Iron in the Biology and Treatment of Gastrointestinal Cancer

Oliver Cheong Tsen Ng

BSc (Hons) MB ChB (Hons) DMCC MRCS (Eng)

4244574

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Declaration:

Except where acknowledged, I declare that this Thesis is the result of my own work which was undertaken during my period of registration for this degree at The University of Nottingham.

Oliver Cheong Tsen Ng.

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Abstract

Background

Iron is intimately related to the biology and pathology of gastrointestinal cancer with both iron excess and iron deficiency influencing disease. This thesis examines the biological and clinical effects of iron replacement in gastrointestinal cancer.

Methods

This thesis reports the effects of iron replacement in colorectal cancers at a biological level using immunohistochemistry, real time polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) during clinical treatment for iron deficiency anaemia prior to surgery. Systematic review of the literature also investigates the clinical evidence for such an approach to pre-operative anaemia. The thesis then examines the natural history and impact of anaemia in oesophagogastric cancer before reporting a pilot randomised control study using intravenous iron to treat anaemia in this setting.

Results

Examining iron administration in colorectal cancer demonstrated that colorectal adenocarcinomas reprogram their iron metabolism to increase the potential labile

iron pool. These changes appear to be decoupled from the normal intracellular iron sensing mechanisms. Route of administration of iron to patients did not alter tumour growth or effect iron transport mechanisms. Differential compartmentalisation of iron was noted however.

Clinical anaemia in oesophagogastric cancer becomes more severe with time and treatment and was associated with poorer survival outcomes. Furthermore, higher initial haemoglobin, rather than just the absence of anaemia, was associated with better survival outcomes. In a randomised control pilot study for intravenous iron use compared to standard care for anaemia in oesophagogastric cancer, intravenous iron effectively replenished iron stores measured using ferritin and transferrin saturations. Despite chemotherapy and the tumour in situ the intravenous iron group saw an increase in haemoglobin. This was despite a significantly lower starting haemoglobin in the intravenous iron group. Quality of life was also significantly improved in the intravenous iron group.

Conclusion

This thesis supports the continued clinical use of intravenous iron for anaemia in gastrointestinal cancer, showing no deleterious effects at a biological level despite replenishment of iron stores and increases in haemoglobin at a clinical level. More research is required to investigate compartmentalisation of iron and conclude clinical efficacy in larger adequately powered studies.

Publications

The following peer reviewed publications are based on work documented within Chapters of this thesis:

1. Iron, microbiota and colorectal cancer. O Ng, Wien Med Wochenschr, 2016. 166(13-14): p. 431-436.
2. Tumour-associated and non-tumour-associated microbiota in colorectal cancer: Letter to the Editor: Ng O, Omar, H, Brookes MJ. Gut 2017;66:633-643. Gut.
3. Anaemia and its effects on tumour regression grade and survival following chemotherapy in adenocarcinoma of the oesophagus. ANC Boucher, O Ng, JH Saunders, AG Acheson, SL Parsons. Journal of Gastrointestinal Oncology 2018; ePublication ahead of print.
4. Feasibility of intravenous iron isomaltoside to improve anaemia and quality of life during palliative chemotherapy for oesophagogastric adenocarcinoma. O Ng, BD Keeler, JA Simpson, S Madhusudan, MJ Brookes, AG Acheson. Nutrition and Cancer 2018. Accepted for publication.
5. Iron therapy for pre-operative anaemia. O Ng, BD Keeler, A Mishra, JA Simpson, K Neal, MJ Brookes, AG Acheson. Cochrane Database Syst Rev, 2015. 12: p. CD011588.

Presentations and Abstracts

The following abstracts are based upon work undertaken during my period of registration for the degree of Doctor of Philosophy and documented in this thesis:

1. Outcomes Following Anaemia in Oesophagogastric Cancer. O Ng, B Oakley, S Holmes, J Catton, A Acheson. Association of Surgeons of Great Britain and Ireland 2015, 23 Apr 15
2. Effect of iron therapy on P-selectin expression in colorectal cancer. O Ng, A Acheson, B Keeler, A Simpson, MJ Brookes. Royal College of Physicians Midlands Gastroenterological Society 13 May 2016.
3. Patient Blood Management Expert Meeting: IRON trial. O Ng, A Acheson. Royal Danish Embassy, 5-6 Jun 15
4. Impact of Haemoglobin on Survival in Oesophagogastric Cancer. B Oakley, O Ng, S Holmes, S Parsons, J Catton, S Madhusudan, E James, MJ Brookes, AG Acheson. Digestive Diseases Federation 15-16 Jun 2015
5. The Effects of MAGIC and Laparoscopic Surgery on Anaemia and Blood Transfusions in Oesophagectomy. O Ng, B Oakley, S Holmes, J Saunders, C Bowman, A Ayantunde, MJ Brookes, AG Acheson, S Parsons. Digestive Diseases Federation 15-16 Jun 2015

6. An Open-label, Randomised Controlled Trial Comparing the Efficacy of Intravenous and Oral Iron in the Preoperative Management of Colorectal Cancer Anaemia: IVICA trial. BD Keeler, JA Simpson, O Ng, H Padmanabhan, MJ Brookes, AG Acheson and IVICA Trial Group. Society of Academic and Research Surgery 5-6 Jan 2016.
7. The Prevalence and Impact of Post-operative Anaemia in Major Surgery. E Leicester, O Ng, A Boucher A Acheson. Association of Surgeons of Great Britain and Ireland, Belfast 11-13 May 2016
8. Anaemia and its Effects on Tumour Regression Grade Following Neoadjuvant Chemotherapy in Oesophageal Cancer. A Boucher, O Ng, J Saunders, S Parsons, E James, A Acheson. Association of Surgeons of Great Britain and Ireland, Belfast 11-13 May 2016
9. The Significance Of Iron Deficiency In Anaemia In Two Week Referrals For Colorectal Cancer. S Mashlab, O Ng, A Acheson, A Banerjea. British Society of Gastroenterology 5-7 Jun 2016
10. Anaemia in Two Week Wait Referrals for Colorectal Cancer; Is it Predictive and Can We Treat it? O Ng, S Mashlab, A Acheson, A Banerjea. The Association of Coloproctology of Great Britain and Ireland 4-6 July 2016

11. Hepcidin does not predict response to iron therapy in pre-operative anaemia in patients with colorectal cancer. O Ng , BD Keeler, JA Simpson, HO Al-Hassi, AG Acheson, MJ Brookes. British Society of Gastroenterology 19-22 Jun 2017
12. Differential Immune Responses Between Proximal And Distal Colorectal Cancer. HO Al-Hassi, GH Lee, A Murugananthan, G Malietzis, ER Mann, J Landy, O Ng, AG Acheson, D Bernardo, MJ Brookes, SC Knight. British Society of Gastroenterology 19-22 Jun 2017
13. The Effects Of Iron Therapy On Iron Transport In Human Colorectal Cancer. O Ng, HO Al-Hassi, R Evstatiev, BD Keeler, T Warr, FB Rowther, V Khare, M Jambrich, AG Acheson, C Gasche, MJ Brookes. British Society of Gastroenterology 19-22 Jun 2017
14. The Effect of Route of Administration of Iron Therapy on Cellular Proliferation and Apoptosis in Human Colorectal Carcinoma. O Ng, HO Al-Hassi, R Evstatiev, BD Keeler, T Warr, FB Rowther, V Khare, M Jambrich, AG Acheson, C Gasche, MJ Brookes. British Society of Gastroenterology 19-22 Jun 2017
15. Iron Replacement In Oesophagogastric Neoplasia. O Ng, BD Keeler, JA Simpson, S Madhusudan, MJ Brookes, AG Acheson. British Society of Gastroenterology 2018
16. Iron therapy for chemotherapy induced anaemia in oesophagogastric cancer. O Ng, BD Keeler, JA Simpson, S Madhusudan, MJ Brookes, AG Acheson.

Royal College of Physicians Midlands Gastroenterological Society 11 May 2018.

17. Feasibility of iron replacement in oesophagogastric neoplasia. O Ng, BD Keeler, JA Simpson, S Madhusudan, MJ Brookes, AG Acheson. Network for the Advancement of Patient Blood Management, Haemostasis and Thrombosis (NATA), Lisbon, Portugal 12-13 April 2018.

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1 Introduction

Iron is essential to all living cells and remains one of the most common nutritional deficiencies worldwide (McLean, Cogswell et al. 2009). Its role in gastrointestinal cancer has been suspected for over a century, but only now can science begin to explain the complex role of iron biology in one of our most common diseases. This thesis examines iron in gastrointestinal cancer at a biological level and iron deficiency, manifest as anaemia, in clinical practice. It adds to the growing debate regarding iron replacement and iron chelation for cancer as clinicians seek to modify iron biology for clinical benefit.

1.1 Historical background

It is postulated that the abundance of iron began in the first 'iron age' almost 200 million years after the Big Bang with the formation of iron in planet Earths inner and outer core. Here, iron is thought to have played a key role in the early chemistry of life, combined with sulphur (Sheftel, Mason et al. 2012). Its biological partnership with sulphur continues to this day in iron-sulphur complexes (Beard 2001).

With the evolution of *Homo sapiens* approximately 200,000 years ago, iron continued to play a key role in metabolism, this time combined with oxygen, although it took until 1931 to discover iron's central role in haemoglobin (Poskitt 2003).

The second Iron Age began in 12th Century BC with the mass production of tools and weapons, combined with carbon in steel. During this period medicinal uses for iron are recorded around the Mediterranean by the Egyptians, Greeks and Romans (Abbaspour, Hurrell et al. 2014). Reports of iron use in disease continued throughout the ages to treat conditions as diverse as acne, gout, tuberculosis and alopecia (Beutler 2002).

Iron was most widely used to treat chlorosis, 'green sickness' in the 17th century. This condition, named after the green complexion that afflicted suffers, was a disease of young women characterised by a pale complexion, headaches, exhaustion and neurasthenia. This condition has since disappeared but is largely thought to have been due to severe iron deficiency and was treated with ferrous sulphate and potassium carbonate despite no understanding of the principles of this therapy (Brumberg 1982).

Only with the growth of modern Chemists in the 18th Century did iron begin to be recognised in human biology, Vincenzo Menghini (1704-1759) the first to demonstrate iron in dried red blood cells using a magnetic blade made of Lodestone (Busacchi 1958) while others were demonstrating the affinity of iron for oxygen and the reversible oxygenation of haemoglobin (Sheftel, Mason et al. 2012). Then in the 19th Century, Fenton demonstrate iron could generate toxic free radical formation by 'Fenton reaction', and began our understanding that iron excess was as dangerous as iron deficiency (Sheftel, Mason et al. 2012).

Only in the last century have we entered a new ‘iron age’, where we have seen an increasing understanding of iron biology and its impact upon human health. Modern interest in iron increased dramatically starting with the discovery of iron’s central role in haemoglobin synthesis in 1931 (Poskitt 2003). This was followed by characterisation of ferritin, crystalized by Laufberger in 1937 (Laufberger 1937). Radioactive iron isotopes in the 1940’s allowed more detailed analysis of ferrokinetics and the important role of iron absorption and mucosal blockage in iron homeostasis. Holmberg and Laurell also discovered plasma iron-binding protein transferrin in 1945 (Holmberg CG 1945) but it took until 1969 for Ewan Morgan to demonstrate transferrin-receptor mediated endocytosis (Morgan and Appleton 1969). The development of molecular biology then led to the understanding of the intracellular regulation of iron in the 1980’s with iron responsive elements and iron responsive element binding protein (IRE/IRP) control (see section 2.1.1) (Hentze, Caughman et al. 1987). Haemochromatosis, first described at autopsy in the mid-1800s, finally revealed a ‘master regulator’ of iron at the turn of the this century when researchers identified hepcidin as central to iron homeostasis and mutations of the hepcidin gene responsible for the disease (Ganz 2003).

In parallel to this increase understanding of iron in human biology, development of intravenous iron preparations began slowly. Medicinal products and food containing iron had been used for thousands of year. Iron-containing chalybeate waters found in famous British spa towns like Bath were known for their healing properties. Oral iron tablets were first introduced in 1832, a combination of 1.39g ferrous sulphate

and 0.1g potassium carbonate known as ‘Blaud’s pills’ after the French physician who used them to treat chlorosis (Poskitt 2003). By 1932 Heath, Strauss and Castle were giving iron solutions to treat hypochromic anaemia (Heath, Strauss et al. 1932). While this corrected anaemia, these intramuscular and subcutaneous injections caused severe acute reactions preventing their use. In 1954 iron dextran was introduced allowing easier administration through the intravenous route. Despite infrequent complications widespread adoption was not favoured due to relative expense and widespread use of oral iron (Auerbach and Macdougall 2014). Clinical need for intravenous iron increased in 1989 with the introduction of erythropoiesis stimulating agents in dialysis patients. Imferon® a high molecular weight iron dextran was the main agent used at the time. In 1991, it was withdrawn from the market due to a contaminated batch of the drug.

Undeterred, Cosmofer® was introduced to the market in the same year, a low molecular weight iron dextran. This was followed by a host of other iron dextran compounds including Dexferrum® (a high molecular weight dextran) and then in 1999 and 2000 two agents with smaller carbohydrate cores, ferric gluconate (Ferrlecit®) and iron sucrose (Venofer®). By the late 1990s, several reports of increased adverse events and anaphylaxis with Dexferrum® led to the removal of high molecular weight iron dextrans from the market in 2009 (Auerbach and Ballard 2010). The legacy of high molecular weight dextran iron causing severe anaphylaxis however has persisted to erroneously impact the perception of all intravenous irons as dangerous.

Widespread adoption of ferric gluconate (Ferrlecit®) and iron sucrose (Venofer®) filled the gap left in the intravenous iron market. Subsequent agents introduced after 2000 have also been manufactured to allow single-dose iron replacement. These include ferumoxytol (Feraheme®), ferric carboxymaltose (Ferinject®) and iron isomaltoside-1000 (Monofer®), all of which promise large doses of iron safely administered over short time frames (Auerbach and Macdougall 2014). With a growing safety profile, intravenous iron agents are now increasingly used outside renal dialysis including inflammatory bowel disease and heart failure with trials in other settings growing year on year (Silverberg, Wexler et al. 2000, Gomollon and Gisbert 2013).

1.2 Epidemiological background

Anaemia affects one-quarter of the world's population, an estimated 1.62 billion (McLean, Cogswell et al. 2009). Iron deficiency is one of the major contributors to this worldwide problem with almost one-third of the population afflicted (DeMaeyer and Adiels-Tegman 1985). Even amongst Western populations with iron-rich diets, 9% of toddlers age 1-2 years, 9-11% of adolescent girls and women of child bearing age and 1% of teenage boys and young men are iron deficient (Looker, Dallman et al. 1997).

Beyond the 'normal' population, anaemia due to iron deficiency increases dramatically with disease. In developed countries, benign and malignant

gastrointestinal causes account for the majority of iron deficiency and anaemia in men and is the second most common cause in women after menstruation (Raje, Mukhtar et al. 2007). Almost 50% of patients diagnosed with colorectal cancer or oesophagogastric cancer have iron deficiency (Ludwig, Muldur et al. 2013). It is associated with more advanced disease and poor Eastern Cooperative Oncology Group (ECOG) performance status (Ludwig, Van Belle et al. 2004). This results in 30-50% of patients having anaemia at diagnosis with gastrointestinal cancer, the majority due to absolute or functional iron deficiency (Ludwig, Muldur et al. 2013). Absolute iron deficiency is where the total amount of iron in iron stores is low. Functional iron deficiency occurs when despite iron stores being adequate iron is sequestered and stored in macrophages and cells limiting the bioavailable iron for metabolism. This is discussed in detail in Chapter 2. In addition to this, treatment with surgery, chemotherapy and radiotherapy further exacerbates the problem resulting in almost universal anaemia and iron deficiency (Ludwig, Muldur et al. 2013).

1.3 Clinical background

Iron deficiency results from an imbalance of absorption versus demand for iron. This results in the depletion of iron stores and finally an iron deficient state. A lack of iron in the diet, malabsorption from the bowel (most commonly coeliac disease), consumption of foods that inhibit iron (calcium, tannins, phytates) and acid reducing

medications (such as proton pump inhibitors, PPIs) are the principal causes of problems with absorption.

Increased demand can result from normal physiology such as pregnancy and menstruation. However, pathological blood loss from gastrointestinal bleeding due to cancer, infection, parasites and inflammatory bowel disease are common causes (Brittenham 2013). Iatrogenic causes are also multiple, chief among these surgery and chemotherapy.

Finally, a functional iron deficiency can result whereby normal iron stores cannot be mobilised due to hepcidin-mediated iron sequestration and restriction of iron. This is thought to be the pathophysiology underlying anaemia of chronic disease in which chronic inflammation leads to chronically high levels of hepcidin (Little 2013).

1.4 Definitions of iron deficiency and anaemia

Iron deficiency is defined as a decrease in the total content of iron in the body (Bermejo and Garcia-Lopez 2009). Criteria for diagnosis vary but a serum ferritin level less than 30 ng/mL in the absence of inflammation would be considered diagnostic (Munoz, Acheson et al. 2017). However, ferritin is often an imprecise measure of total body iron due to inflammation, malignancy, alcoholism and liver disease raising levels of ferritin (Beutler, Hoffbrand et al. 2003). Diagnosis in the presence of inflammation and cancer can therefore be more difficult. Iron deficiency can exist with levels up to 100 ng/mL (Munoz, Acheson et al. 2017). Other studies

have recommended ferritin values <70 and <40 ng/mL in patients with and without inflammation respectively (Guyatt, Oxman et al. 1992). The British Society for Gastroenterology (BSG) suggest as low as 12-15 ng/mL and a 50 ng/mL threshold for ID in the presence of inflammation (Goddard, McIntyre et al. 2000). Bone marrow aspirate is regarded by haematologists as the gold standard for diagnosing ID but is not practicable in routine clinical care (Beutler, Hoffbrand et al. 2003).

Trial of iron therapy has been proposed as a pragmatic approach to diagnosis of iron deficiency, assuming compliance with iron therapy is good (Beutler, Hoffbrand et al. 2003). Response is seen within seven days of parenteral iron therapy and can help separate absolute iron deficiency from functional iron deficiency (Goddard, James et al. 2011). Early indicators of response include increased reticulocyte haemoglobin content (Mast, Blinder et al. 2002).

Other measures for the diagnosis of ID also include transferrin saturations, soluble transferrin receptor, ferritin index (soluble transferrin receptor (sTfR)/log ferritin), hypochromic red cell percentage, reticulocyte haemoglobin concentration (CHr), erythropoietin levels and hepcidin, but these measures are often not routinely measured and still present some diagnostic uncertainty (Bermejo and Garcia-Lopez 2009, Steinmetz, Tsamaloukas et al. 2010).

Transferrin saturation offers a more functional definition of iron deficiency and levels less than 20% would be considered to represent iron deficiency. However, this could represent an absolute or functional iron deficiency and should be interpreted in

light of other tests (Bermejo and Garcia-Lopez 2009). In malnutrition and chronic disease, transferrin synthesis may also be impaired elevating the relative transferrin saturation levels. Significant diurnal variation in transferrin saturations has also been reported limiting its absolute validity (Wish 2006).

Soluble transferrin receptor (sTfR) appears a promising marker of iron deficiency, with ferrokinetic studies demonstrating a strong correlation between sTfR and absolute iron deficiency induced with iron chelation (Borgna-Pignatti and Cohen 1997, Skikne 2008). Combined with ferritin as a ratio of sTfR/log10 serum ferritin several studies report better discrimination than either test alone (Cook, Flowers et al. 2003). However, sTfR is costly and yet to become routinely available.

By contrast, percentage hypochromic red cells and reticulocyte haemoglobin are cheap and widely available on modern multichannel automated haematology analysers. They are indicators of iron deficiency when less than 5% and/or less than 26 pg/cell respectively (Fishbane, Shapiro et al. 2001). Percentage hypochromic red cells, based upon haemoglobin concentration in a red blood cell (and thus corrected for cell size). Reticulocyte haemoglobin reflects haemoglobin in cells only 1-2 days old and is a contemporaneous reflection of iron availability for erythropoiesis, especially when compared to an analysis of the entire red cell mass with cells up to 120 days old. Both tests are less variable than ferritin and transferrin saturations but both tests are seldom used routinely (Wish 2006, Ludwig, Muldur et al. 2013).

Erythropoietin is only useful in specific clinical settings, for example chronic renal failure. Hepcidin, despite its role as 'master regulator' of iron homeostasis is like ferritin an acute phase reactant and is not useful in determining iron status.

Table 1.1 Tests for iron deficiency

	Iron-deficient erythropoiesis	**Absolute tissue iron stores**
Test	Hb Tsat MCV % hypochromic erythrocytes Reticulocyte Hb content Zinc protoporphryin	*Storage iron* Serum ferritin Bone marrow haemosiderin *Tissue iron* Serum transferrin receptor

Adapted from Beutler, Hoffbrand et al. (2003)

Anaemia

Iron deficiency is one of the most common causes of anaemia. However, many people are iron deficient with no clinical manifestation of anaemia. Across a spectrum of iron deficiency they do nevertheless display a multitude of seemingly non-specific symptomatology, see Table 1.3 (Beard 2001).

When iron deficiency anaemia (IDA) does occur, it typically results in microcytic and hypochromic red blood cells with a reduced mean cell haemoglobin (MCH) and reduced mean cell volume (MCV). However, this is not universal with traditional MCV in fact one of the last measures to change in iron-deficient erythropoiesis, with many absolute IDA and functional IDA displaying a normal MCV (Thomas and

Thomas 2002). Reticulocyte haemoglobin content changes much earlier in iron-deficient erythropoiesis due to the relatively short half-life of reticulocyte (1-2 days) as mentioned previously (Brugnara 2000). Several laboratory methods for identifying iron deficiency anaemia are available which broadly test for iron-deficient erythropoiesis (IDE) and absolute measures of tissue iron status, and overlap with those described previously for iron deficiency, see Table 1.1 (Beutler, Hoffbrand et al. 2003).

Anaemia is defined as a total reduction in erythrocyte number, reduced amount of circulating haemoglobin, or decreased circulating red blood cell mass (Perkins 2006), resulting in a pathological state where the oxygen-carrying capacity of blood is insufficient to meet physiological demand (Varat, Adolph et al. 1972). Historically, much emphasis has been placed upon the defining the first half of this definition, while the tools and expertise in establishing the physiological sufficiency of oxygen delivery have been less researched.

Defining the level of haemoglobin whereby anaemia is clinically diagnosed is difficult. Variation within lower limits for haemoglobin related to health status, ethnicity, age and sex of a population can drastically influence what is considered normal. Definitions vary from 130 to 142 g/L for men and 117 to 123 g/L for women (Beutler and Waalen 2006).

The most widely used definition of anaemia is from the World Health Organisation (WHO) who define anaemia as a haemoglobin of less than 130 g/L in men, 120 g/L

in non-pregnant women, see Table 1.2 (WHO 1968). These cut-offs were defined in a study almost 50 years ago by a WHO study group and were based upon surprisingly little data from small studies in India (n=300), Israel (n=266), Mexico (n=364), Poland (n=220) and Venezuela (n=255). This definition by WHO has been criticised for its small sample size and unpublished methodology. Subsequent publications refined severity and corrected for smoking and altitude, but did not alter the absolute cut-offs for anaemia nor the declining 'normal' haemoglobin of the older person (McLean, Cogswell et al. 2009).

Table 1.2 WHO definition of anaemia by haemoglobin at sea level (g/L)

	Non-anaemia	Mild	Moderate	Severe
Children < 5 years	> 110	100-109	70-99	<70
Children 5-11 years	> 115	110-114	80-109	<80
Children 12-14 years	> 120	110-119	80-109	<80
Non-pregnant women >15 years	> 120	110-119	80-109	<80
Pregnant women	> 110	100-109	70-99	<70
Men >15 years	> 130	110-129	80-109	<80

Adapted from WHO (2011)

Beutler proposed new definition of anaemia based on two large US databases, the Scripps-Kaiser and US National Health and Nutrition Examination Survey (NHANES) databases, adjusted for age, sex, health status and ethnicity. This large analysis of normal haemoglobin in a Western population was collected in the San Diego area between 1998 and 2002. They analysed over 32,000 people and furthermore controlled for race, age, renal failure, alcohol intake, CRP, ferritin and transferrin saturations. With good agreement between the two databases they proposed normal limits of 137 g/L for white men age 20 and 59 years and 122 g/L for women of all ages, with a lower 132 for men 60 years and over and a lower

normal limit, between 7-8 g/L less, for black men and women (Beutler and Waalen 2006).

It should be acknowledged that definition of normal haemoglobin and physiological impact of anaemia should be considered separately. Haemoglobin is a means to an end, namely tissue oxygenation. Anaemia tolerance between different patients and in different scenarios is difficult to predict (Meier and Gombotz 2013). Haemoglobin delivers oxygen (DO2) to meet metabolic demand (VO2), with arterial oxygen content according to the formula:

$$CaO2\ (ml/dL) = (SaO2 \times 1.34 \times [Hb]) + (0.0031 \times PaO2)$$

With oxygen delivery thereafter a product of cardiac output:

$$DO2 = CO \times CaO2$$

Where CO = SV x HR

For clinicians, the ideal would be a measure of regional tissue oxygenation (rSO_2) and regional oxygen delivery (rDO2). This measure would help us avoid the pitfall of using normal ranges based upon healthy populations as clinical treatment thresholds for individual patients. It would also remove any bias in administration of treatment, especially in women who despite a lower 'normal' haemoglobin have the same physiological requirements for oxygen but a smaller blood volume and physiological reserve for blood loss that might result for example in surgery or trauma. Non-invasive measurement of regional tissue oxygenation is available using

near infrared spectroscopy (NIRS) and has been widely used for measurement of cerebral tissue oxygenation (Scheeren, Schober et al. 2012), including during major abdominal surgery (Casati, Fanelli et al. 2005). Peripheral tissue oxygenation is less widely studied and routine use of regional tissue oxygenation has not been adopted (Scheeren, Schober et al. 2012).

Table 1.3 Continuum of clinical symptoms due to low ferritin/iron

Serum ferritin level (ng/mL)	Symptom
100	
60-80	Hair loss
50	Fatigue and muscle weakness Restless leg syndrome
40	Brittle Nails and/or spoon-shaped fingernails
35	Headaches
30	Increased infections
20	Depression
15	Irritability, Loss of Concentration, Dizziness
10	Pallor and iron deficiency anaemia
0-5	Craving or eating non-foods (pica) e.g. dirt (geophagia) or ice (pagophagia)

Adapted from Beard (2001)

Table 1.4 Iron deficiency anaemia versus anaemia of chronic disease

	ID	Absolute IDA	Functional IDA (ACD)
Hb	>130 g/L men >120 g/L women	<130 g/L men	<120 g/L women
MCV	80-96 fL/red cell	<80	80-96 fL/red cell
Ferritin	<30 ng/mL	<30ng/mL	>100 ng/mL
Transferrin saturations	>20%	<20%	
Transferrin	Increased	Increased	Decreased or normal
Soluble transferrin receptor	Increased	Increased	Normal
% hypochromic RBC (PHRC)	<5%	<5%	<5%
Reticulocyte Hb (CHr)	<26 pg	<26 pg	<26 pg
Erythropoietin	Normal	Increased	Normal
C-reactive protein	Normal	Normal	Increased

Table adapted from Bermejo (2009)

1.5 Iron metabolism

Iron is essential and ubiquitous to the metabolism of all living organisms from plants to bacteria and humans. It exists in a wide range of oxidation states in nature but rapidly forms highly insoluble iron oxides on contact with oxygen. In humans it is therefore confined to bivalent ferrous iron (Fe^{2+}) and trivalent ferric iron (Fe^{3+}) acting as an electron acceptor and donor respectively (Brittenham 2013).

Two-thirds iron is found in haemoglobin, 25% in iron stores, 15% bound to myoglobin and only 0.1% bound to transferrin (Trumbo, Yates et al. 2001). The amount of iron absorbed from the diet varies from 5-35% depending on body iron status and the type of iron ingested. Total body iron content is 50 mg/kg in adult men and 35 mg/kg in adult women equating to approximately 4-5 grams of iron. About 2 grams is stored as ferritin in macrophages and hepatocytes and 2.6 grams circulating as haemoglobin (Andrews 2004). Iron is found in all cells but stored predominantly in liver, spleen and bone marrow. Macrophages break down and recycle iron from the haemoglobin of red blood cells. Iron can also be stored as hemosiderin.

Iron metabolism is discussed in detail in Chapter 2. In brief, dietary non-haem iron is absorbed in the duodenum and upper jejunum by enterocytes after reduction to ferrous form by duodenal cytochrome *b*-like ferrireductase (Dcytb) (Muir and Hopfer 1985, McKie, Barrow et al. 2001). Subsequent transport into the cell is achieved by divalent metal transport 1 (DMT1). Absorption of haem iron is poorly

understood but thought to be achieved by receptor-mediated endocytosis. After this, liberated iron follows the same pathways as non-haem iron (West and Oates 2008).

After absorption, iron can then be stored as ferritin or exported from cells via basolateral ferroportin, the only known exporter of iron in the body (Brittenham 2013). It is then transported in the extracellular fluid and plasma, bound to transferrin (Brittenham 2013). Cells then obtain iron via binding of the iron-transferrin complex to transferrin-receptor 1 (TfR1) expressed on cell membranes that facilitates the endocytosis. Iron is then released from the endosome via DMT1 to form a labile iron pool which can be taken up for cellular processes (Brittenham 2013).

Iron homeostasis is controlled by hepcidin and executed through ferroportin internalisation (Zhao, Zhang et al. 2013) and at an intracellular transcriptional level by the IRE/IRP control, see Section 2.1.1 (Hentze, Caughman et al. 1987).

1.6 Iron and disease

Iron excess and iron deficiency both have important implications for human disease. Iron is highly toxic and judicious homeostasis is essential to protect the human body from the damaging oxidative effects of iron.

Iron's most widely recognised function in the human is incorporated into haem proteins to form haemoglobin. The deleterious effects of anaemia in cancer and pre-operative settings are discussed at length in other chapters. In short, anaemia is associated with increased morbidity and mortality across a wide range of conditions including cancer and major surgery(Beattie, Karkouti et al. 2009, Acheson, Brookes et al. 2012). Anaemia from iron deficiency also increases blood transfusion (Dunne, Malone et al. 2002). Receiving a blood transfusion is associated with an increase cancer recurrence in colorectal, oesophageal and hepatocellular carcinoma (Motoyama, Okuyama et al. 2004, Amato and Pescatori 2006, Wang, Iyer et al. 2009, Acheson, Brookes et al. 2012). This is thought to be due to transfusion-related immunomodulation (Cata, Wang et al. 2013).

Aside from erythrocytes, iron is found in all cells and involved in a multitude of critical cellular processes acting as a cofactor for enzymes, in iron-sulphur clusters, haem groups (haemoglobin, myoglobin) and in cytochromes essential for oxidative phosphorylation in the mitochondria (Andrews 2004). Subsequently, beyond

anaemia, iron deficiency plays an important role in cancer, oxidative stress, immunity, infection and thrombosis.

Pathways and mechanisms whereby iron promotes carcinogenesis are currently poorly understood. Those related to colorectal cancer and iron transporters are discussed in Chapter 2..

1.6.1 Oxidative stress

Oxidative stress occurs from ferrous irons reacting with hydrogen peroxide to produce free radicals that damage cellular components including DNA, protein and lipids. These effects are manifest in iron overload disorders like haemochromatosis where organs are damaged by free radicals including the heart and liver (Brittenham 2013). The oxidative effects of iron are not limited to haemochromatosis though. In humans, healthy volunteers given oral iron supplementation showed a 40% increase in free radical production in faeces (Lund, Wharf et al. 1999). Induction of oxidative stress at the site of bowel inflammation also occurs with oral iron in inflammatory bowel disease (Oldenburg, van Berge Henegouwen et al. 2000).

1.6.2 Immunity and Infection

Iron is essential for normal immune system development and function (Beard 2001). Innate and adaptive immunity can be altered by iron but many studies are conflicting (Cherayil 2010). Iron allows the formation of toxic hydroxyl radicals (peroxide-

generating enzymes and nitrous oxide-generating enzymes) as part of immune effector pathway (Beard 2001).

In the innate immune system, iron deficiency results in an impairment of natural killer cell activity (Hallquist, McNeil et al. 1992). The natural killer cells are particularly important in tumour growth and metastatic disease progression (Langers, Renoux et al. 2012). In the presence of iron deficiency the bactericidal activity of macrophages is also attenuated (Hallquist, McNeil et al. 1992). TNF alpha reduces both DMT-1 and IREG-1 along with ferritin expression in Caco-2 cells (Sharma, Laftah et al. 2005).

The adaptive immune system is likewise affected, with iron deficiency leading to a reduction in T cells numbers and thymic atrophy with reduced T-cell induction, an effect reversible with iron repletion (Kuvibidila, Kitchens et al. 1999). However, humoral immunity, mediated by B-cells, does not seem to be impaired by iron deficiency (Bagchi, Mohanram et al. 1980).

Iron also has a key role in bacterial infection. Iron is essential for bacterial growth. Bacteria have iron binding siderophores that enable them to absorb free iron or scavenge iron from haemoglobin or transferrin. As a response to infection iron withholding strategies (mediated through hepcidin) play a key role in host defence (Nemeth, Rivera et al. 2004).

Described as hypoferraemia of inflammation, this sequestration of iron in macrophages denies iron to invading microorganisms (Ganz and Nemeth 2015). It

results in functional iron deficiency (also known as anaemia of chronic disease) caused by chronic activation of inflammation and immune pathways. Pathophysiological changes inhibit iron efflux from cells and induce macrophage acquisition of iron (IFN-γ, TNF-α), promote iron storage through increased ferritin (IL-1, IL-6 and IL-10) and decrease enterocyte transfer of iron into the circulation and iron mobilisation from the mononuclear phagocyte system (hepcidin) (Weiss and Goodnough 2005).

Growing research into gut microbiota and dysbiosis all suggests one explanation an association between iron and colorectal cancer. Iron is growth-limiting for many pathogenic bacteria and may promote a shift in the ratio of pathogenic to protective bacteria (Zhu, Gao et al. 2013). This may increase the toxic bacterial metabolites promoting inflammation and carcinogenesis.

Research in the field of gut microbiota has increased in the last 10 years, largely driven by advances in DNA-sequencing (especially of highly conserved hypervariable regions of the 16S rRNA genes in bacteria) which combined with real-time quantitative PCR techniques allow characterisation and quantification of microbiota biodiversity (Matsuki, Watanabe et al. 2004, Ott, Musfeldt et al. 2004).

Specific bacteria have been implicated in carcinogenesis including *Streptococcus bovis, Bacteroides, Enterococcus faecalis* and *Clostridia*. (Moore and Moore 1995, Gold, Bayar et al. 2004, Huycke and Gaskins 2004, Sobhani, Tap et al. 2011). These bacteria produce genotoxic metabolites, such as hydrogen sulphide and secondary

bile-salts, which likely promote inflammation and carcinogenesis (O'Keefe, Chung et al. 2007, Nicholson, Holmes et al. 2012).

In contrast, gut protective *Bifidobacterium longum* and *Lactobacillus acidophilus* appear to inhibit carcinogenesis by forming a protective barrier against colonisation by pathogenic bacteria (Rowland, Bearne et al. 1996, McIntosh, Royle et al. 1999). *Bifidobacteriaceae* bond iron to their surface reducing free radical formation and bioavailable iron for pathogenic bacteria (Kot and Bezkorovainy 1999). *Lactobacilli* also appear to reduce the mutagenic effects of bile acids (Lidbeck, Nord et al. 1992). Butyrate production by these bacteria is also thought to be anti-carcinogenic and may be altered by dysbiosis (Vipperla and O'Keefe 2012).

Eradication of bacteria with antibiotic treatment or breeding germ free mice in colitis-associated cancer models significantly decreases the incidence of colon cancer and alters gut microbiota (Engle, Ormsby et al. 2002, Klimesova, Kverka et al. 2013).

Iron is essential for the growth, virulence and ability to colonise the gut for many of the pathogenic bacterial species (Andrews, Robinson et al. 2003). Bacteria dependent on iron have iron binding siderophores that enable them to absorb free iron or scavenge iron from haemoglobin or transferrin. Importantly the protective Lactobacilli bacteria do not have siderophores and grow independently of iron, instead requiring manganese for growth (Weinberg 1997).

Anaemic African children supplemented with iron demonstrate an increase in the ratio of pathogenic enterobacteria compared to protective bifidobacteria and lactobacilli with an associated increase in inflammation. (Zimmermann, Chassard et al. 2010, Jaeggi, Kortman et al. 2015).

Further support for this theory is found in in vitro and animal studies. Low iron in vitro cultures of faecal microbiota favour the growth of Lactobacillus species and a decrease in Clostridium and Bacteroides species (Dostal, Fehlbaum et al. 2013). Similar findings were seen in rat's gut microbiota with iron deficient and replete diets (Dostal, Chassard et al. 2012). Mice fed iron deprived diets also increased the population of Lactobacilli in the gut (Tompkins, O'Dell et al. 2001).

Genetic modification of iron metabolism genes in mice to increase (iron regulatory protein 2) or decrease iron (hereditary haemachromatosis Hfe), resulted in an abundance of beneficial Lactobacillus species with IRP-2 knockouts and a predominant Enterococcus faecium species in Hfe-/- mice (Buhnik-Rosenblau, Moshe-Belizowski et al. 2012).

The route of iron supplementation also appears to be important. Werner et al. used a Crohn's disease-like ileitis mouse model and gave either iron sulphate containing diet or an iron free diet, with or without iron injections for 11 weeks. They showed that an iron sulphate free diet prevented development of Crohn's ileitis through alteration of the composition of microbiota in the gut. Importantly, systemic iron

repletion via injections did not abrogate the protective effect of luminal iron depletion (Werner, Wagner et al. 2011).

These findings have recently been demonstrated in humans as well. In a study that randomised inflammatory bowel disease patients to oral or intravenous iron therapy for anaemia, those receiving oral iron therapy showed a significant decrease in the abundance of bacterial communities (especially in Crohn's disease) when compared with intravenous iron (Lee, Clavel et al. 2016).

Acknowledgement that iron is pro-inflammatory has already gained recognition within gastroenterology and the treatment of inflammatory bowel disease with oral iron not recommended in active disease (Dignass, Gasche et al. 2015).

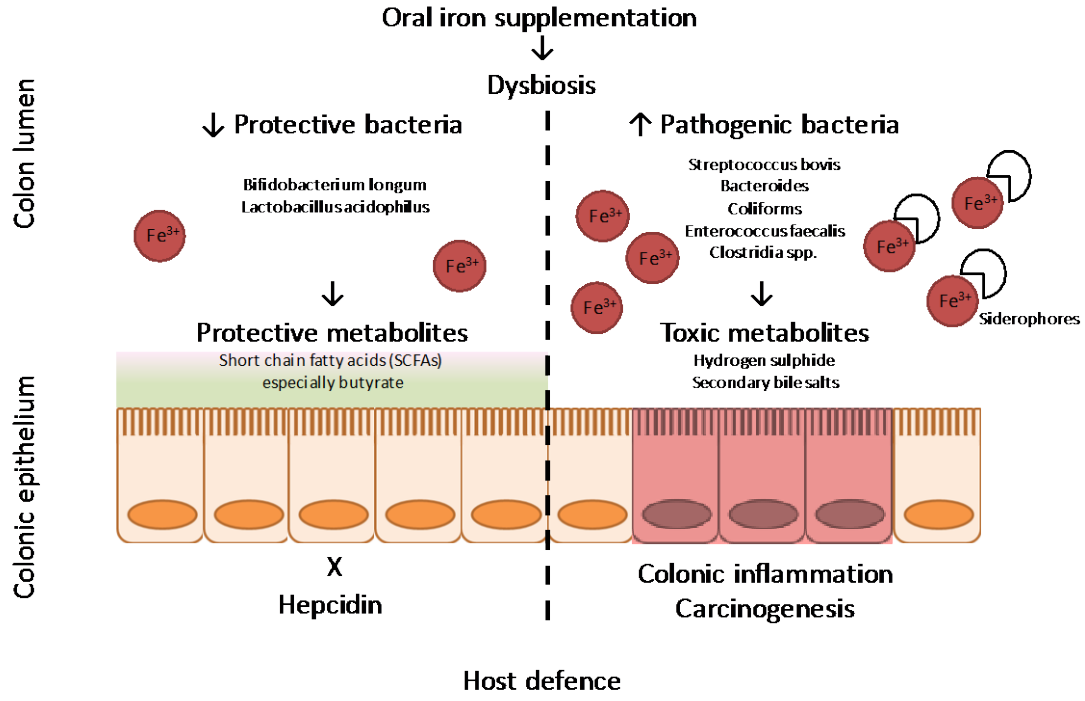


Figure 1.1 Schematic overview of iron induced dysbiosis in the colonic lumen.

1.6.3 Thrombosis

Iron deficiency and iron excess have both been associated with increased thromboembolic events including stroke, coronary heart disease and venous thromboembolism (Franchini, Targher et al. 2008). This is especially important in the pro-thrombotic state of cancer and post-surgery where co-existent iron deficiency is present. Thromboembolic events are also as high as 20-30% in patients receiving chemotherapy (Timp, Braekkan et al. 2013).

The mechanism by which iron causes thrombocytosis is not fully understood, but iron appears to be a potent regulator of thrombopoiesis (Karpatkin, Garg et al. 1974). Mild iron deficiency results in a reactive thrombocytosis, while severe iron deficiency leads to thrombocytopenia (Dan 2005). Platelets greater than 350,000 correspond to a higher risk of thromboembolic events (Khorana, Kuderer et al. 2008).

Iron overload appears equally thrombogenic in experimental models of atherosclerosis. Moderate iron overload promoted thrombus formation, possibly through the oxidative stress and the production of reactive oxygen species (Day, Duquaine et al. 2003). Iron overload from hereditary hemochromatosis also increases cardiovascular events (Ellervik, Tybjaerg-Hansen et al. 2005). Iron chelation and repeated blood donation have consequently been investigated to examine if they reduce cardiovascular risk (Duffy, Biegelsen et al. 2001, Zheng, Cable et al. 2005).

Erythropoietin may also be important, with some amino acid sequence homology with thrombopoietin (Bilic and Bilic 2003). This could explain why IDA results in thrombocytosis. Another theory is that microcytic erythrocytes are less deformable, altering blood flow and promoting coagulation (Hartfield, Lowry et al. 1997).

Others have suggested that platelet aggregation due to decreased antioxidant defence with iron deficiency may increase thrombosis (Tekin, Yavuzer et al. 2001). However, other studies have shown platelets function itself may be impaired. P-selectin, a cell adhesion molecule on activated endothelial cells and activated platelets, is reduced by iron deficiency (Yildirim, Orhan et al. 2011).

Significantly perhaps, over expression of P-selectin may also have a role to play in cancer metastasis with P-selectin binding glycoproteins on cancer cells allowing them to seed (Kohler, Ullrich et al. 2010). Heparin mediated P-selectin inhibition has further been shown to reduce metastases in animal models (Gomes, Kozlowski et al. 2015).

No studies have associated iron therapy (and theoretically more functional platelets) with increases in cancer metastases though. Instead, iron repletion appears to be important in preventing thrombocytosis and the associated risk of venous thromboembolism (Loo and Beguin 1999). In inflammatory bowel disease, administration of iron therapy and correction of iron deficiency decreases platelet count (Kulnigg-Dabsch, Evstatiev et al. 2012). Platelet count also decreases with intravenous iron in renal patients (Dahl, Henry et al. 2008).

1.7 Thesis Aims and Objectives

Iron replacement, in particular intravenous iron, is becoming increasingly common in gastrointestinal cancer despite a paucity of evidence for the efficacy and implications of iron therapy in these conditions.

The aim of this thesis is to examine iron in colorectal cancer at a biological level and iron deficiency, manifest as anaemia, in clinical practice during surgery and chemotherapy for oesophagogastric cancer in order to better understand if iron is effective or potentially disadvantageous in gastrointestinal cancer.

Chapter 2 begins by investigating the effect of luminal and systemic iron therapy on colorectal cancer. The hypothesis was in-vivo increases in cellular iron occur through administration of both enteral and systemic iron supplementation. However, systemic iron reduces cancer proliferation and increases apoptosis whereas enteral iron has the opposite effect mediated in the lumen via upregulation of Wnt signalling in APC mutated colorectal cancer.

Chapter 3, using retrospective data analysis, explores the prevalence and effects of anaemia in oesophagogastric cancer to ascertain the scale and impact of anaemia on outcomes during treatment. It also examines independently the role of chemotherapy in the aetiology of anaemia for oesophagogastric cancer and whether anaemia has implications for tumour response during chemotherapy.

Chapter 4 examines systematically whether current literature for iron therapy supports the use of iron therapy for pre-operative anaemia. The primary objective of this systematic review is to evaluate the efficacy of iron therapy (enteral or parenteral) pre-operatively, in reducing the need for allogeneic blood transfusions in patients undergoing surgery. Secondary objectives are to determine the effects of pre-operative iron therapy for anaemia in patients with pre-operative anaemia.

Chapter 5 reports our original pilot randomised control study of iron therapy for anaemia during chemotherapy for oesophagogastric cancer. This prospective randomised control trial will inform the design of a definitive trial to conclude the efficacy of intravenous iron in the treatment of anaemia in oesophagogastric cancer.

Chapter 6 concludes the thesis with implications for future clinical practice and research plus recommendations and advice for iron therapy based upon the original research presented in this thesis.

2 Iron therapy and its effect on iron transport and cell proliferation in human colorectal carcinoma

Abstract

Background

Iron is essential for the proliferation of all mammalian cells. Oral iron (but not intravenous iron) promotes intestinal tumourigenesis in animal models (Seril, Liao et al. 2005). In humans, expression of iron transport proteins are also altered in colorectal cancer (Brookes, Hughes et al. 2006). This study examines whether iron therapy further alters iron transport expression at both protein and mRNA level in human colorectal cancer and whether route of iron therapy changes proliferation and apoptosis in human colorectal cancer in vivo.

Methods

Thirty patients with colorectal adenocarcinoma and pre-operative iron deficiency anaemia (IDA) received iron therapy a minimum of two weeks before surgery as part of a randomised control trial (15 oral ferrous sulphate, 15 intravenous (IV) ferric carboxymaltose) (Keeler, Simpson et al. 2017). Tumour tissue and adjacent normal tissue from surgical resection were collected. These were analysed for iron loading using Prussian blue staining; iron transporter mechanisms (TfR1, DMT1, ferroportin, IREB2) and proliferation apoptosis and DNA damage (Ki67, beta-catenin, p53,

γH2AX) using immunohistochemistry and real time polymerase chain reaction. Expression was compared with paired normal tissue and differences between treatment groups and MSI-status analysed.

Results

Iron loading was increased in tumour tissues compared to normal tissues ($p<0.05$). Distribution of iron deposits appeared to differ depending on route of iron therapy. Iron transporter proteins TfR1, ferroportin and DMT1 had higher immunoreactivity in tumour compared to normal tissue. Further, all three became partially mislocalised to the cytoplasm whereas in normal tissues expression was membranous. In tumour tissue, RT-PCR showed IREB2 was reduced with a corresponding increase in iron importers TFRC (TFR1) ($p<0.05$) and DMT1 ($p< 0.01$) and a reduction in FTH1 encoding ferritin heavy chain for storage ($p<0.05$). FTH1 mRNA levels were significantly reduced with IV iron treatment in tumour tissues compared with those receiving oral treatment ($p<0.001$). No statistically significant differences in the other iron transporters were seen between treatment groups.

All markers of apoptosis, proliferation and DNA damage were more strongly expressed in tumour tissue compared to normal. Differences between treatment groups were seen in these proteins by immunohistochemistry but did not reach statistical significance. The intravenous group had similar Ki67 (35% oral vs 37% IV, $p=0.77$) and similar nuclear (16% oral vs 13% IV, $p=0.6614$) and membranous beta-catenin (67% oral vs 66% IV, $p=0.89$). Lower p53 (45% oral vs 24% IV,

p=0.09) but similar γH2AX (32% oral vs 31% IV, p=0.91) were seen in the intravenous group. c-MYC mRNA fold-change were significantly higher in tumour cells compared with normal tissue (p<0.0001) and increased in both treatment groups with a smaller fold-change in intravenous 3.2 (1.90-4.50 95% CI) versus oral 4.7 (4.50-8.20 95% CI) iron therapy.

Conclusion

Iron loading is seen in colorectal cancer through an increase in the expression of iron transporters compared to normal colon. When the two treatment arms were compared the only significant difference seen was that tumour ferritin expression was higher in those receiving oral iron. This might suggest that iron supplementation with IV iron compared to oral iron lead to a differential effect on intracellular iron loading. Further work is needed to determine if IV iron supplementation is safer due to differential compartmentalisation of the iron within the mucosa, which might avoid tumour cell iron loading.

IV iron therapy does not cause significant increases in proliferation or apoptosis in tumours when compared to oral iron. This was despite IV iron being more effective in correcting clinical anaemia in the clinical trial (Keeler, Simpson et al. 2017). A trend towards increased proliferation and more apoptosis with oral iron therapy when compared to intravenous iron therapy is seen with c-MYC and p53. Our future studies will include a larger sample number and will also focus on the effect of

intravenous therapy on systemic changes of proliferation and apoptosis markers in human colorectal cancer.

2.1 Background

Iron is one of the most central elements to the biology of all humans' cells and subsequently some of the most common diseases of humans (Abbaspour, Hurrell et al. 2014). The most commonly recognised function of iron is incorporated into haemoglobin and myoglobin for oxygen transport (Abbaspour, Hurrell et al. 2014). However, iron is also essential to many vital cellular functions including electron transport in mitochondrial function, cell cycle progression and de novo DNA synthesis (Eriksson, Graslund et al. 1984). These functions may be pivotal in the growth and progression of malignancy. Iron also acts as a powerful oxidant creating reactive oxygen species (ROS) through the Fenton reaction and has been implicated in tissue damage and carcinogenesis (Bystrom and Rivella 2015). Iron is tightly regulated within the body as a result. Iron is also one of the most common deficiencies, especially in colorectal cancer where 60% of patients are iron deficient at presentation (Beale, Penney et al. 2005). This is even amongst Western populations with iron-rich diets. Oral iron and new intravenous iron therapies offer the promise of treatment but neither clinical or basic science evidence exists to fully understand the consequences of elevated luminal or systemic iron on cancer (Beguin, Aapro et al. 2014).

2.1.1 Normal iron metabolism

Total body iron content is 50mg/kg in adult men and 35 mg/kg in adult women (Andrews 2004). Iron is found in all cells but stored predominantly in liver, spleen and bone marrow. Iron can also be stored as water-insoluble haemosiderin, especially in conditions where ferritin iron storage is exceeded (Saito 2014). Haemosiderin stores appear limitless in ferrokinetic studies and may be an important secondary store when ferritin is exhausted to prevent the toxic effects of excess iron in the absence of iron excretion mechanisms in the body (Saito, Tomita et al. 2012).

Absorption

Around 20-25 mg of iron is required per day, mainly for erythropoiesis. The recycling of iron by macrophages from haemoglobin in senescent erythrocytes is the principal source of bioavailable iron (Hentze, Muckenthaler et al. 2004). The remainder is obtained via iron absorption in the gut from either inorganic (non-haem) or haem-bound iron (Zhang and Enns 2009). Only a fraction (5-35%) of dietary iron is absorbed with around 0.9mg of iron in males and 0.8 mg iron in females. This is usually sufficient to balance the small amount of iron is lost daily through shedding epithelial cells of the gut and skin (approximately 1 mg per day) and can increase three to five-fold in the presence of iron depletion to replenish iron stores and meet the larger quantities of iron required during growth or in women after menstruation, in pregnancy and during lactation (Andrews 2004).

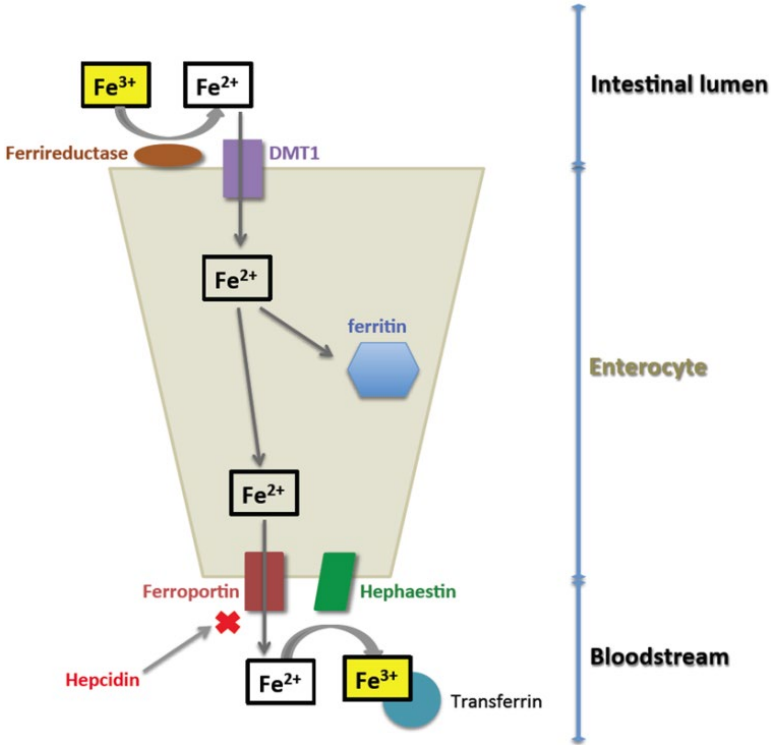


Figure 2.1 Schematic diagram of normal iron absorption

Iron absorption occurs mainly in the duodenum and upper jejunum (Frazer, Wilkins et al. 2005). Dietary non-haem iron is usually ingested in the ferric (Fe^{3+}) form but must be reduced to the ferrous (Fe^{2+}) form to be absorbed. This is achieved by duodenal cytochrome b-like ferrireductase (DcytB) at the enterocyte brush border (McKie, Barrow et al. 2001). Subsequent transport of non-haem iron into the cell is achieved by divalent metal transport 1 (DMT1), an apical proton symporter. Intraluminal pH is an important factor in the absorption of non-haem iron, most likely providing the protons necessary to allow iron transport via DMT1 (Gunshin, Mackenzie et al. 1997, Canonne-Hergaux, Gruenheid et al. 1999). With reduced gastric acidity, iron absorption is impaired, an important factor with the growing use

of proton pump inhibitors (Ajmera, Shastri et al. 2012). Other factors also impact upon the absorption of non-haem iron (Table 2.1).

In enterocytes, high levels of intracellular iron decrease the apical brush border expression of DMT1, the main iron importer. These intracellular levels are mainly determined by the local rate of iron efflux through ferroportin at the basolateral border, itself controlled by systemic hepcidin which links cellular control of iron absorption with body iron homeostasis (Frazer and Anderson 2001).

Heme iron constitutes approximately 30% of dietary iron. Its absorption is pH independent and, unlike non-haem iron, not influenced by gastric pH. Its absorption is poorly understood but is thought to be achieved by receptor-mediated endocytosis via haem-carrier protein 1 (HCP-1) (Latunde-Dada, Takeuchi et al. 2006) and subsequent cleavage from the porphyrin ring by haemoxygenase 1 (HO-1) (Shayeghi, Latunde-Dada et al. 2005). After this, liberated iron follows the same pathways as non-heme iron (West and Oates 2008).

Table 2.1 Factors promoting or inhibiting iron absorption in the gut

Factors inhibiting iron absorption	**Mechanism**
Phytic acid	Insoluble iron complexes
Polyphenols, tannins	Insoluble iron complexes
Bariatric surgery	Duodenal diversion
Divalent metals such as calcium, lead, zinc and magnesium	Competitive inhibition of DMT1
Chronic PPI use	Lowers gastric acid production

Factors promoting iron absorption	**Mechanism**
Ascorbic acid (vitamin C)	Chelation of non-heme ferric iron at acid pH
Citric acid	Chelation of non-heme ferric iron at acid pH
Gastric acid	Promotes stability of ferric iron compounds

Utilisation, Storage and Transport

After absorption, labile iron is rapidly utilised to prevent toxic ROS formation. Iron is either transported into mitochondria, stored as ferritin (through Fe^{3+} binding to apoferritin) or exported from cells via basolateral ferroportin (McKie, Marciani et al. 2000, Brittenham 2013). Although poorly characterised, small iron-chelating metallochaperones, poly (rC) binding protein 1 and frataxin, appear to act as cytoplasmic chaperones for transport of iron into ferritin and mitochondria respectively (Shi, Bencze et al. 2008, Richardson, Lane et al. 2010).

Mitochondria then play a key role in iron utilisation, synthesising the two main forms of functional iron in cells, iron-sulphur clusters (ISC) and haem. These are then exported to act as important co-factors for a host of metabolic processes (Evstatiev and Gasche 2012).

All excess cytosolic iron is stored within ferritin, a spherical 24-unit protein nanocage, made of ferritin heavy chains (FtH) and ferritin light chains (FtL) (Hintze and Theil 2006). Labile ferrous iron is oxidised by ferritin heavy chain or directly by the iron core producing an inert intracellular iron store (Liu and Theil 2005). Iron release in response to low intracellular iron can then occur, although the exact mechanism is still poorly understood (Melman, Bou-Abdallah et al. 2013). Pathways for conversion of ferritin iron to and from haemosiderin iron have not been characterised (Saito 2014).

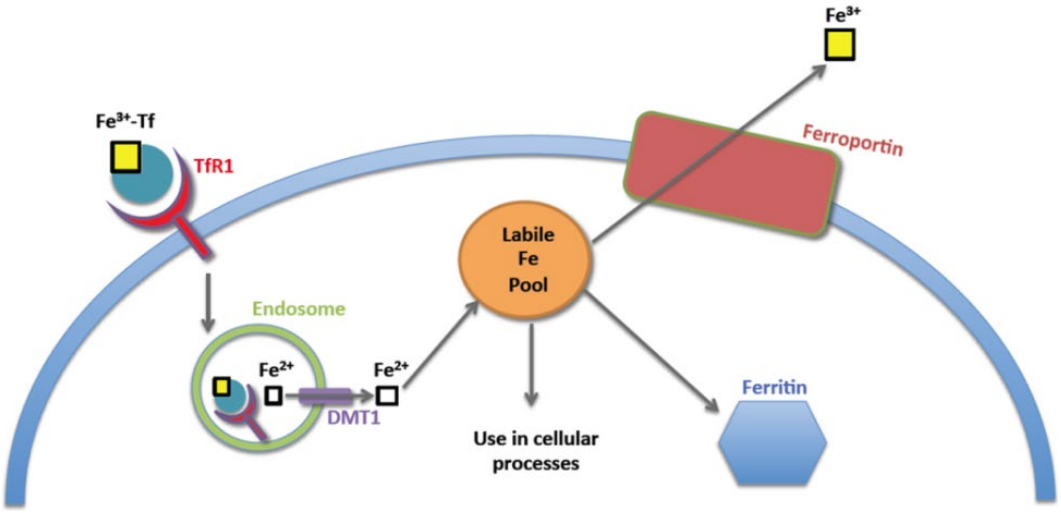


Figure 2.2 Schematic diagram of normal iron transport and storage

Iron export from the cell is facilitated by membrane protein hephaestin (HEPH) or plasma protein ceruloplasmin, which oxidise Fe^{2+} to Fe^{3+} (Vulpe, Kuo et al. 1999, Frazer, Vulpe et al. 2001). Ferroportin then transports iron across the cell membrane. Of note ferroportin is the only known exporter of iron in the body. Once exported, iron can then be transported in the extracellular fluid and plasma, bound to transferrin (Brittenham 2013). Cells then obtain iron via binding of the iron-transferrin complex to transferrin-receptor 1 (TfR1) a ubiquitous membrane bound receptor that facilitates the endocytosis of transferrin (Figure 2.2 Schematic diagram of normal iron transport and storage) (Jeffrey, Basclain et al. 1996, Lee, Oates et al. 2003, Aisen 2004). Iron is then released from the endosome via DMT1 to again form a labile iron pool, which can be taken up for cellular processes as above (Breuer, Shvartsman et al. 2008).

Regulation

Humans have no physiological mechanism for the excretion of iron and given the high potential for toxicity, iron absorption is judiciously controlled and iron distribution tightly regulated within the body (Andrews 2004). Hepcidin is the key mediator for this iron homeostasis (Zhao, Zhang et al. 2013). Hepcidin is a small 25 amino acid peptide produced in hepatocytes of the liver (Ganz 2011). Its control is not entirely understood but iron stores, inflammation, hypoxia and erythropoiesis all influence its secretion (Zhao, Zhang et al. 2013). Hepcidin secretion also appears to be a highly conserved strategy for iron sequestration in the presence of infectious disease, denying bacteria vital iron for their growth (Galaris, Skiada et al. 2008). It is released by the liver (Sharma, Butterworth et al. 2005) in response to inflammatory cytokines (predominantly IL-6 and IL-1) and is inhibited by erythroferrone (Kautz, Jung et al. 2014) (a hormone produced by erythroblasts) effecting a negative feedback mechanism on the regulation of iron (Finch 1994, Zhang and Rovin 2013, Lawen 2015). High levels of hepcidin cause the internalisation of ferroportin thus preventing the export of iron from cells (Knutson, Oukka et al. 2005), inhibiting enteral iron resorption (Nemeth, Tuttle et al. 2004) while simultaneously blocking iron recycling by macrophages from the reticulo-endothelial system (Goodnough, Nemeth et al. 2010). All three types of haemochromatosis are due to abnormalities of this hepcidin-reticulo-endothelial regulatory axis.

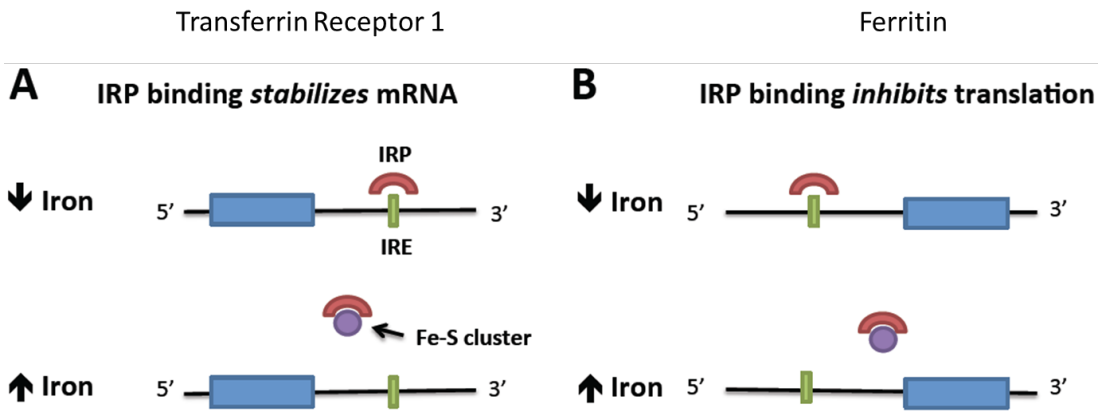


Figure 2.3 Transcriptional control of intracellular iron

Cellular irons levels are registered and controlled at a post-transcriptional level by iron-responsive binding proteins 1 and 2(Constable, Quick et al. 1992). When activated by iron-deficiency, IRPs bind to iron-responsive elements (IREs) in the untranslated regions of messenger RNA (Table 2.2) including TfR1 and ferritin and promote the translation of TfR1 and repression of ferritin, the net effect being to increase the labile intracellular iron pool with decreases in iron export, utilisation and storage (Figure 2.3). When iron levels are high the opposite occurs, with IRP1 binding Fe-sulphur clusters and IRP2 undergoing FBXL5-dependent proteosomal degradation, effecting the repression of TfR1 and translation of ferritin. IRP2 appears to be predominantly involved with iron metabolism, while IRP1 although having some redundancy with IRP2 is bifunctional (also acting as a cytosolic aconitase) involved with erythropoietin production (via HIF2 alpha) and mitochondrial heme and Fe-S cluster formation (via eALAS)(Zhang, Ghosh et al. 2014).

Table 2.2 Targets for iron-responsive binding proteins (adapted from Zhang 2014)

5′UTR of target mRNAs IRP binding inhibits the translation	**3′UTR of target mRNAs** IRP binding increases expression by stabilising the mRNAs
L- and H-ferritin (iron storage protein) – IRP2 FPN1 (iron export protein) – IRP2 Erythroid 5-aminolevulinate synthase (eALAS or ALAS2, the first enzyme for heme synthesis) – IRP1 Mitochondrial aconitase (ACO2, energy production – IRP2 HIF2α (erythropoiesis and hypoxia response) –IRP1 Drosophila succinate dehydrogenase (SDH, citric acid cycle and mitochondrial electron transport chain – IRP2	TfR1 – IRP2 DMT1 (iron import proteins) –IRP2

2.1.2 Iron and colorectal cancer

Epidemiological

Epidemiological data has long suggested a link between increased iron exposure and greater risk of colorectal cancer (Nelson 2001). National Health and Nutrition Examination Survey I Epidemiologic Follow-Up Study showed high transferrin saturation greater than 45% combined with high dietary iron above 18 mg per day were associated with increased risk of cancer (Mainous, Gill et al. 2005). Similar findings for adenoma risk have been shown with ferritin used to measure body iron (Nelson, Davis et al. 1994). Nested case-control analysis of a Women's Health Study in New York analysed 628 patients. Subsite analysis showed proximal colon cancers associated with increasing total iron intake and overall colorectal cancer increasing with iron intake when combined with a high fat diet (Kato, Dnistrian et al. 1999). Wurzelman again identified the proximal colon in females as the subsite for colon cancer most associated with high iron intake. No association was found with rectal cancer. Distal cancers were most closely associated with serum iron. They conclude that iron is associated with colon cancer but mode of epithelial exposure, luminal versus systemic, may be key in pathogenesis (Wurzelmann, Silver et al. 1996).

Further association between iron and colorectal cancer is seen in hereditary haemochromatosis where the HFE gene H63D and C282Y mutations increase iron loading in tissues and was associated with increased risk of colorectal carcinoma

especially in those who consumed high quantities of iron (Shaheen, Silverman et al. 2003).

Conversely, repeated blood donations in healthy donors have been examined as a surrogate measure of iron reduction and although overall showed no association with reduced cancer risk, sub-analysis revealed in men after 3-7 years a reduction in a range of cancers including colorectal (Edgren, Reilly et al. 2008).

Nelson et al. summarised many of these studies in their systematic review and meta-analysis that concluded that iron exposure, body iron stores and hereditary hemochromatosis status were all associated with increased colorectal cancer risk (Nelson 2001).

Animals

Key to theories of iron and its relationship to colorectal cancer are the ability of iron to be absorbed by the colon. This was demonstrated as long ago as 1964 in dogs (Wheby, Jones et al. 1964). Bougle (2002) also demonstrated that beyond the well-recognised role of the duodenum for iron absorption, the proximal colon in rats might be an important site of iron uptake (Bougle, Vaghefi-Vaezzadeh et al. 2002). This is further supported by mouse studies, which demonstrated colon expressed low levels of DcytB but significant levels of DMT-1 and ferroportin especially when mice were iron-deficient (Takeuchi, Bjarnason et al. 2005).

Under experimental conditions for colorectal carcinogenesis, both parenteral and oral iron promoted growth of tumours in rats after 1,2 dimethylhydrazine (DMH). This effect was abrogated by phytates, suggesting a role for dietary fibre in colorectal cancer risk reduction (Nelson, Yoo et al. 1989). Dietary haem iron in particular was highlighted as producing a potent haem-induced cytotoxic factor in rats with subsequent hyperproliferation of epithelium (Sesink, Termont et al. 1999). Liu again demonstrated high iron increases aberrant crypt foci in rats. They also found these effects negated by concomitant high dietary calcium, which also lowers colonic cell proliferation and deoxycholic acid (Liu, Tomotake et al. 2001). Mice ulcerative-colitis models have also shown dose dependant increases in both inflammation and carcinogenesis with oral iron (Seril, Liao et al. 2002). Systemic iron replacement conversely does not increase colitis or carcinogenesis despite adequately replenishing iron stores (Seril, Liao et al. 2005). High dietary iron promoted colorectal cancer growth in mice after the colonotropic carcinogen, azoxymethane (AOM) and did not appear to be due to oxidative stress. However, in the absence of the carcinogen, dietary iron did not initiate tumour formation (Ilsley, Belinsky et al. 2004).

Humans

TfR1 highly expressed in many cancers including melanoma, leukaemia and hepatoma, with a higher rate of iron uptake in these cells (Daniels, Bernabeu et al. 2012). Brookes *et al.* (2006) examined 11 human colorectal carcinomas, 80 paraffin

sections (normal, LGD, HGD, CRC) and 2 cancer cell lines for expression of iron import and export proteins using RT-PCR and western blotting. They demonstrated over-expression of iron importers (DcytB, DMT1 and TfR1, over-expression and intracellular localisation for ferroportin (FPN) and reduced hephaestin (HEPH). Further, they demonstrated increased dysregulation of iron transport along the adenoma-carcinoma sequence from normal through LGD, HGD and carcinoma, with loss of HEPH and FPN expression and increased intracellular iron in more advanced disease. These changes resulted in increased cellular proliferation and E-cadherin repression when studied in in cell lines. (Brookes, Hughes et al. 2006).

2.1.3 Iron in carcinogenesis

Iron has been shown to induce, promote and help progression during carcinogenesis. Induction of cancer, mainly sarcomas, with supra-physiological doses of subcutaneous, intramuscular or intraperitoneal iron has been shown in a variety of animal models (Beguin, Aapro et al. 2014). Locally high concentrations that exceed the capacity to transfer iron to iron body stores are proposed to be the requisite condition for tumour formation (Beguin, Aapro et al. 2014). No studies have shown induction of colorectal cancer.

Promotion of cancer by iron in mouse and rat models treated with cancer inducing agents has been demonstrated in colorectal, oesophageal and hepatocellular cancers. All cancers show increases in tumour incidence or growth with intramuscular, subcutaneous and oral iron (Beguin, Aapro et al. 2014). In contrast, one study compared oral versus intravenous iron supplementation and reported that low dietary iron and intravenous iron supplementation reduced gut inflammation and tumour incidence (Seril, Liao et al. 2005). Progression of established tumours has also been demonstrated in colorectal cancers with tumours in the low iron group growing slower (Hann, Stahlhut et al. 1988).

At the cellular level, iron regulation is central to cell growth and is reprogrammed in cancer to achieve a net increase in the labile iron pool (Torti and Torti 2013). This is achieved by increasing iron importers; DMT1, transferrin receptor 1 (TFR1) (Gatter, Brown et al. 1983, Brookes, Hughes et al. 2006), STEAP3 (Knutson 2007) and

lipocalin 2 (LCN2) (Boult, Roberts et al. 2008, Bao, Clifton et al. 2010); or decreasing iron export through reduced ferroportin or autocrine secretion of hepcidin (Jiang, Elliott et al. 2010, Pinnix, Miller et al. 2010).

Cell cycle

Restriction of iron to iron-dependent enzymes necessary for cell cycle progression and DNA synthesis may be central to the control of cell growth and survival. Iron deplete cells arrest growth in S phases and undergo apoptosis (Lederman, Cohen et al. 1984). This is likely because iron is required to activate the rate-limiting enzyme for de novo DNA synthesis through binding the R2 subunit of ribonucleotide reductase (synthesis of which only occurs in S phase) (Le and Richardson 2002).

Colorectal carcinogenesis

Colorectal carcinogenesis proceeds through one or more of three main mechanisms; chromosomal instability (65-70% sporadic CRC, 1-2% FAP), microsatellite instability (15%, 2-4% Lynch syndrome) and CpG island methylator phenotype (CIMP) (Tariq and Ghias 2016).

The traditional chromosomal instability pathway and adenoma-carcinoma sequence begins with the acquisition of an APC mutation, followed by the activation of the KRAS oncogene and inactivation of tumour suppressor gene TP53. APC mutation in sporadic colorectal cancers occurs in up to 80% (Jasperson, Tuohy et al. 2010). APC mutations lead to accumulation of beta catenin (Clevers 2006). This activates the

Wnt-signalling pathway, the major oncogenic pathway in colorectal cancer (Bienz and Clevers 2000), as a cofactor for T-cell factor (TCF) transcription factors promoting to a cascade of target genes responsible for cell development and stem cell pluripotency (Zhan, Rindtorff et al. 2017). This includes the proto-oncogene c-MYC, a key multifunctional regulator of cell growth (Bienz and Clevers 2000).

Microsatellite instability occurs with mutations in the DNA mismatch repair genes, predominantly MLH1 and MSH2. This in turn can lead to dysfunctional proteins and carcinogenesis as a result (Tariq and Ghias 2016).

Epigenetic changes in DNA promoter regions by methylation especially hypermethylation seen in the CpG island methylator phenotype (CIMP) activate key oncogenes, in particular BRAF, that promote carcinogenesis (Tariq and Ghias 2016).

Mechanistic links between iron metabolism and APC, beta-catenin, c-MYC, p53 and BRAF have been demonstrated.

APC

On the background of APC or beta-catenin mutations, excess iron (both inorganic and heme) may promote tumourigenesis through Wnt-mediated signalling (Brookes, Boult et al. 2008). APC deletion also increases TFR1 and DMT1 in colorectal cancer cell lines and tumourigenesis becomes iron dependant in APC-deficient mice (Radulescu, Brookes et al. 2012).

cMYC

cMYC regulates genes controlling intracellular iron levels through increased IRP2 which represses ferritin and increases TfR1 (Wu, Polack et al. 1999, O'Donnell, Yu et al. 2006). IRP2 promotes tumour growth in vitro, and along with increases in TfR1 appears to have effects that are independent of iron metabolism including through c-MYC and ERK1/2 phosphorylation (Maffettone, Chen et al. 2010).

KRAS

No mechanistic studies exist for KRAS and iron metabolism but an association between heme iron intake and KRAS mutations have been demonstrated (Gilsing, Fransen et al. 2013).

P53

Induction of the tumour suppressor gene p53 in lung and colorectal cancer cells lines increases ferritin and decreases TfR1 by inactivating IRPs, suggesting an iron mediated pathway, independent of cyclin-dependant kinase inhibitors, for cell growth arrest through iron restriction (Zhang, Wang et al. 2008). p53-dependent transcriptional activation of hepcidin has also been demonstrated and may link increased expression of wild-type p53 seen in many cancers with anaemia of chronic disease (Weizer-Stern, Adamsky et al. 2007). Given p53 mutation is present in almost half of cancers this could be central to iron metabolism in cancer (Levine, Momand et al. 1991).

BRAF

Recently, an association with BRAF mutations and IRP2, likely through MEK activation, has also been demonstrated (Horniblow, Bedford et al. 2017).

The IVICA study

The IVICA (IntraVenous Iron for Colorectal Adenocarcinoma) study was a multicentre randomised controlled trial comparing oral versus intravenous iron for pre-operative anaemia in colorectal cancer (Keeler, Simpson et al. 2017). Anaemic adult patients with non-metastatic colorectal adenocarcinoma were recruited preoperatively and randomised to receive either oral ferrous sulphate (OI) or intravenous ferric carboxymaltose (IVI).

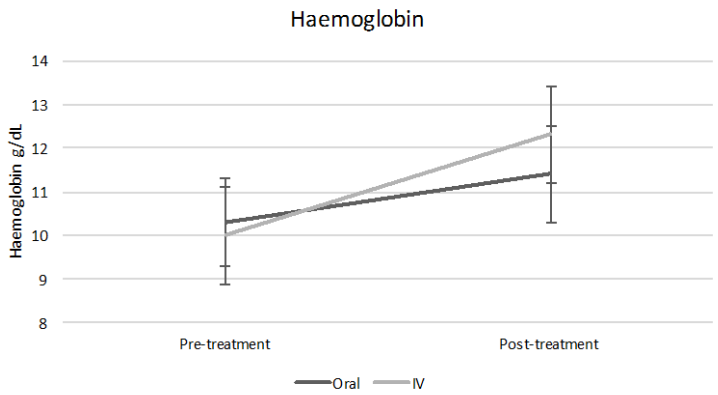
After a median duration of treatment (OI 21 days IQR 15-33; IVI 21 days IQR 15-34, P=0.75), clinical outcomes of the trial (Figure 2.4) demonstrated haemoglobin levels were higher on the day of surgery in IVI (119 g/L IQR 112-129 vs OI 112 g/L IQR 98-120, P<0.01). Median preoperative haemoglobin change in patients not transfused preoperatively was higher in IVI (150 g/L IQR 9-26 vs OI 5g/L IQR-1-13, P<0.01). There were fewer anaemic patients at surgery in the IVI group after treatment (75% vs 90%, P<0.05). There were significant differences in mean units transfused until the 7th postoperative day in patients who underwent surgery with intraoperative blood losses of less than 2 litres (OI 0.6u 95%CI 0.23-0.96; IVI 0.16u 95%CI 0.01-0.3, P< 0.05) and less IVI patients transfused (10 % vs 25%, P<0.05).

Importantly, ferritin levels were significantly higher in the intravenous group at surgery (median 558 (i.q.r. 330–1085) μg/l versus 27·5 (17–51·5) μg/l in oral group; P <0·001), despite parity in recruitment ferritin levels (P =0·224). This same relationship was evident with TSAT levels at surgery (median 19 (16–29) and 9 (5–

14) per cent respectively; P <0·001), despite TSAT levels being significantly lower in the intravenous group at recruitment (P =0·039).

As part of this trial serum (pre-treatment, post-treatment pre-operatively and post-treatment post-operatively) and tissue (colorectal adenocarcinoma and paired normal tissue) were collected to allow comparison of the effects in vivo iron exposure on human colorectal carcinoma via different routes of administration.

A



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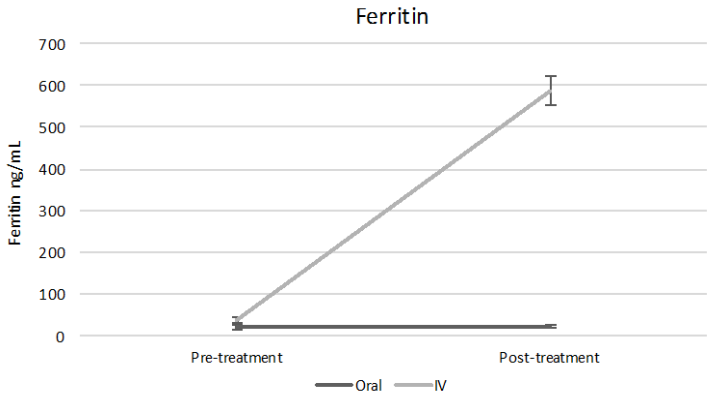

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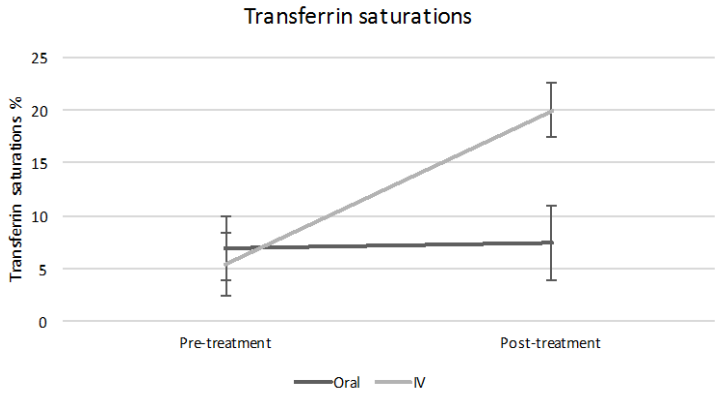


Figure 2.4 IVICA study outcomes (A) Haemoglobin change (B) Ferritin and transferrin saturation change

2.1.4 Summary

In summary, iron is central to cell growth, intimately linked with key pathways for colorectal carcinogenesis and closely associated with promotion and progression of colorectal cancer. Animal studies have demonstrated route of iron administration is important. No studies have allowed for the comparison of oral and intravenous irons effects on colorectal adenocarcinoma in humans until the IVICA study.

2.1.5 Hypothesis

We hypothesised that iron transporters (TfR1, DMT1, ferroportin) were altered in all colorectal adenocarcinomas to promote increases in the labile iron pool.

Further, oral iron induces Wnt activity and c-myc with repression of Ecad and increase tumour motility in non-MSI tumours. In contrast, IV iron does not do this and therefore in this group we do not see an induction of c-myc or repression of Ecad.

MSI tumour groups, whether treated by oral or IV, would not show these effects i.e. no induction in c-myc or repression in ecad.

2.2 Patients, Materials and Methods

The study was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Ethical approval was granted by the East Midlands (Derby) research ethics committee on behalf of the Health Research Authority, reference 13/EM/0069. The study was registered with the Medicines and Healthcare Regulatory Agency, clinical trials.gov (NCT01927328) and EudraCT (2013-000209-22).

2.2.1 Patients

Patients were recruited from two groups in the IVICA trial, randomised to oral or intravenous iron therapy for pre-operative anaemia at least two weeks prior to curative surgical resection. All patients had histologically proven colorectal adenocarcinomas. For both groups information about age, site and size of tumour, histological grade and stage were available. 30 patients were selected based upon previous human studies showing significant differences with this sample size. All patients had to have complete sets of biological samples; tissue and serum from all time points.

2.2.2 Tissues

Tumour tissue samples for gene expression studies and immunohistochemistry were taken intra-operatively for 30 patients. Paired normal tissues from the same patient were also collected. These tissues were either snap frozen and stored at -80°C prior to RNA extraction, cDNA synthesis and RT-PCR or paraffin embedded to be used for immunohistochemistry.

2.2.3 Serum

Blood samples were taken at recruitment (pre-treatment pre-operatively), on the day of surgery (post-treatment pre-operatively) and at follow up 8-12 weeks after surgery (post-treatment post-operatively). Samples were left at room temperature for 30 minutes to clot before centrifugation. Samples were centrifuged at 10,000g for 5 minutes and the serum extracted and stored at -80°C prior to subsequent ELISA assays for P-selectin and hepcidin.

2.2.4 mRNA extraction

Messenger RNA was extracted from snap frozen tissue using the ThermoFisher Scientific mirVana ™ miRNA isolation kit. Lysis and disruption was achieved with three successive additions of denaturing lysis solution and centrifugation. miRNA additive was then added and organic extraction achieved with acid phenol chloroform. Final RNA isolation was performed using a glass-fibre pass through filter and RNA eluted with a low ionic strength solution. RNA purity and

concentration was then determined using a NanoDrop™ 2000/2000c Spectrophotometers (ThermoFisher) and Nanodrop2000/2000c v1.4.2 software was used for checking quality of RNA using A260/280 and A260/230 ratio to measure nucleic acid purity. An A260/280 ratio of ~2.0 was accepted as 'pure' for RNA and A260/230 in the range of 2.0–2.2 consider free of other contaminants.

2.2.5 cDNA synthesis

cDNA synthesis was performed using the Qiagen ® QuantiTect ® Reverse Transcription kit. Genomic DNA was eliminated with genomic DNA wipeout buffer. Template RNA (1 microlitre) was then mixed with reverse-transcription master mix (Quantiscript reverse transcriptase, buffer and dNTP primer mix) as per manufacturer's instructions to give a total volume of 20 microliters and incubated at 42°C for 15 minutes then denatured at 95°C prior to storage at -20°C.

2.2.6 Real time polymerase chain reaction (RT-PCR)

Real time PCR was performed for MYC, IREB2, FTH1, TFRC, SLC11A2, CDH1 using GAPDH control as an internal standard (Thermo Fisher Scientific). Probes and primers are listed in Table 2.3. Probes were validated for specificity using National Center for Biotechnology Information (NCBI) Primer BLAST software. All primers were between exon-exon boundaries. TaqMan ™ Gene expression master mix (Thermo Fisher Scientific) containing AmpliTaq Gold® DNA Polymerase (Ultra Pure), Uracil-DNA glycosylase, dNTPs with dUTP, ROXTM Passive Reference and optimised buffer components, was added to 100 ng of cDNA and dH20 to form a 25

microL reaction mixture. Reactions without cDNA were included as negative controls. All reactions were performed in triplicate. Two-step RT-PCR was conducted using a 7500 Fast Real Time PCR System (Applied Biosystems, UK) and analysis was performed using 7500 Software v2.0.6 (Applied Biosystems, UK).

Optimisation

Primers that span two exons were used to avoid genomic DNA contamination. An additional genomic DNA wipeout buffer step was used. A 'master mix' was employed to reduce sample-to-sample and well-to-well variability and also to improve reproducibility. No template wells were run in parallel to rule out contamination and high quality RNA was ensured using the Nanodrop step above.

Table 2.3 RT-PCR probes

Gene name	Function	Primer, 5'-3'		Probe (5'FAM 3'TAMRA)
		Forward	Reverse	
SLC11A2 (DMT1)	Iron transport	CCA TAT GAA ATA TAA AAT GAA GAG ACA CCT A	CCC CTC TTA ACT TCC ACT GAG AAA	CTC TAT CAG GCT TAG GAT TCT TTG AAC TTA TTT CCA CTT T
SLC40A1 (FPN)	Iron export	AGC AAA TAT GAA TGC CAC AAT ACG	CAA ATG TCA TAA TCT GGC CAA CAG	AGG ATT GAC CAG TTA ACC AAC ATC TTA GCC CC
Ferritin	Iron storage	GGA ACA TGC TGA GAA ACT GAT GAA	CAT CAC AGT CTG GTT TCT TGA TAT CC	CCA ACG AGG TGG CCG AAT CTT CCT T
TFRC (TFR1)	Iron transport	CGT GAT CAA CAT TTT GTT AAG ATT CA	CCA CAT AAC CCC CAG GAT TCT	AAA GAC AGC GCT CAA AAC TCG GTG ATC ATA G
E-Cadherin	Cell adhesion and cell-cell signalling	GGC GCC ACC TCG AGA GA	TGT CGA CCG GTG CAA TCT T	AAA TTC ACT CTG CCC AGG ACG CGG

2.2.7 Immunohistochemistry

Immunohistochemical analysis was conducted at the Medical University of Vienna. Paraffin embedded tissue from matched normal and tumour samples (n=30) were utilized for IHC analysis. Paired normal and tumour tissue were mounted on one slide. Primary antibodies used are described in Table 2.4 Immunohistochemistry antibodies. Tumours were also analysed for loss of MLH1 and MSH2 to determine microsatellite instability (MSI) and microsatellite stable (MSS) phenotypes. For each slide, paraffin was melted at 55-60 °C for 30 minutes. Tissue was then dewaxed twice with xylol for 5 minutes each time. Rehydration was performed with 100% ethanol (1 x 5 minutes, 1 x 1 minute), 96% ethanol (1 minute), 80% ethanol (1 minute), 70% ethanol (1 minute) and VE H_2O (1 minute). Endogenous enzymes were blocked with methanol and 15% H_2O_2 (15 minutes) and then slides washed with tap water 15 times. Antigen retrieval was achieved with heat induction using citrate buffer (450ml dH2) 41ml B 9 ml A) for 20 minutes at 800 watts using a microwave oven. Slides were then washed with tap water 5 times. Non-specific binding was block using 200 µL per slide of normal goat serum (10% goat + 3% BSA in TRIS (BSA 1.5g) 0.01% sodium acetate) for 1 hour in histochamber. 200 µL per slide primary antibody in blocking buffer was then added at the optimised dilution (see Table 2.4) and incubated overnight at 4°C. Slides were then washed with TRIS buffer (2 x 5 minutes). The species specific biotinylated secondary antibody was then added and incubated for 30 minutes (see Table 2.4). Avidin-biotin

enzyme complex (ABC-HRP reagent, 2.5ml TRIS, 1 drop A and 1 drop B, Vectastain ABC Kit, PK-6100; Vector Laboratories) was then added and incubated for 30 minutes. Staining was visualized using 3,3′-diaminobenzidine (32750; Fluka) and nuclear counterstaining was performed using hematoxylin. Slides were dehydrated and embedded in Histofluid (6900002; Marienfeld, Lauda Koenigshofen, Germany). Staining with secondary antibody alone was also performed as control. Images were taken at 40x to 400x magnification using an Olympus BX51 microscope.

Optimisation

A dilution series was performed to determine optimal antibody dilution for each antibody.

2.2.8 Perls Prussian blue staining

Tissue were mounted, rehydrated and dehydrated as per immunohistochemistry. Solution of 0.7g ferrocyanide in 70 ml 0.5% Hcl was applied at room temperature for 60 minutes. Counterstain with nuclear fast red was performed for 1 minute.

Table 2.4 Immunohistochemistry antibodies

Target	Function	Species	Dilution	Company (Cat #)
TfR1	Iron transport	Mouse	1:200	Thermo scientific #13-6800
DMT1	Iron transport	Rabbit	1:250	Biorbyt #orb5976
Ferroportin	Iron transport	Rabbit	1:500	Novus biologicals NBPI 21502
Prussian Blue	Iron loading	N/A	N/A	Sigma-Aldrich #03899
PAK-1	Kinase phosphorylates β catenin	Rabbit	1:50	CST (cell signalling technology)#9664
γH2AX	Histone DNA damage	Rabbit	1:500	CST #9718
Ki67	Proliferation	Rabbit	1:1000	Abcam: ab15580
Cleaved caspase 3	Apoptosis	Rabbit	1:2000	CST #9664
P53	Apoptosis	Rabbit	1:500	Santa Cruz #SC-6243
Beta-catenin	Wnt signalling	Mouse	1:300	BD transduction laboratories #610153

2.2.9 ELISA

Serum were analysed using pre-coated 96 well microplates with antibody specific for human Hepcidin-25 (Peninsula Laboratories® S-1328) and human soluble P-Selectin (Sigma-Aldrich® RAB0426 Sigma) as per manufacturers guidelines. 100 µl of standards and samples were pipetted into the wells in triplicate. Wells were covered and incubated for 2.5 hours at room temperature. The solution was then discarded and microplate wells washed 4 times with 300 µl wash solution. After the last wash, remaining wash was aspirated, the plate inverted and blotted against clean paper towels. 100 µl biotin-conjugated antibody specific for the target was then added to the wells and incubated at for 1 hour at room temperature with gentle agitation. After washing as before, 100 µl avidin conjugated Horseradish Peroxidase (HRP) was added to the wells for 45 minutes. Following a further wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and colour allowed to develop in the dark for 30 minutes at room temperature. 50 µl stop solution was then added to each well and absorbance measured at 450 nm immediately using a Glomax Multi+ Detection System (Promega, UK) plate reader.

2.2.10 Analysis

RT-PCR

Gene expression was normalised to GAPDH and represented as ΔCt and comparison of gene expression compared between tumour and paired normal to give a ΔΔCt

value. Changes in gene expression were represented a negative log of ΔΔCt and 1 regarded as normal.

Immunohistochemistry

Qualitative analysis was performed by two assessors, Oliver Ng and Rayko Estaviev, for all tissue under 10×, 20× and 40× magnification to confirm tissue histology and identify representative areas of normal and tumour tissue. Further, analysis of localisation of staining to cell membrane, cytoplasm and nucleus of crypts cells was performed.

Semi-quantitative analysis of immunoreactivity was performed by three assessors. Methods for semi-quantitative analysis were determined prior to analysis based upon function and localisation of target proteins, see Table 2.5. Two main techniques were used; ratio of positive cells/nuclei in a crypt to all cells/nuclei in a crypt or an analysis of immuno-reactivity with intensity scored from 0-2 analysed on high magnification. Five high magnification fields were assessed per sample. An average score of immunoreactivity was calculated. An independent assessor, resolved discrepancies between scoring. All assessors were blinded to the treatment received.

ELISA

Association between absolute pre-treatment and post-treatment hepcidin levels and mean haemoglobin change from recruitment to day of surgery were tested using the Mann Whitney U test for both oral and intravenous iron groups separately and

combined. Groups were also dichotomised into normal and high hepcidin groups and compared for response, with high hepcidin defined as > 56 ng/mL (Ashby, Gale et al. 2009).

Changes in soluble P-Selectin levels pre-treatment and post-treatment within oral and intravenous iron groups were compared for statistical significance using a pared samples test. Comparisons between groups who received oral iron and intravenous iron were conducted using an independent samples t-test and a p-value of <0.05 was considered significant.

Table 2.5 Immunohistochemistry semi-quantitative assessment

Target	Localisation	Semi-quantitative	Qualitative
TfR1	M C a	Intensity and extent of staining* for each locale as percentage	Localisation
DMT1	M C N a	Intensity and extent of staining* for each locale as percentage	Localisation
Ferroportin	M C b	Intensity and extent of staining* for each locale as percentage	Localisation
Prussian blue	C m S	Intensity and extent of staining* for each locale as percentage	Localisation
PAK-1	C N	Intensity and extent of staining* for each locale as percentage	Localisation
γH2AX	N	Percentage of positive nuclei	
Ki67	N	Percentage of positive nuclei	
Cleaved caspase 3	N	Percentage of positive cells	
p53	N C	Percentage of positive nuclei	
B-catenin	M C N	Intensity and extent of staining* for each locale as percentage	Localisation

M membranous, C cytoplasmic, N nuclear, b basal, a apical, m macrophages, S stroma, * Intensity of staining 0 No staining, 1 weak staining, 2 strong staining e.g. 60% strong and 40% weak cytoplasmic staining = (60 x 2) + (40 x 1) = 160 out of 200 or 80%

Statistics

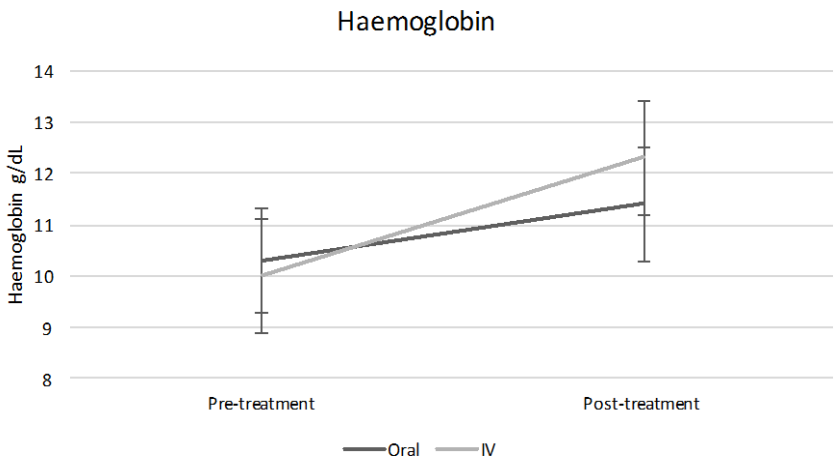
Statistical differences between scores were tested using SPSS (Version 23, IBM). Results were tested using the Shapiro-Wilks normality test. Results for RT-PCR are reported as means with standard deviations. Paired samples T test was used to test for significance between normal and tumour tissue. Independent samples T test was used to test for statistical differences between treatment groups. Linear regression analysis was performed comparing IREB2 expression with other mRNA expression and statistical significance tested with ANOVA. Results for all immunohistochemistry are reported as mean and standard deviation and displayed as dot-plots. Paired samples T test was used to test for differences between normal and tumour, Independent samples T test was used to test for differences between and treatment groups. A p-value of <0.05 was considered significant.

2.3 Results

2.3.1 General

The thirty patients for whom tissue was analysed with RT-PCR and immunohistochemistry from the IVICA trial all had pre-operative iron deficiency anaemia (haemoglobin 103 g/L oral iron group and 100 g/L intravenous iron group, p=0.62) and colorectal adenocarcinoma. Fifteen patients were treated with oral ferrous sulphate 200 mg twice a day for a median of 25 days before surgery and fifteen patients with a single dose intravenous ferric carboxymaltose 1000 mg. All patients reported compliance with oral iron therapy. Patients were similar at recruitment for age, sex, Dukes stage, haemoglobin, ferritin and transferrin saturations (Table 2.6). Patients had a significantly higher increase in ferritin in the intravenous iron group (median ferritin 588 ng/mL IV versus 22 ng/mL oral, p=0.001), Figure 2.5. Haemoglobin and transferrin saturations were also higher in the intravenous iron group by day of surgery (Table 2.6). Patients in the oral iron group remained iron deficient, ferritin 22 ng/mL. No patients had a pre-operative transfusion.

(a)



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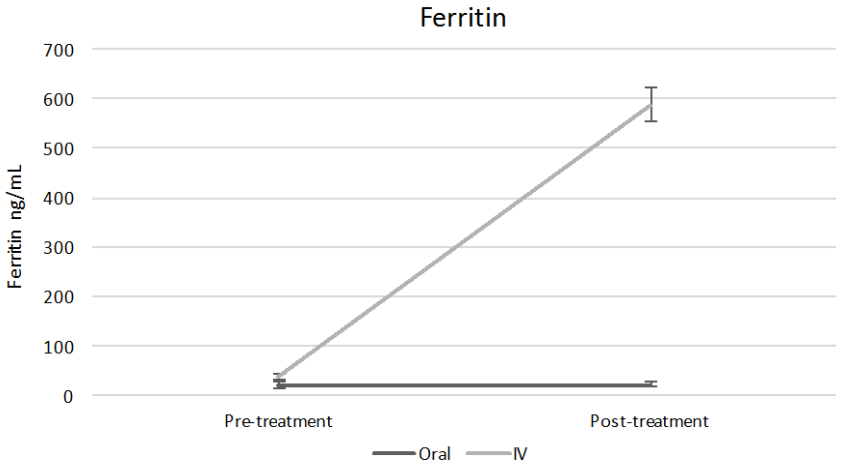

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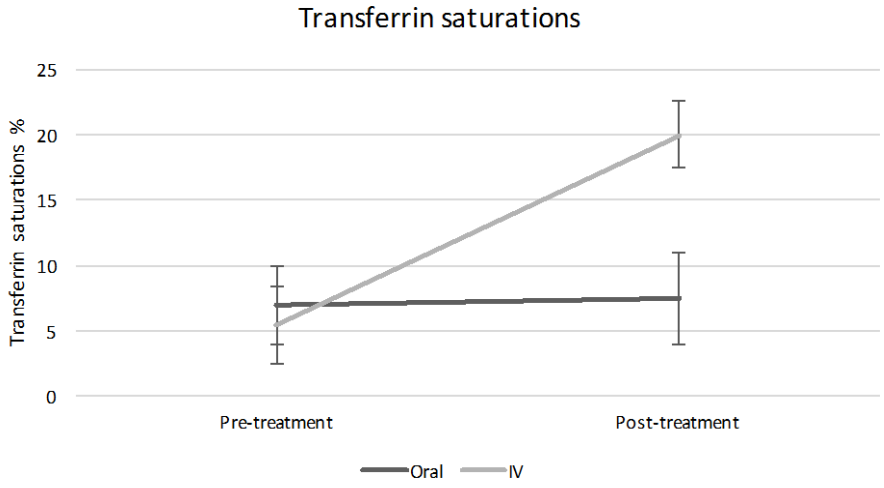

Figure 2.5 Clinical outcomes (a) Haemoglobin (b) Ferritin (c) Transferrin saturations

Table 2.6 Clinical data

	Oral iron (n=15)	IV iron (n=15)	P
Age (years)[1]	74 (46-82)	74 (53-85)	0.662
Sex ratio (M:F)	10:5	8:7	0.758
Haemoglobin (g/L)[2]			
Recruitment	103 (10)	100 (17)	0.616
Day of surgery	114 (11)	123 (21)	0.131
Ferritin (ng/mL)[3]			
Recruitment	21 (14-45)	39 (12-204)	0.384
Day of surgery	22 (16-51)	588 (318-1415)	0.001*
Transferrin saturations (%)[3]			
Recruitment	7 (4-16)	5.5 (2-14)	0.301
Day of surgery	7.5 (4-13)	20 (17-24)	0.290
Dukes n (%)			
A	0	2 (13.3)	
B	9 (60)	10 (66.7)	0.845
C	6 (40)	3 (20)	
Site n (%)			
Caecum	6 (40)	9 (60)	
Ascending colon	0	0	
Hepatic flexure	1 (6.7)	1 (6.7)	
Transverse colon	3 (20)	0	0.938
Splenic flexure	2 (13.3)	1 (6.7)	
Descending colon	0	1 (6.7)	
Sigmoid	2 (13.3)	0	
Rectum	1 (6.7)	3 (20)	
Iron therapy	Ferrous sulphate 200mg BD PO	Ferric carboxymaltose 1000mg Single dose IV	-
Days before surgery[1]	25 (16-36)	26 (15-34)	0.798
Transfusions	0	0	-

1 Median (range) 2 Mean (Standard deviation) 3 Median (Interquartile range) * $p<0.05$

2.3.2 Microsatellite instability

Tumours were analysed for loss of MLH1 and MSH2 to determine microsatellite instability (MSI) and microsatellite stable (MSS) phenotypes. 23% of tumours had MSI, with five cancers demonstrating loss of MLH1 and two cancers demonstrating loss of both MSH2 and MLH1. Of these seven tumours, four of these MSI tumours were in the oral iron group and the remaining three in the intravenous iron group. MSS and MSI tumours were compared for all RT-PCR (Table 2.10) and immunohistochemistry (Table 2.11) outcomes, and across treatment groups. Sub-analysis are discussed below.

2.3.3 Cellular proliferation and apoptosis

Differences in tumour proliferation and apoptosis in human colorectal cancer between patients receiving oral or intravenous iron therapy were compared. c-MYC mRNA was examined using RT-PCR. Protein markers for proliferation (Ki67, beta-catenin, PAK-1), apoptosis (p53, CC3) and DNA damage (γH2AX) were analysed using immunohistochemistry. Expression was compared with paired normal tissue and differences between treatment groups analysed.

In brief, all markers of apoptosis, proliferation and DNA damage were more strongly expressed in tumour tissue compared to normal. No significant differences between tumours in oral and intravenous iron groups were seen. Exclusion of microsatellite instability phenotype did not materially alter results between treatment groups, Table 2.10 and Table 2.11.

Proliferation

c-MYC mRNA fold-change were significantly higher in tumour cells compared with normal tissue ($p<0.0001$) and increased in both treatment groups (Figure 2.6) with a smaller fold-change in intravenous 3.2 (1.90-4.50 95% CI) versus oral 4.7 (4.50-8.20 95% CI) iron therapy (Table 2.8). c-MYC mRNA expression also correlated with IREB2 mRNA expression ($R^2=38\%$, $p=0.010$), Figure 2.7.

Ki67, a widely used protein marker of proliferation, had generally low immuno-reactivity in normal tissue with <10% of normal tissues showing any nuclei positive for Ki67. Tumour, as expected, showed higher nuclear immuno-reactivity than normal tissue. There was a significant difference between normal and tumour tissue ($p=0.002$), Figure 2.9. No difference was seen between treatment groups with tumour tissue in the intravenous iron group showing similar Ki67 to the oral iron group (35% oral versus 37% IV, $p=0.77$), Table 2.9.

Membranous and cytoplasmic β-Catenin, the main intracellular signal transducer in the Wnt signalling pathway, had immunoreactivity in both normal and tumour tissues, Figure 2.9. Nuclear staining was only seen in some tumour tissues and no normal tissues, Figure 2.9. No discernible difference was seen between treatment arms.

Both cytoplasmic and nuclear immunoreactivity for β-Catenin showed significant differences between normal and tumour tissue ($p<0.0001$ and $p=0.0001$ respectively), Figure 2.9. No differences were seen between treatment groups in

either normal or tumour tissue for membranous, cytoplasmic or nuclear staining, Figure 2.9 and Table 2.9 with similar nuclear (16% oral versus 13% IV, p=0.6614) and cytoplasmic beta-catenin (57% oral versus 48% IV, p=0.24).

p21-activated kinase 1 (PAK1), a downstream effector of GTPases overexpressed in many tumours, showed cytoplasmic immunoreactivity in all tissues, both normal and tumour. There were no significant differences between normal and tumour or between treatment groups, Figure 2.9.

No difference was seen in microsatellite instability sub-analysis between treatment groups or in proliferation between stable and microsatellite instability groups, Table 2.10 and Table 2.11.

Apoptosis and DNA damage

Immunohistochemical staining for p53, one of the key tumour suppressor genes mutated in cancer and involved in apoptosis, revealed no positive nuclei in the majority of normal tissue (89%). In comparison, only 20% of tumours had no positive nuclei and there was a statistically significant difference in immunoreactivity between normal and tumour tissue ($p<0.0001$). Between treatment groups, lower p53 immunoreactivity was seen in the intravenous iron group but this was not statistically significant (45% oral versus 24% IV, $p=0.09$). Microsatellite instability was associated with higher p53 compared to the microsatellite stable group ($p=0.011$), Table 2.11.

Cleaved caspase 3 protein, an apoptosis marker, was not seen in normal tissue. All tumours except one had some cleaved caspase 3 immunoreactivity, Figure 2.10 (normal versus tumour $p<0.0001$). However, there were no differences seen between treatment groups, Figure 2.10 and Table 2.9 ($p=0.404$).

DNA double strand breaks, detected through γH2AX protein staining showed no or low nuclear immuno-reactivity in all normal tissue. Tumour, as expected, had higher immuno-reactivity but this was not universal with eight tumours showing <10% positive nuclei. There was a statistically significant difference between normal and tumour tissues ($p<0.0001$), Figure 2.10. No difference was seen between treatment groups in normal or tumour tissue (32% oral versus 31% IV, $p=0.91$)), Figure 2.10.

2.3.1 Tissue iron loading and storage

Tissue iron loading and cellular iron storage were compared in normal and tumour tissue and between treatment groups using Perls Prussian blue staining and RT-PCR for the FTH1 gene.

Iron loading

Prussian blue staining for iron was increased in tumour tissues compared to normal tissues ($p<0.05$). Distribution of iron deposits appeared to differ depending on the route of iron therapy. Perls Prussian blue staining was seen in only 15% (n=4) of normal tissues. Only one case in the normal group had strong Prussian blue staining on the luminal side of crypts, Figure 2.14. This patient was treated with oral iron. Other normal tissues showed Prussian blue in the connective tissue and stromal tissue and had intravenous iron treatment.

In contrast, Perls Prussian blue staining was seen in 63% (n=17) of tumour tissues. Staining in tissue was commonly seen in stroma and adjacent connective tissue rather than within crypt cells. This appeared more frequently with intravenous iron. Three cases had staining in crypt cells (two treated with oral iron and one with intravenous iron), Figure 2.14.

Cellular iron storage

FTH1, the ferritin gene that encodes the heavy subunit of ferritin, was reduced in tumours compared to normal tissues with a similar reduction in the IV iron group 0.43 versus oral iron group 0.44, p=0.977 (Table 2.8).

2.3.2 Cellular iron transport and iron regulation

Differences in iron transport mechanisms and regulation in human colorectal cancer between patients receiving oral or intravenous iron therapy were compared. Paired normal and tumour tissue from surgical resection were analysed for iron genes (TFRC, SLC11A2 and IREB2) using real time PCR and iron transport proteins (TfR1, DMT1 and ferroportin) using immunohistochemistry. Expression was compared between normal and tumour and treatment groups.

Iron transport

In overview, iron transport genes SLC11A2 (encodes DMT1) and TFRC (encodes TfR1) mRNA were increased in tumours along with iron transporter proteins TfR1, DMT1 and ferroportin which had statistically higher immuno-reactivity in tumour compared to normal tissue (Table 2.8 and Table 2.9). Further, all three proteins became partially mislocalised to cytoplasm whereas in normal tissues expression was membranous. No significant differences in the iron transporters were seen between treatment groups and sub-analysis of microsatellite instability did not alter these results.

Expression of TFRC gene which encodes the transferrin receptor protein 1 (TfR1) was increased in tumour compared to normal tissue and in both oral 2.38 (SD 1.38) and intravenous 1.49 (SD 0.89) iron groups, but no statistical difference was seen between groups, p=0.442. Transferrin Receptor 1 (TfR1) protein in normal tissues showed immuno-reactivity at the luminal border of crypts with virtually no immuno-reactivity at the base of crypts (Figure 2.15). In comparison almost all tumours showed widespread cytoplasmic immunoreactivity to TfR1 with no specific localisation to the luminal border, Figure 2.15. There was a statistically significant difference between normal and tumour tissue with tumour tissue showing more intense and extensive immunoreactivity for the TfR1 protein, Figure 2.11 and Table 2.9, $p<0.05$. No difference was seen between treatment groups in tumour tissue (p=0.936), see Table 2.9.

Expression of SLC11A2 mRNA encoding DMT1 was increased in tumour compared to normal, with a similar increase in both oral 1.86 (SD 1.06) and intravenous 1.50 (SD 1.16) iron groups, p=0.974, Table 2.8. Divalent metal transporter 1 (DMT1) protein displayed granular cytoplasmic staining in normal and tumour tissue. In normal tissue DMT1 immunoreactivity was predominantly in the apical cytoplasm and towards the luminal border of the crypts. DMT1 immunoreactivity was more uniform with no polarity in tumour tissue, Figure 2.15. Despite qualitative differences between normal and tumour tissue no statistically significant difference in semi-quantitative scoring was seen for DMT1 protein (p=0.465), Figure 2.11. No

difference between normal and tumour was seen in the intravenous iron group (p=0.376) but were present in the oral iron group (p=0.013), Figure 2.11.

Ferroportin (FPN) in normal tissue showed membranous and cytoplasmic staining predominantly in the basolateral membrane, Figure 2.15. Tumour showed stronger staining and loss or mis-localisation including ferroportin immunoreactivity at the apical membrane, Figure 2.15. Semi-quantitative analysis revealed significant differences between normal and tumour tissue for ferroportin protein with more extensive and intense staining in tumour ($p<0.001$), Table 2.9. No differences between treatment groups were seen in normal or tumour tissue (p=0.616), Figure 2.11 and Table 2.9.

2.3.3 Iron regulation

In tumour compared to paired normal tissue, RT-PCR showed IREB2 was reduced but with an increase in iron importers TFRC (TFR1) and DMT1 and a reduction in FTH1 encoding ferritin heavy chain for storage. FTH1 mRNA levels were significantly reduced with IV iron treatment in tumour tissues compared with those receiving oral treatment ($p<0.001$). Microsatellite stable versus microsatellite instability groups were significantly different with an increase IREB2 (p=0.001) and SLC11A2 (<0.05) in the MSI group, Table 2.10.

IREB2 mRNA levels positively correlated with MYC mRNA levels (R^2=39%, ANOVA p=0.010) and SLC11A2 (R^2=67%, $p<0.001$, Figure 2.7. No correlation was found between FTH1 (p=0.974) and TFRC (p=0.471).

2.3.4 Right versus left sided sub-analysis

Tumours and paired normal tissue from the right colon (defined as caecum to hepatic flexure) were compared to left colon (splenic flexure to rectum) for all RT-PCR (Table 2.12) and immunohistochemistry (Table 2.13) outcomes. No differences were seen between right and left sided normal or tumour tissues, including when stratified by treatment groups.

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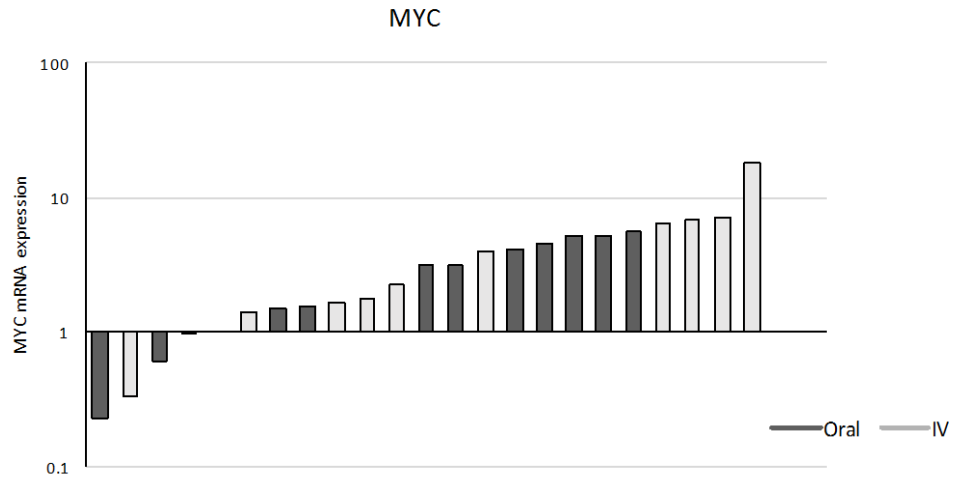

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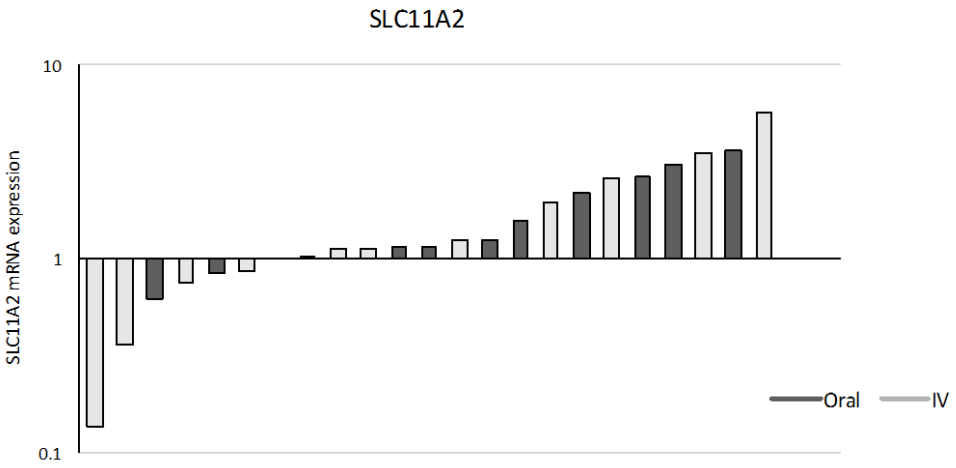


(c)

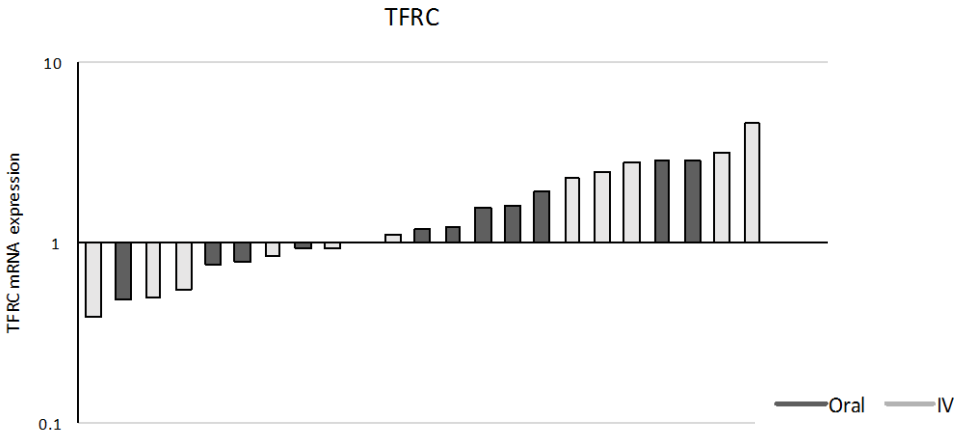
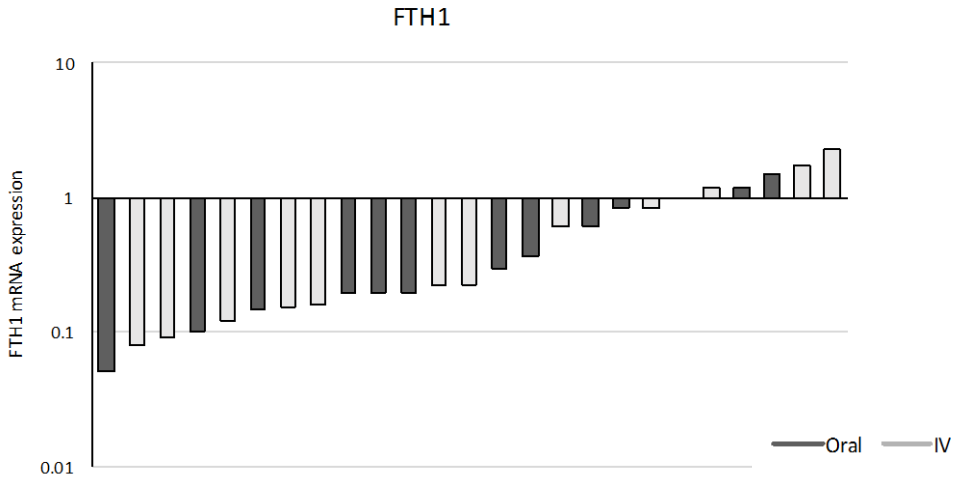


Figure 2.6 Real-time PCR fold change in gene expression comparing oral versus intravenous iron groups (a) MYC (b) SLC11A2 (c) TRFC

(d)



(e)

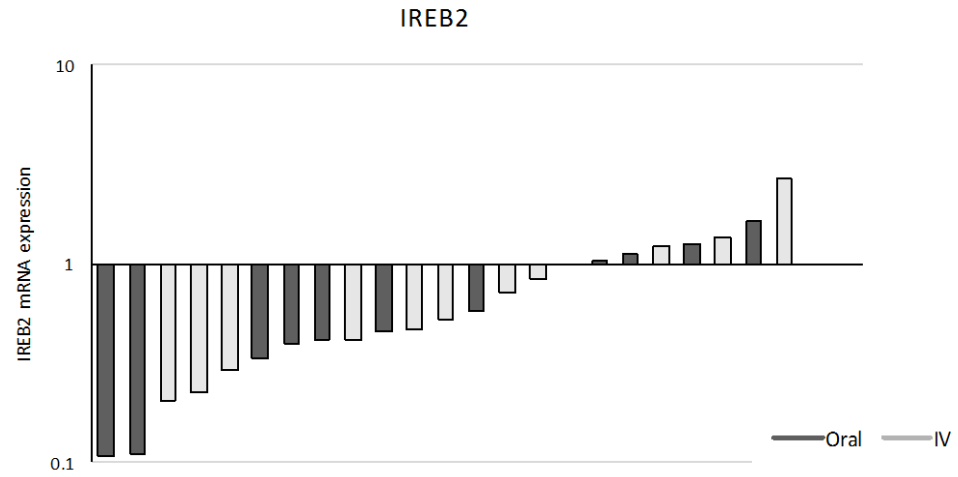


Figure 2.6 Real-time PCR fold change in gene expression comparing oral versus intravenous iron groups (d) FTH1 (e) IREB2

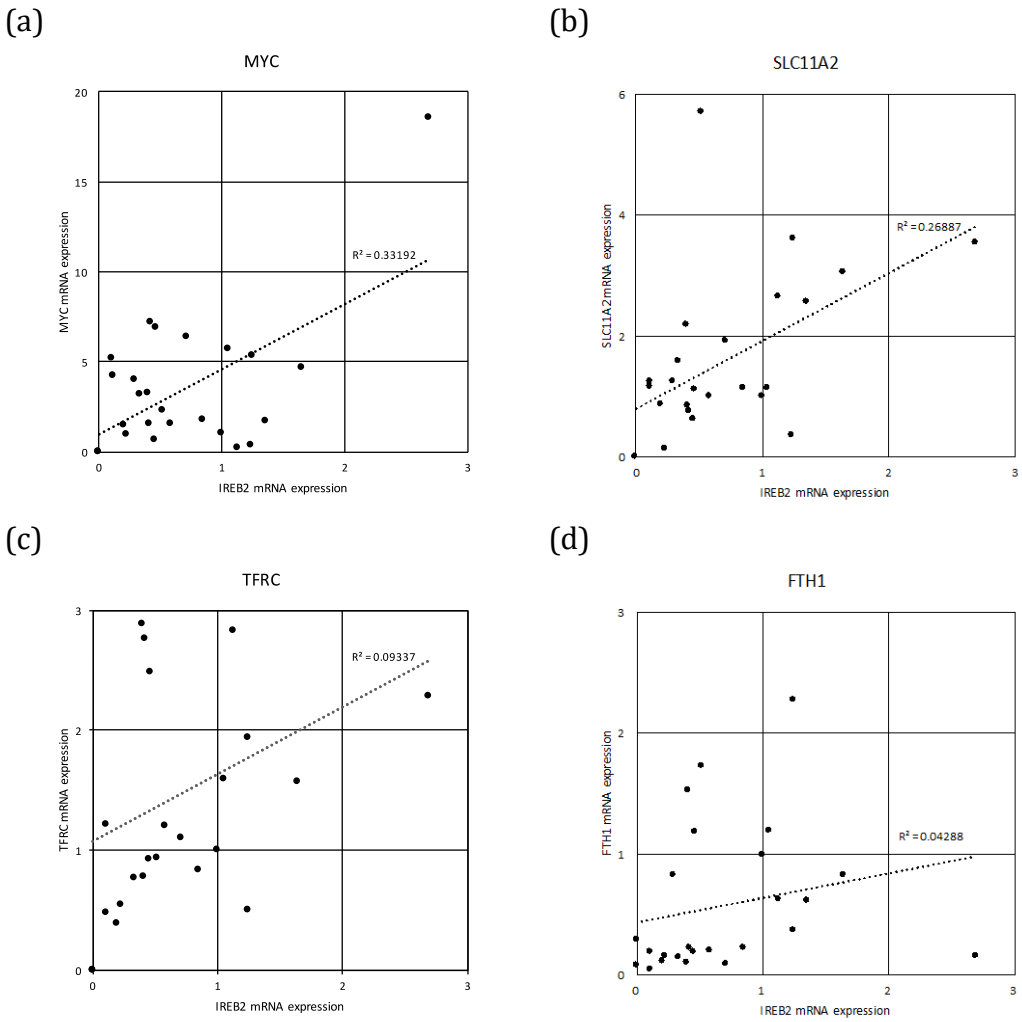
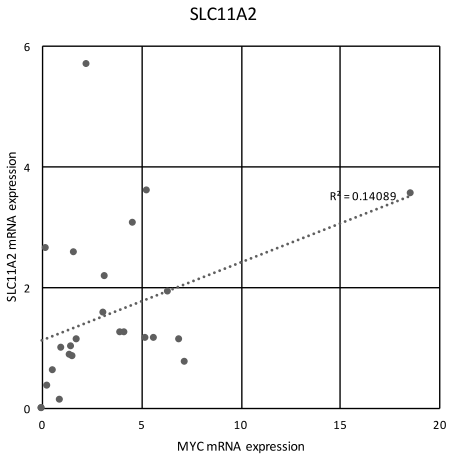


Figure 2.7 IREB2 correlation scatter plots with regression lines (a) MYC (b) SLC11A2 (c) TFRC (d) FTH1

(a)



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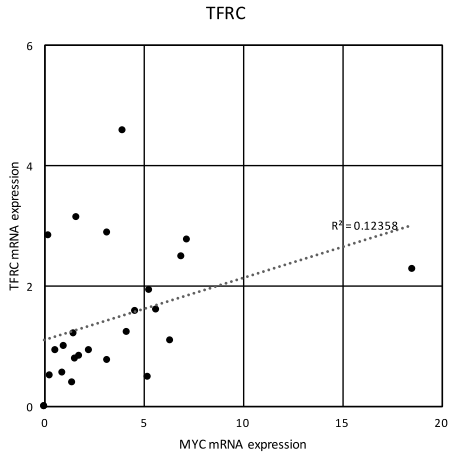


(c)

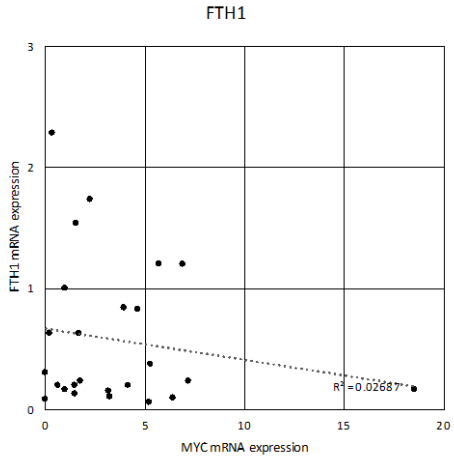


Figure 2.8 MYC correlation scatter plots with regression lines (a) SLC11A2 (b) TFRC (c) FTH1

(1) Proliferation

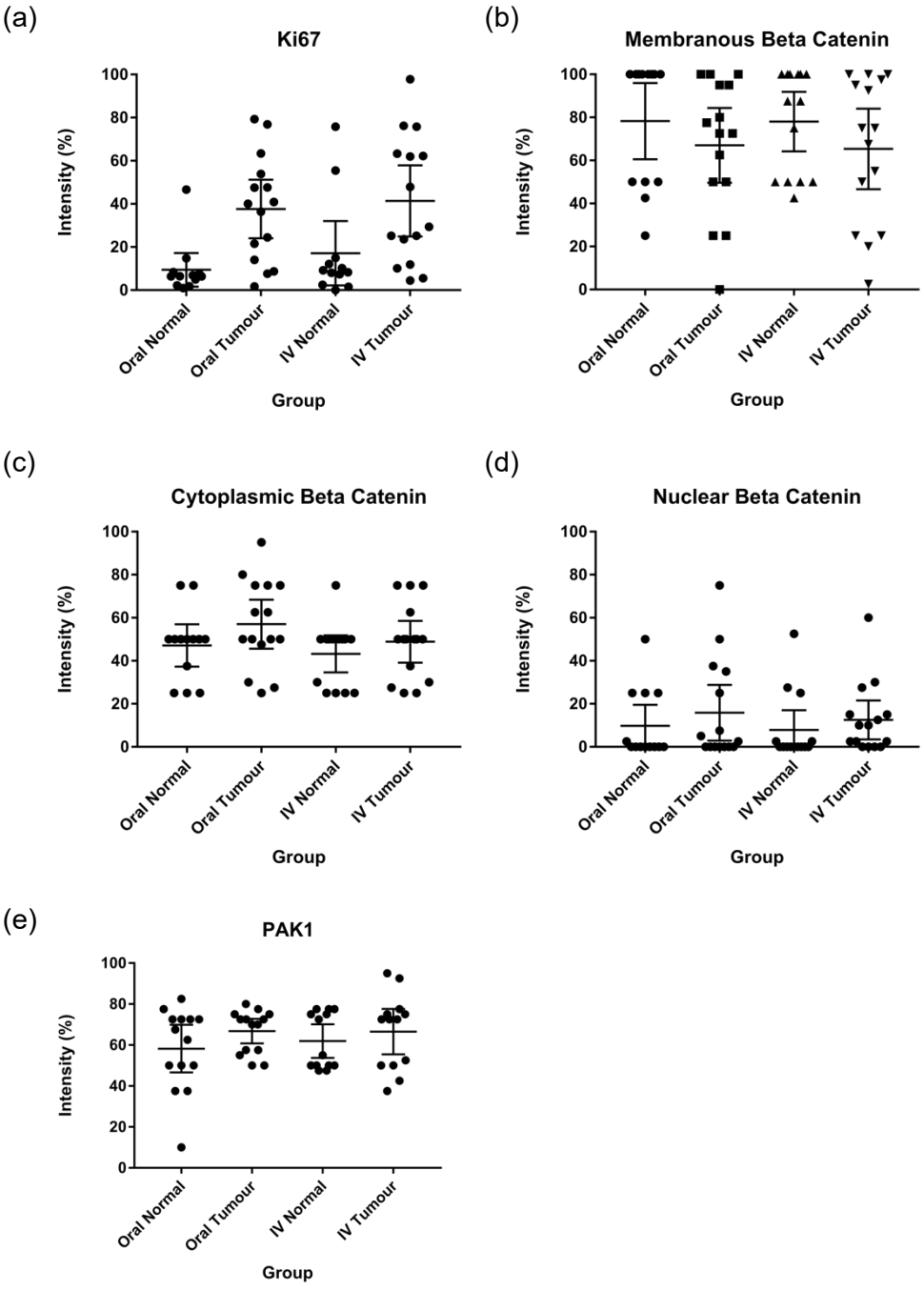
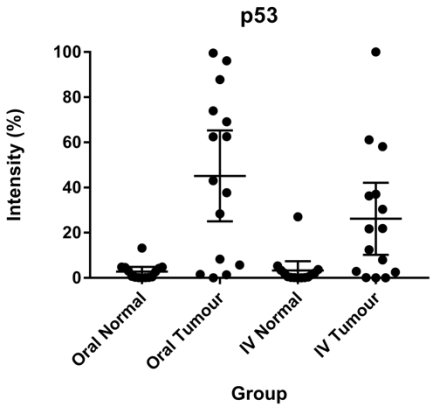


Figure 2.9 Immunohistochemistry semi-quantitative analysis dot plots with mean and standard deviation (1) Proliferation (a) Ki67 (b) Membranous beta-catenin (c) Cytoplasmic beta catenin (d) Nuclear beta catenin (e) PAK1

(2) Apoptosis and DNA damage

(a)



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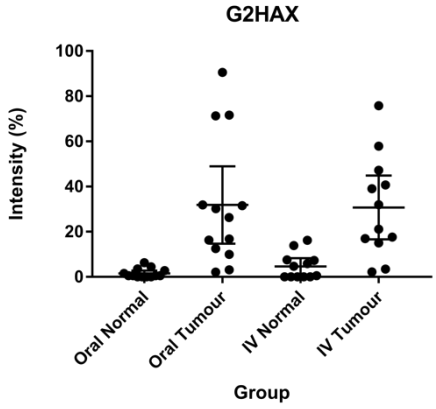


(c)

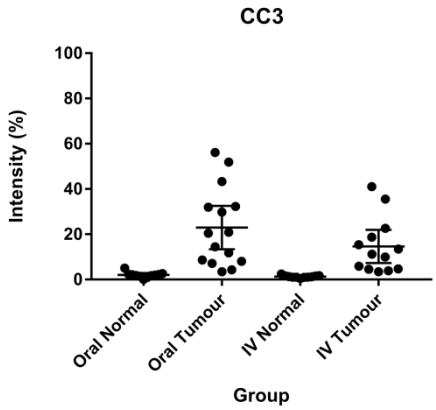
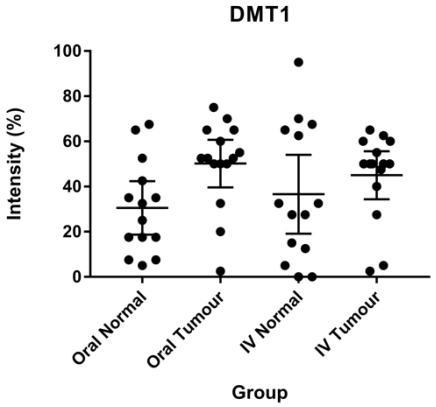


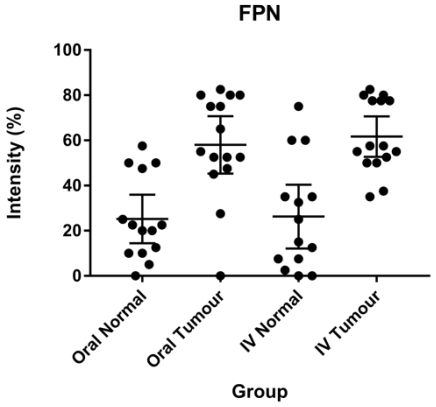
Figure 2.10 Immunohistochemistry semi-quantitative analysis dot plots with mean and standard deviation (2) Apoptosis and DNA damage (a) p53 (b) G2HAX (c) CC3

(3) Iron transport

(a)



(b)



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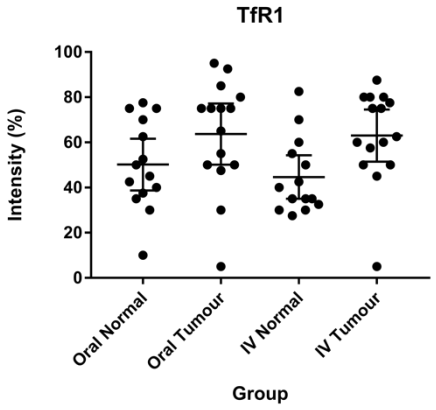
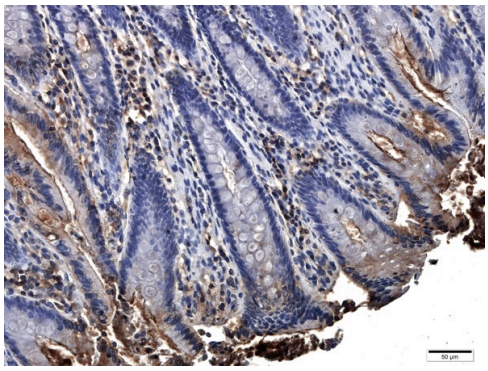


Figure 2.11 Immunohistochemistry semi-quantitative analysis dot plots with mean and standard deviation (3) Iron transport (a) DMT1 (b) FPN (c) TfR1

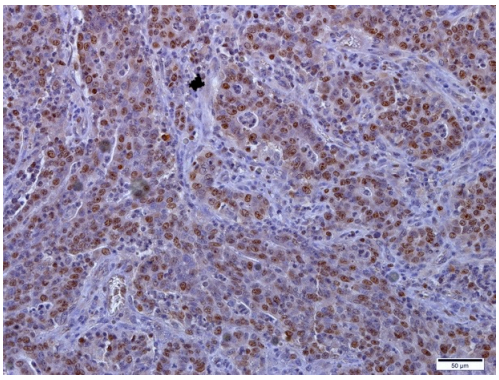
Figure 2.12 Immunohistochemistry photomicrographs for proliferation (a) Ki67 (b) Beta-catenin (c) PAK-1

A

Normal

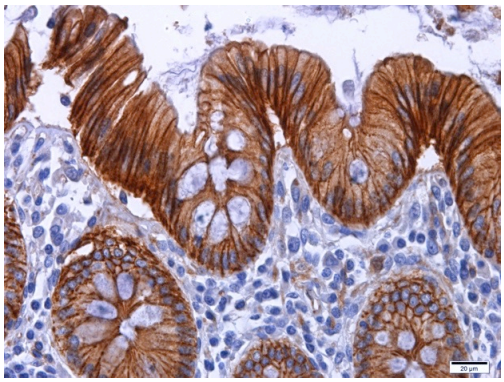


Tumour



B

Normal



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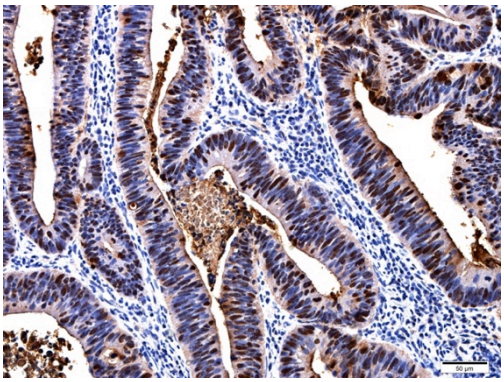

C

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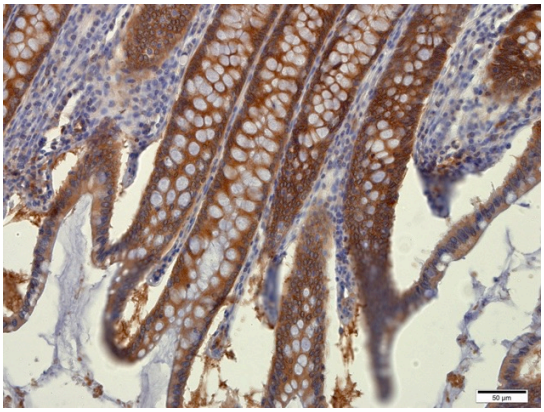


Tumour

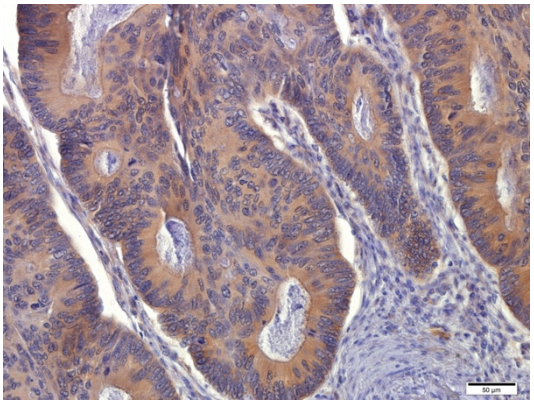
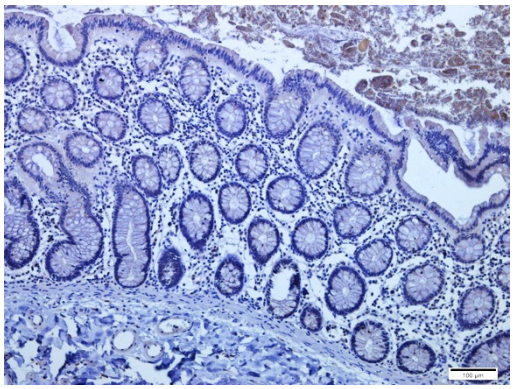


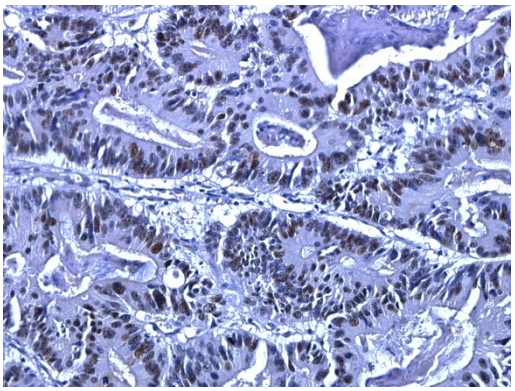
Figure 2.13 Immunohistochemistry photomicrographs for apoptosis and DNA damage (a) P53 (b) Cleaved caspase 3 (c) γH2AX

A

Normal



Tumour



B

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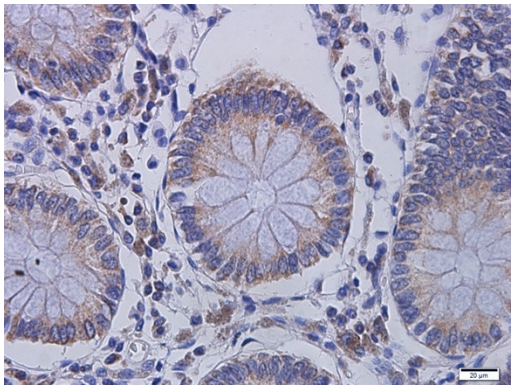
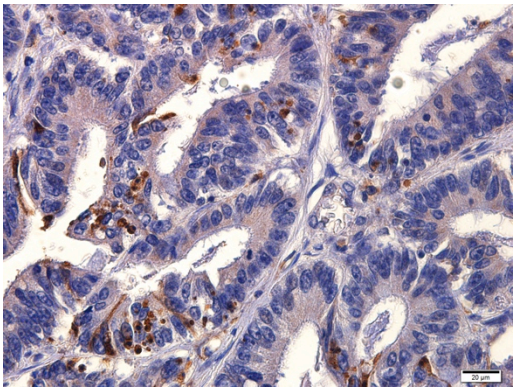
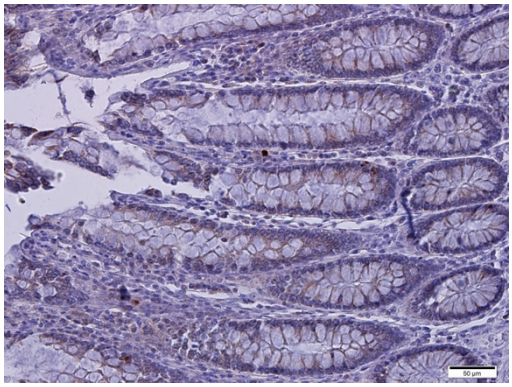


Tumour



C

Normal



Tumour

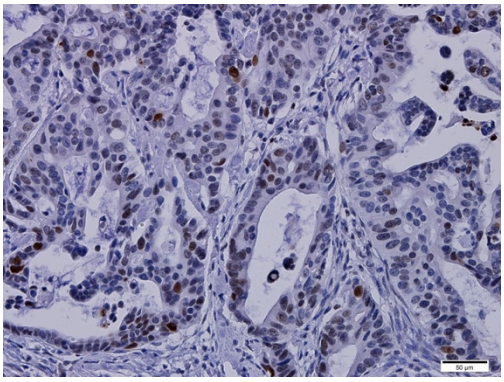
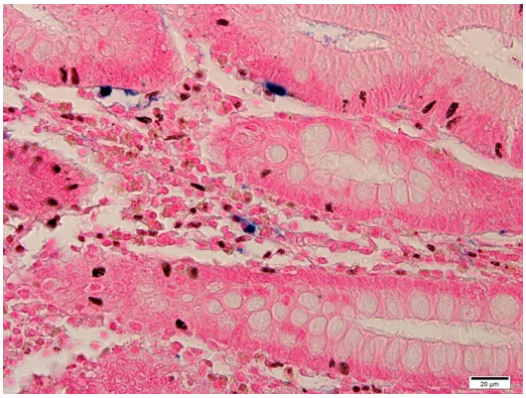


Figure 2.14 Perls Prussian blue photomicrographs for iron loading (a) Normal tissue staining in crypts (b) Tumour tissue staining in crypts (c) Normal tissue staining in lamina propria (d) Tumour tissue staining in deep connective tissue (e) Chi square analysis shown expression of Perl's Prussian blue in tumour and stroma from tumour tissue from patients treated with oral or IV iron

A



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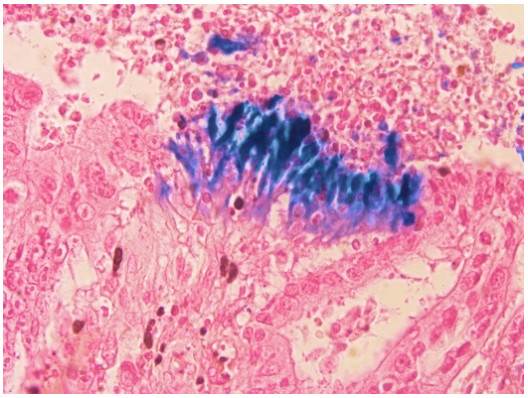
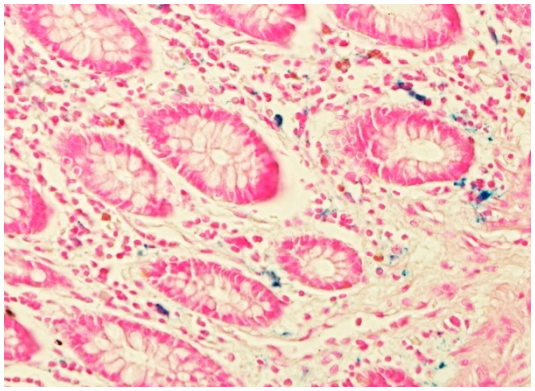
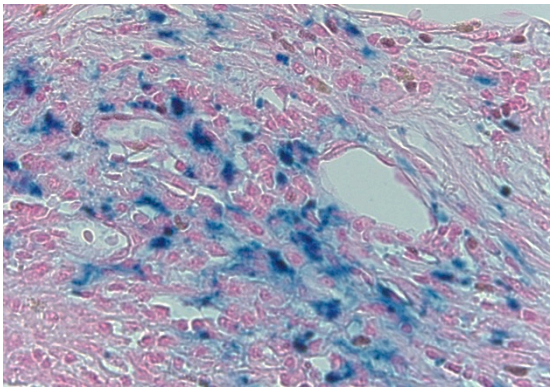


C



D



E

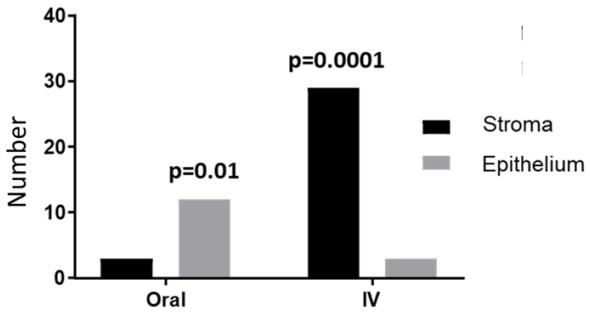
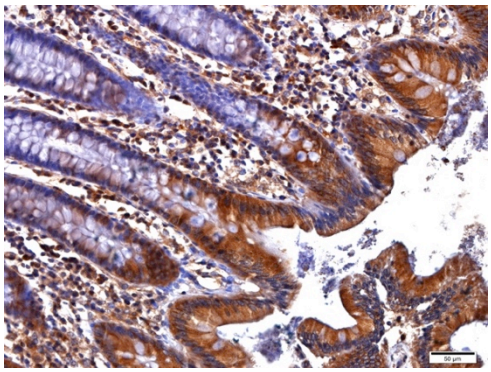


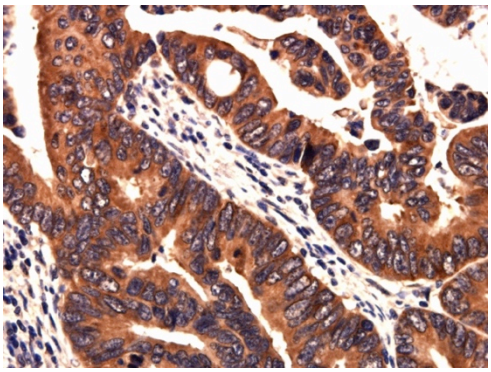
Figure 2.15 Immunohistochemistry photomicrographs for iron transport proteins (a) TfR1 (b) DMT1 (c) FPN

A

Normal

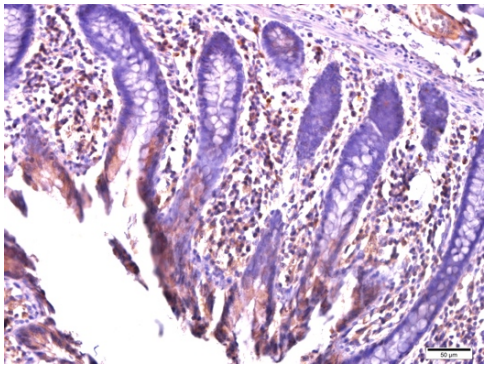


Tumour

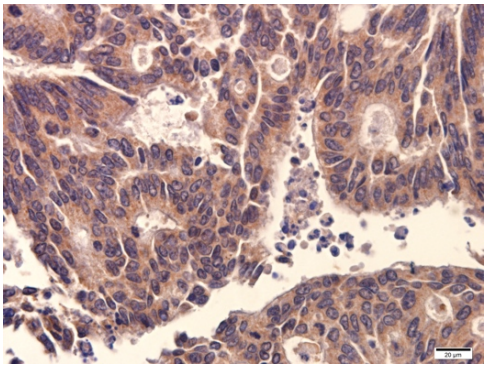


B

Normal

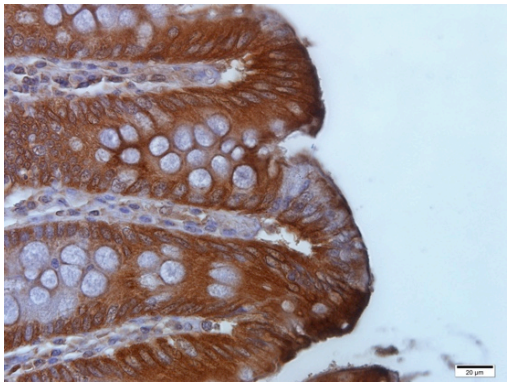


Tumour

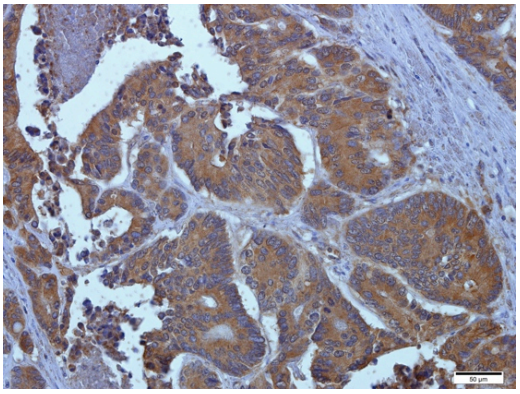


C

Normal



Tumour



2.3.5 Hepcidin ELISA

Median hepcidin levels were 1.9 ng/mL (range 0-86) in the oral iron group and 1.7 ng/mL (range 0-205 ng/mL) in the IV iron group. No correlation was found between hepcidin levels and haemoglobin change in either oral or intravenous groups. Mean haemoglobin change were similar for those with high hepcidin levels (11.4 g/L) compared to low hepcidin levels (11.2 g/L) with no statistical difference found (p=0.949). Subset analysis of oral and intravenous groups also showed no differences (p=0.679 and p=0.775 respectively). Neither absolute hepcidin nor high hepcidin levels >56 ng/ml predicted response to iron therapy, irrespective of the route of administration of iron therapy.

2.3.6 P –selectin ELISA

Soluble P-selectin ELISA showed no significant difference in oral or intravenous iron groups at the time of recruitment (p=0.164) or on day of surgery (p=0.242). Within the oral group there was no significant difference from recruitment to day of surgery (p=0.169). In the IV group no significant difference from recruitment to day of surgery (p=0.227). Sub-group analysis of only patients with a serum ferritin of less than 50 did not then reveal any significant differences between groups. Equally, excluding those who had received pre-trial oral iron therapy again showed no significant difference.

Table 2.7 sP-selectin level changes comparing oral verus intravenous iron groups

	Mean sP-selectin level at recruitment (ng/mL) (SD)	**Mean sP-selectin level on day of surgery (ng/mL) (SD)**	**P**	**Change in sP-selectin level from recruitment to day of surgery (ng/mL) (SD)**
IV	104.8 (30.6)	113.1 (42.7)	0.169 (pair samples T test)	+8.4 (38.3)
Oral	95.7 (26.1)	102.6 (35.6)	0.227 (pair samples T test)	+6.9 (34.2)
p	0.164 (independent samples t test)	0.242 (independent samples t test)		

Table 2.8 Real-time PCR fold change in gene expression in oral and intravenous iron groups compared to normal

	Oral Mean fold change (SD)	IV Mean fold change (SD)	P value[1]
Proliferation			
MYC	5.85 (6.1)	2.82 (1.94)	0.070
Iron transport and storage			
SLC11A2	1.50 (1.16)	1.86 (1.06)	0.974
TFRC	2.38 (1.38)	1.49 (0.89)	0.442
FTH1	0.44 (0.40)	0.43 (0.46)	0.977
Iron regulation			
IREB2	0.90 (0.88)	0.70 (0.51)	0.307

[1] P value for tumour gene expression comparing oral versus intravenous iron groups

Table 2.9 Semi-quantitative analysis of immunohistochemistry

	Oral Mean (SD)			IV Mean (SD)			P value [1]
	Normal	Tumour	p	Normal	Tumour	p	
Proliferation							
Ki67	0.09 (0.12)	0.38 (0.25)	0.006*	0.17 (0.23)	0.41 (0.30)	0.006*	0.708
BCatM	0.78 (0.29)	0.67 (0.31)	0.449	0.74 (0.24)	0.65 (0.34)	0.466	0.890
BCatC	0.47 (0.16)	0.57 (0.21)	0.114	0.43 (0.15)	0.49 (0.18)	0.330	0.252
BCatN	0.10 (0.16)	0.16 (0.23)	0.476	0.08 (0.16)	0.13 (0.16)	0.128	0.655
PAK1	0.58 (0.20)	0.67 (0.10)	0.169	0.62 (0.14)	0.67 (0.18)	0.472	0.966
Apoptosis and DNA damage							
p53	0.03 (0.04)	0.45 (0.36)	<0.001*	0.03 (0.07)	0.26 (0.29)	0.008*	0.124
G2HAX	0.02 (0.02)	0.32 (0.28)	0.001*	0.05 (0.06)	0.31 (0.22)	0.003*	0.915
CC3	0.12 (0.16)	0.08 (0.14)	0.291	0.08 (0.11)	0.11 (0.14)	0.979	0.404
Iron transport							
DMT1	0.31 (0.21)	0.50 (0.19)	0.013*	0.37 (0.30)	0.45 (0.19)	0.376	0.465
FPN	0.25 (0.19)	0.58 (0.23)	<0.001*	0.26 (0.25)	0.62 (0.16)	<0.001*	0.616
TfR1	0.50 (0.20)	0.64 (0.24)	0.016*	0.45 (0.17)	0.63 (0.21)	0.009*	0.936

[1] P value for tumour immunoreactivity comparing oral versus intravenous iron groups

Table 2.10 Microsatellite instability sub-analysis of RT-PCR comparing oral versus intravenous iron groups

	Stable Mean (SD)			**MSI Mean (SD)**			**P value 1**
	Oral (n=6)	IV (n=7)	P	Oral (n=1)	IV (n=2)	p	
Proliferation							
MYC	3.74 (2.75)	2.93 (1.83)	0.628	18.52 (0)	2.42 (3.1)	0.148	0.115
Iron transport and storage							
SLC11A2	1.16 (0.8)	1.57 (1.03)	0.445	3.55 (0)	2.85 (0.29)	0.300	0.009*
FTH1	0.47 (0.41)	0.36 (0.48)	0.635	0.15 (0)	0.72 (0.14)	0.189	0.665
TFRC	2.39 (1.5)	1.28 (0.84)	0.122	2.28 (0)	2.19 (0.88)	0.947	0.585
Iron regulation							
IREB2	0.6 (0.42)	0.5 (0.35)	0.670	2.68 (0)	1.38 (0.36)	0.210	0.001*

[1] P value for tumour gene expression comparing microsatellite stable versus microsatellite instability groups

Table 2.11 Microsatellite instability sub-analysis of immunohistochemistry comparing oral versus intravenous iron groups

	Stable Mean (SD)			**MSI Mean (SD)**			**P value 1**
	Oral (n=11)	IV (n=12)	p	Oral (n=4)	IV (n=3)	p	
Proliferation							
Ki67	0.36 (0.27)	0.42 (0.3)	0.550	0.4 (0.14)	0.37 (0.34)	0.712	0.714
BCatM	0.66 (0.34)	0.66 (0.34)	0.651	0.68 (0.23)	0.6 (0.37)	0.639	0.353
BCatC	0.57 (0.18)	0.5 (0.18)	0.202	0.55 (0.29)	0.43 (0.11)	0.596	0.562
BCatN	0.14 (0.18)	0.12 (0.17)	0.687	0.18 (0.37)	0.14 (0.15)	0.655	0.735
PAK1	0.66 (0.09)	0.64 (0.18)	0.905	0.68 (0.13)	0.72 (0.21)	0.603	0.861
Apoptosis and DNA damage							
p53	0.38 (0.33)	0.16 (0.19)	0.138	0.63 (0.43)	0.62 (0.35)	0.967	0.011*
G2HAX	0.38 (0.31)	0.28 (0.23)	0.807	0.17 (0.13)	0.39 (0.1)	0.447	0.484
CC3	0.02 (0.02)	0.12 (0.15)	0.243	0.25 (0.22)	0.09 (0.12)	0.224	0.079
Iron transport							
DMT1	0.5 (0.19)	0.46 (0.16)	0.982	0.48 (0.2)	0.39 (0.32)	0.941	0.206
FPN	0.6 (0.24)	0.63 (0.15)	0.689	0.48 (0.2)	0.39 (0.32)	0.916	0.736
TfR1	0.57 (0.26)	0.67 (0.14)	0.446	0.79 (0.08)	0.45 (0.36)	0.127	0.955

[1] P value for tumour immunoreactivity comparing microsatellite stable versus microsatellite instability groups

Table 2.12 Right versus left sided cancers sub-analysis of RT-PCR comparing oral versus intravenous iron groups

	Right (caecum to hepatic flexure) Mean (SD)			**Left (splenic flexure to rectum) Mean (SD)**			**P value 1**
	Oral (n11)	IV (n=12)	P	Oral (n=4)	IV (n=3)	p	
Proliferation							
MYC	4.2 (3.29)	3.19 (2.27)	0.463	8.04 (9.14)	2.09 (0.96)	0.462	0.537
Iron transport and storage							
SLC11A2	0.79 (0.47)	2.11 (1.16)	0.045	2.46 (1.15)	1.35 (0.72)	0.340	0.587
FTH1	0.45 (0.49)	0.35 (0.27)	0.813	0.53 (0.34)	0.61 (0.8)	0.455	0.418
TFRC	1.65 (1.12)	1.41 (0.87)	0.111	3.34 (1.16)	1.62 (1.11)	0.768	0.114
Iron regulation							
IREB2	0.49 (0.26)	0.82 (0.6)	0.172	1.44 (1.19)	0.46 (0.1)	0.360	0.467

1 P value for tumour gene expression comparing right versus left sided cancers, all cases

Table 2.13 Right versus left sided cancers sub-analysis of immunohistochemistry comparing oral versus intravenous iron groups

	Right (caecum to hepatic flexure) Mean (SD)			**Left (splenic flexure to rectum) Mean (SD)**			**P value 1**
	Oral (n=11)	IV (n=12)	p	Oral (n=4)	IV (n=3)	p	
Proliferation							
Ki67	0.39 (0.26)	0.47 (0.33)	0.490	0.49 (0.19)	0.32 (0.23)	0.375	0.745
BCatM	0.87 (0.12)	0.66 (0.34)	0.056	0.61 (0.29)	0.63 (0.36)	0.889	0.271
BCatC	0.63 (0.13)	0.46 (0.14)	0.529	0.55 (0.29)	0.52 (0.22)	0.530	0.989
BCatN	0.15 (0.17)	0.14 (0.2)	0.525	0.25 (0.35)	0.09 (0.06)	0.539	0.855
PAK1	0.7 (0.09)	0.69 (0.16)	0.675	0.67 (0.1)	0.62 (0.2)	0.533	0.421
Apoptosis and DNA damage							
p53	0.37 (0.37)	0.33 (0.3)	0.250	0.64 (0.38)	0.15 (0.25)	0.266	0.843
G2HAX	0.41 (0.35)	0.32 (0.24)	0.271	0.21 (0.12)	0.24 (0.14)	0.598	0.218
CC3	0.06 (0.12)	0.08 (0.10)	0.405	0.14 (0.22)	0.17 (0.18)	0.233	0.135
Iron transport							
DMT1	0.48 (0.17)	0.49 (0.17)	0.448	0.55 (0.06)	0.38 (0.2)	0.287	0.654
FPN	0.61 (0.14)	0.67 (0.14)	0.787	0.58 (0.22)	0.52 (0.15)	0.070	0.129
TfR1	0.64 (0.19)	0.68 (0.11)	0.752	0.68 (0.18)	0.54 (0.29)	0.211	0.442

1 P value for tumour immunoreactivity comparing right versus left sided cancers, all cases

2.4 Discussion

This study examined two groups of anaemic patients with colorectal cancer, randomised to oral or intravenous iron therapy. It compared molecular changes between normal and tumour tissue and between treatment groups. Intravenous iron therapy successfully replenished body iron, with increases in ferritin and transferrin saturations. The oral iron group remained iron deficient.

This increase in body iron did not lead to increased proliferation or decrease apoptosis in tumours in the intravenous iron group when compared to the oral iron group and was not associated with changes in proliferation in the paired normal tissues. No previous studies have examined this in humans. Studies in mice by contrast, showed oral iron increases the number and size of tumours when compared to an iron-deficient diet and intravenous iron (Seril, Liao et al. 2005). However, this model of inflammatory colorectal carcinogenesis is unlike most sporadic colorectal cancers in humans (excluding those developing in ulcerative colitis). Further, our human participants were still consuming a normal Western diet commonly replete in dietary iron, rather than the experimental iron-deficient diet of the mouse in Seril et al study. Higher supra-physiological doses of iron were also administered to mice in comparison, over a relatively longer time period when compared to the time period over which carcinogenesis occurs in humans compared to this mouse model of colorectal carcinogenesis.

This study is the first to compare whether the route of iron administration is important in iron loading and demonstrated no difference between oral and intravenous iron groups with all tumours showing increased iron. This is similar to a previous study by Brookes et al (2006). However, qualitative differences between the localisation of iron within the stroma and adjacent connective tissues were noted, occurring more frequently with intravenous iron. The implications of this are uncertain but differential compartmentalisation and the tumour microenvironment could all potentially influence intracellular tumour iron loading, macrophage iron and immune function.

Iron importers (TfR1 and DMT1) at both mRNA and protein level were increased in tumours with no differences seen between treatment groups. Ferritin heavy chain mRNA was also reduced. The net effect of decreased iron storage and increase iron import would be an increase in the labile iron pool. This appears to be occurring due to a change of normal iron sensing mechanisms. IREB2 was decreased in tumours, a normal response to high intracellular iron, but this did not lead to a reduction in TfR1 or an increase in FTH1 as expected. In fact, TfR1 expression increased and FTH1 mRNA expression decreased with no correlation with IREB2 mRNA expression. This is contrary to findings by Horniblow (2017) in which IRP2 and TfR1 expression both increased in colorectal cancer and correlated with each other (Horniblow, Bedford et al. 2017)

This effect had previously been demonstrated in relationship to APC wild type cancer cell lines, whereby the regulation of iron stores appeared to be IRE/IRP dependent with normal iron decreasing IRP2 with subsequent decreases in TFR1 and DMT1 (Radulescu, Brookes et al. 2012). In cancers with a mutation in APC the regulation of colon cancer cells iron stores became IRE/IRP independent and despite high iron, TFR1 and DMT1 expression increased. This could be reversed when APC was transfected into these cells (Radulescu, Brookes et al. 2012). This model would hypothesise that IRE/IRP sensing might be bypassed by beta-catenin TCF signalling and overwhelmed by huge increases in iron. In this study, difference in the microsatellite instability tumours compared to the MSS (and likely APC pathway) tumours no differences in iron regulation were seen.

Changes in iron metabolism were also not related to c-MYC expression, which correlated with IREB2 but showed no relationship with iron transport (SLC11A2 and TFRC) or storage (FTH1). Previous in vitro experiments examining transcriptional targets of c-MYC have been mixed. One study has shown increased c-MYC causes an overexpression of IREB2 and a reduction of FTH1, but had no relationship with TFRC (Wu, Polack et al. 1999). A separate group have shown that c-MYC can independently induce TfR1 (O'Donnell, Yu et al. 2006). Other studies have shown

transfection of c-MYC to colon cancer cell lines increases ferritin heavy chain transcription (Modjtahedi, Frebourg et al. 1992).

Iron export via ferroportin was also altered in tumours with increased expression and mislocalisation, the latter potentially reducing iron export from cells again increasing labile iron. Serum hepcidin in this study was not predictive of haemoglobin response to iron therapy or ferroportin immunoreactivity in tissues. This was also reported by Ward (2008), who also demonstrated hepcidin mRNA expression within one-third of colorectal and subsequent repression of ferroportin (Ward, Roberts et al. 2008).

2.4.1 Limitations

Despite the unique opportunity to examine in vivo differential iron exposure to either oral or intravenous iron therapy in a human clinical randomised control trial there are several limitations to this study. Unlike carefully controlled experiments in animals with relatively homogenous populations due the experimental conditions under which tests were performed, there is likely be marked heterogeneity in the tumours and treatment seen in this trial. This is even while matched for tumour stage, histology and sub-analysed on MSI status. Further, our patients were not fed a controlled diet and likely all patients, both intravenous and oral treatment groups, received an unquantified amount of dietary iron that is usually high in Western diets. Heme iron pathways also play a smaller but significant role in iron absorption and are neither controlled for nor examined in our study. The small window of

intervention, median 21 days treatment, may also be insufficient to alter the biology of a tumour that has developed over the course of maybe five to ten years.

Some technical aspects of the analysis should also be considered. P53 immunohistochemical staining is not indicative of p53 mutation and further analysis would be required to accurately assess this (MacGeoch, Barnes et al. 1993). Likewise APC mutation was assumed in the non-MSI cancers although biological proof of this is not assessed in this study.

We have studied transferrin receptor-mediated iron uptake via TfR1, which is a well-characterised pathway for iron absorption. It is important to note that a wide range of alternate non-transferrin receptor mediated iron uptake pathways exist and have been demonstrated in colorectal and other cancers. This includes iron absorption through heme iron (West and Oates 2008), TfR2 (Kawabata, Germain et al. 2000), oestrogen-inducible transferrin-receptor-like protein (Poola 1997), autocrine transferrin secretion in breast cancer (Vandewalle, Hornez et al. 1989), Scara 5 (Li, Paragas et al. 2009) and TRPC6 (Mwanjewe and Grover 2004). However, their roles in tumour growth are not yet understood (Kwok and Richardson 2002).

The lipocalin 2 (LCN2), also known as neutrophil gelatinase associated lipocalin (NGAL) pathway may be of particular interest. Lipocalin 2 is thought to play a role in the innate immune system sequestering iron via binding bacterial siderophores bound to prevent bacterial growth (Yang, Goetz et al. 2002). Most research into LCN2 has been conducted in breast cancer where the LCN2-catechol complex binds

to cell surface 24p3R receptor (also known as SLC22A17) to deliver iron to cells (Bao, Clifton et al. 2010). This represents a novel non-transferrin mediated mechanism for iron uptake and overexpression is associated with increased proliferation and angiogenesis in breast cancer (Fernandez, Yan et al. 2005). However, lipocalin 2 has also been shown to be ectopically expressed by a range of cancers including in colorectal cancer (Nielsen, Borregaard et al. 1996, Friedl, Stoesz et al. 1999, Lee, Lee et al. 2006). Importantly, it has been associated with tumour size, disease-free survival and overall survival from colorectal cancer (Sun, Yokoi et al. 2011, Marti, Fuster et al. 2013, Maier, Aigner et al. 2014).

We have also not examined IRP1 over-expression, which in contrast to IRP2, reduces tumour growth in vivo. Despite increasing TfR1 it had no effect on intracellular iron levels and high ferritin was noted (Chen, Fillebeen et al. 2007).

Differential compartmentalisation of iron within tissue should also be interpreted carefully. Haemosiderin stains intensely with Prussian blue and ferritin only at high concentrations (Saito 2014). Haemosiderin may be largely inert and biologically inactive, reflecting instead a secondary mechanism for iron storage when ferritin storage is exceeded (Saito, Tomita et al. 2012). The biologically active labile iron pool however is not seen or quantified with Prussian blue, and has instead been inferred. Techniques utilising calcein-acetoxymethyl ester to assay the labile iron pool are available and more accurately reflect intracellular iron (Esposito, Epsztejn et al. 2002).

The constraints of our analysis also give no means to assess causality or direction of any given changes observed, with experimental manipulation of gene or protein expression not possible.

2.4.2 Implications for clinical practice

This study has not revealed any increase in proliferation in colorectal adenocarcinomas when comparing the short-term administration of oral or intravenous iron therapy prior to surgery, despite intravenous iron being more effective in correcting iron deficiency and clinical anaemia in the IVICA trial (Keeler, Simpson et al. 2017). This would support the continued replacement of iron in iron deficiency anaemia to improve pre-operative haemoglobin.

2.4.3 Implications for clinical research

This study has again demonstrated that tumours are iron avid, reprogramming their iron biology to enhance cellular iron acquisition and increase the labile iron pool. The route of administration of iron does not change this propensity for increases in cellular iron acquisition. The effect of differential compartmentalisation of iron within the mucosa and tumour cell iron loading is unclear.

However, growing understanding of dysregulated iron biology in colorectal adenocarcinoma and an emerging body of evidence from cell, tissue and animal work are beginning to reveal the mechanistic foundations for iron and cancer (Nelson 2001, Seril, Liao et al. 2002, Shaheen, Silverman et al. 2003, Ilsley,

Belinsky et al. 2004, Mainous, Gill et al. 2005, Brookes, Hughes et al. 2006). If iron is indeed central to tumourigenesis and intrinsically linked with recognised molecular pathways for carcinogenesis in colorectal cancer such as APC and Wnt signalling, strategies for exploiting tumour iron restriction should be researched. The effectiveness of these strategies will no doubt hinge upon achieving tumour iron restriction without subjecting the patient to the deleterious effects of iron deficiency, such as anaemia and fatigue. One possible strategy would be oral iron chelating agents with intravenous iron supplementation assuming the tumour derives a significant proportion of its iron from luminal content. Body iron might however be accessible to tumours and it may emerge that the tumour has reprogrammed its iron biology to such an advantage that no measures can iron deplete an in situ tumour without inducing the negative effects of systemic iron deficiency in the patient. Equally plausible, the tumours are so iron dependent that even small and temporary interruptions to intracellular labile iron supply may be catastrophic for tumour cells while tolerated by normal colonic epithelium.

To this end, future studies should also examine the differential compartmentalisation of iron, to confirm if this phenomenon is reproducible in more controlled laboratory settings and explore the impact on healthy bowel mucosa, tumour, stromal and immune components that may influence the tumour survival, immune surveillance and metastasis of colorectal cancers.

2.4.4 Conclusion

Iron has for years been thought to be involved in colorectal cancer. Recently, iron therapy has seen resurgence in clinical practice with the advent of safer intravenous iron preparations that allow total body iron dosing and rapid administration times. This has combined with evidence that anaemia and blood transfusions increase mortality and morbidity, especially following surgery. Several recent randomised control clinical trials have consequently investigated the efficacy of intravenous iron in the pre-operative setting (Richards, Clevenger et al. 2015, Froessler, Palm et al. 2016, Keeler, Simpson et al. 2017). This study has investigated the molecular consequences of one of these trials (Keeler, Simpson et al. 2017) and has shown no changes in tumour growth despite iron avid tumours and replenishment of body iron stores with intravenous iron.

3 The prevalence, natural history and impact of anaemia in oesophagogastric cancer

Abstract

Background

The management of oesophageal cancer has evolved significantly in the last decade with the publication of the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial in 2006 and the increasing utilisation of minimally invasive techniques. Anaemia and transfusions are associated with adverse outcomes in oesophagogastric cancer and frequently result from iron deficiency. This study aimed to establish the rates of iron deficiency, natural history of anaemia and implications of this anaemia in all patients diagnosed with oesophagogastric cancer. Two nested studies were conducted to examine the impact of changes in treatment compared to a historical surgical cohort and if anaemia was associated with chemotherapy response.

Methods

All patients diagnosed with oesophagogastric cancer in 2012 to 2013 at a single high-volume referral centre were reviewed. Patient case notes, MDT meetings and electronic records were used to perform a retrospective analysis. Data collected

included treatment received, haemoglobin, MCV, ferritin, iron therapy, blood transfusions, histology and survival. Combining complimentary data sets from a 2003 to 2004 surgical cohort and a database of chemotherapy response (measured as histological tumour response grade), two nested studies were also conducted.

Results

504 patients were included in the study from 2012-2013. Anaemia was present at diagnosis in 227 patients (45%) and increased over time with 64% anaemic prior to treatment and 90% post-operatively in the cohort treated with surgery. One-third of patients (35.4%) had microcytic anaemia. Ferritin was investigated in 28% of patients with anaemia (n=65) and iron deficiency anaemia diagnosed in 12% (n=28) of patients. Of those only 13 patients (6% of those with anaemia) received treatment with oral iron therapy prior to treatment. No patients received intravenous iron therapy.

Those anaemic at diagnosis had a significantly poorer survival compared to those without anaemia ($p=0.035$). Further, moderate and severe anaemia had poorer survival when compared with mild anaemia ($p=0.048$).

There was a significant negative correlation between haemoglobin at diagnosis and blood transfusions ($r=-0.164$, $p<0.001$). Logistic regression analysis revealed that for each 10g/L increase in haemoglobin the risk of transfusion during treatment was reduced in the order of 20% (OR 0.79 [CI 0.72-0.86], $p<0.001$). Adjusting for age, sex, Charlson score and disease stage did not materially alter the odds ratio.

In the surgical subgroup, 285 patients were included, 2003-04 (n=145) and 2012-13 (n=140). Patients were similar in age, sex, site of malignancy and histology. Mean pre-operative haemoglobin was similar, 129 g/L (SD 17) v 123 g/L (SD 17), p=0.416. However, in those who had neoadjuvant chemotherapy, haemoglobin was significantly different ($p<0.05$), 137 g/L (SD 19) v 122 g/L (SD 17). Further, within the 2012-13 group a significant drop in haemoglobin from diagnosis, 135 g/L (SD 21) to pre-operatively 121 g/L (SD 16) was also seen in the neoadjuvant group ($p<0.01$), but not with no neoadjuvant chemotherapy (p= 0.113). Consequently, patients in 2012-13 were significantly more likely to be anaemic pre-operatively (62.9% v 38%, $p<0.01$). There were also trends towards patients having more transfusion (60% v 49%, p=0.062) and more blood per patient 2.5 units v 2.2 units.

Chemotherapy response showed no statistical association between those with pre-chemotherapy anaemia (OR 0.881, CI 0.406-1.914, p=0.931) or those with anaemia at anytime during treatment (OR 0.881, CI 0.406-1.914, p= 0.931).

Conclusion

Anaemia is common in oesophagogastric cancer and becomes worse with time and treatment. It is associated with increased blood transfusions and poorer survival. Anaemia is not associated with tumour response to chemotherapy but has become more common since the introduction of MAGIC regime chemotherapy.

The implications for clinical practice are that haemoglobin should be monitored regularly during treatment for oesophagogastric cancer to allow the early

identification of anaemia. Ferritin should be routinely checked in all patients with anaemia and appropriate treatment considered to address recognised iron deficiency. Initiation of early anaemia treatment could potentially prevent transfusions with even modest increases in haemoglobin associated with fewer transfusions. Further research is required in prospective randomised control trials to examine whether treatment of anaemia improves outcomes.

3.1 Introduction

3.1.1 Epidemiology of oesophagogastric cancer

Oesophagogastric cancers account for over 15,000 new cases of cancer annually in the UK (CRUK 2016). Worldwide it is eighth most common cancers and ranked sixth cause of cancer mortality (Ferlay, Soerjomataram et al. 2015). Almost half (47.3 per cent) of the patients have a tumour of the oesophagus, one-fifth (22.7 per cent) a tumour at the gastro-oesophageal junction (GOJ) and one-third (30 per cent) a stomach tumour (NOCGA 2013). Adenocarcinoma is the predominant histology and for oesophageal cancer has seen a marked increase in the last three decades (Schlansky, Dimarino et al. 2006). Prognosis remains poor with 5 year survival 15% and 19% for oesophageal and gastric cancer respectively (CRUK 2016).

3.1.2 Chemotherapy for oesophagogastric cancer

The management of oesophageal cancer has evolved significantly in the last decade with randomised control trials demonstrating the combination of chemotherapy and surgery improves survival (Allum, Stenning et al. 2009). The 2013 National Oesophagogastric Cancer Audit showed that 63% of patients had chemotherapy and surgery for curative treatment (NOCGA 2013). Following publication of the MAGIC trial, pre-operative and post-operative chemotherapy with epirubicin, cisplatin and infused 5-fluorouracil (ECF) have been widely used (Cunningham, Allum et al. 2006). This trial showed a 25% reduction in the risk of death improving overall and

disease free survival. Use of oral capecitabine (ECX) and oxaliplatin (EOX) are accepted alternatives to reduce toxicity while maintaining efficacy (Cunningham, Starling et al. 2008). In comparison, over a decade ago OE-O2 style chemotherapy using combination cisplatin and fluorouracil achieved a 16% reduction in the risk of death (Medical Research Council Oesophageal Cancer Working 2002).

3.1.3 Surgery for oesophagogastric cancer

In parallel with this, surgical management has changed with the increasing experience and utilisation of minimally invasive techniques (NOCGA 2013). These techniques have equivalent oncological outcomes and may be associated with reductions in peri-operative blood loss and length of stay, although this remains a topic of debate (Braghetto, Csendes et al. 2006, Kauppi, Rasanen et al. 2014, Mu, Yuan et al. 2014). The implementation of enhanced recovery after surgery (ERAS) programmes has also improved hospital length of stay following these procedures (Tang, Humes et al. 2013, Markar, Karthikesalingam et al. 2014). For those treated laparoscopically, it may capitalise on the benefits of less pain and physiological insult.

3.1.4 Epidemiology of anaemia

Anaemia is a common problem during treatment for oesophagogastric cancer (Tachibana, Tabara et al. 1999, Rades, Lang et al. 2006, Melis, McLoughlin et al. 2009, Tanswell, Steed et al. 2011). Prior to surgery between 25.6%-39.9% of patients are anaemic (Shen, Cheong et al. 2005, Ayantunde, Ng et al. 2008, Jung,

Lee et al. 2013). In those receiving chemotherapy it is similar at 31% (Voelter, Schuhmacher et al. 2004). This is exacerbated by treatment, with 54% anaemic during chemotherapy and one study reporting 100% of patients were anaemic after chemotherapy (Voelter, Schuhmacher et al. 2004, Rades, Lang et al. 2006). Studies have even shown that the development of anaemia precedes diagnosis of gastric cancer by three years (Edgren, Reilly et al. 2008).

During the course of neoadjuvant chemotherapy, whilst the tumour remains in situ, dysphagia and anorexia can worsen, chronic bleeding continues, iron metabolism remains impaired and iron stores can be insufficient to satisfy increased demand (Clarke and Pallister 2005). Furthermore, the commonest agents used in chemotherapy are all associated with induction of anaemia (Wood and Hrushesky 1995, Groopman and Itri 1999).

The oesophagectomy procedure itself is associated with significant blood loss and blood usage (Tachibana, Tabara et al. 1999, Langley, Alexiou et al. 2002) increasing the baseline risk of blood transfusion. A previous 2003 study demonstrated 49% of patients required blood transfusion in the peri-operative period for oesophagectomy (Ayantunde, Ng et al. 2008).

3.1.5 Associations of anaemia

Pre-operative anaemia in oesophagectomy increases blood transfusions (Melis, McLoughlin et al. 2009) and blood transfusion alone are an independent risk factor for morbidity and mortality in many other cancers (Knight, Wade et al. 2004, Clarke

and Pallister 2005) and after oesophagectomy (Tachibana, Tabara et al. 1999, Langley, Alexiou et al. 2002). There is also increasing recognition that receiving a blood transfusion may increase cancer recurrence in colorectal, oesophageal and hepatocellular carcinoma (Motoyama, Okuyama et al. 2004, Amato and Pescatori 2006, Wang, Iyer et al. 2009). This may be through transfusion-related immunomodulation (Cata, Wang et al. 2013). Anaemia is also associated with increased local failure with radiotherapy or chemoradiotherapy and poorer overall survival (Voelter, Schuhmacher et al. 2004, Rades, Schild et al. 2005, Rades, Lang et al. 2006, Valencia Julve, Alonso Orduna et al. 2006, Melis, McLoughlin et al. 2009, Tanswell, Steed et al. 2011).

3.1.6 Tumour response grade

Response to neoadjuvant chemotherapy can be measured radiologically and histologically. Mandard described the histopathological response to chemotherapy, grading response from 1-5; and broadly grouping response in chemo-sensitive (TRG 1-3) and chemo-resistant (TRG 4-5), Table 3.1 (Mandard, Dalibard et al. 1994). This grading has been shown to predict disease free survival and is used to inform adjuvant chemotherapy decision-making (Mandard, Dalibard et al. 1994, Fareed, Ilyas et al. 2009, Fareed, Al-Attar et al. 2010).

Table 3.1 Mandard Tumour Regression Grading (Thies and Langer 2013)

Tumour regression grade (TRG)	Description
TRG1	Complete regression (fibrosis without detectable tissue of tumour)
TRG2	Fibrosis with scattered tumour cells
TRG3	Fibrosis and tumour cells with preponderance of fibrosis
TRG4	Fibrosis and tumour cells with preponderance of tumour cells
TRG5	Tissue of tumour without changes of regression

3.1.7 Anaemia and chemosensitivity

No studies have looked at anaemia and neoadjuvant chemotherapy for oesophageal adenocarcinoma. In gastric cancer, anaemia (haemoglobin less than 100 g/L) was prognostic for response to 5-fluorouracil containing first-line chemotherapy regimens, disease free survival and mortality (Park, Lee et al. 2006). Ye et al (2015) showed similar results and went further to show increasing haemoglobin with blood transfusions does not improve chemotherapy response or outcomes (Ye, Liu et al. 2015).

In rectal adenocarcinoma significant association between pre-treatment anaemia and histological response to neoadjuvant chemoradiation has been demonstrated. Lee *et al*. (2009) showed severe anaemia (haemoglobin less than 90 g/L) in their cohort of

490 patients was a prognostic factor for poor tumour response to pre-operative chemoradiation (Lee, Park et al. 2009). Khan *et al*. (2013) reported an inverse correlation between pre-treatment anaemia between the length and size of tumours after chemoradiation. Patients with mild anaemia (haemoglobin of less than 120 g/L) were less responsive to therapy in this study (Khan, Klonizakis et al. 2013).

3.1.8 Mechanisms of chemoresistance

Anaemia ultimately results in less oxygen delivery to tissues and this is especially pronounced in highly metabolically active tumour tissues. This tumour hypoxia may paradoxically promote malignant progression, angiogenesis and resistance to apoptosis thus reducing the efficacy of anti-tumour therapies (Dunst, Pigorsch et al. 1999, Hockel and Vaupel 2001). At a molecular level these changes are mediated by the activation of 'hypoxia induced factors', most notably HIF-1α. When stabilised under hypoxic conditions, HIF-1α up-regulates several genes that promote cell survival and VEGF for angiogenesis (Shannon, Bouchier-Hayes et al. 2003, Brahimi-Horn, Chiche et al. 2007, Wouters, Pauwels et al. 2007). Drug uptake and accumulation might also be altered (Shannon, Bouchier-Hayes et al. 2003). Tumour hypoxia may also create a selective advantage to cells with p53 mutations that are resistance to hypoxia-induced apoptosis resulting in clonal production of resistant and more aggressive cells (Graeber, Osmanian et al. 1996, Shannon, Bouchier-Hayes et al. 2003). Reduction in oxygen free radical generation, essential for the efficacy of radiotherapy, may also explain some of the resistance seen in anaemia to chemoradiation (Gray, Conger et al. 1953). In addition blood transfusions are

thought to cause immunomodulation that may increase infection and cancer recurrence (Blumberg and Heal 1994).

3.1.9 Summary

The importance of anaemia in oesophagogastric cancer cannot be understated with strong associations and plausible mechanisms for its impact upon outcomes following chemotherapy and surgery. Further, it represents a potential target for intervention and another possible step towards continuing to improve outcomes, both from the oncological and surgical aspects of treatment.

3.2 Aims and Objectives

3.2.1 Aims

This study examines clinical anaemia in oesophagogastric cancer. The main study was performed to examine the natural history of anaemia during oesophagogastric cancer and included all patients diagnosed with oesophagogastric cancer in a 2-year period from 1st January 2012 to 31st December 2013.

Two nested studies were performed within the main study. The first combined and compared data from surgically treated oesophageal cancers from a previous study that looked at a similar 2-year period from 1st January 2003 to 31st December 2004 (Ayantunde, Ng et al. 2008). This allowed associations to be examined over nearly a decade, with new chemotherapy regimes and minimally invasive surgical treatment.

The second nested study combined data from a prospectively maintained database of histological tumour response to neoadjuvant chemotherapy. This study examined if pre-operative anaemia impacts upon neoadjuvant chemotherapy efficacy.

3.2.2 Objectives

Main study

- Investigate the natural history of haemoglobin levels during the treatment of gastro-oesophageal cancer

- Establish if anaemia and/or blood transfusions are an independent risk factor for mortality in oesophagogastric cancer
- Identify factors associated with anaemia
- Identify factors associated with blood transfusions
- Examine survival differences between patients with and without anaemia
- Examine survival differences between patients depending on haemoglobin level
- Examine survival differences between patients with and without blood transfusions

Nested study 1

- To examine if treatment changes in chemotherapy and surgery for oesophageal cancer from 2003 to 2012 have altered anaemia and blood transfusions rates

Nested study 2

- To establish whether anaemia is associated with histopathological response to chemotherapy
- Examine whether anaemia is associated with tumour response grade

3.3 Patients and Methods

3.3.1 Ethics

Ethical approval was granted for this study by the Nottingham University Hospitals Clinical Audit and Evaluation board (reference 14-404C). Informed consent was obtained from all patients who were part of the Nottingham University Hospitals tissue biobank and data was held in accordance with the Data Protection Act 1998 and in adherence to Caldicott principles.

3.3.2 Patients

Main study

All patients discussed at the oesophagogastric multi-disciplinary cancer meeting with histologically proven gastro-oesophageal cancer (ICD-10 codes C15 or C16) between the 1st January 2012 to the 31st December 2013 were included. We excluded patients with no data available on haemoglobin and synchronous tumours.

Clinical data were retrospectively gathered from clinical notes and the electronic Notis (Nottingham University Hospital Trust's electronic record system) records. Co-morbidities were recorded and Charlson scores calculated at time of diagnosis (Charlson, Szatrowski et al. 1994). Medications and iron therapy were collated from GP referrals and hospital electronic discharge letters.

Nested study 1

Data from a previous study (Ayantunde, Ng et al. 2008) from 1st of January 2003 to 31st of December 2004 was compared to the subset of surgically treated patients from the above group.

Nested study 2

A prospectively collated independent histological database of resected oesophageal adenocarcinomas up to February 2013 was cross-referenced with clinical and haematological data available from the main study to allow analysis for histological tumour response.

3.3.3 Treatment

Operative details were collected from operation notes. Surgical resection in those patients treated with surgery was conducted at a single specialist centre and performed by a team of consultant upper gastrointestinal surgeons all of whom perform more than 20 resections per year. All patients received standardised perioperative care according to departmental protocols.

Chemotherapy treatment data were extracted from ChemoCare database (CIS oncology, Coventry), including chemotherapy agents, doses, number of cycles, cycle delays and dose reductions. All chemotherapy was received in a single centre and prescribed by a team of three specialist oesophagogastric consultant oncologists.

Assessment of resected specimens was performed by consultant histopathologists and described using the Mandard TRG and TNM staging systems. Tumour regression grade was scored by one of three gastrointestinal pathologists according to RCPath guidelines. A second histopathologist reviewed specimens at an MDT meeting with significant differences in grading reviewed by a third blinded independent pathologist for conclusive grading.

3.3.4 Definitions

Date of diagnosis was recorded as the date on which histological confirmation of oesophagogastric cancer was reported.

Curative treatment included all patients with a multi-disciplinary discussion outcome that included curative surgery with or without neoadjuvant chemotherapy. Palliative treatment included all patients for whom curative surgery was not considered appropriate and included those who received palliative chemotherapy and best supportive care.

Cancer site was defined by TNM7 criteria (Sobin, Gospodarowicz MK et al. 2009), namely that those tumours with an epicentre within 5cm of the GOJ and oesophageal component were considered GOJ tumours, those above and below 5cm (or with only a gastric component) were considered oesophageal and gastric respectively.

Histopathological chemotherapy response was defined as TRG 1-3 while ‘non-responders’ were defined as TRG 4-5.

3.3.5 Haemoglobin

Haemoglobin was retrieved from electronic records in the Nottingham NOTiS system. Haemoglobin and mean corpuscular volume (MCV) were recorded at diagnosis (closest recorded haemoglobin to histological diagnosis), pre-treatment (nadir Hb in the preceding week prior to chemotherapy or surgery), after each individual cycle of chemotherapy and post-treatment (nadir Hb in the month following treatment).

Anaemia was defined using the World Health Organisation (WHO) definition of anaemia (non-pregnant female <120 g/L, male <130 g/L) (WHO 2011). Haemoglobin was also stratified to give severity of anaemia. In both males and females, severe anaemia was defined as <80 g/L and moderate 80-109 g/L. In males, mild anaemia was defined as 110-129 g/L, low normal 130-149 g/L, high normal >150 g/L. In females, mild anaemia was defined as 110-119 g/L, low normal 120-139 g/L and high normal >140 g/L. Patients were grouped into anaemic and non-anaemic based upon haemoglobin at diagnosis. Rates of allogeneic blood transfusion and units cross matched were also recorded. Iron deficiency was defined as ferritin of less than 15 μg/l or less than 50 μg/l in the presence of inflammation (CRP >5) (Goddard, James et al. 2011).

3.3.6 Survival

Survival was calculated from the date of histological diagnosis to the date of death or last confirmed date of survival. In the group treated with curative surgery survival

analysis was also performed from date of surgery to date of death. Survival was censored on the 1st April 2017 for those patients that remained alive.

3.3.7 Statistics

Statistical analysis was performed using SPSS (IBM, version 22). Continuous variables were tested for skewness and kurtosis and presented as means with standard deviation where normally distributed and median values with range otherwise. Statistical significance was calculated using Mann Whitney U-test or student t-test where appropriate.

Median and range were calculated for haemoglobin and age, which were both positively skewed. Baseline characteristics were analysed using Mann Whitney U test for age and one way analysis of variance (ANOVA) for all other variables. Chi-squared ($\chi 2$) was used to test the association of anaemia with TRG.

Survival analysis was performed using Kaplan-Meier estimation of survival functions. Survival was taken from date of histological diagnosis to death. Survival was censored on 1st April 2017. Kaplan Meier survival curves were tested using Breslow-Wilcoxon test to calculate statistical significance. Variables that demonstrated a significant relationship on univariate analysis were included in a Cox proportional hazards regression model. The level of significance was set at a p-value less than 0.05.

3.4 Results

Main study

3.4.1 Patient characteristics

The main study identified 567 patients of which 550 patients met inclusion criteria, see Figure 3.1. 17 patients were excluded from analysis; 13 patients died before a treatment decision and 4 patients had synchronous tumours (two colorectal, one pancreatic, one glioblastoma). A further 46 had no available data on haemoglobin before treatment. Final analysis was performed for 504 patients.

Patients were predominantly male (71.6%) with no differences in sex between anaemic and non-anaemic groups. The median age of patients was significantly higher in the anaemic group 74 years (range 35-97 years) compared to the non-anaemic group 68 years (range 34-98 years). Tumours were predominantly in the oesophagus (56.4%) followed by the stomach (23.7%) and gastro-oesophageal junction (20%). Histologically tumours were adenocarcinoma in 80.7% and squamous cell carcinoma in 17.4%. Patients MDT outcomes are described in Figure 3.1 and intent of treatment determine by the MDT meeting for each patient. 259 patients were treated with curative intent (51.4%), 145 palliative intent (including stenting, palliative chemotherapy and palliative radiotherapy) (28.7%) and 100 patients for best supportive care only (19.8%). Those treated with curative or palliative intent were similar in age (p=0.262) compared to those for best supportive

care who were significantly older ($p < 0.001$ One-way ANOVA). The baseline characteristics are summarised in Table 3.2.

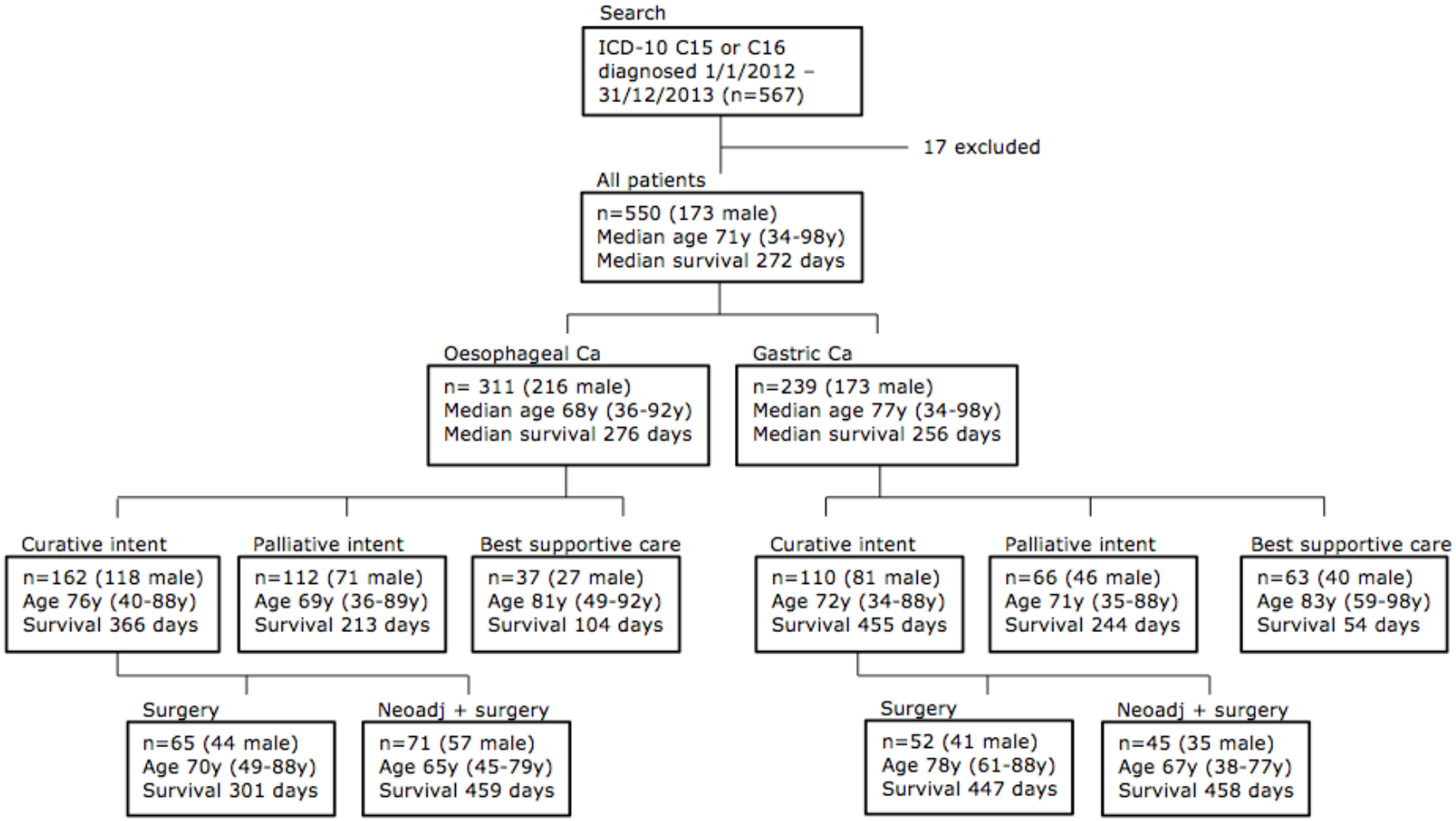


Figure 3.1 MDT outcomes for oesophagogastric cancer from 2012 to 2013

3.4.2 Anaemia

Median haemoglobin at time of diagnosis was 133 g/L (37-188 g/L) for men and 126 g/L (57-161 g/L) for women. 227 patients (45%) were anaemic at diagnosis with higher rates of anaemia in oesophageal cancer compared to gastric cancer (50.7% vs 41.1%, $p<0.05$). About two-thirds of anaemia was normocytic (63.5%) with the other one-third microcytic anaemia (35.4%).

Haematinics were not routinely checked for patients who were anaemic at presentation. Only 32% (n=65) had their ferritin checked, 28% (n=58) had their B12 levels checked and 27% (n=56) had their folate checked. The most common deficiency detected was iron deficiency anaemia, which was present in 43% (n=28) of cases when serum ferritin was measured.

Cancer staging was available for 444 patients. There was no significant relationship between staging of the cancer and rates of anaemia, see Table 3.2. Neoadjuvant chemotherapy (RR 3.605 95% CI 1.765-7.364 $p<0.001$), male sex (RR 3.028 95% CI 1.352-6.780 $p=0.007$) and anaemia at diagnosis (RR 17.726 95% CI 6.804-46.176 $p<0.001$) were the only three predictors of pre-operative anaemia in those undergoing surgery, see Table 3.3 and Table 3.4.

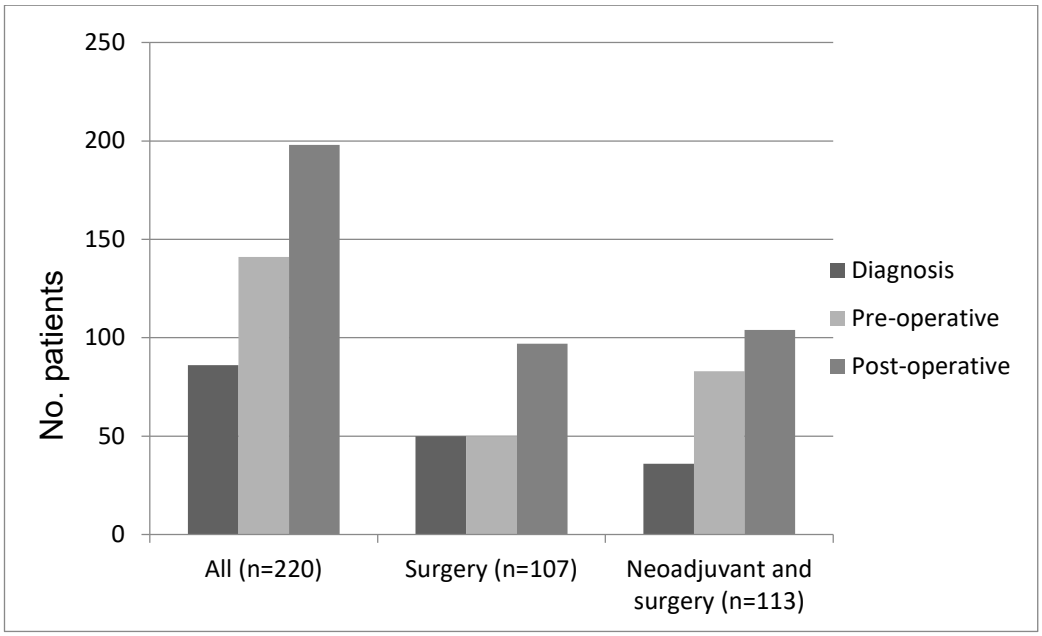
Table 3.2 Demographics and baseline clinical data for the main study

	Anaemia at diagnosis (n= 227)	No anaemic at diagnosis (n=277)	p
Haemoglobin (g/L) Median (range)	112 (37-130)	141 (121-188)	-
Age Median (range)	74 (35-97)	68 (34-98)	<0.001
Sex			
Male	167	194	NS
Female	60	83	NS
Treatment			
Curative	**98**	**161**	-
Surgery only	50	57	
Neoadjuvant CT and surgery	36	77	
Endoscopic mucosal resection	5	15	
Neoadjuvant CT only	6	8	
CRT and surgery	1	4	
Palliative	**129**	**116**	-
Palliative CT	52	58	
Palliative RT	8	7	
Palliative stent	10	11	
Best supportive care	53	31	
Site			
Oesophagus	142	138	0.027
GOJ	36	69	
Stomach	49	70	
Histology			
Adenocarcinoma	176	219	NS
Squamous cell carcinoma	39	46	
Other	12	12	
Stage			
0	7	13	NS
1	29	51	
2	48	46	
3	49	75	
4	54	54	

CT chemotherapy, CRT chemoradiotherapy, RT radiotherapy

Surgery, neo-adjuvant chemotherapy and anaemia

In the main study, 220 patients had curative surgery and 39% (n=89) were anaemic at diagnosis, see Figure 3.2. Prevalence of anaemia increased to 64% (n=141) of patients prior to surgery and was 90% (n=198) post-operatively. Neo-adjuvant chemotherapy increased the number of patients who were anaemic prior to surgery by 26.8%. The average reduction in haemoglobin was also greater, 14.5 g/L compared to 4.7 g/L in those who received neo-adjuvant chemotherapy compared to those who went straight to surgery ($p<0.05$). Only 23 patients had laparoscopic procedures, all of those laparoscopic-assisted Ivor-Lewis oesophagectomy. There were no statistically significant differences in anaemia, transfusions or survival in this group compared to open Ivor-Lewis oesophagectomy or the surgical cohort as a whole.



Anaemia	All patients curative treated (n=220)	Surgery (n=107)	Neoadjuvant and surgery (n=113)
Diagnosis n (%)	86 (39%)	50 (46.7%)	36 (31.8%)
Pre-operative n (%)	141 (64%)	50 (46.7%)	83 (73.5%)
Post-operative n (%)	198 (90%)	97 (90.6%)	104 (92%)

Figure 3.2 Anaemia prior to surgery in patients diagnosed with oesophagogastric cancer in 2012-13

Table 3.3 Risk factors for pre-operative anaemia in those undergoing surgery

Factor	Anaemic	Not anaemic	p-value
Sex			
Male	115	47	p=0.042*
Female	29	23	
Age			
<75 years	100	47	p=0.735
>75 years	44	23	
Anaemic at diagnosis			
Yes	75	7	p<0.001*
No	66	62	
Tumour site			
Oesophageal	80	46	p=0.158
Gastric	64	24	
Neoadjuvant chemotherapy			
No	59	41	p=0.015*
Yes	85	29	
Tumour histology			
AC	124	59	p=0.752
SCC	18	10	
Other	2	1	
T stage			
1	9	5	
2	24	10	p=0.847
3	79	38	
4	19	7	
N stage			
0	61	30	
1	41	16	p=0.754
2	25	13	
3	9	3	

Table 3.4 Independent predictors of pre-operative anaemia by logistic regression analysis

Factor	p-value	RR	95% Confidence Interval
Male sex	0.007	3.028	1.352-6.780
Anaemic at diagnosis	<0.001	17.726	6.804-46.176
Neoadjuvant chemotherapy	<0.001	3.605	1.765-7.364

3.4.3 Blood transfusions

In the main study, patients undergoing treatment for oesophagogastric cancer, 41.5% of patients required a blood transfusion using a total of 851 units of packed red cells, see Table 3.6. The lower haemoglobin was at diagnosis the more likely to receive a blood transfusion, see Table 3.5, $p<0.001$. The vast majority (594 units) of blood transfused were in patients undergoing curative surgery for their disease, with 52.5% of patients requiring transfusion, see Table 3.7. In those patients transfused they received a median of 3 units of blood with one patient receiving a total of 23 units. Anaemia prior to curative surgery increased blood transfusions by 19.7% although this was not statistically significant. Anaemia at diagnosis was the only factor associated with transfusion (RR 2.538 95% CI 1.438-4.479 p=0.001), see Table 3.8 and Table 3.9.

Table 3.5 Odds ratio of receiving a blood transfusion in relation to haemoglobin at diagnosis.

Haemoglobin (g/L)	Odds ratio (95% confidence interval)	P value
>150	1	<0.001*
130-150	1.48 (0.83-2.65)	
110-130	2.01 (1.12-3.61)	
<110	3.77 (1.98-7.16)	
*Denotes statistical significance for overall association and linear trend for logistic regression model.		

Table 3.6 Transfusion rates in oesophagogastric cancer treatment 2012-13

	Number patients transfused (%)	Median number of units transfused	Total number of units transfused
Curative (n=259)	136 (52.5%)	3 (1-23)	594
Palliative (n=245)	73 (29.7%)	3 (1-9)	257

Table 3.7 Transfusions rates comparing those anaemic versus non-anaemic at diagnosis and surgery.

	Transfused (%)	Not transfused (%)	P
Anaemia at diagnosis (n= 227)	106 (46.7%)	121 (53.3%)	<0.05
Not anaemic at diagnosis (n=277)	99 (35.7%)	178 (64.3%)	
Anaemia pre-surgery (n=145)	88 (60.7%)	57 (39.3%)	0.186
No anaemia pre-surgery (n=75)	38 (50.7%)	37 (49.3%)	

Table 3.8 Risk factors for transfusion in those undergoing surgery

Factor	Transfusion	No transfusion	p-value
Sex			
Male	93	84	P=0.549
Female	32	24	
Age			
<75 years	79	76	P=0.249
>75 years	46	32	
Anaemic at diagnosis			
Yes	59	27	P=0.001*
No	62	72	
Tumour site			
Oesophageal	77	59	P=0.284
Gastric	48	49	
Neoadjuvant chemotherapy			
No	65	52	P=0.560
Yes	60	56	
Tumour histology			
AC	103	95	P=0.244
SCC	21	9	
Other	1	2	
T stage			
1	7	9	
2	20	17	P=0.564
3	65	56	
4	19	10	
N stage			
0	53	48	
1	37	24	P=0.846
2	20	18	
3	7	6	

Table 3.9 Independent predictors of transfusion by logistic regression analysis

Factor	p-value	RR	95% Confidence Interval
Anaemia at diagnosis	0.001314	2.538	1.438-4.479

3.4.4 Survival

Median survival, 90 day and 1-year survival were calculated, see Table 3.10. As expected survival was longest in those treated curatively and shortest with best supportive care. Median survival in those treated curatively for oesophageal cancer was 366 days with a 90 day and 1 year survival rate of 90.7% and 49.6%. In gastric cancer these were 455 days, 92.7% and 56.7%. Survival was longer in oesophageal cancer if you received neo-adjuvant chemotherapy before surgery (median survival 459 days vs 301 days p=0.001 Independent samples T test). For gastric cancer survival was similar in this series despite neo-adjuvant therapy (458 days vs 447 days p=0.173 Independent samples T test).

Table 3.10 Survival of patients diagnosed with oesophagogastric cancer in 2012-13.

Oesophageal	Overall (n=311)	Curative (n=162)	Palliative (n=112)	Supportive (n=37)	Surgery (n=65)	Neo +Surg (n=71)
Median survival (days)	276	366	213	104	301	459
90 day	83.6%	90.7%	83.1%	50.0%	86.3%	93.0%
1 year	34.6%	49.6%	21.2%	7.8%	39.3%	61.1%
Gastric	Overall (n=239)	Curative (n=110)	Palliative (n=66)	Supportive (n=63)	Surgery (n=52)	Neo + Surg (n=45)
Median survival (days)	256	455	244	54	447	458
90 day	75.4%	92.7%	80.5%	37.5%	86.7%	95.6%
1 year	37.0%	56.7%	28.3%	10.9%	56.6%	58.6%

Those anaemic at diagnosis had a significantly poorer survival compared to those without anaemia ($p<0.05$), see Figure 3.3. This particularly related to early survival. In those treated with curative surgery the presence of pre-operative anaemia was again associated with poorer survival ($p<0.05$), see Figure 3.4.

When the severity of anaemia was examined, 20.0% of patients had moderate and severe anaemia (Hb < 110 g/L) and 31.9% of patients had mild anaemia (Hb 110-130 g/L). Moderate and severe anaemia was associated with poorer survival when compared with mild anaemia ($p<0.005$), see Figure 3.5. Further, the absolute haemoglobin at diagnosis irrespective of anaemia was associated with survival.

Those patients with a high normal haemoglobin above 150 g/L (14.1% n=71) having a better survival than those with a low normal haemoglobin 130-150 g/L (33.9% n=171), $p<0.001$.

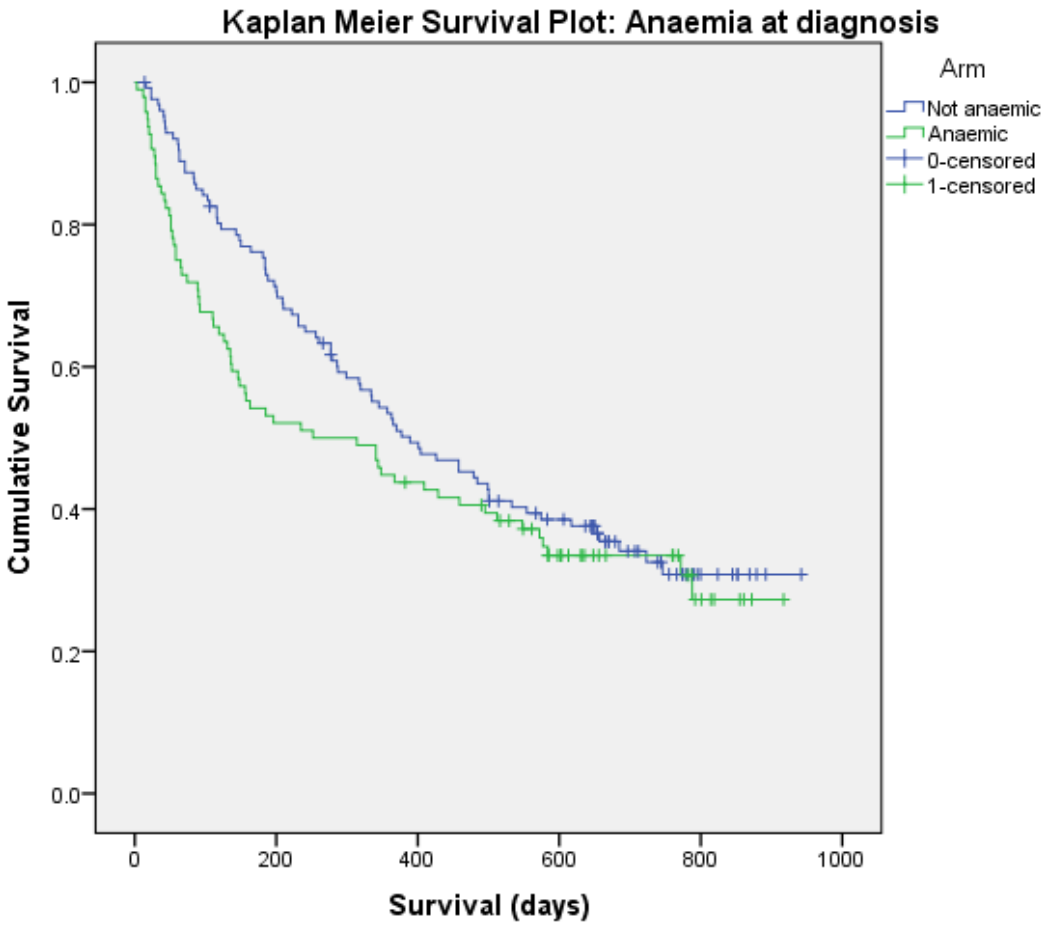


Figure 3.3 Survival of patients diagnosed with oesophagogastric cancer in 2012-13 comparing anaemia with no anaemia at diagnosis (p=0.035).

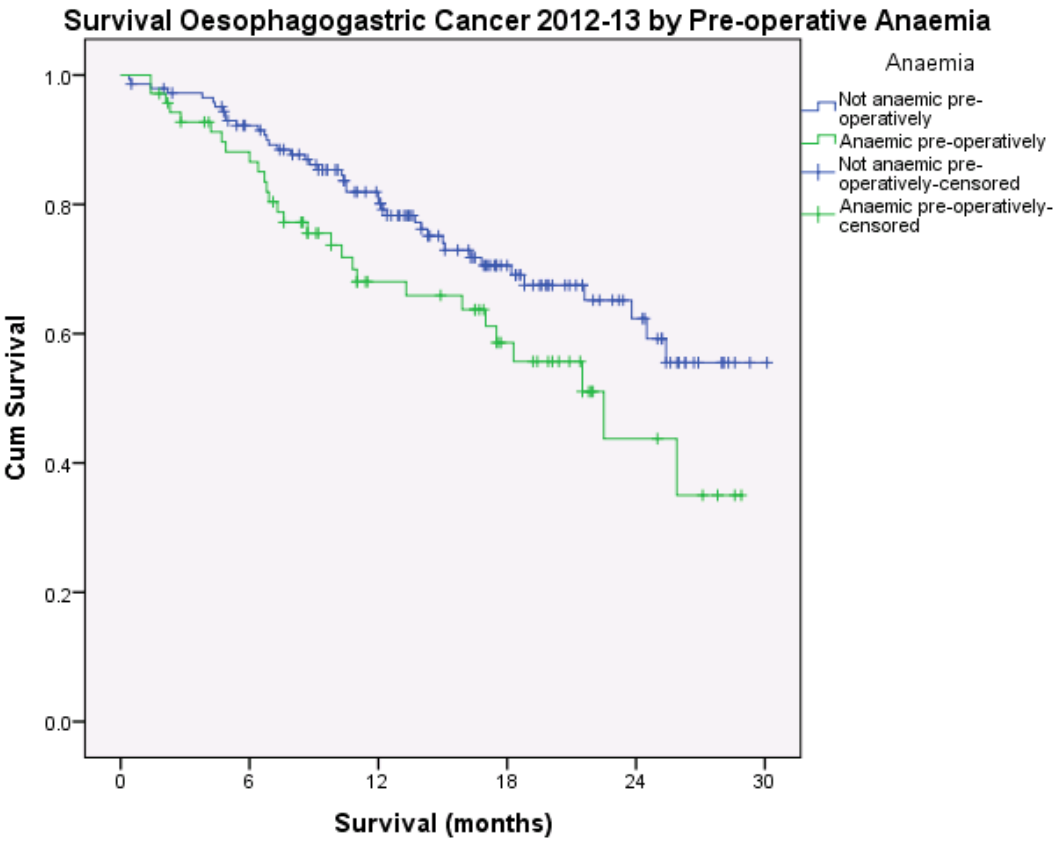


Figure 3.4 Survival of patients diagnosed with oesophagogastric cancer in 2012-13 and treated with surgery comparing pre-operative anaemia (P=0.043).

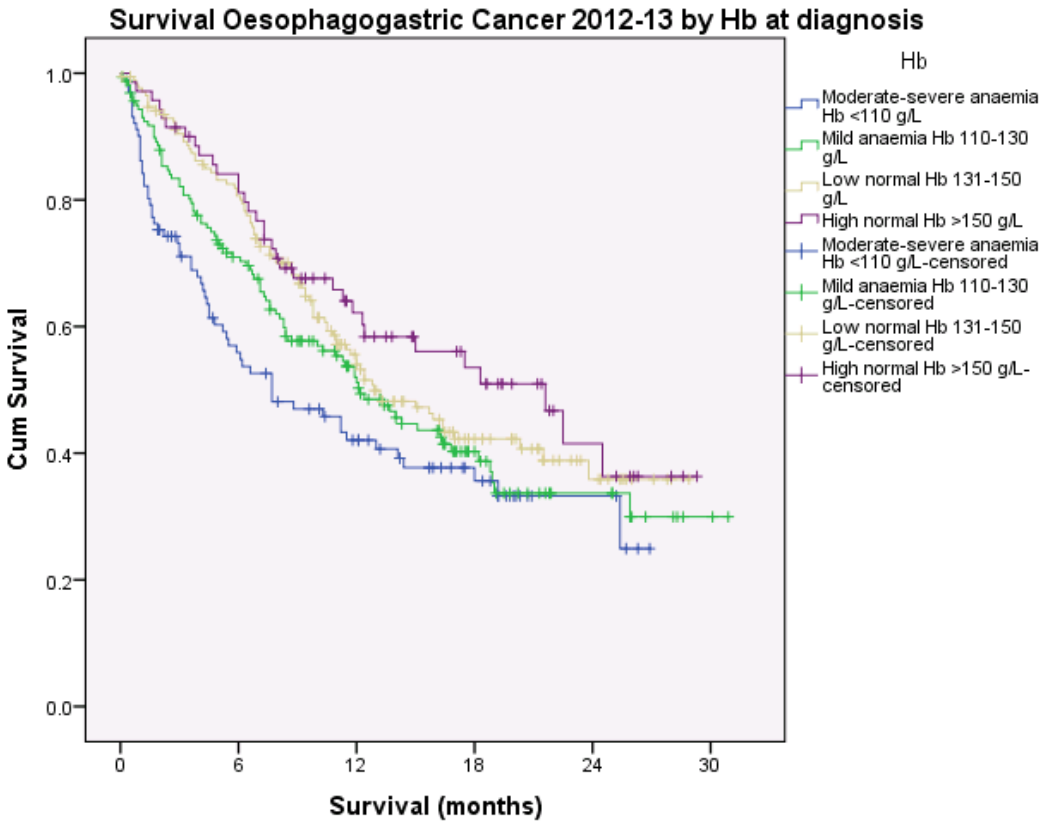


Figure 3.5 Survival of patients diagnosed with oesophagogastric cancer in 2012-13 comparing haemoglobin at diagnosis (P<0.001)

3.4.5 Patient characteristics

A total of 285 patients underwent oesophagectomy during the two two-year periods examined, 2003 to 2004 (n=145) and 2012 to 2013 (n=140). There was no statistical difference in age, sex, tumour site or tumour histology between the two groups, see Table 3.11. Tumours were principally in the lower one-third of the oesophagus and gastro-oesophageal junction (2003-04 88.9% v 2012-13 81.7%) and histologically adenocarcinoma (73.8% v 80.0%).

Treatment

The curative treatment received for oesophageal carcinoma has changed significantly from 2003-2004 to 2012-2013, see Table 3.12. Most apparent is the selection of MAGIC regime ECX chemotherapy over OE-O2 type CF chemotherapy and the increasing numbers of patients receiving chemotherapy. In those with adenocarcinoma, 41.1% (44 of 107) of patients received neoadjuvant chemotherapy in 2003-04 compared to 55.7% (68 of 112) of patients in 2012-13, $p<0.05$. In 2003-04 86.2% of patients completed the prescribed two cycles of CF chemotherapy compared to 78% in 2012-13 who completed the full three cycles of ECX chemotherapy. No data was available for complications or patients who failed to go on to have surgery.

Staging

Patients in 2003-04 had more advanced disease with pathological stage 3 disease in 55.9% (81 patients) compared to 42.1% (59 patients). Only 12.4% (18 patients) had stage 1 disease in 2003-04 compared to 31.4% (44 patients) in 2012-2013. A subset analysis of those who received neoadjuvant chemotherapy showed similar differences in staging, see Table 3.13.

Table 3.11 Patient Characteristics for surgically treated oesophageal cancers from 2003-04 and 2012-13

Characteristic	2003-04 (n=145)	2012-13 (n=140)	p-value
Male	104 (71.7%)	107 (76.4%)	0.365†
Female	41 (28.3%)	33 (23.6%)	
Median age (range)	68 (40-85)	66 (45-84)	0.821‡
>75 years	31 (21%)	28 (20.0%)	
Neoadjuvant chemotherapy (Adenocarcinoma only)	44 of 107 (44%)	68 of 112 (55.7%)	< 0.05*†
Anaemic pre-operative	59 (38.0%)	88 (62.9%)	<0.001*†
Mean Hb (SD) g/L	129 (17)	123 (17)	0.416‡
Tumour histology			
AC	107 (73.8%)	112 (80.0%)	0.236†
SCC	38 (26.2%)	27 (19.3%)	
Other	0	1 (0.7%)	
Pathological TNM stage			
1	18 (12.4%)	44 (31.4%)	
2	43 (29.7%)	36 (25.7%)	0.01*†
3	81 (55.9%)	59 (42.1%)	
4	3 (2.1%)	1 (0.7%)	
Total no. units transfused	316	353	
No. patients transfused	71 (49.0%)	84 (60.0%)	0.062†
Mean units blood received - transfused patients	4.5	4.2	
Mean units blood received - all patients	2.2	2.5	

*p<0.05 † Chi-squared test ‡ Independent samples T-test

Table 3.12 Summary of treatment received for surgically treated oesophageal cancers from 2003-04 and 2012-13

Treatment	2003-2004 No. patients (%)	2012-2013 No. patients (%)
Surgery	(n=145)	(n=140)
Ivor Lewis (2-phase) oesophagectomy	59 (40.6)	89 (63.5) (23 laparoscopic assisted)
Left thoracoabdominal oesophagogastrectomy	32 (22)	29 (20.7)
McKeown (3-phase) oesophagectomy	9 (6.2)	15 (10.7)
Transhiatal oesophagectomy	4 (2.7)	-
Unspecified oesophagectomy	41 (28.2)	7 (5)
Neoadjuvant chemotherapy	(n=64, 44%)	(n=69, 49.2%)
ECF or ECX	8, all ECF (12.5)	59, 2 ECF (85.5)
CF or CX	29, all CF (45.3)	6, 2 CF (8.7)
EOX	-	3 (4.3)
MIC	9 (14)	-
Unspecified	18 (28.1)	1 (1.4)

ECF (epirubicin, cisplatin and fluorouracil 5FU), ECX (Epirubicin, Cisplatin and Capecitabine (Xeloda®)), CF (cisplatin and fluorouracil 5FU), CX (Cisplatin and Capecitabine (Xeloda®)), EOX (Epirubicin, Oxaliplatin, Capecitabine), MIC (mitomycin, ifosfamide and cisplatin)

Table 3.13 Pathological stage in patients who received neoadjuvant chemotherapy.

Stage	2003-04 No. patients	%	2012-13 No. patients	%
1	6	9.4%	18	26.9%
2	22	34.4%	23	34.3%
3	33	51.6%	26	38.8%
4	3	4.7%	0	0.0%

3.4.6 Anaemia

Mean pre-operative haemoglobin was similar in 2003-04 to 2012-13, 129 g/L (SD 17) compared to 123 g/L (SD 17), p=0.416. However, in those who had neoadjuvant chemotherapy for adenocarcinoma, haemoglobin was significantly different (p=0.04), 137 g/L (SD 19) vs 122 g/L (SD 17) from 2003-04 compared to 2012-2013. Within the 2012-13 group a significant drop in haemoglobin from diagnosis, 135 g/L (SD 21) to pre-operatively 121 g/L (SD 16) was also seen in the neoadjuvant group (p<0.01). In those patients who had no neoadjuvant chemotherapy haemoglobin was not significantly different (p= 0.113).

When anaemia rates were examined, patients in 2012-13 were significantly more likely to be anaemic pre-operatively (62.9% vs 38%, p<0.001). This was more pronounced in patients with adenocarcinoma (65.2% vs 34.6%, p<0.001). Patients had more transfusions in 2012-13 (60% vs 49%, p=0.062), received more blood in total (353 units vs 316 units) and more blood per patient (2.5 units vs 2.2 units), but none of these were statistically significant.

Subset analysis of those receiving neoadjuvant chemotherapy during 2012-13 showed 58 of 78 (74%) were anaemic pre-operatively compared to 29 of 64 (45%) in 2003-04 (p<0.05). No data was available for comparison of intra-operative blood loss between the two time periods. Anaemia was similar in severity with most patients experiencing mild anaemia (Hb 110-130 g/L).

3.4.7 Blood transfusions

23 patients (16.4%) had a laparoscopically assisted operations in 2012-13 compared no patients in 2003-04. 11 of 23 patients were transfused (47.8%) compared to 73 of 117 patients who received open procedures (62.4%).

3.4.8 Survival

No difference was seen in overall 2-year survival from day of surgery in 2003-04 compared to 2012-13 (p=0.18). However, analysis of adenocarcinoma separately revealed better survival in 2012-13 (p=0.05), Figure 3.6. This may be due to higher post-operative mortality in 2003-2004. Neoadjuvant treatment looked to improve survival further in the 2012-13 group (p=0.231), but is not statistically significant. There was no significant differences in survival when those with anaemia were compared with no anaemia (p=0.238).

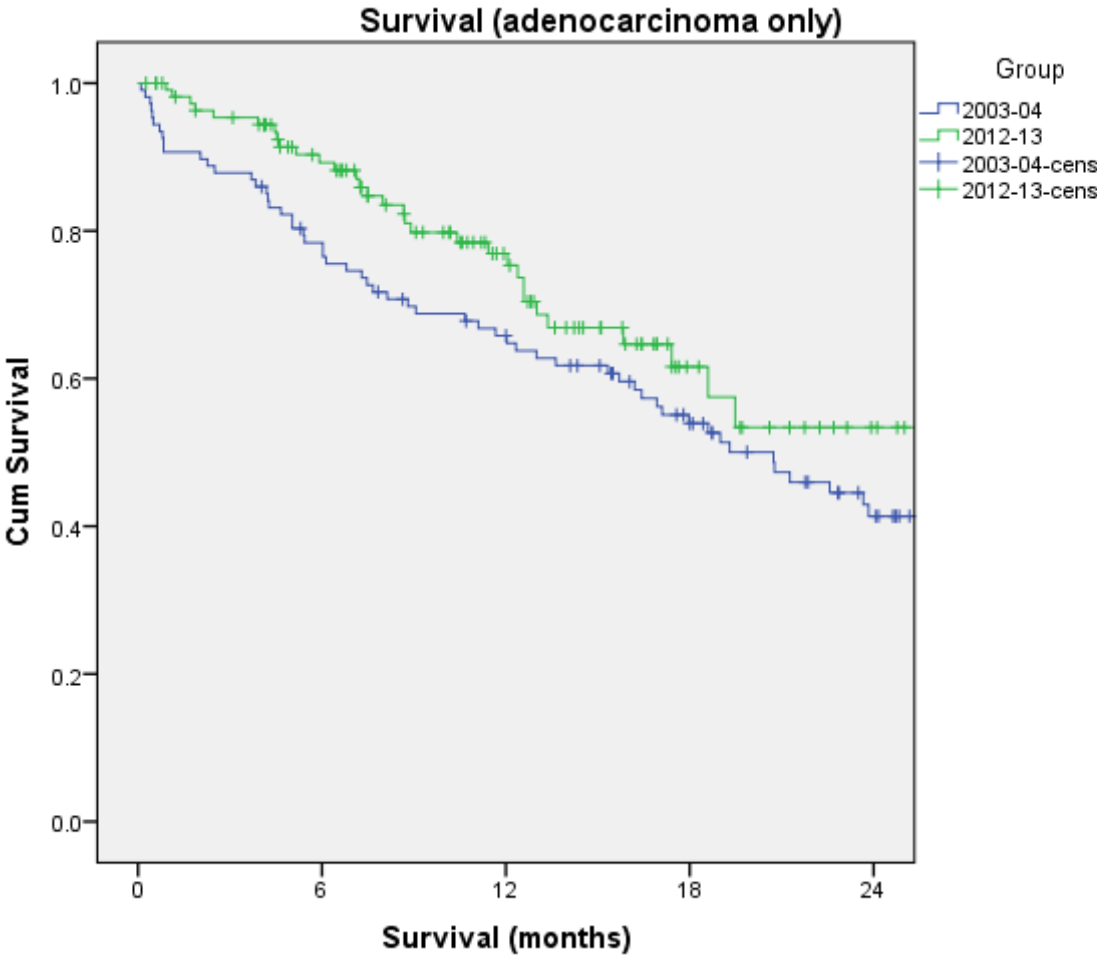


Figure 3.6 Kaplan-Meier survival analysis comparing 2003-04 to 2012-13 for adenocarcinoma only ($p<0.05$).

Nested study 2

3.4.9 Patient characteristics

268 patients had data available for anaemia and histological tumour response grade following chemotherapy. The majority of patients were male (84%, n=226). Chemotherapy received included ECX chemotherapy most commonly (n=228), followed by ECF (n=30) and EOX (n=10).

3.4.1 Anaemia and Tumour response grade

63% (n=169) of patients were classified as histological non-responders (TRG 4-5) with the remaining 27% (n=99) showing some histological response to chemotherapy (TRG 1-3). Anaemia was common in both non-responders (57%, n=57) and responders (43%, n=43). No statistically significant association between pre-treatment anaemia and TRG response was found (OR 0.675 CI 0.420-1.161 P=0.130). Developing anaemia during chemotherapy was also not associated with TRG (OR 0.881 CI 0.406-1.914 P= 0.931). Anaemia severity (p= 0.292) and anaemia at anytime during treatment (p=0.971) were also not associated with TRG, see Table 3.15.

Table 3.14 Demographics and baseline clinical data for the nested study 2

	All patients (n=268)
Haemoglobin (g/L) Median (range)	109 (71-139)
Age Median (range)	67 (31-81)
Sex Male Female	 226 42
Chemotherapy ECF ECX EOX	 30 228 10
Site Oesophagus middle third Oesophagus lower third GOJ Gastric	 7 79 130 52
Histology Adenocarcinoma	 268
Tumour response grade Responder (1-3) Non-responder (4-5)	 99 169

Table 3.15 Statistical analysis of anaemic status, both pre-chemotherapy and anytime against tumour regression grade.

Anaemia status	Responders TRG 1-3	Non-Responders TRG 4-5	Total	Odds ratio/CI,p-value
Pre-chemotherapy Anaemia				
Anaemic	43	57	100	0.68 (CI 0.41- 1.12) P=0.130
Non anaemic	56	110	166	
Anytime anaemia				
Anaemic	83	141	224	0.97 (CI 0.50-1.90) P=0.931
Non anaemic	16	28	44	
Pre-chemotherapy Severity				
Severe/moderate	8	15	23	P=0.292
Mild	35	42	77	
Normal	56	110	166	
Anytime Anaemia Severity				
Severe/moderate	42	67	109	P= 0.902
Mild	41	74	115	
Normal	16	28	44	

3.5 Discussion

3.5.1 Causes and natural history of anaemia

The causes of anaemia in oesophageal cancer are multi-factorial and include bleeding from the tumour site, malnutrition, toxicity from chemotherapy and radiotherapy, chronic inflammation and impaired iron absorption. In our study, anaemia was common amongst patients diagnosed with oesophagogastric cancer with overall rates 46.2% at diagnosis and rates up to 54% in gastric cancer. Anaemia was microcytic in one-third of cases and normocytic in two-thirds, most likely representing a combination of absolute iron deficiency from chronic gastrointestinal blood loss and anaemia of chronic disease.

Anaemia tends to increase dramatically during treatment especially in those patients who receive neo-adjuvant chemotherapy, with over 90% developing anaemia. Those anaemic prior to surgery had poorer survival. Patients who received neoadjuvant chemotherapy were 26.5% more likely to become anaemic and had a much greater drop in their haemoglobin of 14.5 g/L compared to 4.7 g/L ($p<0.05$). Staging does not appear to influence rates of anaemia.

3.5.2 Anaemia and survival

Anaemia at diagnosis in this study was associated with significantly poorer survival and has been associated with poor survival in many other cancers (Knight, Wade et al. 2004, Clarke and Pallister 2005). Further, we demonstrated the more severe the

anaemia at diagnosis the poorer the survival. Higher haemoglobin at diagnosis irrespective of anaemia was also associated with improved survival.

Whether anaemia is confounding or causative is debatable. Tissue hypoxia from anaemia has clearly been shown to lead to induction of 'hypoxia induced factor'. This may further stimulate angiogenesis, disrupt cellular adhesions increasing risk of micro-metastases and alter genes that may lead to more aggressive cells or stimulate oncogenes (Clarke and Pallister 2005, Brahimi-Horn, Chiche et al. 2007). Even mild anemia in oesophageal cancer increases micrometastasises and angiogenesis (Krzystek-Korpacka, Matusiewicz et al. 2009).

The functional effects of anaemia may also explain some of the effects, with anaemic patients showing poorer performance status and exacerbation of their cardiorespiratory comorbidities. These factors are independently prognostic in treatment of oesophageal cancers (Kandaz, Ertekin et al. 2012, Clavier, Antoni et al. 2014).

3.5.3 MAGIC chemotherapy

There was no statistical difference in survival between the OE-O2 and MAGIC chemotherapy regimens for this small cohort of patients, nor would we have expected one in this small study. No difference in survival for anaemia was seen either. However, other studies for chemo-radiotherapy have shown reduced survival outcomes with anaemia and a larger study may have reached significance (Knight, Wade et al. 2004, Shander, Knight et al. 2004, Rades, Lang et al. 2006). Similar

studies in gastric cancer treated with platinum-based chemotherapy or combinations of paclitaxel, platinum and/or 5-FU showed anaemia was associated with decreased survival (Park, Lee et al. 2006, Ye, Liu et al. 2015).

3.5.4 Blood transfusions

Anaemia led to 851 allogeneic red blood cell transfusions in 2012-13 for oesophagogastric cancer and was predominantly associated with surgery. Transfusion rates are higher in those who are anaemic prior to surgery.

In the first nested study, the 2012-13 cohort of surgical patients were more anaemic and received more transfusions than the 2003-04 cohort. However, our 60% rate of transfusion were similar to other studies which report as high as 68.8-76% (Tachibana, Tabara et al. 1999, Langley, Alexiou et al. 2002). This could be due to many factors but did not appear to relate to age, sex, site or histology which were similar across the two groups. The increase in anaemia is unlikely to be due to differences in stage with earlier stage tumours in 2012-13. We do not have data regarding the pre-operative treatment of anaemia and intra-operative blood loss could not be obtained.

More blood transfusions could be as a direct result of these higher rates of pre-operative anaemia. Changes in surgical practice, including the learning curve associated with laparoscopic approach and/or addition of new consultant oesophagogastric surgeons to the previous teams of 2003-04 may have contributed.

The most dramatic change in practice over the last decade has been the increasing use and possibly greater toxicity of neoadjuvant chemotherapy. Those receiving chemotherapy mainly received the MAGIC regimen in the 2012-13 series whilst in 2003-4 the patients would have received two cycles of Cisplatin and 5-flurouracil as per the OE-O2 regimen. Our study suggests that recent MAGIC neoadjuvant chemotherapy is associated with increased frequency (but not severity) of anaemia seen pre-operatively for patients undergoing elective oesophageal resections and is associated with more frequent and numerous blood transfusions.

3.5.5 Anaemia and Response to Chemotherapy

Nested study 2 examined whether anaemia was associated with histological chemotherapy response as one explanation to why anaemia is associated with poorer survival. We found in our group of 268 patients that there was no significant association with anaemia (either before or during chemotherapy) and tumour response grade.

This is despite anaemia being linked with chemotherapy response in gastric cancer (Park, Lee et al. 2006) and radiotherapy (Lee, Park et al. 2009). One explanation for our findings may be the mechanisms of action for the chemotherapy regimen employed here are largely oxygen independent. Rather than the formation of free radicals (for which oxygen supplied by haemoglobin is required), platinum agents such as cisplatin inhibit cell proliferation by direct DNA crosslinking and fluorouracil blocks synthesis of thymidine in DNA replication, both oxygen

independent (Longley, Harkin et al. 2003, Dasari and Tchounwou 2014). Anthracyclines like epirubicin are mixed, have both oxygen dependant and independent mechanisms mode of action, intercalating within DNA strands preventing separation, inhibiting topoisomerase II enzymes, inducing histone eviction and finally generating oxygen free radicals in an iron-mediated process (Gewirtz 1999). For radiotherapy it appears clear that oxygen is essential for radio-sensitivity, which radically reduces when atmospheric oxygen is reduced to less than 25-30 mmHg (Vaupel, Thews et al. 2001). Hypoxia and chemotherapy show much more mixed results (Wouters, Pauwels et al. 2007).

Another plausible explanation is that tumours may be inherently responsive or non-responsive (Tao, Lin et al. 2015). An important study by the OCCAMS consortium using whole genome sequencing demonstrated distinct mutational signatures that correlated well with therapeutic outcomes (Secrier, Li et al. 2016). Hypoxia due to anaemia may therefore only be a minor and negligible determinant of response to chemotherapy in these genetically predetermined in oesophageal cancers.

3.5.6 Limitations

The retrospective conduct of this study is the most important and limiting factor in the interpretation of our results. Due to the methodology, anaemia can be described as an association with outcomes but to imply causality is impossible. Anaemia may be occurring due to more severe disease (although in this study has no association with disease stage or tumour response grade) and is already known to be associated

with increasing age and certain comorbidities. Anaemia could potentially lead to more severe disease through the sequelae of biological events that occur in tumour hypoxia or impact outcomes through its systemic and functional manifestations. Even when correcting analyses for major confounders in anaemia it is important to consider the possibility of important and unknown variables may not have been included.

In survival analysis it is also key to not that covariates might have nonlinear effects on the outcome and there may be interactions between covariates that have affected the outcome. Surgery is a particular covariate to consider, with early mortality due to surgical complications and late survival benefits from successful surgery altering the risk of death over time and potential biasing analysis of survival.

Conclusions regarding anaemia and chemosensitivity should also be caveated with the known limitations of TRG. Histological assessment is subjective but an accepted and validated measure, its validity increased by standardisation of reporting and two reporters. However, it only reflects response in the primary tumour, which has been surgically resected. It therefore may not bear any prognostic weight in nodal disease or reduction in distant micro-metastases. For example, radiological nodal down staging despite no TRG response significantly increases disease-free survival compared to no nodal down-staging (Noble, Nolan et al. 2013).

Finally, no assessment of quality of life or functional outcomes are reported here. This data is not routinely collected. This should not detract from how important

these outcomes are in cancer care, post-operative recovery and survivorship. Longevity of life and quality of life are not necessarily correlated and survival therefore not the sole endpoint for research.

3.6 Conclusion

The associations of anaemia with poorer outcomes in oesophagogastric cancer are well known and have been demonstrated again in this study. In addition we have shown that the initial haemoglobin is important, with high normal haemoglobin favourable, presumably because patients tolerate the inevitable decline in their haemoglobin for longer. We have also shown that a change to the MAGIC regimen of chemotherapy is associated with an increase in peri-operative anaemia and blood transfusions. This is the first study to show anaemia does not appear to influence the efficacy of chemotherapy in oesophageal cancer or impact upon survival benefits from neoadjuvant chemotherapy.

It is unclear how anaemia is currently treated but we suspect that blood transfusions are the mainstay of treatment at present given how few patients were tested for ferritin to look for treatable iron deficiency anaemia. It is also uncertain whether improvement of anaemia and iron deficiency through IV iron therapy would necessarily lead to less morbidity and mortality or prevent the excess morbidity and mortality of allogeneic red blood cell transfusions. However, the possibility should be explored in an attempt to reverse the poor outcomes reported here.

4 Systematic review

4.1 Abstract

Background

Pre-operative anaemia is common and occurs in between 5% to 76% of patients. It is associated with increased peri-operative allogeneic blood transfusions, longer hospital lengths of stay and increased morbidity and mortality. Iron deficiency is one of the most common causes of this anaemia. Oral iron therapy has traditionally been used to treat anaemia but newer, safer parenteral iron preparations have been shown to be more effective in other conditions such as inflammatory bowel disease, chronic heart failure and post-partum haemorrhage. A limited number of studies look at iron therapy for the treatment of pre-operative anaemia. The aim of this Cochrane review is to summarise the evidence for use of iron supplementation, both enteral and parenteral, for the management of pre-operative anaemia.

Objectives

The objective of this review is to evaluate the effects of pre-operative iron therapy (enteral or parenteral) in reducing the need for allogeneic blood transfusions in anaemic patients undergoing surgery.

Search methods

We ran the search on 3 November 2016. We searched the Cochrane Injuries Group's Specialised Register, Cochrane Central Register of Controlled Trials (CENTRAL, The Cochrane Library), Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R), EMBASE Classic and EMBASE (Ovid), CINAHL Plus (EBSCO), PubMed, clinical trials registries, conference abstracts, and we screened reference lists.

Selection criteria

We included all randomised controlled trials (RCTs) which compared pre-operative iron monotherapy to placebo, no treatment, standard of care or another form of iron therapy for anaemic adults undergoing surgery. Anaemia was defined by haemoglobin values less than 130 g/L for males and 120 g/L for non-pregnant females.

Data collection and analysis

Data were collected by two authors, Oliver Ng and Barrie Keeler, on the proportion of patients who receive a blood transfusion, amount of blood transfused per patient (units) and haemoglobin measured as continuous variables at pre-determined time-points: pre-treatment, pre-operatively but post-treatment, and post-operatively.

Statistical analysis was performed using the Cochrane statistical software, Review Manager 5.3. Outcome data were summarised in tables and a forest plot.

Main results

Six prospective randomised controlled studies evaluated pre-operative iron therapy to correct anaemia and included 372 patients in total. Five trials reported the primary outcome (proportion of patients who received allogeneic blood transfusions) for 316 patients (200 iron versus standard care, 116 oral iron versus intravenous iron). Meta-analysis of iron therapy versus standard care showed a non-significant reduction in the proportion of patients who received a blood transfusion (RR 1.21, 95% CI 0.87 to 1.70; participants = 200; studies = 4). Only one study compared oral versus intravenous iron and reported no significant difference in transfusions.

For the secondary outcomes, no difference in haemoglobin at the end of treatment pre-operatively was seen with iron therapy compared to placebo or standard care (MD 0.63, 95% CI -0.07 to 1.34; participants = 83; studies = 2). Intravenous iron versus oral iron showed an increase in haemoglobin with intravenous iron at the end of treatment pre-operatively (MD 1.23, 95% CI 0.80 to 1.65; participants = 172; studies = 2). Ferritin levels were increased by intravenous iron, both when compared to standard care and compared to oral iron (MD 395.03, 95% CI 227.72 to 562.35; participants = 151; studies = 2). Other secondary outcomes including quality of life, short-term mortality and post-operative morbidity were no different between interventions.

Authors' conclusions

The use of iron therapy for pre-operative anaemia does not show a statistically significant reduction in the proportion of patients who received an allogeneic blood transfusion compared to no iron therapy. However, the 372 patients in our analysis falls far short of the 819 patients our information size calculation recommended to detect a 30% reduction in blood transfusions. Intravenous iron is more effective than oral iron at increasing haemoglobin. These conclusions are drawn from only six studies including three very small randomised controlled studies. Further well designed, adequately powered randomised controlled studies are required to determine the true effectiveness of iron therapy for pre-operative anaemia.

4.2 Background

4.2.1 Description of the condition

Anaemia is defined as a total reduction in erythrocyte number, reduced amount of circulating haemoglobin, or decreased circulating red blood cell mass (Perkins 2006), resulting in a pathological state where the oxygen-carrying capacity of blood is insufficient to meet physiological demand (Varat, Adolph et al. 1972). The World Health Organization (WHO) defines anaemia as a haemoglobin level of less than 120 g/L in non-pregnant adult women, less than 110 g/L in pregnant adult women and less than 130 g/L in adult men. Anaemia is a common finding in pre-operative patients, with a prevalence ranging from 5% to 76% depending on the age of the patient, the nature of the condition, and operation planned (Shander, Knight et al. 2004). The most common form of anaemia is secondary to iron deficiency, which can occur secondary to excessive losses, such as chronic haemorrhage, or inadequate intake (Piednoir, Allou et al. 2011).

Anaemia of sufficient severity can cause symptoms such as dizziness, shortness of breath, angina and lethargy. Anaemia in a pre-operative setting is associated with an increased requirement for peri-operative blood transfusion (Benoist, Panis et al. 2001). In patients undergoing colorectal surgery, pre-operative anaemia is an independent risk factor for post-operative complications and a longer post-operative hospital stay (Leichtle, Mouawad et al. 2011). Other studies have shown that peri-

operative anaemia is associated with an increased risk of peri-operative infection and mortality (Dunne, Malone et al. 2002).

4.2.2 Current management of anaemia

Oral iron supplementation and allogeneic blood transfusion are the current standard practice for treatment of anaemia. Erythrocyte stimulating agents and parenteral preparations of iron have also been used to treat anaemia from gastrointestinal cancer but no large blinded randomised control trials conclusively show their efficacy.

4.2.3 Iron therapy

Oral iron therapy (ferrous sulphate, ferrous fumarate) are usually first line treatment for iron deficiency anaemia. Oral iron is associated with a number of gastrointestinal side-effects such as abdominal pain, constipation, diarrhoea and dyspepsia. Non-compliance as a result of these side-effects is a problem.

Parenteral iron was first introduced in the early 20th century in the form of intramuscular and subcutaneous injections (Heath 1932). However, these early formulations caused severe toxic reactions leading to their disuse. Towards the latter half of the 20th century, high molecular weight iron dextran was introduced for both intravenous and intramuscular use. However, the use of high molecular weight iron dextran has been phased out and replaced with low molecular weight iron dextran and other newer formulations of intravenous iron such as iron sucrose, ferric gluconate, ferumoxytol, ferric carboxymaltose and iron isomaltoside. This was due

to reports of anaphylactic-type reactions with the use of high molecular weight iron dextran due to the instability of the molecule as well as the formation of anti-dextran antibodies.

There has been major progress and development of newer formulations of intravenous iron. Earlier high molecular weight iron dextran was linked to fatal anaphylactic-type reactions (Chertow 2004). Iron sucrose, a safer formulation not associated with anaphylactic-type reactions, had to be given in small maximum dosages of 200 mg for each infusion, thus requiring several small dose infusions to achieve the calculated iron deficit.

Newer agents such as ferric carboxymaltose and iron isomaltoside have since been developed which allow total dose infusion and have much higher maximum approved doses and have not been associated with anaphylactic-type reactions (Auerbach, Silberstein et al. 2010).

4.2.4 Description of the intervention

Pre-operative oral iron supplementation has been investigated in colorectal surgery (Lidder, Sanders et al. 2007, Quinn, Drummond et al. 2010) and orthopaedic surgery (Lachance, Savoie et al. 2011) with mixed results. It is cheap, widely available and easily administered but side-effects are common. Oral iron supplementation may also be insufficient to compensate for ongoing blood losses and due to hepcidin mediated reduced intestinal absorption and macrophage sequestration as a result of inflammation (Zhao, Zhang et al. 2013).

The use of intravenous iron has been investigated mainly for the treatment of anaemia in the setting of inflammatory bowel disease and chronic kidney disease. Early studies have shown that intravenous iron is effective in treating anaemia in inflammatory bowel disease, with a quicker result than oral iron and fewer side-effects, an important factor in determining compliance (Kulnigg, Stoinov et al. 2008). The use of intravenous iron in anaemic patients with chronic heart failure has been shown to significantly improve symptoms and quality of life (Okonko, Grzeslo et al. 2008, Anker, Comin Colet et al. 2009). In women with post-partum iron deficiency anaemia, intravenous iron has been shown to be safe and at least as effective as oral iron but with fewer gastrointestinal side-effects (Breymann, Gliga et al. 2008, Seid, Derman et al. 2008). Kim 2009 showed that intravenous iron was more effective than oral iron in the treatment of pre-operative anaemia in women with menorrhagia (Kim, Chung et al. 2009). The use of intravenous iron in patients with chronic kidney disease has been studied and shown to be more effective than oral iron and has fewer side-effects (Qunibi, Martinez et al. 2011).

However, there have been a limited number of studies looking at the use of intravenous iron in a pre-operative setting. The use of intravenous iron for pre-operative anaemia has mainly been studied in orthopaedic surgery. Some of these studies have shown a reduced transfusion rate and infection rate with the use of intravenous iron (Cuenca, Garcia-Erce et al. 2004, Garcia-Erce, Cuenca et al. 2005). An observational study in patients undergoing major surgery (colorectal cancer resections, hysterectomies and lower limb arthroplasties) saw an average increase in

haemoglobin level of 20 g/L within a three- to five-week period (Munoz, Garcia-Erce et al. 2009).

4.2.5 How the intervention might work

The bone marrow requires an internal iron turnover of 20 to 30 mg/day for erythropoiesis. The body absorbs 1 to 2 mg/day of dietary iron, despite the normal diet containing 15 to 20 mg of iron. Ferrous sulphate is one of the most commonly used oral iron supplements and a 200 mg tablet contains 65 mg iron. Oral iron is absorbed most readily on an empty stomach; however, this also increases the risk of gastrointestinal side-effects. Therefore, iron supplements are often taken with food to minimise the side-effects, although this may decrease the absorption by 40% to 66% (Swain, Kaplan et al. 1996). Certain drugs such as antacids, proton pump inhibitors and tetracyclines reduce iron absorption. Oral iron is absorbed mainly in the duodenum where it is reduced into a ferrous state by the duodenal enterocytes and exported via the iron exporter, ferroportin, into the circulation bound to transferrin (Munoz, Garcia-Erce et al. 2009).

Current intravenous iron preparations consist of iron-carbohydrate complexes. Following intravenous injection, the iron-carbohydrate complex is taken up and phagocytosed by the reticulo-endothelial system and the remaining iron core is exported out of the cell and transported for erythropoiesis and storage (Munoz, Garcia-Erce et al. 2009).

The use of intravenous iron bypasses the problems of poor absorption that arise with oral iron supplements. Intravenous iron is also better tolerated with far fewer gastrointestinal side-effects than oral iron (Qunibi 2010). Newer formulations of intravenous iron such as ferric carboxymaltose can be given in large doses (up to 1000 mg) and studies have shown that intravenous iron results in a more rapid rise in haemoglobin when compared with oral iron supplementation (Qunibi, Martinez et al. 2011) and are safe (Auerbach and Macdougall 2014).

New erythrocytes generated following the correction of iron-restricted erythropoeisis in bone marrow will also have a longer half-life than transfused erythrocytes (Kickler, Smith et al. 1985).

4.2.6 Why it is important to do this review

Pre-operative anaemia is a predictor of peri-operative allogeneic blood transfusion (Shander, Knight et al. 2004). Despite screening of blood products, allogeneic blood transfusion carries risks such as viral transmission, immunomodulation, allergic reactions and allo-immunisation (Vamvakas and Blajchman 2009). It has also been independently associated with increased morbidity and mortality (Glance, Dick et al. 2011, Ferraris, Davenport et al. 2012) and reduced cancer-related survival (Acheson, Brookes et al. 2012).

Studies have also associated pre-operative anaemia to increased post-operative morbidity and mortality and increased length of hospital stay (Carson, Duff et al.

1996, Dunne, Malone et al. 2002, Spahn, Moch et al. 2008, Beattie, Karkouti et al. 2009, Acheson, Brookes et al. 2012, Gupta, Sundaram et al. 2013).

Intravenous iron is a method of management of anaemia that is fairly new in the role of managing pre-operative anaemia. It can be given as a large, single-dose regimen with fewer gastrointestinal side-effects than the oral iron tablets (Munoz, Acheson et al. 2017).

The aim of this Cochrane review is to summarise the evidence for use of iron supplementation, both enteral and parenteral, for the management of pre-operative anaemia.

4.3 Objectives

The objective of this review is to evaluate the effects of pre-operative iron therapy (enteral or parenteral) in reducing the need for allogeneic blood transfusions in anaemic patients undergoing surgery.

4.4 Methods

Criteria for considering studies for this review

4.4.1 Types of studies

All randomised controlled trials (RCTs), which compared pre-operative iron monotherapy to placebo, no treatment, standard of care or another form of iron therapy.

Pre-operative iron monotherapy is defined as any iron therapy started after the decision for surgery was made, and initiated before the day of surgery.

4.4.2 Types of participants

Anaemic adults over the age of 18 years undergoing surgery. Anaemia is defined by haemoglobin values less than 130 g/L for males and 120 g/L for non-pregnant females (as per the WHO standard guidelines). We accepted different criteria for anaemia provided that there was a clear definition of anaemia by the study investigators.

We included trials that did not specify anaemic participants if there was stratification of results to an anaemic subgroup. Pregnant women were not eligible for inclusion in this review.

4.4.3 Types of interventions

We included trials that began the administration of iron between the day of decision for surgery and the day before surgery. We included trials with any dose, duration and formulation (enteral or parenteral) of iron.

The types of interventions were:

Oral iron; Parenteral (including intravenous) iron.

We compared between an intervention and placebo/no treatment/standard of care (as per each trial protocol) or between two interventions. Where the effect of iron was combined with another co-intervention, we excluded the trial.

4.4.4 Types of outcome measures

Primary outcomes

Proportion of patients who receive a blood transfusion

Secondary outcomes

Amount of blood transfused per patient (units). Post-operative mortality in the short term (within 30 days) and long term (greater than one year)Post-operative morbidity (including infection rates and adverse events). Any validated measure of quality of life. Measurement value of the following haematologic parameters: haemoglobin, haematocrit, ferritin level and reticulocyte count. Measured as continuous variables at pre-determined time-points: pre-treatment with iron/placebo; pre-operatively but post-treatment with iron/placebo; post-operatively

Information size calculation for the primary outcome

Assuming 20% of patients in the control group require a blood transfusion, and a treatment effect of 30% (i.e. 14% require transfusion following iron therapy), 819 people need to be randomised to receive either iron therapy or control in order to obtain a reliable estimate of the treatment effect (alpha = 0.05, beta = 0.1) (Keeler, Simpson et al. 2017).

4.4.5 Search methods for identification of studies

In order to reduce publication and retrieval bias we did not restrict our search by language, date or publication status.

Electronic searches

The Cochrane Injuries Group's Trials Search Co-ordinator searched the following databases:

Cochrane Injuries Group Specialised Register (25/03/2015);Cochrane Central Register of Controlled Trials (CENTRAL) (Cochrane Library) (issue 3 of 12, 2015);Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R) (1946 to 25/03/2015);EMBASE Classic and EMBASE (Ovid SP) (1947 to 25/03/2015);PubMed (25/03/2015);ISI Web of Science: Science Citation Index Expanded (SCI-EXPANDED) (1970 to 25/03/2015);ISI Web of Science: Conference Proceedings Citation Index-Science (CPCI-S) (1990 to 25/03/2015);ClinicalTrials.gov (https://clinicaltrials.gov/) (25/03/2015);WHO International Clinical Trials Registry Platform (ICTRP) Search Portal (http://apps.who.int/trialsearch/) (25/03/2015).

All search strategies are listed in Appendix 1. We adapted the MEDLINE search strategy as necessary for each of the other databases: the added study filter is a modified version of the Ovid MEDLINE Cochrane Highly Sensitive Search Strategy for identifying randomised trials; to the EMBASE search strategy we added the study design terms as used by the UK Cochrane Centre (Lefebvre 2011).

4.4.6 Data collection and analysis

The Cochrane Injuries Group's Trials Search Co-ordinator ran the searches and collated the search results before passing them on to two authors (ON and BK) for screening.

4.4.7 Selection of studies

Two authors (ON and BK) examined the citations independently and applied pre-agreed selection criteria to identify all potentially eligible studies. Disagreements were resolved through consensus with four authors (ON, BK, MB and AA). We describe the characteristics of excluded studies and reasons for their exclusion in the 'Characteristics of excluded studies' table.

4.4.8 Data extraction and management

One author (ON) extracted data relevant to each study using a standardised data extraction form and presented information in the 'Characteristics of included studies' table. Another author (BK) double-checked the data. We resolved disagreements by discussion between the two authors and involved additional authors (AA, MB) when necessary. When information was unclear, we contacted the study investigators for

further details. Data extraction was also reviewed by the editor of the Cochrane injuries group.

4.4.9 Assessment of risk of bias in included studies

Two authors (ON and BK) independently assessed each study report for risk of bias by making judgements on the following questions according to the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions (Higgins, Altman et al. 2011).

Was the allocation sequence adequately generated (to check for possible selection bias)? Was the allocation sequence adequately concealed (to check for possible selection bias)? Was the study blinded with reference to participants, personnel and outcome assessors (to check for possible performance bias)? Was there a suggestion of incomplete outcome data (to check for possible attrition bias through withdrawals, dropouts and protocol deviations)? Was there an intent-to-treat analysis? Was there any suggestion of selective outcome reporting? Were there any other sources of bias? What was the overall risk of bias?

We assessed the magnitude and direction of bias based upon our assessment of each study. If we considered bias likely to impact on findings, we explored the effect of the bias by undertaking sensitivity analyses. Risk of bias for Keeler 2017 was also reviewed by the editor of the Cochrane injuries group.

4.4.10 Measures of treatment effect

We carried out statistical analysis using the Cochrane statistical software, Review Manager 2014.

For dichotomous data, we present results as summary risk ratios (RRs) with 95% confidence intervals (CIs).

We calculated mean differences (MDs) or standardised mean differences (SMDs) with 95% CIs between the study groups.

4.4.11 Unit of analysis issues

The unit of analysis is the participant.

4.4.12 Dealing with missing data

For included studies, we noted the levels of attrition in the 'Risk of bias' table. We carried out analyses on an intention-to-treat basis as far as possible.

4.4.13 Assessment of heterogeneity

We assessed included trials for heterogeneity by visually examining the forest plots for the estimated treatment effects. We used the I^2 statistic to assess statistical heterogeneity. We regarded heterogeneity as moderate when I^2 was greater than 30%.

4.4.14 Data synthesis

We present outcome data in tables and a forest plot. We created a 'Summary of findings' table according to guidance in the Cochrane Handbook for Systematic Reviews of Interventions (Higgins, Altman et al. 2011). Measures taken at final follow-up were compared between treatment groups.

We used the fixed-effect model in the meta-analysis. The amount of blood transfused per patient was measured in units (where one unit contains approximately 250 millilitres of blood).

In the 'Summary of findings' table, we report the primary and secondary outcome measures.

4.5 Results

4.5.1 Results of the search

The previous search for the 2015 review was performed on 25 March 2015 and retrieved 894 records. An updated search performed on the 3 November 2016 retrieved a further 354 records (see Figure 4.3). From these we identified six studies that used iron therapy as an intervention without the concomitant administration of another therapy (Lidder, Sanders et al. 2007, Edwards, Noble et al. 2009, Kim, Chung et al. 2009, Serrano-Trenas, Ugalde et al. 2011, Froessler, Palm et al. 2016, Keeler, Simpson et al. 2017).

4.5.2 Included studies

We found six prospective randomised controlled studies that evaluated the use of pre-operative iron therapy to correct anaemia. Three studies were in colorectal surgery (Lidder, Sanders et al. 2007, Edwards, Noble et al. 2009, Keeler, Simpson et al. 2017), one in gynaecological surgery (Kim, Chung et al. 2009), one in orthopaedic surgery (Serrano-Trenas, Ugalde et al. 2011) and one in major abdominal surgery (Froessler 2016). Six studies detailed the use of iron therapy prior to surgery for pre-operative anaemia and included a total of 310 participants. One study compared oral iron versus no iron therapy (Lidder, Sanders et al. 2007), three studies compared intravenous iron versus placebo or usual care (Edwards, Noble et

al. 2009, Serrano-Trenas, Ugalde et al. 2011, Froessler, Palm et al. 2016) and two studies compared intravenous iron versus oral iron (Kim, Chung et al. 2009, Keeler, Simpson et al. 2017).

Lidder 2007 conducted an open label prospective randomised controlled trial looking at pre-operative patients undergoing surgery for colorectal cancer. They identified 45 adult patients (28 male). Patients were randomised to receive oral ferrous sulphate (200 mg TDS for 2 weeks) or no iron therapy. 20 patients were anaemic at recruitment (6 in oral iron group, 14 in no iron group). They measured recruitment and pre-operative haemoglobin, ferritin and reticulocyte count, and operative blood transfusions. Only the blood transfusion data are reported separately for the 20 patients with anaemia. This was a pilot study and no power calculation was conducted.

Edwards 2009 conducted a prospective, blinded, placebo-controlled randomised trial involving patients undergoing surgery for colorectal cancer. Sixty patients (39 male) were randomised to receive 600 mg IV iron sucrose in 250 ml 0.9 percent saline or placebo a minimum of two weeks before surgery. Eighteen patients were anaemic at recruitment (nine patients in each arm). The main outcome measures were change in haemoglobin from recruitment to pre-operatively and operative transfusion rates. Study was powered to detect a difference in the haemoglobin change between recruitment and surgery of 5 g/L.

Kim 2009 conducted a phase IV open label prospective randomised controlled trial looking at pre-operative patients undergoing gynaecological surgery for menorrhagia. They included 76 adult female patients with haemoglobin less than 90 g/L and randomised them to receive either intravenous iron sucrose (dose based on calculated total iron deficit) or oral iron (80 mg/day iron protein succinylate) in the three weeks preceding surgery. The study was powered to detect a haemoglobin change of 10 g/L and evaluated change in haemoglobin from recruitment in the study to pre-operatively.

Serrano-Trenas 2011 conducted a prospective randomised controlled trial involving 200 patients, all over 65 years of age, undergoing hip fracture surgery. They did not include anaemia as an inclusion criteria. Patients were randomised to receive either standard treatment or 600mg intravenous iron sucrose. They found no difference in their primary outcome, blood transfusion, for the groups as a whole. Sub-group analysis showed reduced transfusions in intracapsular fractures (standard care 45.7% transfused versus intravenous iron 14.3% $p<0.005$) and interestingly patients with a baseline haemoglobin above 120 g/L (n=110, standard care 35.2% transfused versus intravenous iron 19% $p<0.05$). Presumably those already anaemic and/or with extracapsular fractures, a known risk factor for transfusion, were already at a high risk of transfusion and the effect of intravenous iron did little to reduce this. We include data from the 90 patients who had a haemoglobin less than 120 g/L at recruitment.

Froessler 2016 conducted a prospective randomised control trial involving 72 anaemic patients scheduled for major abdominal surgery (predominantly colorectal surgery 69.4% and hysterectomy 16.7%). Participants were randomised to either intravenous ferric carboxymaltose or usual care. Uniquely, two doses of intravenous iron were administered; the first pre-operatively at a dose of 15mg/kg and the second post-operatively if blood loss exceeded 100 mL at 0.5 mg per mL of intraoperative blood loss. Alongside blood transfusion, haemoglobin and haematinics, this study also reported quality of life. The trial was stopped early due to concerns regarding high blood transfusion rates.

Keeler 2017 conducted an open-label prospective randomised control trial in 116 anaemic patients undergoing curative surgery for colorectal cancer. Participants received either oral ferrous sulphate 200mg twice a day or intravenous ferric carboxymaltose with dose based upon haemoglobin and weight (for example if haemoglobin was less that 100 g/L and weight less than 70 kg the dose given was 1500 mg). The primary outcome was blood transfusions and the study was powered to detect a 1-unit difference in mean blood transfusion. The study also reported haemoglobin, ferritin and transferrin saturations.

4.5.3 Excluded studies

Three studies were excluded. One study excluded anaemic patients (Garrido-Martin 2012), while a second had no subgroup analysis of anaemic patients (Andrews

1997). The third study only randomised non-anaemic participants and gave all anaemic participants iron with no control arm for the group (Crosby 1994).

4.5.4 Risk of bias in included studies

A summary of the review authors' risk of bias judgments can be found in Figure 4.1 and Figure 4.2.

Allocation (selection bias)

All included studies except Lidder 2007 report allocation using a computer-generated randomisation sequence and suitable allocation concealment. The methods of randomisation are not clearly described by Lidder 2007 and the risk of bias is unclear.

Blinding (performance bias and detection bias)

One study was placebo controlled with patients blinded to intervention by means of an opaque sheath over the intravenous giving set (Edwards 2009). All other trials were unblinded with either different routes of administration (Keeler 2017; Kim 2009) or compared to usual care (Serrano-Trenas 2011; Froessler 2016; Lidder 2007.

This absence of blinding is less likely to create bias in the objective outcome measures such as change in haemoglobin and ferritin. It would potentially influence subjective measures like quality of life questionnaires but this was only applicable to one study (Froessler 2016). Blood transfusion, unless administered under a strict transfusion protocol, could potentially be influenced by lack of blinding in these

studies. Three studies report clinicians directly treating participants were blind to the intervention received (Lidder 2007; Froessler 2016; Edwards 2009).

Incomplete outcome data (attrition bias)

One study excluded participants with a compliance of less than 80% from the analysis instead of analysis on an intention-to-treat basis (Kim 2009). This is important, especially when considering oral iron therapy where compliance could be a major factor in the efficacy of the treatment.

Selective reporting (reporting bias)

As reported in incomplete outcome data, one study selectively reported data only from participants in whom compliance was greater than 80%, hence data do not represent all randomised patients on an intention-to-treat basis (Kim 2009). The effect of this is likely to skew the results in favour of intravenous iron therapy.

One study (Froessler 2016) reported early termination of the study after investigators reported higher than expected transfusion rates. Three independent assessors evaluated interim data and two advised termination due to higher than expected poor outcomes. This interim analysis was conducted independently from investigators with data blinded.

Other potential sources of bias

We identified no other source of bias.

Figure 4.1 Risk of bias graph

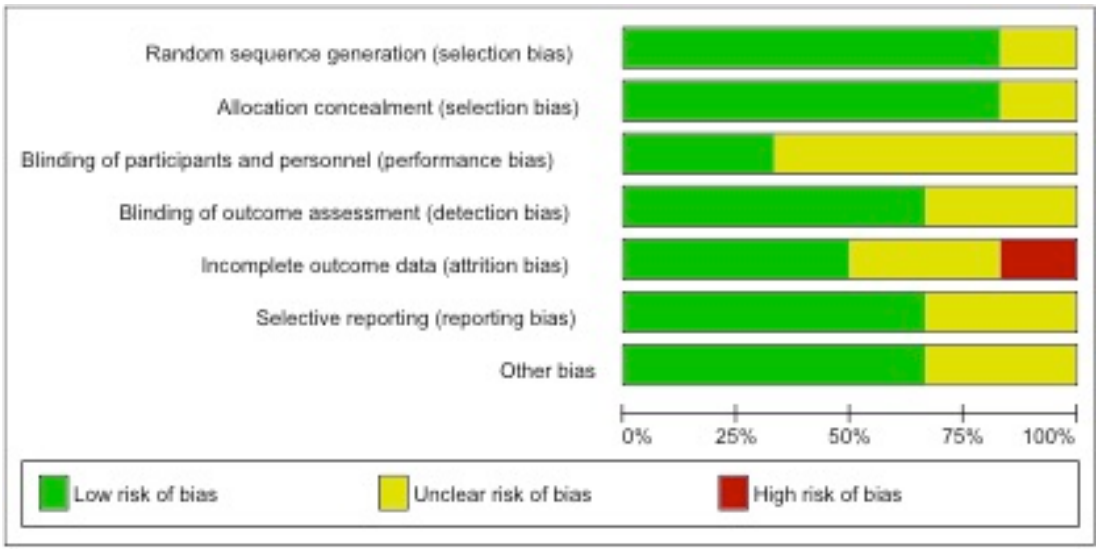
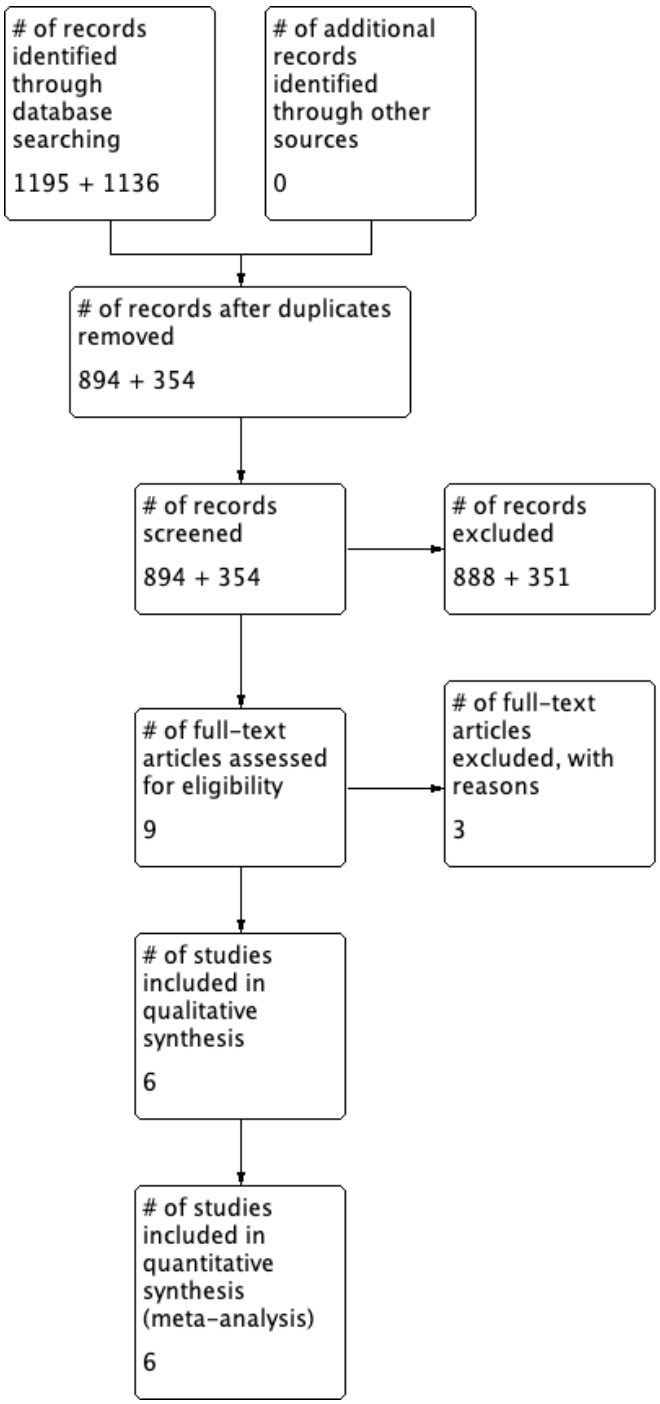


Figure 4.2 Risk of bias summary

Figure 4.3 PRISMA diagram



4.5.5 Outcome 1, Effects of interventions: Iron therapy compared to placebo or standard care

4.5.6 Primary outcome: Proportion of patients who received a blood transfusion

Four studies reported the proportion of patients who received allogeneic blood transfusions (Lidder 2007; Edwards 2009; Froessler 2016; Serrano-Trenas 2011). There was a reduction in the proportion of patients who received a blood transfusion, but the reduction was not statistically significant (RR 1.21, 95% CI 0.87 to 1.70; participants = 200; studies = 4; Analysis 1.1).

4.5.7 Secondary outcomes:

Amount of blood transfused per patient (in units)

Three studies involving 238 participants reported the number of units of blood transfused in each treatment group. However, it was not possible to combine the data because they are skewed and one study did not report subset data for the 90 patients anaemic at recruitment (Serrano-Trenas 2011). The raw data are given in the table below.

Table 4.1 Number of transfusions

Study	Control	Iron group
Edwards 2009	Median 2 units (IQR 3 units) (n=9) (unspecified number of total units transfused)	median 0 units (IQR 1 unit) (n=9) (unspecified number of total units transfused)
Lidder 2007	Median 2.5 units (range 0 to 11 units) (n=14) (39 units transfused in total)	median 1 units (range 0 to 2 units) (n=6) (6 units transfused in total)
Serrano-Trenas 2011	Mean 0.87 units (SD 1.21 units) n=100 (50 anaemic)	Mean 0.76 units (SD 1.16 units) n=100 (40 anaemic)

Post-operative mortality in the short term (within 30 days) and long term (greater than one year)

Two studies did not report mortality (Edwards 2009; Lidder 2007). Two studies reported no difference in short term mortality (Serrano-Trenas 2011; Froessler 2016). No studies reported long term mortality.

Post operative morbidity (including infections and adverse events)

Two studies did not report post-operative morbidity (Edwards 2009; Lidder 2007). Two studies reported no difference in morbidity (Serrano-Trenas 2011; Froessler 2016).

Froessler 2016 reported three minor adverse events (headache, light headedness and back pain). Serrano-Trenas 2011 also report three minor adverse events (one skin rash and two patients with general discomfort). No serious adverse events were reported in any study.

Any validated measure of quality of life

Froessler 2016 reported no difference in quality of life scores after intervention between groups measured using SF-36.

Measurement value of the following haematologic parameters: haemoglobin, haematocrit, ferritin level and reticulocyte count. Pre-specified measurement time points were: pre-treatment, pre-operatively post-treatment, and post-operatively post-treatment.

Haemoglobin level

Both Lidder 2007 and Serrano-Trenas 2011 authors collected data on this outcome at two time points, pre-treatment and pre-operatively post-treatment, but the data are not reported separately for the anaemic patients.

Data were available from Edwards 2009 and Froessler 2016 studies reporting haemoglobin levels for the 83 anaemic patients at the end of treatment, pre-operatively. There was no difference in haemoglobin level between the control and intervention groups (MD 0.63, 95% CI -0.07 to 1.34; participants = 83; studies = 2), Analysis 1.3).

Post-operatively, haemoglobin levels were no different in the iron therapy group (MD 0.17, 95% CI -0.29 to 0.63; participants = 86; studies = 2, Analysis 1.4).

Haematocrit level

The Edwards 2009 study collected data on haematocrit levels pre-treatment, and the end of treatment pre-operatively, and after treatment post-operatively, but no standard deviation values are reported and so it is not possible to analyse the data.

Ferritin

The Lidder 2007 study authors do not report ferritin data separately for the 20 anaemic patients.

The Edwards 2009 study collected data on ferritin levels pre-treatment, and the end of treatment pre-operatively, and after treatment post-operatively, but no standard deviation values are reported and so it is not possible to analyse the data.

Froessler 2016 report a significant difference in ferritin at 4 weeks with intravenous iron therapy (MD 149.00, 95% CI 25.68 to 272.32).

Reticulocyte count

The Lidder 2007 study authors collected data on this outcome at two time points, pre-treatment and pre-operatively post-treatment, but the data are not reported separately for the 20 anaemic patients. No other studies report reticulocyte count.

Figure 4.4 Comparison 1 Iron therapy versus placebo or no iron therapy, Outcome 1 Proportion of patients who received a blood transfusion

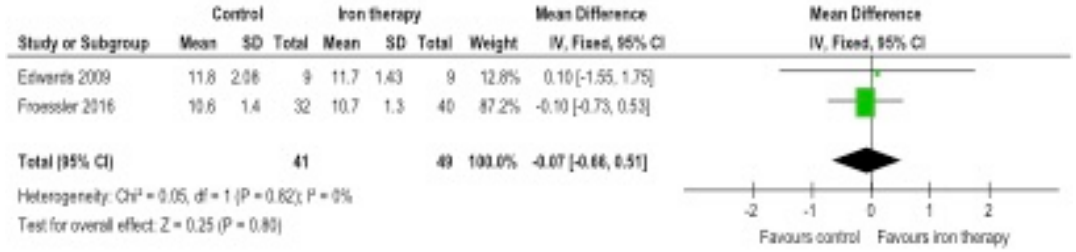


Figure 4.5 Comparison 1 Iron therapy versus placebo or no iron therapy, Outcome 2 Haemoglobin levels pre-treatment (g/dL)

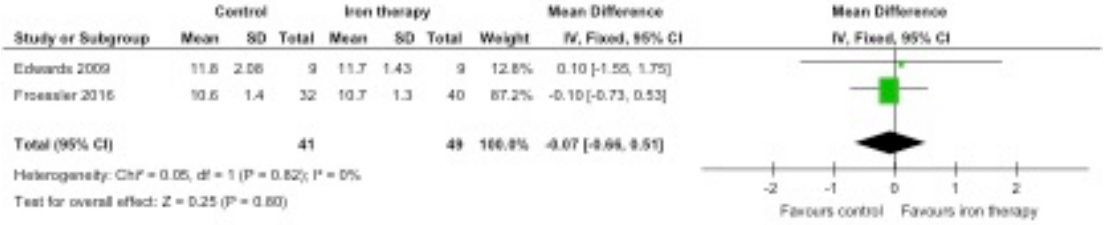


Figure 4.6 Comparison 1 Iron therapy versus placebo or no iron therapy, Outcome 3 Haemoglobin levels post-treatment pre-op (g/dL)

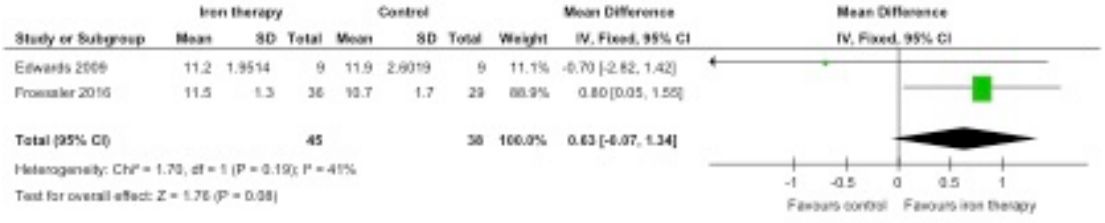


Figure 4.7 Comparison 1 Iron therapy versus placebo or no iron therapy, Outcome 4 Haemoglobin levels after treatment post-op (g/dL)

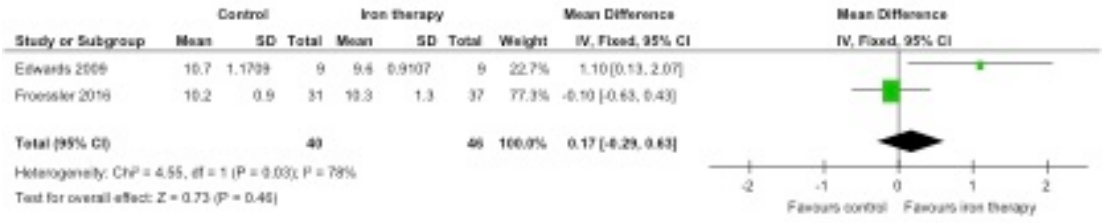


Figure 4.8 Comparison 1 Iron therapy versus placebo or no iron therapy, Outcome 5 Number of units of red blood cells received

Figure 4.9 Comparison 1 Iron therapy versus placebo or no iron therapy, Outcome 6 Ferritin level pre-treatment (ng/mL)

Figure 4.10 Comparison 1 Iron therapy versus placebo or no iron therapy, Outcome 7 Ferritin level post-treatment (ng/mL)

4.5.8 Outcome 2, Effects of Intervention: Intravenous compared with oral iron therapy

Two studies compared intravenous iron therapy with oral iron therapy (Kim 2009; Keeler 2017).

4.5.9 Primary outcome: Proportion of patients who received a blood transfusion

The Kim 2009 study did not measure this outcome. The Keeler 2017 study reports no significant difference in blood transfusions between oral and intravenous iron groups overall.

4.5.10 Secondary outcomes:

Amount of blood transfused per patient (in units)

The Kim 2009 study did not measure this outcome. Keeler 2017 report no significant difference in amount of blood transfused.

Post-operative mortality in the short term (within 30 days) and long term (greater than one year)

The Kim 2009 study did not measure this outcome. Keeler 2017 do not report 30 day mortality but do report no significant difference in 90 day mortality between interventions.

Post-operative morbidity (including infections and adverse events)

No significant difference in grade or rate of all complications or infective complications were found between interventions in the Keeler 2017 study.

The Kim 2009 study report no severe adverse events. Minor adverse events were observed in each group; Two cases of myalgia and one case of injection pain in the intravenous iron group and one event of nausea and one event of dyspepsia in the oral iron group.

Keeler 2017 report one serious adverse event, a rash secondary to intravenous iron and treated with oral anti-histamine. Minor adverse events were observed in both groups; most commonly headaches in the intravenous iron group and 2 patients experiencing dyspepsia and constipation in the oral iron group.

Any validated measure of quality of life

The Kim 2009 study did not measure this outcome. Keeler 2017 study authors did measure quality of life but data is unpublished.

Measurement value of the following haematologic parameters: haemoglobin, haematocrit, ferritin level and reticulocyte count. Pre-specified measurement time points were: pre-treatment, pre-operatively post-treatment, and post-operatively post-treatment.

Haemoglobin level

The Kim 2009 and Keeler 2017 studies reported haemoglobin levels pre-treatment, and there was no difference between the control and intervention groups (MD -0.28, 95% CI -0.65 to 0.10; participants = 172; studies = 2, Analysis 2.4).

Both the Kim 2009 and Keeler 2017 studies measured haemoglobin levels pre-operatively, post-treatment. Haemoglobin levels were significantly higher in the intravenous iron therapy group, compared to the oral iron therapy group (MD 1.23, 95% CI 0.80 to 1.65; participants = 172; studies = 2, Analysis 2.3).

These results are despite Kim 2009 only analysing those with greater than 80% compliance with oral iron therapy and better than widely published compliance with oral iron therapy in the Keeler 2017 study.

Haematocrit level

The Kim 2009 and Keeler 2017 studies do not measure this outcome.

Ferritin

The Kim 2009 and Keeler 2017 studies reported ferritin levels pre-treatment, and there was no difference between the control and intervention groups (MD 6.59, 95% CI -11.75 to 24.93; participants = 151; studies = 2, Analysis 2.4).

The Kim 2009 and Keeler 2017 studies reported ferritin levels pre-operatively post-treatment. Ferritin levels were significantly higher in the intravenous iron therapy

group, compared to the oral iron therapy group (MD 395.03, 95% CI 227.72 to 562.35; participants = 151; studies = 2, Analysis 2.5).

Reticulocyte count

The Kim 2009 and Keeler 2017 studies do not measure this outcome.

Figure 4.11 Comparison 2 Iron therapy: Intravenous versus oral administration, Outcome 1 Proportion of patients who received a blood transfusion

Figure 4.12 Comparison 2 Iron therapy: Intravenous versus oral administration, Outcome 2 Haemoglobin level pre-treatment (g/dL)

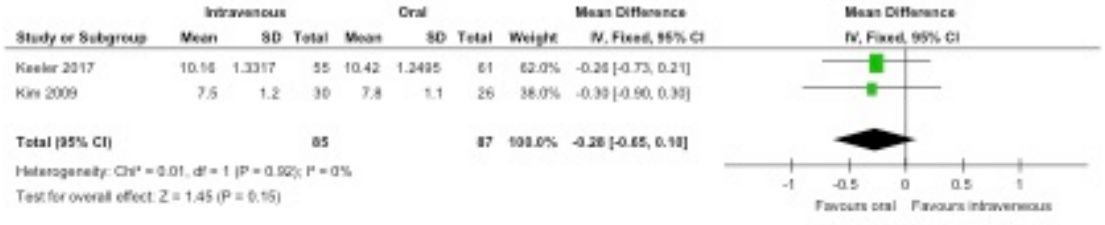


Figure 4.13 Comparison 2 Iron therapy: Intravenous versus oral administration, Outcome 3 Haemoglobin level post-treatment pre-op (g/dL)

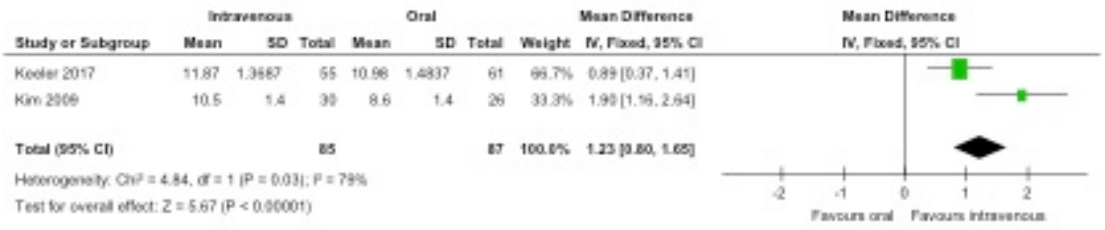


Figure 4.14 Comparison 2 Iron therapy: Intravenous versus oral administration, Outcome 4 Ferritin level pre-treatment (ng/mL)

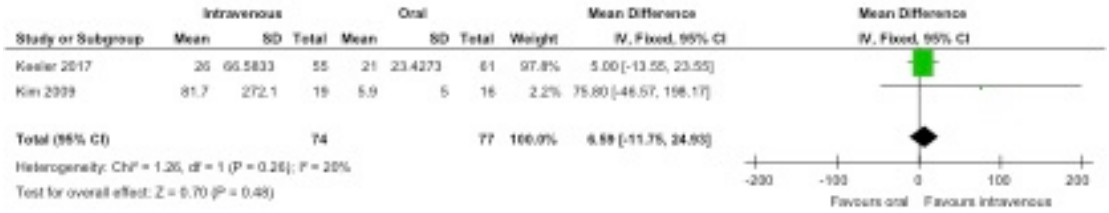
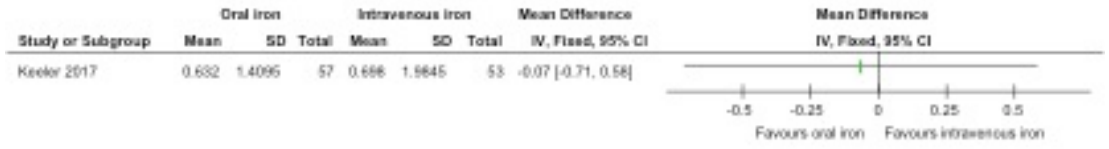


Figure 4.15 Comparison 2 Iron therapy: Intravenous versus oral administration, Outcome 5 Ferritin level post-treatment pre-op (ng/mL)

Figure 4.16 Comparison 2 Iron therapy: Intravenous versus oral administration, Outcome 6 Number of units of red blood cells received



4.6 Discussion

4.6.1 Summary of main results

Six prospective randomised controlled studies evaluated pre-operative iron therapy to correct anaemia, three in colorectal surgery (Lidder, Sanders et al. 2007, Edwards, Noble et al. 2009, Keeler, Simpson et al. 2017), one in gynaecological surgery (Kim, Chung et al. 2009), one in orthopaedic surgery (Serrano-Trenas, Ugalde et al. 2011) and one in major abdominal surgery (Froessler, Palm et al. 2016) and included 372 patients in total. Five trials reported the primary outcome (proportion of patients who received allogeneic blood transfusions) for 316 patients (200 iron versus standard care, 116 oral iron versus intravenous iron). Meta-analysis of iron therapy versus standard care showed a non-significant reduction in the proportion of patients who received a blood transfusion (RR 1.21, 95% CI 0.87 to 1.70; participants = 200; studies = 4; Analysis 1.1). Only one study compared oral versus intravenous iron and report no significant difference in transfusions (Keeler, Simpson et al. 2017).

For the secondary outcomes, all studies except Lidder 2007 and Serrano-Trenas 2011 reported haemoglobin change for the anaemic patients specifically. No difference in haemoglobin at the end of treatment pre-operatively was seen with iron therapy compared to placebo or standard care (MD 0.63, 95% CI -0.07 to 1.34; participants = 83; studies = 2). Intravenous iron versus oral iron showed an increase in haemoglobin with intravenous iron at the end of treatment pre-operatively (MD 1.23, 95% CI 0.80 to 1.65; participants = 172; studies = 2). However, the Kim 2009 study

authors likely bias their results with the exclusion of an important group of patients who had less than 80% compliance with treatment.

Ferritin levels were increased by intravenous iron, both when compared to standard care (Froessler 2016) and compared to oral iron (MD 395.03, 95% CI 227.72 to 562.35; participants = 151; studies = 2, Kim 2009; Keeler 2017).

Other secondary outcomes including quality of life, short-term mortality and post-operative morbidity were no different between interventions.

4.6.2 Overall completeness and applicability of evidence

Evidence regarding iron therapy for pre-operative anaemia is limited with currently only six randomised controlled studies, three of which have a very small sample sizes. Furthermore, the 372 patients available for analysis of the primary outcome does not meet the 819 patients recommended by the information size calculation, preventing us from reaching a reliable conclusion regarding the effects of iron therapy pre-operatively. These studies are also limited in their generalisability with only three surgical specialties represented, albeit specialties where anaemia and blood loss are common.

4.6.3 Quality of the evidence

This update from the previous Cochrane review (Ng, Keeler et al. 2015) has doubled the number of studies and increased the number of patients almost 10-fold from 38 to

372. The three more recent studies have been larger and better designed, reporting fully morbidity, mortality and quality of life.

Kim 2009 made important omissions in not recording blood transfusions and quality of life outcomes. This study also excluded data in the final analysis from participants whose compliance was less than 80%, acknowledging that compliance is a major factor in the efficacy of oral iron therapy, but therefore not reflecting the reality that many patients do not adhere to oral iron due to side-effects.

Edwards 2009, Lidder 2007 and Serrano-Trenas 2011 did not exclude non-anaemic patients or assess for iron deficiency. While they include a subgroup analysis of anaemic patients they do not report all data for this anaemic group and these studies have not been powered to show a difference in the group of patients that require iron to correct their anaemia, namely those with iron deficiency anaemia. As a result these studies have even fewer patients with which to determine the true effect of iron therapy.

4.6.4 Agreements and disagreements with other studies or reviews

The six randomised controlled trials presented here fail to support the conclusions of observational and case-control studies that have demonstrated iron therapy reduces allogeneic blood transfusion and improves pre-operative haemoglobin. These include studies from colorectal surgery (Okuyama 2005; Quinn 2010; Laso-Morales 2016; Calleja 2016), orthopaedics (Cuenca 2004; Cuenca 2005; Theussinger 2007; Munoz 2014), and gynaecological surgery (Breymann 2008). These findings also disagree

with a much larger and broader systematic review of 72 studies including 10,605 patients which included conditions beyond pre-operative anaemia (Litton, Xiao et al. 2013). In this study, meta-analysis showed intravenous iron to be associated with an increase in haemoglobin (standard mean difference 6.5 g/L, 95% confidence interval 5.1 g/L to 7.9 g/L) and a reduced risk of blood transfusion (risk ratio 0.74, 95% confidence interval 0.62 to 0.88).

4.6.5 Implications for practice

Pre-operative anaemia is associated with fatigue, poorer quality of life, increased blood transfusions and an increased risk of post-operative morbidity and mortality. The most common cause of anaemia is due to iron deficiency. Based on the current evidence we cannot conclude that iron therapy improves pre-operative anaemia or reduces the number of patients who receive allogeneic blood transfusions. It may reduce the amount of units of blood received per patient and intravenous iron does appear to be more effective than oral iron at increasing haemoglobin levels prior to surgery. However, these conclusions are based on six studies with a total number of patients well below the number we calculate is required to be conclusive. Further research is very likely to change the results.

4.6.6 Implications for research

Higher quality studies are required to determine the efficacy of iron therapy for the treatment of pre-operative anaemia. Ideally these should be adequately powered large multi-centre trials across the surgical specialities. They should report data for

anaemic patients separately or ideally include only anaemic patients. They should assess for aetiology of the anaemia treated including anaemia of chronic disease and true iron deficiency anaemia. Outcome measurements should include some measure of quality of life, post-operative complications, morbidity and mortality in addition to the haematological parameters and frequency of allogeneic blood transfusion reported in most current studies. It will be important in the design of any future studies to also include strict transfusion guidelines and definitions of iron deficiency and anaemia.

Table 4.2 Characteristics of included studies

Edwards 2009	
Characteristics	
Methods	Prospective randomised blinded placebo-control trial.
Participants	Pre-operative patients undergoing surgery for colorectal cancer (n = 60; note only 18 patients anaemic).
Interventions	IV iron sucrose 600 mg in 2 doses versus placebo.
Outcomes	Transfusion rates and amount of blood transfused, recruitment and admission haemoglobin.
Notes	Study has only 9 anaemic patients in each arm of whom many were not iron deficient.
Risk of bias table	
Random sequence generation (selection bias)	Low risk Quote: "allocated to either the treatment (iron) group or a placebo group, based on a computer-generated randomisation sequence provided by the Research and Development Support Unit. To ensure equal numbers of anaemic patients in each treatment group, randomisation was stratified according to pre-recruitment Hb status: normal (Hb level at least 135 g/L in males and 125 g/L in females), anaemic, or unknown (no test within 2 months of recruitment). Block randomisation was used to ensure similar numbers in each group for each subset."
Allocation concealment (selection bias)	Low risk Quote: "Allocation codes were sealed in sequentially numbered opaque envelopes which were secured within a locked store room in a dedicated research unit."
Blinding of participants and personnel (performance bias)	Low risk Quote: "Although the investigator administering the infusion was not blinded to the treatment group, this was concealed from the patient by using an opaque sheath to cover the drug-giving set."
Blinding of outcome assessment (detection bias)	Low risk Quote: "The chief investigator and clinicians involved in perioperative care also remained blinded to the treatment group for the duration of the trial."
Incomplete outcome data (attrition bias)	Low risk Comment: It appears there was no loss to follow up among people with anaemia.
Selective reporting (reporting bias)	Unclear risk Comment: None identified.
Other bias	Unclear risk Comment: None identified.

Froessler 2016	
Characteristics	
Methods	Prospective randomised control trial of intravenous iron versus usual care
Participants	Adult patients with a ferritin of less than 300 mcg/L, transferrin saturation less than 25% and haemoglobin less than 120 g/L in women and <130 g/L in men undergoing major abdominal surgery (n=72)
Interventions	Single dose intravenous ferric carboxymaltose 15mg/kg up to 1000mg pre-operatively with a second dose post-operatively of 0.5mg/mL blood loss if blood loss was greater than 100mL
Outcomes	Primary outcome was allogeneic blood transfusion. Secondary outcomes haemoglobin, intensive care admission, peri-operative morbidity, mortality, length of stay, iron status and quality of life (SF36)
Notes	Study was stopped early after interim data analysis showed high rates of red blood cell transfusion
Risk of bias table	
Random sequence generation (selection bias)	Low risk Quote: "Randomization followed a computer-generated number sequence and allocation was conducted by telephone"
Allocation concealment (selection bias)	Low risk Comment: computer-generated random allocation
Blinding of participants and personnel (performance bias)	Unclear risk Quote: "The surgeon performing the operation was informed of patient participation in the study but group allocation was not revealed" Comment: No blinding of participants is reported and no placebo administered. It is unclear whether this would influence blood transfusion administration, it would unlikely change haemoglobin levels but could be a major influence on quality of life scores
Blinding of outcome assessment (detection bias)	Unclear risk Comment: No report of who collected outcome data and if they were aware of which intervention participants were allocated to.
Incomplete outcome data (attrition bias)	Unclear risk Comment: No incomplete outcome data was reported.
Selective reporting (reporting bias)	Low risk Comment: None identified.
Other bias	Low risk Comment: None identified.

Keeler 2017	
Characteristics	
Methods	Prospective randomised control trial of intravenous versus oral iron
Participants	Adult patients with haemoglobin less than 110 g/L in women and less than 120 g/L in men undergoing elective surgery for colorectal adenocarcinoma (n=116)
Interventions	Oral ferrous suplhate 200 mg twice a day versus intravenous ferric carboxymaltose (dose calculated on body weight and haemoglobin)
Outcomes	Primary outcome blood transfusions. Secondary outcomes haemoglobin, transferrin, saturations and ferritin
Notes	Inclusion criteria do not include ferritin or transferrin saturations
Risk of bias table	
Random sequence generation (selection bias)	Low risk Quote: "Recruited patients were randomized in a 1:1 fashion via a web-based system using variable block allocation, stratified by patient age and sex"
Allocation concealment (selection bias)	Low risk Comment: computer-generated random allocation
Blinding of participants and personnel (performance bias)	Unclear risk Comment: open-label study with no blinding due to the different routes of intervention administration and the "darkening of stool when ingetsing oral iron". It is unclear whether this would influence blood transfusion administration but it would unlikely change other quantitative measures such as haemoglobin, ferritin or transferrin saturations.
Blinding of outcome assessment (detection bias)	Low risk Comment: Outcome assessors not blind to intervention but unlikely to influence outcome measures.
Incomplete outcome data (attrition bias)	Low risk Comment: Four patients had operation cancelled, one patient died during anaethesia and one patient was deemed inoperable at laparotomy. Patients analysed on an intention to treat basis.
Selective reporting (reporting bias)	Low risk Comment: None identified.
Other bias	Low risk Comment: None identified.

Kim 2009	
Characteristics	
Methods	Prospective randomised control trial, open label.
Participants	Anaemic pre-operative patients with menorrhagia who were due to undergo surgery (n = 76; note only 56 patients > 80% compliance are included in analysis, Hb < 90 g/L).
Interventions	IV iron sucrose (dose according to Ganzoni's formula for cumulative iron deficit) versus oral iron succinylate (dose 80 mg per day for 3 weeks).
Outcomes	Recruitment and admission haemoglobin.
Notes	The study took place between December 2005 and January 2007.
Risk of bias table	
Random sequence generation (selection bias)	Low risk Quote: "computer-generated randomisation table... [to] randomly assign patients."
Allocation concealment (selection bias)	Low risk Quote: "Group allocation was determined by one of the authors not directly involved in patient care."
Blinding of participants and personnel (performance bias)	Low risk Comment: Open label study, no blinding but unlikely to influence the change in haemoglobin.
Blinding of outcome assessment (detection bias)	Low risk Comment: No blinding but objective measurement of haemoglobin unlikely to be influenced.
Incomplete outcome data (attrition bias)	High risk Quote: "Participants who had > 80% compliance were included in the analysis" Comment: Not analysed on intention-to-treat basis. Important because oral iron has reportedly poor tolerance and therefore poor compliance.
Selective reporting (reporting bias)	Low risk Comment: None identified.
Other bias	Low risk Comment: None identified.

Lidder 2007	
Characteristics	
Methods	Prospective randomised control trial.
Participants	Pre-operative patients undergoing surgery for colorectal cancer (n = 49; note only 20 patients anaemic).
Interventions	Oral ferrous sulphate 200 mg TDS versus standard care.
Outcomes	Transfusion rates and amount of blood transfused, pre-treatment and pre-operative haemoglobin.
Notes	Did not exclude non-anaemic patients.
Risk of bias table	
Random sequence generation (selection bias)	Unclear risk Comment: Study does not explain how randomisation was achieved.
Allocation concealment (selection bias)	Unclear risk Quote: "patients were randomised (by telephone to a distant centre)."
Blinding of participants and personnel (performance bias)	Unclear risk Quote: "The clinical team (surgeons, nurses, anaesthetists) wereblinded to treatment allocation. It was not possible to use a placebo and blind the patient, as oral iron alters stool colour."
Blinding of outcome assessment (detection bias)	Unclear risk Quote: "The collection of data was performed by a research fellow not involved in the direct care of the patient."
Incomplete outcome data (attrition bias)	Unclear risk Quote: "Two patients from each group were deemed unsuitable for resective surgery atadmission, two underwent stent insertion and two were referred to the palliative care team." Comment: No incomplete outcome data was reported.
Selective reporting (reporting bias)	Low risk Comment: None identified.
Other bias	Low risk Comment: None identified.

Serrano-Trenas 2011	
Characteristics	
Methods	Prospective randomised control trial.
Participants	Pre-operative patients undergoing hip fracture surgery (n = 200, 90 patients Hb < 120 g/L at baseline)
Interventions	Iron sucrose 600mg IV (in three doses of 200mg IV over 48 hours) versus standard care
Outcomes	Transfusion rates, amount of blood transfused, haematinics, mortality, infections rates and length of hospital stay
Notes	Patients over age of 65 years
Risk of bias table	
Random sequence generation (selection bias)	Low risk Opaque sealed envelopes generated from a randomisation list in blocks of 10
Allocation concealment (selection bias)	Low risk Quote: "neither the patient nor the investigator could know which group the subject was assigned to before his or her consent"
Blinding of participants and personnel (performance bias)	Unclear risk Quote: "Blinding procedures were not used in this trial" Comment: unclear if lack of blinding would influence transfusion practice or other outcomes. Unlikely to influence haemogoblin and haematinics.
Blinding of outcome assessment (detection bias)	Low risk Quote: "Blinded evaluation of trial data by an independent evaluator"
Incomplete outcome data (attrition bias)	Low risk Comment: Data analysed on intention to treat basis
Selective reporting (reporting bias)	Unclear risk Comment: None identified.
Other bias	Unclear risk Comment: None identified.

Table 4.3 Summary of findings iron therapy versus no iron therapy or placebo

Iron therapy versus placebo or no iron therapy for pre-operative anaemia						
Patient or population: Patients with pre-operative anaemia **Settings: Major surgery** **Intervention: Iron therapy versus placebo or no iron therapy**						
Outcomes	**Illustrative comparative risks* (95% CI)**		**Relative effect (95% CI)**	**No of Participants (studies)**	**Quality of the evidence (GRADE)**	**Comments**
	Assumed risk	**Corresponding risk**				
	Control	**Iron therapy**				
Proportion of patients who received a blood transfusion	**635 per 1000**	**356 per 1000** (171 to 749)	**RR 0.56** (0.27 to 1.18)	38 (2 studies)	⊕⊕⊖⊖ **low**[1]	
Amount of blood transfused per patient (in units)	Data from two small studies could not be combined as they were skewed and reported as medians and ranges. One RCT in 18 people reported a difference in medians of 0 (interquartile range: 1) with iron therapy. Another RCT in 20 people reported a median difference of 1 unit with iron therapy (range 0 to 2). -			38 (2 studies)	⊕⊕⊖⊖ **low**[1]	It is not possible to combine the data because they are skewed. These are the raw data.
Post-operative mortality	This outcome was not reported in either of the two studies available.					
Post-operative morbidity	This outcome was not reported in either of the two studies available.					
Any validated measure of quality of life	This outcome was not reported in either of the two studies available.					
Haemoglobin levels at end of treatment pre-op (g/L)	Mean 119 g/L (SD 26)	Mean 112 g/L(SD 19.5)	The mean haemoglobin levels at end of treatment pre-op (g/L) in the intervention groups was **7 g/L lower** (2.82 lower to 1.42 higher)	18 (1 study)	⊕⊕⊖⊖ **low**[2]	Data from one study; the raw data are presented.
***The basis for the assumed risk (e.g. the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).** **CI: Confidence interval; RR: Risk ratio; MD: mean difference; g/L: grams per decilitre of blood.**						

Footnotes

[1] Only two small randomised control trials and a subset of anaemic patients resulting in a very small number of participants.

[2] Only one study with a small number of participants available.

Table 4.4 Summary of findings intravenous iron versus oral iron

Iron therapy: Intravenous versus oral administration for pre-operative anaemia						
Patient or population: Patients with pre-operative anaemia **Settings: Major surgery** **Intervention: Iron therapy: Intravenous versus oral administration**						
Outcomes	**Illustrative comparative risks* (95% CI)**		**Relative effect (95% CI)**	**No of Participants (studies)**	**Quality of the evidence (GRADE)**	**Comments**
	Assumed risk	**Corresponding risk**				
	Oral iron therapy	**Intravenous iron therapy**				
Proportion of patients who received a blood transfusion	This outcome was not reported in the one study available.					
Amount of blood transfused per patient (in units)	This outcome was not reported in the one study available.					
Post-operative mortality	This outcome was not reported in the one study available.					
Post-operative morbidity	This outcome was not reported in the one study available.					
Any validated measure of quality of life	This outcome was not reported in the one study available.					
Haemoglobin levels at end of treatment pre-op (g/L)	mean 86 g/L (SD 14)	mean 105 g/L (SD 14)	The mean haemoglobin levels at end of treatment pre-op (g/L) in the intravenous group was **19 g/L higher** (11.6 to 26.4 higher)	56 (1 study)	⊕⊕⊖⊖ **low**[1,2]	
***The basis for the assumed risk (e.g. the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).** **g/L: grams per decilitre of blood.**						
GRADE Working Group grades of evidence **High quality: Further research is very unlikely to change our confidence in the estimate of effect.** **Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.** **Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.** **Very low quality: We are very uncertain about the estimate.**						

Footnotes

[1] Study excluded those with less than 80% compliance with therapy and compliance was lower in the oral administration group.

[2] Only one study with a small number of participants.

5 Feasibility of intravenous iron isomaltoside to improve anaemia and quality of life during palliative chemotherapy for oesophagogastric adenocarcinoma

5.1 Abstract

Feasibility of intravenous iron isomaltoside to improve anaemia and quality of life during palliative chemotherapy for oesophagogastric adenocarcinoma

5.1.1 Background

Anaemia is common with oesophagogastric adenocarcinoma, increasing mortality, blood transfusions and reducing quality of life (QOL). No clear evidence exists for safe and effective treatment.

5.1.2 Methods

Anaemic patients (Hb <120 g/L women <130 g/L men) with oesophagogastric adenocarcinoma were recruited before initiation of palliative chemotherapy. Patients were randomised to standard care or single dose of intravenous iron isomaltoside (IVI) before chemotherapy. Post-chemotherapy changes in haemoglobin (Hb), ferritin, transferrin saturations (TSAT), blood transfusions and QOL were recorded for three cycles of chemotherapy.

5.1.3 Results

27 patients were randomised to standard care (n=13) or IVI (n=14). No significant change in Hb was seen (standard care MD -6g/L 95%CI -1-11g/L, p=0.336; IVI MD +5 g/L 95%CI -1-11g/L, p=0.903). An increase in ferritin was seen with IVI after cycle one of chemotherapy (standard care 116ng/mL; IVI 770ng/mL, $p<0.05$). No difference in blood transfusions were seen between groups (p=0.851).

IVI improved QOL with physical well-being, emotional well-being, anaemia-specific QOL, trial outcome index and total scores all exceeding minimum clinically important difference. No improvement was seen with standard care.

5.1.4 Conclusion

This feasibility study suggests IVI improves quality of life and ferritin. Larger adequately powered studies are required to definitively conclude if haemoglobin and blood transfusion changes with IVI. However, this would likely need to be a large, multicentre and multinational study and may not be feasible at this scale.

5.2 Background

Anaemia is common in all cancers with 39 per cent of patients anaemic (haemoglobin less than 120 g/L) at presentation and 67 per cent experiencing anaemia within 6 months (Ludwig, Van Belle et al. 2004). This anaemia becomes worse over time and especially during chemotherapy (Groopman and Itri 1999). This is particularly relevant to oesophagogastric cancer where gastrointestinal blood loss exacerbates anaemia, advanced cancer stage and incurable disease is associated with anaemia (Ludwig, Muldur et al. 2013) and chemotherapy has become a major part of treatment, both curative (Cunningham, Allum et al. 2006) and palliative (Wagner, Unverzagt et al. 2010).

Growing evidence supports the negative effects of anaemia in cancer. Anaemia impacts upon prognosis, increasing mortality by as much as 65 per cent (Caro, Salas et al. 2001). It also decreases functional capacity, is associated with lower performance status and reduces quality of life to a level some patients report as worse than death (Groopman and Itri 1999, Harper and Littlewood 2005, Ludwig, Evstatiev et al. 2015). The resulting hypoxia may confer tumours chemoresistance to cytotoxic agents dependant on oxygen reducing the efficacy of treatment (Teicher, Holden et al. 1990, Van Belle and Cocquyt 2003). Symptoms such as fatigue occurs in 58% of patients and result in depression and poor cognitive function (Stone, Richardson et al. 2000). It exacerbates cardiorespiratory symptoms resulting in tachycardia, dyspnoea and arrhythmias (Ludwig and Strasser 2001). Anaemia also

increases blood transfusions, themselves an independent risk factor for increased mortality and venous thromboembolism (Khorana, Francis et al. 2008).

To make matters worse anaemia is often not treated, especially the mild to moderate anaemia (Hb 110-130 g/L) (Ludwig, Van Belle et al. 2004). This is because of limited evidence for effective treatments, under-recognition of its clinical importance and a lack of guidelines (Gordon 2002, Ludwig, Evstatiev et al. 2015).

Anaemia is caused by a multitude of factors including gastrointestinal blood loss, chemotherapy induced-myelosuppression, haemolysis, decreased renal erythropoietin synthesis and nutritional deficiencies, most common amongst these iron deficiency (Grotto 2008). Iron deficiency is common occurring in almost half of all solid tumours (Ludwig, Muldur et al. 2013). Broadly, this iron deficiency can be differentiated into absolute iron deficiency (AID) or functional iron deficiency (FID). Absolute iron deficiency is characterised by depleted iron stores and is defined as a serum ferritin of <30 ng/mL (or <100 ng/mL with inflammation or cancer) with a TSAT <20%. It most commonly occurs due to chronic gastrointestinal blood loss. Functional iron deficiency results in a similar insufficient iron supply (TSAT <20%) but occurs despite replete iron stores (ferritin >30 ng/mL or >100 ng/mL in cancer patients) (Ludwig, Muldur et al. 2013). With functional iron deficiency, iron is retained in the reticuloendothelial system and therefore not biologically available for erythropoiesis. This is mediated by high hepcidin in response to a cascade of inflammatory and immune signals including TNF-α, interferon-γ, IL-1, IL-6 and IL-10. High hepcidin also reduces duodenal iron

resorption rendering oral iron supplementation largely ineffective (Adamson 2008, Goodnough, Nemeth et al. 2010).

Since the late 1980's strategies beyond blood transfusions for anaemia have emerged focussing on erythropoiesis stimulating agents (ESA; epoetin alfa, epoetin beta, darbepoetin). Then from the late 1990's low molecular weight dextran and non-dextran intravenous iron preparations (iron sucrose, iron isomaltoside and ferric carboxymaltose) began to be investigated usually in combination with ESAs.

These new therapies had become necessary as growing evidence for the negative effects of blood transfusions emerged including increased mortality, immunosuppression, venous thromboembolism and cancer progression (Khorana, Francis et al. 2008).

Oral iron had been used, but is seldom nowadays due to the high side effect profile and poor tolerability and efficacy in patients, especially those with functional iron deficiency and thus poor gut absorption of iron. Additionally, no benefit was seen with oral iron when added to ESAs further limiting their utility (Henry, Dahl et al. 2007, Aapro, Osterborg et al. 2012, Steinmetz 2012).

Erythropoiesis stimulating agents have been demonstrated to increase haemoglobin, improve quality of life and reduce blood transfusions (Glaspy 1997, Demetri, Kris et al. 1998, Gabrilove, Cleeland et al. 2001, Littlewood, Bajetta et al. 2001). However, 30-50% of patients do not respond to ESAs and their widespread use has been prevented due to safety issues. ESAs appear to increase tumour growth and reduces

survival (Spivak 2005, Bohlius, Schmidlin et al. 2009). They may also increase venous thromboembolic events especially in relation to ESA induced iron-restricted erythropoiesis (Rizzo, Brouwers et al. 2010, Schrijvers, De Samblanx et al. 2010, Henry, Dahl et al. 2012). At least six trials have demonstrated better haematological response, higher quality of life and reduced transfusions needs if intravenous iron is added to ESA therapy (Ludwig, Evstatiev et al. 2015). Iron stores should therefore be replete and iron replacement a pre-requisite to ESAs otherwise administration exacerbates iron deficiency (Hedenus, Birgegard et al. 2007). International guidelines currently prohibit ESAs if treating with curative intent and only recommend ESAs if receiving chemotherapy and haemoglobin is less than 100-110 g/L and even then at the lowest does to prevent blood transfusion (Bokemeyer, Aapro et al. 2007, Rizzo, Brouwers et al. 2010, Rodgers, Becker et al. 2012). Therefore, ESAs, despite their efficacy, are not suitable for most patients.

No randomised controlled trials have examined the use of intravenous iron monotherapy for the treatment of iron deficiency anaemia in oesophagogastric cancer. However, Steinmetz et al. (2013) conducted a large observational study of 619 patients across 68 centres including a large number of patients with advanced cancers receiving chemotherapy. All patients received ferric carboxymaltose (median dose 1000 mg IQR 600-1500 mg). Those who received ferric carboxymaltose without ESA had a median Haemoglobin increase of 14 g/L (IQR 2–23 g/L; $N = 233$ $p<0.0001$). Patients with high ferritin even greater than 500 ng/mL but low TSAT < 20% also benefited from ferric carboxymaltose. However, only

10.2% had gastric cancers so results are difficult to extrapolate to our patient group (Steinmetz, Tschechne et al. 2013).

Four randomised control trials using intravenous iron monotherapy do exist for other cancers. Three studies examined gynaecological malignancies (Kim, Kim et al. 2007, Dangsuwan and Manchana 2010, Athibovonsuk, Manchana et al. 2013) and the fourth lymphoid malignancy (Hedenus, Karlsson et al. 2014).

Kim (2007) conducted an open-label randomised control trial of 75 anaemic patients (<120 g/L) with cervical cancer receiving platinum-based chemoradiotherapy. Patients received either standard care (n=45) or 200 mg of iron sucrose intravenously (n=30) with each cycle of chemotherapy where mild anaemia was present for up to six cycles. Red blood cell transfusions were less in the intravenous iron arm, 12 (40.0%) patients compared to 29 (64.0%) patients in the control group (P = 0.04) (Kim, Kim et al. 2007).

Dangsuwan (2010) selected 44 anaemic patients (Hb <100 g/L) gynaecological cancer patients who had already received a blood transfusion during chemotherapy and administered 200mg intravenous iron sucrose single dose (n=22) or oral ferrous fumarate 200mg three times a day (n=22) as a preventative strategy for further blood transfusions. Five patients (22.7%) in the intravenous iron arm and 14 patients (63.6%) in the oral iron arm required red blood cell transfusion in consecutive cycles of chemotherapy (p=0.01). They were the only trial to examine quality of life and showed no significant difference between groups (Dangsuwan and Manchana 2010).

The same group followed up this study in 2013, this time examining non-anaemic patients, with an open-label prospective randomised control trial of 64 patients (Hb >105 g/L) with gynaecological malignancy receiving platinum based chemotherapy. Patients were randomised to 200mg intravenous iron sucrose after each chemotherapy for up to six cycles (n=32) or oral ferrous fumarate 200mg three times a day (n=32). Nine patients (28.1%) in the intravenous iron arm and 18 patients (56.3%) in the oral iron arm required blood transfusions ($p = 0.02$) (Athibovonsuk, Manchana et al. 2013).

Of note all three of these studies used a transfusion threshold less than 100 g/L, which in light of research regarding restrictive transfusions would now be consider high. Two studies did not measure ferritin or transferrin saturations either, a significant omission in identifying and understanding the aetiology and efficacy of iron therapy.

The fourth study in lymphoid malignancies, Hedenus et al. (2014) focussed on functional iron deficiency specifically. They recruited 19 patients receiving chemotherapy (Hb <105 g/L, TSAT <20%, ferritin >30 ng/mL women or >40 ng/mL men). Patients received ferric carboxymaltose 1000 mg (n=8) or standard care (n=9). Despite small numbers, the ferric carboxymaltose arm had a mean Hb increase significantly greater than the control arm at week 8 ($p = 0.021$). Hb increased >100 g/L in all ferric carboxymaltose-treated patients and mean TSAT was >20 % after week 2. Of particular note, difficulty in recruitment across 11 sites led to early termination of this study (Hedenus, Karlsson et al. 2014).

In summary, anaemia is common and important. Mild to moderate anaemia is under-recognised and under-treated in palliative cancer care despite its relationship to poorer prognosis and quality of life. Intravenous iron offers potential for treatment where blood transfusions and ESAs are not or should not be used. Iron repletion should also be considered a pre-requisite for ESA therapy, logically making it the first step in anaemia management.

This study aims to assess the feasibility of a single dose intravenous iron therapy to improve anaemia, quality of life and prevent blood transfusions in patients diagnosed with oesophagogastric adenocarcinoma receiving palliative chemotherapy. This would inform a larger multicentre randomised control trial at a later date.

5.3 Methods

An open-label prospective randomised control trial comparing intravenous iron isomaltoside to standard care for anaemia during palliative chemotherapy for oesophagogastric cancer. The study was conducted at two recruiting sites in the UK (Nottingham University Hospitals NHS Trust and the Royal Wolverhampton NHS trust). The study was registered with the Medicines and Healthcare Regulatory Agency, clinical trials.gov (NCT01927328) and EudraCT (2013-000209-22). The study was conducted in accordance with the Declaration of Helsinki and approved by an Independent Ethics Committee.

5.3.1 Enrolment

The CONSORT flowchart illustrates the relative numbers of patients failing screening or declining chemotherapy or trial participation (see Figure 5.1). Included were adult patients with a proven histological diagnosis of oesophagogastric adenocarcinoma, anaemia (<120 g/L in women and <130 g/L in men) and a treatment decision for palliative chemotherapy.

5.3.2 Allocation

Patients were randomised 1:1 to each group using random allocations concealed in opaque envelopes. Patients in the control arm had their anaemia managed by traditional regimens as decided by the clinical oncology team. The patients in the intravenous iron group received a single dose of intravenous iron isomaltoside 1000

(Monofer ®). Dose were calculated using the Ganzoni equation of cumulative iron deficit (Ganzoni 1970). Iron isomaltoside was diluted in 250ml 0.9% sodium chloride and infused over a period of 60 minutes. All subsequent treatment of anaemia was at the discretion of the clinical oncology team.

5.3.3 Follow up

Three follow up visits were performed at the start of each three week cycle of chemotherapy. At each visit, blood analyses (haemoglobin, ferritin, transferrin saturations) and quality of life questionnaires, FACT-An (Cella 1997) and EuroQol EQ-5D (EuroQol 1990) were conducted. Adverse events, complications, blood transfusions and additional iron administration by the clinical oncology team were also recorded.

5.3.4 Analysis

The feasibility outcomes measured included number of eligible patients, study exclusion (screen failure rates), willingness to be recruited and randomised to the study (acceptability), withdrawal of patients (non-concordance) and study retention rates.

Clinical outcomes included haemoglobin, ferritin, TSAT, blood transfusion rate, number of units transfused, mortality, FACT-An and EQ-5D quality of life scores.

5.3.5 Statistics

A descriptive analysis was performed on the data. Numbers and percentages are presented for categorical data, mean and standard deviations for normally distributed continuous data, and median and inter-quartile ranges for non-normally distributed data. The study outcome measures were compared between groups using an independent samples T-test. Changes within groups over time points were compared using a pair samples T-test. All tests were 2-sided, with type I error rates of .05. Minimal clinically important difference (MCID) for quality of life was determined using a distribution method and defined as one standard deviation difference from baseline (Johnston, Ebrahim et al. 2015).

5.3.6 Sample size

No sample size calculation was performed for this feasibility study. We proposed to recruit a total of 40 patients to this trial (20 per group to allow for dropout), based on literature and guidance that 30 or more patients (15 per group) would be sufficient to estimate a parameter (Lancaster, Dodd et al. 2004). Due to poor recruitment and higher than expected exclusion of patients who declined palliative chemotherapy the trial was terminated early.

5.4 Results

5.4.1 Feasibility

From the 23rd September 2013 to 24th July 2017, 901 patients were screened for the IRON trial (see Figure 5.1). Of these 400 (44%) had histologically confirmed adenocarcinoma. 206 patients were anaemic at diagnosis in this group (51.5%). 62 patients were recommended for palliative chemotherapy and therefore eligible for the IRON trial (30%). Pre-screening eligibility was therefore 6.9% and screen failure rate 93.1%. 23 patients (37%) subsequently declined palliative chemotherapy. In comparison, in those offered neoadjuvant chemotherapy only 2 patients of 59 patients declined (3.3%). 39 patients were approached for the trial and acceptability was 66%, with 27 patients willing to participate. The trial was terminated early due to poor recruitment.

Allocated study treatment was received by 25 of the 27 patients (92.5%). Two patients in the intravenous iron group did not receive their intervention. One patient died before administration of iron. The second patient developed a massive upper gastrointestinal haemorrhage, received multiple blood transfusions prior to the administration of iron and was therefore not treated because of concerns regarding iron overload. A third patient in the intravenous iron group died before being administered any chemotherapy and was withdrawn from the trial. Study retention was 88.9% and data were available to analyse 24 patients.

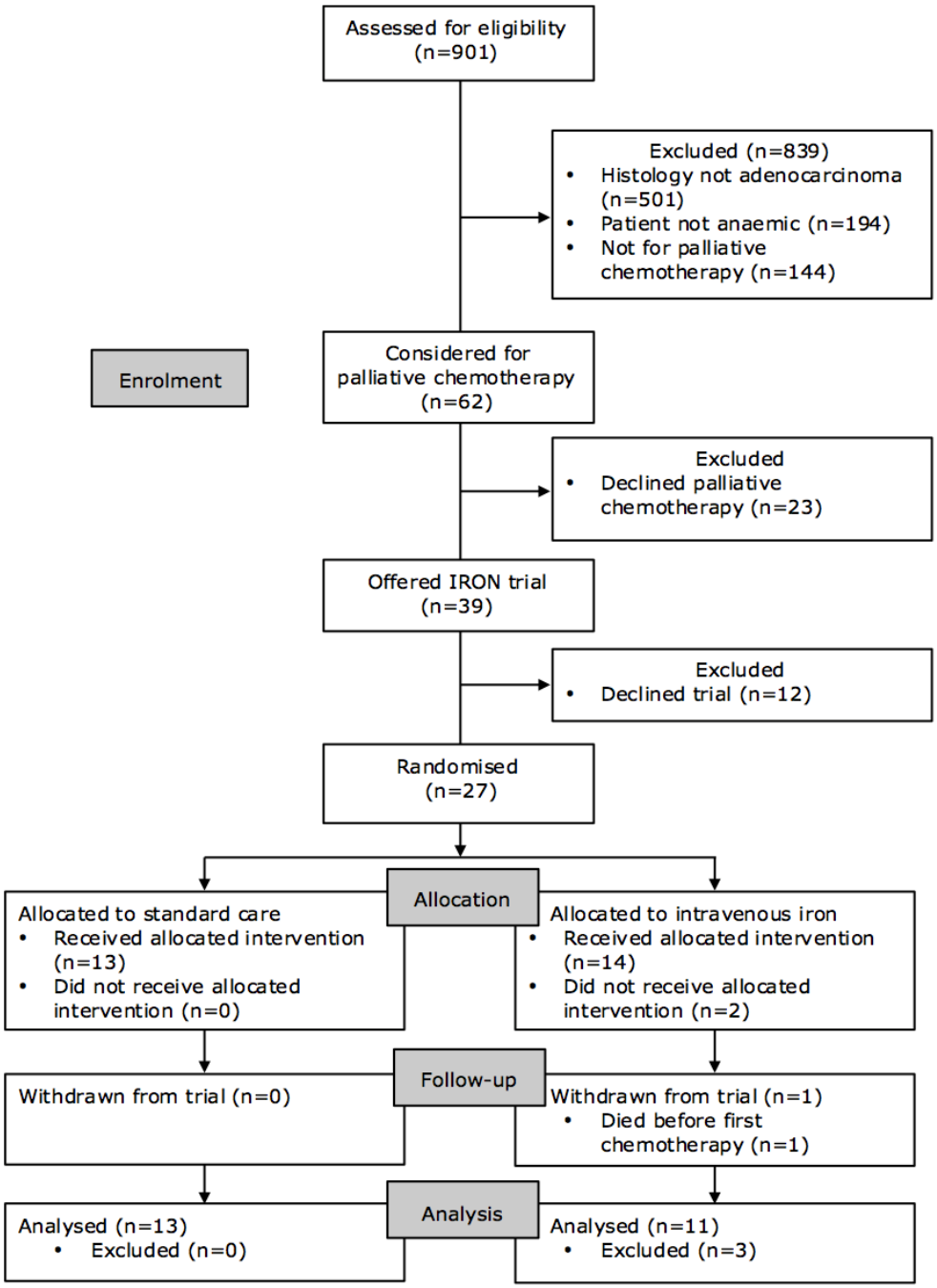


Figure 5.1 CONSORT diagram for IRON trial

5.4.2 General

There were no statistically significant differences in age, sex, body mass index, Charlson score or staging between standard care and intravenous iron groups at recruitment (see Table 5.1). Four patients in the intravenous iron group had received oral iron at some time point in the previous six weeks prior to recruitment to the trial. One of these patients was still taking oral iron at recruitment. This was discontinued prior to administration of intravenous iron. No patients in either group received any oral iron therapy during the trial.

The majority of patients were treated with epirubicin, oxaliplatin and capecitabine (EOX) chemotherapy (standard care group n=11, 84.6% versus intravenous iron group n=10, 90.9%, see Table 5.2 Treatment data). The remainder of patients received cisplatin, capecitabine and Herceptin. 17 patients completed the full three cycles of chemotherapy (62.9%), two patients had chemotherapy stopped after two cycles (7.4%) and five patients received only one cycle of chemotherapy (18.5%). No statistically significant differences were seen between groups and number of chemotherapy cycles completed.

Table 5.1 Demographic and baseline clinical data

	Standard care (n=13)	**Intravenous iron (n=11)**	**P value**
Age median range (years)	68 (38-79)	69 (48-85)	ns
Sex ratio (M:F)	11:2	7:4	ns
BMI (m/kg^2)	27.4 (17.4-41.9)	25.7 (19.9-37.9)	ns
Received oral iron previous 6 weeks	0 (0%)	4 (36%)	
Charlson score	6 (2-8)	6 (6-8)	ns
TNM			
T1-2 T3-4	0 (0%) 13 (100%)	1 (9%) 10 (91%)	ns
N0 N1 N2 N3	0 (0%) 2 (15%) 8 (61.5%) 3 (23%)	1 (9%) 4 (36.3%) 5 (45.4%) 1 (9%)	ns
M0 M1	1 (8%) 12 (92%)	0 (0%) 11 (100%)	ns

ns, P >0.05 statistically non-significant

Table 5.2 Treatment data

	Standard care (n=13)	**Intravenous iron (n=11)**
Chemotherapy regime		
Epirubicin, oxaliplatin and capecitabine (EOX) (n)	11 (84.6%)	10 (90.9%)
Cisplatin, Capecitabine and Herceptin (n)	2 (15.4%)	1 (9.1%)
Dose reductions (n) 20% 25% 50%	 1 (7.7%) 2 (15.4%) 1 (7.7%)	 0 1 (9.1%) 1 (9.1%)
Cycle delays (n)	5 (38%)	2 (18%)
Cycles of chemotherapy completed (n)		
0 1 2 3	0 2 1 10	3** 3 1 7
Iron therapy		
Oral iron (n) Intravenous iron (n)	0 0	0 11
Dose (mg)	-	1200 (880-1500)

5.4.3 Clinical

Haemoglobin

Haemoglobin was significantly higher in the standard care group at recruitment (mean haemoglobin 115 g/L standard care group versus 100 g/L intravenous iron group p=0.044) (see Table 5.1 Demographic and baseline clinical data). Mean haemoglobin decreased by 6 g/L over three cycles of chemotherapy in the standard care group to 108 g/L (see Figure 5.2A, p=0.336). In comparison the haemoglobin in the intravenous iron group increased by 5 g/L during the three cycles of chemotherapy to 105 g/L, resulting in a difference between groups in mean haemoglobin change of 11 g/L (p=0.903). This change in haemoglobin was not significant (p=0.885) and no statistical difference between haemoglobins was seen after recruitment.

Haematinics

Ferritin levels were similar at recruitment between groups (p=0.282) (see Table 5.1). Ferritin showed a significant increase after chemotherapy cycle one in the group treated with intravenous iron 105 ng/mL to 1015 ng/mL ($p<0.05$) and then began to decline with the mean ferritin 558 ng/mL after cycle three (see Figure 5.2B, p=0.366). Ferritin also increased in the standard care group despite no oral or intravenous iron administration from 161 ng/mL at recruitment to 340 ng/mL after cycle three. No statistical differences between groups were seen beyond cycle one of chemotherapy (see Table 5.3 Clinical outcome data).

Transferrin saturations increased above 20% in the intravenous iron group rising from 11.1% to 26.1% after cycle 1 (see Figure 5.2C, p=0.196). Transferrin saturations never exceeded 20% in the standard care group but did rise from 11.9% to 19% after cycle three of chemotherapy. No statistical differences between groups were seen (see Table 5.3 Clinical outcome data).

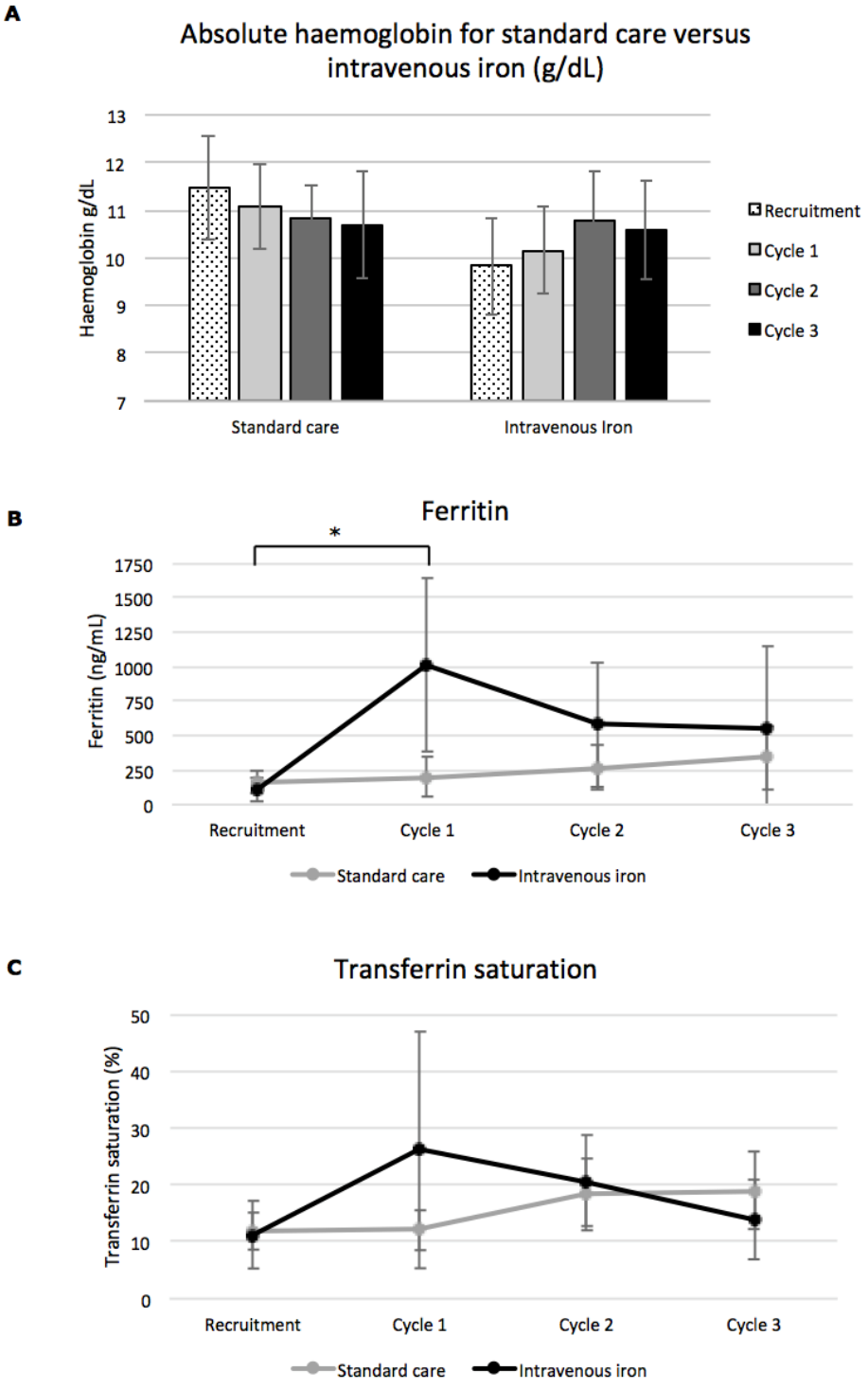


Figure 5.2 Changes in haemoglobin and haematinics by group over 3 cycles of chemotherapy (A) Haemoglobin (B) Ferritin (C) Transferrin saturation

Transfusions

After cycle one of chemotherapy, three patients in the intravenous iron group had received blood transfusions with a mean 5.3 units of blood transfused for this group (see Table 5.3 Clinical outcome data). In comparison only one patient received 3 units of blood in the standard care group at the same time point. No further patients received transfusions in the intravenous iron group while three further patients and one previous patient received an average of 1 unit of blood in the standard care group. No patients required a transfusion after cycle three of chemotherapy. The indication for transfusions were severe anaemia (haemoglobin < 80 g/L) in six patients (one patient from intravenous iron group) and acute upper gastrointestinal haemorrhage in two patients (both in the intravenous iron group).

Table 5.3 Clinical outcome data

	Standard care (n=13)	**Intravenous iron (n=11)**	**P value**
Haemoglobin (g/L) mean (SD)			
Recruitment	114.5 (17.9) (n=13)	99.6 (16.0) (n=11)	0.044 *
After cycle 1	110.8 (11.0) (n=12)	101.5 (14.9) (n=11)	0.101
P value	0.403	0.628	
After cycle 2	108.3 (11.5) (n=10)	107.9 (9.9) (n=8)	0.935
P value	0.318	0.680	
After cycle 3	107.0 (14.9) (n=10)	106.0 (11.9) (n=7)	0.885
P value	0.336	0.903	
MCV	84 (5)	85 (6)	0.789
Platelets	326 (144)	356 (138)	0.616
CRP	56 (87)	40 (30)	0.547
Ferritin mean (SD)			
Recruitment	161 (123)	105 (120)	0.282
After cycle 1	200 (170)	1015 (880)	0.021*
After cycle 2	264 (213)	581 (489)	0.102
After cycle 3	340 (325)	558 (637)	0.366
Transferrin saturations mean (SD)			
Recruitment	11.9 (4.8)	11.1 (8.7)	0.811
After cycle 1	12.1 (4.2)	26.3 (29)	0.196
After cycle 2	18.3 (8.1)	20.7 (8.6)	0.580
After cycle 3	19 (9)	14 (7)	0.260
Blood transfusions (mean number of units transfused)			

After cycle 1	3 (n=1)	5.3 (3.2) (n=3)	0.594
After cycle 2	4 (n=4)	0	
After cycle 3	0	0	
Blood transfusion received (n)	4 (31%)	3 (27%)	0.851 (chi squared)
No blood transfusion (n)	9 (69%)	8 (73%)	
Unplanned hospital admissions (n)	4 (31%)	5 (45%)	0.675 (fishers)
Death (n)	2 (15%)	5 (45%)	0.182 (fishers)

* $p < 0.05$

Subgroup analysis non-transfused

Haemoglobin in subgroup analysis of patients not transfused during the trial again showed a significant difference at recruitment (mean haemoglobin 121 g/L standard care group versus 104 g/L intravenous iron group p=0.021). No difference was seen after cycle two (mean haemoglobin 109 g/L standard care group versus 108 g/L intravenous iron group p=0.737). Haemoglobin dropped with each cycle of chemotherapy in the standard care group from 121 g/L at recruitment to 10.9 g/L after cycle three, mean difference -12 g/L. No drop in haemoglobin was seen in the intravenous iron group from a recruitment haemoglobin of 104 g/L to a haemoglobin after cycle three of 106 g/L, mean difference 2 g/L.

Ferritin again showed a significant increase in the intravenous iron group after cycle one (mean ferritin 62 ng/mL standard care group versus 770 ng/mL intravenous iron group p=0.027). Ferritin then dropped with each cycle of chemotherapy in the intravenous iron group but remains higher than the standard care group throughout. Transferrin saturations also increased in both groups with no significant difference between groups at any time point.

Table 5.4 Subgroup analysis non-transfused

	Standard care (n=9)	**Intravenous iron (n=8)**	**P value**
Haemoglobin (g/L) mean (SD)			
Recruitment	120.6 (9.57) (n=9)	104.4 (15.8) (n=8)	0.021*
After cycle 1	115.8 (8.21) (n=8)	103.1 (12.9) (n=8)	0.035*
After cycle 2	109.6 (10.5) (n=8)	107.9 (9.9) (n=8)	0.737
After cycle 3	109.0 (15.4) (n=8)	106.0 (11.9) (n=7)	0.683
Ferritin mean (SD)			
Recruitment	127 (94)	62 (58)	0.119
After cycle 1	116 (61)	770 (563)	0.027*
After cycle 2	176 (104)	581 (489)	0.054
After cycle 3	296 (331)	558 (637)	0.326
Transferrin saturations mean (SD)			
Recruitment	12.1 (5.3)	11.0 (9.3)	0.934
After cycle 1	14.2 (4.0)	18.3 (10.6)	0.138
After cycle 2	19.0 (9.1)	20.7 (8.6)	0.975
After cycle 3	20.4 (9)	14 (7.6)	0.246

* $p < 0.05$

Adverse events and complications

There were no serious adverse events related to intravenous iron administration. One patient reported some diarrhoea following intravenous iron administration that settled within 24 hours.

There were no significant differences in unplanned hospital admissions between the two groups (p=0.675, see Table 5.3 Clinical outcome data). Seven patient deaths occurred during the study; 2 patients in standard care, 5 patients in the intravenous iron group (p=0.182, see Table 5.3 Clinical outcome data). All deaths related to progression or complications from their oesophagogastric malignancy. No statistical difference was seen between groups.

Quality of life

FACT-An quality of life scores were higher in the standard care group compared to the intravenous iron group at recruitment (total score 128.4 standard care versus 105 intravenous iron). Quality of life scores increased for all dimensions of the FACT-An in the intravenous iron group (see Figure 5.3). In particular physical well-being, emotional well-being, anaemia-specific outcomes, trial outcome index and total scores all exceeded the minimum clinically important difference. No similar increase was seen in the standard care group and no changes reached the minimum clinically important difference.

EQ-5D quality of life scores were again higher in the standard care group compared to the intravenous iron group at baseline in all dimensions except pain and discomfort. Change in EQ-5D scores showed no trend in either group (see Figure 5.4). Usual activity and visual analogue score after cycle two of chemotherapy in the intravenous iron group exceeded the minimum clinically important difference.

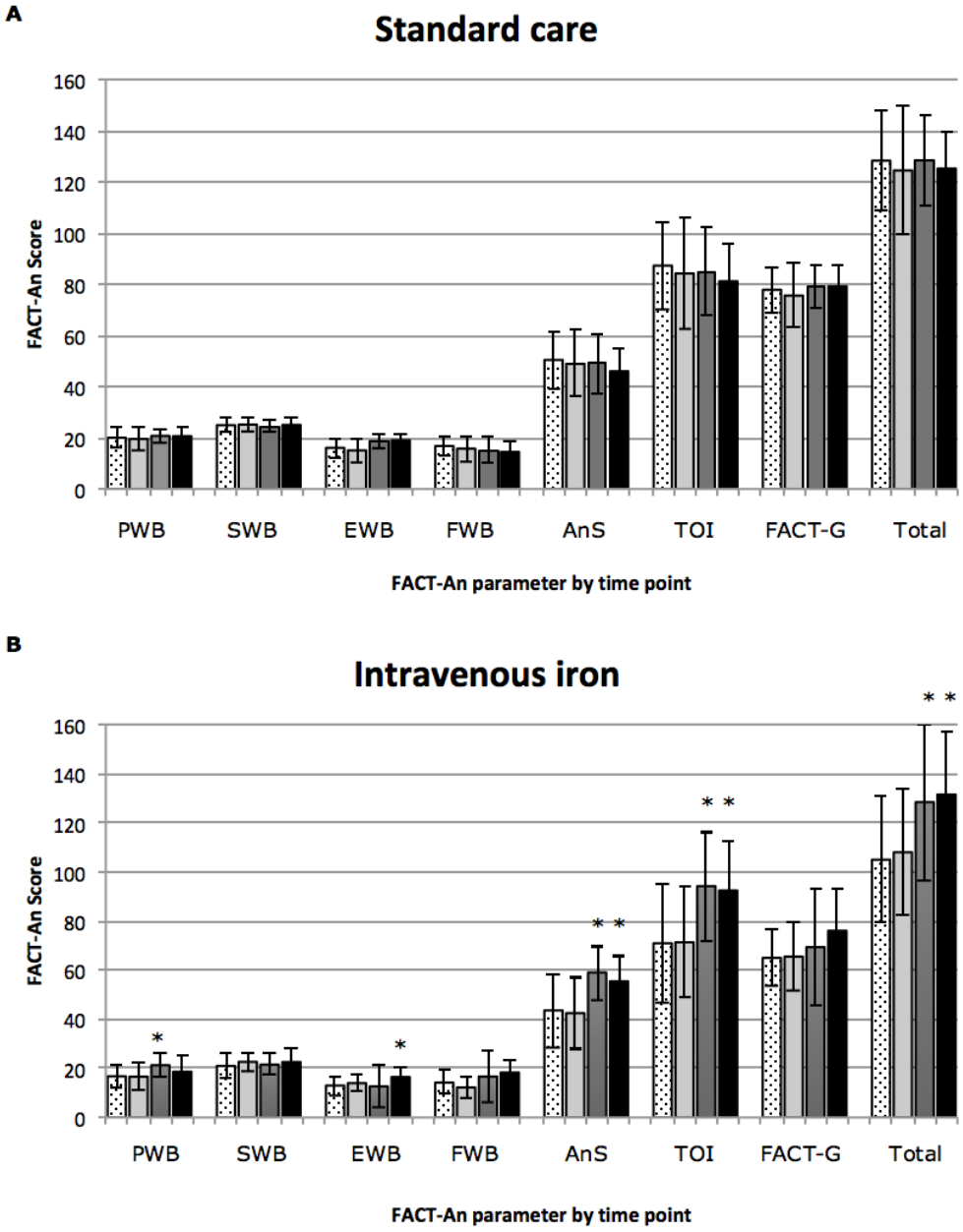


Figure 5.3 FACT-An Quality of Life A. Standard care B. Intravenous iron *Minimally important clinical difference exceeded

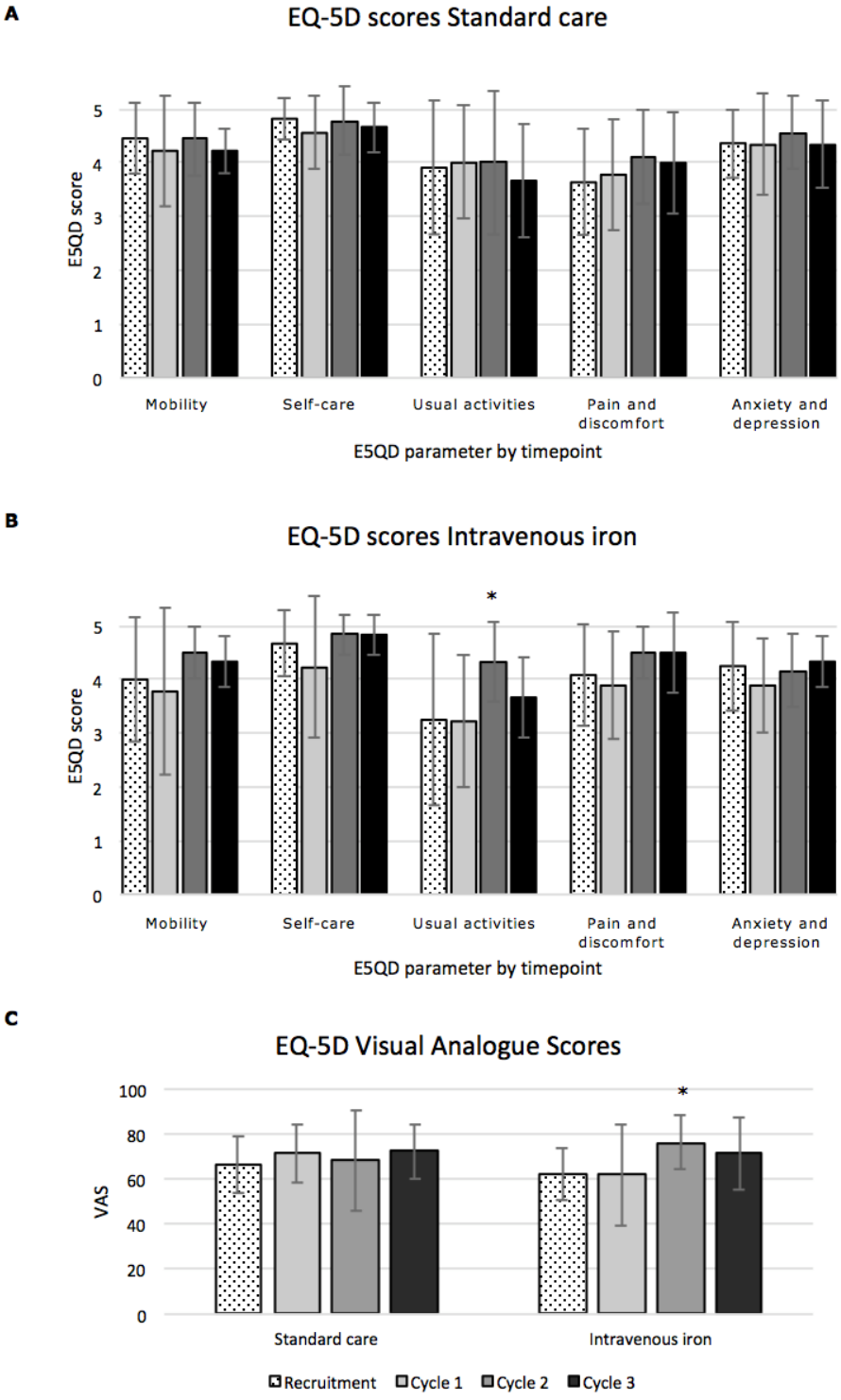


Figure 5.4 EQ-5D scores A. Standard care B. Intravenous iron C. E5QD Visual analogue scores standard care and intravenous iron

Table 5.5 FACT-An quality of life scores

FACT-An Dimension	Time point	Standard care Mean Score (SD)	Intravenous iron Mean Score (SD)
PWB	REC	20 (5.4)	16.8 (6.9)
	C1	19.6 (5.4)	16.6 (6.7)
	C2	20.8 (3.3)	21.3 (4.2)*
	C3	20.7 (4.4)	18.7 (5.7)
SWB	REC	25 (3.7)	21 (7.4)
	C1	25.2 (3.3)	22.7 (4.7)
	C2	24.3 (2.9)	21.6 (3.9)
	C3	25.1 (3.1)	22.6 (4.9)
EWB	REC	16.1 (5.6)	13 (6.1)
	C1	15.1 (5.8)	14 (3.9)
	C2	18.9 (3.6)	12.7 (7.4)
	C3	19 (3)	16.5 (3.3)*
FWB	REC	16.8 (5.5)	14.2 (7.4)
	C1	15.8 (6.3)	12.2 (5.3)
	C2	15.3 (6.3)	16.7 (7.6)
	C3	14.5 (5.1)	18.3 (4.3)
AnS	REC	50.5 (16.3)	43.6 (21.5)
	C1	48.9 (16)	42.5 (18.1)
	C2	49.1 (14.5)	59.1 (9.8)*
	C3	46.1 (11.2)	55.5 (9)*
TOI	REC	87.3 (24.3)	71 (36.5)
	C1	84.3 (26.6)	71.4 (27.4)

	C2	85.2 (21.4)	94.3 (19.7)*
	C3	81.3 (17.7)	92.5 (17.7)*
FACT-G	REC	77.9 (12.9)	65 (17.1)
	C1	75.6 (15.5)	65.6 (17)
	C2	79.3 (10.6)	69.4 (20.6)
	C3	79.3 (10.5)	76.1 (14.8)
Total Score	REC	128.4 (27.5)	105 (38.9)
	C1	124.6 (30.6)	108.1 (31.5)
	C2	128.4 (22)	128.5 (27.8)
	C3	125.3 (17.9)	131.6 (22.7)*

* Minimum clinically important difference exceeded

Table 5.6 EQ-5D quality of life scores

EQ-5D Dimension	Time point	Standard care Mean score (SD)	Intravenous iron Mean score (SD)
Mobility	REC	4.45 (0.66)	4 (1.15)
	C1	4.22 (1.03)	3.78 (1.55)
	C2	4.44 (0.68)	4.5 (0.5)
	C3	4.22 (0.42)	4.33 (0.47)
Self-care	REC	4.82 (0.39)	4.67 (0.62)
	C1	4.56 (0.68)	4.22 (1.31)
	C2	4.78 (0.63)	4.83 (0.37)
	C3	4.67 (0.47)	4.83 (0.37)
Usual activities	REC	3.91 (1.24)	3.25 (1.59)
	C1	4 (1.05)	3.22 (1.23)
	C2	4 (1.33)	4.33 (0.75)*
	C3	3.67 (1.05)	3.67 (0.75)
Pain and discomfort	REC	3.64 (0.98)	4.08 (0.95)
	C1	3.78 (1.03)	3.89 (0.99)
	C2	4.11 (0.87)	4.5 (0.5)
	C3	4 (0.94)	4.5 (0.76)
Anxiety and depression	REC	4.36 (0.64)	4.25 (0.83)
	C1	4.33 (0.94)	3.89 (0.87)
	C2	4.56 (0.68)	4.17 (0.69)
	C3	4.33 (0.82)	4.33 (0.47)

Visual analogue score	REC	66.7 (16.32)	62.27 (17.1)
	C1	71.67 (17.07)	61.88 (26.8)
	C2	68.13 (26.42)	76.5 (11.31)*
	C3	72.44 (15.02)	71.67 (15.46)

* Minimum clinically important difference exceeded

5.5 Discussion

In the UK 21,133 oesophagogastric cancers were diagnosed in 2013-2015 (Chadwick, Varagunam et al. 2016). Of these patients 6,226 received palliative oncology. This study examined intravenous iron for anaemia in 27 patients while undergoing palliative chemotherapy. The feasibility outcomes have highlighted factors that may prevent a definitive study of this design being deliverable on a wider scale. These include the high decline rate of palliative chemotherapy, high transfusion rates, poor prognosis and poor acceptability within this palliative care group. Applied to the national figures, 402 patients per year could potentially be recruited. Based upon our data however, the sample size to detect an expected difference in haemoglobin of 15 g/L by cycle three of chemotherapy (standard deviation 14.8 g/L; effect size 30%) at a 1-sided alpha of 0.05 and a power of 80 % is 774 patients. A study designed to examine the broader subject of chemotherapy-induced anaemia (including those receiving neoadjuvant in whom chemotherapy is rarely declined) or palliative cancer in whom anaemia and fatigue are common may be more pragmatic and generalisable study.

Clinical endpoints however do offer some insight. We have shown higher than reported rates of anaemia in this group of patients with over half of patients anaemic at presentation (Ludwig, Van Belle et al. 2004). Haemoglobin dropped over three cycles of chemotherapy in the standard care group and either increased or remained stable in the intravenous iron group. This appeared to translate into no transfusions

beyond cycle one of chemotherapy in the intravenous iron group. This is in keeping with findings from the two gynaecological studies where iron was effective as a preventative strategy to avoid anaemia and hence blood transfusions at their transfusion threshold of haemoglobin less than 100 g/L (Dangsuwan and Manchana 2010, Athibovonsuk, Manchana et al. 2013). However, our small numbers and no power calculation prevent us from concluding this definitively.

Intravenous iron compared to standard care was effective at replenishing iron stores and restoring transferrin saturations to greater than 20%. After increasing initially, ferritin then declined over the three cycles of chemotherapy and transferrin saturations again fell below 20% by cycle three suggesting that these patients would have become iron deficient again beyond cycle three. A repeat dosing regimen used in other trials (Kim, Kim et al. 2007, Athibovonsuk, Manchana et al. 2013) might therefore be advantageous. We have used a high single dose preparation of iron isomaltoside compared to low dose repeat dosing regimens of iron sucrose in other trials. The merits of both strategies could be further researched but in inflammatory bowel disease, these high dose regimens appear more effective (Evstatiev, Marteau et al. 2011).

Quality of life scores were higher at baseline in the standard care group. The higher haemoglobin at baseline might explain this and reports from other studies suggest that higher haemoglobin is associated with better quality of life. Despite this, intravenous iron improved quality of life while standard care did not. This supports studies that have demonstrated correcting anaemia improves quality of life

(Crawford, Cella et al. 2002, Cella, Kallich et al. 2004, Yakymenko, Frandsen et al. 2017).

No new safety concerns were raised during this trial including no differences in infection or venous thromboembolism. Current intravenous iron preparations already have a well-regarded safety profile (Auerbach and Macdougall 2014).

5.5.1 Limitations

This study was designed primarily for feasibility and in interpreting clinical results we acknowledge the limitations of small numbers and no power calculation. It must also be noted that haemoglobin was significantly different at baseline, with much lower starting haemoglobin in the intravenous iron group. This ironically may favour iron therapy, with Steinmetz (2013) noting that lower starting haemoglobin may improve the effectiveness of any intravenous iron administered, with quicker and larger increases in haemoglobin demonstrated the more anaemic patients were (Steinmetz, Tschechne et al. 2013). Both this lower haemoglobin and the two acute upper gastrointestinal haemorrhages in the intravenous iron group would have resulted in a much higher probability of transfusion. These obligate transfusions we feel should not be considered intravenous iron treatment failure but are important to consider a priori in any study design and sample size calculation for a definitive study. Stratification by age, gender and recruitment haemoglobin may also prevent differences in recruitment haemoglobin impacting upon analysis and conclusions.

5.6 Conclusion

A study designed like this will need adequately powered endpoints, but we caution future investigators of the high screen failure rates and the difficulty in delivering this feasibility study. Our study demonstrated difference in haemoglobin between the two groups of 110 g/L, which could be used to more adequately power an endpoint directed at haemoglobin change. We have also shown trends towards changes in quality of life which could be used to design such studies and in this small group we found no change in transfusion rates but this could be due to the relatively higher haemoglobin in the control arm, and to avoid this for future studies investigators should consider stratification by haemoglobin at recruitment.

6 Conclusions

Iron is as intimately bound to the biology and pathology of gastrointestinal cancer, as it is to the function of all living organisms. The effects of iron excess and deficiency are widely researched but far from complete.

This thesis broadly examined the biological and clinical effects of iron replacement in gastrointestinal cancer. In detail, it reports the important biological effects of iron replacement in colorectal cancers during clinical treatment for iron deficiency anaemia prior to surgery. Systematic review of the literature also investigated the clinical evidence for such an approach to pre-operative anaemia. The thesis then examined the natural history and impact of anaemia in oesophagogastric cancer before reporting a pilot randomised control study to treat anaemia in this setting.

This conclusion will summarise the main findings of this thesis and discuss the implications for current clinical practice, future clinical trials and basic science research.

6.1 Overall summary of research findings

Chapter 1 reviewed our historical and contemporary understanding of the profound and diverse functions iron performs, beyond its most clinically recognised role in anaemia. This included the developing fields of microbiome of the gut and immune function. Together with the introduction to Chapter 4, it also summarised the growth of intravenous iron use as a safe and efficient means by which to correct iron deficiency.

Chapter 2 reported original research examining iron administration in colorectal cancer. It supports previous research that has demonstrated colorectal adenocarcinomas reprogram their iron metabolism to increase the potential labile iron pool. These changes appear to be decoupled from the normal intracellular iron sensing mechanisms. Route of administration of iron to patients in the clinical randomised control trial from which tissue and serum were taken did not alter tumour growth or effect iron transport mechanisms. Differential compartmentalisation of iron was noted though. Hepcidin was not predictive for haemoglobin response and its clinical use is still not supported.

Chapter 3 retrospectively examined anaemia in oesophagogastric cancer, showing similar to other studies that anaemia is common, becomes more severe with time and treatment and is associated with poorer survival outcomes. Furthermore, we demonstrated that higher initial haemoglobin, rather than just absence of anaemia,

was associated with better survival outcomes. We are the first to report that histological chemotherapy response was also not associated with anaemia.

Chapter 4 systematically reviewed the use of iron for pre-operative anaemia. Meta-analysis of data does still not support the clinical use of iron therapy to reduce blood transfusions or increase pre-operative haemoglobin. Iron was effective in replenishing iron stores measured using ferritin.

Chapter 5 reported an original randomised control pilot study for intravenous iron use compared to standard care during palliative chemotherapy. Intravenous iron effectively replenished iron stores measured using ferritin and transferrin saturations. Despite chemotherapy and the tumour in situ the intravenous iron group saw an increase in haemoglobin and no transfusions after cycle 1 of chemotherapy. This was despite a significantly lower starting haemoglobin in the intravenous iron group. Quality of life was also significantly improved in the intravenous iron group. However, the study was not powered and definitive trial designs informed by this pilot study are discussed below.

6.2 Recommendations for clinical practice

There is in inherent logic and wide acceptance that the supplementation of iron in patients with anaemia due to iron deficiency is both clinically justifiable and a reasoned decision. The findings of the research presented in this thesis do not alter this tacit acknowledgement that correction of iron deficiency is beneficial to patients. The goal should remain iron repletion in all those shown to be lacking sufficient iron supply to meet demand. Oral iron has historically and will for the foreseeable future account for the mainstay of this iron therapy.

Where logic is drawn into clinical equipoise begins with the route of administration of this iron therapy. Other key areas for clarification and unification are the threshold of iron deficiency and haemoglobin for intervention, the role of iron in functional iron deficiency and the definitions of iron deficiency.

Intravenous iron therapy as an alternate route for iron has historically been maligned and feared for its propensity to cause severe anaphylaxis. This was mainly due to the legacy of a single high molecular weight agent, Dexferrum®. Modern iterations of the drug are safe and offer rapid correction of body iron stores (Auerbach and Macdougall 2014). Its safe use is more broadly accepted now despite it's past and as a result the clinical use of iron is increasingly seen in renal disease, heart failure and inflammatory bowel disease. There is no widespread adoption in gastrointestinal cancer yet.

For this change in practice towards intravenous iron to occur several key steps need to be in place. The first foundational step must be clinical evidence of the efficacy of intravenous iron therapy. This should ideally take the form of well-designed and powered randomised clinical trials that reflect the population in question. The pilot study presented in this thesis showed promising early results during chemotherapy but requires a larger study to conclude definitive efficacy. Systematic review and meta-analysis of data from smaller trials may also provide valid evidence. The systematic review presented in this thesis is still not sufficiently powered to recommend intravenous iron for pre-operative anaemia. However, with the publication of data from the PREVENTT trial a clear recommendation may be forthcoming. Design considerations for clinical trials are discussed in more detail in another section (see below).

Next a clear framework around which iron therapy is considered should be developed. The retrospective studies in oesophagogastric cancer presented in this thesis highlighted how common but under recognised anaemia was at diagnosis and how frequent subsequent blood transfusions were. Guidance already exists in part under the umbrella of patient blood management (PBM). This does not extend to gastrointestinal cancer not treated with surgical modalities. National and International guidance with position statements from key professional bodies including the BSG, ACPGBI, AUGIS, RCS and NICE will also provide some of the necessary guidance for a broader adoption in gastrointestinal cancer. Some guidance

already exists (Munoz, Acheson et al. 2017). These may help set thresholds for diagnosis and treatment. The studies in this thesis used the WHO definition for anaemia, which while nuanced in what should be considered 'normal' for a particular clinicians population, at least allow a global definition. The WHO thresholds and severity of anaemia still correlated with mortality from oesophagogastric cancer and from a purely pragmatic perspective can be applied in everyday clinical practice. Definitions of iron deficiency could also be better unified through these professional bodies going beyond a definition focussed primarily on ferritin to include other practical and widely available measures of iron status such as transferrin saturations and reticulocyte haemoglobin.

Education and culture change is also required in the sphere of anaemia, with the rebranding of transfusion medicine as seeking to avoid blood transfusions where possible rather than the current emphasis on blood donation by volunteers and the safe administration of ABO compatible blood. While these issues are still important and relevant in modern clinical practice they detract from the acknowledgement that blood transfusions appear to be associated with a host of complications more profound than the immediate transfusions reactions and transmission of blood borne diseases. Re-education regarding the deleterious effects of transfusion, starting ideally in education at medical and nursing school and continued through into continued professional development, would lead to less blood being transfused thoughtlessly and unnecessarily and alternatives to blood as outline in patient blood management principles to be more widely employed.

Education regarding functional iron deficiency should also be updated with the discovery of hepcidin and its role in iron homeostasis. This could replace older descriptions of ‘anaemia of chronic disease’ or ‘anaemia of chronic inflammation’ to emphasise the pathophysiological basis, develop an increased understanding of transferrin saturations and iron homeostasis and explain why intravenous iron appears to be effective in treatment.

Local practice also seems to vary widely depending on perceived ownership of iron deficiency and anaemia. In different hospitals haematologists, gastroenterologists, anaesthetists and surgeons often individually or less frequently collectively take responsibility for the identification and treatment of patients with anaemia and iron deficiency. This is often influenced by an individuals professional experiences and interests or by the institutions logistical and practical experience with intravenous iron administration.

One solution may be developing specific clinical roles to support these measures or reforming existing ‘blood transfusion practitioners’ into ‘patient blood management specialists’ who can safeguard and gate-keep blood products while prescribing and supporting other measures to reduce or eliminate blood transfusions would provide the necessary cross speciality coverage required.

The setting and availability of intravenous iron also varies widely. Local practice will depend not only upon formulary availability and local approval but also partly

upon the real estate and logistics of administration of intravenous iron. The practicalities of administering iron usually dictate that it is best given by staff familiar with its regular administration and is best provided in a day case setting. Despite strong business cases for the health economic benefits of iron individual trusts will still often find need to present individual use cases for each setting. This may be address with future NICE guidance and further clinical trials providing evidence for the monetary savings in pre-emptive treatment of iron deficiency.

Another important change at the governmental, organisational and departmental level should be a shift from reactive to pre-emptive identification and management of anaemia. Current anaemia management is short sighted, often under diagnosing and under treating anaemia in the early stages of gastrointestinal cancer. This leaves no opportunity for pre-emptive red cell mass optimisation. Instead reactive treatment with allogeneic blood transfusions when haemoglobin falls below a threshold of 70-80 g/L becomes obligate to avoid the negative consequences of severe anaemia. Primary care, secondary care referral and secondary care treatment departments should anticipate anaemia given that it occurs in up to 60% of patients diagnosed with gastrointestinal cancer, shaping referral and assessment pathways for its early identification and treatment. This could be unified and incentivised at NHS England level or equivalent with suitable performance indicators and recognition for those trusts meeting targets. Like most preventative measures the health economic and patient benefits would support such intervention. Design of future clinical trials (see

below) should also seek to capture this important health resource and health economic metrics to inform national strategy.

6.3 Design considerations for clinical trials

This thesis presented data from a pilot study for anaemia in oesophagogastric cancer and systematic review of current randomised control studies in pre-operative anaemia. This research, input the IRON trial steering group and Patient and Public Involvement (PPI) through the Independent Patient Voice (IPV) group have helped inform further clinical trial design and a successful application for £212,316 funding from the National Institute for Health Research (NIHR) under the Research for Patient Benefit (RfPB) programme. In this section I summarise some of the key recommendations for clinical trialists designing studies for anaemia and iron therapy.

6.3.1 Population

Clinical trials should target those with known pathology and proven absolute or functional iron deficiency. Inclusion should include mild anaemia because this is the point at which associations with increased mortality and transfusions begin. Within the scope of this thesis, I recommend trials should include all gastrointestinal malignancy from oesophagus to anus, with the possible exception of small bowel due to the rarity of tumours. Areas of particular clinical interest are those patients receiving radiotherapy for low rectal tumours where anaemia appears to reduce the efficacy of treatment and those receiving neoadjuvant chemotherapy and surgery for oesophagogastric cancer where anaemia occurs in over 90% of patients with high rates of blood transfusion.

However, clinical trials using iron therapy should not just focus on patients with anaemia. I put forward this controversial and seemly counterintuitive statement to capture some of the opinion of leading experts in iron trials.

Patients who are iron deficient but yet to develop anaemia are an important and overlooked group of patients in whom the disease and treatment will likely render them anaemic and then unable to regenerate red cell mass due to iron-restricted erythropoiesis. This is particularly common in women who have high rates of undiagnosed iron deficiency due to menstruation. Further research in this area is required to identify how common this scenario is and what impact it has on the patient. Women undergoing surgery with haemoglobins of 120-130 g/L should also be considered for inclusion based upon the reasons details in Chapter 1, namely a smaller blood volume and physiological tolerance for blood loss.

Fatigue is also a common and important symptom due to iron deficiency that does not correlate with anaemia, but has profound effects on recovery and well being essential for both post-operative outcomes and quality of life in palliative settings. A majority of these patients will be anaemic but some may only have iron deficiency and no anaemia while still suffering from fatigue. These iron deficient patients should be included in trials that seek to improve fatigue and quality of life. One of the main outcomes of PPI consultations emphasised how this symptom among all others was the highest priority, and a keystone symptom that impacted upon all other

aspects of their care and well being. It should be suitably address in trial design and inclusion criteria.

The natural history of haemoglobin should also be considered when designing trials. The retrospective analysis of oesophagogastric cancer in this thesis demonstrated clearly that patients not anaemic at diagnosis often become so over time due to a combination of treatment and disease. Trial design that recruits non-anaemic patients and intervenes if patients become anaemic over the trial period better reflects the population of gastrointestinal cancers and clinical reality.

6.3.2 Intervention

Iron therapy for iron deficiency has two potential routes of administration and a variety of formulations of these drugs to consider. Oral iron is an effective and cheap treatment for iron deficiency. Low dose alternate day oral iron therapy may also alleviate some of the negative side effects. However, for a large majority of the patients with gastrointestinal cancers, timing and biology restrict the use of oral iron. Often it takes several months to replenish iron stores in patients whose treatment is being delivered on a timescale of days to weeks. Intravenous iron therapy is therefore indicated. Patients with functional iron deficiency due to their cancer can also not absorb the oral iron and simply suffer the side effects with no efficacy. In these patients intravenous iron is again indicated.

Several intravenous irons are available varying in the maximum dose and time over which the drug can be administered. Broadly this divides them into low dose long administration (for example Cosmofer) and high dose fast administration (for example Ferrinject and Monofer). For gastrointestinal cancers where most of the intravenous iron will be delivered in a day case setting high dose agents that can be administered in under an hour are favourable from a patient and health economic perspective, allowing a single short visit to achieve iron repletion. This opinion was supported by the PPI group who highlighted multiple appointments and hospital admissions place a huge burden on the patient and extended family during already difficult times. Of the two agents currently available I can see no evidence for an advantage to either agent over the other if the objective is to shift the patient from a state of iron deficiency to iron repletion. Monofer does offer a higher maximum single dose so may be advantageous to reduce admissions if repeated dosing is anticipated.

Timing of the intervention should be front loaded towards the earliest practicable time point to minimise the time a patient spends iron deficient and maximise the benefits to red cell mass and functional outcomes. However, in my view an iron replete patient is much better prepared to face treatment and recovery than one left iron deficient. Therefore wherever in the patient pathway iron is given it may still have some benefit, be that pre-chemotherapy, before surgery, peri-operatively or post-operatively. Clinical trials to support this hypothesis could and should be designed, especially if pre-operative iron therapy becomes standard practice, so as

not to disadvantage iron deficient patients who fell outside of a perceived therapeutic window in which iron may have improved pre-operative haemoglobin.

Multiple dosing regimes should also be incorporated into longer clinical trials where the causes of iron deficiency remain. This is particularly pertinent to palliative management of gastrointestinal cancers where the tumour remains in situ and chemotherapy may continue for many months. This repeated dosing may prevent someone from becoming transfusion dependant and we should design trials to look for this benefit.

6.3.3 Comparators

A suitable comparison to intravenous iron for randomised control trials is an on-going debate. Certainly blood transfusions should not be that comparator as some reviewers have historically suggested. The risks of blood transfusions are simply too high to ethically justify their use. Placebo, oral iron and 'standard care' have all been used as comparators in trials. I have discussed the limits of oral iron above. Standard care in the experience of the IRON trial presented in this thesis consisted of administration of allogeneic blood transfusions for major haemorrhage or severe anaemia and so in reality was a no treatment arm. Placebo is an ideal comparator, especially for functional and quality of life outcomes to account for the placebo effect. However, intravenous iron is a dark brown liquid that colours the infusion fluid and giving set. This is quite unlike saline, which is the most appropriate

placebo. Opaque bags and opaque giving sets to cover the infusion and placebo are the best solution I have seen practiced in clinical trials.

6.3.4 Outcomes

Outcomes of clinical trials for iron therapy should continue to use haemoglobin, ferritin and transferrin saturations to assess the efficacy of iron therapy. Reticulocyte haemoglobin should also be considered as an early indicator of effective iron therapy. Additional measures of blood film, B12, folate, erythropoietin, hepcidin, CRP and renal function should also be recorded at baseline to understand the aetiology of anaemia in patients and ensure other causes of anaemia are not be erroneously treated with iron therapy.

All trials should include quality of life and use validated quality of life tools. General quality of life should be measured and can be incorporated into health economic equations including quality adjusted life years. I recommend the inclusion of specific quality of life tools that focus on anaemia and fatigue in addition. These should be administered to capture the more granular improvements expected with treatment of iron deficiency and reflect the broader benefits beyond anaemia.

Health economic and health resource usage data is also vital. This should include all data on use of health care resources in primary and secondary care. This data will clearly add justification to the use of intravenous iron if favourable but is also invaluable for strategic planning and preventative treatment measures as the culture shifts from reactive to pre-emptive treatment of anaemia.

Cognitive and functional outcomes should also be incorporated. In our recent clinical trial applications we have moved beyond traditional measures of functional outcome (sit to stand, shuttle tests, 6 minute walk test) to include real world activity utilising wearable activity trackers. No trials in iron therapy to date have used this approach but the techniques been validated in other settings.

Long-term outcomes should also be built into primary research protocols. These outcomes should include as a minimum disease free survival and overall survival, both of which are predicted to improve if blood transfusions are avoided but to date no evidence exists to support this hypothesis.

6.4 Basic science research hypotheses

The research presented in this thesis has again demonstrated changes in iron metabolism in human colorectal adenocarcinoma to promote a net increase in the labile iron pool. While we have demonstrated the end state, this study has not increased our understanding of the molecular drivers for these changes and how they interface with the tumour suppressor and oncogenes responsible for carcinogenesis. Previous research has suggested part of this may be through Wnt signalling or via HIF2α as a result of tumour hypoxia, both of which have been shown to transcriptionally regulate the expression of iron transporters in iron deficiency. Understanding how pivotal HIF in iron metabolism is may allow selective HIF prolyl-hydroxylase inhibitors to be used to experimentally or clinically to manipulate the labile iron pool.

Further experiments are also required to fully characterise the multifaceted control of intracellular iron metabolism. Knockout models or small interfering RNA (siRNA) have already been used to selectively demonstrate some of the relationships. We should also characterise all other known changes to iron transport mechanisms demonstrated in other cancers such as STEAP 1, 2, 3 (Knutson 2007), lipocalin 2 (LCN2) (Boult, Roberts et al. 2008, Bao, Clifton et al. 2010) and autocrine secretion of hepcidin (Jiang, Elliott et al. 2010, Pinnix, Miller et al. 2010) in order to build a complete theoretical model of iron metabolism in colorectal cancer. Oesophagogastric cancers should also be examined for similar changes in iron

biology using surgical resections to determine if, like colorectal cancer, they are reprogrammed to be iron avid. This could guide potential strategies or adjuvant combinations of iron chelation with chemotherapy and surgery.

High volume molecular techniques such as microarray could also be used to explore whether there are genetic phenotypes particularly strongly associated with altered iron metabolism pathways or linked to some of the key mutations known to exist in colorectal cancers. This would be a better tool for answering whether chromosomal instability and microsatellite instability are associated with difference iron biology.

Experimental mouse models of colorectal cancer could also be used to investigate differential compartmentalisation of iron in the mucosa and the effects of intracellular iron loading by route of iron therapy administration. These could also explore the possibility of oral iron chelation agents combined with intravenous iron replenishment.

Increasing clinical use of intravenous iron may also allow observational studies in patients given intravenous iron and the histopathological analysis of resection specimens to look at iron compartmentalisation.

Immune function or systemic inflammation can also be examined. Building upon our current research we propose investigation of the cytokine and immuno-cellular profile of tumours from our cohort of patients to assess if is altered by route of iron administration.

Iron, microbiota and cancer, discussed in Chapter 1, also forms part of further work by our group to characterise the on tumour and off tumour microbiome in these patients and if this has been altered by oral or intravenous iron therapy.

6.5 Translational research hypotheses

Knowledge that colorectal adenocarcinomas reprogram their iron biology to increase their labile iron pool as demonstrated in Chapter 2 offers an opportunity to target iron for cancer therapy. Most widely research is the prospect of iron chelation therapy but novel methods of Tf-mediated drug delivery and TfR blockade also provide possible means by which to exploit this knowledge.

6.5.1 Iron chelation therapy

The importance of iron for cell cycle progression and intracellular processes was discussed in Chapter 2. Iron chelators are molecules that donate electrons to bind with iron as bidentate, tridentate and hexadentate ligands, doing so with greater affinity than transferrin and rendering iron biological inactive (Liu, Liu et al. 2002). Iron depletion in normal cells halts the cell cycle and affects cell survival related molecules including, cyclins D1, A, and B, cyclin dependent kinase 2 (cdk2) and the cyclin-dependent kinase inhibitors, p21WAF1/CIP1 and p27Kip1p53, p53, N-myc downstream regulated gene-1 (NDRG1), growth arrest and DNA damage (GADD45), the c-Jun amino-terminal kinase (JNK) and p38 mitogen activated protein kinases (MAPK) (Yu, Gutierrez et al. 2012). The net effect of these changes is reduced cell proliferation and increased cell apoptosis. However, whether these occur in colon cancer cells that have already escaped normal mechanisms for iron and cell cycle control is unknown.

Types of iron chelators

There are several commercially available iron chelating agents currently available and used in conditions such as β-thalassemia major and hereditary haemochromatosis to combat the deleterious effects of iron overload. These include desferrioxamine (Desferal®), deferasirox (Exjade®), deferiprone (Ferriprox®) and thiosemicarbazones, (Triapine®). There are also a number of experimental agents that show promise including aroylhydrazones such as pyridoxal isonicotinoyl hydrazone (PIH) (Yu, Gutierrez et al. 2012).

Table 6.1 Iron chelating agents

Agent	**Nature**	**Route of administration**
Desferrioxamine (Desferal®)	Hexadentate ligand	IV
Deferasirox (Exjade®)	Bidentate	Oral
Deferiprone (Ferriprox®).	Bidentate	Oral
Thiosemicarbazones Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 3-A	Tridentate ligand	Oral and IV
Pyridoxal isonicotinoyl hydrazone (PIH)	Tridentate ligand	Oral

Desferrioxamine (DFO, trade name Desferal ®) is a naturally occurring hexadentate bacterial siderophore (Yu, Gutierrez et al. 2012). Its high affinity for iron makes it an effective iron chelation agent. However, this affinity is not sufficient to remove iron

from haemoglobin, myoglobin and cytochromes (Keberle 1964) limiting the side effects of this agent and not markedly reducing haemoglobin levels (Zhang, Wei et al. 2010). However, poor membrane permeability limits its use to intravenous administrations. It also has a short plasma half-life necessitating long intravenous administration times and multiple doses (Yu, Wong et al. 2006). Oral desferrioxamine appears ineffective at preventing even small amounts of oral iron absorption in humans (Jackson, Ling et al. 1995). Trials in neuroblastoma have shown some efficacy and induction of cytotoxicity (Donfrancesco, Deb et al. 1990, Blatt 1994, Donfrancesco, De Bernardi et al. 1995). It has been used experimentally in trials looking to iron deplete cell cultures. Clinical trials have used DFO in colorectal cancer and shown reduced proliferation in cell lines (Cao, Liu et al. 2018). Its apparent inefficacy when administered orally also limits its theoretical use in this setting where the aim would be to achieve local gut iron deficiency and systemic iron repletion.

Deferasirox is a low molecular weight and highly lipophilic oral iron chelation agent with promising potential in anti-cancer therapy. It has been used as effective alternative to desferrioxamine in β-thalassemia major (Porter 2009, Bedford, Ford et al. 2013). It is well tolerated with no significant side effects (Porter 2009). Anti-proliferative activity has been demonstrated in hepatoma cells, selectively inhibiting their growth when compared to normal hepatocytes (Chantrel-Groussard, Gaboriau et al. 2006). It is also effective in gastric, pancreatic, lung multiple myeloma and neuroepithelioma cancer cell lines reducing proliferation (Lui, Obeidy et al. 2013,

Choi, Kim et al. 2016, Harima, Kaino et al. 2016, Kamihara, Takada et al. 2016). Further, it increased the anti-tumour effect of cisplatin when added to gastric cancer cell lines (Choi, Kim et al. 2016). No in vitro or in vivo studies for colorectal cancer have been conducted.

Thiosemicarbazones are a group of tridentate iron ligands with a spectrum of activity as effective iron chelation agents in the setting of cancer treatment. Most widely studied is 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP also known as Triapine ®). Clinical trials have demonstrated anti-proliferative activity in leukaemia (Finch, Liu et al. 2000). Trials in head and neck cancer and non-small cell lung cancer in contrast have shown poor efficacy (Yu, Gutierrez et al. 2012). All trials have also shown high rates of myelosuppression (Yu, Gutierrez et al. 2012). Other intravenous and oral derivatives of the thiosemicarbazones (Dp44mT, Bp44mT) have been investigated (Yuan, Lovejoy et al. 2004). In mice they have shown selective anti-tumour activity. In chemoresistant breast cancer cell lines they have also been effective. They remain potential oral iron chelation agents in cancer therapy (Yu, Gutierrez et al. 2012, Yu, Suryo Rahmanto et al. 2012). In colorectal cancer cell lines, researchers have employed glycoconjugation of thiosemicarbazone to improve targeting to cancer cells (Akam and Tomat 2016).

Aroylhydrazone iron chelators (including pyridoxal isonicotinoyl hydrazone and its derivatives) can be effectively administered orally increasing faecal iron excretion of iron in rats by up to eight times (Hoy, Humphrys et al. 1979). They are synthetic ligands decreasing the cost of manufacturer compared to agents like DFO. Some

derivatives also have higher antiproliferative activity than DFO (Darnell and Richardson 1999). No studies have examined these agents for colorectal cancer. However, antiproliferative activity has been demonstrated in other cancer cell lines (Richardson, Tran et al. 1995).

Side effects

Potential use of iron chelation in colorectal cancer should also note significant side effects seen in clinical trials for other conditions, particularly with Desferrioxamine and 3-AP. These include myelosuppression (thrombocytopenia and neutropenia), cellular hypoxia (and activation of HIF transcription factors), and methaemoglobin (MetHb) formation preventing oxygen transport to dependant tissues (Yu, Gutierrez et al. 2012).

Of significance, as a response to iron chelation and the resultant decrease in intracellular iron, transferrin receptor expression is increased in cells. This is likely more pertinent to normal cells, having demonstrated that tumour cells already express a high level of transferrin receptor despite high intracellular iron levels in Chapter 2.

6.5.2 Transferrin receptor blockers

Transferrin receptor is another potential target by which to reduce bioavailable iron supply to colorectal cancer cells. In 1982, Trowbridge demonstrated the generation of monoclonal antibodies to transferrin receptor using a murine hydridoma. These

anti-transferrin receptor antibodies achieved dose dependant inhibition of growth in a human T cell leukaemia cell line (Trowbridge and Lopez 1982).

Another novel approach to transferrin receptor by Yang et al (2001) used phosphorothioated antisense TfR oligonucleotides (TfR-ODna) to TfR1, reducing both TfR1 mRNA and protein in breast cell lines. Promisingly this agent demonstrated selective anti-cancer activity and was more effective in breast cancer cells than normal breast cells, achieving a 50% reduction in mRNA compared to 14% in normal cells (Yang, Jiang et al. 2001).

No recent research has examined either of these approaches in current anti-cancer therapy.

6.5.3 Tf-linked drug delivery

One novel method of exploiting the endocytosis of Tf and known over-expression of TfR in colorectal cancer is the creation of transferrin carrier molecules that could achieve intracellular delivery of anti-cancer therapy selectively to cancer cells (Daniels, Delgado et al. 2006). Doxorubicin conjugated to transferrin is the most widely studied agent in this context, effective in many cancer cell lines including overcoming doxorubicin resistant cells (Daniels, Delgado et al. 2006). Other drugs including cisplatin, chlorambucil, mytomycin c, gemcitabine and daunorubicin have all been delivered using this method (Daniels, Delgado et al. 2006). This mechanism of drug delivery has also been employed using both transferrin monomers and transferrin oligomers in human colon carcinoma (Caco-2) cells with anti-

proliferative effects greater with transferrin oligomer (Lim and Shen 2004). Beyond drugs, toxic proteins, liposomes, viral vectors and nanoparticles are just some of the molecules that have used transferrin as a vector for intracellular delivery (Daniels, Delgado et al. 2006).

If we can exploit the known overexpression of transferrin receptor by linking transferrin to chemotherapy as a novel delivery method can we also do so combined with chemotherapy agents known to exploit oxygen free radical generation as part of their mechanism of action thereby further exploiting the known excess of labile iron and selectively sparing those iron deficient normal cells, mitigating collateral chemotherapy toxicity.

6.6 Conclusion

Our understanding of iron biology in gastrointestinal cancer has grown considerably in the last 10 years. The parallel advance in clinical and basic science research in the fields of iron therapy and iron biology will no doubt continue into the coming decades. This thesis investigated some of the facets and intersections of these important fields of research. I hope one day these research converge to reveal how we can control and manipulate iron biology to the benefit of patients and scientific understanding.

Abbreviations

ACD	Anaemia of chronic disease
AID	Absolute iron deficiency
BMI	Body mass index
BSG	British Society for Gastroenterology
CI	Confidence interval
CRP	C-reactive protein
ECOG	Eastern Cooperative Oncology Group
ELISA	Enzyme-linked immunosorbent assay
ESA	Erythropoiesis stimulating agents
FID	Functional iron deficiency
FtH	Ferritin heavy chain
FtL	Ferritin light chain
g/L	Grams per litre
Hb	Haemoglobin
Hb	Haemoglobin
Hb	Haemoglobin
ID	Iron deficiency
IDA	Iron deficiency anaemia
IDE	Iron-deficient erythropoiesis
IL	Interleukin
IL	Interleukin
IQR	Interquartile range

IRE	Iron-response elements
IRP	Iron response element binding proteins
IVI	Intravenous iron isomaltoside
MAGIC	Medical Research Council Adjuvant Gastric Infusional Chemotherapy
MCID	Minimal clinically important difference
MCV	Mean corpuscular volume
MDT	Multi-disciplinary team
MSI	Microsatellite instability
ng/mL	Nanograms per millilitre
pg/cell	Picograms per cell
PPI	Proton pump inhibitor
QOL	Quality of life
RDW	Red cell distribution width
sTfR	Soluble transferrin receptor
TNF	Tumour necrosis factor
TNF	Tumour necrosis factor
TRG	Tumour response grade
TSat	Transferrin saturations
WHO	World Health Organisation

Appendix 1

Search strategies for Cochrane Review

Cochrane Injuries Group Specialised Register & Cochrane Central Register of Controlled Trials (CENTRAL) (Cochrane Library) (all years to Issue 10, 2016)#1 MESH DESCRIPTOR iron EXPLODE ALL TREES#2 MeSH descriptor: [Iron Compounds] explode all trees# iron:TI,AB,KY#4 (((ferric OR ferrous):TI,AB,KY#5 MeSH descriptor: [Hematinics] explode all trees#6 (#1 OR #2 OR #3 OR #4 OR #5)#7 MESH DESCRIPTOR preoperative period EXPLODE ALL TREES#8 MESH DESCRIPTOR preoperative care#9 (((prior OR before) adj3 (surg* OR operat*))):TI,AB,KY#10 (preoperat* or perioperati* or preprocedur* or periprocedur* or presurg* or perisurg* or ((pre or peri) next (operat* or procedur* or surgi* or surgu*))):ti,ab,kw#11 (#7 OR #8 OR #9 OR #10)#12 (#6 AND #11)n=268

Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R) (all years to 3/11/2016)1. exp Iron/2. exp Iron Compounds/3. iron.ab,ti,kf.4. (ferric or ferrous) .ab,ti,kf.5. exp Hematinics/6. or/1-57. exp Anemia/8. Iron/df [defciency]9. (anaemi* or anemi*).ti,ab,kf.10. exp Blood Transfusion/11. transfusion.ab.12. or/7-1113. -. (preoperat* or perioperati* or preprocedur* or periprocedur* or presurg* or perisurg* or ((pre or peri) adj (operat* or procedur* or surgi* or

surgu*))).ti,ab,kf.14. ((prior or before) adj3 (surg* or operat*)).ab,ti,kf.15. exp Preoperative Period/16. Preoperative Care/17 or/13-1618 (6 and 12 and 17)19. (randomi#ed or randomi#ation).ab,ti.20. randomized controlled trial.pt.21. controlled clinical trial.pt.22. placebo.ab.23. clinical trials as topic.sh.24. randomly.ab.25. trial.ti.26. Comparative Study/27. or/19-2628. (animals not (humans and animals)).sh.29. 27 not 2830. (18 and 29)n=209 (all years to 3/11/2016)

Ovid EMBASE 1974 to 3/11/20161. Iron/ 2. Iron Derivative/ 3. iron.ab,ti,kf. 4. (ferric or ferrous).ti,ab,kw. 5. exp antianemic agent/ 6. or/1-5 7. exp Anemia/ 8. (anaemi* or anemi*).ti,ab,kw. 9. exp Blood Transfusion/ 10. transfusion.ab. 11. or/7-10 12. (preoperat* or perioperati* or preprocedur* or periprocedur* or presurg* or perisurg* or ((pre or peri) adj (operat* or procedur* or surgi* or surgu*))).ti,ab,kw. 13. ((prior or before) adj3 (surg* or operat*)).ab,ti,kw. 14. exp Preoperative Period/ 15. or/12-14 16. (randomi#ed or randomi#ation).ab,ti. 17. randomized controlled trial/ 18. (RCT or (random* adj3 (administ* or allocat* or assign* or class* or control* or determine* or divide* or distribut* or expose* or fashion* or number* or place* or recruit* or subsitut* or treat*))).ab,kw. 19. placebo/ 20. placebo.ab. 21. randomly.mp. or "at random".ab. 22. trial.ti. 23. or/16-22 24. exp animal/ not (exp human/ and exp animal/) 25. 23 not 24 26. 6 and 11 and 15 and 25n=414

PubMed (to 25/03/2015)(((((((("Comparative Study"[Publication Type]) OR "Randomized Controlled Trial"[Publication Type]) OR "Controlled Clinical Trial"[Publication Type])) OR (((((((randomized[Title/Abstract]) OR randomised[Title/Abstract]) OR placebo[Title/Abstract]) OR randomly[Title/Abstract]) OR trial[Title/Abstract]) OR groups[Title/Abstract]) OR group[Title/Abstract]))) NOT (("Animals"[Mesh]) NOT ("Animals"[Mesh] AND "Humans"[Mesh]))))) AND (((((((("preoperative surgery"[Title/Abstract]) OR "before surgery"[Title/Abstract]) OR "before surgical intervention"[Title/Abstract]) OR "before operation"[Title/Abstract])) OR (("Preoperative Period"[Mesh]) OR "Preoperative Care"[Mesh:noexp]))) AND (((((iron[Title/Abstract]) OR Ferrous compound*[Title/Abstract]) OR ferric compound*[Title/Abstract])) OR ((("Iron"[Mesh]) OR "Ferric Compounds"[Mesh]) OR "Ferrous Compounds"[Mesh])))

Web of Science IndexesSCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI Timespan=All years to 3/11/2016TOPIC Search: #1 (iron or ferric or ferrous)#2 (preoperat* or perioperati* or preprocedur* or periprocedur* or presurg* or perisurg*)#3 (pre-operat* or peri-operati* or pre-procedur* or peri-procedur* or pre-surg* or peri-surg*)#4 (anemi* or anaemi* or transfus*)#5 (#1 and (#2 or #3) and #4)#6 (RCT or random* or placebo)#7 (((singl* OR doubl* OR trebl* OR tripl*) SAME (blind* OR mask*)))#8 (trial)#9 (#6 or #7 or #8)#10 (#4 and #9)n=173

ClinicalTrials.gov 3/11/2016:Basic Search: IRON AND (PREOPERATIVE OR PERIOPERATIVE OR PERIPROCEDURAL OR PRE-OPERATIVE OR PERI-OPERATIVE OR PERI-PROCEDURAL) n=44

WHO International Clinical Trials Registry Platform (ICTRP) Search Portal 3/11/2016:Basic Search: ANEMIA AND IRON AND PREOPERATIVE OR ANEMIA AND IRON AND PERIOPERATIVE OR ANEMIA AND IRON AND PERIPROCEDURAL OR ANEMIA AND IRON AND PRE-OPERATIVE OR ANEMIA AND IRON AND PERI-OPERATIVE OR ANEMIA AND IRON AND PERI-PROCEDURAL OR ANAEMIA AND IRON AND PREOPERATIVE OR ANAEMIA AND IRON AND PERIOPERATIVE OR ANAEMIA AND IRON AND PERIPROCEDURAL OR ANAEMIA AND IRON AND PRE-OPERATIVE OR ANAEMIA AND IRON AND PERI-OPERATIVE OR ANAEMIA AND IRON AND PERI-PROCEDURAL n=28

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