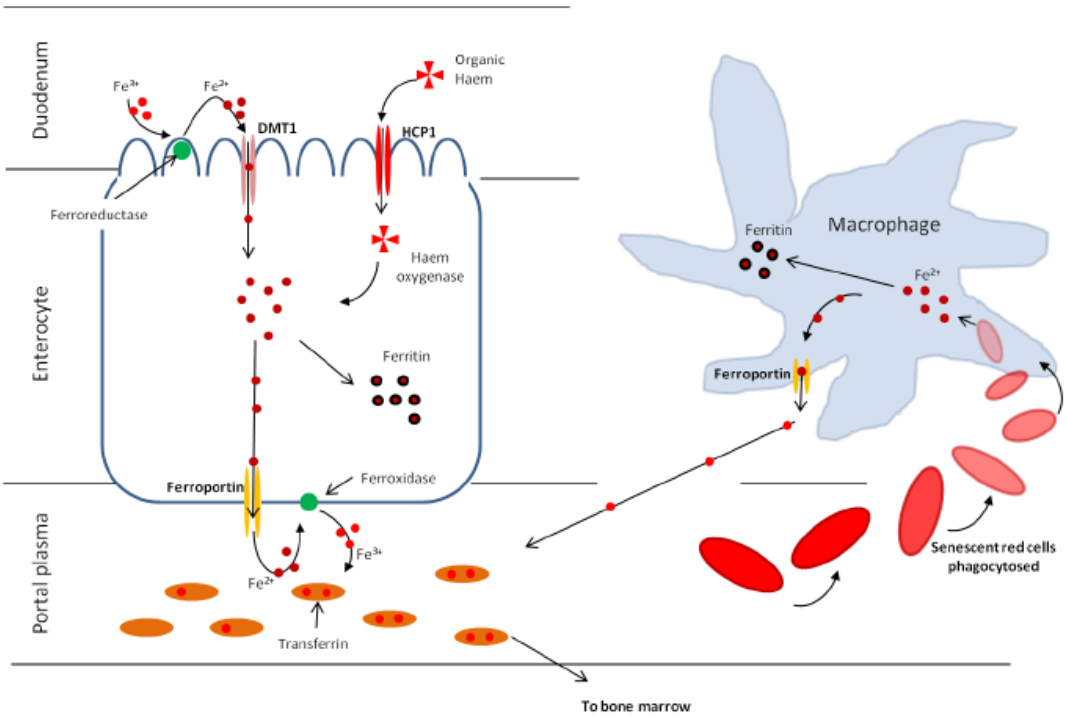


Figure 1.6. Metabolism and absorption of iron. Image from (Svenson, 2015).

1.6.3. Iron metabolism

The metabolism of iron is a complex process (Thomas, et al., 2011). Intestinal absorption of iron is primarily via mature duodenal enterocytes in the upper small intestine although small amounts of iron can be absorbed in other parts of the gastrointestinal tract (Anderson and Frazier, 2017). Iron crosses the apical brush border membrane of the enterocyte entering the cell via the Divalent Metal Transporter 1 (DMT1). Most dietary iron is in the ferric form ($Fe3^{+}$) and must be reduced to its ferrous form ($Fe2^{+}$) before it can enter the cell, due to toxicity. When the body does not need to immediately utilise the iron absorbed it is sequestered within the cell in the iron storage protein ferritin, which is lost only when enterocytes are

sloughed and the end of several days lifespan (Anderson and Frazier, 2017). Ferritin is the major iron storage protein. It is a large protein consisting of 24 subunits arranged in a spherical cell with a large central cavity. Pores in the protein shell allow the import and exit of iron, with each ferritin molecule holding approximately 4500 atoms of iron. Small amounts of ferritin protein secreted from cells has been shown to strongly correlate with the intracellular concentration of iron and is directly proportional to total body iron stores (Addison, et al., 1972, Jacobs, et al., 1972, Cook, et al., 1974, Theil, 2013). Therefore, measurement of serum ferritin concentrations is used as an accurate indicator of body iron stores, although the function of ferritin in serum is unknown.

When required, iron is rapidly exported via ferroportin on the basolateral membrane of the enterocyte. Export is enhanced by the copper-dependent iron oxidase hephaestin, which oxidises the ferrous form ($Fe2^{+}$) by ferroxidase (hephaestin) back to its ferric form ($Fe3^{+}$). Heme from senescent erythrocytes, taken up by macrophages of the spleen, liver and bone marrow and is transported across the cell membrane via the Haem Carrier Protein 13 (HCP1), although this mechanism is less well understood. It is presumed to be absorbed intact being released via the action of heme haemoxygenase 1 (HO1) with export from the enterocyte via the same mechanisms as non-heme iron (Anderson and Frazier, 2017). The absorption of dietary iron for transportation to cells is tightly regulated, dependent on body requirements and the 25 amino acid peptide Hepcidin.

1.6.4. Iron storage and transport

Iron is stored in cells as ferritin, a large water-soluble molecule consisting of 24 subunits arranged in a spherical protein shell with a large iron core. It is the major intracellular iron

storage protein. A single ferritin molecule can hold up to 4500 atoms of iron with entry and exit via pores in the protein (Anderson and Fraizer, 2017). Aggregates of ferritin fuse with lysosomes to become haemosiderin. Free iron is toxic and intracellular chaperones (poly(rC)-binding proteins) have been shown to deliver iron to ferritin (Leidgens, et al., 2013). Both ferrtin and haemosiderin are mobilised when required with small amounts of ferritin being secreted from cells which has been shown to be useful indicator of iron stores (Theil, 2013).

When required, iron is released from cells via ferroportin and transported extracellularly in plasma, bound to transferrin. This is distributed around the body to the site of utilisation, with the bone marrow having high requirements. Less than 3 mg of iron circulates in plasma, bound to transferrin. Each transferrin molecule can bind up to 2 molecules of ferric iron ($Fe3^{+}$) with approximately 30% (i.e.Transferrin Saturation (TSAT) of 30% a useful index of iron supply to the bone marrow) of iron binding sites being occupied normally at any one time (Anderson and Frazier, 2017). Body cells express a transferrin receptor (TFR1) and when required loaded diferric transferrin binds the receptor and is endocytosed into the cytoplasm of the cell via DMT1 on the cell membrane. Here it is internalised via clatherin-mediated endocytosis undergoing a conformational change to release the transferrin complex which when needed for metabolic function. This is delivered to the mitochondria or stored as ferritin until required (Anderson and Frazier, 2017).

1.6.5. Iron regulation

Iron concentrations in the cell are modulated by iron regulatory proteins (IRP's). When IRP's are bound, blocking translation, little ferritin is produced when storage of iron is not required. The IRP's bind to iron responsive elements (IRE's) which protects against degradation and

enables more TFR1 to be expressed to maximise iron intake (Anderson & Frazier, 2017). In times of high iron concentrations IRP's do not bind to IRE's allowing degradation of TFR1 limiting uptake thus promoting storage.

Iron regulation is mediated by hepcidin, a liver-derived 25 amino-acid peptide hormone (den Elzen, et al., 2013; Ganz, 2013; Anderson and Frazier, 2017). Hepcidin binds to target sites on ferroportin internalising it and rendering it to ubiquitin mediated degradation, which blocks release of iron into circulation (Ganz, 2005; Weiss, 2009). When iron is replete hepcidin concentrations are high resulting in reduced supply of iron; when the demand for iron is high, hepcidin concentration is reduced allowing more iron to enter circulation. Acute phase cytokines have also been implicated in the expression of hepcidin (i.e. Interleukin 6 (IL-6) and Tissue Necrosis Factor Alpha (TNF-α)) showing that IL-6 induces hepcidin in inflammatory responses (Nemeth, et al., 2004). The stimulation of hepcidin by IL-6 provides a host protection, controlling the amount of iron available in some extracellular bacterial infections thereby restricting their growth (Armitage, et al., 2011). In severe infections, the onset of anaemia is due to the low iron availability because of this natural body defence mechanism.

1.7. IRON DEFICIENCY ANAEMIA

Iron deficiency (ID) is the most common of the nutritional deficiency worldwide. It affects large numbers of people in developing countries and is prevalent in industrialised countries affecting both wealthy and poor (WHO, 2021). ID contributes to the impairment of red cell production which results in anaemia. Iron deficiency anaemia (IDA) occurs when there are insufficient stores of iron for erythropoiesis due to either decreased absorption, blood loss or increased requirements. The most prominent cause of IDA is attributed to low dietary intake especially those with vegetarian or vegan based diets (Percy, et al., 2017). Other causes may include continued blood loss, anaemia of chronic disease, pregnancy, gastrointestinal related issues and malignancy.

Usually there is a sufficient level of iron for erythropoiesis in the early stages of ID. As iron stores become more depleted anaemia ensues and erythrocytes are not replaced. The equilibrium is affected with no known alternative mechanism being able to compensate for this.

1.7.1 Iron deficiency anaemia in women

Greater than 20% of women experience iron deficiency/iron deficiency anaemia during reproductive age (Percy, et al., 2017). In 2011 the global prevalence of anaemia in non-pregnant women was 29% (Percy, et al., 2017). Anaemia commonly affects more women than men (Kassebaum, et al., 2014; Percy, et al 2017). Blood loss is an important cause of anaemia in women with monthly menstrual sequestration also being the most common cause of ID/IDA. Heavy menstrual bleeding accounts for 20-30% (Patterson, et al., 2000; WHO meeting report, 2012; Percy, et al., 2017).

1.8. VITAMIN B12 (COBALAMIN) AND FOLATE

Anaemia due to vitamin B12 (cobalamin) and folate deficiency is relatively uncommon worldwide with little evidence to suggest that at a population level it increases the prevalence of anaemia with moderate evidence that folate is associated with cognitive impairment (de Benoist, 2008). However, memory and cognitive impairment has been shown in those with severe vitamin B12 deficiency (de Benoist, 2008). Vitamin B12 and folate play a central role in both DNA synthesis and embryogenesis. Deficiency of vitamin B12 develops over time with the most common cause being related to dietary deficiency, commonly seen in the elderly, vegans and vegetarians with poor diets (O'Leary and Samman, 2010). Other attributed causes include inadequate production of intrinsic factor (pernicious anaemia), atrophic gastritis, gastric resection, bacterial overgrowth and some drug interactions interferes with uptake of vitamin B12 in the ileum.

Normal body stores of vitamin B12 are between 1 and 3mg with a small turn over (0.1%) each day. Deficiency ensues when the store of vitamin B12 drops below 300ug. Dietary deficiency can take many years to manifest as a 1mg store meets the needs of the body for approximately 3 years (Allen, 2008). Folate has a higher turnover each day, with store in the average male being around 12mg to 28mg. Deficiency of folate is therefore, detected much more quickly.

Dietary sources of vitamin B12 are derived primarily from animal sources (muscle meat, eggs, dairy and fish). Folate sources include leafy green vegetables, legumes, some fruits and fortified cereal products are primary sources.

1.8.1 Vitamin B12/transcobalamin absorption and transport

The absorption of vitamin B12 is a complex mechanism (figure 1.7). The recommended daily allowance for adults to ensure absorption of 1ug/L is 2.4ug/L to 2.8ug/L, with the average absorption from food being approximately 50% (Stabler and Allen, 2004).

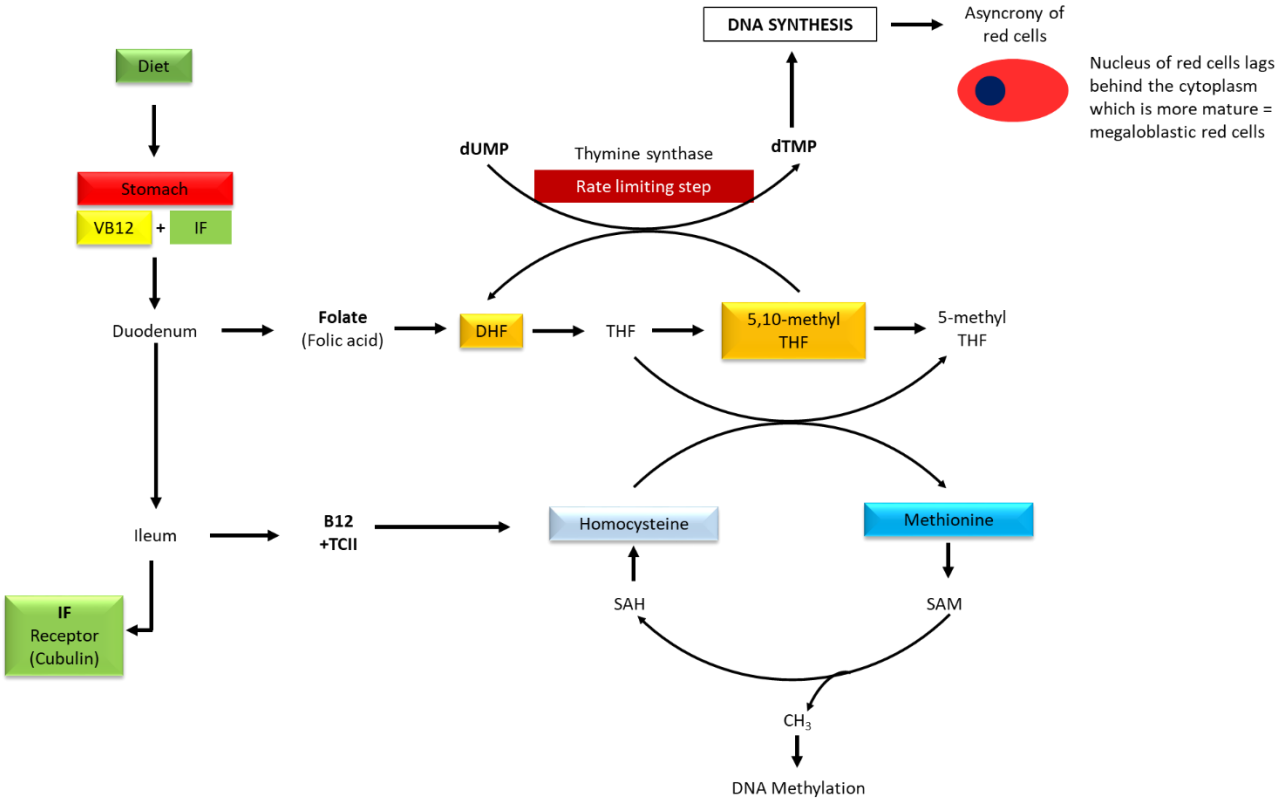


Figure 1.7. Biochemical pathway for metabolism of vitamin B12 and folate in the formation of DNA.

During chewing and swallowing, salivary and oesophageal glands produce haptocorin (a cobalamin-binding protein) (Allen, 2008). In the stomach, gastric epithelial cells secrete hydrochloric acid and pepsin, which releases cobalamin from, recently swallowed food. The parietal cells of the stomach also secrete intrinsic factor (IF) which binds cobalamin forming a VB12-IF complex which is essential for transportation via the duodenum to the terminal ileum (O'Leary and Samman, 2010). In the presence of calcium, the VB12-IF complex is absorbed in

the terminal ileum by the membrane protein cubulin where it is endocytosed, degraded and recycled (Rizzo, et al., 2016). Colbalamin is released to the MRP1 transport protein. In the bloodstream transportation of cobalamin is transported by holotranscobalamin (transcobalamin II), the active form. This represents only 2-20% of circulating cobalamin with the remainder being bound to haptocorin or transcobalamin III, a mechanism which is poorly understood (Rizzo, et al., 2016).

1.8.2 Folate absorption

Food folate occurs manly in a polyglutamate form, which prior to absorption must be hydrolysed to the monoglutmate form, carried out by glutamate carboxypeptidase, on the brush border of the proximal jejunum (Ebara, 2017). The monoglutamate folate is transported across the apical cell membrane via a proton-coupled folate transporter (Zhao, et al., 2011; Visentin, et al., 2014; Ebara, 2017). In the cell folate is metabolised to methyl-tetrahydrofolate (Methyl-THF) and exported to the portal vein via multidrug resistance-associated protein (MRP). It circulates in blood and is then taken up by folate receptors.

1.8.3 Vitamin B12/Transcobalamin and folate metabolism including DNA synthesis

Metabolism of vitamin B12 and folate is a complex involving many biochemical processes. Once absorbed, both vitamin B12 and folate undergo a series of biochemical actions with both being dependent on one another and essential for the synthesis of purines and pyrimidines (O'Leary and Samman, 2010). The synthesis of methionine synthase produced from the vitamin B12 drives the metabolic action (figure 1.7). Cobalamin is delivered to methionine synthase forming cobalamin 1 (most reduced form) binds to the methyl group of the substrate 5-methyltetrahydrofolate transferring it to homocysteine to form methionine and THF products (Froese, et al., 2019). Methylation of methionine forms the methionine

cycle forming homocysteine, which completes the cycle (Froese, et al., 2019). The source of the substrate 5-MTHF is driven by the folate cycle. Folic acid is reduced to dihydrofolate by dihydrofolate reductase with further reductions generating THF (the active co-enzyme form). 5,10 methylene-THF is used in the production of dTMP from deoxyuridine monophosphate (dUMP) being the rate limiting step in the synthesis of DNA, while 10-formal-THF is required for new purine synthesis (Froese, et al., 2019). Deficiencies in either vitamin B12 or folate interrupts these mutually dependent biochemical reactions resulting in a megaloblastic anaemia with asynchrony (delayed maturation of the nucleus) of the erythrocyte due to decreased DNA synthesis.

1.9 LABORATORY DIAGNOSIS OF HAEMATINIC DEFICIENCY

1.9.1. Haematology parameters

For identification of anaemia, haemoglobin levels are widely used initially, however the sensitivity and specificity of determining iron deficiency are low using haemoglobin levels alone (Cook, 2005). In iron deficiency anaemia, low haemoglobin levels only become apparent when there has been a considerable amount of iron depletion. A diagnosis of anaemia can be made from the haemoglobin level alone but further investigations including iron, vitamin B12 and folate measurements are required for a definitive diagnosis (WHO, 2011a).

1.9.2. Red cell indices

Anaemia is characterised by low haemoglobin levels along with other red cell indices such as mean cell haemoglobin (MCH) levels providing an indication as to the cause (figure 1.1). Mean cell volume (MCV may sometime be low but often only in those with severe iron deficiency. The specificity of MCV and MCH for iron deficiency is limited. Microcytic and hypochromic red cells are also seen in many haemoglobinopathies (i.e. in thalassaemia when the MCV is reduced it is out of proportion with the level of anaemia), in sideroblastic anaemia and anaemia of chronic disease. MCV is also known to be affected by preanalytical variables (sample temperature and storage), whereas MCH is much less affected (Fletcher, et al., 2021). In iron deficiency, the MCV can be normal in up to 40% of subjects (Bermejo and Garcia-Lopez, 2009) in addition to states of mixed haematinic deficiency. Jolobe, (2005) reported a sensitivity for MCV at 76fl of 65% and specificity 66% compared with MCH which gave sensitivity of 74% and specificity 59% for iron deficiency. Therefore, MCH is considered the

more reliable marker of iron deficiency (Snook, et al., 2021). Often the presence of a co-existing anaemia (vitamin B12 and folate deficiency or sideroblastic anaemia) confounds the interpretation (Thomas, et al., 2013). MCH is derived from the red blood cell count and haemoglobin concentration and MCV calculated from the haemtocrit (packed cell volume) and red blood cell count. In IDA, MCH levels are low, while in vitamin B12 and folate deficiency the MCV they may be either normal or high. In subjects with combined deficiency the MCV and MCH levels may appear within the normal reference interval thus a normal MCV does not always exclude iron or vitamin B12/folate deficiency.

1.9.3. Reticulocyte Haemoglobin Equivalent (Ret-He)

Reticulocytes, precursors of mature erythrocytes, circulate in peripheral blood for 1-2 days before maturing (Brugnara et al., 1999; Brugnara, 2000; Thomas and Thomas, 2002; Marković, et al., 2007). Modern FBC analysers can measure parameters such as reticulocyte haemoglobin equivalent (Ret-He) or reticulocyte haemoglobin content (CHr). Haemoglobin content can be assessed using these immature erythrocyte (reticulocyte) measurements. These measurements do not give direct indication as to the iron status of a subject but may provide implied information in respect of the adequacy of iron stores, reflecting the iron demand of the cells with changes being seen within a couple of days (Thomas and Thomas, 2002, Cook, 2005, Thomas, et al., 2013). Thresholds for predicting iron deficiency have been suggested which vary between 25pg and 30pg (Canals, et al., 2005; Chinudomwong, et al., 2020).

1.9.4. Immature reticulocyte fraction

Immature reticulocyte fraction indicates younger reticulocytes (Piva, et al., 2015). Being an earlier marker it can be used in the assessment of a regenerating bone marrow, in particular erythrocytosis. The measurement of IRF can be used to assess response of the erythroid during treatment with vitamins and erythropoietin (Sysmex, 2016). Unlike Ret-He where response is usually seen after 2 to 3 days, IFR response can be seen within hours. It can be especially useful in assessing recovery during bone marrow transplantation, red cell stimulating therapy or chemotherapy (Piva, et al., 2015). The IFR reference interval is suggest at 1.6% to 10.5% (Pekelharing, et al., 2010). This reference interval is however, based only from a study conducted in one hospital in the Netherlands using undefined subjects. Reference intervals for different demographic populations and indeed in pregnancy need to be further determined to reflect that of the local populations under study.

1.9.5. Biochemistry assays

Biochemistry assays can provide useful information about haematinic deficiency when levels are low. A typical biochemical pattern of ID shows a decrease in serum ferritin, and transferrin saturation and decreased vitamin B12 and folate level in megaloblastic anaemia in addition to co-existing haematinic deficiency. In an iron deficiency state, an acute phase response or inflammation may not accurately define IDA falsely elevating the ferritin measurement (Oustamanolakis, et al., 2011a).

1.9.6. Serum/plasma ferritin

Ferritin is a water soluble, spherical protein with a hollow shell consisting of 24 sub units and can store a substantial amount of iron in cells (Coyne, 2006). Small amounts of ferritin with little or no iron content are secreted into plasma and cleared from circulation by the liver. As

its concentration in circulation is proportional to iron storage levels, it is used as an indirect measure of iron stores (Coyne, 2006; Oustamanolakis, et al., 2011b). Walters, et al., (1973) established that every 1µg/L of serum ferritin equated to around 8mg of storage iron. This concentration however does not take into consideration different body sizes (Finch, 1986) and more recent studies show levels can vary (Cook, 2005). Finch, 1986 suggests 1µg/L of plasma ferritin is considered equivalent to 120µL of storage iron/kg body weight with an average 75kg male containing around 4 grams of iron (50mg/kg).

Serum ferritin is the most used parameter to diagnose iron deficiency. Ferritin is an acute-phase protein and in the presence of underlying inflammatory processes, the reference value increases suggesting a falsely elevated result. A low serum ferritin level <15µg/L suggests a high likelihood of iron deficiency (Guyatt et al., 1992). Levels between 15 and 30µg/L may still be consistent with and iron deficient state but are less specific (WHO, 2011b). Levels >100µg/L exclude ID (Oustamanolakis, et al., 2011b) although levels between 30µg/L and 100µg/L can be confounded in the presence of inflammatory processes (Mast, 2001; Cook, 2005; Gomollón and Gisbert, 2009).

Serum ferritin, regarded as the most accurate diagnostic measurement of iron storage, is well standardised and routinely available in most clinical laboratories (Cook, 2005). It is a good indicator for ID although the presence of inflammation reduces its sensitivity (Killip, et al., 2007; Cheng, et al., 2011a).

1.9.7 Vitamin B12/Transcobalamin

Vitamin B12, like folate, are essential for embryogenesis, DNA synthesis as well as protein and lipids (Rashid, et al., 2020). Synthesised in the gastrointestinal tract of animals by certain

bacteria, the only source of vitamin B12 in humans is from animal sources (Allen, 2008; Rashid, et al., 2020). Although vitamin B12 can be absorbed from non-meat forms (fish, dairy and some fortified breakfast cereals), large volumes are required to satisfy the same levels. No Bioactive forms of vitamin B12 are found in plant sources (Rashid, et al., 2020). Vitamin B12 is absorbed from animal tissues in the human host, thus those who practice vegetarianism and more so veganism must ensure adequate replacement via synthetic forms.

There is no gold standard test for defining vitamin B12 deficiency in the laboratory and this can be exceptionally challenging. A total vitamin B12 assay is the routine standard test in most laboratories, and measures both the “active” and “inactive” forms. It is a widely available test, is automated as well as being a cheap to perform, although it lacks sensitivity and specificity (Devalia, et al., 2014). Generally, low levels of vitamin B12 signify deficiency and higher levels sufficiency. However, there is an intermediate “grey-area” of interpretation especially in those with borderline levels. The borderline cases often rely on clinical symptoms and further testing using more directed tests such as holotranscobalamin, methylmalonic acid or homocysteine. These additional tests also have limitations as well as being expensive and are therefore are not used routinely. Due to the variation in platforms and assays for total vitamin B12, laboratories are advised to determine their own reference intervals, as a clinically normal total vitamin B12 level is unclear (Devalia, et al., 2014). Establishing an appropriate interval can be challenging for laboratories, because the vitamin B12 (cobalamin) levels are also affected by diet, vitamin supplementation, pregnancy, contraceptive pill, and metformin in addition to others.

1.9.8 Folate

Folates are synthesised by micro-organisms and plants with vegetables, fruit, dairy products and cereals being the primary sources in the diet (Snow, 1999). The recommended daily allowance of folate is approximately 200µg to 300µg per day. The body is unable to maintain high stores of folate so they can easily become depleted within months; hence, it is important to maintain a regular daily intake. Unlike dietary folate, synthetic folate acid is reduced directly by THF without the need for vitamin B12 as a co-factor (Snow, 1999).

Folate deficiency is characterised by low levels detected by laboratory analysis. Laboratory tests for folate can assess either serum levels (most common) or red cell levels. The lower limit for the reference interval can vary but is usually set around 6.8nmol/L (3ng/mL). The levels of folate reflect recent intake and can produce false normal results. Conversely, abnormally low levels can be seen in-patient with anorexia, recent alcohol intake, pregnancy and those taking anticonvulsants (Devalia, et al., 2014). Red cell folate measurement reflect the life span of the red cell but it is affected by pre-analytical variables as well as being is a time consuming and expensive process. Serum folate is therefore the preferred test reflecting current levels. As folate has a close relationship with vitamin B12 in terms of metabolism and clinical symptoms, both analytes should be assessed in conjunction with one another (Devalia, et al., 2014).

1.10 C-Reactive protein (CRP)

Biological mechanisms which underlie the effect of infection and inflammation on iron-status measurements are not well understood (Suchdev, et al., 2017). In medical laboratories, C-Reactive Protein (CRP) is a commonly used test for estimating levels of acute infection and inflammation (Gasche, et al., 2004). CRP is an acute phase protein, like serum ferritin. In the presence of an elevated CRP result, this can indicate that the serum ferritin may be falsely raised thus invalidating the result. This is especially important when trying to determine iron deficiency in the presence of inflammation. Therefore the WHO suggests measuring the CRP level at the same time as the serum ferritin to confirm the presence of an acute phase response (WHO, 2011b; Namaste, et al., 2017). As a result, patients with IDA and raised inflammatory markers may exhibit serum ferritin values within the normal reference interval making a diagnosis of absolute iron deficiency uncertain. Accurate assessment of iron deficiency is important for assessing need for intervention (Namaste, et al., 2017).

1.11 ANAEMIA IN PREGNANCY

Anaemia in pregnancy is a well-known issue worldwide affecting women in both developed and developing countries (Bencaiova, et al., 2012). Prevalence varies ranging from 25% to 92% with higher incidence attributed with socioeconomic background (Bencaiova, et al., 2012). Recent studies have reported increased incidence of iron deficiency in women who are not only anaemic but in those who have no anaemia during pregnancy (Mahdy, et al., 2017). Daru, et al., 2018, reported a high frequency of iron deficiency in pregnancy in the UK. Although true vitamin B12 deficiency, with significant clinical presentation is considered to be rare in the general population, there is limited data available as to its true prevalence. Shields, et al., 2011, however reported 16% of pregnant women in the 1st trimester of pregnancy showed deficiency, rising to 60% in the 3rd trimester in Scotland.

1.11.1 Iron

During the course of pregnancy, increased requirements for iron must be met for foetal development via maternal intake and body stores, with approximately 1200mg of iron required (Percy, et al., 2017). Additionally, during pregnancy there should be an increase in erythrocyte red cell mass from 350mL to 450mL (Miller, 2013; Percy, et al., 2017). Failure of insufficient stores or reduced intake results in iron deficiency and/or iron deficiency anaemia. There is an increased risk of iron deficiency/iron deficiency anaemia in adolescent pregnant women, which is confounded by not only the growth of the foetus, but also their own growth and development (WHO, 2014; Percy, et al., 2017). Furthermore, iron deficiency/iron deficiency anaemia during pregnancy has been associated with maternal depression whilst also having an effect of the foetus due to reduced brain maturation and cognition although whether these effects can be reversed is unknown (Miller, 2013, Percy, et al., 2017).

1.11.2 B12 and Folate

Vitamin B12 and folate is especially important during pregnancy, like iron for general cell development (O'Leary, and Samman, 2010). Furthermore, it is postulated that reduced levels of these vitamins increase the risk of neural tube defects in the foetus, maternal placental abruption, preterm delivery or low birth weight/small-for-gestational-age infants (de Benoist, 2008; O'Leary, and Samman, 2010). Unlike iron, cut-off levels for vitamin B12 and folate are less certain because concentrations of both vitamins decline during the course of pregnancy (de Benoist, 2008).

1.12. INDEX OF MULTIPLE DEPRAVATION DECILE

The Index of Multiple Depravation is an official measure of official depravation in England. It encompasses a wide range of individual living conditions. There are 39 separate indicators across seven distinct domains, which include income, employment, health depravation and disability, education skills and training, crime, barriers to housing and services, and living environment. Neighbourhoods are ranked according to their level of depravation relative to other areas. A score of 10 signifies the least deprived with a score of 1 being most derived (Ministry of Housing, Communities and Local Government, 2019).

In the 2019 IMD Kingston-upon-Hull was ranked 9th of the most deprived local authority in the UK. The East Riding of Yorkshire on the other hand has a more varied distribution of depravation, some with least depravation and some with most depravation (figure 1.8 and 1.9.) (Ministry of Housing, Communities and Local Government, 2019; East Riding of Yorkshire Council, 2021). This wide range of distribution across both Kingston-upon- Hull and the East Riding of Yorkshire provides a good distribution of postcodes to assess whether level of depravation has an impact on haematinic deficiencies and birth outcomes.

Most deprived local authorities based on Rank

1. Blackpool
2. Manchester
3. Knowsley
4. Liverpool
5. Barking and Dagenham
6. Birmingham
7. Hackney
8. Sandwell
9. Kingston upon Hull
10. Nottingham

Most deprived local authorities based on Score

1. Blackpool
2. Knowsley
3. Liverpool
4. Kingston upon Hull
5. Middlesbrough
6. Manchester
7. Birmingham
8. Burnley
9. Blackburn with Darwen
10. Hartlepool

Most deprived local authorities based on the Proportion of LSOAs in the most deprived 10% nationally

1. Middlesbrough
2. Liverpool
3. Knowsley
4. Kingston upon Hull
5. Manchester
6. Blackpool
7. Birmingham
8. Burnley
9. Blackburn with Darwen
10. Hartlepool

Figure 1.8. Lists of most deprived local authorities in the UK 2019. Data from Ministry of Housing, Communities and Local Government, (2019).

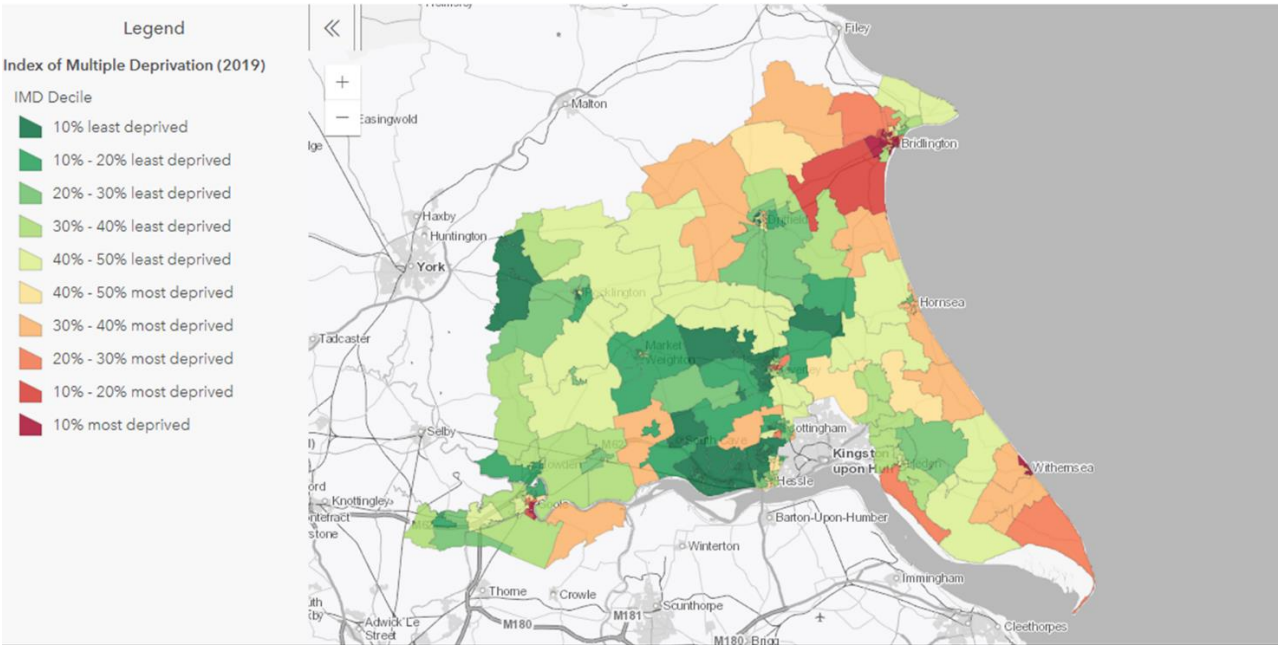


Figure 1.9. Areas of depravation in East Riding of Yorkshire. Source (East Riding of Yorkshire Council, 2021).

1.13. REVIEW OF THE LITERATURE

Prior to starting the study, a full review of available literature was undertaken. The full review can be found in appendix A.

The review found, despite haematinic deficiency being a well-researched area, there were still many significant gaps in knowledge with little evidence of how the haematinic variables linked with birth outcomes. There is no consensus as to the best approach to investigating haematinic deficiency in pregnancy as well as being a lack of longitudinal studies. Additionally there was little literature available as to the most appropriate cut-offs for assessing iron deficiency in pregnancy or the need to adjust for inflammation.

There was limited literature available for screening assessment for haematinic deficiency in the first two trimesters of pregnancy, with studies often used to assess the effects of supplementation, such as ferrous iron. Despite all of these trials, there has been no improvement in women with anaemia in pregnancy, especially in the UK with no clear evidence as to benefit or harm of such supplementation.

The review identified the absence of data affecting those women in pregnancy who were iron deficient but did not result in anaemia. Newer markers, such as Ret-He were acknowledged, although there was no data to support its use in pregnant women.

1.14 AIMS AND OBJECTIVES OF THE PROJECT

1.14.1 AIMS

The overall aim of the project is to determine whether haematinic parameters measured during the 1st and 2nd trimester of pregnancy are useful in predicting birth outcomes (gestational age, birth weight or pregnancy loss).

1.14.2 OBJECTIVES

In order to achieve the aim, the following objectives were required:

- Identify and collect Ethylenediaminetetraacetic acid (EDTA) anticoagulated samples from pregnant women undergoing antenatal screening (booking bloods) and corresponding EDTA samples at 28-week review from the routine laboratory workload after routine full blood counts were complete, obtaining additional reticulocyte variables (Ret-He and IRF).
- Collect the FBC data for each subject from the Laboratory Information Management System (LIMS)
- Obtain the corresponding excess serum sample collected for serology from pregnant women at booking when all routine analysis has been completed.
- Aliquot and freeze the excess plasma and serum for later analysis of plasma ferritin and CRP and serum vitamin B12 and folate.
- Use statistical methods to investigate associations between the haematinic/reticulocyte variables and the three birth outcomes.
- Analyse, using statistical methods, the data collected using univariate and multivariate regression analysis to establish any independent predictors of the three birth outcomes.

- Use the nationally published government Index of Multiple Depravation data to assess the haematinic variables across all social classes to identify if any groups are at increased risk of adverse birth outcomes.

1.14.3 HYPOTHESES

The hypotheses are:

- There is an association between individual haematinic parameters (Haemoglobin, reticulocytes and associated variables, plasma ferritin, vitamin B12 and folate) in the 1st or 2nd trimesters of pregnancy, and one or more of the three birth outcomes.
- There is a significant difference in individual haematinic variables between women with live births versus those with pregnancy loss.
- There is an association between a diagnosis of iron deficiency in the absence of anaemia during pregnancy, and gestational delivery age or birth weight.
- There is predictive value in individual haematinic parameters for the three birth outcomes.
- There is a significant difference in the haematinic parameters measured in pregnant women grouped within the different IMD deciles.
- There is no difference between the deciles for the IMD for any of the haematinic parameters.

2.0 MATERIALS AND METHODS

2.1. Ethical approvals

Ethical approval for the study was obtained from NHS Health Research Authority and Health and Care Research Wales (HCRW) (appendix B). Local approval for the study was obtained from the local research and development team for capacity and capability to deliver the study at Hull University Teaching Hospitals NHS Trust (appendix C). All research was conducted in accordance with the declaration of Helsinki. This was a prospective observational longitudinal study using excess biological material collected during routine clinical care during pregnancy. It involved no deviation from standard clinical care and the subsequent data obtained (Ret-He, IRF, Ferritin, CRP, vitamin B12, and folate) was not used in the clinical management of the pregnancy. All collected material was anonymised for the purpose of the research.

Written consent was not sought from the women under study with implicit consent being provided prior to screening specimens provided. This helped to avoid any selection bias in identifying the samples for the study. Identification of patient details was required to identify the stage and outcomes of pregnancy, however all data was pseudoanonymised throughout sample collection and fully anonymised as soon as possible prior to research analysis. Outcome data was collected and fully anonymised before any further sample analysis performed i.e. plasma ferritin/CRP and serum vitaminB12/folate. A summary of the live birth outcomes can be found in appendix D.

2.2. Sample population

A cohort of n=613 women of reproductive age presenting for antenatal booking were identified from the Women and Children's antenatal services at Hull Royal Infirmary, Kingston-upon-Hull, England. Samples for this cohort were identified from the antenatal booking requests sent to the laboratory as part of the NHS National Screening programme for Sickle Cell and Thalassaemia. Kingston-upon-Hull is a low prevalence area for Sickle Cell and Thalassaemia but all women presenting at booking are offered screening and high risk pregnancies identified from the Family Origin Questionnaire.

2.3. Serum versus plasma comparison and sample stability

Prior to collecting samples, a small comparison study was performed comparing plasma, collected in Ethylenediaminetetraacetic acid (EDTA) with serum clotted samples for ferritin and CRP levels. Plasma was used for the study due to the residual sample availability of the full blood count from the antenatal clinic. Corresponding serum and plasma samples were retrieved from the routine workload after all routine work had been performed. Twenty-six anonymised samples were tested for ferritin and n=22 for CRP.

A small stability study was also undertaken for ferritin using EDTA , vitamin B12 and folate using serum. This was performed to ensure the optimum stability of the samples for flexibility in sample collection times. Plasma samples were retrieved from the routine workload after all routine work had been performed and anonymised. These samples are routinely stored at room temperature (RT) in the local laboratory. Three samples were randomly retrieved and centrifuged at 3500rpm for 10 minutes. Plasma from each sample was removed and aliquoted into four tubes respectively for each of the three samples. Four tubes were stored at room temperature and four tubes at 4^{o}C. One tube from each set was tested on day 2, day 3 day 4 and day 5.

For the clotted serum sample, three samples were collected again from the routine workload after all routine work had been performed and anonymised. These samples are routinely stored at 4^{o}C, therefore the same protocol was employed as for the plasma samples but excluded the assessment of stability at RT. As subject samples would only be retrieved weekly but stored at 4^{o}C, stability was assessed over a longer time period of 1, 3, 5 10 and 15 days. CRP was not tested due to manufacturers published stability data advising 2 months at 2^{o}C to 8^{o}C.

2.4. Sample and data collection

2.4.1. Samples

The Laboratory Information Management System (LIMS) for reporting patient results was used to identify potential samples for inclusion in the study (IBM Corporation, 1982, 2014 – AIX Version 7– 1996 ERICOM Software Ltd, USA Version 5.1.0). All samples for screening had a full blood count performed on the Sysmex XN-10 FBC analyser (Sysmex Corporation, Kobe, Japan). Prior to analysis of the FBC a reticulocyte count request was added to the FBC to provide a Ret-He and IRF result. An algorithm was set up to run on the LIMS to identify the booking and 28 week samples for addition of reticulocyte parameters, anonymisation, aliqoting of plasma and freezing.

Between October and November 2019, after initial screening and testing the EDTA and serum samples (those not identified as high risk of haemoglobinopathy) were retrieved from the routine workload with the study being performed on excess material after all clinically indicated analyses had been performed. The peripheral blood samples for FBC analysis were collected in 4mL K3E EDTA 7.2mg anti-coagulant vacutainers (BD Vacutainer, catalogue number 368860). Serum samples were collected in 5mL SST™ II Advance vacutainers (BD Vacutainer, catalogue number 368860). Both the FBC and Ret-He parameters were performed from the EDTA sample within 24 hours of sample collection.

Retrieved EDTA samples were centrifuged at 3500rpm for 10 minutes. The residual plasma sample material removed was transferred into 2ml micro tube, which were anonymised and frozen at -80^{o}C until required. Serum clotted samples were centrifuged in accordance with laboratory protocols for virology testing and stored at 4^{o}C for 1 week. Prior to freezing, 50µl of serum was also removed and transferred into 2ml micro tube, which were again anonymised and frozen at -80^{o}C until required.

Using the algorithm running in the LIMS, samples were identified for the women attending at 28 weeks gestation antenatal clinic for FBC bloods. EDTA samples were retrieved from the routine workload with residual material being removed, centrifuged and frozen in aliquots as previously described.

Between October and November 2020, and once all pregnancies had been concluded, the EDTA and serum samples were thawed on a roller mixer for approximately half an hour. All samples to tested were realiquoted into hanging cups compatible with the Beckman analysers. The EDTA plasma samples were re-centrifuged at 3500rpm for 10 minutes to exclude any residual cellular material and cryoprecipitate (formed during freezing) prior to testing. Plasma ferritin, serum vitamin B12 and serum folate analysis was performed using a UniCel DxI 800 Access Immunoassay System (Beckman Coulter Inc). CRP analysis was performed on Beckman Coulter AU5800 Clinical Chemistry System (Beckman Coulter Inc).

2.4.2. Outcome Data

Three outcome data variables were identified for the study as:

- gestational delivery date
- birth weight
- live birth or pregnancy loss

Outcome data for the pregnancies was extracted from the Hull University Teaching Hospitals NHS Trust patient management Lorenzo EPR Solution 2.20BASE EFB02 (DXC Technology Company, 2017) maternity system. Data extracted included recorded birth weight at delivery [g], number of babies, week of delivery gestation and status of birth (e.g. live or loss).

The WHO Low Birth Weights Policy Brief (World Health Organisation, 2014) was used to define delivery gestation age and birth weight cut-offs in pregnancy. These are defined as:

Gestational delivery age

- Preterm delivery as less than 37 weeks.
- Full term as 37 through to 41 weeks
- Post term after 42 weeks of pregnancy

Birth weights

- Low <2500g
- Normal 2500g to 3999g
- High ≥4000g

Low birth weights were also further subcategorised as low birth weight (LBW) <2500g, very low birthweight (VLBW) <1500g, and exceptionally low birth weight (ELBW) <1000g.

2.5 Full blood count red cell analysis

2.5.1 Principle of measurement

Haemoglobin, Ret-He and IRF analysis was performed using the Sysmex XN-10 automated full blood count analyser (Sysmex Corporation, Kobe, Japan). The Sysmex XN-10 FBC analyser performs *in vitro* diagnostic analysis of peripheral blood cells, which include erythrocytes and reticulocytes.

Four primary principles are employed by the analyser: Laser flow cytometry, Sheath flow direct current, Radio frequency and sodium lauryl sulphate (SLS) (Sysmex Europe, 2018). An optical detection method of flow cytometry is employed. Blood cells pass through a laser beam generating both forward and side scatter. Red blood cells (RBC) are diluted and pass through an aperture, which is assisted by hydrodynamic focusing with a laminar flow, which ensures the cells are not counted twice (figure 2.1).

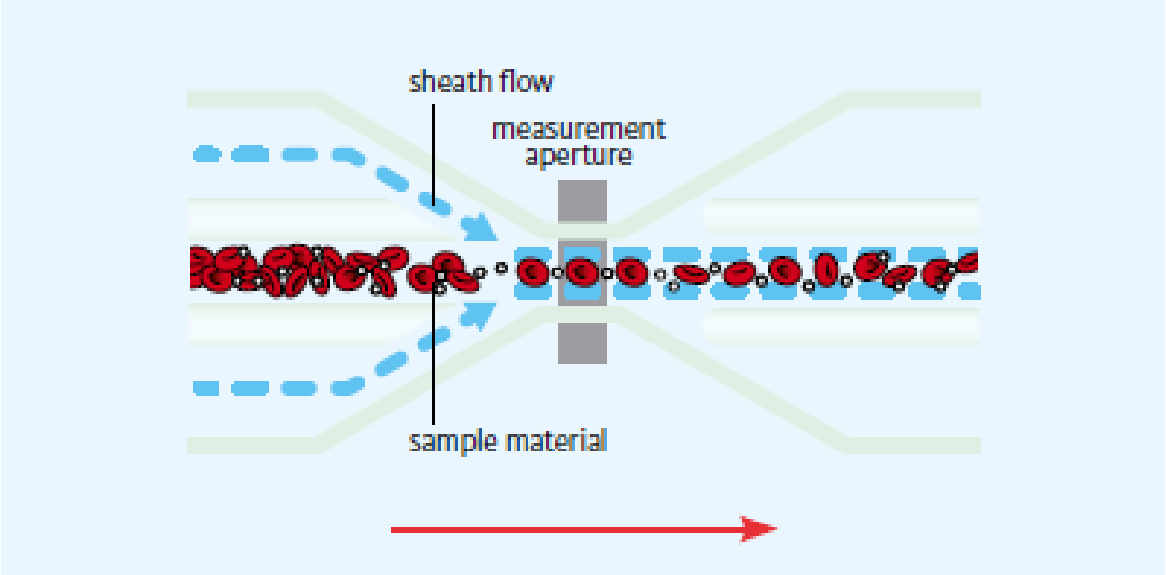


Figure 2.1. Schematic of the impedance principle with hydrodynamic focusing. Image from (Sysmex, 2012).

Cell count is required for the calculation of MCV and MCH. The size of the cells passing through the aperture causes electrical resistance and is recorded as an impedance pulse. The size of

the cell is proportional to the pulse height with haematocrit being calculated via RBC pulse height detection. Radio frequency indicates cellular structure and density with direct current detecting cell volume (Sysmex Europe, 2018).

2.5.2. Principle of measurement of haemoglobin and red cell indices

The aspirated specimen is diluted to a ratio of 1:333 and sent to the analysers flow cell. Sulfolyser (SLS) is added which haemolyses the RBC's resulting in the alteration of globin molecule conformation. The hydrophobic group of the SLS denatures the globin thus allowing oxidation of Fe^{2+} to Fe^{3+}. The hydrophobic group of the SLS binds with Fe^{3+}, which forms a SLS-Hb complex. This is then measured by light absorbance at 555nm.

2.5.3. Principle of measurement of Ret-He

Ret-He measurement is derived from reticulocytes in the reticulocyte channel of the Sysmex XN-10 FBC analyser. It is comprises of a two-step process where nucleic acids are stained in the first instance with a polymethene dye, specific for RNA/DNA. Using impedance, a fluorescent flow cytometry technique (forward and side scatter, against fluorescent intensity) is employed which measures the cellular haemoglobin of the cells. The size of the cell directly correlates with the Hb content (Chinudomwong, et al., 2020). Results are presented as picograms (pg) of each reticulocyte, the parameter of which is termed Ret-He.

2.5.4. Principle of measurement of IRF

IRF is also derived from the reticulocyte channel of the SysmexXN-10 FBC analyser. Using fluorescence intensity, reticulocytes are fractionated into three categories of maturity (figure 2.2):

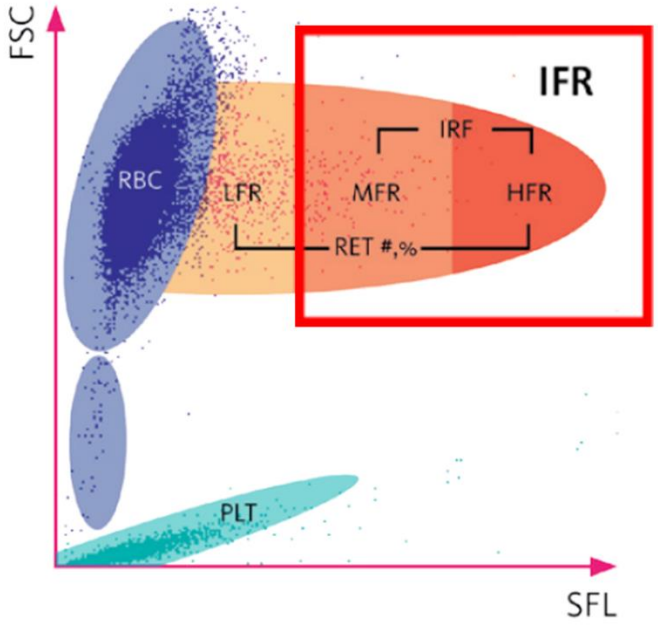


Figure 2.2. Schematic of scattergram showing low medium and high fluorescence of reticulocytes in the reticulocyte channel. Image adapted from (Sysmex, 2016)

On the scattergram:

- Low-fluorescence reticulocytes (LFR) represent ‘mature’ reticulocytes
- Medium-fluorescence reticulocytes (MFR) represent ‘semi-mature’ reticulocytes
- High-fluorescence reticulocytes (HFR) represent ‘immature’ reticulocytes

The IFR is calculated as the sum of MFR plus HFR (IRF=MFR+HFR).

2.6. Biochemistry analysis

2.6.1 Principle of measurement Plasma ferritin

Ferritin analysis was performed using a Beckman Coulter Beckman Coulter UniCel DxI 800 Access Immunoassay System (Beckman Coulter Inc). Plasma ferritin was analysed using a paramagnetic particle in an *in vitro* chemiluminescent immunoassay method for quantitative determination. The assay employs a two-site immunoenzymatic ("sandwich") assay. A pre-defined volume of patient plasma is added to a reaction well coated with goat anti-ferritin-alkaline phosphatase conjugate, and paramagnetic particles coated with goat anti-mouse: mouse anti-ferritin complexes. Plasma binds to the monoclonal anti-ferritin in the solid phase with the goat anti-ferritin enzyme conjugate reacting with a different antigenic site on the ferritin molecule. After incubation, material bound to the solid phase remains bound by the magnetic field while unbound material is washed away. Chemiluminescent substrate is added with the resulting reaction generating light. Results are determined automatically by the system software using a weighted four-parameter logistic curve (4PLC) math model. The light produced is directly proportional to the concentration of ferritin in the sample determined from the measured light production by means of the stored calibration data. (Beckman Coulter, 2019a).

2.6.2 Principle of measurement Vitamin B12/Transcobalamin

Serum Vitamin B12 was measured using the Beckman Coulter Beckman Coulter UniCel DxI 800 Access Immunoassay System (Beckman Coulter Inc). Like plasma ferritin vitamin B12 was analysed using a paramagnetic particle, chemiluminescent immune assay for determination of vitamin B12 (Beckman Coulter Access Vitamin B12 Cobalamin Ref 33000). The assay employs a competitive binding immunoenzymatic principle. Sample is added to a reaction

well along with alkaline potassium cyanide and dithiothreitol. This treatment denatures the B12 binding proteins converting all forms of vitamin B12 to the cyanocobalamin form. Once neutralised, intrinsic factor-alkaline phosphatase conjugate along with paramagnetic particles coated with goat anti-mouse IgG: mouse monoclonal anti-intrinsic factor is added to the sample. Vitamin B12 binds to the intrinsic factor conjugate, which prevents the conjugate from binding to the solid phase anti-intrinsic factor. Following incubation, the material bound to the solid phase is held by a magnetic field with unbound material washed away. Chemiluminescent substrate is added with the resulting reaction generating light. Results are determined automatically by the system software using a weighted four parameter logistic curve (4PLC) math model. The light produced is directly proportional to the concentration of vitamin B12 in the sample determined from the measured light production by means of a stored multi-point calibration curve calibration. (Beckman Coulter, 2019b).

2.6.3 Principle of measurement Folate

Serum folate was measured using the Beckman Coulter Beckman Coulter UniCel DxI 800 Access Immunoassay System (Beckman Coulter Inc) with no sample pre-treatment required as for red cell folate. Serum folate was analysed using a paramagnetic particle, chemiluminescent immune assay for determination of folic acid levels (Beckman Coulter Access Folate Ref A98032). The assay employs a competitive binding immunoenzymatic principle. The sample is treated to release folate from endogenous binding proteins. Folate binding protein, mouse anti-folate binding protein, folic-alkaline phosphatase conjugate, and goat anti-mouse capture antibody coupled to paramagnetic particles are added to the reaction chamber. The folate in the sample competes with the folic-alkaline phosphatase conjugate for binding sites on a limited amount of folate binding protein. The resulting

complex binds to the solid phase via mouse anti-folate binding protein. Following and incubation period in the reaction chamber, materials bound to the solid phase are held in a magnetic field while unbound material is washed away. The chemiluminescent substrate is added with the resulting reaction generating light. Results are determined automatically by the system software using a weighted four parameter logistic curve (4PLC) math model. The light produced is directly proportional to the concentration of vitamin B12 in the sample determined from the measured light production by means of a stored multi-point calibration curve calibration. (Beckman Coulter, 2019c).

2.6.4 Principle of measurement C-Reactive Protein

CRP analysis was performed using a Beckman Coulter AU5800 Clinical Chemistry System (Beckman Coulter Inc). CRP was measured by immune-turbidimetry for quantitative determination of CRP in plasma. Plasma is mixed with a buffer and latex suspension, with CRP reacting specifically with CRP antibodies coated on latex particles to produce insoluble aggregates. Absorbance of the aggregates is proportional to the CRP concentration in the sample (Beckman Coulter, 2019d).

2.6.5 Index of multiple depravation decile

Data for the index of multiple depravation score was accessed via an open data source via Ministry of Housing Communities and Local Government English indices of depravation 2019 (Ministry of Housing, Communities and Local Government, 2019). The local area lookup tool was used to extract the data by postcode for Kingston-upon-Hull and the East Riding of Yorkshire in a Microsoft excel spreadsheet format. Once the postcodes and depravation scores were matched to the study subjects the postcodes were anonymised to HUXX X.

2.6.6 Definition of Anaemia based on NICE guidance

For assessment of anaemia in pregnancy, the National Institute for Health and Care Excellence in the UK was used for haemoglobin cut-off levels, defining anaemia in pregnancy (NICE, 2021). These levels also reflect the levels published by the World Health Organisation (WHO, 2011a), although these were determined more than 30 years ago from pooled longitudinal data from four European studies (Svanberg, et al., 1975, Sjostedt, et al., 1977, Puolakka, 1980, Taylor, et al., 1982, O'Brien and Ru, 2017). There are significant limitations in these studies, which include; 1) the pregnant women were receiving iron supplementation daily; 2) the limited size of the populations studied ranging from 32 to 267 subjects; 3) racial composition was not specified and assumed to be predominantly white; 4) pre-pregnancy BMI (2 out of the 4 studies averaged a BMI 21; and 5) amount of supplemental iron given to different populations varied between 65mg and 200mg, which at the high end is greater than 7 times the current recommended daily allowance. Yet despite these limitations, the following defined levels have been universally adopted:

- Hb <110.0g/L in the 1st trimester of pregnancy
- Hb <105.0g/L in the 2nd and 3rd trimester of pregnancy

The recent UK National Screening Committee external review for 'Screening for Iron Deficiency Anaemia in Pregnancy' (2021) concluded there are large gaps in the current literature.

2.7 Statistical analysis

Microsoft Excel was used to calculate coefficient of variation (CV) percentage for sample stability analysis. The statistical software 'Analyse-it®' for Microsoft®Excel (Version 5.90, Analyse-it Software Ltd, Leeds, UK) was used to analyse distribution of data, correlation, comparison of medians and medians of more than three groups.

All variables evaluated in the study were analysed using a Kolmogorov-Smirnov D statistical test to establish either parametric or non-parametric distribution of the data. As the data did not show parametric distribution, non-parametric methods for analysis of the data was applied.

Correlation studies using Spearman's Rank Correlation were conducted to establish associations between haemoglobin, reticulocyte counts, Ret-He, IRF, plasma ferritin, serum vitamin B12, and serum folate with two birth outcomes (gestational delivery age and birth weight). The Spearman Rank Correlation (the non-parametric version of the Pearson Product Moment Correlation) measures the linear relationship between two ranked variables. The two variables do not need to be measured on the same scale. All statistical comparisons were performed using a 95% confidence interval and statistical significance was defined as $p<0.05$.

To compare median values between 1st and 2nd trimester Wilcoxon-Mann-Whitney U test was used for haemoglobin, reticulocyte counts, Ret-He, IRF, and plasma ferritin to determine any shift in the distributions of the data. It was also used for the assessment of IDWA and comparison of live births with pregnancy losses. The Wilcoxon-Mann-Whitney U test is used to assess two independent groups comparing the median of one sample with the median of the same sample at a different time point. The test takes all observations from both groups

and ranks them in order of size. The observed ranks from the first group are then summed and the test statistic formed. Statistical analysis was performed using a 95% confidence interval and statistical significance defined as $p<0.05$.

The Kruskal-Wallis (ANOVA) test was used to compare vitamin B12 medians and serum folate medians in the 1st trimester bloods using the defined cut-off's for gestational delivery age and birth weight previously described in section 2.4.2. It was also used for the statistical assessment of IMDD data. The Kruskal-Wallis test assesses the analysis of variance of the medians from three or more groups and comparing them. It is considered as the non-parametric alternative to the one-way ANOVA and extension of the Mann-Whitney-U to compare more than two independent groups. Statistical analysis was performed using a 95% confidence interval and statistical significance defined as $p<0.05$.

To evaluate factors associated with birth weight, gestational age and live birth outcome, univariate and multivariate logistic regression (ordinary least squares (OLS)) analyses were performed. OLS regression estimates the relationship between one or more independent variables and a dependent variable by minimising the sum of the squares in the difference between the observed and predicted values of the dependent variable configured as a straight line. These analyses were performed using STATA 17.0 (StataCorp, Lakeway Drive, College Station, Texas 77845 USA). A *p*-value cut-off of 0.05 was used for statistical significance. However, as is standard practice, any variables returning a p-value of < 0.2 upon univariate analysis, were included in the multivariate model to identify independent predictors of birth weight, gestational age and live birth outcome.

3.0. RESULTS

3.1. Sample stability

Prior to starting the study and collection of specimens (plasma and serum) a small study for stability of samples was performed. This was to ensure stability of the collected and any small delays in collecting specimens would not directly affect analysis. Time and temperature can significantly affect results of analysis so optimum time of collection and storage is important. Samples are collected from subjects at varying times during the day and within the study as it was not possible to control for time of sample collection to optimum storage and analysis time. It is widely accepted that plasma and serum samples have longer stability when retained at lower temperatures or frozen.

Within the study laboratory whole blood EDTA samples for FBC's are retained at room temperature, therefore stability was assessed both at RT and 4^{o}C to evaluate optimum concentration levels (in days) before freezing the samples for later analysis.

Three independent plasma specimens and three independent serum specimens with varying concentrations were retrieved from the routine laboratory workload after all clinical testing had been conducted. Each specimen was tested for baseline measurement (plasma for ferritin and CRP and serum for vitamin B12 and folate). Plasma specimens were stored at RT and 4^{o}C. Serum samples were stored at 4^{o}C only. Plasma samples were tested on the following 4 consecutive days with serum specimens tested on days 3, 5, 10 and 15. Excess serum specimens are retained in the virology department at 4^{o}C for 2 weeks then frozen at -21^{o}C for 2 years. As these samples could only be retrieved between 7 to 14 days due to accessibility and clinical testing, the stability study was conducted up to 15 days. Specimens for the study

were however collected within 7 days of collection. The results of the analysis can be found in appendix E.

3.2. Serum and plasma comparison

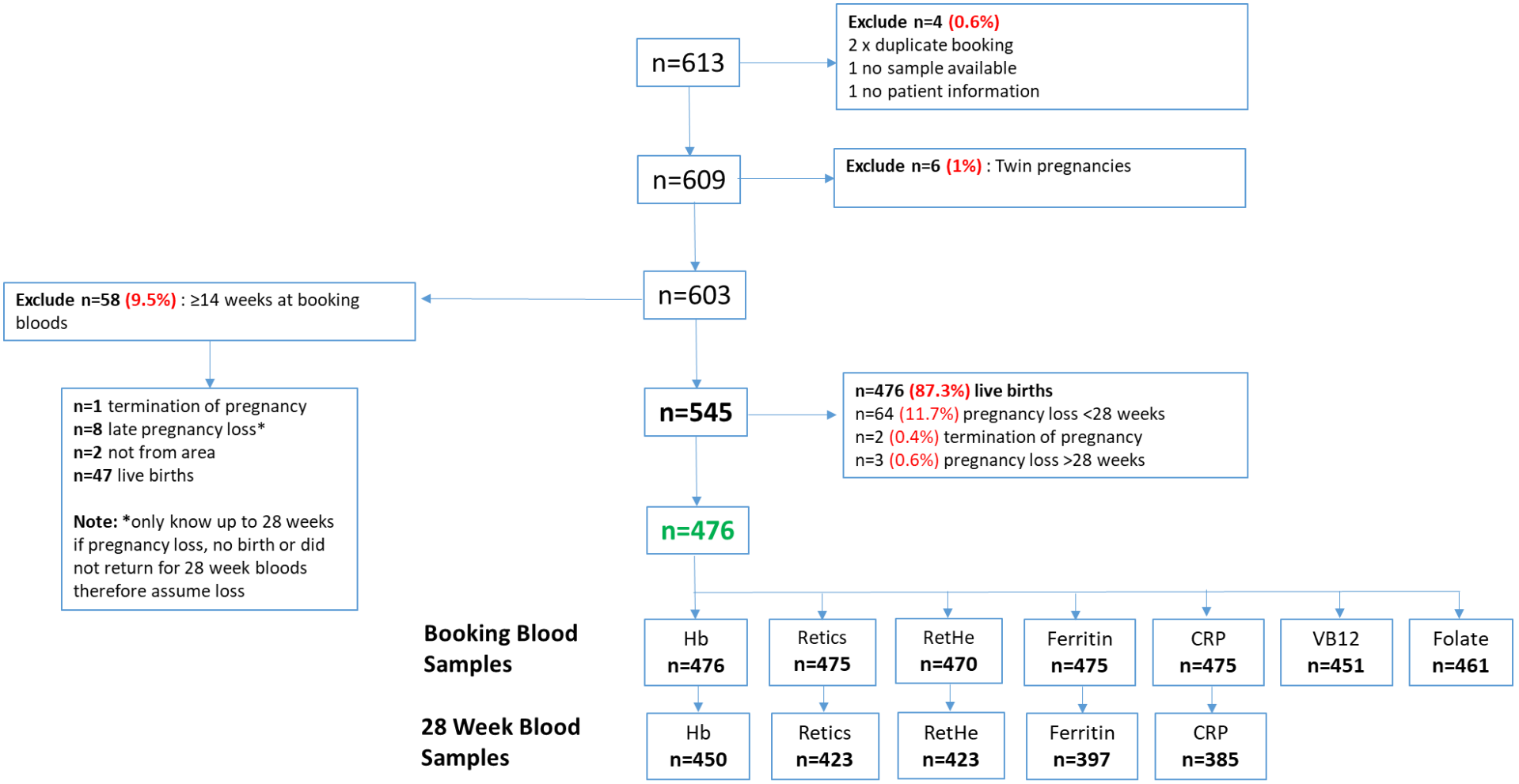
In addition to performing a stability study prior to sample collection, a small comparison study was also performed comparing plasma, collected in Ethylenediaminetetraacetic acid (EDTA) with serum-clotted samples for ferritin and CRP levels. Plasma was used for the study due to the residual sample availability of blood from the antenatal clinic. Corresponding serum and plasma samples collected as described in the materials and methods. The results of the analysis can be found in appendix F, which showed excellent correlation for both ferritin (figure 1) and CRP analysis (figure 3).

Although analysis showed excellent correlation, ($r^2 = 0.993$, $p=0.0001$), using Bland-Altman limits of agreement statistic to show differences between the sample types showed a mean difference of 21.23% for all samples (appendix F, figure 2). However, when excluding the n=5 samples with ferritin values >150µg/L the difference between the samples was 4.2%. For CRP the mean difference was 0.93% (appendix F, figure 4).

3.3. Sample population subject demographics

Data was collected from 613 subjects who presented for antenatal screening (booking bloods) in the 1st trimester of pregnancy. Two samples were excluded as duplicated booking, n=1 had no sample available and n=1 had no patient information. A further n=6 subjects were excluded due to multiple pregnancy. Fifty eight subjects were also removed from the data set as these women presented for booking bloods after 14 weeks gestation (2nd or 3rd trimester). Included in the final analysis were n=545 subjects (figure 3.1). Of these n=476 resulted in live singleton births.

Figure 3.1. Summary of samples collected and excluded from analysis.



Data from n=545 pregnant women was analysed. Of the n=476 women with live births, n=32 (6.72%) had a gestational delivery age <37 weeks, n=380 (83.20%) had a normal delivery gestation and n=48 (10.08%) had a gestational delivery ≥42 weeks. For birth weight, n=28 (5.88%) had a low birth weight <2500g, n=380 (79.83% had a normal birth weight (2500 – 3999g) with n=68 (14.29%) having a high birth weight ≥4000g. Measured variable characteristics of all pregnant women in the study are summarised in table 3.1. Full analysis of data presented in appendix G to U.

Table 3.1. Characteristics of all pregnant women tested for Hb, Reticulocytes, Ret-He, IRF, plasma ferritin, plasma CRP, serum vitamin B12 and serum folate at booking (6 to 14 weeks gestation) and at 28 (+/-2 weeks) gestation. Values are median, 5%,95% percentiles range and IQR.

	Booking bloods (6 - 14 weeks)					28 week bloods (+/- 2 Weeks)				
Variable	**# of samples analysed**	**Median**	5%,95% percentiles	**Range**	**IQR**	**# of samples analysed**	**Median**	5%,95% percentiles	**Range**	**IQR**
Age	**545**	**29**	**19 - 37**	**15 - 47**	**24 - 32**					
Haemoglobin (g/L)	545	129.0	114.0 – 143.0	73.0 - 158.0	123.0 - 135.0	454	115.0	97.0 – 130.0	78.0 - 142.0	109.0 - 121.0
Reticulocytes (x10^9/L)	544	66.8	39.9 – 106.3	28.3 - 133.2	54.8 - 79.7	427	69.6	47.5 – 111.6	33.4 - 146.9	59.9 - 83.8
RetHe (pg)	539	33.5	29.2 – 35.8	5.0 - 38.4	32.2-34.4	427	31.7	25.0 – 34.8	18.6 - 41.5	29.2 - 33.3
Immature Reticulocyte Fraction (%)	536	7.9	3.6 – 14.7	2.5 - 47.6	6.1 - 10.3	427	16.4	9.2 – 28.3	5.4 - 39.0	13.1 - 21.3
Plasma Ferritin (ug/L)	544	28.0	6.0 – 84.5	3.0 - 204.0	16.0 - 47.0	400	7.0	3.0 – 32.6	2.0 - 154.0	5.0 - 11.0
Plasma CRP (mg/L)	544	3.4	0.6 – 19.0	0.3 - 83.0	1.6 - 7.6	394	4.4	0.9 – 17.0	0.3 - 603.0	2.4 - 7.9
Serum Total Vitamin B12 (ug/L)	517	153.5	83.3 – 325.8	28.7 - >1106.7	121.6 - 201.5	-	-	-	-	-
Serum Folate (ug/L)	469	9.1	2.5 – 23.6	1.2 ->23.6	5.2 - 14.4	-	-		-	-

3.4. Statistical analysis for normality

Statistical analysis for normality of distribution was performed using Kolmogorov-Smirnov D (KSD) statistic. This showed the data was not normally distributed for the majority of variables, with the exception of reticulocytes in the 1st trimester and reticulocytes and IRF in the 2nd trimester, which showed normal distribution (appendix G). A summary of the results is displayed in table 3.2. As the majority of the data showed non normal distribution, the non-parametric tests, Spearman Rank Correlation was used for subsequent analysis.

Table 3.2. Summary Kolmogorov-Smirnov D (KSD) statistic testing for normality (logarithmic values) at booking (6 to 14 weeks) and 28 (+/- 2) weeks for live births only. p= assumption of null hypothesis (data is normally distributed).

LOG Data									
Booking Bloods	**Age**	**Hb**	**Retics**	**RetHe**	**IRF%**	**Fer**	**CRP**	**VB12**	**Fol**
Test data available	545	**545**	**544**	**539**	**536**	**544**	**545**	**518**	**528**
Median	29.0	2.1	1.8	1.5	0.90	1.5	0.5	2.2	1.0
Range	15-47	1.9-2.2	1.5-2.1	0.7-1.6	0.4-1.7	0.5-2.3	-0.7-1.9	1.5-3.0	0.1-1.4
Ist Quartile	24.7	2.1	1.7	1.5	0.8	1.2	0.2	2.1	0.8
3rd Quartile	32.0	2.1	1.9	1.5	1.0	1.7	0.9	2.3	1.2
Mean	28.5	2.1	1.8	1.5	0.9	1.4	0.5	2.2	1.0
Mean SE	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SD	5.6	0.0	0.1	0.1	0.2	0.3	0.5	0.2	0.3
Skewness	-	-1.2	-0.1	-8.8	0.1	-0.3	0.1	0.3	-0.5
Kurtosis	-	5.7	-0.2	100.8	0.8	-0.1	-0.3	1.3	-0.6
K-S D Statistic (D)	-	0.06	0.03	0.25	0.05	0.06	0.04	0.05	0.09
P-Value of Test (p)	-	0.0001	1.0000	<0.0001	0.0012	<0.0001	0.0353	0.0078	<0.0001
Accept or reject	-	**Reject**	**Accept**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**
28 week Bloods	**Age**	**Hb**	**Retics**	**RetHe**	**IRF%**	**Fer**	**CRP**	-	-
Test data available	-	**454**	**427**	**427**	**427**	**400**	**404**	-	-
Median	-	2.1	1.8	1.5	1.2	0.9	0.6	-	-
Range	-	1.9-2.2	1.5-2.2	1.3-1.6	0.7-1.6	0.3-2.2	-0.7-2.8	-	-
Ist Quartile	-	2.0	1.8	1.5	1.1	0.7	0.4	-	-
3rd Quartile	-	2.1	1.9	1.5	1.3	1.0	0.9	-	-
Mean	-	2.1	1.9	1.5	1.2	0.9	0.6	-	-
Mean SE	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
SD	-	0.0	0.1	0.1	0.2	0.3	0.4	-	-
Skewness	-	-0.5	0.2	-1.2	-0.3	1.1	-0.3	-	-
Kurtosis	-	1.0	0.0	2.1	0.2	1.4	1.9	-	-
K-S D Statistic (D)	-	0.06	0.04	0.14	0.03	0.13	0.06	-	-
P-Value of Test (p)	-	0.0010	0.0577	<0.0001	1.0000	<0.0001	0.004	-	-
Accept or reject	-	**Reject**	**Accept**	**Reject**	**Accept**	**Reject**	**Reject**	-	-

3.5. General summary of samples collected

A total of n=545 FBC sample data was collected for pregnant women presenting during the booking period (6 to 14 weeks). Sixty-nine FBC sample data was excluded due to pregnancy loss (n=63 <28 weeks and n=4 at >28 weeks as there was no birth outcomes (gestation delivery age or birth weight) recorded). A further n=2 were excluded due to termination of pregnancy. There were n=476 live births from the data collected at booking. At 28 weeks a further n=26 FBC's were not available due to women not attending for their 28 (+/- 2 weeks) week bloods. This resulted in n=450 FBC's being available for analysis at 28 weeks (figure 3.1).

3.6. Haemoglobin analysis

Haemoglobin data for the n=545 women at booking showed a median Hb of 129.0g/L (IQR 123.0 -135.0/range 73.0 – 158.0). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.06 and p value of 0.0001, showing the Hb from the women in this study to be not normally distributed. Of the n= 545 samples collected, there was n=476 women with live birth outcomes available for analysis.

3.6.1 Haemoglobin analysis and delivery outcome

Haemoglobin data for the n=476 women with live birth outcomes showed a median Hb of 129.0g/L (5%-95% percentiles 114.0 – 143.0g/L/range 93.0 – 158.0/IQR 123.0 -135.0). As the data was not normally distributed a Spearman Rank Correlation statistic was used to assess for association of the Hb data against outcomes (gestational delivery age [days] and gestational birth weight) at both booking (6 to 14 weeks) and at 28 weeks (table 3.3, appendix H). The absolute difference between Hb at booking and Hb at 28 weeks was also tested.

Table 3.3. Summary of Spearman Rank Correlation statistic for Hb analysis at booking, 28 weeks and the difference of Hb between Booking Hb and 28 week for association with delivery gestation time [days] and delivery birth weight [g].

	# of results	Median Hb g/L (range)	Spearman's rs	p-value	Accept/reject H0
Hb Booking					
Delivery Gestation [Days]	476	129.0 (93.0-158.0)	0.002	0.9621	Accept H0
Birth Weight [g]			0.041	0.3724	Accept H0
Hb 28 weeks					
Delivery Gestation [Days]	450	115.0 (78.0-142.0)	-0.036	0.4383	Accept H0
Birth Weight [g]			-0.081	0.0867	Accept H0
Difference Booking to 28 weeks					
Delivery Gestation [Days]	450	-14 (-43-18)	-0.046	0.328	Accept H0
Birth Weight [g]			**-0.146**	**0.0018**	**Reject H0**

Anaemia in the 1st trimester of pregnancy, is defined by NICE, (2021) as <110.0g/L. Of the n=476 live births Hb results at booking the median Hb was 129.0g/L (5%-95% percentiles 114.0 – 143.0g/L/range 93.0 – 158.0/ IQR 123.0 – 135.0) with n=11 (2.31%) having an Hb ≤110g/L. The remainder n=465 (97.69%) had an Hb >110.0g/L. In the 2nd trimester anaemia is defined as Hb <105g/L with median Hb 115.0g/L (5%-95% percentiles 97.0 – 130.0g/L/78.0 – 142.0 (IQR 109.0 – 121.0). The data showed that n=69 (15.33%) had an Hb ≤105.0g/L, while n=381 (84.67%) had an Hb >105.0g/L.

Gestational Delivery Outcome at Booking (Hb cut-off 110.0g/L)

Analysis of 1st trimester booking bloods with gestational delivery outcomes (n=476 live births) showed n=32 (6.72%) had a preterm delivery of <37 weeks (24 weeks + 4 days to 36 weeks + 4 days), 429 (90.13%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.15%) had a post term deliver gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The n=465 (97.69%) women with a normal Hb >110.0g/L, n=32 (6.72%) had a preterm gestational delivery (<37w) with n=418 (87.82%) having normal gestation delivery (37 to 41 weeks +6 days) and 15 (3.15%) having post-term gestational delivery (>42 weeks).

All n=11 (2.31%) women with a low Hb ≤110.0g/L had a normal gestational delivery (37 to 41 weeks +6 days).

The data showed there was no correlation/association between Hb and delivery gestation age [days] (R^2= 0.002, p= 0.9621) in the 1st trimester (table 3.3, appendix H).

Gestational Birth Weight Outcome at Booking (Hb cut-off 110.0g/L)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes (n=476 live births) showed n=28 (5.88%) had a low birth weight <2500g at delivery. There were n=380 (79.83%) live births with normal birth weight (2500g to 3999g) and n=68 (14.28%) had a high birth weight >4000g (4000g to 4885g).

Of the n=465 (97.69%) with Hb >110.0g/L n=28 (5.94%) had low birth weight (<2500g), n=369 (77.52%) had a normal birth weight (>2500g to 3999g) with n=68 (14.28%) having a high gestational birth weight (>4000g).

All n=11 (2.31%) women with Hb ≤110.0g/L at booking, had normal gestational birth weight (2500g to 3999g).

The data showed there was no correlation/association between Hb and birth weight [g] (R^2= 0.041, p= 0.3724) in the 1st trimester (table 3.3, appendix H).

Gestational Delivery Outcome at 28 weeks (Hb cut-off 105.0g/L)

Analysis of 2nd trimester booking bloods with gestational delivery outcomes (n=450 live births) showed n=28 (6.22%) had a preterm delivery of <37 weeks (24 weeks + 4 days to 36 weeks + 4 days), n=407 (90.45%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.33%) had a post term delivery gestation >42 weeks (42 weeks to 42 weeks + 2 days).

Of the n=381 (84.67%) women with Hb >105.0g/L, n=26 (5.78%) had a preterm gestational delivery (<37w) with n=341 (75.78%) having normal gestation delivery time (37 to 41 weeks + 6 days) and 14 (3.11%) having post-term gestational delivery (>42 weeks (42weeks to 42 weeks + 2 days)).

Women with a Hb ≤105.0g/L (n=69 (15.33%)) at 28 weeks showed n=2 (0.44%) had a preterm delivery gestation (<37 weeks), n=66 (14.67%) had a normal gestational delivery (37 to 41 weeks + 6 days), and n=1 (0.22%) had a post-term gestational delivery (>42 weeks (42 weeks + 2 days)).

The data showed there was no correlation/association between Hb and delivery gestation age [days] (R^2= -0.036, p= 0.4383) in the 2nd trimester (table 3.3, appendix H).

<u>Gestational Birth Weight Outcome at 28 weeks (Hb cut-off 105.0g/L)</u>

Analysis of 2nd trimester booking bloods with gestational birth weight outcomes (n=450 live births) showed n=26 (5.78%) had a low birth weight <2500g at delivery. There were 358 (79.56%) live births with normal birth weight (2500g to 3999g) and n=66 (14.67%) had a high birth weight >4000g (4000g to 4885g).

Women with a Hb >105.0g/L at 28 weeks n=381 (84.67%) showed n=25 (5.56%) had a gestational birth weight of <2500g, n=301 (71.56%) had a normal birth weight (>2500g to 3999g) with n=55 (12.22%) having a high gestational birth weight (>4000g).

Of the n=69 (15.33%) of women with Hb <105.0g/L at 28 weeks only n=1 (0.22%) had a gestational birth weight of <2500g, with n=57 (12.67%) having a normal birth weight (>2500g to 3999g) and n=11 (2.44%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between Hb and birth weight [g] (R^2= -0.081, p= 0.0867) in the 2nd trimester (table 3.3, appendix H).

Difference in Hb levels between 1st trimester booking bloods and 2nd trimester 28 weeks bloods

The median Hb of the 1st trimester booking bloods (n=545) were compared with the median Hb 2nd trimester 28 week bloods (n=450). The results showed that there was a decrease in the median Hb from booking from 129.0g/L to 115.0g/L at 28 weeks. This showed an overall median decrease of 14.0g/L (10.85%) between 1st and 2nd trimester bloods, range -43.0g/L to + 18.0g/L (-30.36% to +19.35%) (figure 3.2, appendix H. The absolute difference between 1st trimester booking bloods and 2nd trimester 28 week bloods were correlated with birth outcomes (gestational delivery time [days] and gestational birth weight [g] using Spearman Rank Correlation.

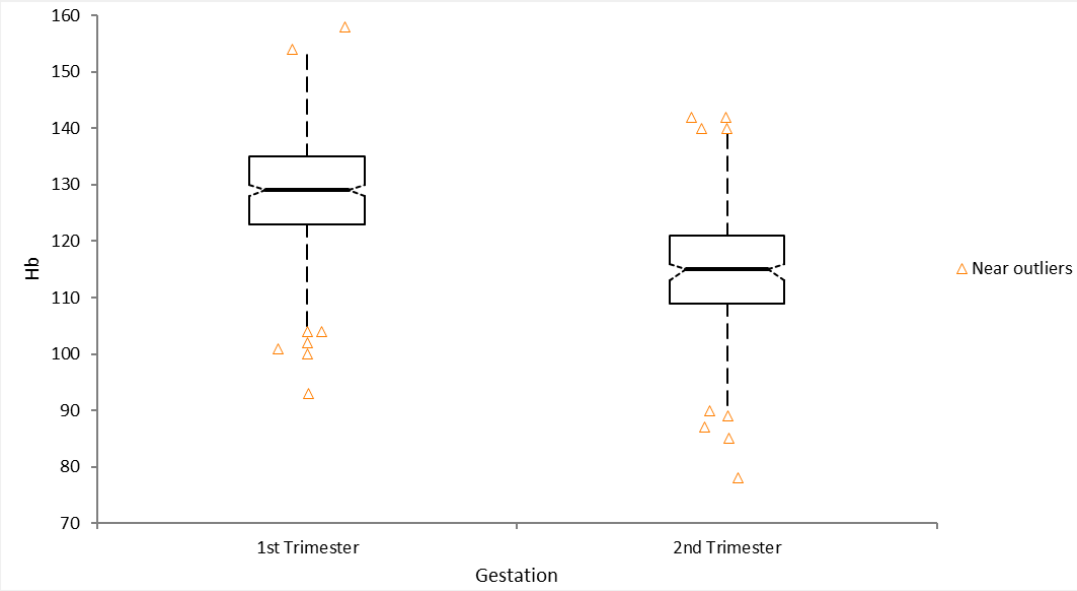


Figure 3.2. Difference of median Hb count from 1st trimester booking bloods to 2nd trimester 28 week bloods using Wilcoxon-Mann-Whitney test p=<0.0001 rejecting the null hypothesis of the shift in location between the distributions of the populations being equal to 0.

Difference of Hb between booking and 28 weeks

Analysis of the n=450 data sets were analysed for differences between 1st trimester booking bloods and 2nd trimester 28 week bloods, n=16 (3.55%) had an increase in Hb between 1st trimester and 2nd trimester. The remaining n=434 (96.45%) all showed a decrease in Hb ranging from 1.0g/L to 43.0g/L at 28 weeks.

Difference of Hb between booking and 28 weeks and gestational delivery outcome

Women with a decrease in Hb n=434 (96.45%), n=24 (5.33%) had a gestational delivery date <37 weeks (28w+3 days to 36w+4 days), n=396 (88.01%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=14 (3.11%) having a post term gestational delivery >42 weeks (42 to 42weeks +2 days).

Those with an increase or no difference in Hb the n=16 (3.55%), n=4 (0.89%) had a gestational delivery date <37 weeks (32w+1 day to 35w+4 days), n=11 (2.44%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=1 (0.22%) having a post term delivery (>42 weeks).

The data showed there was no correlation/association between the difference between Hb at booking and 28 weeks with gestational delivery time (R^2= -0.046, p= 0.3280) (table 3.3, appendix H).

Difference of Hb between booking and 28 weeks and gestational birth weight outcome

Women with a decrease in Hb n=434 (96.45%), n=22 (5.33%) had a low gestational birth weight (<2500g), n=348 (77.33%) had a normal birth weight (>2500g to 3999g) with 64 (14.22%) having a high gestational birth weight (>4000g (4000g to 4885g)).

Those with an increase or no difference in Hb n=16 (3.55%), n=4 (0.88%) had a low gestational birth weight (<2500g), n=10 (2.33%) had a normal delivery birth weight (2500g to 3999g) with n=2 (0.44%) having a high gestational birth weight (>4000g (4000g to 4885g)).

The data showed a very weak correlation/association between the difference between Hb at 1st trimester booking and 2nd trimester 28 weeks with gestational birth weight (R^2= -0.046, p= 0.0018) (table 3.3, appendix H).

3.6.2 Summary

The data for Hb analysis showed there were low numbers of anaemic women using the NICE generally accepted cut-off of 110.0g/L in the 1st trimester, 2.31% increasing to 15.33% in the 2nd trimester. For 1st trimester booking bloods 6.72% of women had pre-term delivery gestational delivery outcomes (<37 weeks) with 6.22% in the 2nd trimester (28 weeks) table 3.3/appendix H Results were similar for low birth weight (<2500g) gestational delivery outcomes with 5.88% and 5.78% respectively for booking and 28 week Hb.

Overall the data for Hb showed no association with birth outcomes, either preterm delivery or low gestational birth weight with the exception of difference in Hb levels between booking bloods and 28 weeks bloods for birth weight outcomes although this correlation was very weak R^2= -0.046.

3.7. Reticulocyte analysis

Reticulocyte data for the n=544 women at booking showed a median reticulocyte count of $66.8x10^9$/L (IQR 54.8 -79.7/range 73.0 – 158.0). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.03 and p value of 1.000, showing the reticulocyte count from the women in this study to be normally distributed. However as all other parameters tested showed non normal distribution therefore a non-parametric analysis was used for consistency of analysis. Of the n=544 samples collected, there was n=475 women with live birth outcomes available for analysis.

3.7.1 Reticulocyte analysis and delivery outcome

Reticulocyte data for the n=475 women with live birth outcomes showed a median reticulocyte count of $66.8x10^9$/L (IQR 55.0 -80.9/range 28.3 – 133.20). A Spearman Rank Correlation statistic was used to assess for association of the reticulocyte data against outcomes (gestational delivery age [days] and gestational birth weight) at both booking (6 to 14 weeks) and at 28 weeks (table 3.4, appendix I). The absolute difference between reticulocyte count at booking and at 28 weeks was also tested.

Table 3.4. Summary of Spearman Rank Correlation statistic for reticulocyte count analysis at booking, 28 weeks and the difference between booking reticulocyte count and 28 week counts for association with delivery gestation age [days] and delivery birth weight [g].

	# of results	Median Retics x10^9/L (Range)	Spearman's rs	p-value	Accept/reject H0
Retics Booking					
Delivery Gestation	475	66.8 (28.3 - 133.2)	**-0.109**	**0.0176**	**Reject H0**
Birth Weight			0.012	0.7979	Accept H0
Retics 28 weeks					
Delivery Gestation	423	69.4 (33.4 - 146.9)	-0.055	0.2623	Accept H0
Birth Weight			0.044	0.3617	Accept H0
Difference Booking to 28 weeks					
Delivery Gestation	422	3.7 (-62.2 - 54.7)	0.06	0.2194	Accept H0
Birth Weight			0.018	0.7177	Accept H0

There are no published or consensus values available for reticulocyte counts during pregnancy, therefore the cut-off used (80.0x10^9/L) based on locally defined laboratory reference intervals was used for the normal population.

Of the n=475 reticulocyte count results at booking the median reticulocyte count was 66.80x10^9/L (28.3 – 133.20 (IQR 55.0 – 80.9)) with n=356 (74.95%) having a reticulocyte count ≤80x10^9/L (normal). There were no women with reticulocytopenia (<20x10^9/L). The remainder n=119 (25.05%) had a reticulocyte count >80x10^9/L. In the 2nd trimester the median reticulocyte count was 69.4x10^9/L (33.40 – 146.90 (IQR 59.9 – 82.8)). The data showed that n=303 (71.63%) had a reticulocyte count ≤80x10^9/L (normal) and no reticulocytopenia, while n=120 (28.37%) had a reticulocyte count >80x10^9/L.

Gestational Delivery Outcome at booking (reticulocyte count cut-off 80x10^9/L)

Analysis of 1st trimester booking bloods with gestational delivery outcomes (n=475 live births) showed n=33 (6.74%) had a preterm delivery of <37 weeks (24 weeks + 4 days to 36 weeks + 4 days), 428 (90.10%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.16%) had a post term delivery gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The n=119 (25.05%) subjects with a reticulocyte count >80x10^9/L, n=11 (2.32%) had a preterm gestational delivery (<37w) with n=104 (21.89%) having normal gestation delivery (37 to 41 weeks +6 days) and 4 (0.84%) having post-term gestational delivery (>42 weeks).

Women with a reticulocyte count ≤80x10^9/L (n=356 (74.95%) showed, n=21 (4.42%) had a preterm gestational delivery (<37w) with n=324 (68.21%) having normal gestation delivery (37 to 41 weeks +6 days) and 11 (2.32%) having post-term gestational delivery (>42 weeks).

The data showed a very weak correlation/association between reticulocyte count and delivery gestation [days] (R^2= -0.109, p= 0.0176) in the 1st trimester (table 3.4, appendix I).

Gestational Birth Weight Outcome at booking (reticulocyte count cut-off 80x10^9/L)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes (n=475 live births) showed n=28 (5.89%) had a low birth weight <2500g at delivery. There were 379 (79.80%) live births with normal birth weight (2500g to 3999g) and n=68 (14.31%) had a high birth weight >4000g (4000g to 4885g).

Of the n=119 (25.05%) with reticulocyte count >*80x10^9/L,* n=9 (1.89%) had low birth weight (<2500g), n=90 (18.95%) had a normal birth weight (>2500g to 3999g) with n=20 (4.21%) having a high gestational birth weight (>4000g).

Women with a reticulocyte count ≤80x10^9/L (n=356 (74.95%) showed, n=19 (4.00%) had low birth weight (<2500g), n=289 (60.85%) had a normal birth weight (>2500g to 3999g) with n=48 (10.10%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between reticulocyte count and birth weight [g] (R^2= 0.012, p= 0.7979) in the 1st trimester (table 3.4, appendix I).

Gestational Delivery Outcome at 28 weeks (reticulocyte count cut-off 80x10^9/L)

Analysis of 2nd trimester booking bloods with gestational delivery outcomes (n=423 live births) showed n=26 (6.41%) had a preterm delivery of (<37 weeks), n=383 (90.55%) had a normal gestation delivery (37 -41 weeks +6 days) and n=14 (3.31%) had a post term delivery gestation (>42 weeks).

Of the n=120 (28.37%) women with a reticulocyte count >80x10^9/L, n=10 (2.36%) had a preterm gestational delivery (<37w) with n=105 (24.83%) having normal gestation delivery time (37 to 41 weeks + 6 days) and n= 5 (1.18%) having post-term gestational delivery (>42 weeks (42weeks to 42 weeks + 2 days)).

Women with a reticulocyte count ≤80x10^9/L (n=303 (71.63%)) at 28 weeks showed n=16 (3.78%) had a preterm delivery gestation (<37 weeks), n=278 (65.72%) had a normal gestational delivery (37 to 41 weeks + 6 days), and n=9 (2.13%) had a post-term gestational delivery (>42 weeks (42 weeks + 2 days)).

The data showed there was no correlation/association between reticulocyte count and delivery gestation [days] (R^2= -0.055, p= 0.2623) in the 2nd trimester (table 3.4, appendix I).

Gestational Birth Weight Outcome at 28 weeks (reticulocyte count cut-off 80x10^9/L)

Analysis of 2nd trimester booking bloods with gestational birth weight outcomes (n=423 live births) showed n=24 (5.68%) had a low birth weight <2500g at delivery. There were n=337 (79.67%) live births with normal birth weight (2500g to 3999g) and n=62 (14.65%) had a high birth weight >4000g (4000g to 4885g).

Women with a reticulocyte count >80x10^9/L at 28 weeks (n=120 (28.37%)) showed n=8 (1.90%) had a gestational birth weight of <2500g, n=91 (21.51%) had a normal birth weight (>2500g to 3999g) with n=21 (4.96%) having a high gestational birth weight (>4000g).

Of the n=303 (71.63%) of subjects with reticulocyte count ≤80x10^9/L at 28 weeks, n=16 (3.78%) had a gestational birth weight of <2500g, with n=246 (58.16%) having a normal birth weight (>2500g to 3999g) and n=41 (9.69%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between reticulocyte count and birth weight [g] (R^2= -0.0.44, p= 0.9100) in the 2nd trimester (table 3.4, appendix I).

Difference in reticulocyte count between 1st trimester booking bloods and 2nd trimester 28 weeks bloods

The median reticulocyte of the 1st trimester booking bloods were compared with the median reticulocyte count of the 2nd trimester 28-week bloods. The results showed that there was an increase in the median reticulocyte count from booking from 66.75x10^9/L to 69.35x10^9/L at 28 weeks. This showed an overall median increase of 2.60 x10^9/L (0.04%) between 1st and 2nd trimester bloods, range -62.2x10^9/L to + 54.7x10^9/L (-63.53% to +98.04%) (figure 3.3, appendix I). The absolute difference between 1st trimester booking bloods and 2nd trimester 28 week bloods were correlated with birth outcomes (gestational delivery age [days] and gestational birth weight [g] using Spearman Rank Correlation.

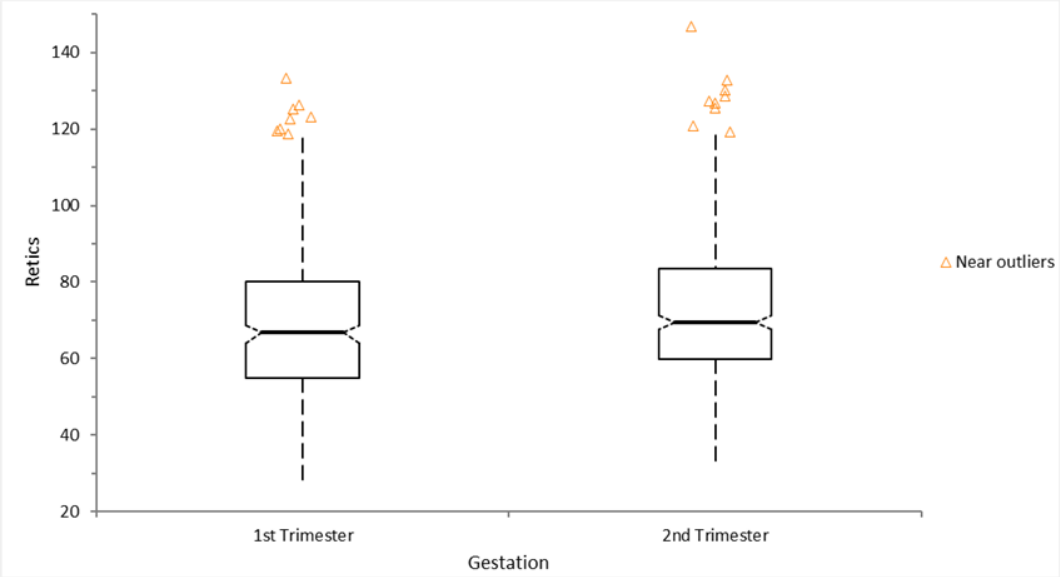


Figure 3.3. Difference of median reticulocyte count from 1st trimester booking bloods to 2nd trimester 28 week bloods using Wilcoxon-Mann-Whitney test p=0.0017 rejecting the null hypothesis of the shift in location between the distributions of the populations being equal to 0.

Difference of* reticulocyte count *between booking and 28 weeks

Analysis of the n=422 data sets were analysed for differences between 1st trimester booking bloods and 2nd trimester 28 week bloods, n=254 (60.19%) had an increase in reticulocyte count between 1st trimester and 2nd trimester. The remaining n=168 (39.81%) all showed a decrease in reticulocyte count.

Difference of* reticulocyte count *between booking and 28 weeks and gestational delivery outcome

Subjects with a decrease in reticulocyte count (n=168 (39.81%)), n=12 (2.84%) had a gestational delivery date (<37 weeks), n=151 (35.79%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=5 (1.18%) having a post term gestational delivery (>42 weeks).

Those with an increase or no difference in reticulocyte count (n=254 (60.19%), n=14 (3.32%) had a gestational delivery date <37 weeks, n=231 (54.74%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=9 (2.13%) having a post term delivery (>42 weeks).

The data showed there was no correlation/association between the difference between reticulocyte count at booking and 28 weeks with gestational delivery time (R^2= 0.06, p= 0.2194) (table 3.4, appendix I).

Difference of reticulocyte count between booking and 28 weeks and gestational birth weight outcome

Women with a decrease in reticulocyte count (n=168 (39.81%)), n=10 (2.37%) had a low gestational birth weight (<2500g), n=133 (31.52%) had a normal birth weight (>2500g to 3999g) with n=25 (5.92%) having a high gestational birth weight (>4000g (4000g to 4885g)).

Those with an increase or no difference in reticulocyte count (n=254 (60.19%)), n=14 (3.32%) had a low gestational birth weight (<2500g), n=203 (48.10%) had a normal delivery birth weight (2500g to 3999g) with n=37 (8.77%) having a high gestational birth weight (>4000g (4000g to 4885g)).

The data showed there was no correlation/association between the difference between reticulocyte count at booking and 28 weeks with gestational birth weight (R^2= 0.018, p= 0.7177) (table 3.4, appendix I).

3.7.2. Summary

The data for reticulocyte count analysis showed overall there was little difference of reticulocyte counts at booking to 28 weeks, 25.05% had raised reticulocytes at booking with 28.37 % having raised counts at 28 weeks. The mean difference was 0.04%.

For 1st trimester booking bloods 6.74% of women had pre-term delivery gestational delivery outcomes (<37 weeks) with 6.41% in the 2nd trimester (28 weeks) table 3.4/appendix I. Results were similar for low birth weight (<2500g) gestational delivery outcomes with 5.89% and 5.68% respectively for booking and 28 week reticulocyte counts.

Overall the data for reticulocyte counts showed no association with birth outcomes, either preterm delivery or low gestational birth weight with the exception of reticulocyte counts and delivery gestation in the 1st trimester booking bloods although this correlation was very weak R^2= -0.109.

3.8. Reticulocyte Haemoglobin Equivalent (Ret-He) analysis

Ret-He data for the n=539 women at booking showed a median Ret-He of 33.5pg (IQR 32.2 - 34.4/range 5.0 – 38.4). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.25 and p value of <0.0001, showing the reticulocyte count from the women in this study to be not normally distributed. Of the n=539 samples collected, there was n=470 women with live birth outcomes available for analysis.

3.8.1. Ret-He analysis and delivery outcome

Ret-He data for the n=470 women with live birth outcomes showed a median Ret-He count of 31.7pg (IQR 29.3 -33.3/range 24.5 – 38.4). A Spearman Rank Correlation statistic was used to assess for association of the Ret-He data against outcomes (gestational delivery age [days] and gestational birth weight) at both booking (6 to 14 weeks) and at 28 weeks (table 3.5, appendix J). The absolute difference between reticulocyte count at booking and at 28 weeks was also tested.

Table 3.5. Summary of Spearman Rank Correlation statistic for Ret-He count analysis at booking, 28 weeks and the difference between booking Ret-He count and 28 week counts for association with delivery gestation age [days] and delivery birth weight [g].

	# of results	Median RetHe pg (range)	Spearman's rs	p-value	Accept/reject H0
RetHe Booking					
Delivery Gestation [Days]	470	33.5 (24.5-38.4)	0.05	0.281	Accept H0
Birth Weight [g]			-0.047	0.312	Accept H0
RetHe 28 weeks					
Delivery Gestation [Days]	423	31.7 (18.6 - 41.5)	0.04	0.4081	Accept H0
Birth Weight [g]			**-0.149**	**0.0021**	**Reject H0**
Difference Booking to 28 weeks					
Delivery Gestation [Days]	417	-1.60 (-11.0 - 6.4)	0.025	0.6107	Accept H0
Birth Weight [g]			**-0.167**	**0.0006**	**Reject H0**

There are no published or consensus values available for Ret-He counts during pregnancy, therefore the cut-off used was 29pg. Variable cut-off's in the normal population have been used with a pragmatic value of 29pg being suggested (Fletcher, et al., 2021)

Of the n=470 Ret-He count results at booking the median Ret-He count was 33.5pg (24.5 – 38.4 (IQR 32.4 – 34.4)) with n=15 (3.19%) having a Ret-He count ≤29pg. The remainder n=455 (96.81%) had a Ret-He count >29pg. In the 2nd trimester, the median reticulocyte count was 31.7pg (18.6 - 41.5 (IQR 29.3 – 33.3)).

Gestational Delivery Outcome at booking (Ret-He count cut-off 29pg)

Analysis of 1st trimester booking bloods with gestational delivery outcomes (n=470 live births) showed n=32 (6.81%) had a preterm delivery of <37 weeks (24 weeks + 4 days to 36 weeks + 4 days), n=423 (90.00%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.19%) had a post term delivery gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The n=455 (96.81%) subjects with a normal Ret-He count >29pg, n=30 (6.38%) had a preterm gestational delivery (<37w) with n=410 (87.23%) having normal gestation delivery (37 to 41 weeks +6 days) and n=4 (3.19%) having post-term gestational delivery (>42 weeks).

Women with a Ret-He count ≤29pg (n=15 (3.19%) showed, n=2 (0.43%) had a preterm gestational delivery (<37w) with n=13 (2.77%) having normal gestation delivery (37 to 41 weeks +6 days) and n=0 (0.00%) having post-term gestational delivery (>42 weeks).

The data showed no correlation/association between Ret-He count and delivery gestation [days] (R^2= 0.05, p= 0.281) in the 1st trimester (table 3.5, appendix J).

Gestational Birth Weight Outcome at booking (Ret-He count cut-off 29pg)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes (n=470 live births) showed n=28 (5.96%) had a low birth weight <2500g at delivery. There were n=375 (79.79%) live births with normal birth weight (2500g to 3999g) and n=67 (14.25%) had a high birth weight >4000g (4000g to 4885g).

Of the n=455 (96.81%) with Ret-He count >*29pg,* n=27 (5.75%) had low birth weight (<2500g), n=362 (77.02%) had a normal birth weight (>2500g to 3999g) with n=66 (14.04%) having a high gestational birth weight (>4000g).

Women with a Ret-He count ≤29pg (n=15 (3.19%) showed, n=1 (0.21%) had low birth weight (<2500g), n=13 (2.77%) had a normal birth weight (>2500g to 3999g) with n=1 (0.21%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between reticulocyte count and birth weight [g] (R^2= -0.047, p= 0.312) in the 1st trimester (table 3.5, appendix J).

Gestational Delivery Outcome at 28 weeks (Ret-He count cut-off 29pg)

Analysis of 2nd trimester booking bloods with gestational delivery outcomes (n=423 live births) showed n=26 (6.14%) had a preterm delivery of (<37 weeks), n=382 (90.32%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.54%) had a post term delivery gestation (>42 weeks).

Of the n=323 (76.36%) women with a Ret-He count >29pg, n=23 (5.44%) had a preterm gestational delivery (<37w) with n=287 (67.85%) having normal gestation delivery time (37 to

41 weeks + 6 days) and n=13 (3.07%) having post-term gestational delivery (>42 weeks (42weeks to 42 weeks + 2 days)).

Women with a Ret-He count ≤29pg (n=100 (23.64%)) at 28 weeks showed n=3 (0.71%) had a preterm delivery gestation (<37 weeks), n=95 (22.46%) had a normal gestational delivery (37 to 41 weeks + 6 days), and n=2 (0.47%) had a post-term gestational delivery (>42 weeks (42 weeks + 2 days)).

The data showed there was no correlation/association between reticulocyte count and delivery gestation [days] (R^2= -0.040, p= 0.4081) in the 2nd trimester (table 3.5, appendix J).

Gestational Birth Weight Outcome at 28 weeks (Ret-He count cut-off 29pg)

Analysis of 2nd trimester booking bloods with gestational birth weight outcomes (n=423 live births) showed n=24 (5.68%) had a low birth weight <2500g at delivery. There were n=337 (79.66%) live births with normal birth weight (2500g to 3999g) and n=62 (14.65%) had a high birth weight >4000g (4000g to 4885g).

Women with a Ret-He count >29pg at 28 weeks (n=323 (76.36%)) showed n=23 (5.44%) had a gestational birth weight of <2500g, n=261 (61.70%) had a normal birth weight (>2500g to 3999g) with n=39 (9.22%) having a high gestational birth weight (>4000g).

Of the n=100 (23.64%) of women with a Ret-He count ≤29pg at 28 weeks, n=1 (0.24%) had a gestational birth weight of <2500g, with n=76 (17.96%) having a normal birth weight (>2500g to 3999g) and n=23 (5.44%) having a high gestational birth weight (>4000g).

The data showed there was a very weak correlation/association between Ret-He count and birth weight [g] (R^2= -0.149, p= 0.0021) in the 2nd trimester (table 3.5, appendix J).

Difference in Ret-He count between 1st trimester booking bloods and 28 weeks bloods

The median Ret-He of the 1st trimester booking bloods were compared with the median Ret-He count of the 2nd trimester 28-week bloods. The results showed that there was a decrease in the median Ret-He count from booking from 33.50pg to 31.70pg at 28 weeks. This showed an overall median increase of 1.8pg (5.37%) between 1st and 2nd trimester bloods, range -11.02pg to + 6.4pg (-34.92% to +26.12%) (figure 3.4, appendix J). The absolute difference between 1st trimester booking bloods and 2nd trimester 28 week bloods were correlated with birth outcomes (gestational delivery age [days] and gestational birth weight [g] using Spearman Rank Correlation.

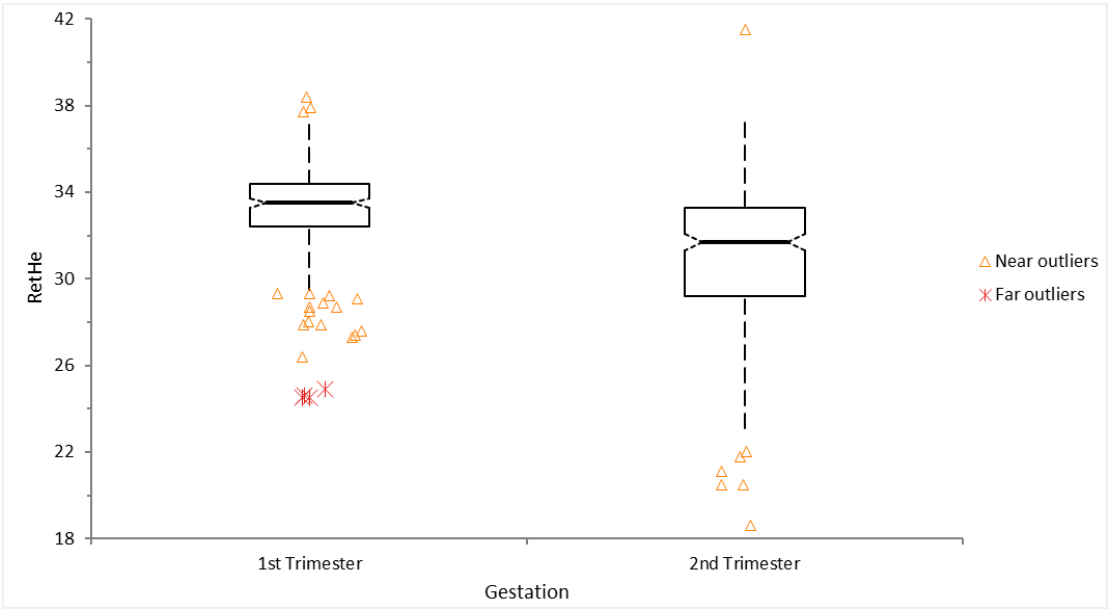


Figure 3.4. Difference of median Ret-He count from 1st trimester booking bloods to 2nd trimester 28 week bloods using Wilcoxon-Mann-Whitney test p=<0.0001 rejecting the null hypothesis of the shift in location between the distributions of the populations being equal to 0.

Difference of* Ret-He count *between booking and 28 weeks

Analysis of the n=417 data sets were analysed for differences between 1st trimester booking bloods and 2nd trimester 28 week bloods, n=71 (17.02%) had an increase in reticulocyte count between 1st trimester and 2nd trimester. The remaining n=346 (82.98%) all showed a decrease in reticulocyte count.

Difference of* Ret-He count *between booking and 28 weeks and gestational delivery outcome

Women with a decrease in Ret-He count (n=346 (82.98%)), n=25 (5.99%) had a gestational delivery date (<37 weeks), n=310 (74.35%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=11 (2.64%) having a post term gestational delivery (>42 weeks).

Those with an increase or no difference in Ret-He count (n=71 (17.02%), n=1 (0.24%) had a gestational delivery date <37 weeks, n=67 (16.06%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=3 (0.72%) having a post term delivery (>42 weeks).

The data showed there was no correlation/association between the difference between Ret-He count at booking and 28 weeks with gestational delivery time (R^2= 0.025, p= 0.6107) (table 3.5, appendix J).

Difference of Ret-He count between booking and 28 weeks and gestational birth weight outcome

Women with a decrease in Ret-He count (n=346 (82.98%)), n=22 (5.27%) had a low gestational birth weight (<2500g), n=268 (64.28%) had a normal birth weight (>2500g to 3999g) with n=56 (13.43%) having a high gestational birth weight (>4000g (4000g to 4885g)).

Those with an increase or no difference in reticulocyte count (n=71 (17.02%)), n=2 (0.48%) had a low gestational birth weight (<2500g), n=64 (15.34%) had a normal delivery birth weight (2500g to 3999g) with n=5 (1.20%) having a high gestational birth weight (>4000g (4000g to 4885g)).

The data showed there was a weak correlation/association between the difference between Ret-He count at booking and 28 weeks with gestational birth weight (R^2= -0.167, p= 0.0006) (table 3.5, appendix J).

3.8.2 Summary

The data for Ret-He count analysis showed overall there was almost no median difference of Ret-He counts at booking to 28 weeks, 96.81% had a normal Ret-He at booking with 76.36 % having normal counts at 28 weeks. The mean difference was 21.12%.

For 1st trimester booking bloods 6.81% of women had pre-term delivery gestational delivery outcomes (<37 weeks) with 6.14% in the 2nd trimester (28 weeks) table 3.5/appendix J. Results were similar for low birth weight (<2500g) gestational delivery outcomes with 5.96% and 5.68% respectively for booking and 28 week Ret-He counts.

Overall the data for Ret-He counts showed no association with birth outcomes, either preterm delivery or for low gestational birth weight with the exception of Ret-He counts and birth weight delivery outcome in the 2nd trimester which showed a weak correlation weak R^2= -0.149. A weak correlation R2= -0.167 for the difference between booking and 28 weeks and birth weight delivery was also seen.

3.9. Immature Reticulocyte Fraction (IRF) analysis

IRF data for the n=536 women at booking showed a median IFR of 7.9% (IQR 6.1- 10.3/range 2.5-47.6). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.05 and p value of 0.0012, showing the IRF from the women in this study to be not normally distributed. Of the n=536 samples collected, there was n=469 women with live birth outcomes available for analysis.

3.9.1. IRF analysis and delivery outcome

IRF data for the n=469 women with live birth outcomes showed a median IRF of 7.9% (IQR 6.2 -10.2/range 2.6 – 47.6). A Spearman Rank Correlation statistic was used to assess for association of the IRF against outcomes (gestational delivery age [days] and gestational birth weight) at both booking (6 to 14 weeks) and at 28 weeks (table 3.6, appendix K). The absolute difference between IRF at booking and at 28 weeks was also tested.

Table 3.6. Summary of Spearman Rank Correlation statistic for IRF count analysis at booking, 28 weeks and the difference between booking IRF and 28 week counts for association with delivery gestation age [days] and delivery birth weight [g].

	# of results	Median IRF% (range)	Spearman's rs	p-value	Accept/reject H0
IRF% Booking					
Delivery Gestation [Days]	469	7.6 (2.6 - 47.6)	**-0.173**	**0.0002**	**Reject H0**
Birth Weight [g]			-0.064	0.1665	Accept H0
IRF% 28 weeks					
Delivery Gestation [Days]	423	16.3 (5.4 - 39.0)	**-0.096**	**0.0481**	**Reject H0**
Birth Weight [g]			**0.108**	**0.027**	**Reject H0**
Difference Booking to 28 weeks					
Delivery Gestation [Days]	416	8.3 (-26.8 - 32.2)	0.021	0.6659	Accept H0
Birth Weight [g]			**0.172**	**0.0004**	**Reject H0**

There are no published or consensus values available for IRF counts during pregnancy. Values for normal non-pregnant population are suggested of 1.6% to 10.5% (Pekelharing, et al.,

2010). Of the n=469 IRF results at booking the median IRF count was 7.9% (IQR 6.2 -10.2/range 2.6 – 47.6) with n=362 (77.19%) having a IRF ≤10.5%. The remainder n=107 (22.81%) had an IRF >10.5%. In the 2nd trimester the median IRF was 16.3 (5.4 – 39.0 (IQR 13.10 – 21.28)).

Gestational Delivery Outcome at Booking (IRF normal range 1.6% to 10.5%)

Analysis of 1st trimester booking bloods with gestational delivery outcomes (n=469 live births) showed n=32(6.82%) had a preterm delivery of <37 weeks (24 weeks + 4 days to 36 weeks + 4 days), 422 (89.99%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.19%) had a post term delivery gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The n=362 (77.19%) subjects with a normal IRF 1.6% to 10.5%, n=17 (3.62%) had a preterm gestational delivery (<37w) with n=335 (71.44%) having normal gestation delivery (37 to 41 weeks +6 days) and n=10 (2.13%) having post-term gestational delivery (>42 weeks).

Women with an IRF >10.5% (n=107 (22.81%) showed, n=15 (3.20%) had a preterm gestational delivery (<37w) with n=87 (18.55%) having normal gestation delivery (37 to 41 weeks +6 days) and n=5 (1.06%) having post-term gestational delivery (>42 weeks).

The data showed an association between IRF and delivery gestation [days] (R^2= -0.173, p= 0.0002) in the 1st trimester (table 3.6, appendix K).

Gestational Birth Weight Outcome at Booking (IRF normal range 1.6% to 10.5%)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes (n=469 live births) showed n=28 (5.97%) had a low birth weight <2500g at delivery. There were n=374 (79.74%) live births with normal birth weight (2500g to 3999g) and n=67 (14.29%) with a high birth weight >4000g (4000g to 4885g).

Of the n=362 (77.19%) with IRF 1.6% to 10.5%, n=18 (3.84%) had low birth weight (<2500g), n=291 (62.05%) had a normal birth weight (>2500g to 3999g) with n=53 (11.30%) having a high gestational birth weight (>4000g).

Women with an IRF >10.5% (n=107 (22.81%) showed, n=10 (2.13%) had low birth weight (<2500g), n=83 (17.70%) had a normal birth weight (>2500g to 3999g) with n=14 (2.98%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between IRF and birth weight [g] (R^2= -0.064, p= 0.1665) in the 1st trimester (table 3.6, appendix K).

Gestational Delivery Outcome at 28 weeks (IRF normal range 1.6% to 10.5%)

Analysis of 2nd trimester booking bloods with gestational delivery outcomes (n=423 live births) showed n=26 (6.14%) had a preterm delivery of (<37 weeks), n=383 (90.56%) had a normal gestation delivery (37 -41 weeks +6 days) and n=14 (3.30%) had a post term delivery gestation (>42 weeks).

Of the n=47 (11.11%) women with a IRF 1.6% to 10.5%, n=2 (0.47%) had a preterm gestational delivery (<37w) with n=42 (9.94%) having normal gestation delivery time (37 to 41 weeks + 6 days) and n=3 (0.70%) having post-term gestational delivery (>42 weeks (42weeks to 42 weeks + 2 days)).

Women with an IFR >10.5% (n=376 (88.89%)) at 28 weeks showed n=24 (5.67%) had a preterm delivery gestation (<37 weeks), n=341 (80.62%) had a normal gestational delivery (37 to 41 weeks + 6 days), and n=11 (2.60%) had a post-term gestational delivery (>42 weeks (42 weeks + 2 days)).

The data showed there was an association between IRF and delivery gestation [days] (R^2= -0.096, p= 0.0481) in the 2nd trimester (table 3.6, appendix K).

Gestational Birth Weight Outcome at 28 weeks (IRF normal range 1.6% to 10.5%)

Analysis of 2nd trimester bloods with gestational birth weight outcomes (n=423 live births) showed n=24 (5.67%) had a low birth weight <2500g at delivery. There were n=337 (79.68%) live births with normal birth weight (2500g to 3999g) and n=62 (14.65%) had a high birth weight >4000g (4000g to 4885g).

Women with a normal IRF 1.6% to 10.5% at 28 weeks (n=47 (11.11%)) showed n=4 (0.94%) had a gestational birth weight of <2500g, n=40 (9.46%) had a normal birth weight (>2500g to 3999g) with n=3 (0.71%) having a high gestational birth weight (>4000g).

Of the n=376 (88.89%) of women with an IRF >10.5% at 28 weeks, n=20 (4.73%) had a gestational birth weight of <2500g, with n=297 (70.22%) having a normal birth weight (>2500g to 3999g) and n=59 (13.94%) having a high gestational birth weight (>4000g).

The data showed there was an association between IRF and birth weight [g] (R^2= -0.108, p= 0.0270) in the 2nd trimester (table 3.6, appendix K).

Difference in IRF between 1st trimester booking bloods and 28 weeks bloods

The median IRF of the 1st trimester booking bloods were compared with the median IRF of the 2nd trimester 28-week bloods. The results showed that there was an increase in the median IRF from booking from 7.90% to 16.30% at 28 weeks. This showed an overall median increase of IRF of 8.4% (48.46%) between 1st and 2nd trimester bloods, range -26.8% to 32.2% (-56.30% to +874.07%) (figure 3.5, appendix K). The absolute difference between 1st trimester

booking bloods and 2nd trimester 28 week bloods were correlated with birth outcomes (gestational delivery age [days] and gestational birth weight [g] using Spearman Rank Correlation.

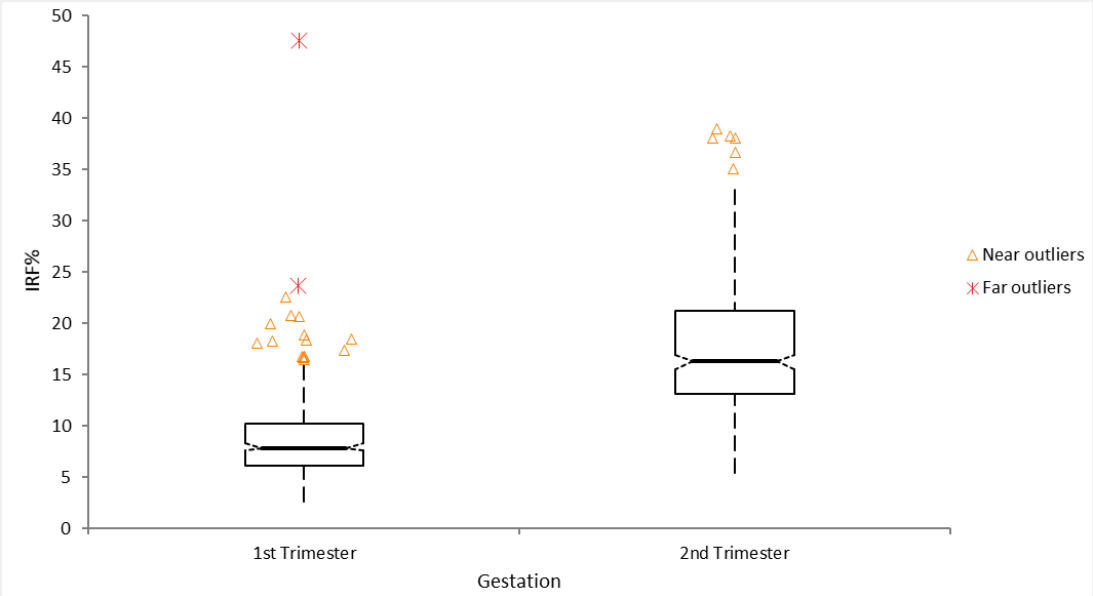


Figure 3.5. Difference of median IRF from 1st trimester booking bloods to 2nd trimester 28 week bloods using Wilcoxon-Mann-Whitney test p=<0.0001 rejecting the null hypothesis of the shift in location between the distributions of the populations being equal to 0.

Difference of* IRF *between booking and 28 weeks

Analysis of the n=416 data sets were analysed for differences between 1st trimester booking bloods and 2nd trimester 28 week bloods, n=405 (97.35%) had an increase in IRF between 1st trimester and 2nd trimester. The remaining n=11 (2.64%) all showed a decrease in IRF.

Difference of* IRF *between booking and 28 weeks and gestational delivery outcome

Women with a decrease in IRF (n=11 (2.64%)), n=0 (0.00%) had a gestational delivery date (<37 weeks), n=11 (2.64%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=0 (0.00%) having a post term gestational delivery (>42 weeks).

Those with an increase or no difference in IRF (n=405 (97.35%), n=24 (5.76%) had a gestational delivery date <37 weeks, n=320 (76.94%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=61 (14.66%) having a post term delivery (>42 weeks).

The data showed there was an association between the difference between IRF at booking and 28 weeks with gestational delivery time (R^2= 0.172, p= 0.0004) (table 3.6, appendix K).

Difference of IRF between booking and 28 weeks and gestational birth weight outcome

Women with a decrease in IRF (n=11 (2.64%)), n=0 (0.00%) had a low gestational birth weight (<2500g), n=11 (2.64%) had a normal birth weight (>2500g to 3999g) with n=0 (0.00%) having a high gestational birth weight (>4000g (4000g to 4885g)).

Those with an increase or no difference in reticulocyte count (n=405 (97.35%)), n=26 (6.25%) had a low gestational birth weight (<2500g), n=365 (87.75%) had a normal delivery birth weight (2500g to 3999g) with n=14 (3.36%) having a high gestational birth weight (>4000g (4000g to 4885g)).

The data showed there was no correlation/association between the difference between IRF at booking and 28 weeks with gestational birth weight (R^2= -0.021, p= 0.6659) (table 3.6, appendix K).

3.9.2 Summary

The data for IRF analysis showed overall, there was a significant difference in the median of IRF between booking and 28 weeks, 77.19% had a normal IRF at booking with only 11.11% having normal counts at 28 weeks. The mean difference was 66.68%.

For 1st trimester booking bloods 6.82% of women had pre-term delivery gestational delivery outcomes (<37 weeks) with 6.14% in the 2nd trimester (28 weeks) table 3.6/appendix K. Results were similar for low birth weight (<2500g) gestational delivery outcomes with 5.97% and 5.67% respectively for booking and 28 week IRF%.

Overall the data for IRF showed an association with birth outcomes overall with the exception of IRF and birth weight delivery outcome in the 1st trimester which showed a weak correlation weak R^2= -0.064. There was also a weak correlation R^2= 0.021 for the difference between booking and 28 weeks and gestational delivery.

3.10. Ferritin analysis

Plasma ferritin data for the n=544 women at booking showed a median ferritin level of 28.0µg/L (IQR 16.0 – 47.0/range 3.0 – 204.0). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.06 and p value of <0.0001, showing the plasma ferritin level from the women in this study is not normally distributed. Of the n=544 samples collected, there was n=475 women with live birth outcomes available for analysis.

3.10.1 Ferritin analysis and delivery outcome

Plasma ferritin data for the n=475 women with live births showed a median ferritin of 28µg/L (IQR 17.0 – 48.0/range 3.0 - 204). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.06 and p value of 0.0012, showing the ferritin from the women in this study to be not normally distributed.

As the data was not normally distributed a Spearman Rank Correlation statistic was used to assess for association of the plasma ferritin data against outcomes (gestational delivery age [days] and gestational birth weight) at both booking (6 to 14 weeks) and at 28 weeks (table 3.7, appendix L). The absolute difference between plasma ferritin at booking and plasma ferritin at 28 weeks was also tested.

Table 3.7. Summary of Spearman Rank Correlation statistic for plasma ferritin analysis at booking, 28 weeks and the difference of plasma ferritin between booking plasma ferritin and 28 week for association with delivery gestation age [days] and delivery birth weight [g].

	# of results	Median Ferritin ug/L (range)	Spearman's rs	p-value	Accept/reject H0
Ferritin Booking					
Delivery Gestation [Days]	475	28 (3.0-204.0)	0.078	0.0886	Accept H0
Birth Weight [g]			-0.026	0.5757	Accept H0
Ferritin 28 weeks					
Delivery Gestation [Days]	397	7 (2.0-154.0)	0.017	0.7329	Accept H0
Birth Weight [g]			**-0.156**	**0.0019**	**Reject H0**
Difference Booking to 28 weeks					
Delivery Gestation [Days]	396	-19 (-149.0-34.0)	**-0.116**	**0.021**	**Reject H0**
Birth Weight [g]			-0.021	0.6732	Accept H0

There is currently no good quality evidence during pregnancy to suggest a suitable cut-off for ferritin measurement in determination of iron deficiency in the 1st or 2nd trimester of pregnancy. The British Society for Haematology advises a cut-off 30 µg/L be used (Pavord, et al., 2019). Of the n=475 plasma ferritin results at booking the median plasma ferritin was 28.0 µg/L (3.0 – 204.0 (IQR 17.0 – 48.0)) with n=258 (54.32%) having a plasma ferritin ≤30µg/L. The remaining n=45.68 (45.68%) had a plasma ferritin >30µg/L. In the 2nd trimester, the median plasma ferritin was 7.0µg/L (2.0 – 154.0 (IQR 5.0 – 11.0)). The data showed that n=375 (94.46%) had a plasma ferritin ≤30µg/L, while n=22 (5.54%) had a plasma ferritin >30µg/L.

Gestational Delivery Outcome at booking (plasma ferritin cut-off 30µg/L)

Analysis of 1st trimester booking bloods with gestational delivery outcomes (n=475 live births) showed n=31 (6.52%) had a preterm delivery of (<37 weeks), n=429 (90.33%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.15%) had a post term deliver gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The n=217 (45.68%) women with a plasma ferritin >30µg/L, n=16 (3.37%) had a preterm gestational delivery (<37w) with n=193 (40.63%) having normal gestation delivery (37 to 41 weeks +6 days) and n=8 (1.68%) having post-term gestational delivery (>42 weeks).

Of the n=258 (54.32%) women with a plasma ferritin ≤30µg/L, n=15 (3.16%) had a preterm gestational delivery (<37w) with n=236 (49.69%) having normal gestation delivery (37 to 41 weeks +6 days) and n=7 (1.47%) having post-term gestational delivery (>42 weeks).

The data showed there was no correlation/association between plasma ferritin and delivery gestation [days] (R^2= 0.078, p= 0.0886) in the 1st trimester (table 3.7, appendix L).

Gestational Birth Weight Outcome at booking (plasma ferritin cut-off 30µg/L)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes (n=475 live births) showed n=27 (5.68%) had a low birth weight <2500g at delivery. There were n=379 (79.79%) live births with normal birth weight (2500g to 3999g) and n=69 (14.53%) had a high birth weight >4000g (4000g to 4885g).

Of the n=217 (45.68%) with plasma ferritin >30µg/L, n=13 (2.53%) had low birth weight (<2500g), n=175 (37.05%) had a normal birth weight (>2500g to 3999g) with n=29 (6.10%) having a high gestational birth weight (>4000g).

The remaining n=258 (54.32%) women with a plasma ferritin ≤30µg/L at booking, n=14 (2.95%) had low birth weight (<2500g), n=205 (43.16%) had a normal birth weight (>2500g to 3999g) with n=39 (8.21%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between plasma ferritin and birth weight [g] (R^2= -0.026, p= 0.5757) in the 1st trimester (table 3.7, appendix L).

Gestational Delivery Outcome at 28 weeks (plasma ferritin cut-off 30µg/L)

Analysis of 2nd trimester booking bloods with gestational delivery outcomes (n=397 live births) showed n=23 (5.79%) had a preterm delivery of (<37 weeks), n=361 (90.93%) had a normal gestation delivery (37 -41 weeks +6 days) and n=12 (3.27%) had a post term delivery gestation (>42 weeks).

Of the n=22 (5.54%) women with a plasma ferritin >30µg/L, n=2 (0.50%) had a preterm gestational delivery (<37w) with n=19 (4.79%) having normal gestation delivery time (37 to 41 weeks + 6 days) and n=1 (0.25%) having post-term gestational delivery (>42 weeks).

Women with a plasma ferritin ≤30µg/L (n=375 (94.46%)) at 28 weeks showed n=21 (5.29%) had a preterm delivery gestation (<37 weeks), n=342 (86.15%) had a normal gestational delivery (37 to 41 weeks + 6 days), and n=12 (3.02%) had a post-term gestational delivery (>42 weeks).

The data showed there was no correlation/association between plasma ferritin and delivery gestation [days] (R^2= 0.017, p= 0.7329) in the 2nd trimester (table 3.7, appendix L).

Gestational Birth Weight Outcome at 28 weeks (plasma ferritin cut-off 30µg/L)

Analysis of 2nd trimester booking bloods with gestational birth weight outcomes (n=397 live births) showed n=22 (5.54%) had a low birth weight <2500g at delivery. There were n=319 (80.35%) live births with normal birth weight (2500g to 3999g) and n=56 (14.11%) had a high birth weight >4000g (4000g to 4885g).

Women with a plasma ferritin >30µg/L at 28 weeks n=22 (5.54%) showed n=3 (0.76%) had a gestational birth weight of <2500g, n=16 (4.02%) had a normal birth weight (>2500g to 3999g) with n=3 (0.76%) having a high gestational birth weight (>4000g).

Of the n=375 (94.46%) of women with a plasma ferritin >30µg/L at 28 weeks n=19 (4.78%) had a gestational birth weight of <2500g, with n=303 (76.33%) having a normal birth weight (>2500g to 3999g) and n=53 (13.35%) having a high gestational birth weight (>4000g).

The data showed a weak correlation/association between the difference between plasma ferritin at 1st trimester booking and 2nd trimester 28 weeks with gestational birth weight (R^2= -0.156, p= 0.0019) (table 3.7, appendix L).

Difference in plasma ferritin levels between 1st trimester booking bloods and 2nd trimester 28 weeks bloods

The median plasma ferritin of the 1st trimester booking bloods were compared with the median plasma ferritin 2nd trimester 28-week bloods. The results showed that there was a decrease in the median plasma ferritin from booking from 28 µg/L to 7µg/L at 28 weeks. This showed an overall median decrease of 21µg/L (75.0%) between 1st and 2nd trimester bloods, range -149.0µg/L to + 34.0µg/L (-94.62% to +125.00%) (figure 3.6, appendix L). The absolute difference between 1st trimester booking bloods and 2nd trimester 28 week bloods were correlated with birth outcomes (gestational delivery age [days] and gestational birth weight [g] using Spearman Rank Correlation.

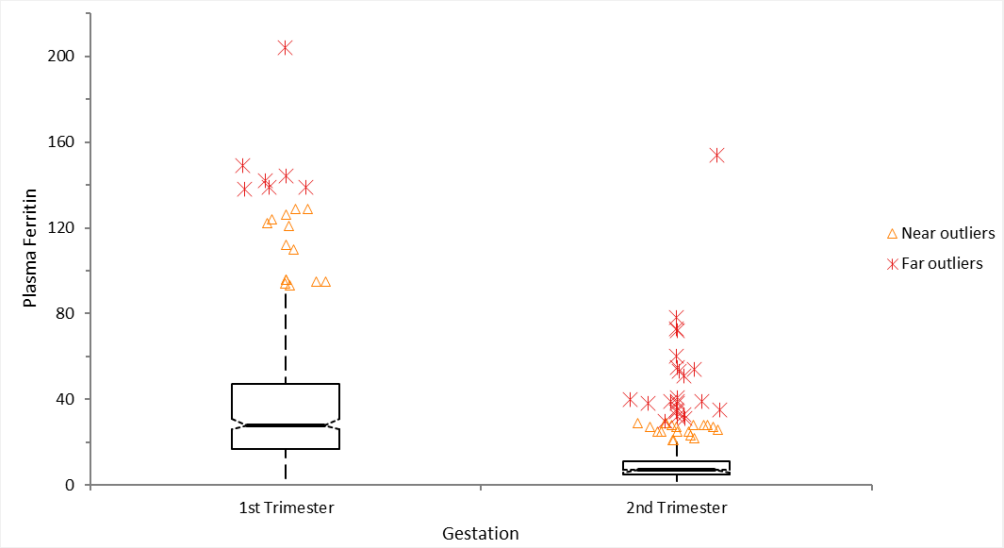


Figure 3.6. Difference of median plasma ferritin count from 1st trimester booking bloods to 2nd trimester 28 week bloods using Wilcoxon-Mann-Whitney test p=<0.0001 rejecting the null hypothesis of the shift in location between the distributions of the populations being equal to 0.

Difference of plasma ferritin between booking and 28 weeks

Analysis of the n=396 data sets were analysed for differences between 1st trimester booking bloods and 2nd trimester 28 week bloods, n=14 (3.53%) had an increase in plasma ferritin between1st trimester and 2nd trimester. The remaining n=382 (96.47%) all showed a decrease in plasma ferritin ranging from 2µg/L to 154µg/L at 28 weeks.

Difference of plasma ferritin between booking and 28 weeks and gestational delivery outcome

Women with a decrease in plasma ferritin n=382 (96.47%), n=21 (5.30%) had a gestational delivery date <37 weeks, n=349 (88.14%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=12 (3.03%) having a post term gestational delivery (>42 weeks).

Those with an increase or no difference in plasma ferritin (n=14 (3.53%)), n=1 (0.25%) had a gestational delivery date <37 weeks, n=12 (3.03%) had a normal gestational delivery (37 to 41 weeks + 6 days), with n=1 (0.25%) having a post term delivery (>42 weeks).

The data showed a weak correlation/association between the difference between plasma ferritin at 1st trimester booking and 2nd trimester 28 weeks with delivery gestation [days] (R^2= -0.116, p= 0.0021) (table 3.7, appendix L).

Difference of plasma ferritin between booking and 28 weeks and gestational birth weight outcome

Women with a decrease in plasma ferritin n=382 (96.47%), n=19 (4.80%) had a low gestational birth weight (<2500g), n=310 (78.29%) had a normal birth weight (>2500g to 3999g) with n=53 (13.38%) having a high gestational birth weight (>4000g).

Those with an increase or no difference in plasma ferritin n=14 (3.53%), n=2 (0.50%) had a low gestational birth weight (<2500g), n=9 (2.27%) had a normal delivery birth weight (2500g to 3999g) with n=3 (0.76%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between plasma ferritin and birth weight [g] (R^2= -0.021, p= 0.6732) in the 1st trimester (table 3.7, appendix L).

3.10.2 Plasma ferritin with raised C-reactive protein

With ferritin being an acute phase protein, levels can be affected by infection or inflammatory processes. To account for this CRP analysis was requested and analyse to aid interpretation of any ferritin result >30µg/L. The data showed at 1st trimester booking there were n=110 (23.15%) had a raised CRP with n=53 (11.13%) of women having both a raised CRP and ferritin

>30µg/L (30 to >100 µg/L) therefore suggesting that the true number of women with lower ferritin levels at booking is n=311 (65.47%) appendix M. Interestingly at 28 weeks, although n=96 (24.18%) women had a raised CRP only n=5 (1.26%) had a raised CRP and raised plasma ferritin (appendix M).

There was no correlation between a plasma ferritin >30µg/L and raised CRP >8mg/L either at 1st trimester booking or 28 weeks n=53 (R^2= -0.124, p= 0.3778) and n=5 (R^2= 0.149, p= 0.1506) respectively although sample numbers are small.

3.10.3 Summary

The data for plasma ferritin analysis showed there were 54.32% of women with low plasma ferritin levels using the BSH guideline cut-off of 30µg/L in the 1st trimester, increasing to 94.46% in the 2nd trimester showing that the vast majority of women in the 2nd trimester have low ferritin levels suggesting iron deficiency. For 1st trimester booking bloods 6.52% of women had pre-term delivery gestational delivery outcomes (<37 weeks) with 5.79% in the 2nd trimester (28 weeks) table 3.7/appendix L. Results were similar for low birth weight (<2500g) gestational delivery outcomes with 5.68% and 5.54% respectively for booking and 28 week Hb.

Overall the data for plasma ferritin showed no association with birth outcomes, either preterm delivery or low gestational birth weight at booking or 28 weeks except for birth weight at 28 weeks. For difference in plasma ferritin levels between booking bloods and 28 weeks there was an association with gestational delivery outcomes although this correlation was weak R^2= -0.116. There was no correlation between the difference of plasma ferritin between booking and 28 weeks for birth weight outcomes R^2= -0.021.

3.11. Vitamin B12 analysis

Data for vitamin B12 analysis were only collected for booking bloods, as serum samples are not routinely taken at 28 weeks (2nd trimester) of pregnancy.

Vitamin B12 data for the n=519 women at booking showed a median vitamin B12 level of 153.55pmol/L (IQR 121.60 – 201.63/range 28.7 - >1160.70). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.05 and p value of 0.0078, showing the vitamin B12 level from the women in this study is not normally distributed.

Of the 519 samples collected, there was n=451 women with live birth outcomes available for analysis. As the difference between booking and 28 week blood could not be assessed, levels of vitamin B12 were therefore assessed between gestational delivery cut-offs (<37 weeks, 37 to 41 weeks and >42 weeks), and birth weight cut-offs (<2500g, 2500 to 3999g, and >4000g).

3.11.1. Vitamin B12 analysis and delivery outcome

Vitamin B12 data for n=451 women showed a median vitamin B12 of 28pmol/L (IQR 119.75 – 197.36/range 28.7 - >1106.7). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.05 and p value of 0.012, showing the ferritin from the women in this study to be not normally distributed.

As the data was not normally distributed a Spearman Rank Correlation statistic was used to assess for association of the Vitamin B12 data against outcomes (gestational delivery age [days] and gestational birth weight) at booking only (table 3.8, appendix N).

Table 3.8. Summary of Spearman Rank Correlation statistic for Vitamin B12 analysis at booking for association with delivery gestation age [days] and delivery birth weight [g].

	# of results	Median VB12 pmol/L (range)	Spearman's rs	p-value	Accept/reject H0
VB12 Booking					
Delivery Gestation [Days]	451	151.30 (28.70 - >1106.70)	0.09	0.0566	Accept H0
Birth Weight [g]			0.062	0.188	Accept H0

The locally derived cut-off level for the determination of total vitamin B12 in respect of analysis method for the local population was used to assess for vitamin B12 deficiency. The locally derived cut-off level is determined as <115pmol/L. Of the 451 vitamin B12 results available at 1st trimester booking the median vitamin B12 was 151.30pmol/L (28.70 - >1106.70 (IQR 119.75 – 197.36)) with n=92 (20.39%) having a Vitamin B12 ≤115pmol/L. The remaining n=359 (79.61%) had a vitamin B12 >115pmol/L.

Gestational Delivery Outcome at Booking (Vitamin B12 cut-off 115pmol/L)

Analysis of 1st trimester booking bloods with gestational delivery outcomes (n=451 live births) showed n=31 (6.87%) had a preterm delivery of (<37 weeks), n=405 (90.60%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (2.53%) had a post term deliver gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The n=359 (79.61%) women with a plasma ferritin >115pmol/L, n=26 (5.76%) had a preterm gestational delivery (<37w) with n=324 (72.65%) having normal gestation delivery (37 to 41 weeks +6 days) and n=9 (1.20%) having post-term gestational delivery (>42 weeks).

Of the n=92 (20.39%) women with a plasma ferritin ≤115pmol/L, n=5 (1.11%) had a preterm gestational delivery (<37w) with n=81 (17.95%) having normal gestation delivery (37 to 41 weeks +6 days) and n=6 (1.33%) having post-term gestational delivery (>42 weeks).

The data showed there was no correlation/association between Vitamin B12 and delivery gestation [days] (R^2= 0.051, P= 0.0566) in the 1st trimester (table 3.8, appendix N). There was no statistically significant difference between gestational delivery dates for preterm, normal or late delivery and Vitamin B12 levels (p=0.4161) (figure 3.7, appendix N).

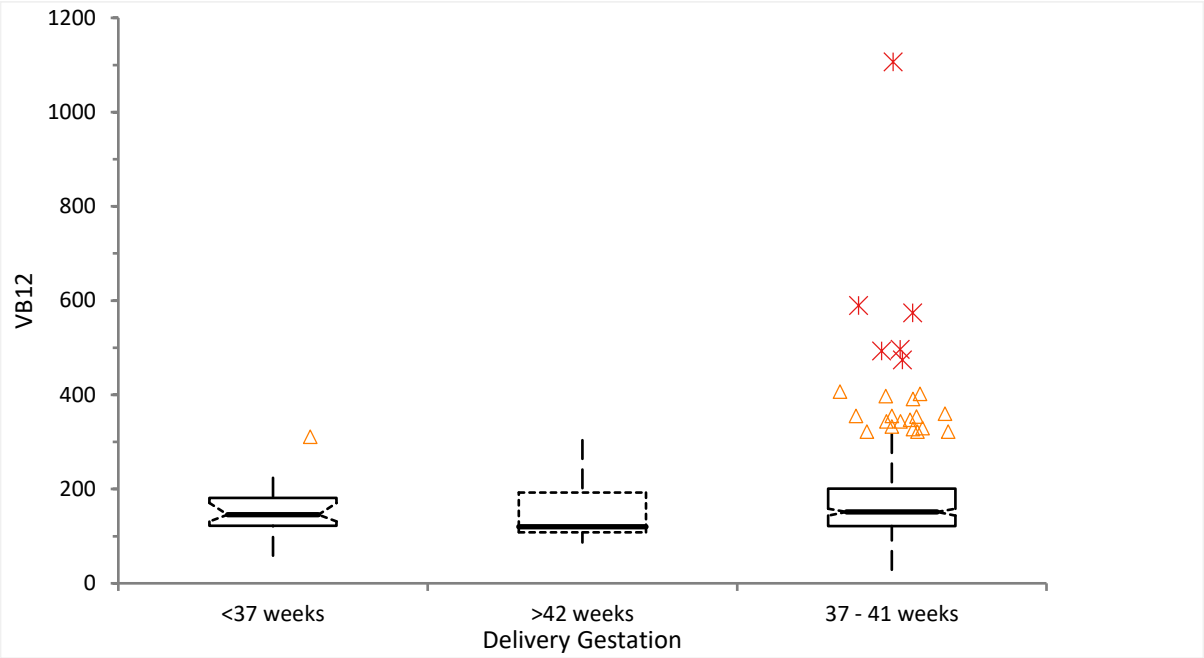


Figure 3.7. Difference between gestational delivery age and median Vitamin B12 levels at 1st trimester booking Kruskal-Wallis (ANOVA), p=0.4161 accepting the null hypothesis of no difference between the gestation delivery age groups.

Gestational Birth Weight Outcome at Booking (Vitamin B12 cut-off 115pmol/L)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes (n=451 live births) showed n=31 (6.87%) had a low birth weight <2500g at delivery. There were n=356 (78.95%) live births with normal birth weight (2500g to 3999g) and n=64 (18.18%) had a high birth weight >4000g (4000g to 4885g).

Of the n=359 (79.61%) with a Vitamin B12 >115pmol/L, n=26 (5.76%) had low birth weight (<2500g), n=282 (62.55%) had a normal birth weight (>2500g to 3999g) with n=51 (11.30%) having a high gestational birth weight (>4000g).

The remaining n=92 (20.39%) women with a Vitamin B12 ≤115pmol/L at booking, n=5 (1.11%) had low birth weight (<2500g), n=74 (16.40%) had a normal birth weight (>2500g to 3999g) with n=13 (2.88%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between Vitamin B12 and birth weight [g] (R^2= -0.062, P= 0.188) in the 1st trimester (table 3.8, appendix N). There was no statistically significant difference between gestational birth weight for low, normal or high and vitamin B12 levels (p=0.5545) (figure 3.8, appendix N).

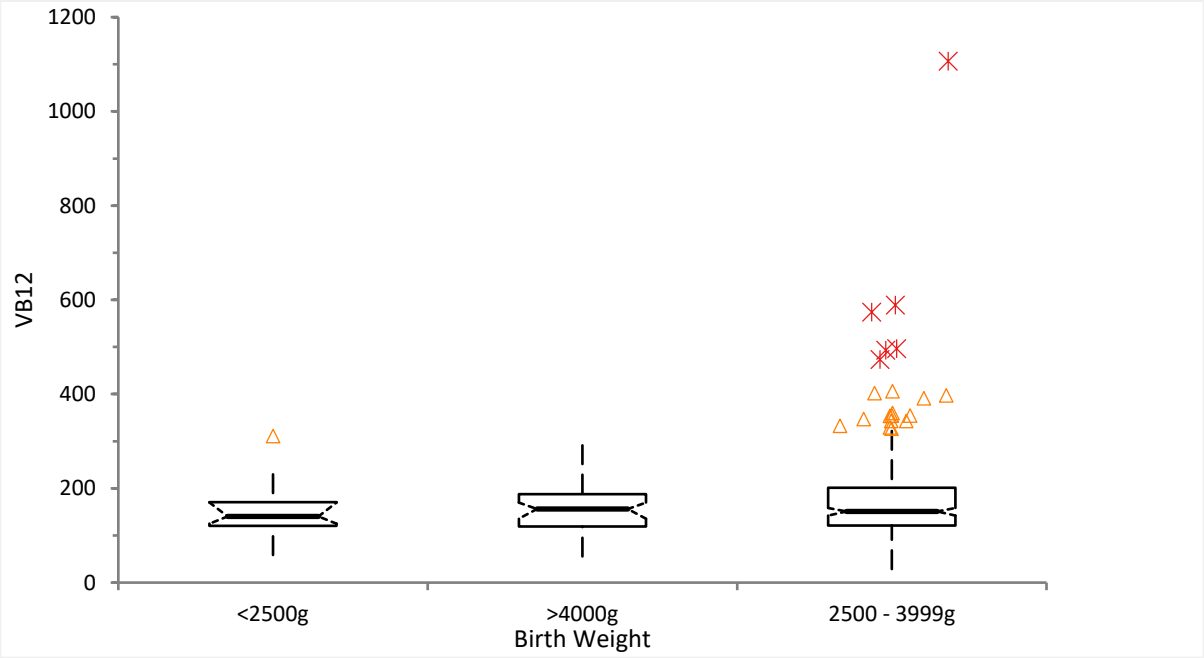


Figure 3.8. Difference between gestational birth weight and median Vitamin B12 levels at 1st trimester booking Kruskal-Wallis (ANOVA), p=0.5545 accepting the null hypothesis of no difference between the gestation birth weight groups.

3.11.2 Summary

The data for vitamin B12 analysis showed there were 20.39% of women with low vitamin B12 levels using the locally derived cut-off 115pmol/L in the 1st trimester. For 1st trimester booking bloods 5.56% of women had pre-term delivery gestational delivery outcomes (<37 weeks) (table 3.8, appendix N).

Overall the data for vitamin B12 showed no association with birth outcomes, either gestational delivery (R^2 = 0.09) or gestational birth weight (R^2=0.062).

3.12. Serum folate analysis

Data for serum folate analysis were only collected for booking bloods, as serum samples are not routinely taken at 28 weeks (2nd trimester) of pregnancy.

Serum folate data for the n=528 women at booking showed a median serum folate level of 10.37ug/L (IQR 5.58 – 16.77/range 1.15 – 23.60). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.09 and p value of <0.0001, showing the serum folate level from the women in this study is not normally distributed.

Of the n=528 samples collected, there was 461 women with live birth outcomes available for analysis. As the difference between booking and 28 week blood could not be assessed, levels of serum folate were therefore assessed between gestational delivery cut-offs (<37 weeks, 37 to 41 weeks and >42 weeks), and birth weight cut-offs (<2500g, 2500 to 3999g, and >4000g).

3.12.1 Serum folate analysis and delivery outcome

Serum folate data for n=461 women with live birth outcomes showed a median serum folate of 10.7µg/L (IQR 5.53 – 17.01/range 1.15 - >23.6). Using a logarithmic scale, the Kolmogorov-Smirnov D (KSD) statistic returned a value of 0.09 and P value of <0.0001, showing the serum folate from the women in this study to be not normally distributed.

As the data was not normally distributed a Spearman Rank Correlation statistic was used to assess for association of the serum folate data against outcomes (gestational delivery age [days] and gestational birth weight) at booking only (table 3.9, appendix O).

Table 3.9. Summary of Spearman Rank Correlation statistic for serum folate analysis at booking for association with delivery gestation age [days] and delivery birth weight [g].

	# of results	Median Folate µg/L (range)	Spearman's rs	p-value	Accept/reject H0
Folate Booking					
Delivery Gestation [Days]	461	10.71 (1.15 - >23.6)	0.051	0.2778	Accept H0
Birth Weight [g]			0.042	0.3695	Accept H0

The locally derived cut-off level for the determination of serum folate in respect of analysis method for the local population was used to assess for folate deficiency. The locally derived cut-off level is determined as <3µg/L. Of the n=461 serum folate results available at 1st trimester booking the median serum folate was 10.7pmol/L (IQR 5.54 – 17.01/range 1.15 - >23.6) with n=31 (6.72%) having a folate ≤3µg/L. The remaining n=430 (93.28%) had a serum folate >3µg/L.

Gestational Delivery Outcome at Booking (Serum folate cut-off 3µg/L)

Analysis of 1st trimester booking bloods with gestational delivery outcomes (n=461 live births) showed n=32 (6.93%) had a preterm delivery of (<37 weeks), n=414 (89.82%) had a normal gestation delivery (37 -41 weeks +6 days) and n=15 (3.25%) had a post term deliver gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The n=430 (93.28%) women with a serum folate >3µg/L, n=31 (6.72%) had a preterm gestational delivery (<37w) with n=384 (83.31%) having normal gestation delivery (37 to 41 weeks +6 days) and n=15 (3.25%) having post-term gestational delivery (>42 weeks).

Of the n=31 (6.72%) women with a serum folate ≤3µg/L, n=1 (0.21%) had a preterm gestational delivery (<37w) with n=30 (6.51%) having normal gestation delivery (37 to 41 weeks +6 days) and n=0 (0%) having post-term gestational delivery (>42 weeks).

The data showed there was no correlation/association between serum folate and delivery gestation [days] (R^2= 0.051, P= 0.2778) in the 1st trimester (table 3.9, appendix O). There was no statistically significant difference between gestational delivery date for preterm, normal or late delivery and serum folate levels (p=0.7709) (figure 3.9, appendix O).

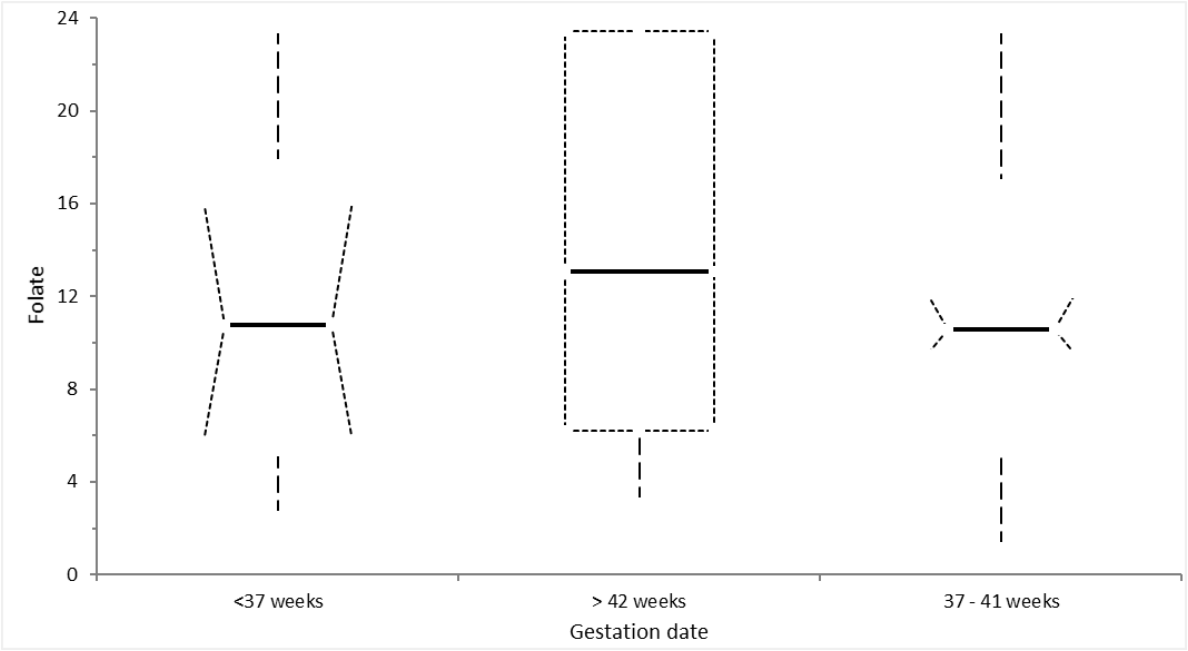


Figure 3.9. Difference between gestational delivery age and serum folate levels at 1st trimester booking Kruskal- Wallis (ANOVA), p=0.7709 accepting the null hypothesis of no difference between the gestation delivery age groups.

Gestational Birth Weight Outcome at Booking (Serum folate cut-off 3µg/L)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes (n=461 live births) showed n=28 (0.43%) had a low birth weight <2500g at delivery. There were n=366 (78.46%) live births with normal birth weight (2500g to 3999g) and n=67 (15.47%) had a high birth weight >4000g (4000g to 4885g).

Of the n=430 (93.28%) with a serum folate >3µg/L, n=26 (5.64%) had low birth weight (<2500g), n=344 (73.69%) had a normal birth weight (>2500g to 3999g) with n=60 (13.95%) having a high gestational birth weight (>4000g).

The remaining n=31 (6.72%) women with a serum folate ≤3µg/L at booking, n=2 (0.43%) had low birth weight (<2500g), n=22 (4.77%) had a normal birth weight (>2500g to 3999g) with n=7 (1.52%) having a high gestational birth weight (>4000g).

The data showed there was no correlation/association between folate and birth weight [g] (R^2= -0.042, P= 0.3695) in the 1st trimester (table 3.9, appendix O). There was no statistically significant difference between gestational birth weight for low, normal or high weights and serum folate (p=0.6537) (figure 3.10, appendix O).

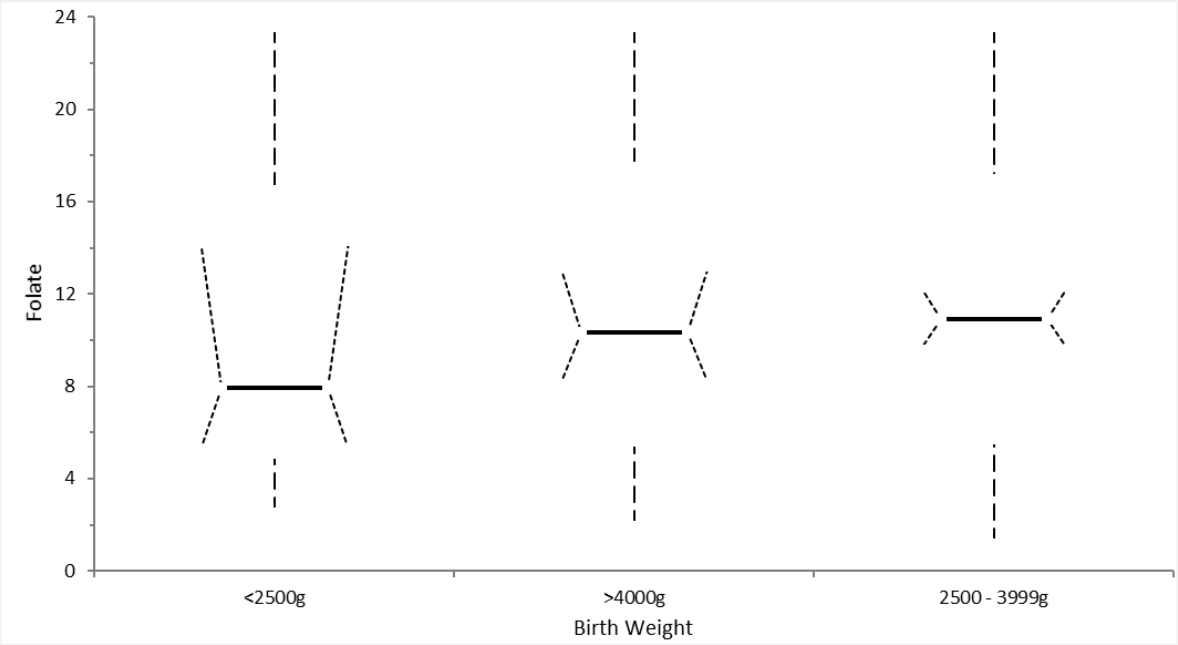


Figure 3.10 Difference between gestational birth weight and serum folate levels at 1st trimester booking Kruskal-Wallis (ANOVA), p=0.6537 accepting the null hypothesis of no difference between the gestation birth weight groups.

3.12.2 Summary

The data for serum folate analysis showed there were 6.72% of women with low serum folate levels using the locally derived cut-off 3µg/L in the 1st trimester. For 1st trimester booking bloods 6.93% of women had pre-term delivery gestational delivery outcomes (<37 weeks) (table 3.9, appendix O).

Overall the data for serum folate showed no association with birth outcomes, either preterm delivery or low gestational birth.

3.13. Iron deficiency without anaemia and delivery outcomes

Iron deficiency without anaemia is defined as a low serum ferritin with a haemoglobin level within normal intervals. There are currently no defined cut-off's in pregnancy to define IDWA therefore the parameters used for assessment of serum ferritin ≤30.0µg/L and Hb >110.0g/L in the 1st trimester booking blood and >105.0g/L in the 2nd trimester 28 weeks bloods was used for assessment of the data.

There were n=249 (52.42%) women in the 1st trimester at booking with plasma ferritin ≤30.0µg/L and Hb >110.0g/L suggesting IDWA. This rose to n=312 (69.33%) in the 2nd trimester. The median plasma ferritin level in this group at 1st trimester booking was 18.0µg/L (IQR 12.0 – 24.0 /range 3.0 – 30.0) and the median Hb was 128.0g/L (IQR 122.0 – 133.0 /range 112.0 – 147.0). In the 2nd trimester the plasma ferritin was 7.0µg/L (IQR 5.0 – 10.0 (Range 2.0 – 29.0) and the median Hb was 116.0µg/L (IQR 111.0 – 122.0 /range 106.0 – 142.0).

As the data was not normally distributed a Spearman Rank Correlation statistic was used to assess for association of those women with IDWA against outcomes (gestational delivery age [days] and gestational birth weight) at both booking (6 to 14 weeks) and at 28 weeks (table 3.10, appendix P). The absolute difference between ferritin at booking and ferritin at 28 weeks was also tested.

Table 3.10. Summary of Spearman Rank Correlation statistic for ferritin analysis at booking, 28 weeks and the difference of ferritin between Booking ferritin and 28 week for association with delivery gestation age [days] and delivery birth weight [g].

	# of results	Median Hb g/L (range) / Ferritin ug/L (range)	Spearmen's rs	t statistic	p-value	Accept/reject H0
IDWA Booking						
Delivery Gestation [Days]	249	128 (112 - 147) / 18 (3.0 - 30.0)	0.043	0.67	0.5014	Accept H0
Birth Weight [g]			**0.151**	**2.4**	**0.173**	**Reject H0**
IDWA 28 weeks						
Delivery Gestation [Days]	312	116 (106 - 142) / 7.0 (2.0 - 29.0)	-0.029	-0.51	0.6121	Accept H0
Birth Weight [g]			-0.107	-1.89	0.0591	Accept H0

Gestational Delivery Outcome at Booking (IDWA)

Analysis of 1st trimester booking bloods with gestational delivery outcomes for women with IDWA (n=249 live births) showed n=15 (3.15%) had a preterm delivery of (<37 weeks), n=227 (47.69%) had a normal gestation delivery (37 -41 weeks +6 days) and n=7 (1.47%) had a post term deliver gestation >42 weeks (42 weeks to 42 weeks + 2 days).

The data showed there was no correlation/association between IDWA and delivery gestation age [days] (R^2= 0.043, p= 0.5014) in the 1st trimester (table 3.10, appendix P).

Gestational Birth Weight Outcome at Booking (IDWA)

Analysis of 1st trimester booking bloods with gestational birth weight outcomes for women with IDWA (n=249 live births) showed n=14 (2.94%) had a low birth weight <2500g at delivery. There were n=196 (41.18%) live births with normal birth weight (2500g to 3999g) and n=39 (8.19%) had a high birth weight >4000g (4000g to 4885g).

The data showed there was a weak correlation/association between IDWA and birth weight [g] (R^2= -0.151, P= 0.0173) in the 1st trimester (table 3.10, appendix P).

Gestational Delivery Outcome at 28 weeks (IDWA)

Analysis of 2nd trimester booking bloods with gestational delivery outcomes for women with IDWA (n=312 live births) showed n=19 (4.22%) had a preterm delivery of (<37 weeks), n=282 (62.67%) had a normal gestation delivery (37 -41 weeks +6 days) and n=11 (2.44%) had a post term delivery gestation (>42 weeks).

The data showed there was no correlation/association between IDWA and delivery gestation age [days] (R^2= -0.029, p= 0.6121) in the 2nd trimester (table 3.10, appendix P).

Gestational Birth Weight Outcome at 28 weeks (IDWA)

Analysis of 2nd trimester booking bloods with gestational birth weight outcomes for women with IDWA (n=312 live births) showed n=18 (4.00%) had a low birth weight <2500g at delivery. There were n=249 (55.33%) live births with normal birth weight (2500g to 3999g) and n=45 (10.00%) had a high birth weight >4000g (4000g to 4885g).

The data showed there was no correlation/association between IDWA and birth weight [g] (R^2= -0.107, P= 0.0591) in the 2nd trimester (table 3.10, appendix P).

Difference in plasma ferritin levels and Hb between 1st trimester booking bloods and 2nd trimester 28 weeks bloods

The median plasma ferritin and Hb of the 1st trimester booking bloods for women with IDWA were compared with the median plasma ferritin and Hb of 2nd trimester 28-week bloods (figure 3.11, appendix P). The results showed that there was a decrease in the median plasma ferritin from booking from 18.0µg/L to 7.0µg/L at 28 weeks. This showed an overall median decrease of 11.0µg/L (61.11%) between 1st and 2nd trimester bloods. For Hb there was a

decrease from booking from 128.0g/L to 116.0g/L at 28 weeks. This showed an overall median decrease of 12.0g/L (9.37%) between 1st and 2nd trimester bloods.

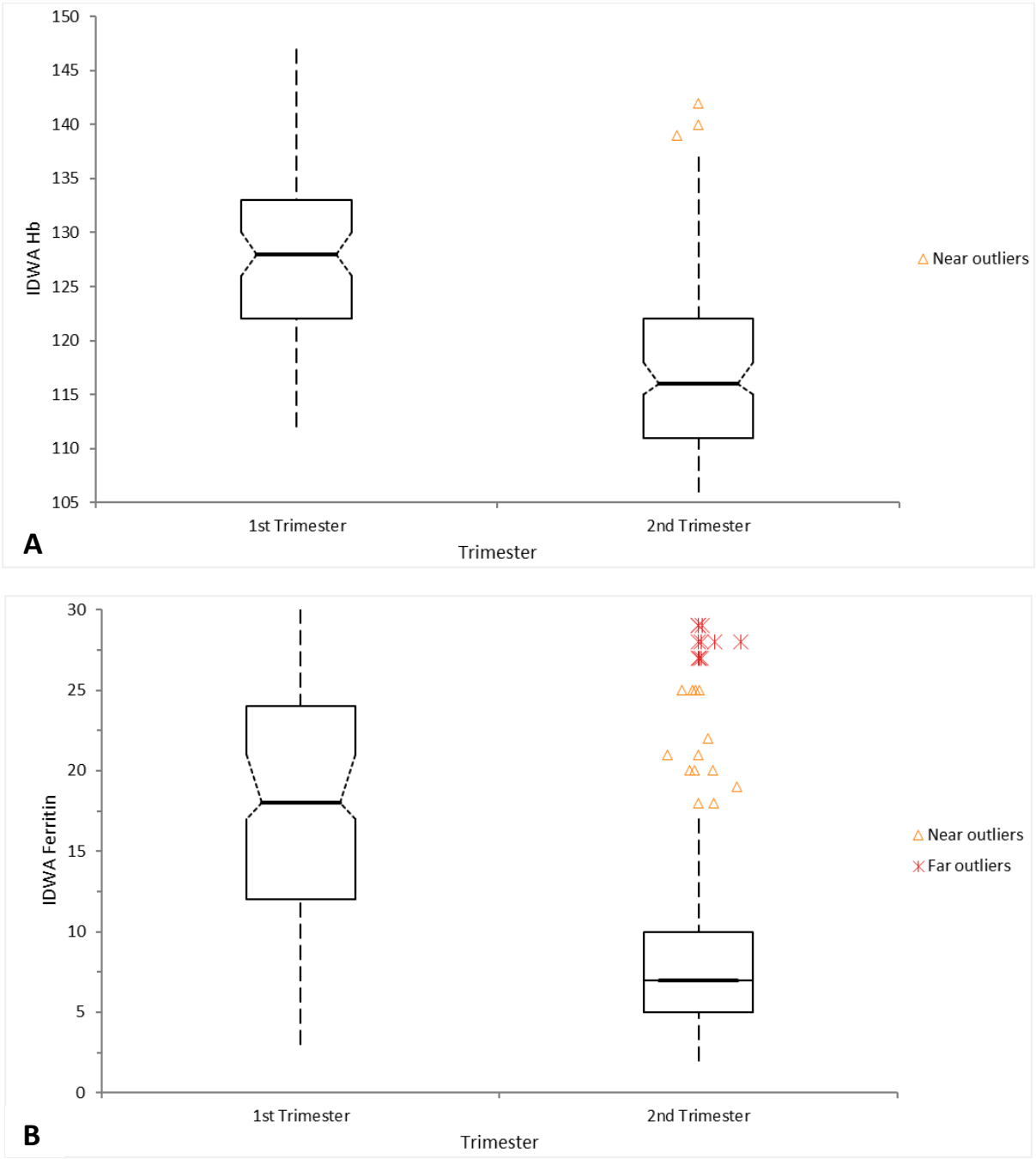


Figure 3.11. (A) Difference between IDWA Hb >110.0g/L with serum ferritin <30.0µg/L at 1st trimester booking to 2nd trimester. (B) Difference between IDWA Hb <105.0g/L with serum ferritin <30.0µg/L at 2nd trimester bloods. For IDWA Haemoglobin using Wilcoxon-Mann-

Whitney test p=<0.0001 rejecting the null hypothesis of the shift in location between the distributions of the populations being equal to 0. For IDWA serum ferritin using Wilcoxon-Mann-Whitney test p=<0.0001 rejecting the null hypothesis of the shift in location between the distributions of the populations being equal to 0.

3.13.6. Summary

The data for plasma ferritin analysis showed there were 52.31% of women with low ferritin levels using the BSH guideline cut-off of 30.0µg/L in the 1st trimester who were not anaemic (Hb>110.0g/L), increasing to 69.33% in the 2nd trimester showing that the majority of women in the 2nd trimester have low serum ferritin levels suggesting iron deficiency although they do not have anaemia. Both serum ferritin and Hb decrease between 1st trimester booking and 2nd trimester 28 weeks.

For 1st trimester booking bloods 3.15% of women had pre-term delivery gestational delivery outcomes (<37 weeks) with 4.22% in the 2nd trimester (28 weeks) (table 3.10, appendix P). Results were similar for low birth weight (<2500g) gestational delivery outcomes with 2.94% and 4.00% respectively for booking and 28 weeks.

Overall the data for IDWA showed no association with birth outcomes, either preterm delivery or low gestational birth weight although there was a weak association with IDWA compared with birth weight at booking (R^2= 0.151).

3.14. Live births and pregnancy loss

During the course of the study and sample collection, n=69 women experienced pregnancy loss (n=63 <28 weeks and n=4 at >28 weeks as there was no birth outcomes (gestation delivery age or birth weight) recorded). A further n=2 were excluded due to termination of pregnancy. There were n=476 live births from the data collected at booking (figure 3.1). Data from the n=63 women with pregnancy loss <28 weeks was analysed for all parameters under investigation (table 3.11, appendix Q). The n=2 TOP have no relevance to the outcome and the n=4 with pregnancy loss >28 weeks were excluded as the number is so low as to draw any useful conclusions.

As the data previously showed non normal distribution the non-parametric test Wilcoxon-Mann-Whitney test was used to compare the two groups for all the parameters of interest in the study (Hb, Reticulocytes, Ret-He, IRF, ferritin, vitamin B12 and folate).

Table 3.11. Summary of Wilcoxon-Mann-Whitney statistic for Hb Reticulocytes, Ret-He, IRF, Ferritin, Vitamin B12 and Folate comparison of pregnancy losses.

Pregnancy loss v live births	# of results	Median Loss (IQR/range)	Median Live(IQR/range)	W statistic	z approximation	p-value	Accept/reject H0
Hb [g/L]	540	(n= 64) 130.00 (121.0 - 137.0 / 73.0 - 150.0)	(n=476) 129.00 (123.0 - 135.0 / 93.0 - 158.0)	18275	0.82	0.411	Accept H0
Reticulocytes [x10^9/L]	539	(n=64) 67.10 (50.85 - 76.23 / 33.30 - 129.60)	(n=475) 66.80 (55.02 - 80.07 / 28.30 - 133.20)	15994	-1.10	0.2715	Accept H0
RetHe [pg]	**533**	**(n=63) 32.80 (31.07 - 34.08 / 16.9 - 35.5)**	**(n=470) 33.50 (32.40 - 34.40 /24.50 - 38.40)**	**13762**	**-2.67**	**0.0077**	**Reject H0**
Ferritin [µg/L]	539	(n=64) 27.0 (12.0 - 50.0 / 3.0 - 129.0)	(n=475) 28.0 (17.0 - 47.0 / 3.0 - 204.0)	16407.5	-0.75	0.4556	Accept H0
IRF%	**485**	**(n=62) 7.40 (5.28 - 11.03 / 2.50 - 33.90)**	**(n=423) 16.3 (13.10 - 21.28 / 5.4 - 39.0)**	**4090**	**-10.65**	**<0.0001**	**Reject H0**
Vitamin B12 [pmol/L]	**513**	**(n=62) 164.60 (128.26 - 238.59/ 63.10 - 503.50)**	**(n=451) 151.30 (120.18 - 197.38 / 28.70 - 1106.70)**	**18206.5**	**2.08**	**0.0378**	**Reject H0**
Folate [µg/L)	523	(n=62) 9.25 (5.31 - 15.47 / 1.66 - >23.60)	(n=461) 10.71 (5.54 - 17.01 / 1.15 - >23.60)	15033	-1.08	0.278	Accept H0

3.14.1 Haemoglobin analysis pregnancy loss and live births

Haemoglobin data for the n=64 women with pregnancy loss <28 weeks showed a median Hb of 130.0g/L (IQR 121.0 – 137.0/range 73.0 – 150.0). Women with live births (n=476) at 1st trimester booking showed a median Hb of 129.0g/L (IQR 123.0 – 135.0 / range 93.0 – 158.0) (table 3.11, appendix Q).

There was no statistically significant difference of Hb between pregnancy loss and live births (p=0.4110) Figure 3.12.

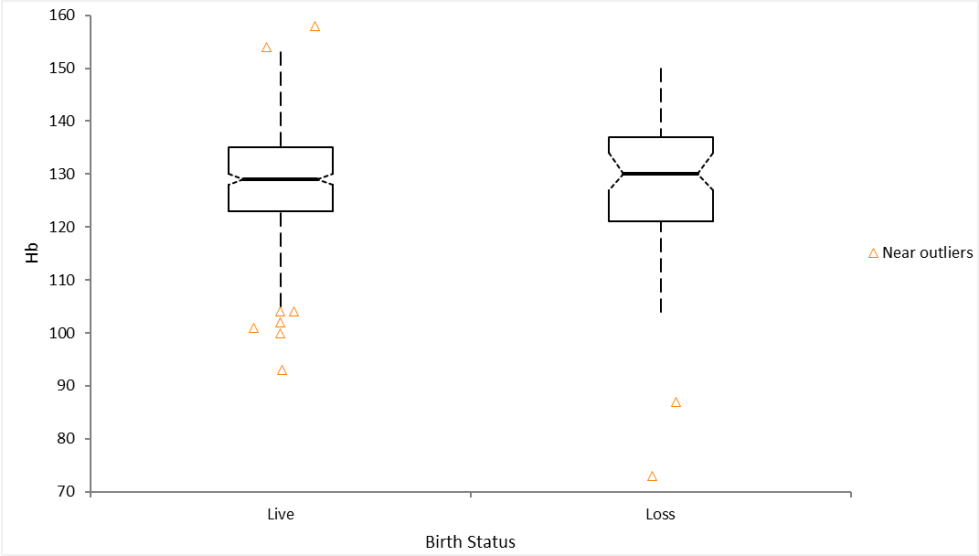


Figure 3.12. Difference in Hb between pregnancy losses and live births <28 weeks using Wilcoxon-Mann-Whitney test p=0.2780 accepting the null hypothesis of the shift in location between the distributions of the populations is equal to 0 (i.e. there is no difference).

3.14.2 Reticulocyte analysis pregnancy loss and live births

Reticulocyte data for the n=64 women with pregnancy loss <28 weeks showed a median reticulocyte counts of 67.10x10^9/L (IQR 50.85 – 76.23/range 33.30 – 129.60). Women with live births (n=475) at 1st trimester booking showed a median reticulocyte count of 66.80x10^9/L (IQR 55.02 – 80.07 / range 28.30 – 133.20) (table 3.11, appendix Q).

There was no statistically significant difference of reticulocyte count between pregnancy loss and live births (p=0.2715) (figure 3.13, appendix Q).

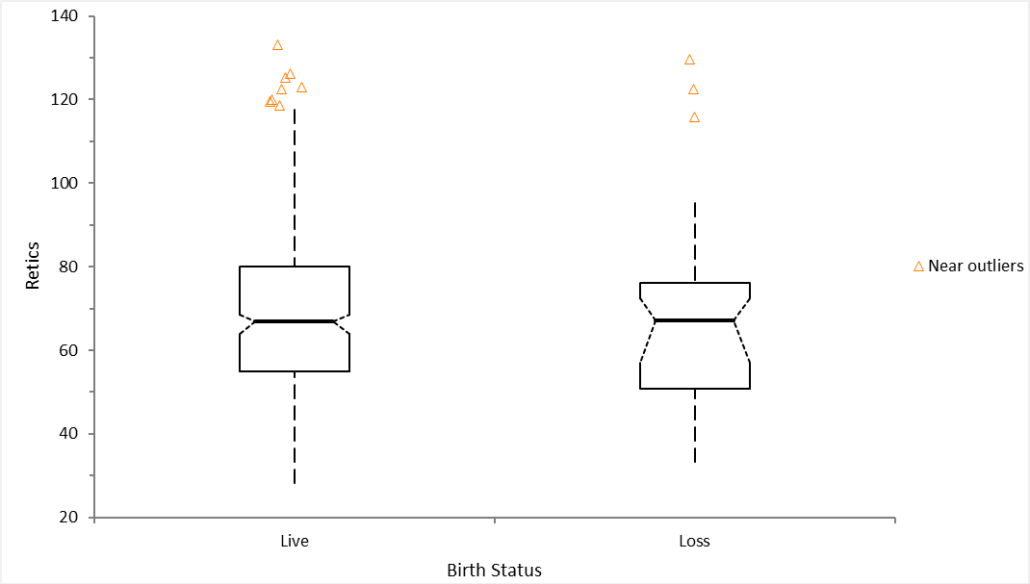


Figure 3.13. Difference in reticulocyte count between pregnancy losses and live births <28 weeks using Wilcoxon-Mann-Whitney test p=0.2715 accepting the null hypothesis of the shift in location between the distributions of the populations is equal to 0 (i.e. there is no difference).

3.14.3. Ret-He analysis pregnancy loss and live births

Ret-He data for the n=63 women with pregnancy loss <28 weeks showed a median Ret-He of 32.80pmol/L (IQR 31.07 – 34.08/range 16.9 – 35.5). Women with live births (n=470) at 1st trimester booking showed a median Ret-He of 33.50pmol/L (IQR 32.40 – 34.40 / range 24.5 – 38.4) (table 3.11, appendix Q).

The analysis showed a statistically significant difference for Ret-He between pregnancy loss and live births (p=0.0077) figure 3.14, appendix Q

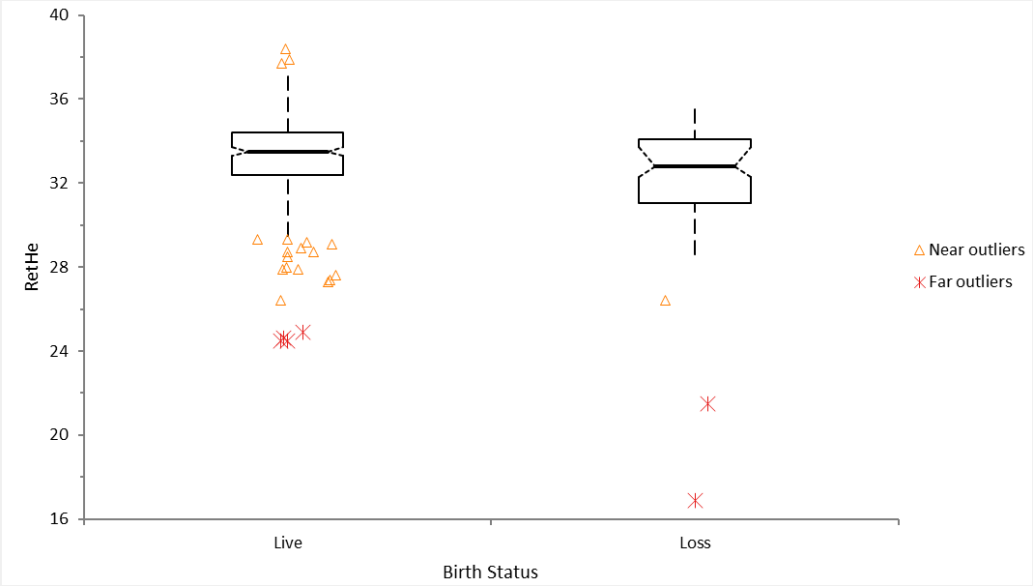


Figure 3.14. Difference in Ret-He count between pregnancy losses and live births <28 weeks using Wilcoxon-Mann-Whitney test p=0.0077 rejecting the null hypothesis of the shift in location between the distributions of the populations is not equal to 0 (i.e. there is a difference).

3.14.4. IRF analysis pregnancy loss and live births

IRF data for the n=63 women with pregnancy loss <28 weeks showed a median IRF of 7.40% (5%-95% percentiles 3.05 – 14.42%/IQR 5.28 – 11.03/range 2.5 – 33.9). Women with live births (n=470) at 1st trimester booking showed a median IRF of 16.3% (5%-95% percentiles 9.20 – 28.35%/IQR 13.10 – 21.28 / range 5.4 – 39.0) (table 3.11, appendix Q).

The analysis showed a statistically significant difference of IRF between pregnancy loss and live births (p=<0.0001) figure 3.15, appendix Q.

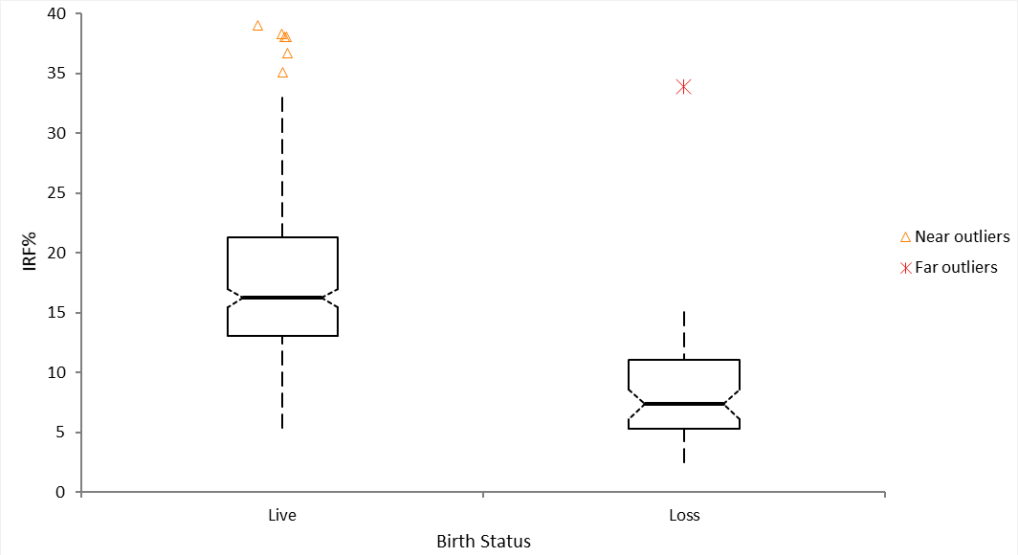


Figure 3.15. Difference in IRF count between pregnancy losses and live births <28 weeks using Wilcoxon-Mann-Whitney test p=<0.0001 rejecting the null hypothesis of the shift in location between the distributions of the populations is not equal to 0 (i.e. there is a difference between IRF values for live births and losses).

3.14.5. Plasma ferritin analysis pregnancy loss and live births

Plasma ferritin data for the n=64 women with pregnancy loss <28 weeks showed a median plasma ferritin of 27.0µg/L (IQR 12.0 – 50.0/range 3.0 – 129.0). Women with live births (n=475) at 1st trimester booking showed a median plasma ferritin count of 28.0µg/L (IQR 17.0 – 47.0 / range 3.0 – 204.0) (table 3.11, appendix Q).

There was no statistically significant difference of plasma ferritin levels between pregnancy loss and live births (p=0.4556) figure 3.16, appendix Q.

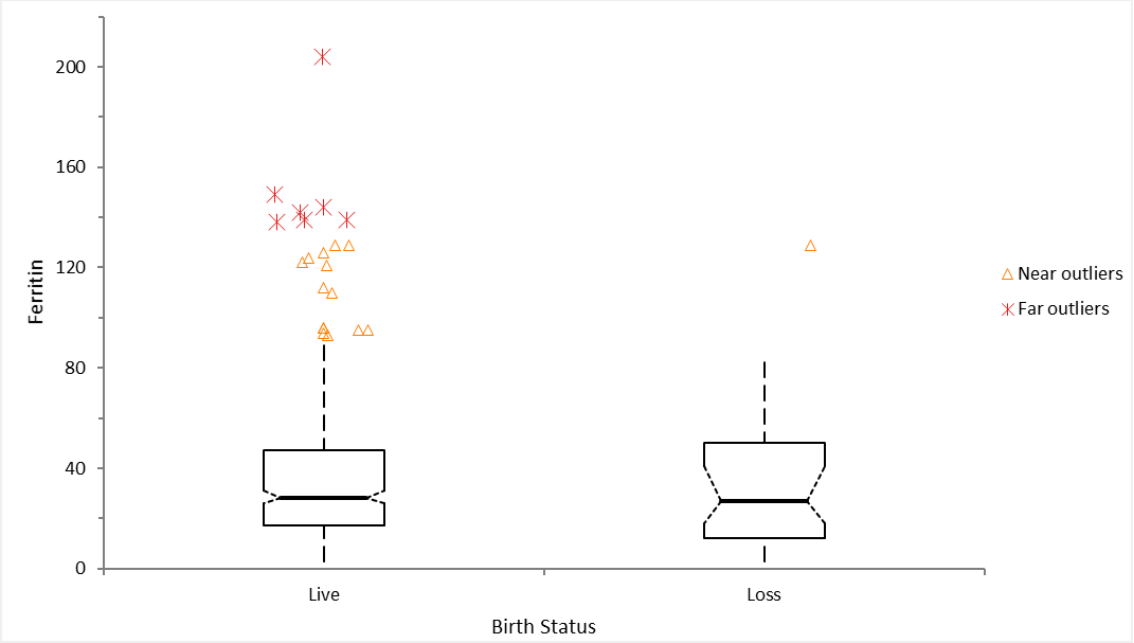


Figure 3.16. Difference in plasma ferritin levels between pregnancy losses and live births <28 weeks using Wilcoxon-Mann-Whitney test p=0.4556 accepting the null hypothesis of the shift in location between the distributions of the populations is equal to 0 (i.e. there is no a difference).

3.14.6. Vitamin B12 analysis pregnancy loss and live births

Vitamin B12 data for the n=62 women with pregnancy loss <28 weeks showed a median vitamin B12 level of 164.60pmol/L (IQR 128.26 – 238.59/range 63.10 – 503.50). Women with live births (n=451) at 1st trimester booking showed a median vitamin B12 count of 151.30pmol/L (IQR 120.18– 197.37 / range 28.70 - >1106.70) (table 3.11, appendix Q).

There was no statistically significant difference for vitamin B12 levels between pregnancy loss and live births (p=0.0378) figure 3.17, appendix Q.

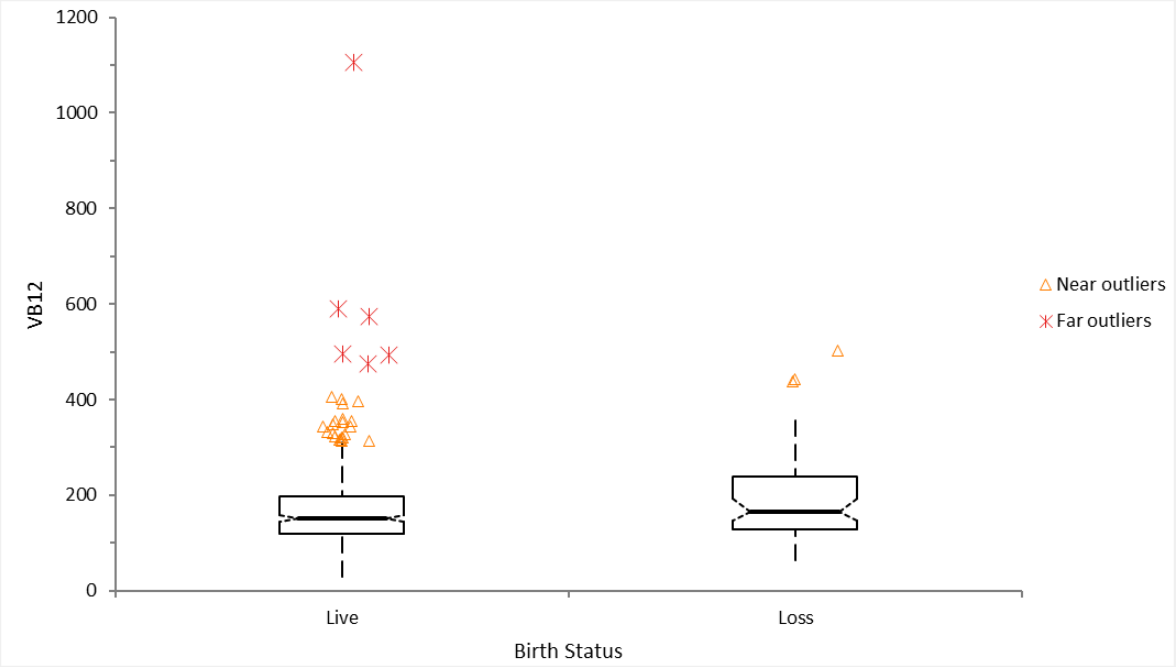


Figure 3.17. Difference in vitamin B12 levels between pregnancy losses and live births <28 weeks using Wilcoxon-Mann-Whitney test p=0.0378 rejecting the null hypothesis of the shift in location between the distributions of the populations is not equal to 0 (i.e. there is a difference).

3.14.7. Serum folate analysis pregnancy loss and live births

Serum folate data for the n=62 women with pregnancy loss <28 weeks showed a median serum folate level of 9.25µg/L (IQR 5.13 – 15.47/range 1.66 - >23.60). Women with live births (n=461) at 1st trimester booking showed a median serum folate count of 10.71µg//L (IQR 5.54 – 17.01 / range 1.15 - >23.60) (table 3.11, appendix Q).

There was no statistically significant difference for serum folate levels between pregnancy loss and live births (p=0.2780) figure 3.18, appendix Q.

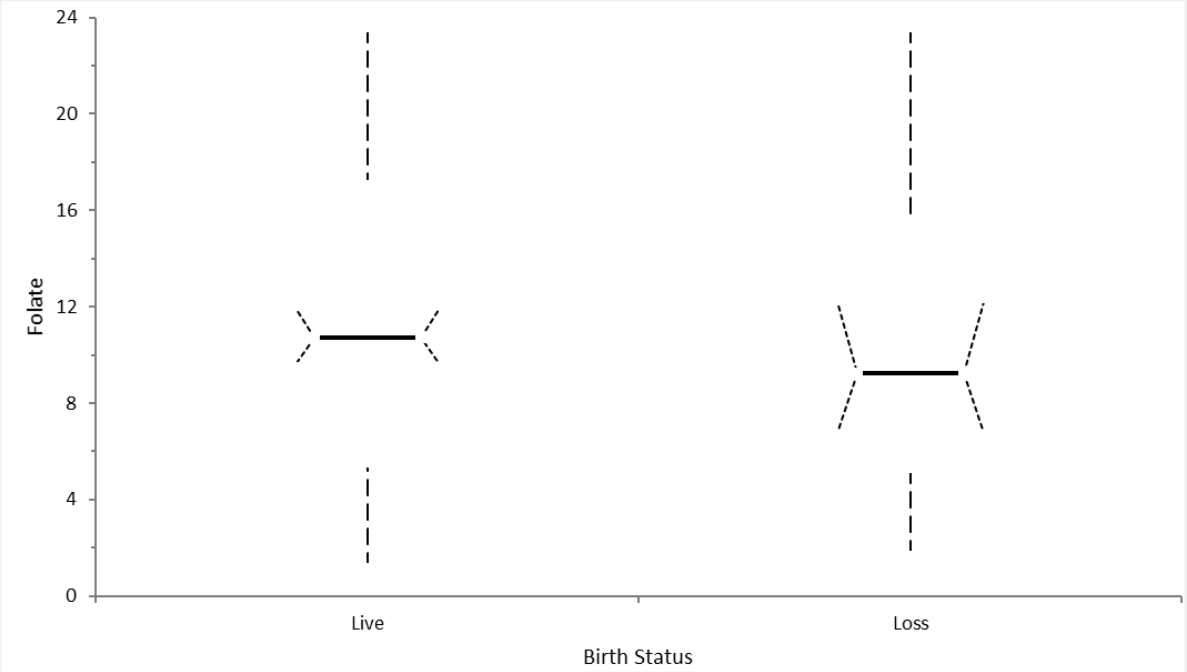


Figure 3.18. Difference in serum folate levels between pregnancy losses and live births <28 weeks using Wilcoxon-Mann-Whitney test p=0.2780 accepting the null hypothesis of the shift in location between the distributions of the populations is equal to 0 (i.e. there is no difference).

3.14.8. Live birth and pregnancy loss summary

Generally, the data showed there is no statistically significant difference between Hb, reticulocytes, plasma ferritin, and folate between those women with pregnancy loss before 28 weeks. The data did however show a statistically significant difference for Ret-He (p=0.0077), IRF (p=<0.0001 and vitamin B12 (p=0.0378) although the median values for Ret-He were similar (0.70pmol/L difference). For IRF women with pregnancy loss the data showed a statistically significant lower median percentage (7.40%) compared to the women with live births (16.30%). For vitamin B12 those with women with pregnancy loss showed a higher median concentration (164.60pmol/L) compared with women with live births (151.30pmol/L)

with a median difference between the two groups of 13.30pmol/L.

3.15. Summary of univariate analysis of associations with outcomes

Three outcomes analysed were:

- gestational delivery,
- birth weight
- pregnancy loss <28 weeks

3.15.1. Gestational delivery

Using Spearman Rank statistic, reticulocytes and IRF both showed a negative association for gestational delivery in the 1st trimester, R^2 = -0.109, p=0.0176 and R^2=-0.173, p=0.0002 respectively, with IRF showing the most association. IRF showed a positive association for in the 2nd trimester, R^2=0.096, p=0.0481. The difference in serum ferritin between the 1st and 2nd trimester also showed a negative association for gestational delivery. Although reticulocytes, IRF and serum ferritin showed an association, this was weak (table 3.12).

Table 3.12. Summary table of parameters showing most association and with birth outcomes (gestational delivery and birth weight).

	# of results	Median (range)	Spearman's rs	p-value	Accept/reject H0
Booking					
Retics Delivery Gestation	475	66.8 (28.3 - 133.2)	-0.109	0.0176	Reject H0
IRF% Delivery Gestation [Days]	469	7.6 (2.6 - 47.6)	-0.173	0.0002	Reject H0
IDWA Birth Weight [g] (Hb/ferritin)	249	128 (112 - 147) / 18 (3.0 - 30.0)	0.151	0.0173	Reject H0
28 weeks					
RetHe Birth Weight [g]	423	31.7 (18.6 - 41.5)	-0.149	0.0021	Reject H0
IRF% Delivery Gestation [Days]	423	16.3 (5.4 - 39.0)	-0.096	0.0481	Reject H0
IRF% Birth Weight [g]			0.108	0.027	Reject H0
Ferritin Birth Weight [g]	397	7 (2.0-154.0)	-0.156	0.0019	Reject H0
Difference Booking to 28 weeks					
Hb Birth Weight [g]	450	-14 (-43-18)	-0.146	0.0018	Reject H0
RetHe Birth Weight [g]	417	-1.60 (-11.0 - 6.4)	-0.167	0.0006	Reject H0
IRF% Birth Weight [g]	416	8.3 (-26.8 - 32.2)	0.172	0.0004	Reject H0
Ferritin Delivery Gestation [Days]	396	-19 (-149.0-34.0)	-0.116	0.021	Reject H0

3.15.2. Birth Weight Outcomes

Using Spearman Rank statistic , in the 1st trimester, only those with iron deficiency without anaemia showed any association with birth weight outcome, showing a positive association R^2=0.151, p=0.0173. In the 2nd trimester Ret-He and serum ferritin showed negative associations, R^2=-0.149, p=0.0021 and R^2=-0.156, p=0.0019 respectively. IRF showed positive association in the 2nd trimester, R^2=0.108, p=0.027. The differences between 1st trimester and 2nd trimester bloods for Hb and Ret-He both showed negative associations, R^2= -0.146, p= 0.0018 and R^2=-0.167, p=0.0006 respectively with IRF showing a positive association, R^2=0.172, p=0.0004. Again all associations were weak (table 3.12).

3.15.3. Pregnancy Losses

Using Wilcoxon-Mann-Whitney, pregnancy losses were compared with those women with live births for all parameters tested. There were three outcomes which showed a statistically significant difference between the two groups, Ret-He (p=0.0077), IRF (p=<0.0001) and serum vitamin B12 (p=0.0378) (table 3.13).

Table 3.13. Summary table of parameters showing most significance for pregnancy losses compared to live births.

Pregnancy loss v live births	# of results	Median Loss (IQR/range)	Median Live(IQR/range)	W statistic	z approximation	p-value	Accept/reject H0
RetHe [pg]	533	(n=63) 32.80 (31.07 - 34.08 / 16.9 - 35.5)	(n=470) 33.50 (32.40 - 34.40 /24.50 - 38.40)	13762	-2.67	0.0077	Reject H0
IRF%	485	(n=62) 7.40 (5.28 - 11.03 / 2.50 - 33.90)	(n=423) 16.3 (13.10 - 21.28 / 5.4 - 39.0)	4090	-10.65	<0.0001	Reject H0
Vitamin B12 [pmol/L]	513	(n=62) 164.60 (128.26 - 238.59/ 63.10 - 503.50)	(n=451) 151.30 (120.18 - 197.38 / 28.70 - 1106.70)	18206.5	2.08	0.0378	Reject H0

3.16. Multivariate analysis

To evaluate any predictors of the three birth outcomes tested for in the study separate univariate and multivariate analysis was performed using logistic regression (ordinary least squares (OLS)) for 1st trimester booking bloods, 2nd trimester 28 week bloods and difference between 1st and 2nd trimester bloods. Data for all n=545 samples was analysed using all variables collected (mothers age, delivery gestation, index of multiple depravation, Hb, reticulocytes, Ret-He, IRF, plasma ferritin, CRP, vitamin B12 and folate) during the study.

3.16.1 Gestational Delivery Outcome (univariate and multivariate analysis)

Univariate analysis was performed for delivery gestation outcome, which showed reticulocytes at booking, IRF at booking, vitamin B12 at booking, reticulocytes at 28 weeks and delta Hb to be statistically significant and some predictive value in the 1st trimester (table 3.14).

Table 3.14: Univariate logistic regression analysis for predictors of gestational age at delivery.

	R Squared	95% Confidence Interval (CI)	Probability (p)
Mother's Age	0.0023	-0.3384484 0.1090071	0.3141
Index of Multiple Deprivation	0.0016	-0.2235048 0.5549124	0.4032
Hb	0.0013	-0.0791382 0.1807893	0.4426
Reticulocytes	0.0115	-0.1312368 -0.0098075	0.0229
Ret-He	0.0025	-0.2996722 0.9908792	0.2931
IRF	0.0149	-0.7173458 -0.0979426	0.0100
Ferritin	0.0019	-0.022903 0.0641585	0.3522
CRP	0.0006	-0.1814476 0.1055341	0.6034
VB12	0.0095	0.0004117 0.0282659	0.0436
Folate	0.0013	-0.1133552 0.2500286	0.4602
Hb at 28 weeks	0.0020	-0.1853199 0.0651073	0.3460
Retics at 28 weeks	0.0136	-0.1415727 -0.014322	0.0165
Ret-He at 28 weeks	0.0001	-0.4236694 0.348169	0.8476
IRF at 28 weeks	0.0046	-0.3420128 0.0572639	0.1617
Ferritin at 28 weeks	0.0013	-0.1292274 0.0600925	0.4732
CRP at 28 weeks	0.0002	-0.0452535 0.0334649	0.7686
Δ Hb	0.0087	0.0014243 -0.2944674	0.0478
Δ Reticulocytes	0.0033	-0.0862092 0.0200325	0.2215
Δ Ret-He	0.0029	-0.2240659 0.0588372	0.2517
Δ IRF	0.0035	-0.2897961 0.0634954	0.2087
Δ Ferritin	0.0044	-0.0148352 0.0892164	0.1608
Δ CRP	0.0000	-0.0410188 0.0413939	0.9929

Shaded rows highlight those variables with predictive value.

The significant predictors (reticulocytes at booking, IRF at booking, vitamin B12 at booking, reticulocytes at 28 weeks and delta Hb) from univariate analysis was assessed by multivariate logistic regression. Only delta Hb for gestational delivery outcome showed statistical significance as an independent predictor (table 3.15).

Table 3.15: Multivariate logistic regression analysis for independent predictors of gestational age at delivery.

	95% Confidence Interval (CI)	Probability (p)
Reticulocytes	-0.1469774 0.0337502	0.219
IRF	-0.3825188 0.4046248	0.956
VB12	-0.0004278 0.0277256	0.057
Reticulocytes at 28 weeks	-0.137268 0.0556769	0.406
IRF at 28 weeks	-0.4444118 0.1372467	0.300
Δ Hb	0.0229001 0.3835988	0.027
Δ Ferritin	-0.0003248 0.1127934	0.051

Shaded rows highlight those variables with predictive value.

3.16.2. Birth Weight Outcome (univariate and multivariate analysis)

Univariate analysis was performed for birth weight outcome, which showed gestational delivery days, Hb at 28 weeks, Ret-He at 28 weeks, IRF at 28 weeks, plasma ferritin at 28 weeks, delta Hb and deltaIRF to be statistically significant and some predictive value in the 1st trimester (table 3.16). Index of multiple depravation score was borderline.

Table 3.16: Univariate logistic regression analysis for predictors of birth weight.

	R-squared	95% Confidence Interval (CI)	Probability (p)
Mother's Age	0.001	-6.056729-13.66811	0.449
Delivery Gestation	0.428	25.73125 - 31.9194	0.000
Index of Multiple Deprivation	0.005	-4.673256 - 29.61356	0.154
Hb	0.002	-2.829109 - 8.618391	0.321
Reticulocytes	0.001	-3.465024 - 1.910276	0.570
Ret-He	0.000	-31.98627 - 24.84493	0.805
IRF	0.005	-23.46929 - 3.876405	0.160
Plasma ferritin	0.000	-2.122972 - 1.709593	0.832
CRP	0.000	-6.969837 - 5.678001	0.841
VB12	0.003	-0.2505683 - 0.9867829	0.243
Folate	0.002	-3.785106 - 12.20179	0.301
Hb at 28 weeks	0.011	-11.63431 - 0.6484188	0.029
Reticulocytes at 28 weeks	0.000	-3.412058 - 2.368914	0.723
Ret-He at 28 weeks	0.025	-46.17503 - -11.7883	0.001
IRF at 28 weeks	0.019	1.788166 - 19.73024	0.019
Ferritin at 28 weeks	0.016	-9.852788 - -1.269134	0.011
CRP at 28 weeks	0.001	-2.284401 - 1.315366	0.597
Δ Hb	0.0304	5.788281 - 18.55802	0.0002
Δ Reticulocytes	0.0006	-2.942034 - 1.745545	0.6162
Δ Ret-He	0.0010	-4.15862 - 8.318601	0.5127
Δ IRF	0.0191	-19.32231 - -3.878077	0.0033
Δ Ferritin	0.0028	-.9732599 - 3.614855	0.2584
Δ CRP	0.0004	-1.426152 - 2.204284	0.6738

Shaded rows highlight those variables with predictive value.

The significant predictors (delivery gestation, Hb at 28 weeks, Ret-He at 28 weeks, IRF at 28 weeks, delta Hb and deltaIRF) from univariate analysis was assessed by multivariate logistic regression. Delivery gestation, Index of multiple depravation, Ret-He at28 weeks, IRF at 28 weeks and deltaIRF for birth weight outcome showed statistical significance as independent predictors (table 3.17). IMDD was shown to be borderline using univariate analysis but showed itself to be an independent predictor of birthweight using the multivariate model.

Table 3.17: Multivariate logistic regression analysis for independent predictors of birth weight.

	95% Confidence Interval (CI)	Probability (p)
Delivery Gestation	24.89983 - 31.8889	0.000
Index of Multiple Deprivation	2.875538 - 31.27197	0.019
IRF at booking	-18.55483 - 7.034715	0.377
Hb at 28 weeks	-2.540731 - 8.940909	0.274
Ret-He at 28 weeks	-38.27611 - -0.7027016	0.042
IRF at 28 weeks	1.20615 - 20.53403	0.028
Ferritin at 28 weeks	-6.014079 - 1.263396	0.200
Δ Hb	-1.632836 - 11.47309	0.141
Δ IRF	-15.26467 - -3.319305	0.002

Shaded rows highlight those variables with predictive value.

3.16.3. Pregnancy loss and live Outcome (univariate and multivariate analysis)

Univariate analysis was performed for birth weight outcome, which showed Ret-He at booking, Reticulocytes at 28 weeks, delta Hb, delta reticulocytes, delta Ret-He, and deltaIRF to be statistically significant and some predictive value in the 1st trimester (table 3.18).

Table 3.18: Univariate logistic regression analysis for predictors of birth outcome.

	R Squared	95% Confidence Interval (CI)	Probability (p)
Mother's Age	0.0053	-0.0091952 - 0.0006874	0.092
Index of Multiple Deprivation	0.0011	-0.0053318 - 0.012303	0.437
Hb	0.0000	-0.0027102 - 0.0029581	0.931
Reticulocytes	0.0014	-0.0007828 - 0.0020205	0.386
Ret-He	0.0201	0.0063673 - 0.0249716	0.004
IRF	0.0015	-0.0033984 - 0.0092146	0.365
Ferritin	0.0015	-0.0005514 - 0.0014995	0.364
CRP	0.0016	-0.0018032 - 0.0050024	0.357
VB12	0.0059	-0.0006122 - 0.0000354	0.081
Folate	0.0025	-0.0017152 - 0.0065078	0.253
Hb at 28 weeks	0.0001	-0.000694 - 0.0008541	0.838
Reticulocytes at 28 weeks	0.0093	-0.0008355 - -0.0000072	0.046
Ret-He at 28 weeks	0.0028	-0.001129 - 0.0039369	0.277
IRF at 28 weeks	0.0019	-0.0019146 - 0.0007202	0.376
Ferritin at 28 weeks	0.0000	-0.0005408 - 0.0005532	0.982
CRP at 28 weeks	0.0000	-0.0002239 - 0.0002323	0.972
Δ Hb	0.6067	-0.006288 - -0.0054873	0.000
Δ Reticulocytes	0.3339	-0.0063945 -0.0050316	0.000
Δ Ret-He	0.3759	-0.0167279 - -0.0134445	0.000
Δ IRF	0.2627	-0.0216584 -0.016289	0.000
Δ Ferritin	0.0059	-0.0022631 - 0.0001029	0.074
Δ CRP	0.0039	-0.0017809 - 0.0002687	0.148

Shaded rows highlight those variables with predictive value.

The significant predictors (Ret-He at booking, Reticulocytes at 28 weeks, delta Hb, delta reticulocytes, delta Ret-He, and deltaIRF) from univariate analysis was assessed by multivariate logistic regression. Only Reticulocytes at 28 weeks for gestational delivery outcome showed statistical significance as an independent predictor of live birth (table 3.19).

Table 3.19: Multivariate logistic regression analysis for independent predictors of birth outcome.

	95% Confidence Interval (CI)	Probability (p)
Mother's Age	-0.0010576 - 0.0040818	0.248
Ret-He	-0.0038573 - 0.0101506	0.377
VB12	-0.0002175 - 0.0001192	0.566
Retics at 28 weeks	-.0014185 -.0000171	0.045
Δ Hb	-0.0012418 - 0.0025273	0.502
Δ Reticulocytes	-0.0008934 - 0.0009205	0.977
Δ Ret-He	-0.0008934 - 0.0009205	0.442
Δ IRF	-0.0029235 - 0.0033256	0.899
Δ Ferritin	-0.0004411 - 0.0007061	0.650
Δ CRP	-0.0003932 - 0.0002945	0.778

Shaded rows highlight those variables with predictive value.

3.16.4. Summary of multivariate analysis

Using multivariate logistic regression analyses showed only delta Hb to be an independent predictor of delivery gestation. Independent birth weight predictors were delivery gestation and Hb, Ret-He, and IRF in addition to delta Hb and deltaIRF. For birth outcome (live versus loss) only reticulocytes at 28weeks was shown to be an independent predictor.

3.17. Comparison of Hb and reticulocyte parameters with plasma ferritin

All samples collected for Haemoglobin, reticulocytes and associated variables (Ret-He and IRF) were compared, using Spearman Rank Correlation statistic, with plasma ferritin in both the 1st and 2nd trimesters. All variables showed statistical significance p<0.05 (table 3.20).

Table 3.20. Comparison of plasma ferritin with Hb, reticulocytes and associated parameters in 1st and 2nd trimesters.

	R Squared	95% Confidence Interval (CI)	Probability (p)
Hb	0.241	0.158 – 0.321	<0.0001
Reticulocytes	0.170	0.085 – 0.253	<0.0001
Ret-He	0.292	0.210 – 0.370	<0.0001
IRF	-0.157	-0.241 - -0.071	0.0003
Hb 28 weeks	0.426	0.339 – 0.506	<0.0001
Reticulocytes 28 weeks	0.275	0.178 – 0.366	<0.0001
Ret-He at 28 weeks	0.561	0.487 – 0.627	<0.0001
IRF at 28 weeks	-0.304	-0.394 - -0.209	<0.0001

All variables show significant correlation.

For those women with pregnancy loss (excluding TOP), Haemoglobin, reticulocytes and associated variables (Ret-He and IRF) were compared, using Spearman Rank Correlation statistic, with plasma ferritin in both the 1st and 2nd trimesters. Hb, Ret-He and IRF showed statistical significance p<0.05, but reticulocyte count did not show any significance (table 3.21).

Table 3.21: Comparison of plasma ferritin with Hb, reticulocytes and associated parameters in women with pregnancy loss <28 weeks.

	R Squared	95% Confidence Interval (CI)	Probability (p)
Hb	0.288	0.044 – 0.499	0.0183
Reticulocytes	0.149	-0.101 – 0.382	0.2279
Ret-He	0.353	0.114 – 0.554	0.0036
IRF	-0.276	-0.492 - -0.027	0.0262

Shaded rows highlight those variables with significant correlation.

3.18. Comparison of 1st trimester booking bloods (<14 weeks) with Index of Multiple Depravation Decile

Data was collected for n=545 women across the Hull and East Yorkshire Region (figure 3.19), with n=1 being excluded from analysis as was out of area. There were n=346 pregnancies within the Kingston-upon-Hull City region and n=198 pregnancies within the East Riding of Yorkshire geographical region. Data for n=475 women in the 1st trimester of pregnancy booking bloods with live births was compared across the Index of Multiple Depravation Decile across Kingston-upon-Hull and the East Riding of Yorkshire with 1 being the most deprived group and 10 being the least deprived (appendix R). Data was compared for all analyses within the study, Hb, reticulocytes, Ret-He, IRF, plasma ferritin, vitamin B12 and serum folate.

Figure 3.19. Geographical spread of all pregnant women in 1st trimester within Hull and East Riding of Yorkshire, n=544.

The data showed that there we more pregnancies in the lowest decile (1) than in any other groups, representing 33.70% of all live births (table 3.22).

Table 3.22: Summary of n=475 live births and n=64 pregnancy losses by Index of Multiple Depravation Decile at 1st trimester booking and at 2nd trimester 28 weeks.

# of live births by IMDD	N	%		# of pregnancy loss by IMDD	N	%
1	160	33.68%		1	24	37.50%
2	22	4.63%		2	6	9.38%
3	52	10.95%		3	3	4.69%
4	43	9.05%		4	10	15.63%
5	19	4.00%		5	2	3.13%
6	33	6.95%		6	2	3.13%
7	35	7.37%		7	3	4.69%
8	42	8.84%		8	4	6.25%
9	33	6.95%		9	4	6.25%
10	36	7.58%		10	6	9.38%
Pooled	475	100.00%		Pooled	64	100.00%

Similarly, n=64 women with pregnancy loss <28 weeks was also compared across the Index of Multiple Depravation Decile across Hull and East Riding of Yorkshire which showed more pregnancy losses in the Decile <5 than in those >5 with 37.50% being in the most deprived group.

Kruskal-Wallis statistical test for all comparisons statistic was used for analysis across all groups for both live births and pregnancy loss <28 weeks.

3.18.1.Comparison of Multiple Deprivation Decile for live births at 1st trimester booking bloods, 2nd trimester 28 weeks and pregnancy losses <28 weeks

1st Trimester booking bloods

Haemoglobin by Index of Multiple Deprivation Decile for live births in 1st trimester <14 weeks

Analysis of 1st trimester booking bloods for Hb (n=475 live births) showed the lowest median value was seen in decile 10 of 126.5g/L. There was no statistically significant difference between the population groups from 1 to 10 decile, p=0.6020, with similar median values ranging from 126.50g/L to 133.00g/L (figure 3.20, appendix S).

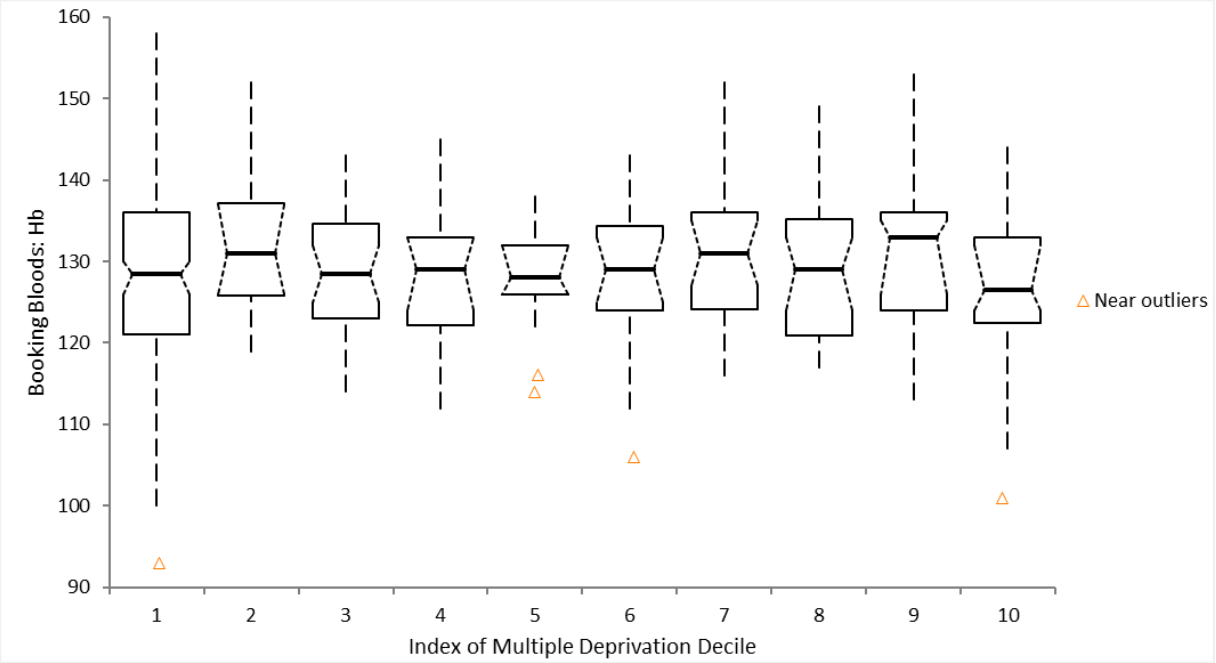


Figure 3.20. Summary of median Hb for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 1st Trimester bloods, p=0.6020, accepting the null hypothesis of no difference between the deciles.

Reticulocytes by Index of Multiple Deprivation Decile for live births in 1st Trimester <14 weeks

Analysis of 1st trimester booking bloods for reticulocytes (n=474 live births) showed the median reticulocyte value across all groups was similar with only a difference of $10x10^9$/L between the lowest and highest groups. There was no significant difference between the population groups from 1 to 10 decile, p=0.1229, with similar median values ranging from 59.10 x10^9/L to 69.60 x10^9/L (figure 3.21, appendix S).

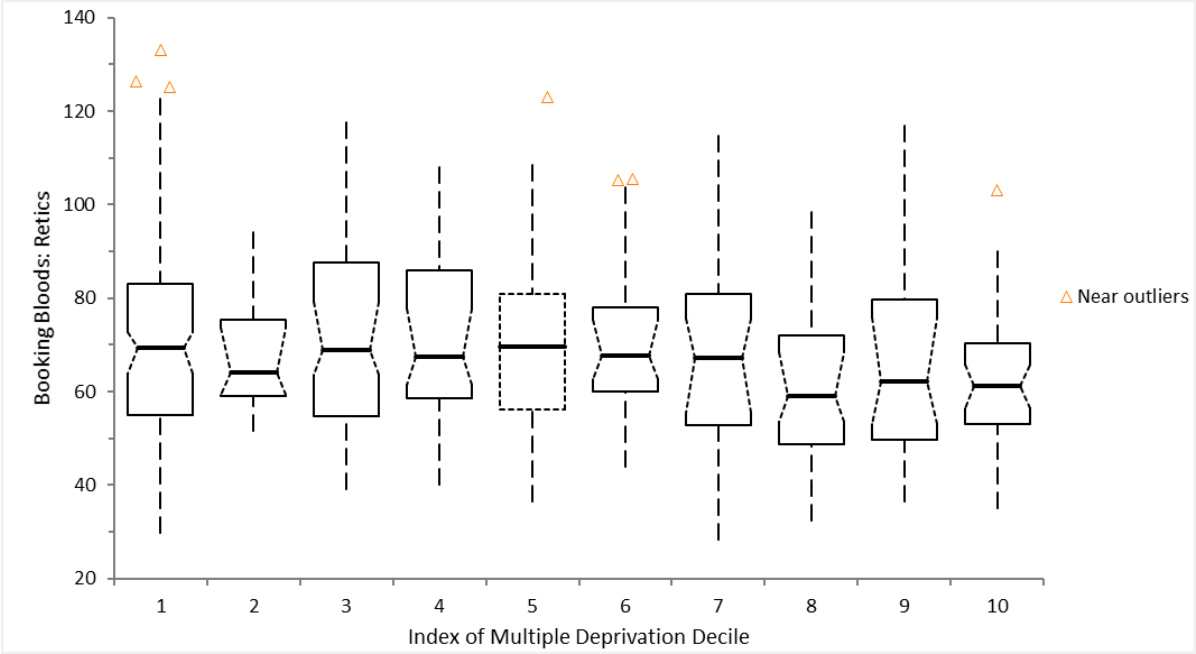


Figure 3.21. Summary of median reticulocytes for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 1st trimester bloods, p=0.1229, accepting the null hypothesis of no difference between the deciles.

Ret-He by Index of Multiple Deprivation Decile for live births in 1st trimester <14 weeks

Analysis of 1st trimester booking bloods for Ret-He (n=470 live births) showed the median Ret-He value across all groups was similar with only a difference of 0.9pg between the lowest and highest groups. There was a significant difference between the population groups from 1 to

10 decile, p=0.0358, although the median only ranged from 33.2pg to 34.10pg (figure 3.22, appendix S).

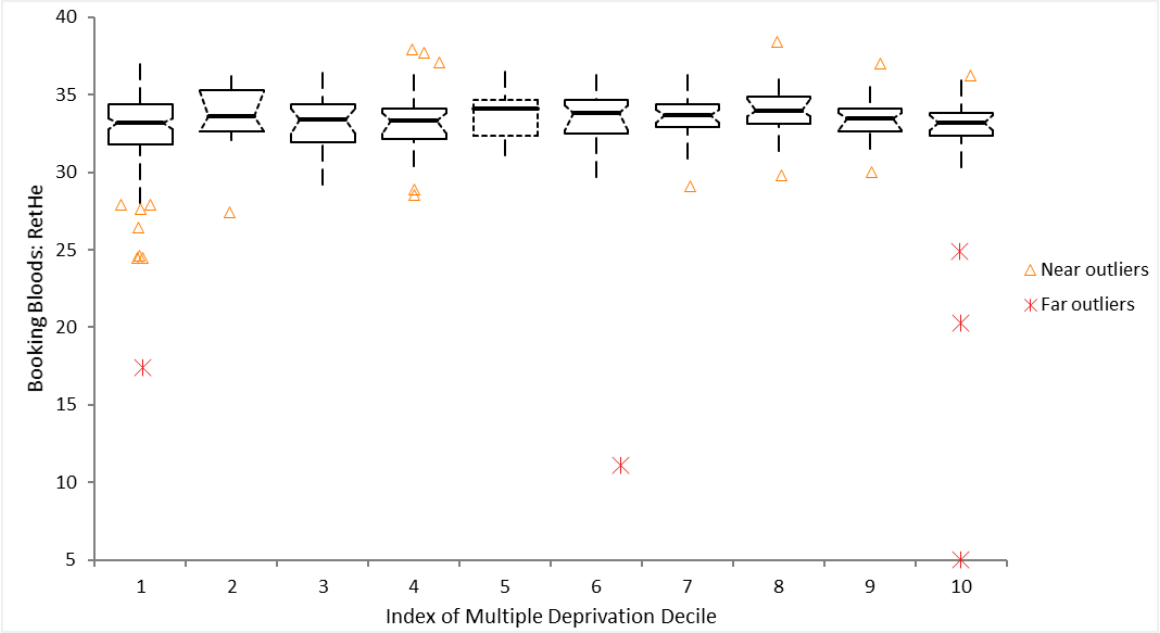


Figure 3.22. Summary of median Ret-He for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 1st trimester bloods, p=0.0358, rejecting the null hypothesis that there is a difference between the deciles.

IRF by Index of Multiple Deprivation Decile for live births in 1st trimester <14 weeks

Analysis of 1st trimester booking bloods for IRF (n=468 live births) showed the median IRF value across all groups was similar with only a difference of 2.7% between the lowest and highest groups. There was a significant difference between the population groups from 1 to 10 decile, p=0.0415 although median values ranged from 6.50% to 9.20% (figure 3.23, appendix S).

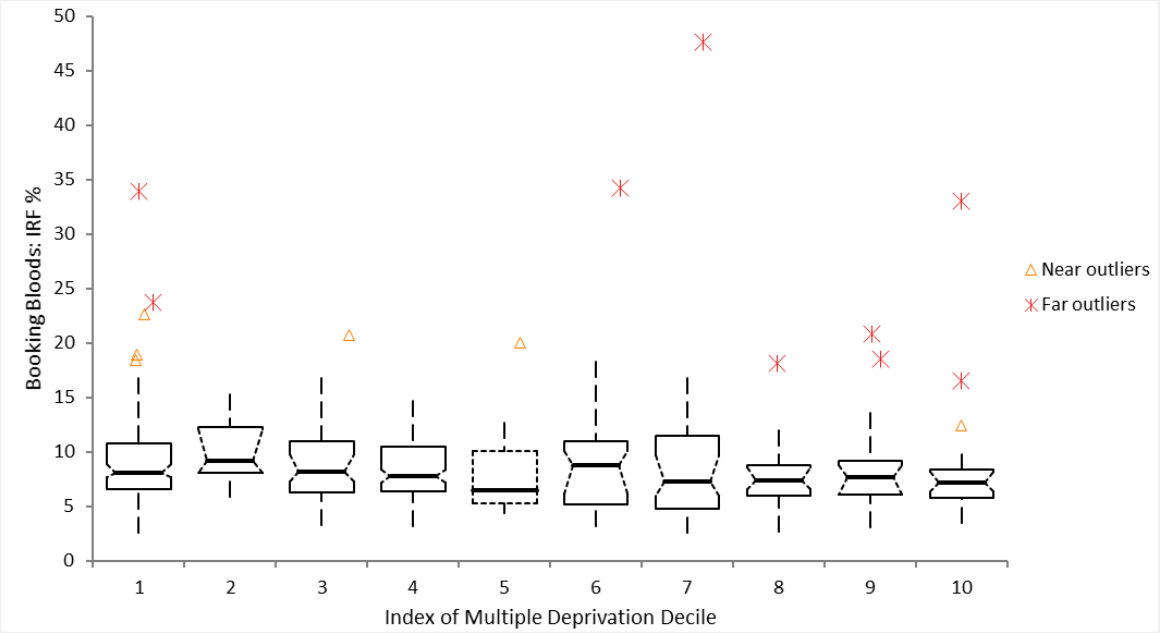


Figure 3.23. Summary of median IRF for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 1st trimester bloods, p=0.0415, rejecting the null hypothesis that there is a difference between the deciles.

Plasma ferritin by Index of Multiple Deprivation Decile for live births in 1st trimester <14 weeks

Analysis of 1st trimester booking bloods for plasma ferritin (n=474 live births) showed the lowest median ferritin values were seen in deciles 1, 2, 9 and 10 (24.0, 24.5, 28.0 and 26.5ug/L respectively). This indicated the most deprived areas and least deprived areas had lower plasma ferritin levels. There was a significant difference between the population groups from 1 to 10 decile, p=0.0003, with median values ranging from 24.0ug/L to 37.0ug/L (figure 3.24, appendix S).

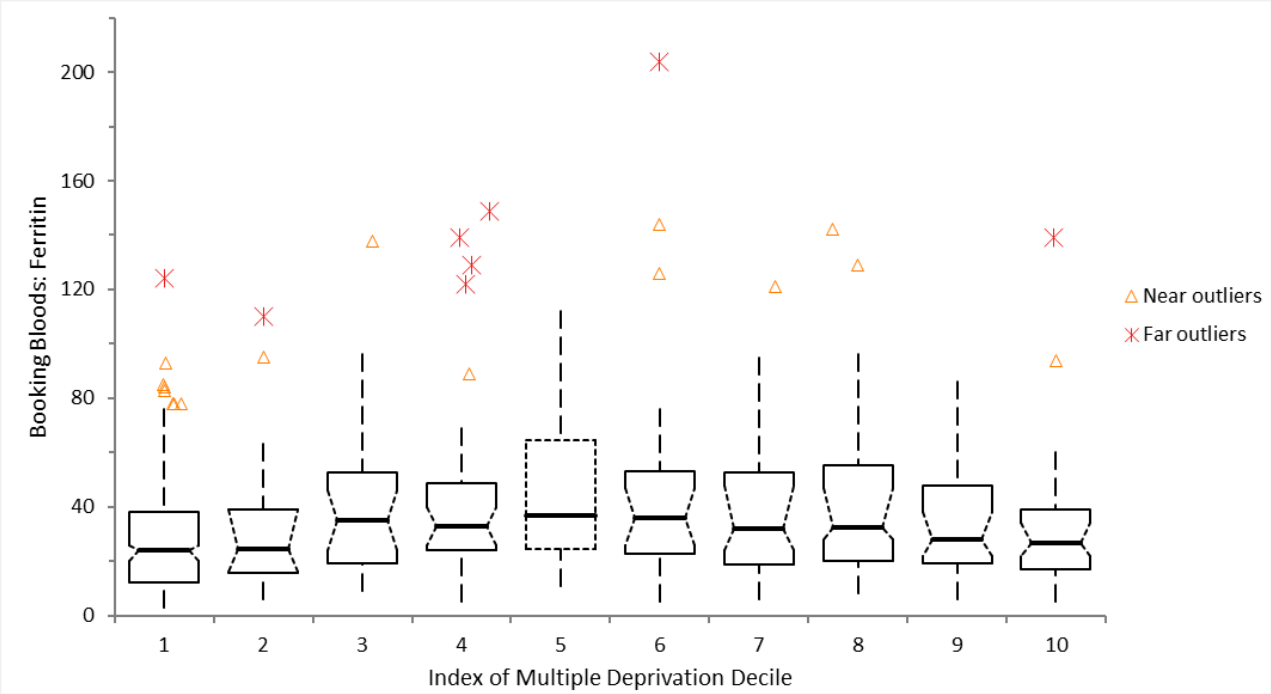


Figure 3.24. Summary of median plasma ferritin for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 1st Trimester bloods, p=0.0003, rejecting the null hypothesis that there is a difference between the deciles.

Vitamin B12 by Index of Multiple Deprivation Decile for live births in 1st trimester <14 weeks

Analysis of 1st trimester booking bloods for vitamin B12 (n=450 live births) showed the lowest median serum vitamin B12 was seen in decile 6 of 141.00ug/L while the highest was seen in decile 10 of 188.80ug/L. There was no significant difference between the population groups from 1 to 10 decile with median values ranging from 141.00pmol/L to 188.80pmol/L (figure 3.25, appendix S).

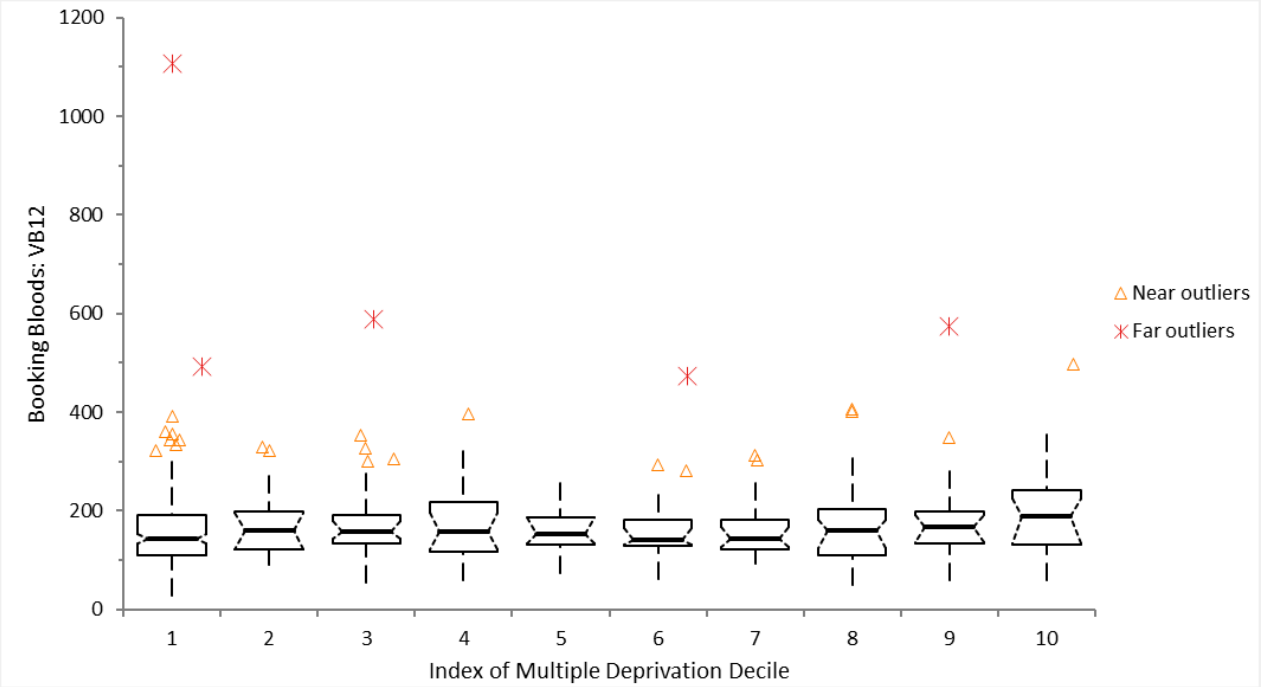


Figure 3.25. Summary of median Vitamin B12 for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 1st trimester bloods, p=0.2453, accepting the null hypothesis that there was no difference between the deciles.

Serum folate by Index of Multiple Deprivation Decile for live births in 1st trimester <14 weeks

Analysis of 1st trimester booking bloods for serum folate (n=408 live births) showed the lowest median serum folate levels were seen in deciles 1 and 2 of 7.34 and 8.16ug/L respectively. There was a significant difference between the population groups from 1 to 10 decile, p=0.0047, with median values ranging from 6.78ug/L to 14.24ug/L (figure 3.26, appendix S), although all values were within the 5%, 95% range (range 2.5 – 23.6µg/L).

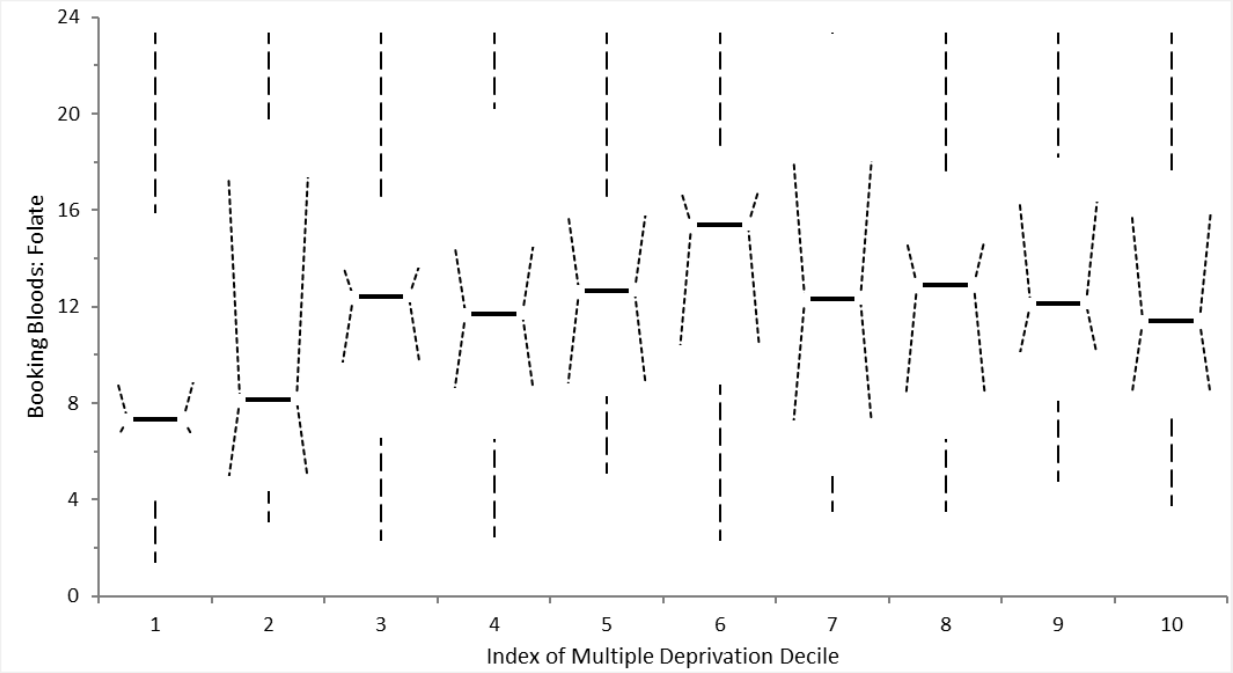


Figure 3.26. Summary of median serum folate for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 1st trimester bloods, p=0.0047, rejecting the null hypothesis that there is a difference between the deciles, although deciles 1 and 2 had lower levels that the other groups (expected 5% - 95% range 2.5 – 23.6µg/L) .

2nd Trimester 28 week bloods

Haemoglobin by Index of Multiple Deprivation Decile for live births in 2nd trimester 28 weeks

Analysis of 2nd trimester (28 week) bloods for Hb (n=450 live births) showed the lowest median Hb of 110.0g/L in decile 2, while the highest was seen in decile 9 of 118.0g/L. There was no significant difference between the population groups from 1 to 10 decile, p=0.1109 (figure 3.27, appendix T).

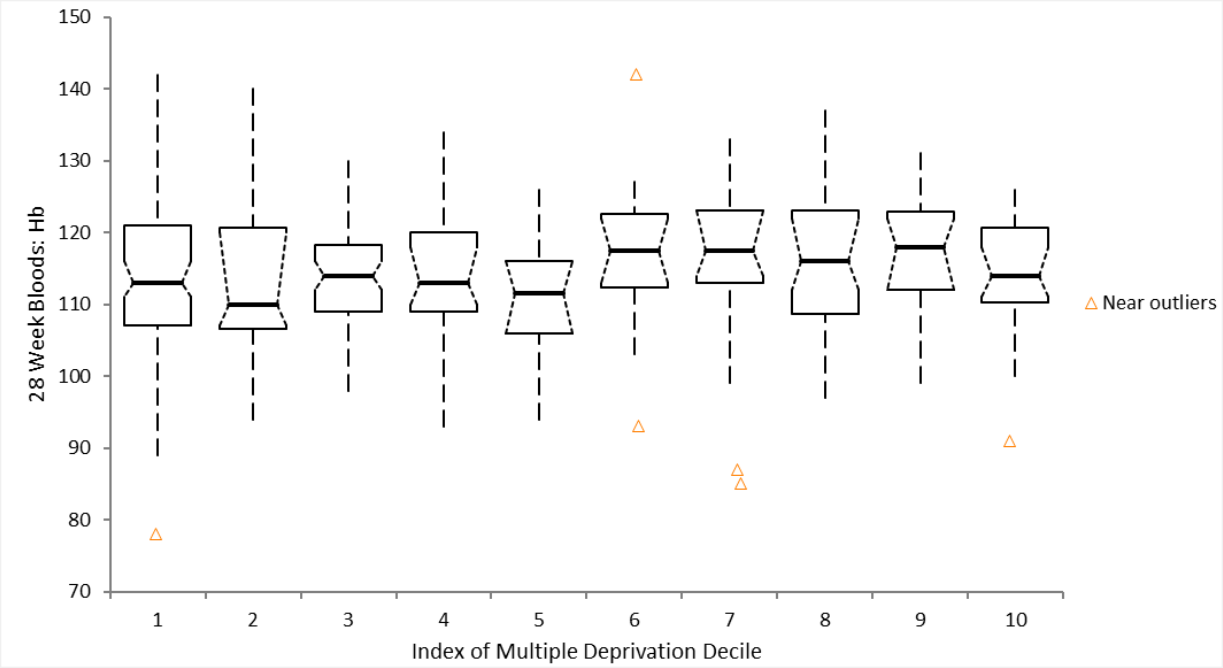


Figure 3.27. Summary of median Hb for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 2nd trimester bloods, p=0.1109, accepting the null hypothesis of no difference between the deciles.

Reticulocytes by Index of Multiple Deprivation Decile for live births in 2nd trimester 28 weeks

Analysis of 2nd trimester (28 week) bloods for reticulocytes (n=423 live births) showed a median difference between all deciles of $9.45x10^9$/L. There was no statistically significant

difference between the population groups from 1 to 10 decile, p=0.4571, with median values ranging from 64.50 x10^9/L to 73.95 x10^9/L (figure 3.28, appendix T).

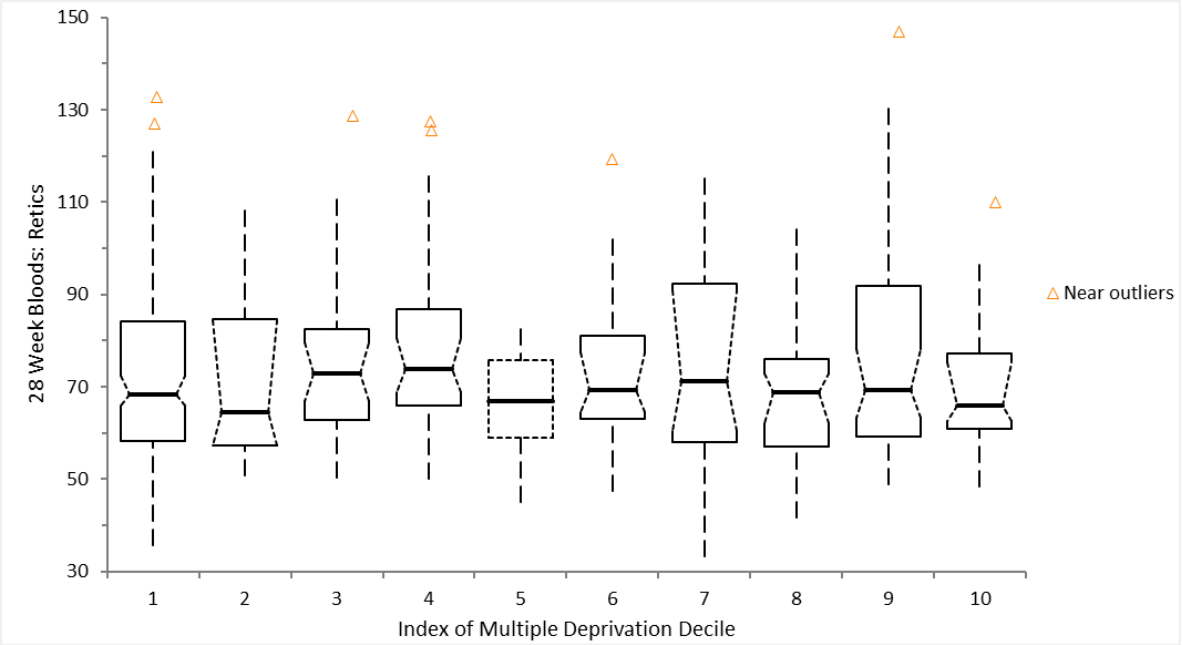


Figure 3.28. Summary of median reticulocytes for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 2nd Trimester bloods, p=0.4571, accepting the null hypothesis of no difference between the deciles.

Ret-He by Index of Multiple Deprivation Decile for live births in 2nd trimester 28 weeks

Analysis of 2nd trimester (28 week) bloods for Ret-He (n=423 live births) showed a median difference of 1.45pg between all deciles. There was a statistically significant difference between the population groups from 1 to 10 decile, p=0.0277 with medians ranging from 31.10pg to 32.55pg (figure 3.29, appendix T).

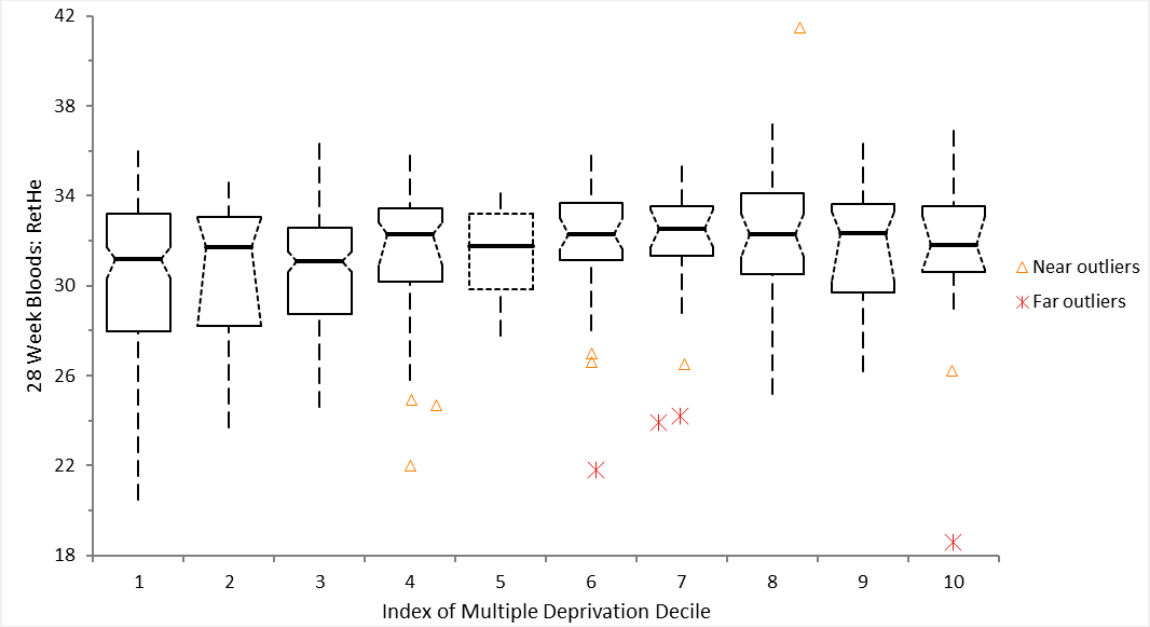


Figure 3.29. Summary of median Ret-He for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 2nd trimester bloods, p=0.0277, rejecting the null hypothesis that there was no difference between the deciles.

IRF by Index of Multiple Deprivation Decile for live births in 2nd Trimester 28 weeks

Analysis of 2nd trimester (28 week) bloods for IRF (n=423 live births) showed a median difference of 4.6% between all deciles. There was no statistically significant difference between the population groups from 1 to 10 decile, p=0.1545, with median values ranging from 14.50% to 18.75% (figure 3.30, appendix T).

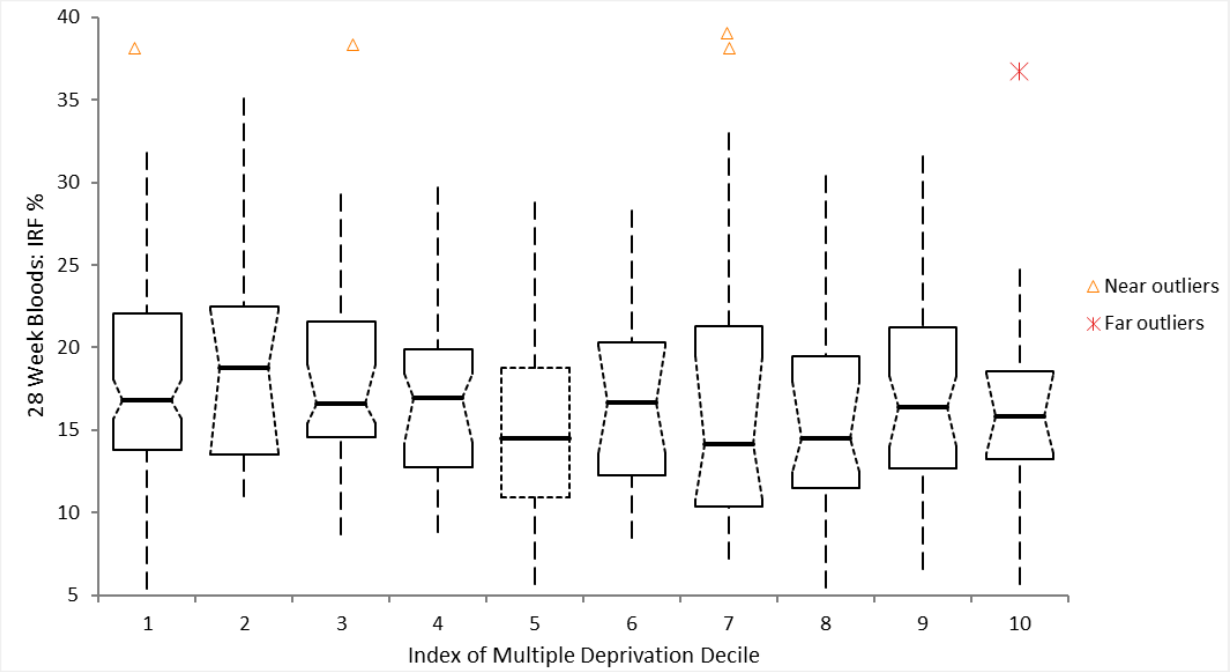


Figure 3.30. Summary of median IRF for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 2nd trimester bloods, p=0.1545, accepting the null hypothesis of no difference between the deciles.

Plasma ferritin by Index of Multiple Deprivation Decile for live births in 2nd trimester 28 weeks

Analysis of 2nd trimester booking bloods for ferritin (n=397 live births) showed a difference between deciles of 3ug/L with the lowest plasma ferritin level ween in deciles 1 and 10 (6.00ug/L and 6.50ug/L respectively). There was no statistically significant difference between the population groups from 1 to 10 decile, p=0.1881, with median values ranging from 4.20ug/L to 6.00ug/L (figure 3.31, appendix T).

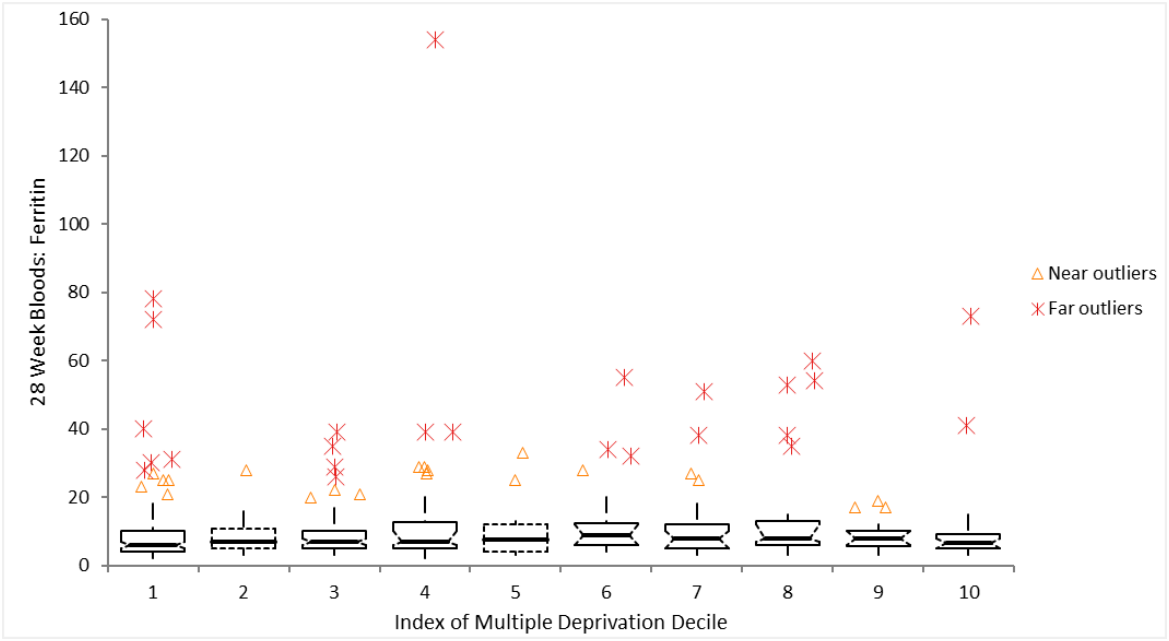


Figure 3.31. Summary of median plasma ferritin for all live births across all Index of Multiple Depravation Deciles by Kruskal-Wallis for 2nd trimester bloods, p=0.1881, accepting the null hypothesis that there was no difference between the deciles.

3.18.2. Pregnancy losses <28 weeks

Haemoglobin by Index of Multiple Deprivation Decile for pregnancy losses in < 28 weeks

Analysis of Hb in 1st Trimester bloods with pregnancy losses <28 weeks (n=64 losses) showed the lowest median Hb (122.5g/L) was in decile 8, while the highest (142.0g/L) was in decile 6. There was no significant difference between the population groups from 1 to 10 decile, p=04744, with median values ranging from 122.50g/L to 142.0/L (figure 3.32, appendix U).

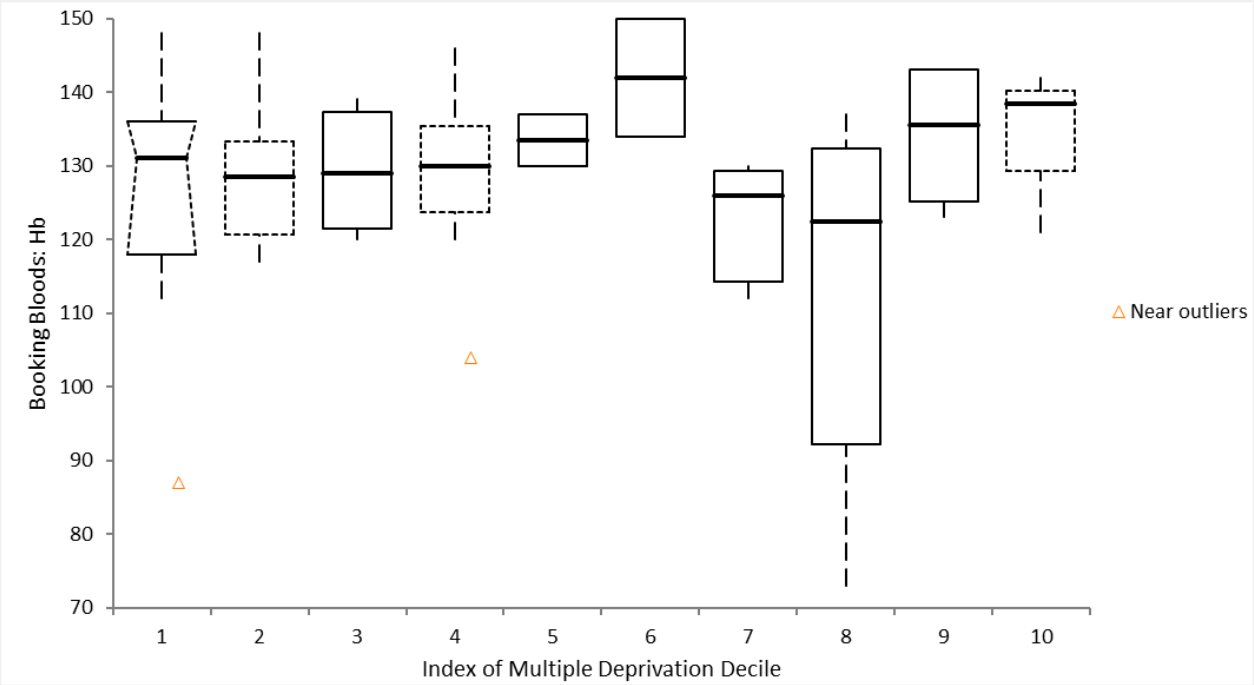


Figure 3.32. Summary of median Hb for all pregnancy losses <28 weeks across all Index of Multiple Depravation Deciles by Kruskal-Wallis, p=0.4744, accepting the null hypothesis of no difference between the deciles.

Reticulocytes by Index of Multiple Deprivation Decile for pregnancy losses in < 28 weeks

Analysis of reticulocytes in 1st trimester bloods with pregnancy losses <28 weeks (n=64 live births) showed a median difference between deciles of 26.45x10^9/L. There was no statistically

significant difference between the population groups from 1 to 10 decile, p=0.6126, with median values ranging from 56.90 x10^9/L to 80.25 x10^9/L (figure 3.33, appendix U).

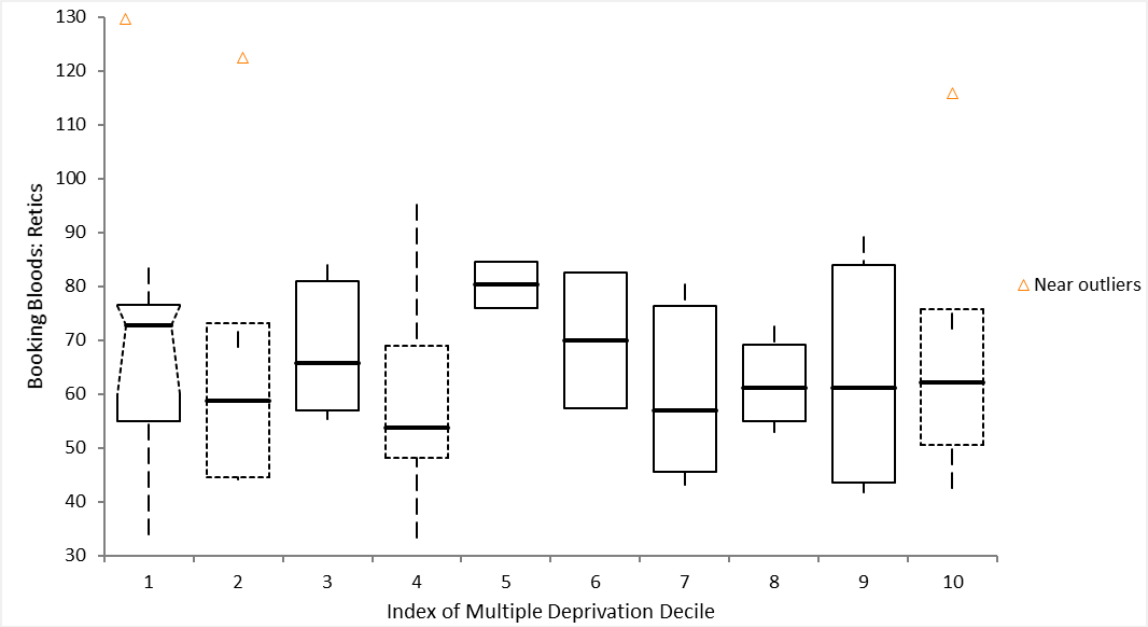


Figure 3.33. Summary of median reticulocytes for all pregnancy losses <28 weeks across all Index of Multiple Depravation Deciles by Kruskal-Wallis, p=0.6126, accepting the null hypothesis of no difference between the deciles.

Ret-He by Index of Multiple Deprivation Decile for pregnancy losses in < 28 weeks

Analysis of Ret-He in 1st trimester bloods with pregnancy losses <28 weeks (n=63 live births) showed a median difference between deciles of 3.05pg. There was no statistically significant difference between the population groups from 1 to 10 decile, p=0.5588, with medians ranging from 31.20pg to 33.90pg (figure 3.34, appendix U).

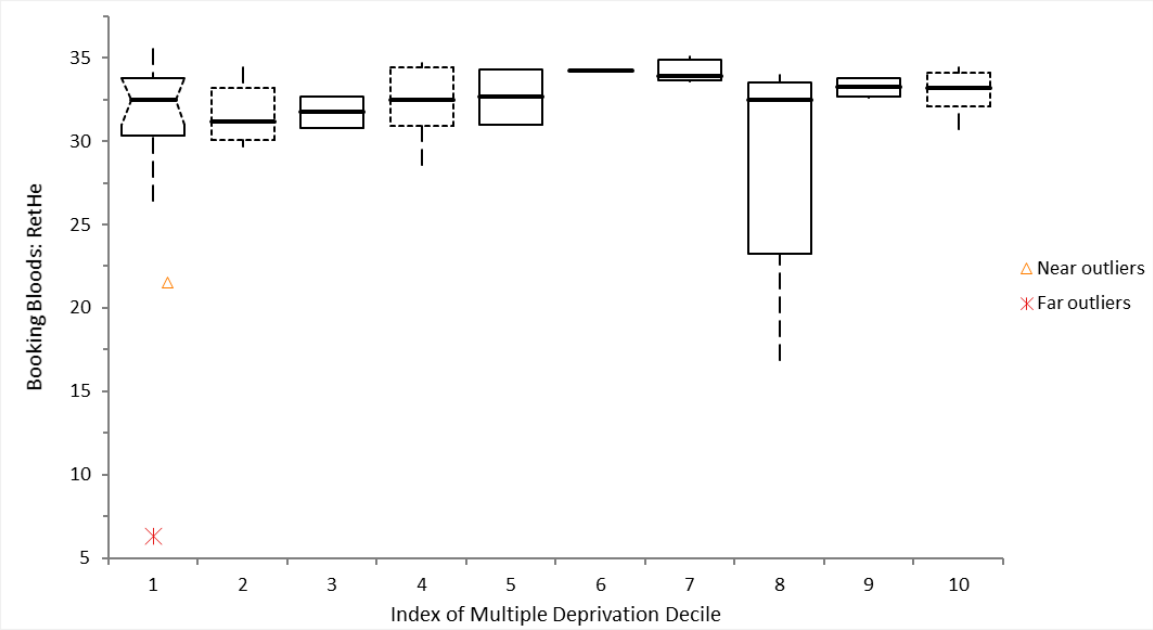


Figure 3.34. Summary of median Ret-He for all pregnancy losses <28 weeks across all Index of Multiple Depravation Deciles by Kruskal-Wallis, p=0.5588, accepting the null hypothesis of no difference between the deciles.

IRF by Index of Multiple Deprivation Decile for pregnancy losses in < 28 weeks

Analysis of IRF in 1st Trimester bloods with pregnancy losses <28 weeks (n=62 live births) showed a median difference of 6.9% between deciles. The lowest was seen in decile 9 (4.70%), while the highest was seen in decile 5 (11.60%). There was no significant difference between the population groups from 1 to 10 decile, p=0.0948, with median values ranging from 4.70% to 11.60% (figure 3.35, appendix U).

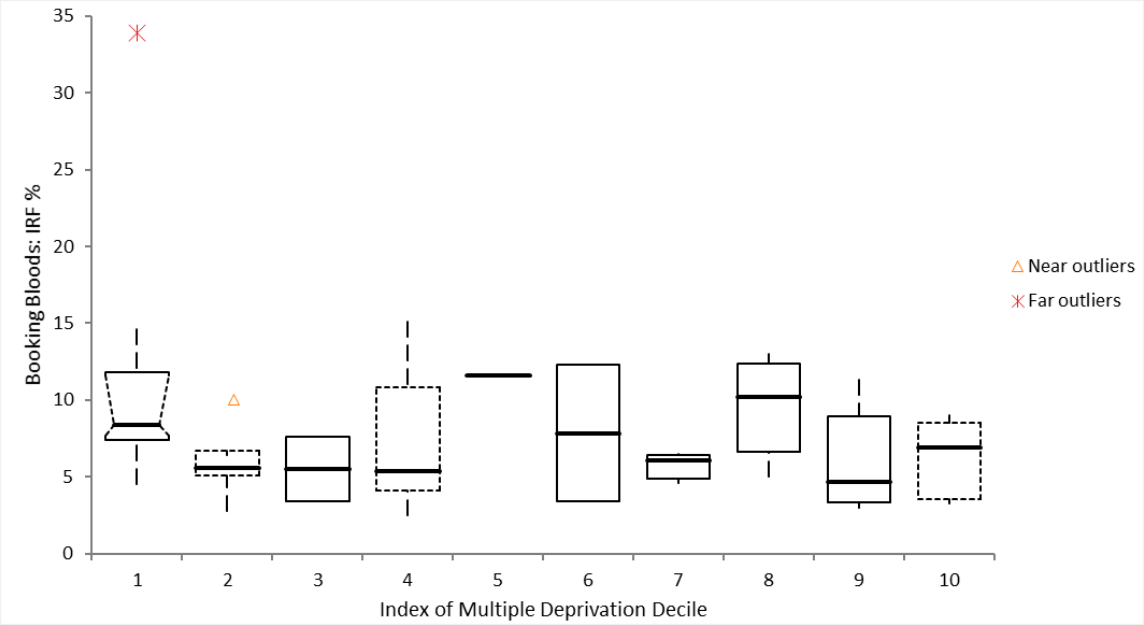


Figure 3.35. Summary of median IRF for all pregnancy losses <28 weeks across all Index of Multiple Depravation Deciles by Kruskal-Wallis, p=0.0948, accepting the null hypothesis of no difference between the deciles.

Plasma ferritin by Index of Multiple Deprivation Decile for pregnancy losses in < 28 weeks

Analysis of plasma ferritin in 1st trimester bloods with pregnancy losses <28 weeks (n=64 live births) showed a median difference of 41.5ug/L between deciles. The lowest plasma ferritin level was seen in deciles 2 and 5 (both 17.5ug/L), while the highest was seen in decile 6 of 59ug/L. There was no statistically significant difference between the population groups from 1 to 10 decile, p=0.6329, with median values ranging from 17.50ug/L to 59.00ug/L (figure 3.36, appendix U).

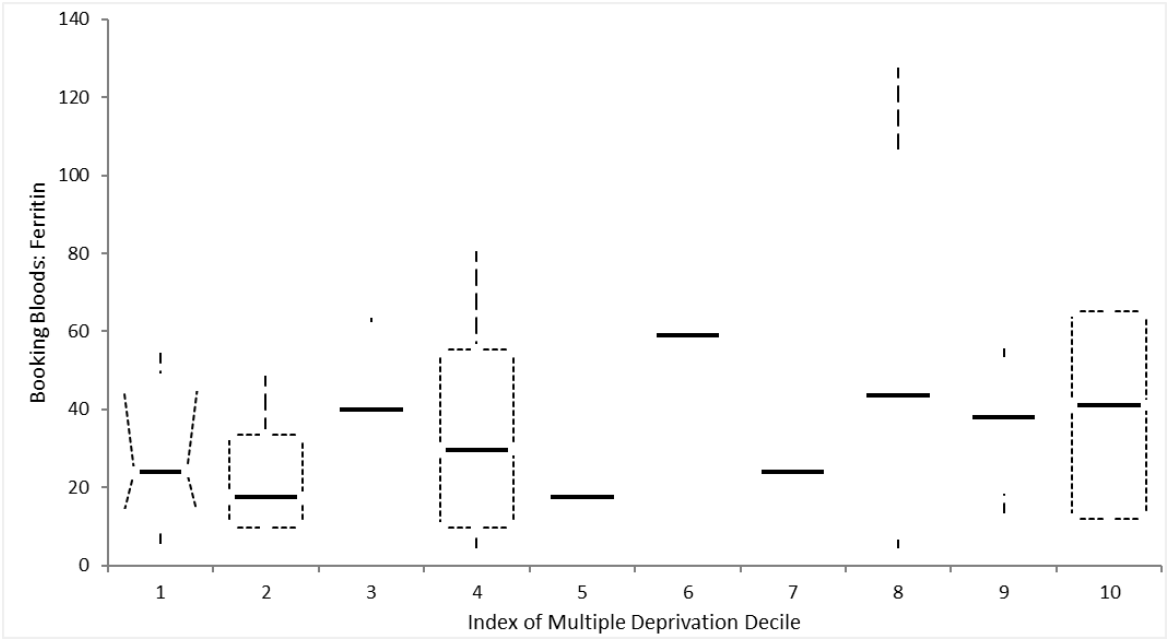


Figure 3.36. Summary of median plasma ferritin for all pregnancy losses <28 weeks across all Index of Multiple Depravation Deciles by Kruskal-Wallis, p=0.6329, accepting the null hypothesis of no difference between the deciles.

Vitamin B12 by Index of Multiple Deprivation Decile for pregnancy losses in < 28 weeks

Analysis of vitamin B12 in 1st Trimester bloods with pregnancy losses <28 weeks (n=62 live births) showed the lowest values seen in decile 6 (132.9ug/L), while the highest was seen in decile 8 (270.0ug/L). There was no statistically significant difference between the population groups from 1 to 10 decile, p=0.6734, with median values ranging from 132.90ug/L to 270.55ug/L (figure 3.37, appendix U).

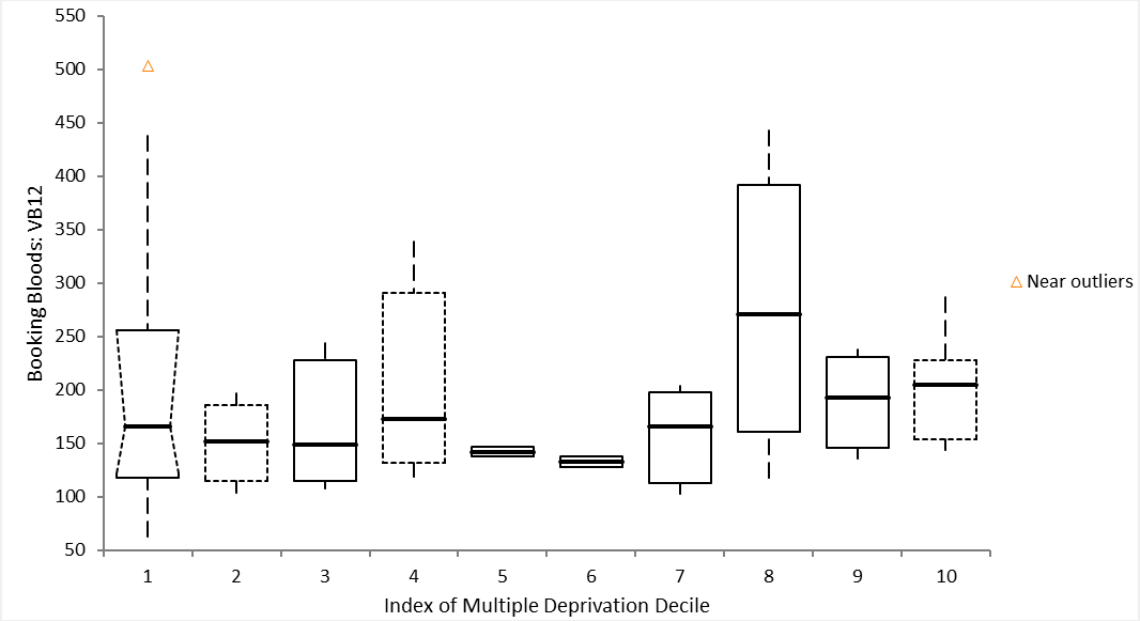


Figure 3.37. Summary of median vitamin B12 for all pregnancy losses <28 weeks across all Index of Multiple Depravation Deciles by Kruskal-Wallis, p=0.6734, accepting the null hypothesis of no difference between the deciles.

Serum folate by Index of Multiple Deprivation Decile for pregnancy losses in < 28 weeks

Analysis of serum folate in 1st trimester bloods with pregnancy losses <28 weeks (n=55 live births) showed the lowest values in decile 1 (5.49ug/L), while the highest was seen in decile 7 (11.72ug/L). There was no statistically significant difference between the population groups from 1 to 10 decile, p=0.7790, with median values ranging from 5.49ug/L to 11.72ug/L (figure 3.38, appendix U).

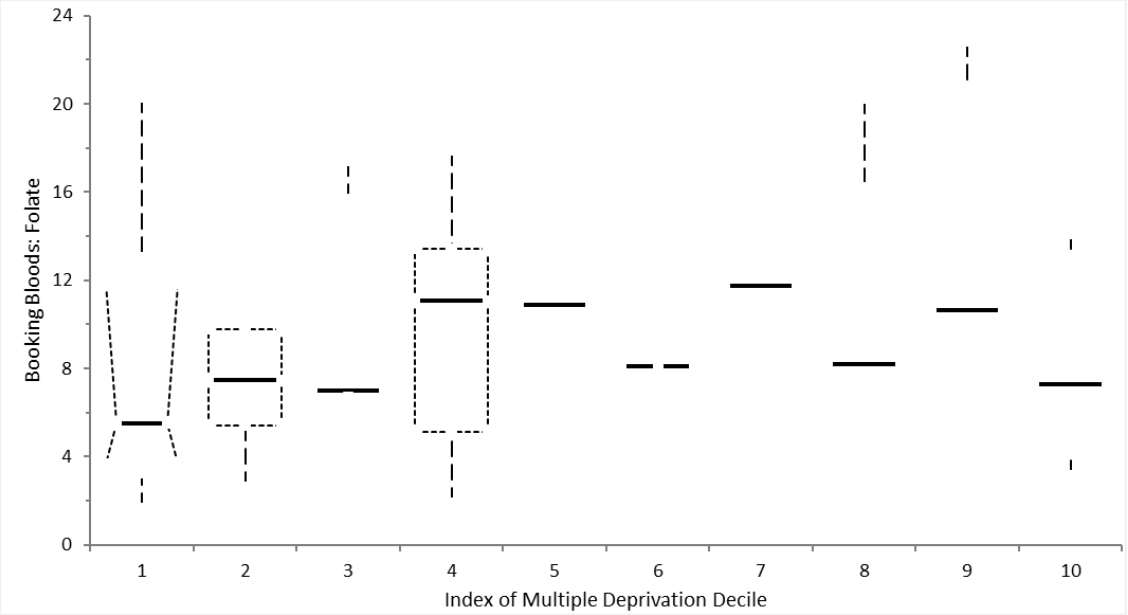


Figure 3.38. Summary of median serum folate for all pregnancy losses <28 weeks across all Index of Multiple Depravation Deciles by Kruskal-Wallis, p=0.7790, accepting the null hypothesis of no difference between the deciles.

3.18.3. Summary

In the 1st trimester, booking bloods there was no significant difference between the deciles for Hb, reticulocytes or vitamin B12. There was significant differences between the deciles for Ret-He, IRF, plasma ferritin and folate (summary table 3.23).

Table 3.23. Summary of Kruskall-Wallis Analysis for significant difference between the IMDD's (p values).

	1st Trimester	2nd Trimester	Birth Losses
Hb	0.6020	0.1109	0.4744
Reticulocytes	0.1229	0.4571	0.6126
RetHe	**0.0358**	**0.0277**	0.5588
IRF%	**0.0415**	0.1545	0.0948
Plasma Ferritin	**0.0003**	0.1881	0.6329
Serum VB12	0.2453	-	0.6734
Serum folate	**0.0047**	-	0.7790

In the 2nd trimester (28-week bloods), there was no significant differences between the deciles except for Ret-He (summary table 3.23). For pregnancy losses, there was no significant differences between the deciles.

4.0 DISCUSSION

Anaemia in pregnancy is a well-known public health issue, affecting many women. It has been attributed to nutritional deficiencies, especially in the westernised world (Bresani, et al., 2013). The WHO estimates a global prevalence of anaemia in pregnancy at around 40% (Parker, et al., 2012; Haider, et al., 2013) ranging from 5.4% in developed countries to 80% in developing countries (Sun, et al 2017). It has been well studied and reviewed by many with reports of prevalence in European countries ranging between 25% and 92% (Bencaiova, et al., 2012). In this single centre study of pregnant women within Kingston-upon-Hull and East Yorkshire region, 613 women presenting for 1st trimester booking bloods were identified. Four were excluded due to no available sample, no patient information or duplicate booking. A further 6 women were excluded due to twin pregnancies with no further analysis conducted due to the low number. A subsequent 58 women were excluded as these women presented for booking bloods after the 1st trimester (>14 weeks). This resulted in 545 1st trimester pregnancy-booking bloods for analysis. Sixty-seven of these women encountered pregnancy loss (64 <28 weeks and 3 >28 weeks) with a further 2 having a termination of pregnancy, thus there were 476 live births. These women were followed up in the 2nd trimester at 28-week bloods, which yielded 454 women with live births. Four were excluded due to pregnancy loss >28 weeks.

Of the live births 476 women, the median gestation for delivery outcomes was 39 weeks + 5 days (range 24+4 to 41+6 weeks, IQR 38+5 to 40+4 weeks). There was 32 (6.72%) women with low gestational delivery age <37 weeks and 178 (37.39%) with deliveries > 40 weeks. The global preterm birth rate is estimated to be around 11% for babies born before 37 weeks gestation with significant variations between and within countries (Walani, 2020). Lower

income countries, in particular South East Asia and sub-Saharan Africa have higher preterm birth rates than higher income countries (Chawanpaiboon, et al., 2019; Walani, 2020). In 2016 the USA had 14% preterm births in the African-American population, but only 9 % in white women. One study from India showed higher prevalence for preterm births <37 weeks of 34.75% (Kumari, et al., 2019). Another study from Pakistan also reports a high prevalence of preterm birth at 21.64% (Hanif, et al., 2020). In contrast in high-income countries, the low prevalence in the East Yorkshire population likely reflects the high white British population of the region. In contrast, there was a higher rate of late term deliveries. Although not further explored in this study, there are reports of increased risk of neonatal complications in neonates and infants with late gestational delivery (Cheng, et al., 2011b; Heslehurst, 2017). Murzakanova, et al., (2020) however, reports there being more perinatal morbidity in early term infants compared with those born later term. Further investigation is warranted in the population under investigation in this study.

For birth weight, 28 (5.88%) of women had babies with low birth weights <2500g and 68 (14.28%) >4000g. The characteristics of the women in relation to the study outcomes are shown in table 3.1. The highest proportion of LBW neonates are seen in developing countries (96.5%) (Adam, et al., 2019). In African states such as Burkina Faso, Ghana and Uganda, the prevalence of low birth weights was between 10% and 15.7%. In India, another low-income country, almost 20% of newborns have low birth weights. In contrast, in the UK the proportion of low birth weight babies in 2018 was reported as 6.9%, a decrease from 7.5% in 2000 (Nuffield Trust, 2021).

The prevalence in the East Yorkshire region for LBW was lower than the national average reported. The incidence of high birth weight babies (fetal macrosomia), those weighting

>4000g accounts for approximately 9% of babies worldwide (Mayo Clinic, 2020). Genetic risk factors have been associated with fetal macrosomia in addition to maternal conditions such as obesity and diabetes or unknown factors (Mayo Clinic, 2020). Assessment of these high birth weight babies is beyond the scope of this study although further research may be of benefit and association with measured variables such as Hb, Reticulocytes, ferritin, B12 and serum folate.

4.1 Haemoglobin, gestational delivery and birth weight outcomes

Five hundred and forty five women presented in the 1st trimester (<14 weeks) (age range 15 – 47) with the median maternal age in pregnancy being 29. Using the WHO definition of anaemia in pregnancy (Hb <110.0g/L), 14 women (2.56%) were anaemic with levels ranging from 73.0g/L to 110.0g/L. Interestingly when compared to the cut-off for anaemia in non-pregnant women (Hb <120.0g/L) this rose to 86 women (15.78%). Using this cut-off agrees with the work of Walsh, et al., (2011) who reported 13.2% of pregnant women at booking had a Hb <120.0g/L. Cut-off levels for anaemia (Hb <110.0g/L and 105.0g/L), were determined over 30 years ago and universally accepted, despite many confounding factors, including women taking iron supplementation. It is has been stated that the evidence from these dated studies is not substantial enough to determine what is normal during pregnancy (O'Brien & Ru, 2017). Barroso, et al., (2011) reported a prevalence of anaemia in the UK at some point during pregnancy of 24.4%, although the prevalence for each trimester of pregnancy was not stated. A further two-centre English study reported a higher prevalence of 46%, again at some point during the pregnancy (Nair, et al., 2017). A USA study estimated anaemia in the 1st trimester to increase from 1.8% to 8.2% in the 2nd trimester (Scholl, 2005; Goonewardene, et al., 2012), while Nair, et al., reports an increase from 5% to 22%. These latter levels reflect the prevalence in the 1st trimester seen in this study. In the 2nd trimester (~28 weeks), the present study found n=69 women (15.33%) were anaemic using the WHO cut-off for Hb <105.0g/L, ranging from 78.0g/L to 105.0g/L. When a higher cut-off <110.0g/L was used the number of anaemic women (n=146) rose to 32.44%, and when compared with non-pregnant cut-off of <120.0g/L n=310 pregnant women (68.88%) were anaemic.

It has long been reported that there is an association between low haemoglobin levels and poor birth outcomes (preterm delivery age and low birth weight). The WHO publishes cut-off levels for Hb in the three trimesters as <110.0g/L 1st trimester and <105.0g/L in the 2nd and 3rd trimesters of pregnancy gestation. While these cut-offs were established decades ago, they were defined from pooled longitudinal data from women on iron supplementation in European studies (O'Brien & Ru, 2017). There is still however, uncertainty as to how levels of Hb link to clinical outcomes.

1st trimester

In this study n=476 subjects with live birth outcomes were analysed, and showed a median Hb in the 1st trimester of 129.0g/L (93.0 – 158.0) well above the cut-off level for anaemia in pregnancy published by WHO, but in agreement with Nair, et al., (2017) who reported a median Hb of 125.0g/L. The data from this current study showed no association with either gestational delivery age (R^2 = 0.002, p=0.9621) or birth weight outcomes (R^2=0.041, p=0.3724). Thirty two women (6.72%) had a preterm delivery and none of these women had an Hb <110.0g/L. Low haemoglobins and adverse outcomes may be reported by many, especially low to middle income countries, but this was not seen in this study, suggesting that the cause of the preterm birth and low birth weight in higher income countries is not necessarily attributed to low Hb. However, Nair, et al., (2017) (an English study of haemoglobin versus stillbirths and perinatal death) reported 46% of women have anaemia (<110.0g/L) at some point during the pregnancy and those with moderate-severe anaemia (Hb<110.0g/L) were at increased risk of stillbirth or perinatal death (Odds Ratio 4.97% stillbirth and 5.16% perinatal death). Dewey & Oakes, (2017) stated a link with low Hb in early pregnancy and adverse outcomes with the risk reducing in the 2nd and 3rd trimesters, although

they comment the cut-off Hb concentrations does require re-evaluation, a point eluded to by Walsh, et al., (2011). Given the low numbers of women in this current study with Hb <110.0g/L, a higher Hb threshold is probably more useful in developed countries where levels of nutrition are improved compared with those in lower income countries. In fact, Walsh, et al., (2011) found a poor relationship between Hb and iron status (discussed later), especially in the 1st trimester booking bloods with only 4.42% (20/492) having low Hb, a percentage which is reflected in this current study (2.56% (14/545)). Walsh, et al., indicates Hb to be an insensitive marker for iron deficiency, missing over 90% of low serum ferritin values using Hb alone and observing that only 2% of women had a low Hb <110.0g/L with low serum ferritin, compared with 13.2% when a higher Hb of 120.0g/L was applied. Thus, it is perhaps not unexpected to see no correlation between Hb, low gestational delivery age and low birth weight in more affluent countries with reduced number of women with low Hb in the 1st trimester. A more recent Australian study in 2019 by Randall, et al., also saw only 4% of 31,906 women with a Hb <110.0g/L at <20 weeks gestation, again reflecting the levels seen in this study. In 29 low to middle income countries, Daru, et al., (2018) in a study of 312,281 women, saw higher rates of maternal death both ante- and postnatal with Hb <70.0g/L, reflecting the differences of Hb levels between developed and developing countries. Using univariate and multivariate logistic regression analysis in the present study, Hb showed no predictive capacity in the 1st trimester for either gestational delivery age or birth weight outcomes.

2nd trimester and difference from 1st to 2nd trimester Hb

In the 2nd trimester (~28 week), 450 women with live birth outcomes were analysed showing a decreased median Hb of 115.0g/L (78.0-142.0) when compared to that in the 1st trimester.

This again is well above the established value for anaemia published by WHO for the 2nd trimester (105.0g/L), and again agreed with Nair, et al., (2017) who reported a median of 113.0g/L. The data from the current study did not show any association with either gestational delivery age (R^2=0.036, p=0.4383) or birth weight outcomes (R^2=0.081, p=0.0867). Only n=2 (0.44%) women had a Hb <105.0g/L with gestational delivery age <37weeks and only n=1 (0.22%) woman had a low birth weight baby <2500g. Few studies could be found comparing the 2nd trimester Hb with birth outcomes (gestational delivery and low birth weight) (Dewey & Oakes, 2017; Nair, et al., 2017; Symington, et al., 2019).

Of the n=450 sets of 1st and 2nd trimester bloods available, the median difference between 1st and 2nd trimester bloods returned a Mann-Whitney-U statistical significance p=<0.0001 with a median Hb difference of 14.0g/L (10.85% decrease) between the two trimesters. Of the women with a decrease in Hb between 1st and 2nd trimester n=24 (5.33%) had a gestational delivery age <37 weeks, while those with either an increase or no difference in Hb, n=4 (0.89%), had a preterm delivery <37 weeks. For gestational delivery age there was no association with Hb (R^2=0.046, p=0.3280). However, a negative association was seen between birth weight and difference in Hb between 1st and 2nd trimester, albeit weak (R^2=-0.046, p=0.0018). There were n=22 (5.33%) women with low gestational birth weights <2500g. Delta Hb showed some predictive capacity for gestational age at delivery and was the only variable to show prognostic significance at multivariate analysis as an independent predictor. For birth weight, there was some predictive capacity at 28 weeks, but it did not show predictive capacity at multivariate level.

It would appear, from the data published by Nair, et al., (2017), that the risk of adverse outcomes due to moderate-severe anaemia reduces (still birth and perinatal death) in the 2nd

trimester from 5 fold in the 1st trimester to two and a half fold in the 2nd trimester. Again, it is questionable whether the WHO cut-off levels for anaemia in pregnancy in higher income countries are still valid or should be re-evaluated.

Expansion of plasma during pregnancy, a physiological effect, also plays a part in defining expected Hb levels (Vricella, 2017). Over the course of a normal pregnancy, plasma expands by approximately 6% in the 1st trimester, 18% to 29% in the 2nd trimester and by 42% in the 3rd trimester, which needs to be taken into account when assessing the variable measurements during pregnancy (Vricella, 2017). Further work is required to understand the association of plasma expansion and haematological variable, in addition to iron and haematinic parameters. Also of note is haemoglobin being a late marker of anaemia, especially in iron deficient subjects. When analysing Hb, it must be acknowledged that the measurement is the average of the life cycle of an un-nucleated erythrocyte, approximately 120 days, thus evaluating both old and new red cells.

4.2 Reticulocyte count, gestational delivery and birth weight outcomes

Reticulocytes, the precursors to mature red cells, circulate in peripheral blood approximately two days before maturing to erythrocytes. The data showed no reticulocytopenia in either the 1st or the 2nd trimester. In the 1st trimester of the n=545 specimens collected from pregnant women n=544 were analysed (one specimen was not tested for unknown reason). Of these, n=410 women had normal reticulocyte levels (20 $x10^9$/L to 80$x10^9$/L - reference interval based on local population) in the 1st trimester accounting for 75.37% of women, indicating normal supply of haemoglobinised erythrocytes. The remainder (24.63%) had a raised reticulocyte count, suggesting increased erythropoiesis and thus increased supply of new erythrocytes. In the 2nd trimester n=427 specimens were analysed with n=304 (71.19%) having a normal reticulocyte level (20-80 x 10^9/L) and the remainder (28.81%) having a raised count.

For the associated reticulocyte parameter, Ret-He, measuring the early haemoglobinisation of erythrocytes, in the 1st trimester there were n=539 measurements (the Ret-He for 6 women was not measured). Using a pragmatic cut-off of 29pg (Fletcher, et al., 2021), as there is no defined absolute value in either pregnant or non-pregnant women or in men, 29 women (5.69%) had a Ret-He <29pg suggesting inadequate haemoglobinisation of early reticulocytes, indicating low iron availability and possible iron deficiency. In the 2nd trimester 99 women (23.18%) had a Ret-He <29pg.

Interestingly, comparing the RetHe levels for different Hb cut-offs defined in pregnancy showed those with a Hb <110g/L (n=11 at booking) had a lower RetHe (median 24.60pg) than those with a Hb >110g/L (n=528) which gave a median value of 32.80pg. There was no median difference RetHe when analysing cut-off values 110.0 – 120.0g/L and >120g/L at booking. In

the second trimester, applying cut-off values of <105g/L, 105 – 120g/L and >120g/L showed those with Hb <105g/L (n=141) had a lower median RetHe (27.20pg) than those with an Hb>105g/L. There was a smaller median difference between those with an Hb between 105 – 120g/L and those >120g/L (31.50pg and 32.80pg respectively). This suggests RetHe to be an excellent additional marker when Hb levels are low. However, the sample numbers (n=11) for Hb <110g/L in the first trimester are low, compared with those <105g/L in the second trimester (n=141).

Another extended parameter IRF, evaluates the regeneration of erythropoiesis in the bone marrow, and like Ret-He shows response, especially to supplementation, within 24 to 48 hours. The normal universally published interval for this Sysmex FBC analyser-specific parameter is 1.6% to 10.5% (Pekelharing, et al., 2010). In the 1st trimester there were n=536 measurements (9 measurements were unavailable) showing n=406 women (75.74% (range 2.5-10.4%)) had a normal IRF <10.5%. The remainder (24.26%) had a raised IRF suggesting increased regeneration of erythrocytes, and showed similar percentage values to the reticulocyte counts in the 1st trimester. In the 2nd trimester only n=46 women (10.77%) had a IRF within the normal range (5.4 – 10.4%), the remainder (89.23%) showing increased erythropoietic regeneration.

There is little data suggesting the use of reticulocyte counts or associated parameters in pregnancy, although a few studies have eluded to its usefulness especially in monitoring the effects of supplementation (Ervasti, et al., 2007; Schoorl, et al., 2012; Kumar, et al., 2016; Bó, et al., 2021). There are no established reference intervals for the reticulocyte counts or associated parameters in pregnancy. The published intervals available are based on normal

male/female populations, as there is limited data available for more robust intervals (Fletcher, et al., 2021).

Reticulocytes along with Ret-He and IRF are newer extended red cell parameters used on modern FBC analysers such as Sysmex XN series (Sysmex Corporation, Kobe, Japan). They are able to provide useful information about early erythrocytes in addition to being more sensitive 'real-time' markers of erythropoietic activity, in contrast to measuring the Hb of mature erythrocytes, which provides information of an average 120-day life span. Abnormal reticulocyte counts are not specific to one condition, i.e. haemolysis with an increased response being seen in blood loss and treatment of iron, vitamin B12 or folate. They are however useful in measuring bone marrow response. At any one time, erythrocytes will contain approximately half of the body's iron (Ganz, 2013). There is currently limited information for Ret-He and IRF as to their usefulness in pregnancy.

1st trimester

In this study, n=475 reticulocyte counts from the 1st trimester, which were associated with live birth outcomes, were available for analysis (1 subject excluded as not tested). The data showed a median reticulocyte count of 66.8 $x10^9$/L (range 73.0-158.0). There was a weak negative association with gestational delivery outcome which was statistically significant (R^2=-0.109, p=0.0176). However no association was seen with birth weight outcome (R^2=0.012, p=0.7979). The results reflect those of the Hb in terms of women with low gestational delivery date (<37w) n= 33 (6.74%) 1st trimester and n= 28 (5.89%) for low birth weight <2500g. Using univariate and multivariate logistic regression analysis of reticulocytes showed some predictive capacity for gestational age, but not birth weight at delivery in the 1st trimester.

However, it was not shown to be an independent predictor for either gestational delivery age or birth weight.

No studies could be found to compare this data for these birth outcomes. Kumar, et al., (2016) assessed n=155 women comparing three groups in the 1st trimester, those with severe anaemia, those with borderline anaemia and those with normal blood counts. They found no difference between the three groups.

2nd trimester and difference from 1st to 2nd trimester Reticulocyte counts

No associations were found in either the 2nd trimester or the difference between 1st and 2nd trimester bloods (n=423) for either gestational delivery age or birth weight outcomes. In fact the median difference between 1st and 2nd trimester reticulocyte bloods (n=422) was (66.75 – 69.35x10^9/L) and increase of 2.6x10^9/L (0.04%), thus showing that in early and midterm pregnancy reticulocyte counts are stable. The median difference between 1st and 2nd trimester bloods returned a Mann-Whitney-U statistical significance p=0.0017 with a median reticulocyte difference of 2.60x10^9/L (0.04% increase) between the two trimesters. Again the results reflected to those of the Hb in terms of women with preterm births (<37w) n= 26 (6.41%) 1st trimester and n= 24 (5.68%) for low birth weight <2500g. Using univariate and multivariate logistic regression reticulocytes showed some predictive capacity for gestational age at delivery at 28 weeks, but was not shown to be an independent predictor. It did not show any predictive capacity for birth weight.

Reticulocytes have long been shown to be useful in assessment of haemolytic conditions where patients have increased erythrocytes due to accelerated red cell turnover, and other erythrocyte disorders such as thalassaemia. In 2011, Urrechaga, et al, assessed n=352 women

in Spain (90 normal subjects, 136 with Beta Thalassaemia Trait and 126 with severe anaemia) reporting the mean reticulocyte count in each group to be $47.8x10^9$/L normal, $77.2x10^9$/L beta thalassaemia trait, $53.4x10^9$/L mild IDA and $54.9x10^9$/L severe IDA. The data showed that those with Beta Thalassaemia Trait had increased reticulocyte counts compatible with the expansion of the erythroid to compensate for the ineffective erythropoiesis. For the mild and severe IDA group the reticulocyte counts were lower, in the normal reticulocyte range. Wagner et al., (2011) and Noronha & Grotto 2005 also reported increased reticulocytes in thalassaemia.

It would be expected in pregnancy that the reticulocyte count be mildly elevated, especially in those women taking iron supplementation. Of the n=544 women assessed in the 1st trimester, 24.63% had a raised reticulocyte count (>$80x10^9$/L, locally derived cut-off) with 28.80% being raised in the 2nd trimester. Further studies are required to assess the usefulness of reticulocyte count in pregnancy, especially responses to iron supplementation.

4.3 Reticulocyte Haemoglobin Equivalent, gestational delivery and birth weight outcomes

Ret-He, an extended reticulocyte parameter is useful for estimating the haemoglobin content of reticulocytes and iron availability in the bone marrow of new erythrocytes. In pregnancy, the use of Ret-He has not been extensively investigated and there is insufficient data to currently support its use (Kumar, et al., 2016), although other studies have recommended it use, especially in a cancer setting and pregnancy (Peerschke, et al., 2014 and Schoorl, et al., 2012), the latter study investigating its use for monitoring iron supplementation. With reticulocytes being early erythrocytes, maturing within 1-2 days, this newer Ret-He parameter is likely a more accurate marker of Hb in erythrocytes. No studies could be found evaluating Ret-He and its association with birth outcomes.

1st trimester

In this study, n=470 reticulocyte counts from the 1st trimester that were associated with live birth outcomes were available for analysis. The data showed the median Ret-He was 33.5pg (range 5.0pg – 38.4pg). There was no association of Ret-He with either gestational delivery date or birth weight outcomes. The results reflected those of the Hb and reticulocytes in terms of women with low gestational delivery date (<37w) n= 32 (6.81%) 1st trimester and n= 28 (5.96%) for low birth weight <2500g. Univariate and multivariate logistic regression showed no predictive capacity for either gestational delivery age or birth weight in the 1st trimester.

2nd trimester and difference from 1st to 2nd trimester Ret-He

At 28 weeks, there were n=417 values available with a median Ret-He of 31.7pg (24.5-38.4). There was an observed association between Ret-He and birth weight. It showed a weak positive correlation (R^2=0.149, p=0.0021) showing statistical significance. This was also reflected in the difference between 1st and 2nd trimester bloods for birth weight (R^2=-0.167, p=0.0006). No associations were shown for delivery gestation date. There was a median difference of 1.8pg between 1st and 2nd trimester bloods, with Mann Whitney U returning a statistically significance of p=<0.0001. However, clinically this difference is very low and the median values in both 1st and 2nd trimester suggests most women still retain a Ret-He above a value which would not suggest iron deficiency (>29pg). Again the results reflected to those of the Hb and reticulocytes in terms of women with low gestational delivery date (<37w) n= 26 (6.14%) 1st trimester and n= 24 (5.68%) for low birth weight <2500g. Univariate and multivariate logistic regression showed no predictive capacity for gestational delivery age at 28 weeks or in delta Ret-He between the two trimesters. It did show some predictive capacity for birth weight at 28 weeks, but not for delta Ret-He. Therefore, Ret-He can be said to be an independent predictor of birth weight at 28 weeks.

A few studies assessing Ret-He in pregnancy were found, but did not show correlation with birth outcomes. Kumar, et al., (2016) categorised 3 groups of pregnant women (n=155) in the 1st trimester of pregnancy. Using a cut-off for anaemia as 110.0g/L, category 1 (severe anaemia) showed a median Ret-He of 28.6pg, category 2 (borderline anaemia) 30.3pg and category 3 (normal) 31.7pg. Thus suggesting those with borderline or severe anaemia have a lower Ret-He than those without anaemia. This current study found similar results (26.5pg, 32.5pg and 33.2pg respectively), although the number of subjects in the severe anaemia

group was low (n=13). This is perhaps not unexpected and those with anaemia are likely to have lower Ret-He values. Kumar, et al., further assessed a reference interval suggesting an optimal cut-off in pregnancy to be 27.2pg (26.0pg – 30.9pg). Previous studies, although limited, have suggested a cut-off in the non-pregnant population with anaemia of between 25pg to 30pg, (Canals, et al., 2005; Chinudomwong, et al., 2020). This reflects the suggested limits observed by Kumar, et al., (2016) are probably more likely. A more recent Brazilian study by Bó, et al., (2021), sought to assess diagnostic performance of Ret-He and also establish a reference interval assessing pregnant and non-pregnant (male and female) participants. They found no difference in Ret-He between the 3 trimesters (median Ret-He 35.5pg, 34.9pg and 33.9pg respectively) and control group (35.0pg). This reflected similar findings to an unpublished study by Giddings, (2014) (data available at the study hospital), finding no statistical difference in Ret-He between all three trimesters of pregnancy and with the normal population, suggesting a universal cut-off in both normal subjects and in pregnancy. Ervasti, (2007) also reported a median Ret-He of 32.5pg (using CHr on Advia FBC analyser, the equivalent of Ret-He on Sysmex FBC analysers) in full-term pregnancies, agreeing with these studies in the 3rd trimester, although 80% of women in the study had some form of iron supplementation during pregnancy. Pragmatically a cut-off of 29pg for defining iron deficient individuals suggested by Fletcher, et al. (2021), albeit not specific for pregnancy, seems appropriate. Parodi, et al., 2016 found Ret-He to be a predictor of response to oral iron in children but there are limited numbers of studies in the adult population, especially in higher income countries. More studies are needed.

4.4 Immature Reticulocyte Fraction, gestational delivery and birth weight outcomes

IRF, another novel reticulocyte parameter on modern FBC analysers, can also be a useful measure of bone marrow erythropoietic regeneration, which can increase after only a few hours compared with Ret-He (2-3 days) and Hb (up to 120 days). It has been shown to be useful in monitoring patients undergoing bone marrow and stem cell transplantation. In successful transplants, 80% of cases, showed IRF has responded earlier than granulocytes (D'onofrio, 1998). Again very few studies are reported for IRF use in assessing anaemia, despite an early study, of this novel parameter, more than 2 decades ago which reported its potential usefulness (Chang & Kass, 1997).

1st trimester

In the 1st trimester of this study, n=469 IRF analyses with live birth outcomes were available for analysis. The data showed a median IRF of 7.9% (range 2.5% to 47.6%). The data showed a negative, but clinically significant association with gestational delivery days (R^2=-0.173, p=0.0002). There was no association with birth weight outcomes. The results reflected those of the Hb, reticulocytes and Ret-He in terms of women with low gestational delivery date (<37w) and low birth weight (<2500g). Univariate logistic regression analysis showed some predictive capacity for gestational age at delivery in the 1st trimester only but was not retained an independent predictor at multivariate analysis. There was no predictive capacity for birth weight.

2nd trimester and difference from 1st to 2nd trimester IRF

At 28 weeks, there were n=423 values available with a median IRF of 16.3% (5.4% to 39.0%). There was an observed association between IRF for both gestational delivery date and birth weight. For delivery gestation there was a weak negative correlation (R^2=-0.096, p=0.0481) and a positive correlation with birth weight (R^2=0.108, p=0.027), both also showing statistical significance. Difference between 1st and 2nd trimester (n=416) saw no association with birth outcome for gestational delivery date but positive association for birth weight (R2=0.172, p=0.0004) again showing statistical significance. The median difference between 1st and 2nd trimester bloods returned a Mann-Whitney-U statistical significance p=<0.0001 with a median IRF difference of 8.4% (a 48.46% increase) between the two trimesters. The results reflected those of the Hb, reticulocytes and Ret-He in terms of women with low gestational delivery date (<37w) and low birth weight (<2500g). There was no predictive capacity for IRF at 28 weeks or delta IRF between the two trimesters for gestational delivery age. However, there was predictive capacity for birth weight for both IRF at 28 weeks and delta IRF, both of which can therefore, be said to be independent predictors of birth weight outcomes.

Only one study could be found assessing IRF in pregnant individuals and two studies assessing the same in normal and anaemic subjects. Chang & Kass, (1997) compared IFR with absolute reticulocyte counts concluding that there was a weak but significant positive correlation between IRF and absolute reticulocyte counts in healthy persons (R^2=0.27, p=0.018 (simple regression)). In this study of pregnancy, using Spearman correlation, 1st trimester bloods also gave a positive, statistically significant correlation between absolute reticulocyte count and IRF (R^2=0.469, p=<0.0001). IRF ≥23% generally indicated an adequate erythroid response to anaemia but recommended further examination of the cause of the anaemia be sought

(Chang & Kass, 1997). Values <23% indicated non-responsive or unresponsive bone marrow in the presence of anaemia.

Urrechaga, et al., (2011) reported on the clinical importance of using reticulocyte related parameters to assess erythropoietic activity of the bone marrow and their use in the diagnosis of anaemia. They assessed IRF n=90 healthy male and females, n=136 Beta Thalassaemia Trait, n=121 mild IDA and n=126 severe IDA. IFR% mean results were 4.4%, 8.7%, 12.9% and 16.7% respectively showing IRF% increasing with the level of anaemia. Interestingly the IRF in beta thalassaemia subjects is only mildly raised compared with the reticulocyte count observed in the same study, perhaps reflecting the difference in the pathophysiology of the differing anaemic conditions. This could potentially be used to differentiate Beta Thalassaemia trait from iron deficiency. Iron status can influence the reticulocyte subsets with subsequent erythroid expansion resulting in premature release of immature reticulocytes from the bone marrow into the peripheral blood in an attempt to compensate for the anaemia. Urrechaga, et al., (2011) suggesting elevated IRF values of 12.9% and 16.7% reflect reducing iron stores.

Kumar, et al., (2016) also observed IRF in subjects with anaemia in pregnancy in an Indian population; the only pregnancy study that could be found. They categorised between normal, borderline and anaemic pregnant females according to Hb in the 1st trimester of pregnancy (severe anaemia <110.0g/L, borderline 110.0g/L – 120.0g/L and normal >120.0g/L). An increased IRF was observed in severe anaemia (13.5%), borderline anaemia (11.2%) and no anaemia (9.5%), while those with anaemia reflected values similar to Urrechaga, et al. (2011). Those with no anaemia had a higher IRF in pregnancy than those in the non-pregnancy study. This could be a reflection of the pregnancy status and pathophysiology of pregnancy where a

mildly raised IRF could be observed. Both Chang and Kass and Urrechaga, et al., were studies of high-income countries (USA and Spain respectively), while Kumar, et al., (2016) was low to middle-income country, thus suggesting levels of anaemia are irrelevant in assessment of IRF. Further studies of the usefulness of IFR in pregnancy are required.

4.5 Plasma ferritin, gestational delivery and birth weight outcomes

Serum ferritin, when low is a valuable marker of iron deficiency. Of the n=545 women presenting in the 1st trimester, one specimen was not tested due to insufficient sample availability. This resulted in n=544 specimens being available for analysis. Of these n=544 specimens n=296 (54.41%) of women had a plasma ferritin level ≤30.0ug/L (range 3.0 ug/L – 30.0ug/L), suggesting iron deficiency, although many of these had no signs of anaemia (discussed in a later section). The remaining n=248 (45.59%) had plasma ferritin levels between 31.0ug/L and 204.0ug/L.

In the 2nd trimester, n=400 specimens were available for analysis. Of these n=378 women (94.50%) had a plasma ferritin level ≤30.0ug/L, with the remaining n=22 women having plasma ferritin levels between 31.0ug/L and 154.0ug/L. This data suggests that almost all women in pregnancy by the 2nd trimester (28 weeks) encounter iron deficiency with an estimated median drop in plasma ferritin from 28.0ug/L to 7.0ug/L (75%).

There is an abundance of reviews and data in the literature of iron deficiency in pregnancy (Breyman, 2002; Pasricha, 2012; Api, et al., 2015; Rukuni, et al., 2015; Daru, et al., 2017a; Daru, et al., 2017b; O'Brien & Ru, 2017). Anaemia in pregnancy has been mainly attributed to nutritional iron deficiency, although in this study the data from the 1st and 2nd trimesters does not indicate the levels of anaemia reported by other studies with only 2.56% being anaemic in the 1st trimester and 15.33% in the 2nd trimester. This adds weight to ferritin being a more useful marker of iron deficiency than the haemoglobin levels currently used in assessment of women during pregnancy (Alper, et al., 2000). However, as highlighted by Ervasti, et al., (2007), a low ferritin does not necessarily reflect exhausting iron stores. It is also worth remembering that ferritin (a water soluble protein) has little or no iron content but is secreted

into plasma with its concentration correlating with levels of iron storage and is thus used as a surrogate marker (Coyne, 2006). Haemoglobin is a late marker of iron deficiency due to the 120-day red cell lifespan. Again, there is limited data reflecting the prevalence of iron deficiency without anaemia, which part of this study aims to identify.

The requirements for increased iron for both mother and developing fetus are not disputed, with diet alone most likely being unable to meet the increased need, although the underlying mechanisms in the mother and fetus still need to be established (Singhal, et al., 2018). Ferritin is also an acute phase protein, therefore in the presence of infection/inflammation may under report the true incidence of iron deficiency. Again, there is limited literature in which to establish this.

Serum and plasma ferritin levels have been well studied and are known to deplete during pregnancy. Although used as a surrogate marker of iron stores, ferritin is commonly used as a measure of iron deficiency. During pregnancy iron absorption is increased with an increase in fetal receptors for iron especially in 3rd trimester (Gambling, et al., 2011, Balesaria, et al., 2012). The effects of low or iron deficient women and the effect on birth outcomes (premature birth and low birth weights) is still relatively unknown, despite many authors reporting it to be the case (Rukuni, et al., 2015).

1st trimester

In this study, 475 plasma ferritins from the 1st trimester, associated with live birth outcomes were available for analysis. The data showed a median plasma ferritin of 28.0ug/L (range 3.0ug/L to 204.0ug/L). The data showed no association with live birth outcomes, either gestational delivery age or birth weight. The results reflected those of the Hb, reticulocytes

and Ret-He in terms of women with low gestational delivery date (<37w) and low birth weight (<2500g). Using univariate and multivariate logistic regression analysis showed no predictive capacity in the 1st trimester for either gestational delivery age or birth weight outcomes, which was surprising given the number of women with low/borderline ferritin levels. Only three studies could be found reporting prevalence of low ferritin levels in the 1st trimester (Alper, et al., 2000; Walsh, et al., 2011; and Srour, et al., 2018). The remainder examined ferritin levels in the 2nd or 3rd trimesters.

Ferritin levels used in clinical practice are the consensus of experts. Most studies have used a serum or plasma ferritin cut-off of 15µg/L or less (Alper, et al., 2000, Uberos, et al., 2000; de Azevedo Paiva, et al., 2003; Walsh, 2011; Kaestrel, et al., 2015; and Srour, et al., 2018) while a few have used higher cut-offs of 20µg/L or 30µg/L (Turgeon O'Brien, et al., 2000; Weintraub, et al., 2005; and Abdel-Malek, et al., 2018) and this most likely reflects the differences in the reported prevalence of iron deficiency using ferritin markers along with social status of each country.

In this study, using cut-offs of 12.0µg/L, 15.0µg/L and 30.0µg/L in the 1st trimester, found 17.24%, 23.48% and 54.31% respectively had low plasma ferritin levels. In the 2nd trimester, using the same cut-off values, 79.15%, 85.11% and 93.79% respectively had low plasma ferritin levels, corresponding with the overall decrease in ferritin levels between the two trimesters with the higher cut-off identifying more iron deficient women. As previously stated, the British Society for Haematology UK guideline, consensus is to use a cut-off value of 30.0µg/L, although serum ferritin is not currently recommended for screening iron deficiency but is based on the Hb alone, with only ferritin being investigated in those with very low haemoglobin levels. This would suggest the majority of women are being missed

although it had not been proved that the level of Hb (unless very low) or ferritin significantly affects birth outcomes. This study only found a statistically significant association for plasma ferritin in the 2nd trimester (p=0.0019).

No definitive cut-off for ferritin levels in pregnancy has been evaluated. However, in contrast in a randomised control trial of ferrous iron supplementation, Milman, (2006) suggests using a cut-off of 70.0µg/L with those having levels above this not requiring iron supplementation. Applying this to the numbers of women with low ferritin levels in this study, 90.82% of women in the 1st trimester and 90.57% in the 2nd trimester would all require iron supplementation. This agrees with O'Brien, et al., (2017) who commented, "to avoid adverse birth outcomes associated with maternal IDA, Canada and USA recommend universal iron supplementation for pregnant women".

Srour, et al., (2018) studied the prevalence of anaemia and birth outcomes in Palestine. In the 1st trimester of pregnancy 52% of women with depleted iron stores (ferritin ≤15.0ng/mL) were observed, with all cases of anaemia having a low ferritin. This was double the numbers observed in the current study, which showed women with plasma ferritin ≤15.0µg/L was 23% and ≤30.0µg/L was 54%. The percentage of iron deficiency (low ferritin values), varied between studies, depending on the cut-off value applied for ferritin. The current study used a cut-off value of 30µg/L as suggested by the existing national UK guidance (Pavord, et al., 2020). In the Palastinian study (Srour, et al., 2018) there were high numbers of anaemic women, which most likely will have included women with thalassaemia in addition to being conducted in a low middle-income country, although the authors report no significant difference in anaemia incidence between socioeconomic status. Interestingly, the mean birth weight for infants in Palestine was 3022g, compared with 3390g in this study (368g higher)

and gestational delivery was 38.5 vs 39+1 week, that could be accounted for given the levels of anaemia.

In contrast, in a high-income country study of n=236 pregnant women < 30 weeks gestation and using a cut-off 31.0µg/L, Abdel-Malek, et al., (2018), found no significant correlation between serum ferritin preterm or full-term deliveries or with gestational age at birth. They acknowledged their results agreed with Weinthraub, et al., (2005) although the latter study had a very low study sample size (50 pregnancies) and the authors acknowledged they were unable to reach statistical significance because of this.

Turgeon O'Brien, et al., (2000), another high-income country, found no association between ferritin and pregnancy outcomes but reported women with serum ferritin <20.0µg/L or >59.0µg/L had a shorter gestational delivery thus recommending evaluation of iron status early in pregnancy. This would agree in some way with Dewey and Oaks, (2017) who reported adverse outcomes with pregnant women who had either low or high Hb levels, the so-called U-shaped curve.

Interestingly, Uberos, et al., (2000) reported that women with a ferritin >13.0ng/mL were 4.5 times more likely to have a small for gestational age baby at 38 weeks. Using the same cut-off in the 1st trimester, this was not reflected in this present study.

2nd trimester and difference from 1st to 2nd trimester plasma ferritin

At 28 weeks, there were n=397 values available with a median plasma ferritin of 7.0µg/L (2.0 µg/L to 154.0 µg/L). There was no observed association for delivery gestation but there was a statistically significant negative association with birth weight (R^2=-0.156, p=0.0019). In contrast, the difference between 1st and 2nd trimester (n=396) (delta ferritin) saw a

statistically significant negative association for gestational delivery (R^2=-0.116, p=0.021) but no association was seen for birth weight. The median delta Ferritin returned a Mann-Whitney-U statistical significance of p=<0.0001, with a median delta ferritin of 21.0µg/L (a 75.0% decrease). The results reflected those of the Hb, and reticulocyte parameters in terms of women with low gestational delivery date (<37w) and low birth weight (<2500g). Using univariate and multivariate logistic regression showed no predictive capacity either at 28 weeks or between the two trimesters. However, at 28 weeks, ferritin showed some predictive capacity for birth weight, although it was not shown to be an independent predictor.

In an Indian study of 100 women in the 3rd trimester, 56% of women were reported to have a serum ferritin <20.0µg/mL (Sumathi, et al., 2017), although the exclusion of women with haemoglobinopathies was not highlighted given the higher incidence of these in India. Additionally, only anaemic pregnant women were selected suggesting a selection bias and making the 56% of women with low ferritin levels an underrepresentation and the numbers studied were low (100 women, 50 in each group).

4.6 Plasma ferritin with raised CRP

CRP is used as a direct measure of acute inflammatory status. In this current study, CRP was measured using plasma instead of serum due to availability. In the 1st trimester of the 545 women presenting, n=544 specimens were available for analysis. Of these, using a locally established laboratory cut-off level of 8.0mg/L, n=420 women (77.06%) had a low inflammatory status (range 0.28mg/L to 8.0mg/L). The remaining n=125 (22.94%) had a raised inflammatory marker ranging from 8.1 mg/L to 83mg/L. In the 2nd trimester, n=307 women (75.80%) had a low inflammatory status, with the remaining n=98 (24.2%) having a raised marker (range 8.1 mg/L to 603mg/L). When comparing these with women with higher ferritin levels (>30µg/L), n=63 (11.56%) in the 1st trimester, they also had raised CRP levels, suggesting the ferritin level was falsely elevated and therefore would increase the level of women with iron deficiency from 54.41% to 66.00%. In the 2nd trimester there was lower numbers of women with a raised CRP (n=5) 1.23% increasing the numbers with iron deficiency to 95.75%. One possible reason for the lower numbers of raised CRPs in 2nd trimester was the time of year the samples were collected. 1st trimester bloods were collected between September and December while the 2nd trimester bloods were collected in the spring when typical rates of infection from winter cold and flu viruses are significantly less than the autumn and winter months.

No literature could be found establishing ferritin levels in the presence of a raised inflammatory marker, especially in pregnancy. The interpretation of ferritin levels in the presence of inflammation is challenging. Formulas have been proposed (Namaste, et al., 2017) but these are not yet robust enough to be employed (Fletcher, et al., 2021) and have not been evaluated in pregnancy. Namaste, et al., (2017) in the BRNDRA project concluded

that using an internal regression correlation to estimate depleted iron stores (not in pregnancy) in the presence of inflammation, did not reflect a linear relationship between ferritin and inflammatory markers. Further research is therefore needed to validate any relationship. Other studies, not in pregnancy, found no correlation between mean ferritin and high sensitivity CRP levels (Partyka, et al., 2014).

In this current study n=64 women (11.56%) were identified in the 1st trimester as having a raised ferritin and raised CRP but in the 2nd trimester this was substantially reduced 1.23% (n=5). Indeed, an Indian study also found the prevalence of inflammation and anaemia of inflammation in pregnancy was low (Finkelstein, et al., 2020). There was no associations in the 1st trimester between raised CRP >8.0µg/L and raised ferritin with either gestational delivery (p=0.7707) or birth weight (p=0.3343). The exceptionally small numbers 1.24% (n=5) seen in the 2nd trimester were too small to test for any significant association. Further research is required with or without pregnancy. Using univariate and multivariate logistic regression analysis showed no predictive capacity.

4.7 Vitamin B12, folate, gestational delivery and birth weight outcomes

Vitamin B12, along with folate is an important constituent in the production of DNA synthesis and embryogenesis. Vitamin B12 is essential for red cell formation. Although less studied, during pregnancy, like iron, requirements for vitamin B12 increase with measured levels declining as the plasma expands until term of pregnancy when it is shunted to the placenta (Goonewardene, et al., 2012). Of the n=545 women presenting for 1st trimester booking bloods only n=517 specimens were available for analysis. The exclusion of n=28 specimens was due to either no sample being available or insufficient serum for analysis. It was not possible to collect any samples for analysis in the 2nd trimester, as women do not routinely provide a serum sample at the 2nd trimester 28-week bloods (a full blood count only is usually requested). From the specimens analysed n=101 (19.53%) showed a low vitamin B12 level below the locally established cut-off by method (115.0pmol/L). In 2012, Shields, et al., reported 16% of women in 1st trimester had a low vitamin B12 level, agreeing with the findings of this present study, although the number of women studied was much smaller (n=113). Higher levels (60%) were reported in the 3rd trimester in the Shield, et al., study but again this was a small group (n=77 women). As this data was not collected during this existing study, agreement cannot be confirmed.

Due to importance in embryogenesis and for DNA synthesis, deficiencies in vitamin B12 can result in congenital abnormalities. Due to the co-existing biochemical mechanisms of vitamin B12 and folate, supplementation with folate can mask the measurement and effects of vitamin B12 (Goonewardene, et al., 2012). During pregnancy however, folate supplementation is recommended. There is very limited literature available in respect of vitamin B12 and pregnancy outcomes of low birth weight or gestational delivery. There are

reports of vitamin B12 deficiency being rare in pregnancy (Goonewardene, et al., 2012). However, Shields, et al., 2011 found 34% of pregnant women had co-existing deficiency with folate and iron. It is likely that when combined with iron deficiency and folate (probably due to poor dietary intake, especially in those with vegan diets) would lead to poor outcomes in pregnancy. This was highlighted in an African-American study in 1991 by Knight, et al., although there has been no further studies to back up this data. In pregnancy, vitamin B12 levels decline due to maternal plasma expansion but in 2018, Kaur, et al., (2018) reported an association of low vitamin B12 with recurrent pregnancy loss concluding all women in pregnancy regardless of anaemic status should be screened though the women in this study were not pregnant at the time.

1st trimester only

In this study n=451 vitamin B12 levels from the 1st trimester, associated with live birth outcomes were available for analysis. The data showed a median vitamin B12 of 115.30pmol/L (range 28.7pmol/L to >1106.7pmol/L). Despite n=31 women (6.87%) having both a low birth weight and early gestational delivery age, there was no association overall with live birth outcomes, either gestational delivery age or birth weight. The numbers of women with low gestational delivery date (<37w) and low birth weight (<2500g) were marginally higher than that of Hb, reticulocytes and Ret-He but very low numbers of these women had vitamin B12 levels <115.30pmol/L (n=5, 1.11%). Using Kruskal-Wallis (ANOVA) for the difference between gestational delivery age (<37 weeks, 37 – 41 weeks and >41 weeks) and vitamin B12 showed no statistical significant difference in mean concentration levels (p=0.2983). The same observation was seen for birth weight (<2500g, 2500 – 3999g and > 4000g) with Kruskal-Wallis (ANOVA) p=0.1616. Univariate logistic regression analysis showed

some predictive capacity for gestational age at delivery in the 1st trimester only but was not retained as an independent predictor at multivariate level. There was no predictive capacity in terms of birth weight outcome. A Taiwanese study back in 1987 only reported n=3/221 (1.36%) full term pregnancies as having vitamin B12 deficiency, with the same number having folate deficiency. This value seems low, especially when reports expect this to be at a higher frequency at term due to vitamin B12 being shunted to the placenta (Goonewardene, et al., 2012).

Kalem, et al., (2016) (a retrospective study) measured 3rd trimester outcomes associated with vitamin B12, folate and iron. All women in this study were questioned as to whether they were taking additional supplementation. The study found no statistically significant difference in the mean birth weight and concluded there was no effect in the birth outcomes. What should be noted is the small sample group (n=72) which was acknowledged by the authors. Conversely, the authors of a n=466 sample study of women referred for thalassaemia screening, concluded vitamin B12 and iron were the main depleted micronutrients (Karabulut, et al., 2015). Thalassaemia trait was reported in 3% of the group presenting with anaemia (24.9%) with 21.6% having a low vitamin B12 based on local cut-off levels. The increased number presenting with vitamin B12 can be attributed to the nutritional deficiency seen in this population, mainly a plant-based Mediterranean diet in a middle-high income country. This may also be reflective of the high numbers of women seen with iron deficiency (serum ferritin <12.0µg/L, 46.1%) a higher percentage than was seen in the current study (17.24%) using the same cut-off value.

Measurement of serum vitamin B12 levels is complicated due to the differences between analyte measurement and analyser. These differences have been reported in the literature

(Devalia, et al., 2014). This may account for the lower levels seen in the current study population, although local cut-off levels have been previously evaluated using local population data. The difficulty in measuring any changes during pregnancy may be further complicated by maternal and placental changes that can affect circulating micronutrient concentrations and their functional biomarkers (Wheeler, 2008).

4.8 Serum folate, gestational delivery and birth weight outcomes

As for vitamin B12, folate also has the same importance for embryogenesis and DNA synthesis. Both micronutrients are highly dependent on each other for rate limiting biochemical reactions. Like the other haematinics, folate requirements increase during pregnancy due to increased cell turnover and low body stores. Both vitamin B12 and folate share the same signs and symptoms with increased requirements during pregnancy. In contrast to vitamin B12, the body store of folate in normal healthy individuals is approximately 4-5 months. As such, folate supplementation is required during pregnancy, due to increased requirements, especially in the latter months of term (Kalem, et al., 2016).

Of the n=545 women identified for 1st trimester booking bloods only n=469 specimens were available for analysis as n=76 were excluded due to either no sample or insufficient sample available. Using the local laboratory cut-off for serum folate of 3.0ug/L, n=38 (8.10%) specimens showed low levels suggesting folate deficiency. As for vitamin B12, no serum samples were available in the 2nd trimester for serum folate analysis. Kalem, et al., (2016) reports folate levels are not likely to be low in the 1st and 2nd trimesters due to folate store availability based on data from a Cochrane Review of folic acid supplementation during pregnancy (Lassi, et al., 2013). However, Kalem, et al., (2016) only evaluated n=72 pregnant women in the 3rd trimester of pregnancy and only 1 woman was determined as having folate deficiency. This could be due to the awareness among pregnant women for the need of folate supplementation and associated neurological defects that may be encountered. However, Siega-Riz, et al., (2004) did demonstrated low folate levels in the 2nd trimester of pregnancy. As this data was not collected during this study in the 2nd trimester, agreement cannot be confirmed.

1st trimester only

In this study, n=461 folate levels from the 1st trimester, associated with live birth outcomes were available for analysis. The data showed a median serum folate of 10.71µg/L (range 1.15 µg/L to >23.6µg/L). Only n=32 women (6.93%) had a low birth weight and n=28 (6.07%) had an early gestational delivery age. There was no association, or statistical significance overall with either gestational delivery age or birth weight (p=0.2778 and p=0.3695 respectively). The numbers of women with low gestational delivery date (<37w) and low birth weight (<2500g) were lower any of the other variables tested. Gestational delivery age was 0.21% and low birth weight was 0.43%. Using Kruskal-Wallis (ANOVA) for the difference between gestational delivery age (<37 weeks, 37 – 41 weeks and >41 weeks) and serum folate showed no statistical significant difference in mean concentration levels (p=0.06997). The same observation was seen for birth weight (<2500g, 2500 – 3999g and > 4000g) with Kruskal-Wallis (ANOVA) p=0.7160. Using univariate and multivariate logistic regression analysis folate showed no predictive capacity for either gestational delivery age or birth weight outcomes.

While there is significantly more literature available investigating folate levels during pregnancy, surprisingly there is little describing the association with gestational delivery age or birth weight. Ho, et al., (1987) reported a very low number of women with folate deficiency associated with anaemia. Karabulut, et al., (2014) also saw low numbers of women with folate deficiency, from a sample population of n=466 in 1st trimester pregnant women. Using a cut-off 4.0µg/mL the authors found only 3.4% were deficient. This was lower than the numbers seen in the current study, which saw 6.72% of women with folate deficiency, with or without anaemia, using a cut-off 3.0µg/L.

Folate supplementation is well supported both pre pregnancy and during pregnancy. The links with birth defects such as neural tube abnormalities are well known. The importance of supplementation communicated to pregnant women from the outset of pregnancy by midwives and healthcare professionals most likely accounts for the low numbers of folate deficient women seen, especially in middle-high income countries.

The Cochrane Review of data in 2013 documented folate supplementation during pregnancy, compared with a placebo, but showed no association with reducing the chances of pre-term births, still births, neonatal deaths, low birth weight babies, or pre-delivery anaemia (Lassi, et al., 2013). They did report however, pre-delivery serum folate levels were improved. This would agree with the data from this study in the 1st trimester, although 2nd trimester and term delivery bloods were not available for analysis, as these are not routinely taken in clinical practice, based on current UK guidelines.

In contrast, Siega-Riz, et al., (2004), did report an association of folate deficiency with preterm birth in a large study of serum and red cell folate (total n=3,060). They found a low dietary intake ≤500.0µg was associated with increase preterm delivery. However, thresholds for defining deficiency were much higher than those in other studies (serum folate <16.3ng/mL and red cell folate ≤626.6ng/mL) and other micronutrient and iron status was not measured. The authors acknowledge numerous limitations within the study, including selection bias and supplementation compliance.

4.9 Iron deficiency without anaemia (IDWA)

Literature reporting iron deficiency in the absence of anaemia is limited, both in general population and in pregnancy. There are many reviews but very little primary data (Clénin, 2017, Juul, et al., 2019; Castelo-Branco and Quintas, 2020; Georgieff, 2020; and Al-Naseem, et al., 2021). This is surprising given Al-Naseem, et al., (2021) reports that IDWA is twice as common in the general population. In fact, only one study could be found assessing non-anaemic pregnant women (Ribot, et al., 2012). Their study reports iron deficiency in 20% of women in the 1st trimester, 54% in the 2nd trimester and 66% in the 3rd trimester, using a serum ferritin cut-off <12.0µg/L and the WHO defined cut-off for Hb in pregnancy.

1st trimester

In this study, n=249 (52.42%) women with live birth outcome were found to have IDWA using a ferritin cut-off <30.0µg/L and Hb>110.0g/L in the 1st trimester. The data showed a median plasma ferritin level of 18.0µg/L (range 3.0µg/L to 30.0µg/L) and median Hb of 128.0g/L (range 112.0g/L to 147.0g/L). Fifteen women (3.15%) had low gestational delivery date and n=14 (2.94%) had a baby with low birth weight. There was no statistically significant association for plasma ferritin in respect of gestational delivery date, but there was a significant positive association for birth weight (p=0.173).

In the 2nd trimester, n=312 (69.33%) of women had IDWA using ferritin cut-off 30.0µg/L and Hb >105.0g/L. The data showed a median plasma ferritin level of 7.0ug/L (range 2.0µg/L to 29.0µg/L) and median Hb of 116.0g/L (range 106.0g/L to 142.0g/L). Nineteen women (4.22%) had low gestational delivery date and n=18 (4.00%) had a baby with low birth weight. There

was no statistically significant association for plasma ferritin in respect of gestational delivery date or birth weight.

Between the 1st and 2nd trimester bloods there was a median decrease in Hb levels of 12.0g/L (9.37%) with Mann-Whitney-U statistic returning a statistically significant p value of <0.0001. The difference between 1st and 2nd trimester plasma ferritin levels was 11.0µg/L (61.11%) which also returned a statistically significant Mann-Whitney-U statistic of p=<0.0001. The results showed that either with or without anaemia, plasma ferritin dropped significantly between 1st and 2nd trimester bloods.

With such little research of IDWA in both the general population and in pregnancy, it is difficult to assess comparisons between studies. Most research has been conducted on women with anaemia, but as the results of this study show, ferritin drops significantly between 1st and 2nd trimesters but does not appear to have a statistically significant effect, with the exception of birth weight in the 1st trimester.

Subjects with iron deficiency may still present with symptoms associated with low iron levels which is an important consideration when assessing patients, however many go undiagnosed for long periods of time (Soppi, 2018). Relying solely on haemoglobin level alone misses a substantial number of diagnoses, such as is highlighted in this study, from the number of women presenting without anaemia but low ferritin and developing iron deficiency. Indeed, plasma volume expansion may also be a contributing cause as well as low levels due to the physiological process. Defining more appropriate cut-off levels for ferritin in pregnancy are required as use of general limits may indicate iron replacement is needed, although some studies have shown that raising the iron level and in turn, the haemoglobin may also contribute to adverse outcomes (Dewey & Oaks, 2017). Consideration should also be given to

looking at the whole picture rather than being reliant on one single test. Most women in pregnancy are not investigated for iron deficiency unless they are anaemic and even then, reliance is on the assumption that low Hb levels are indicative of IDA (Juul, et al., 2019). With Hb being a late marker of iron deficiency there is missed opportunities for earlier intervention before onset of anaemia.

4.10 Live birth compared with pregnancy loss <28 weeks

Of the n=545 pregnant women in the study, n=69 (12.66%) had a pregnancy loss with n=64 (11.60%) having a pregnancy loss before 28 weeks, n=3 (0.7%) after 28 weeks and n=2 (0.4%) termination of pregnancy. As there were only n=3 pregnancy losses >28 weeks, this was too low for analysis. Only n=3 (4.34%) women with a pregnancy loss had anaemia (Hb<110g/L), with n=14 (20.28%) having a Hb level <120.0g/L.

Using Mann-Whitney-U statistic for those <28 weeks, showed the best predictors of pregnancy loss to be Ret-He, IRF, and vitamin B12, (p=0.0077, p=<0.0001 and p=0.0378 respectively). Of the three variables, IRF was the most significant. There was no statistical significance for Hb, reticulocyte count, plasma ferritin or folate, thereby being poor predictors of birth outcome. Clinically Ret-He and vitamin B12 would need further investigation as the difference between the absolute concentration values for live births and pregnancy losses was low. For Ret-He the median loss level was 32.8pg whereas for live births was 33.5pg. For vitamin B12 pregnancy losses showed a higher value 164.6pmol/L compared to live births 151.3pmol/L, clinically indicating most women had adequate vitamin B12 levels. Ret-He, Retics at 28 weeks, delta Hb, delta Reticulocytes, delta Ret-He, delta IRF all show some predictive capacity for live birth outcome, but only Reticulocytes at 28 weeks can be said to be an independent predictor of live birth.

Haemoglobin

The data showed no association between pregnancy loss and Hb (table 3.11). This did not agree with any other published data, although there is limited data available investigating pregnancy loss <28 weeks and correlation with Hb levels. Interestingly, a number of studies

report adverse outcomes in pregnancy women with either low Hb or high Hb (Scholl & Reilly, 2000; Randall, et al., 2019; and Díaz-López, et al., 2021). Tomashek, et al., 2006 on the other hand did not find such an association with those women with mild anaemia or high Hb in either 1st or 2nd trimester of pregnancy. In the limited literature available, adverse outcomes was seen in those with moderate or severe anaemia when tested preconception. (Ronnenberg, et al., 2004). Díaz-López,, et al., 2021 report the same U-shaped association with Hb as Dewey and Oaks, (2017). Unfortunately, the data from this study only showed low numbers of women in the 1st trimester with moderate or severe anaemia and pregnancy loss (n=3 <110.0g/L and n=14 <120.0g/L) too low to assess any statistical significance. Similarly, there were no pregnancy losses in this cohort with high Hb's. The highest Hb seen was 150.0g/L.

Indeed, Nair, et al., (2017) saw more pregnancy losses in their UK study compared to this present study, but there were variations in the populations studied. In particular, Nair, et al., had a higher proportion of ethnic minority groups (Wolverhampton 64.5% and London 44.9% white British) which may likely account for the difference with Hull East Yorkshire groups (93% white British).

Reticulocytes, Ret-He, IRF

No significant association was seen between pregnancy loss and reticulocyte count, however, the newer extended parameters showed statistical significance for Ret-He and IRF. IRF showed the most significance (p=0.0001). For those women with pregnancy loss (n=64) the median IRF level was 7.40% which was more than half the value of those with live births (n=475 (16.3%)), however there was overlap of the 5%, 95% values (live 9.20 – 28.35% verses Loss 3.05 – 14.42%). It is possible the low IRF in those women experiencing spontaneous

pregnancy loss is because the pregnancy is not viable, although it was difficult to determine in the study, as the exact time of pregnancy loss was not recorded due to most women failing to return for assessment after booking bloods. This suggests, in these women, a reduced activity of the bone marrow erythroid and thus restriction of iron does not occur. Unfortunately, no reported studies could be found documenting reticulocytes and associated parameters in pregnancy with adverse outcomes. Given the significance of Ret-He and IRF seen during this study, these extended measurements warrant further investigation. Given that they indicate not only the supply of iron to the erythroid, but also reflect bone marrow activity of erythrocytes, their practicality could be useful in both pre and during pregnancy as predictors of outcome, especially risk of pregnancy loss. In addition, they may also be an excellent replacement or additional marker for iron deficiency as comparison of these parameters with plasma ferritin showed not only statistical significance but also a strong positive correlation with Ret-He and IRF (Tables 3.20 and 3.21).

Plasma ferritin

No association was seen between pregnancy loss and plasma ferritin. This was surprising given the high numbers of women with plasma ferritin levels <30.0µg/L in the 1st trimester and significant drop in the 2nd trimester <28 weeks (see section 3.14). Again, no studies could be found reporting the association of low ferritin levels in the 1st trimester and pregnancy loss. Several studies reported findings of low ferritin levels and adverse birth outcomes (preterm birth and low birth weight) and as reported in section 3.14 findings (Alwan, et al., 2015; Finkelstein, et al., 2019; and Iqbal, et al., 2019). On the contrary, Goldenberg, et al., (1996) found high plasma ferritin levels had a strong association with pre term delivery and birth weight, especially at 28 weeks but did not report on pregnancy losses.

Serum Vitamin B12 & serum folate

Interestingly, a positive association was found between pregnancy loss and serum vitamin B12 (p=0.0378) despite low numbers of women having vitamin B12 deficiency. Of the n=64 women with pregnancy loss in this study n=8 (12.5%) women had a vitamin B12 level <115.0pmol/L (local cut-off) compared with n=92 (20.39%) women with no pregnancy loss. A few reports were found investigating vitamin B12 and adverse outcomes including pregnancy loss but no associations were reported (Liu, et al., 2020; de Weerd, et al., 2003). Equally, Neumann, et al., (2013) concluded vitamin B12 deficiency may adversely affect pregnancy outcomes (intrauterine growth restriction and stillbirth) although the sample population for the study was low (n=138). In addition, this was a Kenyan study where higher levels of vitamin B12 deficiency would be likely due to diet, which largely consists of rice, pasta, lentils, porridge and vegetables.

Folate showed no association with pregnancy loss, most likely for reason previously stated. Again, little data could be found examining folate levels and pregnancy loss. Gaskins, et al., (2014) showed in a sample population of n=15,950 pregnancies with n=2,756 (17.3%) spontaneous abortions and n=120 (0.8%) stillbirths, therefore the higher the intake of folate the lower the risk of adverse outcome. Given the focus that is given to folate deficiency (and vitamin B12 deficiency) and its effect on embryogenesis and DNA synthesis, it was surprising not to find more studies investigating associations with pregnancy loss. Most studies concentrated on either pre-term delivery or birth weights.

There are low numbers of studies investigating laboratory parameters with the three birth outcomes assessed during this study, in addition limited or no studies of reticulocytes and associated extended parameter were found. This study showed Ret-He and IRF to be potential

future diagnostic parameters with or without the need to assess serum or plasma ferritin. Hb, reticulocytes, Ret-He and IRF showed significant correlation with plasma ferritin. This would mean these indices could be performed using the same sample as for the FBC at booking and at 28 weeks. For the delta Hb and delta reticulocytes, delta Ret-He and delta IRF cut-off levels between the two trimesters need to be established and could be used as predictive indicators during pregnancy for either supplementation or further investigations.

Comparison of plasma ferritin with reticulocytes and associated variables

With reticulocyte variables showing association and predictive capacity for birth outcomes, particularly birth weight and pregnancy loss, plasma ferritin was correlated with Hb, reticulocytes, Ret-He and IRF. All variables showed good correlation and statistical significance with only reticulocytes verses plasma ferritin not showing any statistical significance (tables 3.19 and 3.20).

It would not be surprising for Hb to be correlated with ferritin with this being a marker of iron stores, so concurrently the Hb will fall due to low supply albeit with a delay. No studies could be found evaluating this during pregnancy, although there were a few studies found examining the relationship in paediatrics (Davidkova, et al., 2016; Di Pinto, et al., 2020). Davidkova, et al., found a negative correlation between Hb and ferritin (R^2=-0.14, p=0.002), with Di Pinto, et al., reporting the same (R^2=-0.19, p=0.002). In pregnancy, this study found a positive correlation in 1st and 2nd trimesters (R^2=0.241, p=<0.0001 and R^2=0.426, p=<0.0001 respectively) with those women with pregnancy losses also showing positive correlation (R^2=0.298, p=00167).

For reticulocytes, no studies were found assessing the correlation of reticulocytes with ferritin. In fact, no literature could be identified in non-pregnant groups assessing the same.

The current study found a positive association in both 1st and 2nd trimester (R^2=0.170, p=<0.0001 and R^2=0.275, p=<0.0001 respectively). Interestingly, those women with pregnancy loss showed a weak association and weak statistical significance (R^2=0.141, p=0.2655). The highest association was seen in the 28 weeks bloods.

More studies were found for the association of Ret-He and its comparable marker CHr (Bayer Advia FBC analyser) with ferritin (Davidkova, et al., 2016, Mehta, et al., 2016, Di Pinto, et al., 2020, Ogawa, et al., 2020, Sany, et al., 2020, A summary of their findings can be seen in Table 4.1.

Table 4.1 Summary of studies examining correlation of Ret-He or CHr with ferritin.

Study	Study population	Study number	R-squared	Probability (p)
Davidkova, et al., 2016	Paediatric on haemodiaylsis	606 observations	0.09	0.04
Mehta, et al., 2016	Bone marrow (adults)	142	0.7860	0.0000
Di Pinto, et al., 2020	Paediatric on haemodialysis	40 children 164 observations	0.35	<0.0001
Ogawa, et al., 2020	Haemodialysis patients on erythropoietin	181	0.47 (SF<50ng/mL) 0.22 (SF>50ng/ml)	Not stated
Sany, et al., 2020	Haemodialysis patients	50	Not stated	<0.0001
Neef, et al., 2021	ID & IDA in children	970	0.41 (SF<100ng/mL)	<0.0001
Suria, et al., 2021	Blood donors 1st time & regular donors	410 (205 each group)	0.756 (group 1) 0.707 (group 2)	Not stated

SF = Serum ferritin

No literature could be found examining the correlation of Ret-He with ferritin in pregnancy, however a positive correlation was seen in both 1st and 2nd trimesters (R^2= 0.292, p=<0.0001 and R^2=0.561, p=<0.0001). A positive correlation was also seen in those women with pregnancy loss (R^2=0.406, p=0.0010). The strongest correlations were seen at 28 weeks and in the pregnancy loss groups.

Finally, for the IRF variable; again no studies could be found exploring this variable in pregnancy with ferritin, or in any other groups except Suria, et al., (2021). Suria, et al., studied n=410 (205 each group) of first time and regular donors finding IRF negatively correlates with ferritin (-0.563 (group 1) and -0.576 (group 2)). During pregnancy, in 1st and 2nd trimesters the current study data showed negative correlation between IRF and ferritin (R^2= -0.157, p=0.0003 and R^2=-0.304, p=<0.0001). A significant negative correlation was also seen in the pregnancy loss group (R^2= -0.302, p=0.0172).

The data from this analysis suggests the reticulocyte counts and associated extended parameters (Ret-He and IRF) could be used to suggest iron deficiency in pregnancy and indeed adds weight to them being used as predictors of outcome, although further research is needed in terms of cut-off levels but not in pregnancy (Neef, et al., 2021). Bó, et al., (2021) in contrast, investigated pregnant women suggesting a normal reference range for Ret-He of 29.75pg to 38.42pg with a median of 35.0pg. They concluded Ret-He to be an excellent auxiliary tool for assessing iron deficiency in pregnancy.

4.11 Comparison of Index of Multiple Depravation Decile (IMDD) and live births, 1st trimester (booking), 2nd trimester (28 weeks), and pregnancy losses <28 weeks

Index of multiple depravation decile (IMDD) is widely used to officially measure assessing relative depravation in distinct geographical regions in England. The index is generated from a series of indicators measuring a range of socially derived measures including health depravation and disability. The deciles are graded 1 to 10, 10 being the least deprived and 1 being the most deprived. Kingston upon Hull is known to be one of the most deprived cities in the UK ranking 9th in 2019. In contrast, the East Riding of Yorkshire has a more varied distribution. The geographical distribution of the pregnant women within the study in each decile is detailed in appendix R. As can be seen, there is a wide geographical distribution of pregnant women in the region, although the majority of births are those in the lowest deciles, which centre mostly around the city. Women in the higher deciles are seen in the more rural East Riding.

More pregnancy losses <28 weeks were seen in those in the lower deciles <5 with 37.5% of these being in the least deprived group (decile1). A summary of the numbers of live births and losses by decile is detailed in table 3.22. This data agrees with that of Opondo, et al., (2019), an English and Welsh study of 4.6 million singleton births assessing outcome measures, which included neonatal death, infant death, and preterm birth. The study assessed different ethnic groups showing those in the most deprived areas had a 47% to 129% greater risk of those in the most deprived areas having adverse birth outcomes. Indeed, those in the ethnic minority groups showed a greater risk (48% to 138%) compared with white British birth outcomes. Smith, et al., (2011), also an English study (East Midlands and South Yorkshire) reported on the risk of congenital abnormalities in babies and whether these were

associated with depravation. They found there was a significant socioeconomic inequality between the rates of neonatal mortality compared with the rate of live births for nine of the abnormalities they studied; concluding those in the least deprived areas had a 61% higher rate of live births. In contrast, Hirst, et al., (2019) (National Health Service for England Hospital Episode Statistic) reported on small for gestational age (SGA) births finding those in the most deprived areas were twice as likely to have a SGA baby compared to those in least deprived areas. Another study found associations between risk of preterm delivery and small for gestational age births in the most deprived groups (Beyerlein, et al., 2020).

In this current single centre study, covering Kingston-upon-Hull and the East Riding of Yorkshire, variables of Hb, reticulocytes and extended parameters (Ret-He and IRF), plasma ferritin, vitamin B12 and serum folate were investigated against the IMDD for women during 1st and 2nd trimesters of pregnancy. A review of the literature did not identify any study assessing these variables against IMDD. Using the Kruskal-Wallis statistic, all variables were assessed across all deciles for differences levels for live births and pregnancy losses. For Hb no significant difference was observed between the deciles in either the 1st or 2nd trimesters returning a p value of 0.6020 and 0.1109 respectively. Similarly, there was also no significant difference in those with pregnancy losses.

For reticulocytes, no statistically significant differences were seen in either 1st or 2nd trimester or for those with pregnancy losses. Interestingly, Ret-He did show statistical significance in both 1st and 2nd trimesters, but not for pregnancy losses. IRF showed statistical significance in the 1st trimester only. This is a novel finding that the newer extended variables of reticulocyte counts could be used as a predictor of birth outcomes in socioeconomic distributions although

exact cut-off levels would need to be established. These variables could potentially be used as a screening measure to identify women most at risk.

Plasma ferritin also showed a statistically significant difference for those in the 1st trimester but not in the 2nd trimester or for pregnancy loss. This suggests the use of ferritin levels in the 1st trimester could potentially be used to identify those women who are most at risk in the lower IMDDs.

Vitamin B12 measures showed no statistical significance but serum folate did show statistical significance in the 1st trimester. Again, this could potentially be used as a screening measure to identify those women most at risk of folate deficiency. This is extremely important and could help in further reducing the risk of adverse outcomes such as neural tube defects. Only one study could be found examining folate intake and socioeconomic depravation (Campbell, et al., 2013). In this Scottish study, reporting on 1536 pregnant women with epilepsy, there tended to be a lower rate of education and employment (assessed as part of the IMDD), but the authors found no difference across all decile groups with 4.7% of births having a major congenital malformation. Those in the higher decile groups were more likely to take folic acid supplementation 56.8% compared with 14% in the lower deciles and therefore less likely to have seizures during pregnancy. The authors conclude that improving folic acid supplementation during pregnancy improves healthcare delivery for women with epilepsy.

Of interest, using multivariate analysis (tables 3.16 and 3.17) IMDD was shown to be borderline at univariate analysis for predictors of birth weight, but was shown to be an independent predictor of birth weight at multivariate level. Although this was shown to be an independent predictor, many other factors are likely to be involved contributing to birth

weights such as obesity, smoking, and some of the other measures, which make up the IMDD. Further investigations are needed to assess other factors.

4.12 Limitations of the study

There was a number of limitations to the study. From a sample and collection testing aspect, specimens were collected from the excess biological material collected after all clinical testing was complete. As only excess material was collected, on occasions there was insufficient sample remaining to perform all additional testing. Where the plasma samples were used, all full blood counts were performed, although testing for plasma ferritin and/or CRP may not have been carried out. This did reduce the numbers of full sets of data being available (see figure 3.1). Additionally, although there was an LIMS algorithm in place to add reticulocytes to the full blood count, there were instances where these were not actioned. This however, only affected a relatively small number of samples. As the EDTA samples were collected after all routine full blood counts and haemoglobinopathy screening was performed, it was often difficult to be accurate as to the time the samples was collected i.e within 24 hours. All steps to minimise sample deterioration were taken, which included storing the identified samples at 2^{o}C to 8^{o}C for optimum preservation. A samples stability study was performed to ensure optimum sample retrieval times and least deterioration of sample variables under study (section 3.1, appendix E).

Residual serum samples were collected after all serology testing from 1st trimester booking bloods had been performed. These samples were tested and stored within the Virology department. As the Virology department was situated at an alternative site within the Trust, samples could only be collected once weekly. As such control of storage and collection was not optimum, although they were stored routinely at 2^{o}C to 8^{o}C for two weeks. Similarly, a number of sample had insufficient volumes and could not be retrieved. All steps to minimise

the effects of sample deterioration were taken and a sample stability study was also undertaken (section 3.1, appendix E).

As the study focused on laboratory screening and testing of specimens of all women presenting for 1st trimester booking bloods (excluding those with a haemoglobinopathy), confounding factors such as smoking, diabetes, weight, pre-existing conditions etc were not accounted for. Confounding factors affecting gestational delivery dates and birth weights (Basso, et al., 2006; Abraham, et al., 2017; Luecke, et al., 2019; Santos-Antonio, et al., 2020) are known and it may be useful to account for these factors in future studies.

In this study, multiple pregnancies were excluded from analysis. Separate analysis of women with multiple pregnancies in the future would be useful, although based on this study numbers would be low, hence the lack of analysis. It is also acknowledged that women in the study ranged from those with prima gravida to those with multi gravida pregnancies. There have been reports that women with multi gravida pregnancies are more likely to experience anaemia that those with prima gravida (Al-Farsi, et al., 2011; Imai, 2020). Further analysis examining the differences of these women and the effect on the variables in this study is required, especially the extended reticulocyte variables.

It is acknowledged that a major limitation of the study is women taking vitamin or iron supplementation were not identified prior to, or documented during the pregnancy. The use of iron and vitamin supplementation as well as diet during pregnancy interferes with the laboratory concentration results and will no doubt have had an effect that is unable to be accounted for. Of note is that all women during the study had considerably lower levels of plasma ferritin in the 2nd trimester compared with the 1st trimester, therefore the effect of those taking any supplementation is difficult to assess given the low levels seen.

It is clear from the data, in the current study, both the Hb and Ferritin decrease between the first and second trimesters (the ferritin falling significantly), yet only 5.3% had a gestational delivery <37 weeks and 5.33% having a low birth weight <2500g. Interestingly, none of the women with a low Hb had low birth weight babies. Stangret, et al., 2017 hypothesises the plasma expansion seen in pregnancy causes a physiologic anaemia by reducing the blood viscosity of the mother to enhance placental perfusion, thus enabling increased nutrient delivery to the fetus in turn expanding erythrocyte mass (Means, 2020). Milman, et al., (1999) estimates the approximate transfer of maternal iron to the fetus during pregnancy to be 500-800mg. Consequently, it is not surprising this physiological process, of diversion of iron, is to the detriment of the mother with the priority given the developing fetus. Perhaps addressing pre-pregnancy iron deficiency should be more of a focus to improve physiological effects encountered in both ante- and post-partum periods. Furthermore, it is still unclear as to whether maternal iron supplementation during pregnancy has any substantial effect on maternal iron deficiency, with the prevalence of iron deficiency being largely unchanged over the last 30 years, especially in the UK (Rukuni, et al., 2015).

4.13 Future Direction

In this study of pregnant women during the 1st and 2nd trimesters of pregnancy, new variables have been identified showing potential use as predictors of the three birth outcomes (gestational delivery age, birth weight and pregnancy losses). Identifying these how these existing variables may be used in the future directs opportunities for further work.

Late gestational delivery age

A number of other studies eluded to late gestational delivery age increasing the risk of neonatal complications (Cheng, et al., 2011b; Heslehurst, 2017). With limited literature, further exploration is warranted.

High birth weight babies

Investigation of women with high birth weight babies (>4000g) was not in the scope of this current study, however the data showed there to be a significant number of babies in the population under study which would benefit from further investigation. This would be especially useful in assessing the association of Hb, Reticulocytes and extended parameters, ferritin, vitamin B12 and serum folate.

Haemoglobin

Although Hb has been the most studied variable, as well as the current one assessed as per current UK guideline, there is still uncertainty as to how Hb levels are linked with clinical outcomes, especially gestational delivery age, birth weight and pregnancy loss in developed countries.

Plasma expansion in pregnancy may account for the haemodilution effect and this further work into the effect on Hb concentration and how this affects the Hb levels during each gestation. Doing so may provide further insight into more appropriate Hb thresholds guiding the assessment of those with anaemia. The plasma expansion effect should also be further investigated for the other haematological variables, iron and haematinics.

Reticulocytes, Ret-He and IRF

There is currently limited literature as to the usefulness of Ret-He and IRF in pregnancy and further studies as to the usefulness of reticulocyte counts in assessment of iron supplementation are needed. The investigations in this study show both Ret-He and IRF parameters are not only useful and predictive for birth outcomes but also their potential to complement or replace traditional markers such as ferritin. Using these variables would also negate the use of additional blood samples needing to be provided as the extended variables can be performed from the one FBC sample routinely assessed during pregnancy in both the 1^{st} and 2^{nd} trimesters.

For Ret-He and IRF, reference intervals for pregnancy in each trimester need to be established but in the interim the levels used for the normal population should be used as a guide. In addition, more studies are required evaluating the usefulness of Ret-He and IRF and their association with birth outcomes, particularly gestational delivery age, birth weight and pregnancy losses.

The difference in the variables between the two trimester, equally provides potential as predictors of birth outcomes, as demonstrated in this study. Cut-off levels for delta Hb and delta reticulocytes, delta Ret-He and delta IRF need to be established and could provide

predictive indicators during pregnancy to guide either supplementation or further investigations.

Iron Deficiency without Anaemia

Iron deficiency in the absence of anaemia is understudied, although is acknowledged as an issue. Even in the non-pregnant population, especially women the effects of iron deficiency can be debilitating. With such limited data available, further work is required and especially the association with clinical features, not only in pregnancy but in the general population.

Inflammatory markers

There was no available literature establish raised ferritin cut-off levels in the presence of an inflammatory marker, especially in pregnancy. The relationship between ferritin and inflammatory markers needs to be validated in both the non-pregnant population and during pregnancy.

Index of Multiple Depravation Decile

There is an opportunity to make use of IMDD for pregnant women to aid identification of those women most at risk. With the multivariate finding that IMDD can be an independent predictor of birth weight, further studies are needed to identify which women are most at risk and how they can be targeted in addition to other factors, which may be contributing to adverse outcomes.

5.0 CONCLUSION

In conclusion, this study showed reticulocytes and especially the extended reticulocyte variables are associated with birth outcomes, especially birth weight. No associations were seen for vitamin B12 or folate in the group under study. In summary:

Associations with gestational delivery age outcome were seen for:

- Reticulocyte count and IRF in the 1st trimester
- IRF% only in the 2nd trimester
- delta ferritin between 1st and 2nd trimester bloods
- delta Hb was the only independent predictor at multivariate level

Associations with birth weight outcome were seen for:

- Iron deficiency without anaemia in the 1st trimester
- Ret-He, IRF and ferritin in the 2nd trimester
- Delta Hb, delta Ret-He and delta IRF
- Hb, Ret-He, IRF, delta Hb and delta IFR were all shown to be independent predictors at multivariate level

Comparison of live births with pregnancy losses were seen for:

- Ret-He, IRF and vitamin B12 showed statistically significant difference between the two groups.
- Reticulocyte count only in the 2nd trimester was shown to be an independent predictor at multivariate level.

The comparison of Hb and reticulocyte parameters with plasma ferritin showed statistically significant correlation in both the 1st and 2nd trimesters. These extended variables of

reticulocyte analysis provide excellent opportunities to redefine testing pathways for women during pregnancy. This could revolutionise antenatal care in respect of iron deficiency in pregnancy, providing more targeted assessment and appropriate treatment of those most at risk, and aid improvement of birth outcomes by identifying women at risk earlier. In addition, the reticulocyte parameters may also provide a low cost alternative to ferritin, especially Ret-He and IFR, which can be performed on existing EDTA samples already collected for routine FBC and haemoglobinopathy screening.

The study also concluded:

- Significant differences across IMDD groups for Ret-He, IFR, plasma ferritin and serum folate in the 1st trimester but only for Ret-He in the 2nd trimester.
- At multivariate level analysis, IMDD was shown to be an independent predictor of birth weight.
- No differences were seen across the groups for those with pregnancy losses.

Identfying differences between the socioeconomic groups may aid targeted interventions for those most at risk, especially for birth weight outcomes.

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