Programme:

MSc Human Nutrition

Module title:

ADV716 - Research Dissertation

Assignment title:

THE EFFECT OF ORAL $FESO_4$ SUPPLEMENTATION ON TOTAL PLASMA IRON AND NON-TRANSFERRIN BOUND IRON IN HEALTHY ADULTS WITH VARIOUS NATURAL LEVELS OF ASCORBIC ACID.

Word Count:

14391

Submission Date:

17th August 2018 at 10pm

By Charlotte Woods

Student Number: 10407047

Supervisor:

Desley White

UNIVERSITY OF PLYMOUTH

The effect of oral $FeSO_4$ supplementation on total plasma iron and non-transferrin bound iron in healthy adults with various natural levels of ascorbic acid

Submitted by
Charlotte Marie Woods

To the University of Plymouth as a dissertation for the degree of
MSc Human Nutrition

Declaration

I declare that the submitted dissertation is the result of my own research and has been completed by me. I also declare that all references and other sources used by me have been appropriately acknowledged in the work. Furthermore, no part of this work has been submitted for the purpose of academic examination, either in its original or similar format, either in this University or elsewhere. I am solely responsible for errors in this work. This study has been carried out as part of a larger study using the same participants, methodology and laboratory analysis as another student, however the work in this dissertation and conclusions drawn are by myself only. It is our intention to combine our results in the future.

Signed:  Printed: Charlotte Marie Woods Date: 17th August 2018

Abstract

Introduction:

Oral iron supplementation may cause the production of non-transferrin bound iron (NTBI) which can cause oxidative damage. Ascorbic acid (AA) can act as an antioxidant against reactive oxygen species. The aim of this study was to investigate the effect of long-term oral iron supplementation on total plasma iron (TPI), NTBI and AA, to analyse associations between variables and dietary intake and explore the role of AA as an antioxidant.

Methods:

Eleven healthy adults aged 18-55 participated in a pre-post experimental study where they consumed oral $FeSO_4$ supplementation on alternate days for twenty-eight days. A food frequency questionnaire was taken to assess iron modulators and antioxidant intakes. Blood biomarkers including TPI, total iron binding capacity (TIBC), transferrin saturation, NTBI and total, reduced and oxidised AA were measured at baseline and post supplementation. Variables were statistically analysed for pre-post differences and correlations.

Results:

Mean TPI increased significantly in participants with baseline TPI levels below 30µmol/l ($p = 0.03$). Mean reduced AA increased significantly from baseline to post supplementation ($p = 0.03$). Baseline TIBC had a statistically significant negative correlation with total and oxidised AA ($p = 0.02$; $p = 0.02$). Baseline reduced AA had a statistically significant negative correlation with iron and vitamin C intake ($p = 0.02$; $p = 0.04$). Iron intake correlated positively with all other nutrient intakes.

Conclusion:

TPI only appears to increase in participants who have lower baseline TPI levels due to adequate systemic iron status reducing absorption. NTBI appears to be transient, only being produced in circulating blood short-term following ingestion of oral iron supplementation. Findings are suggestive of the activity of AA as an antioxidant but further investigation is necessary before conclusions can be drawn. Dietary analysis highlighted the possibility of vitamins A and E working as antioxidants against iron related reactive oxygen species.

Table of Contents

Tables and Figures

Tables

Figures

Abbreviations

AA – Ascorbic Acid

BMI – Body Mass Index

CASP – Critical Appraisal Skills Programme

EFSA - European Food Safety Authority

FeSO4 – Ferrous Sulphate

FFQ – Food Frequency Questionnaire

HPLC – High-Performance Liquid Chromatography

ICSH – International Committee for Standardization in Haematology

MDA – Malondialdehyde

NDNS – National Diet and Nutrition Survey

NICE – National Institute of Health and Care Excellence

NIH – National Institutes of Health

NIST – National Institute of Standards and Technology

NTBI – Non-transferrin Bound Iron

RNI – Reference Nutrient Intake

SACN – Scientific Advisory Committee on Nutrition

SD – Standard Deviation

TBD - Total Base Damage

TIBC - Total Iron Binding Capacity

TPI – Total Plasma Iron

UV-Vis – Ultraviolet Visible

Acknowledgements

I would especially like to thank Desley White and Maria-Anne Gaitanou for their great help, input and support with this study as well as all of the individuals who participated in the study and enabled this study to take place. I would like to thank Steve Shaw for his helpful statistics advice. Lastly, I would like to thank Ajen Limbu, Stephanie Amil and Karen Burton for their endless support throughout.

Introduction

Background

Oral iron supplementation is an over the counter medication used as a treatment method for iron deficiency anaemia (Scientific Advisory Committee on Nutrition (SACN), 2010). Many individuals take over the counter oral iron supplementation after suffering with symptoms of iron deficiency anaemia, such as fatigue (SACN, 2010; National Institute of Health and Care Excellence (NICE), 2018). Iron has no mechanism of excretion once it has been absorbed in the intestines, therefore it is tightly controlled by the body to prevent toxicity and tissue damage from iron build up (Bhagavan, 2015.) Despite this tight control, there is evidence to suggest that oral iron supplementation may be causing the production of non-transferrin bound iron (NTBI), resulting in oxidative damage (Brissot *et al*, 2012). Ascorbic Acid (AA) has been shown to increase the absorption of non-haem iron and act as an important antioxidant against reactive oxygen species (National Institutes of Health (NIH), 2018). Thus, the aim of this dissertation was to investigate the effect of oral iron supplementation on the production of NTBI and the association with AA.

Anaemia affects 14% of women and 2-5% of men in the United Kingdom and is one of the most common nutritional deficiency diseases (NICE, 2018). It is diagnosed as an individual having haemoglobin below 12g/100ml for women and 13g/100ml for men (NICE, 2018). Iron deficiency anaemia can result from poor dietary iron, a complication with iron absorption or as a result of blood loss from menstruation or intestines (Bhagavan, 2015). A lack of iron can reduce the synthesis of haemoglobin which results in poor distribution of oxygen to body tissues and subsequently poor physical, reproductive, neurological and immune function (SACN, 2010).

Iron is a metal that exists in the body as ferric (Fe^{3+}), which is oxidised, and ferrous (Fe^{2+}) which readily receives and donates electrons. Fe^{2+} can cause the formation of reactive oxygen species (Bhagavan, 2015; Roghi *et al*, 2015). Therefore, it is bound to proteins for storage and transportation which include transferrin, ferritin, haemoglobin and myoglobin (Bhagavan, 2015). Transferrin accounts for about 0.2% of total body iron and is involved in the transportation of iron in blood plasma and extracellular fluid. The majority of stored iron is bound to ferritin, a large molecule in

the liver, bone marrow, spleen and muscles (20-30%) (SACN, 2010). Iron is used as a part of the synthesis of haemoglobin (60-70%) in erythrocytes and myoglobin (10%) in the muscle. These are molecules involved in the transportation and storage of oxygen (Geisser and Burckhardt, 2011). The concentrations of iron between each of these compartments varies due to the constant production and breakdown of erythrocytes in the bone marrow, liver and spleen (SACN, 2010).

The body's systemic need for iron can determine the amount of iron absorbed by ferroportin in enterocytes in the small intestine (Bhagavan, 2015). Enterocytes respond to liver mechanisms that control the release of iron from the iron pool into the portal circulation. Hepcidin is produced by the liver when iron stores are high and is a down regulator of iron absorption by the degrading of ferroportin (Bhagavan, 2015). Reasons for iron absorption are to replace lost iron from the shedding of skin cells, hair, urine, gastrointestinal secretions, menstruation, pregnancy and lactation (SACN, 2010).

The reference nutrient intake (RNI) for iron is 8.7mg for males and 14.8mg for females aged 19-50 years which can be achieved through diet (Department of Health, 1991). The average intake of iron for adults aged 19-64 is 11.6mg per day for men and 9.3mg per day for women, showing women are consuming less iron than recommended (National Diet and Nutrition Survey (NDNS), 2018). Iron supplements made up a small amount of iron intake per day in most groups, with the exception of women aged 35-49 years of which 21% used some form of iron supplement (SACN, 2010). AA is thought to increase the absorption of non-haem iron whilst calcium, polyphenols, zinc and fibre are thought to be inhibitors (SACN, 2010).

Supplements do not necessarily resolve the underlying cause of iron deficiency anaemia (SACN, 2010). Types of supplements available to the public are ferrous sulphate ($FeSO_4$), ferrous fumarate, ferrous gluconate, ferrous glycine sulphate and iron polysaccharide (NICE, 2018). The dosages vary from 7-60mg per day, of which the higher doses are commonly used but not advised in the United Kingdom (SACN, 2010). A guidance level of 17mg of iron supplementation per day is recommended (SACN, 2010). Side effects of high doses can include constipation, nausea, diarrhoea and vomiting (SACN, 2010; Leonard *et al*, 2014). Despite these

recommendations, the clinical practice guideline by NICE (2018) still currently recommends high doses for individuals diagnosed with iron deficiency anaemia, showing a discrepancy between contemporary policies.

Previous studies have found that iron supplementation can cause a release of NTBI in the blood (Brittenham *et al*, 2014). NTBI is a type of iron that has been associated with oxidative damage to lipid membranes of cells, in particular cardiac and hepatic cells (Brissot *et al*, 2012; Roghi *et al*, 2015). Oxidative damage may occur as NTBI is not bound to an iron associated protein, leaving it available to donate or receive electrons, which causes the production of reactive oxygen species (Roghi *et al*, 2015). This oxidative damage has been suggested to be carcinogenic to the body, subsequently increasing cancer risk, in particular colorectal cancer (SACN, 2010). NTBI may also be increasing the risk of heart disease by contributing to the development of atherosclerosis by oxidising low density lipoproteins or causing cardiac cell damage (Roghi *et al*, 2015). The lack of knowledge surrounding the production of NTBI and its activity following iron supplementation advocates the need for further investigation.

AA, also known as vitamin C, is an essential vitamin used in the body to synthesise collagen, L-cartine and neurotransmitters and is involved in protein metabolism and immune function (NIH, 2018). Fruits, vegetables and some fortified grains are good sources of AA (NIH, 2018). The recommended intake for AA in adults aged 19-50 years is 40mg per day which is achieved easily by the current population (Department of Health, 1991). AA works as an antioxidant as it can readily donate electrons to stabilise reactive oxygen species without becoming unstable itself. Additionally, it works to regenerate other antioxidants including vitamin E (Lane and Richardson, 2014). Once donated its electron, AA becomes oxidised but is rapidly converted back to its reduced form intracellularly (Lane and Richardson, 2014). Therefore, it has been suggested that AA could reduce NTBI to a stable molecule, thus counteracting its effect of oxidative damage (Chakraborty and Jana, 2017). AA also enhances non-haem iron absorption (Lane and Richardson, 2014). Thus, increased iron absorption may be increasing NTBI production. The ultimate role of AA here is unclear, and warrants further investigation.

Iron deficiency anaemia is a problematic condition with a treatment method of oral iron supplementation that has questionable safety with dosages that may be too high. Yet it is still available over the counter without the necessity of a pre-assessment. Extra to this, discrepancies in current policies need to be addressed. The potentially damaging role of NTBI has been explored, but not yet fully investigated, as with the association of AA and its antioxidant activity in relation to iron. Literature reviews are commonly used to critically analyse the current evidence base surrounding a relevant topic which can be applied to policy, practice and future research (Baker, 2016). Therefore, a literature review has been carried out to critically analyse current literature surrounding this topic.

Search Strategy

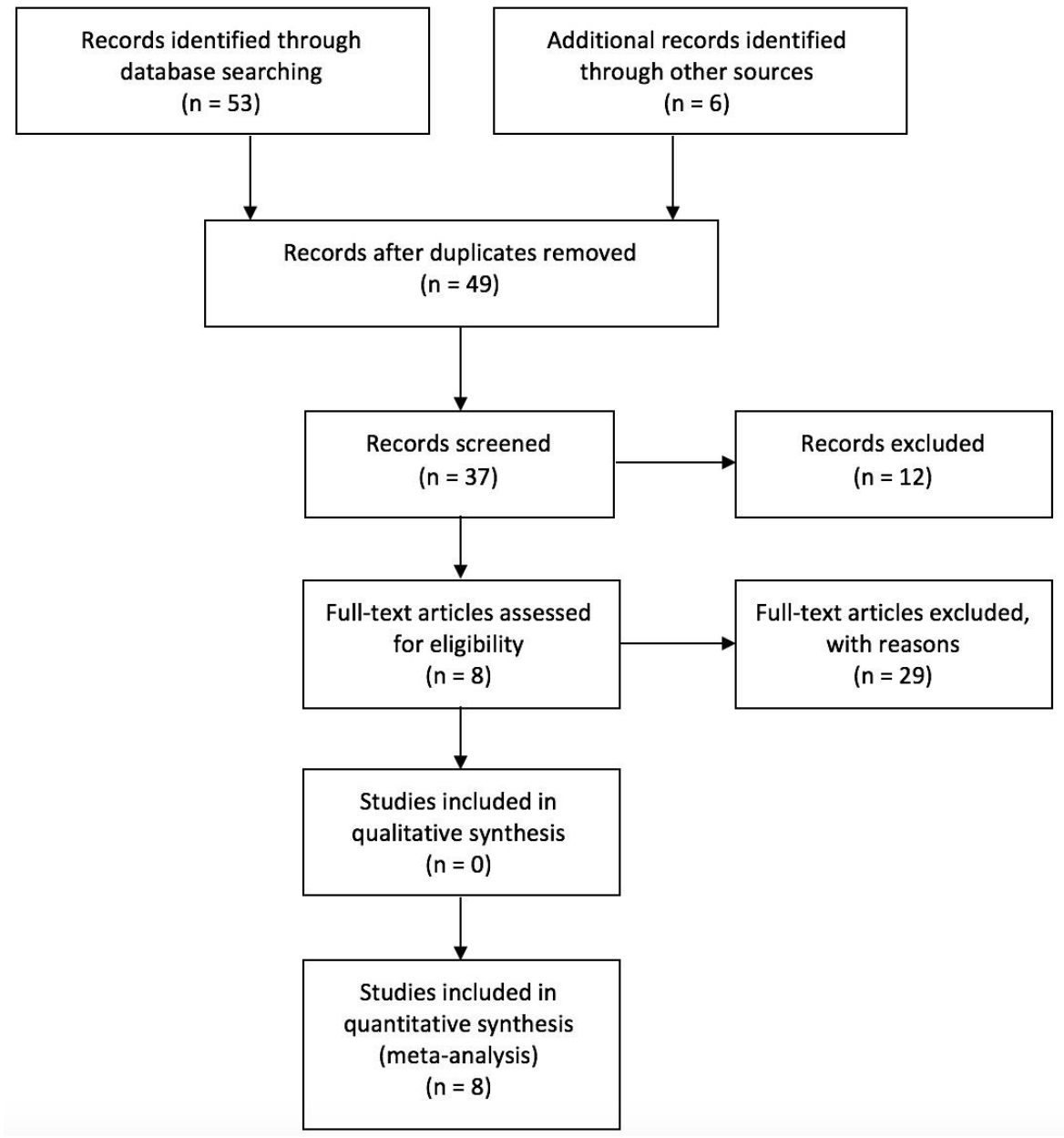
The PICO Framework (Williamson and Whittaker, 2017) illustrated in Table 1 was used to structure the literature search. Limits were set to 'humans'. Older research was considered to inform this review due to minimal research available from the last twenty years. Pubmed, Medline, Cochrane, Web of Science, Cinahl, Embase and The Joanna Briggs Institute were searched using the terms illustrated in Table 1. Boolean operators 'OR' and 'AND' were used to connect terms. An additional Google Scholar search was carried out to explore a wider selection of literature. The literature search ceased when no new articles were found.

Table 1 Search Criteria

PICO FRAMEWORK	SEARCH TERMS	INCLUSION CRITERIA	EXCLUSION CRITERIA
POPULATION (P)	Healthy, Individuals, Adults, Participants, Cohort, Men, Women	Healthy, Adults, 18-65 Years Old	Chronic or Acute Illness, Anaemia, Pregnant or Lactating, Children, Elderly
INTERVENTION (I)	Oral iron supplementation, Fe, $FeSO_4$, iron tablets, fortification, iron challenge	Oral Iron Supplementa-tion	Haemodialysis, Intravenous Iron, Iron Supplementation with Added Medication, Alternative Supplementation
OUTCOME (O)	Dietary intake, plasma/serum iron, total iron binding capacity (TIBC), transferrin, transferrin saturation, non-transferrin bound iron (NTBI), AA, oxidative damage, lipid peroxidation, free radical, reactive oxygen species	Iron Status, NTBI, AA (with NTBI or oxidative damage)	Absorption Only (without NTBI or AA consideration)

AA – Ascorbic Acid, Fe – Iron, $FeSO_4$ – Ferrous Sulphate, NTBI – Non-transferrin Bound Iron, TIBC – Total Iron Binding Capacity

The screening process is shown in Figure 1. Forty-nine articles were found following the removal or duplicates. Forty-three from literature databases and six from other sources, including reference searches. Following screening, a total of eight full text articles were assessed for their eligibility, following the inclusion and exclusion criteria set in Table 1.



(The PRISMA Group, 2009)

Figure 1 PRISMA Flow Diagram

Reasons for excluding articles during the screening process included articles that:

- Did not directly measure NTBI or AA
- Included pregnant or lactating women
- Had a lack of information regarding the characteristics of the study
- Studies that only gave a placebo to healthy subjects

Table 2 provides a detailed outline of the literature found. The critical analysis of the studies are detailed below. Studies investigating oral iron supplementation and NTBI are presented first, followed by those investigating oral iron supplementation and AA. The review is then summarised in terms of the study findings, overall quality, gaps in literature and the contribution of literature within the local and national context. Following this are the aims, objectives and research question for future research.

Table 2 Summary of Literature

STUDY	TITLE	DESIGN	PARTICIPANTS	METHODS	DATA ANALYSIS	STATISTICAL ANALYSIS	RESULTS
Rehman *et al* (1998)	The effects of iron and vitamin C co-supplementation on oxidative damage to DNA in healthy volunteers	Longitudinal Prospective Randomised Study	N = 38 Males and Females English 21-45 Years Old Healthy, No Supplements – time not stated, Smoking, Medication, High Alcohol Intake, Pregnancy, Oral Contraceptives	Twelve-week supplementation of $FeSO_4$ and AA. No control. Blood samples taken three times throughout the study. Dietary analysis completed.	Gas Chromatography Mass Spectrometry (TBD) Micro-fluorometry (AA), Ultraviolet Visible Spectrophotometry (Transferrin Bound Iron) Calculation – Transferrin Bound Iron and UIBC (Transferrin Saturation)	Statistical tests not stated	Participants with higher baseline levels of AA had lower TBD. Not statistically significantly significant. No statistically significant differences in Transferrin Bound Iron in participants who had higher baseline AA levels. Participants with lower baseline AA levels had a significant increase in Transferrin Bound Iron and higher but not statistically significant initial TBD.
Proteggente *et al* (2001)	Iron supplementation and oxidative damage to DNA in healthy individuals with high plasma ascorbate	Pre-Post Longitudinal Prospective Experimental Study	N = 37 Males and Females English 23-46 Years Old, Healthy No Supplements – time not stated, Medication, Smoking, High Alcohol Intake, Pregnancy, Oral Contraceptives	Six-week supplementation of **ferrous glycine sulphate.** No control. Blood samples taken before and after supplementation. Dietary analysis completed.	Micro-fluorometry (AA), Ultraviolet Visible Spectrophotometry (Transferrin Bound Iron) Calculation – Transferrin Bound Iron and UIBC (Transferrin Saturation), Gas Chromatography Mass Spectrometry (TBD)	ANOVA Pearson's Correlations	Iron supplementation had no effect on oxidative damage to DNA in the presence of high plasma AA. Effects of AA acting as a pro-oxidant in the presence or iron appears to be minimal.
Colpo *et al* (2008)	A single high dose of ascorbic acid and iron is not correlated with oxidative stress in healthy volunteers	Experimental Crossover Study	N = 9 Males Brazilian 20-31 Years Old Healthy No Supplements – time not stated, No Smoking No High Alcohol Intake No Medication	Three supplementations of AA, **iron carbonyl** or both were given over three days separated by fifteen days washout. No control. Blood samples were taken four times over twenty-four hours following supplementation. No dietary analysis.	Ultraviolet Visible Spectrophotometry (TPI), Electron Paramagnetic Resonance (AA), Ultraviolet Visible Spectrophotometry (MDA)	ANOVA	Total Plasma Iron (TPI) significantly increased in the group supplemented with iron and AA, compared to pre-treatment levels. MDA levels significantly decreased after AA supplementation.
Troesch *et al* (2011)	Fortification iron as ferrous sulfate plus ascorbic acid is more rapidly absorbed than as sodium iron EDTA but neither increases serum nontransferrin-bound iron in women	Experimental Crossover Study	N = 16 Females Swiss 18-40 Years Old Healthy No Supplements – time not stated, Pregnancy or Lactation, Blood Donation – 4 Months, Medication Adequate C-reactive Protein Oral Contraceptives Allowed	Two supplementations of iron, one with **$FeSO_4$**, with and without AA were given over two days separated by seven-day washout. No control. Blood samples were taken six times over eight hours following supplementation. No dietary analysis.	Exchange Chromatography (Hepcidin), Ultraviolet Visible Spectrophotometry (TPI, TIBC) Calculation – TPI/TIBCx100 (Transferrin Saturation), Graphite Furnace Atomic Absorption Spectrometry (NTBI)	ANOVA Kruskal-Wallis Test Paired T-Test Pearson's Correlation	Iron was more rapidly absorbed from $FeSO_4$ and AA than another iron solution without AA. Hepcidin increased significantly following the $FeSO_4$ and AA supplementation. No significant increase in NTBI after either supplementation.

Schumann *et al* (2012a)	Impact of oral iron challenges on circulating non-transferrin-bound iron in healthy Guatemalan males	Longitudinal Prospective Cohort Study	N = 8 Males Guatemalan 23-54 Years Old Healthy No Supplements – 6 Months	Nine week bi-weekly **$FeSO_4$** supplementation in increasing quantities including control. Blood samples taken at baseline and hourly for three hours following supplementation. No dietary analysis	Enzyme Immunoassay Spectrophotometry (TPI) Calculation – Plasma Transferrin and TPI (Transferrin Saturation) Fluorometric Competitive Binding Assay (NTBI)	ANOVA Students T-Test Pearson's Correlation	A dose-dependent progression in NTBI was observed with increasing dosages of iron and is reflected by TPI. This increase is disproportionate. In general, NTBI was not detectible in the fasting state.
Schumann *et al* (2012b)	Oral administration of ferrous sulfate, but not of iron polymaltose or sodium ironethylenediamine tetraacetic acid (NaFeEDTA), results in a substantial increase of non-transferrin-bound iron in healthy iron-adequate men	Experimental Crossover Study	N = 10 Males Guatemalan 18-55 Years Old Healthy No Supplements – 'recent use' Adequate Haemoglobin	Four forms of iron supplementation, including **$FeSO_4$,** given to participants over four fays separated by seven day intervals including control. Blood samples taken four times over four and a half hours following supplementation. No dietary analysis.	Ultraviolet Visible Spectrophotometry (TPI) Fluorometric Competitive Binding Assay (NTBI)	MANOVA Pearson's Correlation	Rise in NTBI following $FeSO_4$ was statistically significant compared to water. Correlation (r = 0.87) of TPI and NTBI.
Schumann *et al* (2013)	Differences in circulating non-transferrin-bound iron after oral administration of ferrous sulfate, sodium iron EDTA, or iron polymaltose in women with marginal iron stores	Experimental Crossover Study	N = 10 Females Guatemalan 22-49 Years Old Healthy (Marginal Iron Stores) No Supplements – 'recent use' No Pregnancy No Abnormal Menstrual Cycles	Four forms of iron supplementation, including **$FeSO_4$**, given to participants over four fays separated by seven day intervals including control. Blood samples taken four times over four and a half hours following supplement-ation. No dietary analysis.	Ultraviolet Visible Spectrophotometry (TPI) Fluorometric Competitive Binding Assay (NTBI)	MANOVA Pearson's Correlation	Statistically significant rise in NTBI following $FeSO_4$ supplementation. Correlation (r = 0.88) of NTBI and TPI.
Brittenham *et al* (2014)	Circulating non-transferrin-bound iron after oral administration of supplemental and fortification doses of iron to healthy women: a randomized study	Experimental Crossover Study	N = 32 Females Swiss 18-39 Years Old Healthy Normal BMI No Supplements – 2 Weeks, Pregnancy or Lactation, Inflammation, Medication, Blood Donations – 4 Months Oral Contraceptives Allowed	Three forms of iron supplementation, including **$FeSO_4$**, given to participants over three days in a randomised order. No control. Blood samples taken five times over eight hours following supplementation. No dietary analysis.	Ultraviolet Visible Spectrophotometry (TPI, TIBC), Calculation – TPI/TIBCx100 (Transferrin Saturation), Graphite Furnace Atomic Absorption Spectrometry (NTBI) Enzyme Immunoassay Spectrophotometry (Hepcidin)	Unpaired T-Test Mann-Whitney ANOVA Pearson's Correlation	With 60mg doses of iron, NTBI increased to reach peaks at 4 hours and progressively declined until last sample at 8 hours. For this dose, the amount of iron absorbed significantly correlated with NTBI. Hepcidin increased after NTBI peak at 4 hours.

AA – Ascorbic Acid, $FeSO_4$ – Ferrous Sulphate, MDA - Malondialdehyde, NTBI – Non-transferrin Bound Iron, TBD - Total Base Damage, TIBC - Total Iron Binding Capacity, TPI – Total Plasma Iron, UIBC – Unsaturated Iron Binding Capacity

This literature review included five experimental crossover studies and three longitudinal experimental studies. Five studies investigated NTBI (Troesch *et al*, 2011; Schumann *et al*, 2012a; Schumann *et al*, 2012b; Schumann *et al*, 2013; Brittenham *et al*, 2014) whilst three investigated AA (Rehman *et al*, 1998; Proteggente *et al*, 2001; Colpo *et al*, 2008). Therefore, NTBI and AA had not been investigated using healthy individuals within the same study. Furthermore, there were no studies investigating NTBI that supplemented iron long-term. This highlights gaps in the literature. The literature was appraised with the assistance of the Critical Appraisal Skills Programme (CASP) (2018). Rehman *et al* (1998), Proteggente *et al* (2001) and Brittenham *et al* (2014) scored eleven out of twelve using the Cohort Study Checklist. Colpo *et al* (2008) and Troesch *et al* (2011) scored nine and Schumann *et al* (2012a), Schumann *et al* (2012b) and Schumann *et al* (2013) scored eight. Overall scores were high indicating reasonable quality literature.

Critical Appraisal of Non-Transferrin Bound Iron Literature

Experimental Crossover Studies

Troesch *et al* (2011), Schumann *et al* (2012b), Schumann *et al* (2013) and Brittenham *et al* (2014) performed experimental prospective crossover studies to test the effects of orally supplemented $FeSO_4$ on NTBI. Troesch *et al* (2011) found an increase in total plasma iron (TPI) but no significant increase in NTBI following supplementation of $FeSO_4$ with AA. Hepcidin increased significantly following $FeSO_4$. In contrast, Schumann *et al* (2012b) and Schumann *et al* (2013) both found a statistically significant rise in NTBI following $FeSO_4$ and this correlated with TPI ($r = 0.87$; $r = 0.88$). Brittenham *et al* (2014) also found an increase in NTBI following 60mg iron and this correlated with TPI. Hepcidin increased after NTBI peaked at four hours post supplementation.

Although randomised controlled trials are considered ‘gold standard’, the crossover design allowed for a smaller number of participants to be used, as each participant was able to take part in all branches of the study, keeping participant variables constant (Parahoo, 2014). Crossover designs can be affected by order effects (Parahoo, 2014). Troesch *et al* (2011), Schumann *et al* (2012b) and Schumann *et al*

(2013) allowed for a seven-day washout period between study days which is considered sufficient (Geisser and Burckhardt, 2011). Brittenham *et al* (2014) used random sequence allocations of their treatment arms therefore any order effects should have been carried forward.

Longitudinal Prospective Cohort Study

Schumann *et al* (2012a) performed a nine-week longitudinal prospective study giving participants increasing doses of $FeSO_4$. A dose-dependent progression in NTBI was observed and is reflected by a dose-dependent progression of TPI. Despite being labelled a longitudinal study, each study day was analysed individually, therefore it was not useful in detecting changes over time (Parahoo, 2014). In addition, the dose-dependent progression on plasma iron and NTBI may have been a result of the accumulation of iron absorbed over time as doses were given in close proximity of each other. The results of this study contributed strongly to literature, as dosage of supplementation and NTBI have not been studied previously.

Participants

Troesch *et al* (2011) and Brittenham *et al* (2014) utilised a power calculation to enable them to detect a difference of 30% in iron absorption. This was justified due to the absence of data necessary to perform a power calculation for NTBI. No other study justified for their sample size. The participants were Guatemalan and Swiss, reducing the generalisability to the English population due to potential genetic and environmental differences such as diet and exercise (Parahoo, 2014). No study looked at both males and females together highlighting a gap in research. Papers that used women ensured they were not pregnant or lactating. This was beneficial as pregnancy and lactation can increase women's iron loss and subsequent need to absorb more iron from their diet, potentially increasing NTBI production (Geisser and Burckhardt, 2011; Lane and Richardson, 2011).

Troesch *et al* (2011) and Brittenham *et al* (2014) excluded individuals who had given blood donations less than four months prior to the study. This reduced confounding effects as blood donations can have a significant effect on iron status due to sudden

increased iron loss (Smith *et al*, 2014). An extensive inclusion and exclusion criteria from all studies allowed for increased control of variables which increased replicability and validity from reduced participant bias (Parahoo, 2014).

Methods

All studies took careful consideration to standardise each supplementation which reduced variables and increased internal validity (Parahoo, 2014). Schumann *et al* (2012a) supplemented participants with high doses of $FeSO_4$, including 120mg and 240mg which may have been unnecessary. Common supplementation doses are rarely above 60mg and doses above 17mg can have serious side effects including constipation, nausea, diarrhoea and vomiting (SACN, 2010; Leonard *et al*, 2014). Troesch *et al* (2011) used incomparable treatment arms of $FeSO_4$ with AA and $Na^{57}FeEDTA$ without AA. Therefore, changes in absorption or NTBI production could have been attributed to the difference in the type of iron and/or the added AA (Parahoo, 2014). Brittenham *et al* (2014) took samples up to eight hours following supplementation and found noteworthy results. Considering this, Schumann *et al* (2012a), Schumann *et al* (2012b) and Schumann *et al* (2013) may have underestimated the results by only sampling for four and a half hours. No study took a dietary analysis from their participants, despite diet being a major influence on iron absorption and utilisation (Bhagavan, 2015).

Table 3 Literature Outcome Measures

STUDY	OUTCOME MEASURES								
-	TPI	TIBC	Transferrin Bound Iron	Transferrin Saturation	NTBI	Hepcidin	AA	MDA	TBD
Rehman *et al* (1998)	-	Y	Y	Y	-	-	Y	-	Y
Proteggente *et al* (2001)	-	-	Y	Y	-	-	Y	-	Y
Colpo *et al* (2008)	Y	-	-	-	-	-	Y	Y	-
Troesch *et al* (2011)	Y	Y	-	Y	Y	Y	-	-	-
Schumann *et al* (2012a)	Y	-	-	Y	Y	-	-	-	-
Schumann *et al* (2012b)	Y	-	-	-	Y	-	-	-	-
Schumann *et al* (2013)	Y	-	-	-	Y	-	-	-	-
Brittenham *et al* (2014)	Y	Y	-	Y	Y	Y	-	-	-

AA – Ascorbic Acid, NTBI – Non-transferrin Bound Iron, MDA - Malondialdehyde, TBD - Total Base Damage, TIBC - Total Iron Binding Capacity, TPI – Total Plasma Iron

Multiple different biomarkers have been used by the literature. All studies used TPI for at least one biomarker for iron status. Using biomarkers such as TPI alone can provide a limited picture of the body's true iron status and minor changes may have little clinical meaning (Geisser and Burckhardt, 2011). However, TPI does not require the measurement of CRP or leucocytes to rule out inflammation, which can provide an inaccurate picture of iron status in biomarkers such as ferritin (Khan *et al*, 2016).

Total Iron Binding Capacity (TIBC) is a measure of the capacity of iron associated proteins to bind to iron (Zhou *et al*, 2014). TIBC is a commonly used measure of iron status, used in combination with TPI and transferrin saturation for a reliable measure of iron status (Nunes *et al*, 2014). A disadvantage of this biomarker is that it does not take into account which proteins are bound to the iron (Eslayed *et al*, 2016).

Transferrin saturation represents the quantity of iron bound to transferrin in respect to the total transferrin in the plasma (Eslayed *et al*, 2016). It is an important biomarker for iron status as it is central to iron homeostasis but is only useful when

used alongside other biomarkers such as TPI and TIBC (Eslayed *et al*, 2016; Porter *et al*, 2016). A disadvantage of transferrin saturation is that simple reporting may obscure important trends in results (Porter *et al*, 2016).

Hepcidin is an important diagnostic biomarker for iron metabolism and homeostasis due to its connected role with ferroportin (Ruchala and Nemeth, 2014; Kali *et al*, 2015). However, the validation of hepcidin as a biomarker has been criticised due to the lack of effective assays to measure it (Miseta *et al*, 2015).

As the focus of these studies is on NTBI, it is expected that a blood biomarker of NTBI will be used. NTBI has been shown to rapidly increase and decrease following iron loading (Ito *et al*, 2017). However, there is still limited knowledge about the activity of NTBI, what NTBI is bound to or how many iron complexes make up the 'NTBI' that is currently detected (Maas *et al*, 2013; De Swart *et al*, 2016). Therefore, there is a lack of confidence to determine whether NTBI is being detected accurately and laboratory methods require further improvements (Pfeiffer and Looker, 2017).

Laboratory Data Analysis

Table 4 Literature Laboratory Assays

LABORATORY ASSAY	DESCRIPTION	OUTCOME MEASURE
Ultraviolet Visible Spectrophotometry	A measure of light wavelength at 535nm from a material or substance in the ultraviolet or visible spectrum (NIST), 2018).	TPI, TIBC, Transferrin Bound Iron, MDA
Enzyme Immunoassay Spectrophotometry	This method utilises enzyme linked reactions to create coloured light. This light is then measured by spectrophotometry at a particular wavelength (NIST, 2018).	Hepcidin, TPI
Exchange Chromatography	This method separates constituents in multiple phases through columns based on their ion charges which enables them to be measured (McMurry, 2015).	Hepcidin
Graphite Furnace Atomic Absorption Spectrometry	This method vaporises the material or substance using a graphite coated furnace. Atoms absorb light at specific wavelengths which are measured (Taylor *et al*, 2013).	NTBI
Fluorometric Competitive Binding Assay	Specific constituents are bound to a ligand and a measure of fluorescence is taken by exciting the electrons which causes them to emit light (Sanderson *et al*, 2014).	NTBI
Gas Chromatography Mass Spectrometry	This method uses a carrier gas such as helium during one phase and a liquid during another and measures constituents according to their mass (Pavia *et al*, 2018).	TBD
Micro-fluorometry	Micro-fluorometry is a variation of fluorometry, with the addition of a microscope to detect fluorescence that cannot be seen by the human eye (Sanderson *et al*, 2014).	AA
Electron Paramagnetic Resonance Spectroscopy	This process works with materials with unpaired electrons, exciting them to enable them to be measured (McMurry, 2015).	AA

AA – Ascorbic Acid, NIST – National Institute of Standards and Technology, NTBI – Non-transferrin Bound Iron, MDA = Malondialdehyde, TBD = Total Base Damage, TIBC = Total Iron Binding Capacity, TPI – Total Plasma Iron

Four studies used Ultraviolet Visible (UV-Vis) Spectrophotometry (Table 4) to measure TPI, with the use of ferrozine as the chromogen. Troesch *et al* (2011) and Brittenham *et al* (2014) also used this assay to measure TIBC. This is a commonly used, reliable method which is selective, accurate and reproducible (Elgailani and Alsakka, 2016). Ferrozine has been shown to have 25% increased sensitivity in comparison to previous chromogens (International Committee for Standardization in Haematology (ICSH), 1990). A disadvantage of this assay is that the reagents also react with copper, however this influence is predicted to be minor (ICSH, 1978).

Brittenham *et al* (2014) used Enzyme Immunoassay Spectrophotometry and Troesch *et al* (2011) used Exchange Chromatography (Table 4) to measure hepcidin. Enzyme Immunoassay Spectrophotometry is highly reliable as the use of monoclonal immunologic reagents provides the user accurate measurement and control over the assay with limited interference from blood plasma and background absorbance (Flowers *et al*, 1989; Siemens Healthcare Limited, 2018). Exchange Chromatography requires only one interaction for separation of substances which reduces the risk of error such as those from multiple stage techniques (Cabanne and Santarelli, 2014). Overall, Miseta *et al* (2015) suggests that both methods are not effective to quantify hepcidin.

Troesch *et al* (2011) and Brittenham *et al* (2014) used Graphite Furnace Atomic Absorption Spectrometry (Table 4) whilst the others used Fluorometric Competitive Binding Assay (Table 4) to measure NTBI. Graphite Furnace Atomic Absorption Spectrometry can determine the concentrations of a variety of constituents, is sensitive, precise and has minimal interference (Taylor *et al*, 2013). Fluorometric Competitive Binding Assay is simple, reliable and also has little interference (Breuer and Cabantchik, 2001). It uses fluorescein-labelled apo-transferrin which is specifically designed to bind to NTBI. However, this assay is thought to underestimate NTBI, be unable to detect it at low levels and be affected by stray light (Breuer and Cabantchik, 2001; Sanderson *et al*, 2014).

Statistical Data Analysis

Schumann *et al* (2012a), Schumann *et al* (2012b) and Schumann *et al* (2013) did not test for normality. Normality tests are an important part of analysis of data. Parametric statistical tests are more sensitive but rely on assumptions, of which may not apply to non-parametric data (Bland, 2015). Therefore, the results from the statistical testing may not accurately reflect the true findings of these studies. Troesch *et al* (2011), Schumann *et al* (2012a) and Brittenham *et al* (2014) used t-tests to measure the difference between baseline and peak for normally distributed data. This test can also provide precise data, as long as the assumptions of the test are upheld (parametric data) (McDonald, 2014). All studies used Pearson's Correlation to measure the associations between variables. This is a common method used by many researchers because of its efficacy (Currell, 2015). Nonetheless, with small sample sizes, the use of Pearson's Correlation may be misleading when outliers are present. A non-parametric test, such as Spearman's Rank Correlation Coefficient may have been more appropriate (Bland, 2015).

Critical Appraisal of Ascorbic Acid Literature

Longitudinal Prospective Studies

Rehman *et al* (1998) and Proteggente *et al* (2001) used longitudinal prospective studies to test the effects of iron supplementation and AA on oxidative damage. Rehman *et al* (1998) supplemented participants with $FeSO_4$ and AA for twelve weeks. They found that participants whom had higher baseline levels of AA had lower total base damage (TBD) and participants who had lower baseline AA levels had a significant increase in transferrin bound iron following six weeks of supplementation.

Proteggente *et al* (2001) supplemented participants with $FeSO_4$ for six weeks. They found that participants who had higher baseline AA had no change in TBD. This suggested that AA was unlikely acting as a pro-oxidant when it was associated with increased iron absorption. Due to the time difference between blood samples, there was a chance that differences between pre-post measurements were due to maturation effects instead of the independent variable (Parahoo, 2014).

Experimental Crossover Study

Colpo *et al* (2008) carried out an experimental crossover study over three separate days to test the effects of different combinations of iron carbonyl and AA supplementation on oxidative damage. They found that TPI increased significantly following supplementation and malondialdehyde (MDA) decreased following supplementation. The crossover design allowed for different treatments to be tested using the same participants as natural levels of oxidative damage can vary with each individual (De Lucca *et al*, 2016). Colpo *et al* (2008) ensured an acceptable washout period of fifteen days between treatments (Geisser and Burckhardt, 2011). The results of these studies suggest that baseline AA plays an important role in determining iron absorption and oxidative damage.

Participants

The studies used samples sizes of 9-38 participants. None of which justified the power of their sample to detect changes in variables following supplementation (SACN, 2010). All studies used participants with no medical conditions or taking medication. This is an important advantage as acute and chronic disease and their associated treatments can increase the occurrence of oxidative damage (Rodrigues *et al*, 2018). No study excluded participants who exercise heavily which has been shown to increase oxidative stress (Pingitore *et al*, 2015). In addition, participants who had recently donated blood were not excluded. This has been shown to reduce iron stores (Smith *et al*, 2014). These could be confounding factors.

Rehman *et al* (1998) and Proteggente *et al* (2001) used English participants, increasing the generalisability of the sample to the local population (Parahoo, 2014). The studies used an overall age range of 20-46 years old which ruled out confounders from the older population who are more likely to have oxidative damage (Cencioni *et al*, 2013). All studies excluded smokers and participants who had a high alcohol intake. This increased the validity of the results as smoking and alcohol have been shown to increase oxidative damage in the body (Zuo *et al*, 2014).

Methods

Rehman *et al* (1998) used a commercial capsule form of $FeSO_4$ which ensured standardisation and increased the studies internal validity (Parahoo, 2014). Rehman *et al* (1998) and Proteggente *et al* (2001) used a validated eight-day food diary and validated food frequency questionnaire (FFQ) respectively to assess the dietary intake of their participants. Colpo *et al* (2008) did not consider the dietary influence despite diet having an effect on iron absorption and oxidative stress (Bhagavan, 2015). Proteggente *et al* (2001) did not use an FFQ specifically designed to measure iron intake meaning that the sensitivity of this tool may not be high enough to provide an accurate estimation. As well as this it did not measure modifiers of iron intake such as phytates and polyphenols, limiting the data provided by this dietary analysis tool (Bhagavan, 2015).

Rehman *et al* (1998) and Proteggente *et al* (2001) used transferrin bound iron as a biomarker. TPI is more commonly used than transferrin bound iron in recent studies as transferrin bound iron only accounts for about 0.2% of the body's total iron, therefore provided a limited representation of iron status (Geisser and Burckhardt, 2011).

All three studies used total AA as a biomarker for vitamin C status. Plasma AA is a good biomarker of tissue vitamin C levels when interpreted alongside the physiological processes that can affect its plasma levels (Pollard *et al*, 2003). However, vitamin C can exist in different forms within the blood including reduced and oxidised AA (Lane and Richardson, 2014). Therefore, it can be recognized that measuring AA both as a whole and in its different forms can provide a clearer picture on its antioxidant activity. Without accurate measurements of these, misleading conclusions can be formed (Lykkesfeldt, 2007).

As a marker of oxidative damage, Rehman *et al* (1998) and Proteggente *et al* (2001) used TBD. Frijhoff *et al* (2015) describes this measure of oxidative stress as one of the best measures when linked to predictors of disease, however states that commercial assays used to detect this measure have questionable clinical significance. MDA, used by Colpo *et al* (2008), has been one of the most investigated lipid peroxidation end products. However, it has its limitations (Frijhoff *et al* (2015). MDA can be created during the measurement process and the assay used is not sensitive enough to measure MDA bound to other proteins (Herrera *et al*, 2014; Frijhoff *et al*, 2015).

Laboratory Data Analysis

Colpo *et al* (2008) used UV-Vis Spectrophotometry (Table 4) to measure TPI, with the use of ferrozine as the chromogen whilst Rehman *et al* (1998) and Proteggente *et al* (2001) used the chromogen ferene to measure transferrin bound iron. Although ferrozine is highly sensitive, ferene is described by ICSH (1990) to be 50% more sensitive than other methods.

Rehman *et al* (1998) and Proteggente *et al* (2001) used Micro-fluorometry (Table 4) to measure AA. Micro-fluorometry has the advantage of being able to detect constituents at low amounts (Tee *et al*, 1988). Nonetheless, it requires a lengthy procedure, which introduces greater opportunity for human error and interference from stray light (Sanderson *et al*, 2014). Colpo *et al* (2008) used Electron Paramagnetic Resonance Spectroscopy (Table 4) to measure AA. This method is useful for measuring and identifying organic radicals with unpaired electrons in comparison to fluorescence which only detects them indirectly (Bruker, 2018).

Rehman *et al* (1998) and Proteggente *et al* (2001) used Gas Chromatography Mass Spectrometry (Table 4) to measure TBD. This method is adequate for the measurement of low concentrations of constituents and can positively identify the presence of specific substances even in small quantities, however it is a time-consuming technique (Pavia *et al*, 2018). Colpo *et al* (2009) used UV-Vis Spectrophotometry to measure MDA with the use of thiobarbituric acid reaction. Agarwal and Chase (2002) suggest that this method is not specific enough to adequately detect MDA and it is subject to multiple interferences.

Statistical Data Analysis

Rehman *et al* (1998) did not state which statistical tests were used. This reduces the replicability of the study and the reader's ability to interpret the results. Statistical tests used by Proteggente *et al* (2001) and Colpo *et al* (2008) have been discussed above.

Summary and Rationale for Further Research

This literature review found that four out of five studies detected an increase in NTBI following supplementation of $FeSO_4$. Additionally, one study recognised a dose-dependent progression of TPI and NTBI. Two studies found that participants who had higher baseline AA had lower TBD. In addition, participants who had a lower baseline AA had a significant increase in transferrin bound iron following supplementation. Furthermore, MDA reduced following supplementation of iron carbonyl and AA. This suggests that NTBI is being produced following oral iron supplementation and that AA is acting as an antioxidant always and as a modulator of iron absorption only when baseline levels are low.

Overall, a CASP (2018) analysis indicated reasonable quality literature. Strengths of the literature were the extensive participant criteria and the ruling out of many confounding factors. Careful precautions were taken with biomarkers, such as ruling out inflammation. Limitations of the literature were the use of giving increasing doses within close proximity of each other, making it unclear if the dose-dependent progression was from increasing doses or accumulation of iron over time (Schumann *et al*, 2012a). The two treatment branches used by Troesch *et al* (2011) were not comparable which decreased the internal validity of the study (Parahoo, 2014). Rehman *et al* (1998), Proteggente *et al* (2001) and Colpo *et al* (2008) only investigated total AA.

This literature review highlighted some significant gaps in literature. No study investigated NTBI long term, NTBI and AA together in healthy adults, NTBI alongside a dietary analysis and both males and females in one study. Therefore, this provided a rationale to investigate the effects of long term oral iron supplementation on healthy males and females looking at NTBI and the role of AA as an antioxidant, with an additional dietary analysis. Carrying out this study will contribute to current literature, policy and practice surrounding the use, safety and dosage of oral iron supplementation. This can inform local and national policy surrounding the use of iron supplementation as over the counter medication and in healthy individuals who are not iron deficient. It may also provide insight into the potential use of AA as an antioxidant in the case of iron supplement use.

Aims, Objectives and Research Question

Aims:

- To investigate the effect of long-term oral iron supplementation on total plasma iron, non-transferrin bound iron and ascorbic acid
- To investigate any associations between total plasma iron, non-transferrin bound iron and ascorbic acid
- To explore the role of ascorbic acid as an antioxidant for iron related reactive oxygen species
- To analyse the association between diet and total plasma iron, non-transferrin bound iron and ascorbic acid

Objectives:

- To give healthy adults oral $FeSO_4$ supplementation on alternate days for twenty-eight days
- To collect data on participant's dietary intake using a food frequency questionnaire
- To obtain baseline and post supplementation blood samples for the analysis of iron related biomarkers, including non-transferrin bound iron and ascorbic acid
- To statistically analyse differences and associations between baseline and post supplementation biomarkers
- To interpret and critically discuss the results and their contribution to current literature, policy and practice

Research Question:

Does twenty-eight day oral $FeSO_4$ supplementation have an effect on total plasma iron and non-transferrin bound iron in healthy adults with various natural levels of ascorbic acid?

Methodology

Research Design and Methodological Considerations

This study adopted a quantitative approach with a prospective, pre-post experimental design using a single cohort of participants. A quantitative, experimental approach was taken as iron supplementation has not previously been studied for its effects on NTBI and associations with natural levels of AA. Experimental research is regarded as one of the optimum forms of research to inform evidence based practice (Parahoo, 2014). This project facilitated a quantitative approach on the assumption that the physiological data obtained can be objectively quantified for statistical analysis to determine relationships between variables (Parahoo, 2014). The quantitative experimental approached has been used and seen to be effective in producing results in previous studies regarding iron supplementation, NTBI and AA outlined previously in the literature review.

Despite randomised controlled trials being the gold standard within scientific research, the pre-post design was considered appropriate for the nature of the study as it can measure the degree of change from the long-term supplementation, satisfying the aims and objectives whilst providing feasibility within time and cost restraints (Parahoo, 2014). This approach has been described as deterministic when used with experimental studies, as it looks at cause and effect, therefore appropriate for the use with this particular project (Parahoo, 2014). The design enabled the participants to act as their own controls which maximised the potential number of participants for testing as well as increasing the internal validity due to minimisation of participant variables (Parahoo, 2014). In addition, the approach allowed for ethical reduction where the total number of participants involved was reduced (Parahoo, 2014). A criticism of the quantitative approach is the view that human phenomena cannot be quantified, and observations often lack the full picture, only taking into account what is being observed (Parahoo, 2014). Also, maturation effects or behaviour change between the baseline and post supplementation data can cause a change in the results unrelated to the intervention (Parahoo, 2014).

The independent variable was the consumption of oral iron supplementation and the dependent variables were the levels of blood biomarkers associated with iron. This

approach did not support randomisation and blinding which are useful tools in scientific research (Parahoo, 2014). The non-randomised and blinded approach mimicked the natural environment where healthy participants may knowingly choose to take iron supplementation as part of their every diet and lifestyle increasing ecological validity.

Participant Recruitment

Inclusion and Exclusion Criteria

The participant sample was carefully selected to ensure that variables were minimised. While this narrow selection was beneficial for demonstrating cause and effect in experimental design, the generalisability to the wider population was reduced (Parahoo, 2014). The inclusion and exclusion criteria outlined in Table 5 was strictly followed to increase reliability and replicability of the study. The criteria were based on that used by previous literature outlined in the literature review, as well as to reduce variables foreseen by the researchers.

Table 5 Participant Criteria

INCLUSION CRITERIA	EXCLUSION CRITERIA
Male and Females	Pregnancy or Lactation
18-55 Years Old	Anaemia or Low Iron Stores
Regular Menstrual Cycles	Acute or Chronic Illness
Acceptable BMI	Eating Disorders
Use of Contraceptives (if applicable and not necessary)	Use of Medication or Supplements (previous two weeks)
	Blood Donation (previous six months)

BMI – Body Mass Index

Healthy adults were chosen as no previous study supplemented healthy adults with iron and tested both NTBI and AA. In addition, abnormal menstrual cycles, abnormal BMI, anaemia, infection, acute and chronic illness and the use of some medications have been shown to be problematic confounders in previous studies, therefore excluded for this study (SACN, 2010). No previous study has investigated the effects of iron supplementation on NTBI in both males and females. Yet both sexes take iron

supplementation, exposing them to the potential damaging effects (Low *et al*, 2016). Therefore, both were included in this study. An age range of 18 to 55 was set in line with previous studies, to include only young to middle aged adults as iron status can fluctuate naturally with age (Geisser and Burckhardt, 2011; Stucchi *et al*, 2018). The use of contraceptives has been allowed in previous literature as they help to regulate women's menstrual cycles preventing increased iron loss (Halie *et al*, 2016). Pregnancy and lactation have been shown to decrease women's iron stores following the increased loss of iron, therefore the increased absorption of iron may have confounding effects on the results (Pena-Rosas *et al*, 2015). Eating disorders may result in a dietary deficiency of iron or AA which could increase the risk of iron deficiency anaemia (NICE, 2018). Lastly, individuals who have donated blood in the last six months were excluded due to the dramatic loss of iron stores which will result in the body increasing its iron absorption in response to the demand (Smith *et al*, 2014).

Recruitment Strategy

The sampling method to recruit participants involved a volunteer technique, with the use of advertisement within the setting of the university aimed at students, lecturers, and other members of the population working in and around the university. Volunteer sampling has been described as the weakest type of sampling as researchers have little control over the sample composition (Parahoo, 2014). However, this technique was chosen as it was feasible, inexpensive, could be done easily within time restraints and reached a broad range of individuals. When individuals showed interest in taking part, they were sent a Participant Information Sheet (Appendix 1) with the inclusion and exclusion criteria, which they were asked to look over before continuing. Participants were then invited to attend a baseline data collection day.

Sample Size

A power calculation using previous literature was attempted to determine the number of participants needed to calculate statistical significance. However, there was a lack of sufficient data to carry out the calculation as no previous study has tested the effects of twenty-eight day iron supplementation on NTBI. Instead an aim of twenty

participants was used based on feasibility and previous literature, two of which had adequate justifications for their sample size.

Data Collection

Data Collection Days

Participants attended a total of two morning data collection days. They attended the Clinical Assessment Laboratory at the university site in a fasted state, were briefed and asked if they had any questions before signing a Consent Form (Appendix 2). They each completed a FFQ (Appendix 3) and an information questionnaire (Appendix 4) whilst waiting to have their blood sampled.

The qualified and experienced phlebotomist used sterile needles and lithium heparin vacutainers to extract six millilitres of blood from each participant. The phlebotomist received 'Venepuncture Training' by S. Frost, R.G.N, R.S.C.N, DipN Cert, Ed on 02/07/2009 and passed their 'Venepuncture Practical Assessment' with R. Ellis, Sato – Phlebotomy Training Officer on 12/08/2009 and has been in regular practice since. Lithium heparin vacutainers are used commonly in clinical practice for the determination of constituents in plasma due to the anticoagulation factor and are acceptable to use for total AA measurement (Lykkesfeldt, 2012).

AA is instable if left unprocessed as it has a high tendency to oxidise (Lykkesfeldt, 2012). Therefore, blood samples were centrifuged at 5000g for ten minutes and plasma extracted less than twenty minutes following venepuncture. 200µl was then extracted and re-centrifuged at 20,000g for ten minutes at 4^{o}C. Samples were then stored at -80^{o}C for future use. All collected blood was processed to plasma on the day of collection. No whole blood or material classed a 'relevant human material' in the Human Tissue Act (2004) were stored. Samples were allowed to thaw naturally at room temperature before laboratory analysis.

Once samples were taken, participants were immediately offered breakfast which consisted of a glass of water alongside toast with a choice of olive spread, honey or strawberry jam. Participants were required to take 60mg $FeSO_4$ in tablet form on

alternate days of twenty-eight days. $FeSO_4$ is one of the most common forms or iron used as a supplement (SACN, 2010). It has been shown to produce a large increase in TPI concentrations following supplementation, which has been associated with NTBI, making it particularly useful for this study (Schumann *et al*, 2012a; Schumann *et al*, 2012b; Schumann *et al*, 2013; Brittenham *et al*, 2014).

Participants were then checked to ensure they did not feel unwell and given another opportunity to ask questions before they left, with encouragement to contact the researchers at any point should they need to. A 'check-up' email was sent to the participants midway through the study, which also detailed the requirements for the second data collection day and reminded them to bring with them their empty tablet strips so that compliance with the supplementation could be assessed.

The second data collection day ran similar to the first, with the absence of the completion of questionnaires. Samples took slightly longer to process on the first day compared to the second due to researchers becoming more comfortable with the process. Participants were debriefed before leaving the second data collection day and informed that results can be emailed to them should they wish. The researchers contact details were provided to the participants should they have any concerns following the project.

Outcome Measures

Quantitative blood plasma biomarkers and nutrient intakes have been used. These outcome measures are objective which provided increased validity and reliability (Parahoo, 2014). TPI is a measure of all iron contained in plasma and was used as a representation of iron status (Porter *et al*, 2016). Multiple studies in the literature review used TPI for at least one biomarker for iron status. To directly measure iron absorption from supplementation, TPI is a more useful biomarker than haemoglobin and ferritin which are used for diagnosis (NICE, 2018). In addition, the need to rule out inflammation or infection was not necessary with TPI, as it is with a biomarker such as ferritin (Khan *et al*, 2016).

TIBC a measure of the capacity of iron associated proteins to become saturated with iron (Zhou *et al*, 2014). TIBC is a commonly used measure of iron status (Nunes *et*

al, 2014) and has been used by previous studies in the literature review. TIBC can provide a better clinical understanding on the activity of iron and iron absorption from the diet (Zhou *et al*, 2014). A disadvantage of this biomarker when used alone is that it does not take into account which proteins are bound to the iron (Eslayed *et al*, 2016).

Transferrin saturation is the percentage of iron bound to transferrin in relation to total transferrin in plasma (Eslayed *et al*, 2016) and has been used by previous studies. Transferrin saturation can provide clarity on iron homeostasis but is only useful when used in conjunction with other biomarkers, such as TPI and TIBC (Eslayed *et al*, 2016). Transferrin saturation can be affected by physiological conditions such as infection and inflammation, which were ruled out in this study by extensive participant criteria (Eslayed *et al*, 2016).

NTBI is a measure of any iron within the blood that is 'unbound' or bound to smaller (low molecular weight) proteins and has been described as a unique but potential marker of iron metabolism (Ito *et al*, 2017). The exact chemical makeup of NTBI is yet to be known (Maas *et al*, 2013; De Swart *et al*, 2016). Due to this, there is a lack of confidence that laboratory assays are correctly identifying NTBI (Pfeiffer and Looker, 2017). However as NTBI was the focus of this study, the use as a biomarker was essential.

AA was measured in three forms, total, oxidised and reduced. Total AA is a measure of all AA present in a blood plasma sample and a good biomarker for vitamin C tissue levels (Pollard *et al*, 2003). Reduced AA is absorbed from food and is available in the body for use. Oxidised AA is the AA that has donated an electron via antioxidant activity (Lane and Richardson, 2014). Multiple measurements of AA have not been taken in previous studies concerning oral iron supplementation in healthy participants. Therefore, this study provided a clearer picture of AA activity not seen in previous literature (Lykkesfeldt, 2011).

Dietary analysis was taken as diet has an effect on iron absorption and oxidative stress (SACN, 2010). Specific dietary components were considered for analysis. These included iron and vitamin C intake, as these are direct mediators of iron

absorption (Lane and Richardson, 2014). Zinc, fibre and calcium were taken as they have been shown to reduce iron absorption (Olivares *et al*, 2013). The mechanism for calcium as an inhibitor is unknown, however thought to be due to calcium competing of binding to substances in the absorption pathway (SACN, 2010). Additional dietary antioxidants included were vitamin A, D and E as these are commonly known antioxidants in the body (Madhikarmi and Murthy, 2014). AA has been found to regenerate other antioxidants, in particular vitamin E which is important for protection of lipid membranes against oxidative stress (Lane and Richardson, 2014). Polyphenols have also been shown to reduce dietary iron absorption, however the FFQ did not measure polyphenol intake (SACN, 2010; Cercamondi *et al*, 2014).

Data Analysis

Laboratory Data Analysis

Table 6 shows a description of the laboratory assays used to measure the blood biomarkers which were the most recent tested and developed assays available for use within the limits of the university laboratory. All baseline and post supplementation samples were ran side by side to reduce variables and keep potential errors constant. Standards received the same treatment as biological samples to reduce variability. To reduce the risk of pipetting errors, the same pipettes were used throughout the analysis and two researchers worked together when working on a set of samples, visually ensuring all samples were identical.

Table 6 Laboratory Data Analysis

LABORATORY ASSAY	DESCRIPTION	REAGENTS
Ultraviolet Visible Spectrophotometry using the CamSpec M302 Ultraviolet Visible Spectrophotometer for **TPI** and **TIPC**	A measure of light wavelength in the ultraviolet and visible spectrum from a substance (NIST, 2018). Uses a wavelength of 593nm. The value obtained will be used to calculate TPI and TIBC values as follows: Plasma Specimen – (Blank x 200 Standard – Blank)	TPI - Trichloroacetic Acid and Thioglycollic Acid, Ferene in Sodium Acetate. TIBC - Ferric Chloride, Iron Saturating Solution, Magnesium Carbonate, Ferene in Sodium Acetate
	Transferrin saturation was calculated using as per the calculation by (Beilby *et al*, 1992) which is: (TPI x 100) / TIBC	
Ultraviolet Visible High-Performance Liquid Chromatography using the Dionex UVD170S Detector for **NTBI**	Separation of mixture through speed of travel. A nitrilotriacetic acid ligand forms a complex with NTBI. Larger proteins removed by centrifugation using Millipore Amicon ultra filters (Kime *et al*, 1996). Uses wavelength of 450nm (McMurry, 2015).	Phosphate Buffered Saline Nitrilotriacetic Acid Dihydropyran Iron Chromophore Ferric Iron
Electrochemical High-Performance Liquid Chromatography using the BAS LCD 40 Electrochemical Detector for **Total AA, Reduced AA** and **Oxidised AA**	Measurement using an electrical column, where substances undergo electrochemical oxidation or reduction reactions which creates a current which is measured (Mitton and Trevithick, 1994). AA values were calculated as follows: Curve Measure (mm) / (Curve Standard Thirty (mm) / 30)	Standard Ascorbate Metaphosphoric Acid and Ethylenediaminetetraacetic Acid Tris(2-carboxyethyl)phosphine

AA – Ascorbic Acid, NIST – National Institute of Standards and Technology, NTBI – Non-transferrin Bound Iron, TIBC - Total Iron Binding Capacity, TPI – Total Plasma Iron

Total Plasma Iron and Total Iron Binding Capacity

UV-Vis Spectrophotometry was used to measure TPI and TIBC following the method by (ICSH, 1990). This is a common method used in clinical laboratories to perform quantitative analysis due to its universal application (Vo, 2015) and was used multiple times by studies outlined in the literature review. It is a selective, accurate and reproducible method to measure TPI and TIBC (Elgailani and Alsakka, 2016).

Ferene was used for the chromogen for this assay as this has been shown to increase sensitivity by 50% (ICSH, 1990).

TPI was measured by adding 300µl plasma to 350µl protein precipitation solution and mixing thoroughly for precisely one minute. Samples were heated at 56ºC for 15 minutes in a dry water bath. Following this, samples were centrifuged at 1000g for five minutes. 400µl of the supernatant was extracted and 400µl of ferene was added. Samples were incubated for five minutes at room temperature before being read in the UV-Vis Spectrophotometer.

TIBC was measured by adding 350µl plasma to 350µl iron saturating solution and mixing thoroughly before standing for five minutes. 36mg of light magnesium carbonate was added and the solution was agitated frequently over a 60-minute period. The samples were then centrifuged at 1000g for five minutes before having the supernatant removed and being re-centrifuged for a further five minutes. The supernatant was removed again and read in the UV-Vis Spectrophotometer. Standards were used for this method with a strong r-value for both biomarkers (TPI - $y = 0.0054x - 0.007$, $R^2 = 0.92297$; TIBC - $y = 0.0055x - 0.0017$, $R^2 = 0.99779$).

NTBI

UV-Vis (High-Performance Liquid Chromatography) (HPLC) was used to measure NTBI following the method by Kime *et al* (1996). This method is the gold standard method for measuring NTBI, due to its sensitivity (Sasaki *et al*, 2011). The highly sensitive HPLC system has the potential to detect values as low as 0.02µmol/l with a good level of precision (Kime *et al*, 1996). The risk of contaminants was reduced with the use of non-metallic polyether-ethyl ketone tubing throughout the HPLC system (Sasaki *et al*, 2011; Ito *et al*, 2014).

NTBI was measured by extracting 150µl plasma to 15µl nitrilotriacetic acid and incubating the solution at room temperature for 20 minutes. 150 µl of phosphate buffered saline was added and the solution was mixed well before being placed in the millipore amicon ultra filters. The units were centrifuged at 13,000g for 30 minutes at 4ºC. 150µl of the ultra-filtrate was added to 15µl of dihydropyran iron chromophore and incubated at room temperature for five minutes before being

injected into the HPLC system. The blank sample had a very minimal value of 0.009 which suggests that there was minimal contamination from outside sources of iron. A strong standard suggests minimal error in measurement ($y = 0.0049x + 0.015$, $R^2 = 0.9959$).

Ascorbic Acid

Electrochemical HPLC was used to measure total and reduced AA, based on the method by Sato *et al* (2010). The method is fast, simple, selective and sensitive for use with biological samples (Sato *et al*, 2010). An advantage of this method is that the MPA/EDTA solution has been shown to stabilise AA (Sato *et al*, 2010). HPLC grade H_2O was used to decrease the risk of impurities contaminating the sample.

Plasma was added to metaphosphoric acid and ethylenediaminetetraacetic acid (MPA and EDTA) and centrifuged at 20,000g at 4^{o}C for ten minutes. The supernatant was removed and diluted with MPA and EDTA and inserted into the HPLC for measurement of reduced AA. A further aliquot of supernatant was added to tris(2-carboxyethyl)phosphine in MPA and EDTA, incubated at room temperature for twenty minutes before adding more MPA and EDTA and insertion into the HPLC for measurement of total AA.

Food Frequency Questionnaire

To analyse dietary intake, an FFQ (Appendix 3) was used similar to previous literature (Proteggente *et al*, 2001). FFQs can be used to assess micronutrients, such as iron and vitamin C, when longer dietary analysis tools are not available (Emmett, 2009). It was chosen specifically as it captured a variation of diet throughout time which other dietary analysis tools do not provide and allows a standardisation of responses (Medical Research Council, 2015). It was easy for participants to use and only one completed FFQ was required per participant, reducing participant burden (Medical Research Council, 2015). A disadvantage of this tool is that it is subject to response bias from social desirability which has been shown previously with the use of doubly labelled water (Rhee *et al*, 2015). The FFQ and its associated analysis software was used and validated by Bingham *et al* (1997).

Statistical Data Analysis

All data has been analysed using the Statistics Package for the Social Sciences. Data was tested for normality using the Shapiro-Wilk test, used previously by Troesch *et al* (2011) and Brittenham *et al* (2014) however due to the small sample size, most data was analysed using non-parametric tests. Baseline participant characteristics and data will be presented using median +/- range, mean (standard deviation (SD)) or percentage where appropriate. A paired t-test was used to test each variable for differences between baseline and post supplementation data. This test is simplistic but can provide precise data, as long as the assumptions of the test are upheld (McDonald, 2014). Spearman's Rank Correlation Coefficient was considered an appropriate test for a small sample (McDonald, 2014). A p-value of $p < 0.05$ was considered statistically significant.

Ethical Considerations

Approval

Ethical approval was obtained from the university's Ethics Committee of the Faculty of Health, Education and Society prior to the commencement of the project (Ref: 17/18-362, Appendix 5). All research was carried out in accordance with World Medical Association (2013) guidelines.

Consent

To ensure that informed consent was gained, a simple but extensive brief was given to the participants before data was collected. Informed consent is essential so that participants know the risks and benefits of the study before deciding whether to take part (Williamson and Whittaker, 2017). Briefing followed comprehensive criteria to ensure consistency between participants. This included:

- A brief explanation of the project, with reference to the Participant Information Sheet (Appendix 1)
- An explanation of the role of the participants and researchers
- Specific timings of when the iron supplementation should be consumed

- A reminder that the blood test may cause minimal pain and bruising afterwards
- A reminder that taking the iron supplementation has a risk of causing gastrointestinal problems
- A reminder that the participant has a right to withdraw at any time without giving a reason and that this would not affect their relationship with the researchers or the university.
- A request for the participants to bring back their tablet test strips and inform the researchers of any problems with taking the supplementation as this information could be valuable to the analysis of the study results
- An open opportunity to ask questions

All the participants were healthy independent adults who appeared to have capacity. Not blinding the participants had the ethical advantage of providing openness and honesty to the participants. However, participants were asked not to change their diet and lifestyle throughout the study as a result of the information they received from participating in the study (Parahoo, 2014).

Professional Issues

The researchers were familiar with many of the participants outside of the setting of the study due to the nature of the sampling technique. Actions were taken to ensure work on the project that could involve participant details were always carried out in a safe quiet place to ensure confidentiality. In addition, researchers never talked about participants in regard to the study to other participants or the public. All findings were discussed in an anonymised manner where coded data was used for confidentiality.

Participant Burden

Participant burden was minimised as much as possible. Participants were sent all information via email to read at their own convenience, including the questionnaires should they wish to bring to the sampling days to reduce their time at the laboratory. They were given staggered times to attend the lab to ensure they would not be waiting around longer than necessary. In addition, as participants were required to

attend the data collection days fasted for a minimum of eight hours, all sessions began at 9am so they could fast overnight, and a small breakfast was provided after venepuncture.

Data Protection and Confidentiality

At the time of blood sampling, the participant's names were coded confidentially by one of the researchers, the codes and names of the participants were kept in an encrypted pass-worded file on a pass-worded computer. All other participant information was kept on a separate computer in a different, safe location with only the identifying participant codes. In addition, hard copies of data were kept secure in a locked cabinet at the Clinical Assessment Laboratory. This was to ensure data protection and confidentiality were upheld and reduce researcher bias when analysing the results. Participant contact details were not passed on to anybody else. As per policy, data will be kept securely for ten years following the study completion by the university. The Data Protection Act (2018) was followed and upheld for the whole process.

Risks and Hazards

Both the University Codes of Practice for Control of Substances Hazardous to Health and the Control of Substances Hazardous to Health (COSHH) Regulation (2002) were followed rigorously. Waste and used whole blood fractions and derivatives were disposed of according to the university health and safety policies and the requirements of the Human Tissue Act (2004). Human tissue derived waste and contaminated equipment were bagged separately to other laboratory waste and disposed of through the local laboratory disposal route. A qualified first aider was available at all times in case of an adverse event.

NTBI has been associated with oxidative damage, posing a risk to the participants, however eight-week oral iron supplementation in anaemic women has shown no negative long-term effects in previous literature (King *et al*, 2008). Therefore, a twenty-eight day supplementation period was deemed safe for this project.

The iron supplementation tablets can increase the risk of constipation, gastrointestinal upset and discoloured faeces (Leonard *et al*, 2014). It is unethical to put participants at risk of harm (Parahoo, 2014). Therefore, supplementation was given every other day as it has been shown by evidence to reduce negative side effects without compromising iron absorption (Stoffel *et al*, 2017).

Venepuncture, a commonly used but invasive technique used to sample the blood also posed a minor risk to the participants, such as bruising, bleeding or infection (Assi and Baz, 2014). Venepuncture was performed by a qualified and experienced phlebotomist wearing a protective coat and disposable gloves, using sterile equipment to minimise infection risk. Bruising was minimised with the use of pressure at the venepuncture site for one minute before the application of an allergy-sensitive plaster. To reduce the risk of sharps injury to the phlebotomist or the participants, standard precautions were taken as per the risk assessment for the laboratory. No injuries or adverse events occurred during this study.

Results

Descriptive statistics have been used to demonstrate the baseline characteristics of the participants with median ± range used for non-parametric data and mean (standard deviation (SD)) used for parametric data. Inferential statistics have been used to explore differences and correlations between baseline and post supplementation outcome measures. The results are presented below in terms of the aims of the study:

- To investigate the effect of long-term oral iron supplementation on total plasma iron, non-transferrin bound iron and ascorbic acid
- To investigate any associations between total plasma iron, non-transferrin bound iron and ascorbic acid
- To explore the role of ascorbic acid as an antioxidant for iron related reactive oxygen species
- To analyse the association between diet and total plasma iron, non-transferrin bound iron and ascorbic acid

Participant Characteristics

Table 7 Characteristics of Participant Sample

CHARACTERISTICS	**PARTICIPANTS**		
	Baseline TPI (<30µmol/l)	**Baseline TPI (>30µmol/l)**	**All**
Age (y) (median ± range)	23.0 (21-26)	37.0 (22-52)	23.0 (21-52)
Height (cm) (median ± range)	167.5 (158-195)	178.0 (163-182)	177.0 (158-195)
Weight (kg) (median ± range)	68.8 (49-92)	75.0 (58-82.5)	69.7 (49-92)
BMI (median ± range)	23.9 (18.6-24.9)	23.1 (18.5-26)	23.7 (18.5-26)
Gender			
Male (n (%))	n = 4 (36)	n = 3 (27)	n = 7 (64)
Female (n (%))	n = 2 (18)	n = 2 (18)	n = 4 (36)
Previous Supplement Use (n (%))	n = 4 (36)	n = 3 (27)	n = 7 (64)
Occupation			
Student (n (%))	n = 5 (45)	n = 3 (27)	n = 8 (73)
Other Employment (n (%))	n = 1 (9)	n = 2 (18)	n = 3 (27)

BMI – Body Mass Index, TPI – Total Plasma Iron

Table 7 shows the baseline characteristics of the participants. The participants were analysed as a whole and in two groups for those who had TPI above 30µmol/l and those who had TPI below 30µmol/l. The reason for this was to see if there was any correlation in absorption of iron, its subsequent effect on NTBI and association with natural levels of AA in terms of baseline iron status. However only participants who had baseline levels of TPI below 30µmol/l had a statistically significant increase in post supplementation TPI ($p = 0.03$) (Table 9). The 30µmol/l cut off was based on the recommended SACN (2010) reference values for TPI, with healthy values from 10-30µmol/l. As five out of eleven participants had values well above the upper end of this reference, it was deemed appropriate to use this as a cut off value.

Originally, twenty participants were recruited for the project. Five dropped out before the commencement of the study due to time commitments, recent blood donation and suspicion of anaemia. Three participants were unable to continue with the project as venepuncture was unsuccessful. One participant withdrew from the project midway due to personal crisis. Eleven participants, seven males and four females, completed the project with an age range of 21-52 years old. The mean age of participants was 29.4y (males 29.4y and females 29.3y). The average height of participants was 174.1cm with a range of 158cm to 195cm. The average weight was 69.9kg and BMI 22.7, however females had an average BMI of 20.0 whilst males had an average of 24.2. One male participant was marginally overweight (BMI 26) but still considered to be healthy.

Seven participants had previously used supplements, however all were stopped a minimum of one week prior to the beginning of the project. Eight participants were students known to the university, from various courses, whilst three worked in general employment. Two participants stated they had low activity levels (less than one hour per week), two stated they had high activity levels (above three hours per week) and seven stated they had medium activity levels (one to three hours per week). Participant characteristics did not differ significantly between baseline TPI above and below 30µmol/l. Age was slightly higher (23.5 to 36.4y) in participants who had a higher baseline level of TPI, however this was not significant.

Blood Biomarkers

Table 8 Baseline and Post Intervention Blood Biomarkers (All Participants)

BIOMARKER	BASELINE (Day 0)	POST INTERVENTION (Day 28)
TPI (µmol/l) (mean (SD))	29.33 (14.55)	32.05 (11.53)
TIBC (µmol/l) (mean (SD))	58.51 (25.47)	50.20 (17.37)
Transferrin Saturation (%) (mean (SD))	59.37 (31.69)	70.49 (33.89)
NTBI (µmol/l) (median ± range)	0.52 (0-2.73)	0.26 (0-1.04)
Total AA (µmol/l) (mean (SD))	26.69 (3.12)	25.58 (6.25)
Reduced AA (µmol/l) (mean (SD))	0.88 (0.34)	1.00 (0.37)*
Oxidised AA (µmol/l) (mean (SD))	25.82 (3.17)	24.58 (6.53)

*Value significantly different than baseline ($p < 0.05$), AA – Ascorbic Acid, NTBI – Non-transferrin Bound Iron, SD – Standard Deviation, TIBC – Total Iron Binding Capacity, TPI – Total Plasma Iron

Table 8 shows the baseline and post intervention blood biomarkers for all participants. One participant had baseline TI levels below the recommended reference values by SACN (2010). Five participants had adequate baseline TI levels and five had TI levels above the higher threshold. All participants had total AA levels below the recommended reference values (<50µmol//) (European Food Safety Authority (EFSA), 2013). TPI increased slightly from baseline to post supplementation, however this increase was not statistically significant. TIBC reduced overall after supplementation. However, this was highly variable, with some participants having increased TIBC, some having decreased and some not changing. Transferrin saturation was higher than normal values (20-45%) (Elsayed *et al*, 2016) and increased non-significantly following supplementation, again this was highly variable. NTBI decreased non-significantly, and once again was highly variable.

Total AA remained approximately the same, with slightly more variability post supplementation than at baseline. Reduced AA increased significantly from baseline to post supplementation ($p = 0.03$). A visual representation of this can be seen in Figure 2, which includes standard deviation bars.

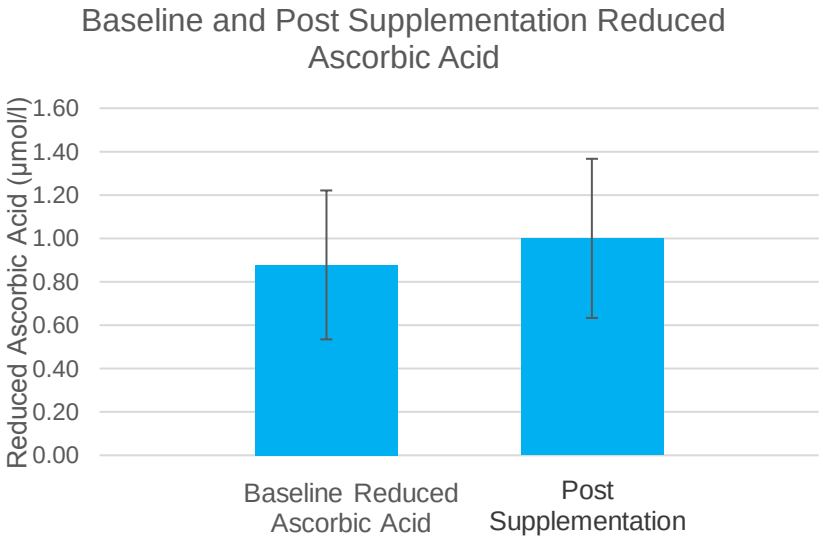


Figure 2 Baseline and Post Supplementation Reduced Ascorbic Acid

Lastly, oxidised AA remained approximately the same, once again with more variability in levels following supplementation. A visual representation of the changes in all measures of AA can be seen in Figure 3. This graph shows that in total, AA reduced non-significantly, however the proportion of reduced AA increased significantly and this can be seen against oxidised AA changes.

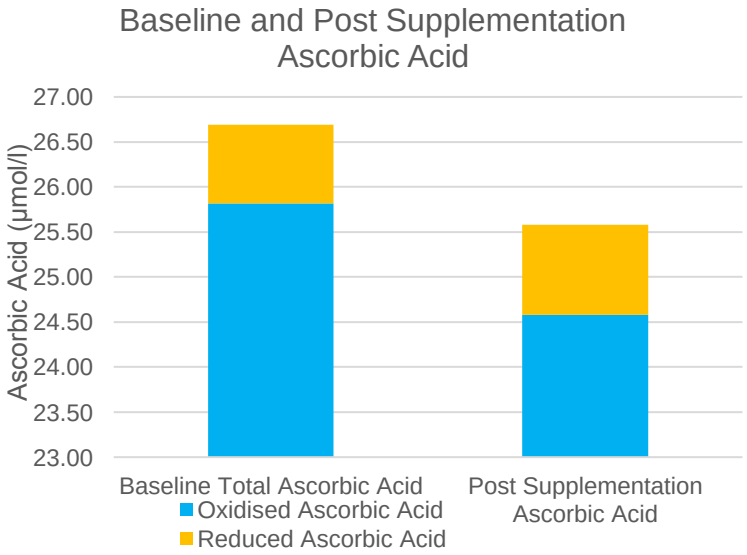


Figure 3 Baseline and Post Supplementation Ascorbic Acid

Table 9 Baseline and Post Intervention Blood Biomarkers (Separated by Plasma Total Iron)

BIOMARKER	BASELINE (Day 0)		POST INTERVENTION (Day 28)	
	Baseline TPI (<30µmol/l)	**Baseline TPI (>30µmol/l)**	**Baseline TPI (<30µmol/l)**	**Baseline TPI (>30µmol/l)**
TPI (µmol/l) (mean (SD))	19.0 (9.5)	41.8 (7.9)	30.1 (13.0)*	34.4 (10.5)
TIBC (µmol/l) (mean (SD))	57.4 (24.5)	59.8 (29.5)	56.6 (10.2)	42.5 (22.1)
Transferrin Saturation (%) (mean (SD))	44.0 (31.8)	77.8 (21.6)	51.8 (20.6)	92.9 (34.4)
NTBI (µmol/l) (mean (SD))	1.02 (1.02)	0.36 (0.38)	0.26 (0.36)	0.39 (0.41)
Total AA (µmol/l) (mean (SD))	27.7 (3.4)	25.5 (2.5)	24.2 (6.5)	27.3 (6.2)
Reduced AA (µmol/l) (mean (SD))	0.89 (0.38)	0.86 (0.34)	1.01 (0.42)	0.99 (0.34)
Oxidised AA (µmol/l) (mean (SD))	26.8 (3.4)	24.6 (2.7)	23.2 (6.7)	26.3 (6.6)

*Value significantly different than baseline ($p < 0.05$), AA – Ascorbic Acid, NTBI – Non-transferrin Bound Iron, SD – Standard Deviation, TIBC – Total Iron Binding Capacity, TPI – Total Plasma Iron

Table 9 shows the baseline and post supplementation blood biomarkers separated by TPI above and below 30µmol/l. Participants with lower mean baseline levels of TPI (19.0µmol/l), had a statistically significant increase in TPI following supplementation ($p = 0.03$) compared to participants who had higher mean baseline levels (41.8µmol/l) whose TPI levels did not increase following supplementation ($p = 0.13$). This is shown in Figure 4 below. In addition, TIBC did not change following supplementation in participants with lower levels of baseline TPI, however decreased non-significantly in participants with higher baseline TPI (51.8 to 42.5µmol/l). NTBI was higher at baseline in people with lower baseline TPI levels (1.02µmol/l) than with people with higher baseline TPI levels (0.36µmol/l), however this result was not significantly different. NTBI levels decreased non-significantly in these participants following supplementation. None of the AA biomarkers appeared to be different between both groups.

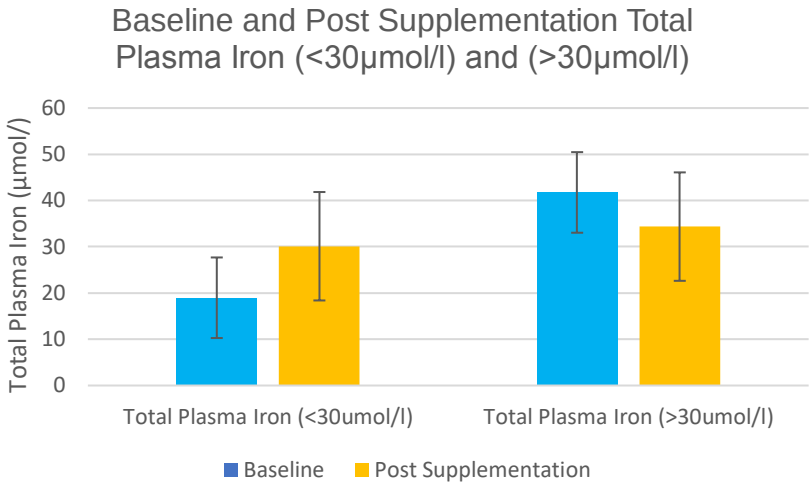


Figure 4 Baseline and Post Supplementation Total Plasma Iron (<30µmol/l) and (>30µmol/l)

Nutrient Intake

Table 10 Nutrient Intake from Food Frequency Questionnaire

NUTRIENT	PARTICIPANT INTAKE (estimates per day)		
	Baseline TPI (<30µmol/l)	**Baseline TPI (>30µmol/l)**	**All**
Iron (mg) (mean (SD))	9.7 (4.0)	10.6 (4.3)	10.1 (4.0)
Vitamin A (µg) (mean (SD))	871.3 (665.0)	819.4 (486.5)	847.7 (562.6)
Vitamin C (mg) (mean (SD))	101.0 (64.5)	116.2 (65.5)	108.0 (62.1)
Vitamin D (µg) (mean (SD))	2.0 (1.3)	3.0 (2.4)	2.5 (1.8)
Vitamin E (mg) (mean (SD))	10.1 (3.9)	10.3 (4.4)	10.2 (3.9)
Zinc (mg) (mean (SD))	8.2 (3.8)	8.9 (2.7)	8.5 (3.2)
Calcium (mg) (mean (SD))	1021.1 (472.7)	1019.0 (387.5)	1020.1 (414.5)
Fibre (g) (mean (SD))	14.3 (6.7)	17.3 (8.9)	15.7 (7.6)

SD – Standard Deviation, TPI – Total Plasma Iron

Table 10 shows the participant nutrient intakes per day. Results from the dietary analysis show that on average, participants consumed 10.1mg iron per day which is acceptable according to (Department of Health, 1991) for males, however females should be consuming 14.8mg per day. Vitamin C intake was 108.0mg per day on average, with the recommended consumption at 40mg (Department of Health, 1991). Alternatively, vitamin D intake was 2.5µg per day. No participant was achieving the recommended dietary intake of 10µg per day (SACN, 2016). Vitamin A, vitamin E, zinc and calcium intake were adequate for this age group (Department of Health, 1991). Fibre intake was 15.7g whereas the updated recommendation by SACN (2015) is 30g. Nutrient intakes did not differ significantly between participants who had baseline TPI below and above 30µmol/l. However, iron and vitamin C intake was slightly higher in the group who had higher baseline TPI levels.

Correlations

Blood Biomarkers

Baseline TIBC had a statistically significant negative correlation with baseline transferrin saturation, total AA and oxidised AA ($p = 0.01$, $r = -0.75$; $p = 0.02$, $r = -0.71$; $p = 0.02$, $r = -0.67$). Post supplementation TPI had a statistically significant positive correlation with post supplementation TIBC ($p = 0.01$, $r = 0.74$).

Blood Biomarkers and Nutrient Intakes

Baseline and post supplementation TPI and NTBI did not correlate significantly with any of the nutrient intakes. Baseline reduced AA had a statistically significant negative correlation with iron intake, vitamin C intake and fibre intake ($p = 0.02$, $r = -0.68$; $p = 0.04$, $r = -0.63$; $p = 0.01$, $r = -0.73$).

Nutrient Intakes

Table 11 Correlations of Nutrient Intakes

NUTRIENT	Fibre (g)	Calcium (mg)	Zinc (mg)	Vitamin E (mg)	Vitamin D (µg)	Vitamin C (mg)	Vitamin A (µg)
Iron (mg) (p-value, r-value)	C (0.003, 0.80)	C (0.001, 0.86)	C (0.005, 0.77)	C (0.00, 0.90)	C (0.003, 0.80)	C (0.01, 0.73)	C (0.004, 0.79)
Vitamin A (µg) (p-value, r-value)	C (0.04, 0.64)	C (0.002, 0.82)	-	-	-	C (0.02, 0.69)	
Vitamin C (mg) (p-value, r-value)	C (0.00, 0.93)	-	-	C (0.02, 0.68)	-		
Vitamin D (µg) (p-value, r-value)	-	C (0.02, 0.71)	C (0.003, 0.80)	C (0.002, 0.83)			
Vitamin E (mg) (p-value, r-value)	C (0.003, 0.80)	C (0.16, 0.70)	C (0.01, 0.75)				
Zinc (mg) (p-value, r-value)	-	C (0.01. 0.74)					
Calcium (mg) (p-value, r-value)	-						

C = statistically significant correlation ($p < 0.05$)

Table 11 shows the correlations between nutrient intakes with their respective p-values and r-values. Iron intake correlated positively with all other nutrient intakes ($p < 0.05$).

Results Summary

Participant characteristics did not differ significantly between groups who had baseline TPI above and below 30µmol/l. All participants had total AA levels below the recommended reference values (EFSA, 2013). Mean reduced AA increased significantly from baseline to post supplementation (0.88-1.00µmol/l, $p = 0.03$) in the cohort as whole. Mean TPI did not increase significantly in the whole cohort following supplementation, but increased significantly in the group with lower baseline TPI levels (19.0-41.8µmol/l, $p = 0.03$). There were no significant changes in other blood biomarkers.

For the whole cohort, men were achieving the recommended iron intake per day but not women. Vitamin C intake per day was higher than recommended whereas vitamin D intake was lower than recommended. Baseline TIBC had a statistically significant negative correlation with total AA and oxidised AA ($p = 0.02$, $r = -0.71$; $p = 0.02$, $r = -0.67$). Baseline reduced AA had a statistically significant negative correlation with iron intake and vitamin C intake ($p = 0.02$, $r = -0.68$; $p = 0.04$, $r = -0.63$). Multiple nutrients correlated with each other, as seen in Table 11. Iron intake correlated positively with all other nutrient intakes ($P < 0.05$).

Discussion

The main results summarised at the end of the previous section will be discussed in terms of the aims of the study with respect to the research question. The discussion will consider the results in light of biological and biochemical theory and previous literature. It will go on to discuss this in terms of limitations and strengths of the study, contribution to current policy and practice and discuss questions posed for future research. The aims of this study were:

- To investigate the effect of long-term oral iron supplementation on total plasma iron, non-transferrin bound iron and ascorbic acid
- To investigate any associations between total plasma iron, non-transferrin bound iron and ascorbic acid
- To explore the role of ascorbic acid as an antioxidant for iron related reactive oxygen species
- To analyse the association between diet and total plasma iron, non-transferrin bound iron and ascorbic acid

Research Question:

Does twenty-eight day oral $FeSO_4$ supplementation have an effect on total plasma iron and non-transferrin bound iron in healthy adults with various natural levels of ascorbic acid?

Biological and Biochemical Theory in Relation to Previous Literature

Effect of Oral Iron Supplementation

The first aim of the study was to investigate the effect of long-term oral iron supplementation on TPI, NTBI and AA. The study found that mean TPI did not increase significantly in the whole cohort following supplementation, but increased significantly in the cohort with lower baseline TPI levels (<30µmol/l) (19.0-41.8µmol/l, $p = 0.03$). SACN (2010) recommends that a TPI level between 10-30µmol/l is indicative of a good iron status. Iron absorption is tightly controlled by hepcidin and ferroportin in response to systemic iron status (Bhagavan, 2015). Subsequently, it is unlikely that an observable amount of iron will be absorbed following

supplementation in healthy individuals who have an adequate iron status as hepcidin is likely degrading ferroportin (Bhagavan, 2015). This could explain the significant increase in TPI in participants who had baseline TPI below 30µmol/l but not in participants who had baseline TPI above 30µmol/l.

Another reason for lack of significant increase in TPI could be due to enterocytes developing a mucosal block to prevent the absorption of excess iron following a large intake of dietary iron, in this case from the oral supplementation (SACN, 2010). Previous literature found that healthy adults who had adequate TPI levels had a significant increase in TPI following $FeSO_4$ supplementation (Troesch *et al*, 2011; Schumann *et al*, 2012a; Schumann *et al*, 2012b; Schumann *et al*, 2013; Brittenham *et al*, 2014). Brittenham *et al* (2014) found a larger TPI increase in participants who had slightly reduced baseline TPI in line with the results in this study.

The study found no change in NTBI from baseline to post supplementation. At face value, this suggests that NTBI was not produced as a result of long-term oral iron supplementation. A previous short-term study found that NTBI increased significantly in the eight hours following ingestion of $FeSO_4$ and reached its peak at four hours (Brittenham *et al*, 2014). In this study, hepcidin increased significantly from four to eight hours post supplementation as NTBI decreased, which is suggestive that hepcidin is taking up NTBI from blood to the liver (Lane and Richardson, 2014). This gives rise to the possibility that NTBI is only circulating the body for a short-term period following supplement ingestion, which would give an explanation as to why no change was found in this study, with venepuncture sample taken over twenty-four hours following the last $FeSO_4$ ingestion. Therefore, this study postulates that NTBI is transient. This finding warrants further investigation as it is a new phenomenon not found in previous literature.

Participants had mean total AA level (26.69µmol/l) below the recommended reference values indicative of an inadequate vitamin C status (EFSA, 2013). A lack of vitamin C in the whole cohort could be an explanation for the minimal increase in TPI following supplementation. A low total AA level may result in a reduced non-haem iron absorption through the lack of reduction of Fe^{3+} to Fe^{2+} preventing iron from being taken up by enterocytes (Lane and Richardson, 2014). In addition, low

AA levels may reduce the possibility of AA binding to iron to create a complex that is more soluble for absorption (Lane and Richardson, 2014). In comparison to previous literature, participants had ranges of approximately 50-70µmol/l (Rehman *et al*, 1998; Proteggente *et al*, 2001) and approximately 10µmol/l (Colpo *et al*, 2008). This shows that despite finding low values for this study, previously literature has a wide range of acceptable values. It must be noted that Colpo *et al* (2008) used a different laboratory assay to Rehman *et al* (1998) and Proteggente *et al* (2001). Therefore, lower values could be attributed to an underestimation from the analysis technique.

TPI only appears to increase in participants who have low to adequate baseline levels of TPI and increases are small in comparison to the 60mg dosage. In addition, NTBI appears to only be circulating the blood short-term following supplementation. This is suggestive that any risks of oxidative damage from oral iron supplementation are associated short-term in healthy individuals who have a low-adequate iron status. Should these individuals feel the need to take oral iron supplementation, a suggestion from this study is that they should be pre-assessed to ensure they are not undergoing any unnecessary risks and to ensure that any symptoms they have are not associated with another underlying disease (NICE, 2018). In addition, risks could be reduced by a reduction in common dosage from 60mg, as used in this study, to 17mg as recommended by SACN (2010).

Individuals who have a high iron status are unlikely to be affected by the damaging effects of NTBI, however these participants still have the opportunity to buy this supplementation over the counter, the use of which is unnecessary. As shown previously, the use of iron supplementation can result in many negative gastrointestinal side effects including associations with colorectal cancer, likely due to the exposure of the gut to iron associated reactive oxygen species (Leonard *et al*, 2014). This gives a rationale for the reduction dosage in common oral iron supplementation and a revision of policy that allows oral iron supplementation to be sold over the counter without previous assessment of iron status.

Associations between Blood Biomarkers

A second aim of the study was to investigate any associations between TPI, NTBI and AA. The study found that baseline TIBC had a statistically significant negative correlation with total AA and oxidised AA ($p = 0.02$, $r = -0.71$; $p = 0.02$, $r = -0.67$) but no other correlations between blood biomarkers. TIBC increases following transferrin synthesis and release from the liver in response to an increased systemic need for iron (Bhagavan, 2015). This suggests that as total AA is lower, absorption of dietary iron is less, resulting in a higher systemic need for iron and subsequently an increase in TIBC. It is common knowledge that AA assists with non-haem iron absorption (Lane and Richardson, 2014). Nonetheless, this provides confidence that ascorbic acid is playing an important role in iron absorption despite the minimal increase in TPI following supplementation in this study. No previous literature measured TIBC alongside AA.

Antioxidant Activity of Ascorbic Acid

The third aim of the study was to explore the role of AA as an antioxidant for iron related reactive oxygen species. The study found that mean reduced AA increased significantly from baseline to post supplementation (0.88-1.00μmol/l, $p = 0.03$) in the cohort as whole. However total and oxidised AA did not change. No change in total AA provides confidence that participants did not change their diet throughout the study and is in line with previous literature (Proteggente *et al* 2001, Colpo *et al*, 2008).

No change in oxidised AA overall reduces the likelihood that AA was acting as an antioxidant as AA becomes oxidised following its donation of an electron in antioxidant activity (Lane and Richardson, 2014). However, the significant increase in reduced AA puts doubt in this theory. The study postulates that reduced AA increased significantly as a result of increased AA recycling following antioxidant activity, possibility related to the production of NTBI (Lane and Richardson, 2014). Previous literature states that oxidised AA is rapidly converted back to reduced AA and maintained in this form within intracellular and extracellular fluid (Lane and Richardson, 2014). As previous literature did not measure reduced AA, this is the

first study of its kind to receive this finding. This unusual finding poses questions for further investigation into this topic.

The negative correlation between TIBC and oxidised AA is suggestive that a high oxidised AA results from high systemic iron (a low TIBC and transferrin synthesis is being inhibited) as AA is becoming oxidised from iron related reactive oxygen species, such as NTBI (Lane and Richardson, 2014). This proposal is supported by previous studies who found that participants who had higher baseline total AA had higher transferrin bound iron. These studies also found that participants with higher baseline total AA had lower levels of TBD as AA was likely working as an antioxidant (theoretically becoming oxidised should this biomarker have been measured) (Rehman *et al*, 1998; Proteggente *et al*, 2001).

Overall it still remains unclear as to the activity of AA as an antioxidant in relation to iron related reactive oxygen species due to minimal increase in TPI, no change in NTBI and subsequently no possible correlation with NTBI and AA post supplementation. The finding that reduced AA increase significantly is unusual and warrants further investigation. Previous literature has suggested that AA is unlikely to be working as a pro-oxidant. The evidence here points towards AA working as an antioxidant but the findings are speculative and warrant further investigation.

Associations between Blood Biomarkers and Dietary Intake

The last aim of the study was to analyse the association between diet and TPI, NTBI and AA. The study found that men were achieving the recommended iron intake per day but not women. Women were receiving 10.9mg dietary iron on average, whereas the recommendation for their age group is 14.8mg per day (Department of Health, 1991). The implications for this are that women were at risk of iron deficiency anaemia, however this did not appear to be the case when considering baseline TPI. All females had TPI above 10µmol/l and two females had TPI above 30µmol/l. The dietary intakes were in line with results from previous studies (Rehman *et al*, 1998; Proteggente *et al*, 2001).

In addition, mean vitamin C intake per day was higher than recommended (108.0mg/day) yet this was not reflected in the total AA levels which appeared to be

low. Pollard et al (2003) suggests that this may be due to many physiological processes that vitamin C is involved in, causing it to appear uncorrelated. Previous studies also found high intakes of vitamin C (29-290mg and 24-131mg) (Rehman et al, 1998; Proteggente et al, 2001). It was suggested previously that the low levels of total AA were the cause of the minimal increase in TPI, however the high dietary intake of vitamin C counteracts this theory, promoting the conclusion that a minimal increase was seen post supplementation simply due to the participants having an adequate baseline iron status.

There was no correlation between TPI and iron intake. This is unsurprising as the relationship between iron status and iron intake can be affected by age, metabolic responses, genetics and menstrual losses (Bhagavan, 2015). As well as this, as iron is tightly controlled in the body, an individual with adequate iron stores is unlikely to absorb much iron from the diet, even when dietary iron intake is high, thus iron status does not always reflect intake in its entirety, possibly accounting for the lack of correlation (SACN, 2010; NDNS, 2018).

Baseline reduced AA had a statistically significant negative correlation with iron intake and vitamin C intake ($p = 0.02$, $r = -0.68$; $p = 0.04$, $r = -0.63$). When dietary iron and vitamin C are high, iron status is also likely to be high. This suggests that the higher dietary iron intake and vitamin C reduced AA may be lower as it is acting to reduce iron for cellular uptake and/or donating electrons following a subsequent rise in NTBI. Thus becoming oxidised (Lane and Richardson, 2014). This is suggesting that not only is AA increasing absorption but it is then acting against the reactive oxygen species subsequently created. This is only a speculation on the complex physiology interplay of AA that is still under investigation (Lane and Richardson, 2014). Overall AA may be working simultaneously as a modulator of iron absorption but also as an antioxidant, ultimately in a protective manor.

Multiple nutrients correlated with each other, as seen in Table 11. Iron intake correlated positively with all other nutrient intakes ($P < 0.05$). This meant that the higher iron intake, equally high were inhibitors of iron absorption, such as fibre, zinc and calcium. Despite this, mean baseline TPI (29.33µmol/l) appeared to be adequate in participants (SACN, 2010) suggesting that dietary inhibitors were not

affecting iron absorption and subsequently the results. Previous literature did not measure intake of modulators of iron absorption.

Vitamin D intake was lower than recommended (2.5µg/day), however this does not take into account vitamin D synthesised by the sun (SACN, 2016). This low intake provides confidence that vitamin D was not acting as an antioxidant in place of vitamin C. Iron intake correlated with vitamins A and E, suggesting that these could have been acting as antioxidants alongside vitamin C. This suggestion is supported by Madhikarmi and Murthy (2014) who found a significant decrease in lipid peroxidation following supplementation of vitamins A, C and E. No study in the literature review measured intakes of other dietary antioxidants. This finding needs to be investigated further.

Despite dietary intake of iron being low for women, iron status appeared to be adequate in participants. The high vitamin C intake was suggestive that minimal increase in TPI following supplementation was not due to low total AA levels, but due to participants having an adequate baseline systemic iron. The statistically significant negative correlation of baseline reduced AA and iron and vitamin C intake is suggestive that AA may be working simultaneously as a modulator of iron absorption but also as an antioxidant, ultimately in a protective manor. Iron intake was shown to correlate with its inhibitors of absorption, however this is unlikely to have majorly affected absorption due to participants having adequate iron status. Lastly, vitamins A and E may be working alongside vitamin C as an antioxidant due their correlation with iron intake. This needs to be investigated further.

Strengths and Limitations of Study

Limitations

The research design did not account for maturation effects or behaviour change. Participants were asked not to change their diet or lifestyle in any way, however the possibility of this cannot be ruled out and must be considered in the results. Possible actions that participants may have taken were to decrease their dietary iron intake as a result of learning about its associated risks. This is unlikely, but could have reduced the overall measure of TPI post supplementation and have resulted in the minimal increase seen baseline to post supplementation. In addition, the study relied heavily on compliance of the participants to take the supplementation. Participants consumed all supplementation to the researcher's knowledge.

The external validity of this design is compromised due to the small sample size (Parahoo, 2014). A larger sample size may have been more beneficial to detect changes and stronger correlations between biomarkers (SACN, 2010). The use of the volunteer technique could have resulted in a biased sample of participants, thus reducing the generalisability to the wider population (Parahoo, 2014).

The use of healthy individuals was purposeful for this project, as no previous research has investigated the effect of oral iron supplementation on NTBI and its association with natural levels of AA in this population. However, it can be seen as a limiting factor as the absorption of supplemented iron by individuals with adequate iron status was likely smaller than individuals with low iron status (SACN, 2010). A large age range reduced the generalisability of the results to a specific age group.

Blood samples took longer to process on the first data collection day in comparison to the second, this could have resulted in changes in AA from baseline to post supplementation due to the instability of AA and its tendency to oxidise following venepuncture. This could explain the statistically significant increase in reduced AA following supplementation, as baseline AA was left longer some reduced AA may have oxidised following venepuncture.

It appears that the participants included in this study had suboptimal levels of total AA. However dietary intakes show that mean vitamin C intake (108.0mg/day) was above the recommendations by the Department of Health (1991). Previous literature has shown fluctuations in total AA measurement depending on the laboratory assay used (Rehman *et al*, 1998; Proteggente *et al*, 2001; Colpo *et al*, 2008). The laboratory assay used in this study could have underestimated the measurement of total AA giving an explanation for the unusual low levels in all participants. This underestimation could be due to the electrode used in this assay drifting over time, which is common and requires more frequent calibration with standards (Mitton and Trevithick, 1994). The FFQ used was not specifically designed to measure iron intake which is a limitation in many studies highlighted by SACN (2010). In addition, the FFQ did not measure polyphenols which have been shown to influence dietary iron absorption (Cercamondi *et al*, 2014).

Strengths

The strict adhesion to participant criteria ensured the reduction of confounding factors and provided confidence that the results were without participant bias, giving the results increased validity and improving the replicability of this study. The lower number of participant allowed more attention to detail regarding the participants individual characteristics (Parahoo, 2014). As the first study considering NTBI long term, a power calculation was not possible, however data provided by this study can be used to inform future studies.

A strength of the study was the use of three forms of AA biomarker. Multiple forms of AA provide a clearer picture of AA antioxidant activity and this has not been seen in previous literature regarding oral iron supplementation. Furthermore, the dietary analysis was not previously carried out in studies looking at the effects of oral iron supplementation and NTBI. In addition, studies that looked at oral iron supplementation and AA only considered iron and vitamin C intake, despite there being many other modulators of iron. No study outlined in the literature review considered the influence of other antioxidants and iron.

Another strength of this study was the use of more up to date laboratory techniques to measure NTBI and AA. The techniques used in the assay were not used in pervious literature studying healthy adults outlined in the literature review. This implies that results may be more accurate and precise that previous studies. A major strength of this study is that it addresses multiple gaps in literature previously identified. These will be discussed below in terms of the study's contribution to literature, policy and practice.

Contribution of Study to Literature, Policy and Practice

This study contributes to current literature surrounding the topic of oral iron supplementation and NTBI production. Previous to this study, there was no literature investigating the effects of oral iron supplementation on NTBI with consideration of AA. In addition, there was no literature investigating the effects of long term oral iron supplementation in healthy individuals with the use of a dietary analysis.

The small increase in TPI following 60mg $FeSO_4$ supplementation as well as its associated negative side effects suggest that the dosage should be lowered in line with recommendations by SACN (2010). The study suggests that risks of oxidative damage from oral iron supplementation are associated short-term in healthy individuals who have a low-adequate iron status. Due to these risks, a medical assessment could be beneficial before oral iron supplementation is taken to reduce the possibility of individuals causing themselves unnecessary harm. Furthermore, this may help to rule out health problems with similar symptoms to iron deficiency anaemia. This medical assessment can be in conjunction with a revised policy of the availability of oral iron as an over the counter medication. The combination of previous literature, the significant increase in reduced AA, the correlation with total and oxidised AA and TIBC and the correlation of baseline reduced AA and iron and vitamin C intake are suggestive of AA antioxidant activity with an overall protective behaviour. Vitamins A and E may also be working as antioxidants. These findings contribute to literature but it is stressed that further investigation needs to be carried out on this subject.

This knowledge can help to improve policy and practice surrounding risks and use of oral iron supplementation as a treatment of suspected iron deficiency anaemia. Policies such as SACN (2010) could be updated to include new found knowledge and this could work in harmony with clinical practice guidelines such as NICE (2018). Suggestions for dosage, over the counter availability and medical assessment have been made. Before this knowledge can be translated into policy and subsequently practice, further investigations are required. These are discussed below.

Future Research

It is common that experimental research opens up more questions than answers them (Parahoo, 2014). This project is the first of its kind to look at these particular variables and it can provide a valuable insight for future research (Parahoo, 2014). Future research could study healthy participants with lower baseline levels of TPI to ensure absorption is seen. To improve upon the limitations of this study, future studies should aim for a larger sample size and utilise a FFQ that is specifically designed and validated for the use with iron supplementation and its associated modulators. The finding that NTBI may be transient warrants further research to find out exactly how long NTBI circulates the blood and how long the resulting oxidative damage is present. In addition, the significant increase in reduced AA was an unusual finding and warrants further research. The theory that AA is working as an antioxidant against iron related reactive oxygen species remains mostly unanswered. As NTBI was not detected, it was unable to be correlated with AA. Consequently this should be further investigated. The potential activity of vitamins A and E as antioxidants against iron related reactive oxygen species is a rationale for further research on not only vitamin C but multiple known antioxidants.

Conclusion

Does twenty-eight day oral $FeSO_4$ supplementation have an effect on total plasma iron and non-transferrin bound iron in healthy adults with various natural levels of ascorbic acid?

In conclusion, this study found that TPI only appears to increase in participants who have lower baseline TPI levels due to adequate systemic iron status reducing the need for absorption. NTBI appears to be transient, only being produced in circulating blood short-term following ingestion of oral iron supplementation. Study findings are suggestive of the activity of AA as a protective antioxidant but further investigation is necessary before conclusions can be drawn. Dietary analysis provided valuable information towards the results of this study and highlighted the possibility of vitamins A and E also working as an antioxidant against iron related reactive oxygen species, such as NTBI.

Carrying out this study has contributed to current knowledge surrounding the risks of oral iron supplementation and questioned its dosage and availability as an over the counter medication, with a suggestion that it only be taken following medical assessment. The use of AA as an antioxidant in relation to iron related reactive oxygen species remains unclear. Findings are speculative but promising and contribute to current literature. Multiple recommendations for future research were made, including the use of participants with lower baseline TPI values, a study design that allows transient NTBI to be detected and further investigation of the antioxidant activity AA.

Appendices

Appendix 1 - Participant Information Sheet

Appendix 2 – Consent Form

Appendix 3 – Food Frequency Questionnaire

Appendix 4 – Participant Questionnaire

Appendix 5 – Ethical Approval

Appendix 1 Participant Information Sheet

PARTICIPANT INFORMATION SHEET
STUDY TITLE: The effect of $FeSO_4$ iron supplementation on lipid peroxidation and redox active plasma iron in humans.

Invitation to participate
We would like to invite you to participate in a new research study. Before you decide whether or not to participate, it is important for you to understand why the research is being done and what it will involve. This information sheet explains the background and aims of the study. Please take time to read it carefully and discuss it with others if you wish. If there is anything that is unclear, or if you would like more information, please ask us. Your participation in this study is entirely voluntary.

What is the overall aim of the study?
To investigate the relationship between iron supplementation and levels of non-transferrin bound iron (NTBI) in the blood and natural levels of vitamin C. NTBI is a type of free iron and has been shown to cause damage in the body and vitamin C has been shown to work as an antioxidant, potentially counteracting this damage. Blood will be measured for iron in three forms, NTBI, vitamin C and malondialdehyde (MDA) which is a product of this damage.

What would I have to do if I took part in this study?
If you decide to take part you will be required to provide 4ml of venous blood, conducted by an experienced sympathetic phlebotomist. You will also be required to fill in a three-day food frequency questionnaire prior to arrival and a short question sheet on the day.

Participant Inclusion and Exclusion Criteria

Inclusion:

- Male or female
- 18-55 Years Old
- Regular Menstrual Cycles (if applicable)
- BMI in healthy range
- Contraceptives accepted (if applicable)

Exclusion:

- Pregnancy or Lactation
- Anaemia or Low Iron Stores
- Acute or Chronic Illness
- Eating Disorders

- Use of Medication or Supplements (previous two weeks)
- Blood Donation (previous six months)

Will any expenses be paid?
We are unable to pay expenses for this project. However, breakfast will be provided after the collection of blood samples.

Do I have to take part?
No. It is entirely up to you whether or not to take part. If you decide to take part you may choose to withdraw at any time without giving any reason. If you decide not to take part your usual healthcare will not be affected in any way.
If you decide to take part you will be asked to sign a consent form.

Will my records be confidential?
All information collected about you during the course of this research will be kept strictly confidential. All information will be stored electronically on a computer which is password protected, in a document file that is also password protected. The data will be stored for a maximum of 10 years, after which it will be destroyed.
All information will be handled in compliance with the Data Protection Act (2018).
Your name and address (which we need in order to contact you) will be stored separately from the other information you supply during the project so that you cannot be identified from your study records.

What are the possible benefits of taking part in this study?
You will be able to contribute to research that will influence the control and the usage of iron supplements, to reduce the risk of oxidative stress in the healthy population.

What are the possible disadvantages of taking part in this study?
There are no known disadvantages of taking part in the study other than the risks below, inconvenience of giving a blood sample and the time taken to complete a food diary.

Risks:
Iron Supplementation:
Common: Constipation dark stools, abdominal pain (mild)
Rare: Nausea, vomiting, diarrhea,
Venipuncture:
Mild pain, bruising, bleeding, infection, dizziness, fainting

Who is organizing the study?
The research is being organized by the

Who has reviewed this research study?
The study has been reviewed by the Ethics Committee of the Faculty of Health, Education and Society.

What will happen to the data obtained from this study?
The results of the study will be written up in our dissertations with the potential for publishing in a journal.

How will I hear about the results of the study?
When the results of the whole study are completed, those who wish to find out how it went will be provided with a summary of the major findings and what they mean for future research and potential future treatment. This will be discussed with you when you attend the lab to provide your blood samples. We will make a note of all who would like a report and provide a summary of the major findings in layman's terms at the end of the study.

Your rights
Your participation in this study is entirely voluntary. You may withdraw at any time without it affecting your relationship with the. If you withdraw after we have taken blood from you, all data from your samples will be withdrawn from the study.

Contact for further information
If you require any further information about this study, or have any questions please contact either:

Maria-Anne Gaitanou
maria-anne.gaitanou@students.plymouth.ac.uk

Charlotte Woods
charlotte.m.woods@students.plymouth.ac.uk

If you have any concerns or complaints as to how the study was conducted, please contact:

Project supervisor:
Dr Desley White desley.white@plymouth.ac.uk

Appendix 2 Consent Form

HEALTH PROFESSIONS WITH PLYMOUTH UNIVERSITY

Title of project: The effect of $FeSO_4$ iron supplementation on lipid peroxidation and redox active plasma iron in humans.

Name of researchers:
Charlotte Woods

Please initial boxes below:

1. I confirm that I have read and understand the information sheet for the above study and that I am able to ask any questions. ☐

2. I confirm that I satisfy the participant inclusion and exclusion criteria in the Participation Information Sheet. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time. ☐

4. I understand and am aware that: ☐
   - 4 mL of my blood will be taken by a trained phlebotomist ☐
   - My blood will be used for research in the above study. ☐
   - Any information given E.g. Food diary, Personal information will be analyzed and used in the above study. ☐

5. I understand that all sample and data information will be recorded anonymously and will not be used for anything other than the above study. ☐

6. I agree to take part in the above study. ☐

Name of participant ……. Signature…….. Date……

Name of investigator…….. Signature……...Date……

Please estimate your average food use as best you can, and please answer every question - do not leave ANY lines blank. PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stew or slices									
Lamb: roast, chops or stew									
Chicken or other poultry eg. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, eg. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls									
Liver, liver paté, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, eg. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, eg. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, eg. crab, prawns, mussels									
Fish roe, taramasalata									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crispbread, eg. Ryvita									
CEREALS (one bowl)									
Porridge, Readybrek									
Breakfast cereal such as cornflakes, muesli etc.									
POTATOES, RICE AND PASTA (medium serving)									
Boiled, mashed, instant or jacket potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, eg. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DAIRY PRODUCTS AND FATS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, eg. Cheddar, Brie, Edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream(tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block margarine, eg. Stork, Krona (teaspoon)									
Polyunsaturated margarine (tub), eg. Flora, sunflower (teaspoon)									
Other soft margarine,dairy spreads (tub), eg. Blue Band, Clover (teaspoon)									
Low fat spread (tub), eg. Outline, Gold (teaspoon)									
Very low fat spread (tub) (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
SWEETS AND SNACKS (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate , eg. digestive (one)									
Sweet biscuits, plain, eg. Nice, ginger (one)									
Cakes eg. fruit, sponge, home baked									
Cakes eg. fruit, sponge, ready made									
Buns, pastries eg. scones, flapjacks, home baked									
Buns, pastries eg. croissants, doughnuts, ready made									
Fruit pies, tarts, crumbles, home baked									
Fruit pies, tarts, crumbles, ready made									
Sponge puddings, home baked									
Sponge puddings, ready made									
Milk puddings, eg. rice, custard, trifle									
Ice cream, choc ices									
Chocolates, single or squares									
Chocolate snack bars eg. Mars, Crunchie									
Sweets, toffees, mints									
Sugar added to tea, coffee, cereal (teaspoon)									
Crisps or other packet snacks, eg. Wotsits									
Peanuts or other nuts									
SOUPS, SAUCES, AND SPREADS									
Vegetable soups (bowl)									
Meat soups (bowl)									
Sauces, eg. white sauce, cheese sauce, gravy (tablespoon)									
Tomato ketchup (tablespoon)									
Pickles, chutney (tablespoon)									
Marmite, Bovril (teaspoon)									
Jam, marmalade, honey (teaspoon)									
Peanut butter (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DRINKS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)									
Coffee, instant or ground (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener, eg. Coffee-mate (teaspoon)									
Cocoa, hot chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits, eg. gin, brandy, whisky, vodka (single)									
Low calorie or diet fizzy soft drinks (glass)									
Fizzy soft drinks, eg. Coca cola, lemonade (glass)									
Pure fruit juice (100%) eg. orange, apple juice (glass)									
Fruit squash or cordial (glass)									
FRUIT **For seasonal fruits marked *, please estimate your average use when the fruit is in season**									
Apples (1 fruit)									
Pears (1 fruit)									
Oranges, satsumas, mandarins (1 fruit)									
Grapefruit (half)									
Bananas (1 fruit)									
Grapes (medium serving)									
Melon (1 slice)									
* Peaches, plums, apricots (1 fruit)									
* Strawberries, raspberries, kiwi fruit (medium serving)									
Tinned fruit (medium serving)									
Dried fruit, eg. raisins, prunes (medium serving)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
VEGETABLES Fresh, frozen or tinned (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots									
Spinach									
Broccoli, spring greens, kale									
Brussels sprouts									
Cabbage									
Peas									
Green beans, broad beans, runner beans									
Marrow, courgettes									
Cauliflower									
Parsnips, turnips, swedes									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu , soya meat, TVP, Vegeburger									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

Appendix 4 Participant Questionnaire

PARTICIPANT INFORMATION

STUDY TITLE: The effect of $FeSO_4$ iron supplementation on lipid peroxidation and redox active plasma iron in humans.

Name:

……..

Date of Birth:

……..

Preferred Email:

……..……

Height (m): ……

Weight (kg): ……...

Job Title/Degree Studying:

……..

Physical Activity Levels:

Low (less than 1 hour per week)
Medium (1-3 hours per week)
High (more than 3 hours per week)

……..

If you have previously taken supplements, when did you last take them?

……..

Females - Do you currently take contraceptives?

……..

Any other information about you that you believe would be relevant to the study?

……..

Appendix 5 Ethical Approval

Plymouth University
Faculty of Health and Human Sciences and Peninsula Schools of Medicine and Dentistry Student Ethics Committee
APPLICATION FOR ETHICAL APPROVAL OF RESEARCH
Review Panel; Andrew Wills; Miriam McMullan; Garry Farnham
25/04/2018

Title of research: The effect of $FeSO_4$ iron supplementation on lipid peroxidation and redox active plasma iron in humans.

Ref. Number: 17/18-362 and 17/18-363

Student Names: Charlotte Woods, Maria-Anne Gaitanou
Course/Programme for which project is being carried out: MSc Human Nutrition
Email address and other contact details:
maria-anne.gaitanou@students.plymouth.ac.uk;
charlotte.m.woods@students.plymouth.ac.uk
Supervisor/Chief Investigator/Independent researcher Name: Desley White

Dear Charlotte, Dear Maria-Anne,
Thank you for your Ethics application.

Decision: Supervisor to be given delegated responsibility to check the revised submission. When your supervisor is happy with the proposal you have permission to proceed. You do not need to resubmit a proposal to the committee.

Comments:
a) In methodology section include a statement that:

"all collected blood will be processed to plasma within 7 days (or on the day) of collection. No whole blood or material classed a "relevant human material" in the Human Tissue Act 2004 will be stored in this project".

(Contd.)

Also include:

"Waste and used whole blood fractions and derivatives will be disposed of according to the University health and safety policies and the requirements of the Human Tissue Act 2004. Specifically Human Tissue derived waste and contaminated equipment will be bagged separately to other laboratory waste and subsequently disposed of through the local laboratory disposal route."

b) Additional info needed concerning Phlebotomy qualifications

c) Inclusion of possible Iron sulphate side effects in Patient information sheet, and also possible side effects of taking blood (i.e., fainting). Include a section on **"Risks"**

d) In the information sheet the researchers still need to add a contact to whom a complaint about the conduct of the research can be directed.

e) In the consent form (for number 3) there needs to be a tick box for every statement made. There are 3 statements there presently with only one tick box.

f) The PIS should be revised to give a layperson's account of the overall aim of the study.

g) The PIS and consent form should contain a declaration that participants have none of the specific exclusion criteria.

Wishing you every success with your studies.

Kind Regards

Sylvia
Dr Sylvia Terbeck (Assistant Professor/Lecturer in Psychology)
Chair, Faculty of Health and Human Sciences and Peninsula Schools of Medicine and Dentistry Student Ethics Committee
Email : sylvia.terbeck@plymouth.ac.uk

Reference List

Agarwal, R. and Chase, S. (2002) 'Rapid, fluorimetric-liquid chromatographic determination of malondialdehyde in biological samples', *Journal of Chromatography B Analytical Technologies in the Biomedical and the Life Sciences,* 775 (1), pp. 121-126.

Assi, T. and Baz, E. (2014) 'Current applications of therapeutic phlebotomy', *Blood Transfusion*, 12 (1), pp. S75-S83.

Baker, J. (2016) 'The Purpose, Process, and Methods of Writing a Literature Review', *Association of perioperative Registered Nurses*, 103 (3), pp. 265-269.

Beilby, J., Olynyk, J., Ching, S., Prins, A., Swanson, N., Reed, W., Harley, H. and Garcia-Webb, P. (1992) 'Transferrin index: an alternative method for calculating the iron saturation of transferrin', *Clinical Chemistry*, 38 (10) pp. 2078-2081.

Bhagavan, N. (2015) *Essentials of Medical Biochemistry: With Clinical Cases.* London: Academic Press.

Bingham, S., Gill, C., Welch, A., Cassidy, A., Runswick, S., Oakes, S., Lubin, R., Thurnham, D., Key, T., Roe, L., Khaw, K. and Day, N. (1997) 'Validation of dietary assessment methods in the UK arm of EPIC using weighed records, and 24-hour urinary nitrogen and potassium and serum vitamin C and carotenoids as biomarkers', *International Journal of Epidemiology*, 26 (1), pp. S137-151.

Bland, M. (2015) *An Introduction to Medical Statistics.* Oxford: Oxford University Press.

Bouzid, K., Bahlous, A., Ferjani, F., Kalai, E., Ducrocq, R., Ben Mami, F. and Abdelmoula, J. (2012) 'Advantage of HbA1c assay by HPLC D-10 versus cobas integra 400 in a population carrier for HbS and HbC', *Clinical Laboratory,* 58 (7-8), pp. 821-828.

Breuer, W. and Cabantchik, Z. (2001) ‘A Fluorescence-Based One-Step Assay for Serum Non-Transferrin-Bound’, *Analytical Biochemistry,* 299, pp. 194-202.

Brissot, P., Ropert, M., Le Lan, C. and Loreal, O. (2012) ‘Non-transferrin bound iron: A key role in iron overload and iron toxicity’, *Biochimica et Biophysica Acta,* 1820 (3), pp. 403-410.

Brittenham, G., Anderson, M., Egli, I., Forman, J., Zeder, C., Westerman, M. and Hurrell, R. (2014) ‘Circulating non-transferrin-bound iron after oral administration of supplemental and fortification doses of iron to healthy women: a randomized study’, *The American Journal of Clinical Nutrition*, 100 (3), pp. 813-820.

Bruker (2018) *What is EPR?* Available at: https://www.bruker.com/products/mr/epr/what-is-epr.html (Accessed: 15 August 2018).

Cabanne, C. and Santarelli, X. (2014) ‘Media Selection in ion-exchange chromatography in a single microplate’, *Methods in Molecular Biology*, 1129, pp. 45-51.

Critical Appraisal Skills Programme (CASP) (2018) *CASP Cohort Study Checklist.* Available at: https://casp-uk.net/casp-tools-checklists/ (Accessed: 7 August 2018).

Cencioni, C., Spallotta, F., Martelli, F., Valente, S., Mai, A., Zeiher, A. and Gaetano, C. (2013) ‘Oxidative stress and epigenetic regulation in ageing and age-related diseases’, *International Journal of Molecular Sciences,* 14 (9), pp. 17643-17663.

Cercamondi, C., Egli, I., Zeder, C. and Hurrell, R. (2014) ‘Sodium iron EDTA and ascorbic acid, but not polyphenol oxidase treatment, counteract the strong inhibitory effect of polyphenols from brown sorghum on the absorption of fortification iron in young women’, *The British Journal of Nutrition*, 111 (3), pp. 481-489.

Chakraborty, A. and Jana, N. (2017) ‘Vitamin C Conjugated Nanoparticle Protects Cells from Oxidative Stress at Low Dose but Induces Oxidative Stress at Low Dose

but Induces Oxidative Stress and Cell Death at High Dose', *ACS Applied Material and Interfaces,* 10.1021/acsami.7b16055.

Colpo, E., de Bem, A., Pieniz, S., Schettert, S., dos Santos, R., Farias, I., Bertoncello, I., Moreira, C., Barbosa, N., Moretto, M. and Rocha, J. (2008) 'A single high dose of ascorbic acid and iron is not correlated with oxidative stress in healthy volunteers', *Annals of Nutrition and Metabolism*, 53 (2), pp. 79-85.

Control of Substances Hazardous to Health (COSHH) (2002) *Control of Substances Hazardous to Health.* London: Health and Safety Executive.

Currell, G. (2015) *Scientific Data Analysis.* Oxford: Oxford University Press.

Data Protection Act (2018) *Data Protection Act.* Available at: http://www.legislation.gov.uk/ukpga/2018/12/contents/enacted (Accessed: 16 August 2018).

De Lucca, L., Rodrigues, F., Jantsch, L., Meme, W., Gallarreta, F. and Goncalves, T. (2016) 'Oxidative Profile and Aminolevulinate Dehydratase Activity in Healthy Pregnant Women with Iron Supplementation', *International Journal of Environmental Research and Public Health,* 13 (5), pp. E463.

Department of Health (1991) *Dietary reference values for food and energy and nutrients for the United Kingdom.* London: Department of Health.

De Swart, L., Hendriks, J., Van Der Vorm, L., Cabantchik, Z., Evans, P., Hod, E., Brittenham, G., Furman, Wojczyk, B., Janssen, M., Porter, J., Mattijssen, V., Biemond, B., MacKenzie, M., Origa, R., Galanello, R., Hider, R. and Swinkels, D. (2016) 'Second international round robin for the quantification of serum non-transferrin-bound iron and labile plasma iron in patients with iron-overload disorders', *Haematologica,* 101 (1), pp. 28-45.

DiaSys (2015) *DiaSys Immunoturbidimetric Tests No Doubt.* Holzheim: DiaSys Diagnostic Systems.

Elgailani, I. and Alsakka, M. (2016) ‘Determination of iron content if different haemoglobin samples from some patients by UV-Visible Spectrophotometer’, *Advances on Analytical Chemistry,* 6 (2), pp. 35-40.

Elsayed, M., Sharif, M. and Stack, A. (2016) ‘Chapter Four - Transferrin Saturation: A Body Iron Biomarker’, *Advances in Clinical Chemistry*, 75, pp. 71-97.

Emmett, P. (2009) ‘Dietary assessment in the Avon Longitudinal Study of Parents and Children’, *European Journal of Clinical Nutrition,* 63 (1), pp. S38-S44.

European Food Safety Authority (EFSA) (2013) ‘Scientific Opinion on Dietary Reference Values for vitamin C EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)’, *EFSA Journal,* 11 (11), pp. 3418.

Flowers, C., Kuizon, M., Beard, J., Skikne, B., Covell, A. and Cook, J. (1986) ‘A Serum Ferritin Assay for Prevalence Studies of Iron Deficiency’, *American Journal of Haematology,* 23, pp. 141-151.

Frijhoff, J., Winyard, P., Zarkovic, N., Davies, S., Stocker, R., Cheng, D., Knight, A., Taylor, E., Oettrich, J., Ruskovska, T., Gasparovic, A., Cuadrado, A., Weber, D., Poulsen, H., Grune, T., Schmidt, H. and Ghezzi, P. (2015) ‘Clinical relevance of biomarkers of oxidative stress’, *Antioxidants and Redox Signalling*, 23 (14), pp. 1144-1170.

Geisser, P. and Burckhardt, S. (2010) ‘The Pharmacokinetics and Pharmacodynamics of Iron Preparations’, *Pharmaceutics,* 3 (1), pp. 12-33.

Halie, Z., Teweldeberhan, A. and Chertok, I. (2016) ‘Association between oral contraceptive use and markers of iron deficiency in a cross-sectional study of Tanzanian women’, *International Journal of Gynaecology and Obstetrics,* 132 (1), pp. 50-54.

Herrera, A., Victorino, V., Campos, F., Verenitach, B., Lemos, L., Aranome, A., Oliveira, S., Cecchini, A., Simao, A., Abdelhay, E., Panis, C. and Cecchini, R. (2014) 'Impact of tumor removal on the systemic oxidative profile of patients with breast cancer discloses lipid peroxidation at diagnosis as a putative marker of disease recurrence', *Clinical Breast Cancer*, 14, pp. 451–459.

Human Tissue Act (2004) *Human Tissue Act.* Available at: http://www.legislation.gov.uk/ukpga/2004/30/contents (Accessed: 13 August 2018).

International Committee for Standardisation in Haematology (ICSH) (1978) 'Recommendations for the measurements of the serum iron in human blood', *British Journal of Haematology,* 38, pp. 291.

International Panel of the International Committee for Standardisation in Haematology (ICSH) (1990) 'Revised recommendations for the measurements of the serum iron in human blood', *British Journal of Haematology,* 75, pp. 615-616.

Ito, S., Ikuta, K., Kato, D., Lynda, A, Shibusa, K., Niizeki, N., Toki, Y., Hatayama, M., Yamamoto, M., Shindo, M., Iizuka, N., Kohgo, Y. and Fujiya, M. (2016) 'In vivo behavior of NTBI revealed by automated quantification system', *International Journal of Haematology,* 104 (2), pp. 175-181.

Ito, S., Ikuta, K., Kato, D., Shibusa, K., Niizeki, N., Tanaka, H., Addo, L., Toki, Y., Hatayama, M., Inamura, J., Shindo, M., Sasaki, K., Iizuka, n., Fujiya, M., Torimoto, Y. and Kohgo, Y. (2014) 'Non-transferrin-bound iron assay system utilizing a conventional automated analyzer', *International Journal of Clinical Chemistry*, 437, pp. 129-135.

Jacobs, E., Hendriks, J., van Tits, B., Evans, P., Breuer, W., Liu, D., Jansen, E., Jauhiainen, K., Sturm, B., Porter, J., Scheiber-Mojdehkar, B., von Bonsdorff, L., Cabantchik, Z., Hider, R. and Swinkles, D. (2005) 'Results of an international round robin for the quantification of serum non-transferrin-bound iron: Need for defining standardization and a clinically relevant isoform', *Analytical Biochemistry*, 341 (2), pp. 241-250.

Kali, A., Charles, M. and Seetharam, R. (2015) ‘Hepcidin - A novel biomarker with changing trends’, *Pharmacognosy Reviews*, 9 (17), pp. 35-40.

Khan, A., Khan, W., Ayub, M., Humayun, M. and Haroon, M. (2016) ‘Ferritin Is a Marker of Inflammation rather than Iron Deficiency in Overweight and Obese People’, *Journal of Obesity,* 1937320.

Kime, R., Gibson, A., Yong, W., Hider, R. and Powers, H. (1996) ‘Chromatographic method for the determination of non-transferrin-bound iron suitable for use on the plasma and bronchoalveolar lavage fluid of preterm babies’, *Clinical Science*, 91; pp. 633-639.

King, S., Donangelo, C., Knutson, M., Walter, P., Ames, B., Viteri, F. and King, J. (2008) ‘Daily supplementation with iron increases lipid peroxidation in young women with low iron stores’, *Experimental Biology and Medicine*, 233 (6), pp. 701-707.

Lane, D. and Richardson, D. (2014) ‘The active role of vitamin C in mammalian iron metabolism: much more than just enhanced iron absorption!’, *Free Radical Biology and Medicine,* 75, pp. 69-83.

Leonard, A., Chalmers, K., Collins, C. and Patterson, A. (2014) ‘Comparison of two doses of elemental iron in the treatment of latent iron deficiency: efficacy, side effects and blinding capabilities’, *Nutrients,* 6 (4), pp.1394-1405.

Low, M., Speedy, J., Styles, C., De-Regil, L. and Pasricha, S. (2016) ‘Daily iron supplementation for improving anaemia, iron status and health in menstruating women’, *Cochrane Database of Systematic Reviews*, 4, CD009747.

Lykkesfeldt, J. (2007) ‘Ascorbate and Dehydroascorbic Acid as Reliable Biomarkers of Oxidative Stress: Analytical Reproducibility and Long-term Stability of Plasma Samples Subjected to Acidic Deproteinization’, *Cancer Epidemiology Biomarkers and Prevention*, 16 (11), pp. 2175-2520.

Lykkesfeldt, J. (2012) ‘Ascorbate and dehydroascorbic acid as biomarkers of oxidative stress: validity of clinical data depends on vacutainer system used’, *Nutrition research,* 32 (1), pp. 66-69.

Madhikarmi, N. and Murthy, K. (2014) ‘Antioxidant enzymes and oxidative stress in the erythrocytes of iron deficiency anemic patients supplemented with vitamins’, *Iran Biomed Journal,* 18 (2), pp. 82-87.

Maas, R., Voets, P., De Swart, L. and Swinkels, D. (2013) ‘Non-transferrin-bound iron: a promising biomarker in iron overload disorders’, *Nederlands Tijdschrift Voor Geneeskunde,* 157 (49), pp. A6258.

Medical Research Council (2015) *Food Frequency Questionnaires.* Available at: http://www.measurement-toolkit.mrc.ac.uk/diet/subjective-methods/food-frequency-questionnaire (Accessed: 13 August 2018).

McDonald, J. (2014) *Handbook of Biological Statistics.* Maryland: Sparky House Publishing.

McMurry, J. (2015) *Organic chemistry: with biological applications*. Stamford: Cengage Learning.

Meyland, I. (2004) *Ferrous Glycinate (Processed with Citric Acid) Chemical and Technical Assessment (CTA).* Available at: http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/61/FERROUSGLYCINATE.pdf (Accessed: 3rd July 2018).

Miseta, A., Nagy, J., Nagy, T., Poo, V., Fekete, Z. and Sipos, K. (2015) ‘Hepcidin and its potential clinical utility’, *Cell Biology International*, 39, pp. 1191-1202.

Mitton, K. and Trevithick, J. (1994) ‘High-performance liquid chromatography-electrochemical detection of antioxidants in vertebrate lens: glutathione, tocopherol, and ascorbate’, *Methods in Enzymology*, 233, pp. 523-539.

National Diet and Nutrition Survey (NDNS) (2018) *Results from Years 7 and 8 (combined) of the Rolling Programme (2014/2015 to 2015/2016).* London: Public Health England.

National Institute of Health and Care Excellence (NICE) (2018) *Anaemia – Iron Deficiency.* London: National Institute of Health and Care Excellence (Clinical Practice Guidelines).

National Institutes of Health (NIH) (2018) *Vitamin C.* Available at: https://ods.od.nih.gov/factsheets/VitaminC-HealthProfessional/ (Accessed: 23rd July 2018).

National Institute of Standards and Technology (NIST) (2018) *Spectrophotometry.* Available at: https://www.nist.gov/programs-projects/spectrophotometry (Accessed 29 July 2018).

Nunes, L., Grotto, H., Brenzikofer, R. and Macedo, D. (2014) 'Hematological and Biochemical Markers of Iron Status in a Male, Young, Physically Active Population', *BioMed Research International*, 349182.

Olivares, M., Castro, C., Pizarro, F. and De Romana, D. (2013) 'Effect of increasing levels of zinc fortificant on the iron absorption of bread co-fortified with iron and zinc consumed with a black tea', *Biological Trace Element Research*, 154 (3), pp. 321-325.

Parahoo, K. (2014) *Nursing Research Principles, Process and Issues.* Basingstoke: Palgrave Macmillan.

Pavia, D., Kriz, G., Lampman, G. and Engel, R. (2018) *A Microscale Approach to Organic Laboratory Techniques.* Boston: Cengage Learning.

Pena-Rosas, J., De-Regil, L., Garcia-Casal, M. and Downswell, T. (2015) 'Daily oral iron supplementation during pregnancy', *Cochrane Database of Systematic Reviews,* 22 (7), pp. CD004736.

Pfeiffer, C. and Looker, A. (2017) ‘Laboratory methodologies for indicators of iron status: strengths, limitations, and analytical challenges’, *The American Journal of Clinical Nutrition*, 106 (6), pp. 1606S-1614S.

Pingitore, A., Lima, G., Mastorci, F., Quinones, A., Iervasi, G. and Vassalle, C. (2015) ‘Exercise and oxidative stress: potential effects of antioxidant dietary strategies in sports’, *Nutrition*, 31 (7-8), pp. 916-922.

Pollard, J., Wild, C., White, K., Greenwood, D., Cade, J. and Kirk, S. (2003) ‘Comparison of plasma biomarkers with dietary assessment methods for fruit and vegetable intake’, *European Journal of Clinical Nutrition*, 57 (8), pp. 988-998.

Porter, J., El-Alfy, M., Viprakasit, V., Giraudier, S., Chan, L., Lai, Y., El-Ali, A., Han, J. and Cappellini, M. (2016) ‘Utility of labile plasma iron and transferrin saturation in addition to serum ferritin as iron overload markers in different underlying anaemias before and after deferasirox treatment’, *European Journal of Haematology*, 96 (1), pp. 19-26.

Proteggente, A., England, T., Rice_evans, C. and Halliwell, B. (2001) ‘Iron supplementation and oxidative damage to DNA in healthy individuals with high plasma ascorbate’, *Biochemical and Biophysical Research Communications,* 288 (1), pp. 254-251.

Rehman, A., Collis, C., Yang, M., Kelly, M., Diplock, A., Halliwell, B. and Rice-Evans, C. (1998) ‘The Effects of Iron and Vitamin C Co-supplementation on Oxidative Damage to DNA in Healthy Volunteers’, *Biochemical and Biophysical Research Communications,* 246, pp. 293-298.

Rhee, J., Sampson, L., Cho, E., Hughes, M., Hu, F. and Willett, W. (2015) ‘Comparison of Methods to Account for Implausible Reporting of Energy Intake in Epidemiologic Studies’, *Practice of Epidemiology,* 181 (4), pp. 225-233.

Rodrigues, F., De Lucca, L., Neme, W. and Goncalves, T. (2018) ‘Influence of gestational diabetes on the activity of aminolevulinate dehydratase and oxidative stress biomarkers’, *Redox Report,* 23 (1), pp. 63-67.

Ruchala, P. and Nemeth, E. (2014) ‘The pathophysiology and pharmacology of hepcidin’, *Trends in Pharmacological Sciences*, 35, pp. 155–161.

Sanderson, M., Smith, I., Parker, I. and Bootman, M. (2014) ‘Fluorescence microscopy’, *Cold Spring Harbor Protocols*, 2014 (10), pp. 1-36.

Sasaki, K., Ikuta, K., Tanaka, H., Ohtake, T., Torimoto, Y., Fujiya, M. and Kohgo, Y. (2011) ‘Improved quantification for non-transferrin-bound iron measurement using high-performance liquid chromatography by reducing iron contamination’, *Molecular Medicine Reports*, 4, pp. 913-918.

Sato, Y., Uchiki, T., Iwama, M., Kishimoto, Y., Takahashi, R. and Ishigami, A. (2010) ‘Determination of dehydroascorbic acid in mouse tissues and plasma by using tris(2-carboxyethyl)phosphine hydrochloride as reductant in metaphosphoric acid/ethylenediaminetetraacetic acid solution’, *Biological and Pharmaceutical Bulletin*, 33 (3), pp. 364-369.

Schumann, K., Kroll, S., Romero-Abal, M., Georgiou, N., Marx, J., Weiss, G. and Solomons, N. (2012a) ‘Impact of oral iron challenges on circulating non-transferrin-bound iron in healthy Guatemalan males’ *Annals of Nutrition and Metabolism*, 60 (2), pp. 98-107.

Schumann, K., Solomons, N., Romero-Abal, M., Orozco, M., Weiss, G. and Marx, J. (2012b) ‘Oral administration of ferrous sulfate, but not of iron polymaltose or sodium ironethylenediaminetetraacetic acid (NaFeEDTA), results in a substantial increase of non-transferrin-bound iron in healthy iron-adequate men’, *Food and Nutrition Bulletin,* 33 (2), pp. 128-136.

Schumann, K., Solomons, N., Orozco, M., Romero-Abal, M. and Weiss, G. (2013) ‘Differences in circulating non-transferrin-bound iron after oral administration of

ferrous sulfate, sodium iron EDTA, or iron polymaltose in women with marginal iron stores', *Food and Nutrition Bulletin*, 34 (2), pp. 185-193.

Scientific Advisory Committee on Nutrition (SACN) (2015) *Carbohydrates and Health.* London: The Stationary Office.

Scientific Advisory Committee on Nutrition (SACN) (2010) *Iron and Health.* London: The Stationary Office.

Scientific Advisory Committee on Nutrition (SACN) (2016) *Vitamin D and Health.* London: The Stationary Office.

Siemens Healthcare Limited (2018) *IMMULITE 2000 XPi Immunoassay System.* Available at: https://www.healthcare.siemens.co.uk/immunoassay/systems/immulite-2000-xpi-immunoassay-system (Accessed: 2 August 2018).

Smith, G., Fisher, S., Doree, C., Di Angelantonio, E. and Roberts, D. (2014) 'Oral or parental iron supplementation to reduce deferral, iron deficiency and/or anaemia in blood donors', *Cochrane Database of Systematic Reviews*, 3 (7), CD009532.

Stoffel, N., Cercamondi, C., Brittenham, G., Zeder, C., Geurts-Moespot, A., Swinkels, D., Moretti, D. and Zimmerman, M. (2017) 'Iron absorption from oral iron supplements given on consecutive versus alternate days and as single morning doses versus twice-daily split dosing in iron-depleted women: two open-label, randomised controlled trials', *The Lancet Haematology*, 4 (11), pp. e524-e533.

Stucchi, M., Cantoni, S., Piccinelli, E., Savonitto, S. and Morici, N. (2018) 'Anemia and acute coronary syndrome: current perspectives', *Vascular Health Risk Management*, 14, pp. 109-118.

Taylor, A., Day, M., Hill, S., Marshall, J., Patriarca, M. and White, M. (2013) 'Atomic spectrometry update. Clinical and biological materials, foods and beverages', *Journal of Analytical Atomic Spectrometry,* 28, pp. 435-429.

Tee, E., Young, E. Ho, S. and Siti Mizura, S. (1988) ‘Determination of Vitamin C in Fresh Fruits and Vegetables Using the Dye-titration and Mircrofluorometric Methods’, *Pertanika*, 11 (1), pp. 39-44.

The PRISMA Group (2009) 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement', *Public Library of Science*, 6 (7): e1000097.

Troesch, B., Elgi, I., Zeder, C., Hurrell, R. and Zimmermann, M. (2011) ‘Fortification iron as ferrous sulfate plus ascorbic acid is more rapidly absorbed than as sodium iron EDTA but neither increases serum non transferrin-bound iron in women’, *The Journal of Nutrition,* 141 (5), pp. 822-827.

Vo, K. (2015) *Spectrophotometry.* Available at: https://chem.libretexts.org/Core/Physical_and_Theoretical_Chemistry/Kinetics/Reaction_Rates/Experimental_Determination_of_Kinetcs/Spectrophotometry (Accessed: 7 August 2018).

Williamson, G. and Whittaker, A. (2017) *Succeeding in Literature Reviews and Research Project Plans for Nursing Students.* London: SAGE Publications Ltd.

World Medical Association (2013) 'World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects', *The Journal of the American Medical Association*, 310 (20), pp 2191-2914.

Zhou, Y., Liu, T., Kang, P. and Jia, C. (2014) ‘Association of better iron status biomarkers and coronary artery disease risk’, *Internal Medicine Journal*, 44, pp. 846-850.

Zuo, L., He., F., Sergakis, G., Koozehchian, M., Stimpfl, J., Rong, Y., Diaz, P. and Best, T. (2014) ‘Interrelated role of cigarette smoking, oxidative stress, and immune response in COPD and corresponding treatments’, *American Journal of Physiology*, 307 (3), pp. L205-L218.