The effect of maternal iron status and intake during pregnancy on cardiovascular disease risk in the offspring

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Chapter 2:

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Abstract

Iron is an important micronutrient essential in carrying oxygen and maintaining the function of many body enzymes. It is of particular importance during gestation as body demands increase leading to iron deficiency in women with inadequate iron stores at the start of pregnancy. Animal studies have shown that iron deficiency in pregnancy leads to offspring with adverse cardiovascular risk profiles compared to offspring of iron replete mothers. This thesis aimed to examine the association of maternal iron intake and status in pregnancy with short and long term birth outcomes that are considered cardiovascular risk indicators later in life.

Analysis of data from three cohorts and one Mendelian randomisation study was included in this thesis. Total maternal iron intake in early, but not late, pregnancy was positively associated with birth size. There was no evidence of association between taking iron-containing supplements in pregnancy and size at birth. However, taking multivitamin-mineral supplements, which contain iron, in late pregnancy was associated with an increased risk of preterm birth. Also taking iron supplements up to 32 weeks gestation was associated with lower offspring systolic blood pressure at 10 years. Maternal iron deficiency and anaemia in early pregnancy were associated with an increased risk of giving birth to a SGA baby. Infant brachio-femoral PWV measured at 2-6 weeks of age was found to be higher in women who were anaemic in early pregnancy, but not in those who were only iron deficient. Finally, using a Mendelian randomisation design, maternal iron status measured by serum ferritin with C282Y mutation as an instrumental variable, was not found to be associated with adult offspring BP and adiposity.

In conclusion, maternal iron intake and status in early pregnancy seem to be associated with short term birth outcomes like size at birth, while associations with long term offspring cardiovascular indicators were not detected in this thesis.

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Abbreviations

AGA	Appropriate for gestational age
ALSPAC	Avon Longitudinal Study of Parents and Children
Baby VIP	Baby's Vascular health and Iron in Pregnancy study
BP	Blood pressure
BMI	Body mass index
CARE	Caffeine and Reproductive Health study
CVD	Cardiovascular disease
CI	Confidence interval
FMD	Flow mediated dilatation
FFQ	Food frequency questionnaire
GDM	Gestational diabetes
Hb	Haemoglobin
ID	Iron deficiency
IDA	Iron deficiency anaemia
IDE	Iron deficient erythropoiesis
IMD	Index of Multiple Deprivation
IUGR	Intra-uterine growth restriction
IV	Instrumental variable
LBW	Low birth weight
LRNI	Lower Reference Nutrient Intake
MCV	Mean corpuscular volume
MR	Mendelian randomization
MVM	Multivitamin-mineral supplements
NICE	National Institute for Health and Care Excellence
OLS	Ordinary least squares
OR	Odds ratio
PWV	Pulse wave velocity
RNI	Reference Nutrient intake
sF	Serum ferritin
sTfR	Serum transferrin receptor
R/F	Serum transferrin receptor to serum ferritin ratio
RCT	Randomised controlled trial
SGA	Small for gestational age
SD	Standard deviation
TS	Transferrin saturation
UKWCS	UK Women's Cohort Study
UKWCS-IBPS	UK Women's Cohort Study – Iron and Blood Pressure Sub-cohort
WC	Waist circumference
WHO	World Health Organization

1 Introduction

1.1 Setting the scene

Obesity, high blood pressure (BP) and arterial stiffness are major risk factors that contribute to premature death from heart disease and stroke. Factors affecting the development of the fetus before birth, including maternal nutrition in pregnancy, may influence these risk factors later in life. Diet deficient in essential nutrients including iron is a common problem during pregnancy. Iron is an essential micronutrient and is important not only in carrying oxygen, but also to the catalytic activity of a variety of enzymes. In the fetus, it is vital to the synthesis of haemoglobin (Hb) and brain development. Iron deficiency anaemia (IDA) in pregnancy is a common problem, even in high income country settings. Around 50% of pregnant women worldwide are anaemic, with at least half of this burden due to iron deficiency (ID).

IDA is associated with adverse short-term maternal and birth outcomes, particularly if present during the first half of pregnancy. Iron supplements are widely recommended and used during pregnancy globally. However, the evidence on the extent of benefit they contribute to the offspring's health is not well established, and their routine use has its side effects and drawbacks. Dietary iron intake is difficult to assess accurately and it is unlikely to be sufficient to meet the demands of pregnancy if women start with inadequate body iron stores at conception.

Evidence from experimental animal models suggests that maternal ID during pregnancy is associated with fetal growth restriction, as well as offspring obesity and high BP later in life. The possible biological mechanisms for this observed association

may be due to ID-induced changes in placental structure and function, enzyme expression, nutrient absorption and fetal organ development. These observed causal associations in animal models need to be investigated in humans using epidemiological study designs high up in the evidence hierarchy.

This thesis describes a lifecourse approach to exploring the associations between maternal iron intake and status during pregnancy and offspring potential markers of cardiovascular disease (CVD) risk in population studies. These markers include both short-term outcomes (size at birth, preterm birth, infant arterial elasticity) and longer-term outcomes (child and adult BP, adiposity, arterial stiffness and endothelial function). In this thesis, different epidemiological study designs were utilised to examine these associations using data collected specifically for this project, as well as data from multiple existing longitudinal UK birth cohorts.

1.2 **Hypothesis**

Maternal ID during pregnancy is associated with increased cardiovascular risk in the offspring.

1.3 **Aim & objectives**

The work included in this thesis aims to assess the relationship between both maternal iron status and intake during pregnancy and potential cardiovascular indicators in the offspring.

The objectives are to:

1. Examine the relationship of maternal dietary iron intake and iron-containing supplements with immediate birth outcomes including birth weight and preterm birth (<37 weeks gestation) (chapter 3)
2. Examine the relationship of maternal iron status measured in early pregnancy, using serum ferritin (sF) and serum transferrin receptor (sTfR) levels, with birth weight, preterm birth and infant arterial stiffness (chapter 4)
3. Examine the relationship of maternal iron intake from diet and supplements during pregnancy with offspring's vascular profile (BP, arterial stiffness and endothelial function) and body size in childhood at around 10 years (chapter 5)
4. Examine the relationship of maternal iron status, using *HFE* genotype as an instrumental variable (IV), with BP and measures of adiposity including body mass index (BMI) and waist circumference (WC) in the adult offspring (chapter 6)
5. Examine the relationship of maternal Hb levels and anaemia during pregnancy with short term birth outcomes (birth weight and preterm birth) and cardiovascular indicators in the offspring including BP, endothelial function and adiposity at age 10, and arterial stiffness in infancy and at age 10 (chapters 3, 4, 5)

1.4 Thesis overview

This thesis includes four studies using multiple epidemiological research designs including Mendelian randomisation, historical and prospective cohort study designs. The research databases investigated in this thesis come from four sources:

1. CARE:

   The Caffeine and Reproductive Health study

2. Baby VIP:

   Baby's Vascular health and Iron in Pregnancy study

3. ALSPAC:

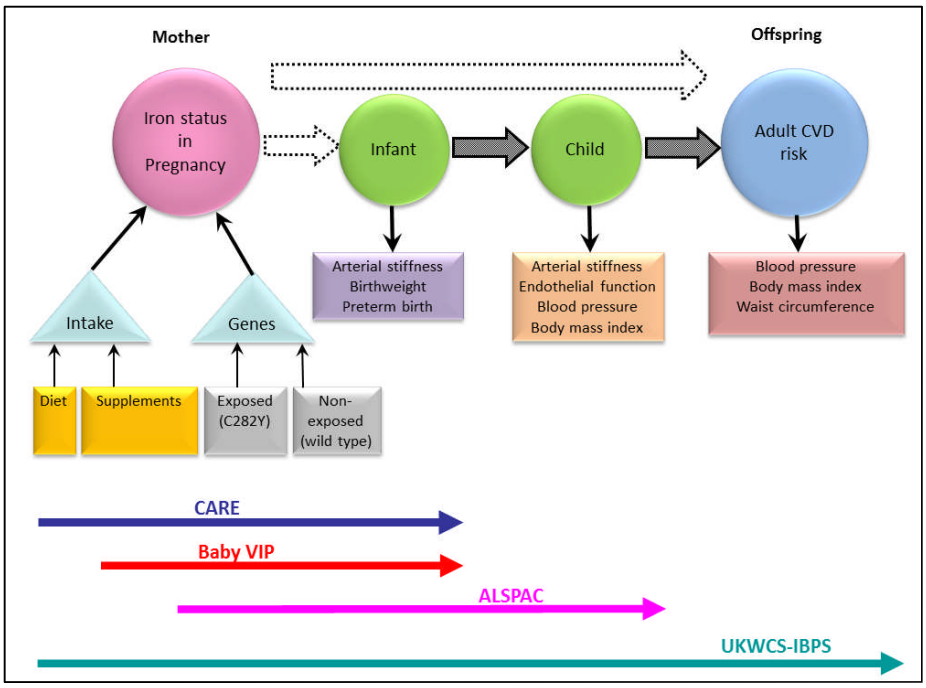
   The Avon Longitudinal Study of Parents and Children

4. UKWCS-IBPS:

   The UK Women's Cohort Study – Iron and Blood Pressure Sub-cohort

An outline of how these studies fit together to provide a lifecourse approach to addressing the hypothesis under investigation in this thesis is illustrated in Figure 1.

The second chapter is a summary of the relevant background evidence linked to the thesis objectives. Chapters 3 - 6 will describe the four component studies of this research programme, each going through a study-specific introduction, methodology, results, discussion and conclusion. In chapter 7, the results of the four studies will be pulled together in a general discussion chapter, followed by the conclusion in chapter 8.



CARE: Caffeine and Reproductive Health study (chapter 3)

Baby VIP: Baby's Vascular health and Iron in Pregnancy study (chapter 4)

ALSPAC: The Avon Longitudinal Study of Parents and Children (chapter 5)

UKWCS-IBPS: The UK Women's Cohort Study – Iron and Blood Pressure Sub-cohort (chapter 6)

Figure 1: Thesis overview

2 Background

2.1 Chapter summary

This chapter investigates the research evidence relevant to the hypothesis of interest in this thesis. It first examines iron status in pregnancy (section 2.2). It then investigates the evidence linking maternal ID during pregnancy with short-term adverse birth outcomes including low birth weight (LBW) and preterm birth (sections 2.2.4 and 2.2.5). Intake of iron from the diet and supplements is discussed, and the evidence linking it to birth outcomes is presented (sections 2.2.7 and 2.2.8).

This is followed by an evaluation of the experimental and the epidemiological evidence supporting associations between maternal ID in pregnancy and cardiovascular outcomes in the offspring, and the biological mechanisms potentially underlying such associations (sections 2.3). It then explores the advantages and limitations of the available biomarkers to measure iron status (section 2.4), and reviews the cardiovascular indicators used as outcomes in this thesis, with a particular focus on arterial stiffness measured by pulse wave velocity (PWV) as a predictor of future CVD risk (section 2.5). This chapter concludes with a note on the originality and significance of the research included in this thesis (section 2.6).

2.2 Iron status in pregnancy

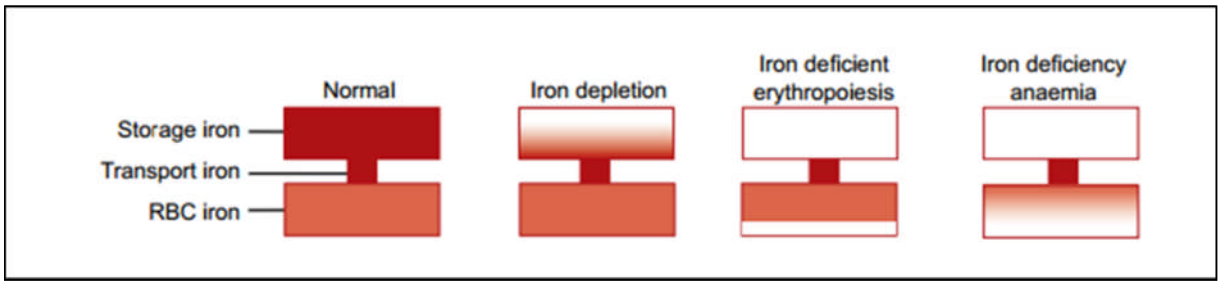
The human body requires iron for essential physiological functions including oxygen transport, Hb and myoglobin synthesis, and cell growth and differentiation (Cetin et al., 2009). It is vital for the function of body enzymes necessary for electron transfer and oxidation-reduction reactions (Vijayaraghaven, 2004). Its deficiency limits oxygen delivery to cells. In the fetus, iron is used to synthesize Hb (Milman, 2006b), and is essential in brain development (Lozoff, 2000). The size of iron stores required at each stage of pregnancy to ensure an optimal outcome for the mother and the child is still not exactly known (Lynch, 2011).

Iron balance in the body is determined by several elements: iron intake and absorption, iron loss and body iron stores. Inadequate iron intake leads to enhancing dietary iron absorption, mobilizing body iron stores, reducing iron transport to the bone marrow and eventually lowering Hb levels leading finally to anaemia (Vijayaraghaven, 2004). Genetics also influence body iron. Women who carry a C282Y mutation in the *HFE* gene are more likely, in the homozygous state, to suffer from haemochromatosis, a condition which is characterised by iron overload in the liver (Rhodes et al., 1997, Willis et al., 1999). About 12-20% of Northern Europeans are heterozygotes for this mutation (Rhodes et al., 1997). These *HFE* gene mutation carriers are usually asymptomatic. However, they tend to have higher total body iron stores (Chan et al., 2005, Cade et al., 2005, Beutler et al., 2002, Jackson et al., 2001)

During pregnancy, all the iron delivered to the fetus comes from maternal stores, absorption of dietary iron, or turnover of maternal erythrocytes (Wu et al., 2004). As there is an increased demand for iron during this period to cover the mother's and the baby's needs, this is likely to affect iron balance in the body leading to deficiency, particularly if the pregnancy starts with inadequate iron stores.

2.2.1 Stages of iron deficiency

ID refers to a spectrum ranging from iron depletion to IDA as illustrated in Figure 2. Women can experience one or more of these stages at different time points prior to conception, during pregnancy and post-partum. Biomarkers referred to below that are used to measure iron status are discussed in detail in section 2.4.



Source: *Guidelines for control of iron deficiency anaemia; Ministry of Health, India (National Iron+ Initiative 2013)*

Figure 2: Stages of iron deficiency

2.2.1.1 Iron depletion

In iron depletion the amount of stored iron which is measured by sF concentrations is reduced, however the amount of transport and functional iron may not be affected. Those with iron depletion do not have iron stores to mobilise if the body requires additional iron, as in the case of pregnancy (Pavord et al., 2011). This leads to iron-

deficient erythropoiesis (IDE). In this thesis, a WHO cut-off of 15 ug/l in sF was used to indicate depleted iron stores in pregnancy (WHO, 2011).

2.2.1.2 Iron deficient erythropoiesis

In IDE, stored iron is depleted and transport iron, measured by transferrin saturation (TS), is reduced further. The amount of iron absorbed is not sufficient to replace the amount lost or to provide the amount needed for growth and function. In this stage the shortage of iron limits red blood cell production and results in increased erythrocyte protoporphyrin concentration and increase sTfR levels (Pavord et al., 2011, Vijayaraghaven, 2004). This in turn leads to the development of IDA.

2.2.1.3 Iron deficiency anaemia

Anaemia accounts for 9% of the total disability from all conditions in 2010, with children under 5 years and women having the highest burden. IDA is the most common aetiology of anaemia. It is defined as anaemia accompanied by depleted iron stores and signs of a compromised iron supply to the tissues. It is the most severe form of ID. Shortage of iron stores, transport, and functional iron result in reduced Hb production leading to a fall in its blood levels, in addition to low sF, low TS, increased sTfR and erythrocyte protoporphyrin concentrations (Pavord et al., 2011).

The WHO defines anaemia in pregnancy as a Hb concentration of <11 g/dl (WHO, 2001), while the Centers for Disease Control and Prevention (CDC) defines the cut-off at 10.5 g/dl from 12 weeks gestation on (Dowdle, 1989, Ramsay et al., 2000). In the UK, the National Institute for Health and Care Excellence (NICE) defines the cut-off as 11 g/dl at around 12 weeks gestation, and 10.5 g/dl at around 28 weeks gestation,

which are the two routine antenatal screening points for anaemia in the UK (National Institute for Clinical Excellence (NICE), 2008).

2.2.2 Iron requirements during pregnancy

During pregnancy, extra iron is required to cover the increasing red cell mass, plasma volume and the growth of the fetoplacental unit. The body's capacity to increase absorption during pregnancy starts with around 8% of ingested iron in the first trimester and progressively increases to 37% by 36 weeks gestation (Whittaker et al., 1991). One study when all women had sF >12 ug/l at recruitment in the first trimester found the proportion of ferrous iron absorbed to be 7, 36 and 66% in gestational weeks 12, 24 and 36 respectively (Barrett et al., 1994), compared to around 11% in non-pregnant women (Milman, 2006a). It appears that the increased absorption of iron during pregnancy is elicited by depleted iron stores. This is shown by the demonstration of an inverse relationship between sF and iron absorption during pregnancy (Barrett et al., 1994, O'Brien et al., 1999, O'Brien et al., 2003).

The average total amount of iron which a women needs to mobilize during her pregnancy is 1200 mg (Milman, 2006a, McMahon, 2010). The fetus takes up about 400 mg over full gestation, with up to 175 mg accumulating in the placenta. Pregnant women require an extra 1 mg/day in the first trimester, 4-5 mg/day in the second trimester, and a minimum of an extra 6 mg/day in the third trimester if they were to meet their pregnancy iron demands (Whittaker et al., 1991). However, it is still unlikely that iron requirements during late pregnancy can be met through diet alone, even with optimal absorption, if the pregnancy starts with inadequate iron stores (Milman, 2008). Therefore, a woman must enter pregnancy with iron stores of ≥300

mg if she is to meet her requirements fully (Bothwell, 2000). In fact, in a study that assessed iron status in early pregnancy, women with initial iron depletion (sF<12 ug/l) were more likely to have iron depletion and ID (sF<12 ug/l and TS<16%) throughout pregnancy compared to women who start their pregnancy non–iron depleted, despite the iron-depleted women receiving iron supplements (Ribot et al., 2012).

2.2.3 Epidemiology of iron deficiency in pregnancy

ID remains the leading single nutrient deficiency in the world (WHO, 2010). It is estimated to affect 1 to 2 billion people, with women of child-bearing age, infants, and young children particularly at risk. Around 50% of pregnant women worldwide are anaemic, with at least half of this burden due to ID (WHO, 2010, WHO, 2001). The prevalence of IDA varies from 31% of pregnant women in South America to 64% in south Asia, with about 88% in India alone (Vijayaraghaven, 2004). Up to 40% of women worldwide have very low iron stores (Scientific Advisory Committee on Nutrition, 2010). ID during pregnancy is not just a problem in low and middle income countries. It is also common in high income countries (Beard, 1994, Milman et al., 1998, Robinson et al., 1998). About 25 to 40% of pregnant women in Western societies are estimated to have ID (Bergmann et al., 2002), with this problem being more pronounced in lower socioeconomic groups (Godfrey et al., 1991). In Denmark, 42% of women of child-bearing age were found to have small iron reserves (Milman et al., 1998).

2.2.4 Iron deficiency anaemia in pregnancy and birth outcomes

ID can cause an increased production of norepinephrine, which then stimulates production of corticotropin-releasing hormone and in turn possibly restricts fetal

growth (Allen, 2001). There is research evidence that links IDA at different stages of pregnancy with size at birth and gestational age. A few studies have assessed early pregnancy Hb in relation to birth outcomes. In a cohort of Chinese textile workers likely to become pregnant, preconceptional anaemia was associated with reductions in birth weight, birth length, and head circumference, with an increased risk of LBW and fetal growth restriction (Ronnenberg et al., 2004). In another Chinese study, rates of LBW and preterm birth, but not small for gestational age (SGA), were related to early pregnancy Hb concentration in a U-shaped manner (Zhou et al., 1998). In a cohort from the United States, IDA in early but not in late pregnancy was associated with greater risk of LBW and preterm birth (Scholl et al., 1992, Scholl and Hediger, 1994). Another historical cohort study of around 173,000 pregnant US women found an increased risk of preterm birth with low Hb levels in the first and second trimester. In this study, however, high Hb level (>14 g/dl) during the first and second trimester was associated with SGA (Scanlon et al., 2000).

The mechanism for the association between maternal IDA in early pregnancy and LBW or prematurity is not clear. However, a few mechanisms have been postulated such as the role of chronic hypoxia in increasing stress hormones production, increased oxidative stress and higher risk of maternal infections (Allen, 2001). The main clinical significance of LBW as an outcome is that it is associated with adverse health consequences both in the short and long terms. Adverse effects in the short term include increased risk of infant morbidity and mortality (Richardson et al., 1993). LBW is associated with around 60–80% of neonatal deaths in low income countries (Lawn et al.). In the long term, LBW has been shown to be strongly associated with increased

risk of CVD (Gluckman and Hanson, 2006, Forsén et al., 1999, Poulter et al., 1999, Rich-Edwards et al., 2005, Stene et al., 2001, Hyppönen et al., 2001, Reilly et al., 2005, Barker, 1995).

On the other hand, the relationship between maternal IDA in late pregnancy and birth outcomes seems to be different. In a prospective UK study, the risk of SGA birth was lower in women with IDA in the third trimester compared to non-anaemic women (Baker et al., 2009). A retrospective British study of about 154,000 women found that the lowest incidence of LBW and preterm birth occurred with Hb concentrations between 9.5 – 10.5 g/100 ml in the third trimester (Steer et al., 1995). High Hb concentration in late pregnancy has been shown to be associated with adverse birth outcomes including LBW and preterm birth. One suggested mechanism for this observed association is the potential for iron to lead to a high-level of production of reactive oxygen species and increased blood viscosity leading to reduced placental perfusion (Steer et al., 1995, Milman, 2006a, Casanueva and Viteri, 2003, Casanueva et al., 2006, Goldenberg et al., 1996).

2.2.5 Iron depletion without anaemia in pregnancy and birth outcomes

Not much research has explored the effect of iron depletion without anaemia on birth outcomes, particularly at the start of pregnancy. Ronnenberg et al. reported an association between preconceptional maternal ferritin with birth weight (Ronnenberg et al., 2004). Both low (<12 ug/l) and high (≥60 ug/l) ferritin were associated with lower birth weight. sTfR was not associated with adverse birth outcome, but elevated sF was associated with increased risk of LBW. A Spanish prospective study of 205

pregnant women found those with iron depletion (sF<12 ug/l) in the first antenatal visit delivered babies weighing around 200 g less than women without iron depletion, despite receiving iron supplements during gestation (Ribot et al., 2012).

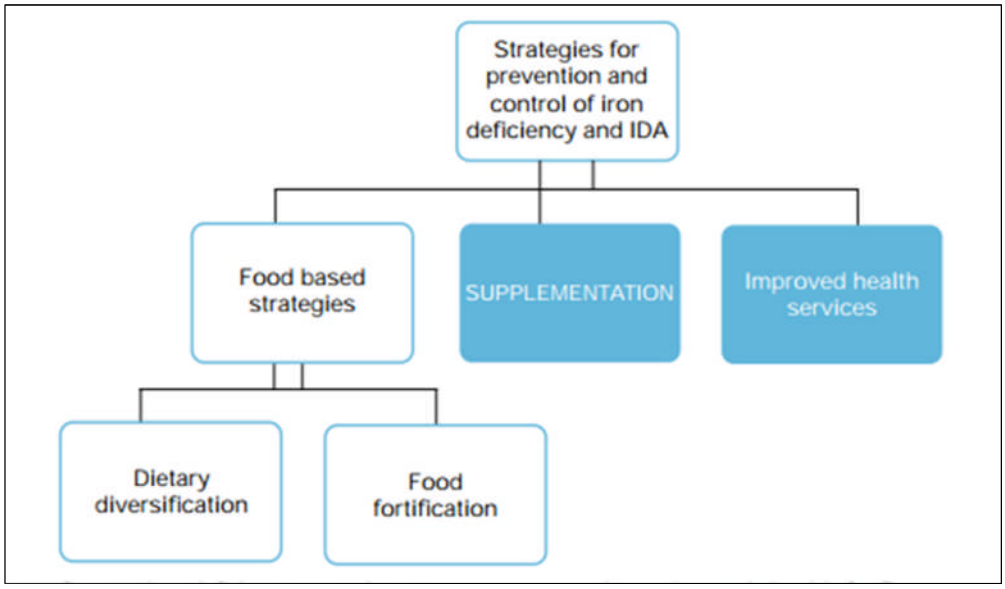
There is some evidence from another Spanish cohort, where all pregnant women are recommended iron supplements, linking maternal ID during pregnancy with neonatal neurological outcomes including general autonomous response, motor performance and self-regulation capabilities (Ribot et al., 2012). This could be a direct relationship or mediated by infant iron status. Maternal ID in pregnancy reduces fetal iron stores well after birth and into the first year of life. Insufficient iron accretion into liver storage as a result of preterm birth or maternal ID during pregnancy is a risk factor for developing infant ID (Kelleher, 2006).

2.2.6 Infant iron deficiency

Infant ID may have adverse effects on infant development (Allen, 2000). Perinatal ID and IDA during infancy has been associated with an increased risk of delayed psychomotor and cognitive development as well as behavioural problems into mid-childhood (Beard, 2008, de Ungria et al., 2000). Some studies suggest that these changes cannot be reversed with iron repletion therapy beyond 2 years of age (Grantham-McGregor and Ani, 2001). There is evidence that exclusively breast-fed infants of ID mothers are at higher risk of developing IDA, with weekly or daily iron supplementation in the first three months of life not reducing this risk (Yurdakök et al., 2004).

2.2.7 Dietary iron intake during pregnancy

In this section, I will review sources of dietary iron, dietary recommendations during pregnancy, associations of dietary iron with birth outcomes and some possible interventions to prevent and correct ID and IDA during pregnancy including fortification. Figure 3 illustrates the types of such interventions in the general population.



Source: Iron deficiency anaemia: assessment, prevention and control. A guide for programme managers (WHO, 2001).

Figure 3: Interventions to prevent and correct iron deficiency and iron deficiency anaemia

2.2.7.1 Sources of dietary iron

The modern British diet is thought to be lower in total iron than that consumed 30 years ago (Heath and Fairweather-Tait, 2002). Dietary iron occurs in two forms: haem, which comes from meat sources, and non-haem. Iron status is influenced by dietary iron intake, particularly haem iron, as well as factors affecting its bioavailability for absorption (Brussaard et al., 1997).

Recent evidence shows that haem and non-haem iron potentially have different effects on individual health outcomes (Tzoulaki et al., 2008). Haem iron, which comes from Hb and myoglobin in animal sources, has a higher bioavailability and is well-absorbed. Its absorption is further facilitated by organic compounds present in meat called meat-factors, and is influenced little by other dietary constituents. It also enhances non-haem iron absorption from other foods consumed at the same time (Skikne and Baynes, 1994).

About 95% of iron in the average British diet is in the form of non-haem iron (Food Standards Agency, 2003). The extent to which non-haem iron is absorbed is highly variable and depends on the individual's iron status and other dietary components. Vitamin C enhances non-haem iron absorption when consumed as part of a meal (Skikne and Baynes, 1994), while high calcium intakes from milk/dairy products during pregnancy may reduce non-haem iron absorption. In a sample of 576 pregnant women, ferritin concentrations fell with increasing calcium intake with the proportion of women with sF values $\leq$12 ug/l rising from 14% of the women in the lowest quarter of calcium intake to 29% of the women in the highest quarter (Robinson et al., 1998). Other potential inhibitors of non-haem iron absorption include phytate in cereal grains/wholemeal bread, oxalic acid in some vegetables such as spinach and beetroot, seeds and nuts, and polyphenols in tea/coffee (Skikne and Baynes, 1994). However, a recent analysis using data from 4 complete diet studies found that sF was the most important factor in explaining differences in non-haem iron absorption, and that between-person variations explain a large proportion of the differences in non-haem iron absorption (Armah et al., 2013). These findings were supported by a recent

systematic review, with diet having a stronger effect on absorption at low sF levels (Collings et al., 2013). It is worth noting that inhibitory effects of dietary components on iron absorption are extended to the absorption of iron supplements as well. The absorption of iron supplements has been shown to be 40% lower when taken with a meal (Brise, 1962).

Intake of animal tissue was shown to be directly related to women's iron stores (Heath et al., 2007). There is evidence that sF is only positively associated with haem iron intake and not non-haem or total dietary iron (Cade et al., 2005). Therefore the rising number of vegetarians in high income countries may increase the prevalence of ID. In addition to the absence of haem iron from their diet, vegetarians usually have a high intake of beans, lentils, cereals and fruit which contain inhibitors of non-haem iron absorption such as phytates and polyphenols (Hallberg, 2002). Consumption of a lacto-ovo vegetarian diet results in 70% lower non-haem iron absorption compared to a non-vegetarian diet (Hunt et al. 1999).

2.2.7.2 Dietary iron recommendations during pregnancy

In the UK, the Reference Nutrient Intake (RNI) for women aged 19-50 years is 14.8 mg/day with no specific recommended increment during pregnancy (Department of Health, 1991). The RNI is the amount of a nutrient that is enough to ensure that the needs of 97.5% of the population are being met. The Lower Reference Nutrient Intake (LRNI) is the amount adequate for only the small number of people who have low requirements (2.5%), which is 8 mg/day for iron in women of child-bearing age (Department of Health, 1991). National-level data on iron intake of pregnant women in the UK are not available, however the average intake in the UK for women of

childbearing age is below the RNI. The mean daily dietary intake of total iron from the 2001 National Diet and Nutrition Survey (NDNS) in Great Britain is 10 mg/day for women aged 19-64 years, with an average of 0.5 mg/day from haem-iron (Foods Standards Agency, 2003). Around 25% of all women aged 19-64 years, 41% of women aged <34 years and 53% of women receiving income-benefits had daily dietary iron intakes lower than the LRNI of 8 mg/day (Scientific Advisory Committee on Nutrition, 2008).

In the United States, the recommended iron intake during pregnancy is 27 mg/day (IOM, 2001). However, median iron intake in pregnant women was 15 mg/day in the US National Health and Nutrition Examination Survey (NHANES III) (National Center for Health Statistics, 1994). Relatively low iron intake is also seen in other European countries, for example, Denmark (Andersen et al., 1995). There is also evidence from a nutritional survey in Norway suggesting that women's dietary patterns do not change with pregnancy (Crozier et al., 2009, Trygg et al., 1995). In this survey, 96% of pregnant women had an iron intake <18 mg/day with an average iron intake of 11 mg/day (Trygg et al., 1995). In the 4th edition of Nordic Nutrition Recommendations (NNR), the recommended daily intake of dietary iron was 15 mg/day for women of childbearing age, including pregnant and lactating women, even though it stated that the physiological need of some women for iron during the last two thirds of pregnancy cannot be satisfied from food alone (without supplements) (Nordic Council of Ministers, 2004). The 5th edition of NNR did not change this recommendation due to insufficient available evidence (Domellöf et al., 2013).

The Scientific Advisory Committee on Nutrition (SACN) report on Iron and Health recommends reducing red and processed meat consumption due to links with colorectal cancer incidence (Scientific Advisory Committee on Nutrition, 2010). The report states that setting a maximum recommended intake of 70 g cooked meat/day would have little impact on the proportion of the adult population with low iron intakes. The above recommendation is largely based on evidence from prospective studies of diet and colorectal cancer in middle-aged participants. However, a pooled analysis of around 61,566 younger participants from the EIPC-Oxford cohort aged 35-69 years at baseline observed no significant difference in colorectal cancer incidence between meat eaters and vegetarians (Key et al., 2009). Therefore, recommending meat as the source of haem iron during the limited span of pregnancy is unlikely to have adverse effects in relation to the lifetime risk of colorectal cancer given the available research evidence and potentially positive benefits due to iron availability.

2.2.7.3 Iron fortification

Fortified foods may have the potential to reduce micronutrient deficiencies including iron during pregnancy (Yang and Huffman, 2011). In the Americas, iron-fortified flour is wide-spread (Walter et al., 2001). In the UK, the addition of iron to white and brown flour is mandatory (Heath and Fairweather-Tait, 2002). Breakfast cereals are commonly iron-fortified as well. However, fortification-iron is regarded to have poor bio-availability (Hurrell, 1997). Despite the increase in breakfast cereals consumption, the apparent decrease of red meat intake may be more influential on population iron levels as it has much better absorption (Heath and Fairweather-Tait, 2002). A study in Denmark found that 40% of women of child-bearing age had low iron status despite

having a national iron fortification program of flour (Milman et al., 2003). In order to meet the iron demand in pregnancy, women should make considerable changes in their dietary pattern which some argue to be unrealistic, hence the recommendation of iron supplements.

2.2.7.4 Dietary iron in pregnancy and birth outcomes

Few studies have explored the association of iron intake from dietary sources and birth outcomes compared to those that have explored the effect of iron supplements. This section attempts to review the available evidence linking dietary iron intake and two main birth outcomes, birth weight and preterm birth. The studies described below were retrieved as a result of an electronic database search (Appendix 10.1). The search was limited to the English Language and human research. A total of 654 papers were retrieved, and their titles were scanned for relevance. The references of retrieved relevant papers were also hand-searched.

There were no interventional studies found investigating these relationships. In terms of observational studies, there were five UK studies that examined the relationship of dietary iron intake in pregnancy with birth outcomes. A cohort study in London, which included 513 pregnant women at 12 weeks gestation and assessed intake via a 7-day food diary, found a social gradient in median iron intake from the diet ranging from 11.4 mg/day in women from social class V to 14.7 mg/day in women from social class I (Wynn et al., 1994). The median iron intake was 11.8 mg/day in mothers with babies weighing 3500-4500 g (n=165) and 9.8 mg/day in mothers of LBW babies (<2500 g) (n=28). The correlation between total dietary iron intake and birth weight was stronger

in mothers with babies weighing less than the median birth weight of 3270 g (r=0.25) compared to those weighing more than 3270 g (r=0.08) (Doyle et al., 1990).

Another British cohort study undertaken in Southampton measured dietary intake during pregnancy using a 100-item food frequency questionnaire (FFQ) and a 4-day diary (Godfrey et al., 1996). The women in this study had a mean iron intake (from food and supplements) of 15.5 and 16.8 mg/day in early and late pregnancy respectively. These levels were higher than that observed in other studies and the British NDNS levels (Food Standards Agency, 2003). For every 1 mg/day decrease in iron intake in late pregnancy, birth weight fell by 63 g. However, a cohort undertaken in Portsmouth showed no evidence of association between maternal iron intake and birth weight. This study used two methods to assess dietary iron intake; a 7-day food diary at antenatal booking, and a FFQ at 28 weeks gestation. The median iron intake was 10.2 mg/day from food alone and 10.8 mg/day in total using the diary, compared to 12.4 mg/day from food alone and 15.7 in total mg/day using the FFQ (Mathews et al., 1999). These results suggest that FFQs may lead to overestimation.

A prospective study in London and Manchester recruited 500 pregnant adolescents (14-18 years) and assessed micronutrient dietary intake by another dietary assessment method; three 24-hour recalls in the third trimester (Baker et al., 2009). The median intake of iron of 10.8 mg/day, including supplements, was similar to that described by Mathews et al. using the 7-day diary. Participants with dietary iron intake (from food only) below the lowest quartile of 7.7mg/day were almost 4 times more likely to deliver SGA infants (OR=3.7, 95% confidence interval (CI) 1.2, 11.8) (Baker et al., 2009).

The association of iron intake with smoking was examined in a London study that assessed maternal dietary intake using a 7-day weighed diary at 28 and 36 weeks gestation (Haste et al., 1991). The mean iron intake was 9.3 mg/day for smokers, 11.7 mg/day for non-smokers at 28 weeks, and 8.5 mg/day for smokers and 11.2 mg/day for non-smokers at 36 weeks. Lower dietary iron intake between 28 and 36 weeks of pregnancy was associated with higher risk of SGA, reinforcing the findings by Baker et al. However, there was no association detected in this study between iron intake and gestational age at delivery (Haste et al., 1991).

In terms of studies outside the UK, a prospective study of 826 pregnant women aged 12-29 years in Camden, New Jersey, ascertained dietary intake using two 24-hour recalls and supplement use by interview at each trimester of pregnancy. This study investigated the association between maternal iron status and intake. Lower sF was associated with lower iron intake in early pregnancy (Scholl and Hediger, 1994, Scholl et al., 1992). There was a mean difference of about 4.5 mg/day in iron intake between anaemic and non-anaemic women (defined by sF <12 ug/l). These associations were not observed in the third trimester. There was a significant association between ID and both LBW and preterm birth in this study. This is in contrast to another US study performed in Boston which found no evidence of association between iron intake and birth size. This study assessed micronutrients intake from food and supplements at 27 weeks gestation using a FFQ (Lagiou et al., 2005). A longitudinal study in Iran which assessed dietary iron intake among other micronutrients using a 24-hour recall found a significant positive association between iron intake in the third trimester of pregnancy with birth weight (Tabrizi and Saraswathi, 2011). Regarding other study designs such as

case-control studies, one study in New Zealand which assessed dietary intake at birth using a FFQ, reported an association of iron supplements only with a reduced risk of SGA (Mitchell et al., 2004). However, a study in Saudi Arabia, also estimating maternal iron intake from a FFQ administered after delivery, found no difference in mean birth weight between mothers with above and below the recommended dietary intake of iron of 30 mg/day using univariable analysis (Al-Shoshan, 2007).

There is recent evidence from a Korean birth cohort which included 1087 pregnant women of an effect modification of the relationship between maternal iron intake during pregnancy and birth weight by maternal genotype (*GSTM1* polymorphism) (Hur et al., 2013). This suggests that the potential effect of increasing dietary iron intake in pregnancy on offspring outcome might be selective depending on the mother's genotype. Table 1 summarises the characteristics of the studies discussed above.

2.2.7.4.1 Limitations of studies on maternal iron intake and birth outcomes

Many studies that assessed total iron intake did not model the relationships separately for iron from food and that from dietary supplements (Doyle et al., 1990, Godfrey et al., 1996, Mathews et al., 1999). Studies that combine dietary iron intake with a measure of iron status in pregnant women are rare (Scholl et al., 1992). These studies have not considered the potential different effects of haem and non-haem iron considering their different bioavailability profile. Also, the potential of effect modification by other micronutrients such as vitamin C has not been investigated. Some of the studies reviewed above had design and statistical analysis limitations which are likely to affect the generalisability of their results. The research described in chapters 3 and 5 of this thesis takes the above-mentioned limitations into account.

Study ID	Design	Study size (n)	Exposure assessment	Main outcome	Main finding
Al-Shoshan 2007	Retrospective	1771	FFQ* 24 hours after delivery	BW** (grams)	No evidence of association
Baker 2009	Prospective	500	three non-consecutive 24-hour recalls in trimester 3	SGA** & preterm birth	Higher SGA risk in lowest quartile of iron intake
Godfrey 1996	Prospective	538	Two FFQs at < 17 weeks and 32 weeks	BW & placental weight	Total iron intake positively associated with BW
Haste 1991	Prospective	169	7-day weighed food diaries at 28 & 36 weeks	SGA & preterm birth	Lower iron intake associated with higher SGA risk
Hur 2013	Prospective	1087	24-hour recall	BW (grams)	Positive association stratified by genotype (GSTM1)
Lagiou 2005	Prospective	222	FFQ at 27 weeks	BW (grams)	No evidence of association
Mathews 1999, 2004	Prospective	693	7-day food diary at booking & FFQ at 28 weeks	BW & placental weight (grams)	No evidence of association
Mitchell 2004	Case-control	844	FFQ at birth	SGA	Iron supplements associated with higher SGA risk
Scholl 1992, 1994	Prospective	826	Two 24-hour recall "early in pregnancy" 3 interviews at first, second & third trimesters	BW (grams) & preterm birth	Association between ID with low BW and preterm birth
Tabrizi 2011	Prospective	450	Three 24-hour recalls for each trimester	BW (kg)	Positive association
Wynn 1994, Doyle 1990	Prospective	513	7-day food diary at booking	BW (grams)	Total iron intake positively correlated with BW

*Food frequency questionnaire **Birth weight *** Small of gestational age

Table 1: Summary of studies of dietary iron intake in pregnancy and birth outcome

2.2.8 Iron supplementation in pregnancy

Iron supplements are widely recommended and used during pregnancy worldwide. Iron supplementation is commonly recommended from the second trimester of pregnancy because iron demand starts to increase at around that period. However, the timing of supplementation is critical in terms of evidence of effectiveness or harm.

2.2.8.1 Routine iron supplementation programmes

World Health Organization (WHO) guidelines for pregnant women recommend a standard daily dose of 60 mg of iron for 6 months or 120 mg iron daily if taken for less than 6 months. WHO also recommends weekly folic acid and iron (60 mg) supplementations to all women of reproductive age in areas were the prevalence of anaemia is higher than 20% of women in this age group , or higher than 40% in pregnant women (WHO, 2006).

In the USA, routine low-dose iron supplementation (30 mg/day) is recommended for all pregnant women (CDC, 1998). In Canada, the current recommendation is to provide supplements of ≈16 mg/day throughout pregnancy (Cockell et al., 2009). European Union guidelines recommend iron supplements in the second half of pregnancy (Commission of the European Communities, 1993). Recently, a review by the 5th Nordic Nutrition Recommendations concluded that 40-60 mg/day of iron supplementation should be offered from week 18-20 of gestation or earlier, depending on sF measured in early pregnancy. The purpose is to prevent ID and IDA at delivery as the review acknowledges that there is no evidence of benefit on the offspring (Domellöf et al., 2013).

In the UK, NICE does not recommend routine iron supplementation during pregnancy. Rather it recommends that Hb levels less than 11 g/dl in the first trimester and 10.5 g/dl at 28 weeks gestation are investigated by testing for sF levels and iron supplementation considered if indicated (National Institute for Clinical Excellence (NICE), 2008). The Scientific Advisory Committee on Nutrition's (SACN) Iron and Health Report supports the NICE guidelines in not recommending routine iron supplements to pregnant women (Scientific Advisory Committee on Nutrition, 2010). However, there is no recommendation for detection or intervention in pregnant women who are iron-deficient but not anaemic.

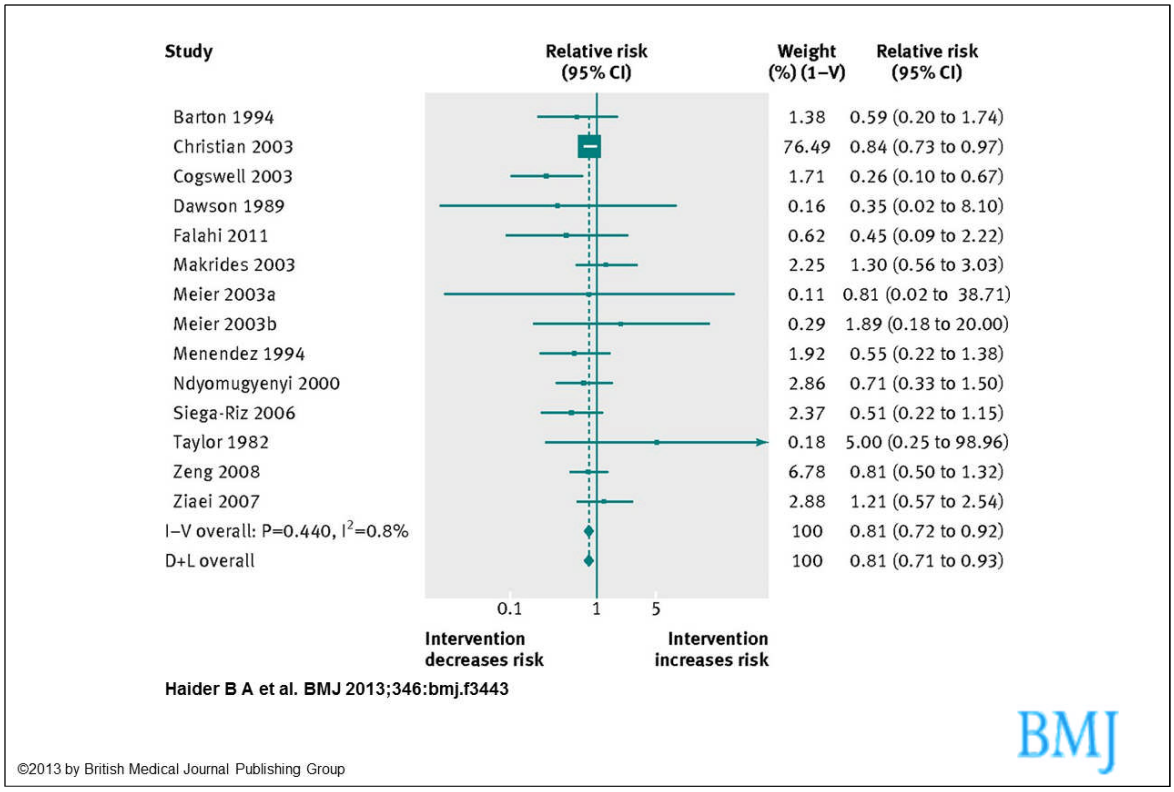
2.2.8.2 Experimental evidence of effectiveness/harm of iron supplementation

Most randomised controlled trials of iron supplementation in pregnancy have concentrated on maternal indices of ID and/or anaemia as outcomes. Many have shown positive effects on maternal iron status (Pena-Rosas and Viteri, 2009). One randomised controlled trial (RCT) showed that a supplement of at least 40 mg/day iron from 18 weeks gestation onwards prevented IDA in 90% of women during pregnancy and post-partum (Milman et al., 2005). However, other studies show that routine iron supplementation of non-anaemic women in pregnancy does not prevent iron depletion by late pregnancy, particularly in women who start their pregnancy with inadequate iron stores (Ribot et al., 2012, Cogswell et al., 2003).

The evidence surrounding the effects of iron supplements in pregnancy on infant outcomes is less consistent (Scholl and Reilly, 2000, Rasmussen, 2001, Scholl, 1998, Haram et al., 2001, Lao et al., 2001, Allen, 2000). The evidence in favour of iron

supplementation to increase average birth weight and significantly reduce the incidence of LBW is observed more clearly in studies conducted in low income country settings where the prevalence of ID is substantially high (Mishra et al., 2005). Many studies examining the relationship between routine iron supplements and birth weight have not differentiated between iron-deficient and non-iron-deficient mothers. However, one small study found that supplementation with iron during pregnancy improves newborn birth weight only in those women who start pregnancy with ID (Aranda et al., 2011). A Cochrane review found that a mother is less likely to have IDA if taking iron supplements. Higher infant ferritin concentrations at 3 and 6 months and birth length were also observed in the supplemented group. However, it concluded that the evidence for benefit was inconclusive in relation to clinically-important infant outcomes (Pena-Rosas and Viteri, 2009).

A placebo-controlled RCT in Hong-Kong published after the Cochrane review showed favourable effects of taking iron supplements in the second trimester on birth weight and incidence of SGA (Chan et al., 2009). In a recent systematic review by Haider et al. of 48 RCTs and 44 cohort studies, iron supplement intake during pregnancy was associated with higher Hb levels and lower risk of anaemia in the mother, and lower risk of LBW in the infant (Figure 4). There was also a dose-response positive relationship between iron dose and birth weight. However, intake of iron supplements was not associated with duration of gestation, risk of preterm birth or SGA births (Haider, 2013).



I–V=inverse variance method D+L=DerSimonian and Laird method

Source: Anaemia, prenatal iron use, and risk of adverse pregnancy outcomes: systematic review and meta-analysis (Haider, 2013).

Figure 4: Forest plot for effect of iron supplement use on low birth weight

2.2.8.3 Potential adverse effects of iron supplements

Despite the inconclusive evidence on the benefit of routine iron supplementation during pregnancy on infant outcomes, it remains widely recommended on a national and international level. The assumption underlying this recommendation is that supplementation, even if not beneficial, would be harmless to mother and baby. However, based on some emerging research evidence, this assumption may not be true. There are potential drawbacks of taking routine iron supplements, particularly in late pregnancy, that need to be weighed against the benefits of correcting maternal IDA. High-dose iron supplements (>100 mg/day) are associated with side-effects such as nausea, vomiting and constipation (British Medical Association and Royal

Pharmaceutical Society of Great Britain, 2010). The United States' Institute of Medicine has established an upper tolerable dose of 45 mg/day to minimize the risk of side-effects (IOM, 2001).

In addition to gastrointestinal side effects, iron can inhibit the absorption of other minerals such as manganese, copper and zinc (O'Brien et al., 2000, Rossander-Hulten et al., 1991, O'Brien et al., 1999, Gambling et al., 2011). There is concern that giving iron supplements could lead to other micronutrient deficiencies by competing for gastrointestinal uptake (Langley-Evans, 2009). Hence, the interaction of different micronutrients needs to be taken into consideration, and the potential for negative interactions is likely to increase if iron is taken as part of a multivitamin-mineral supplement (MVM). Iron supplements can also reduce the absorption of dietary non-haem iron (Roughead and Hunt, 2000), and can increase oxidative stress and the production of free radicals (Casanueva and Viteri, 2003, Scholl, 2005). It has also been shown that iron transfer to the fetus is better in non-iron-supplemented than in supplemented women (O'Brien et al., 2003). A better understanding of iron regulation and its interactions with other micronutrients would be useful to maximise effectiveness of iron supplementation programmes.

Iron in supplements can be found in two forms: ferrous and ferric. Ferrous iron supplements such as ferrous fumarate, ferrous sulphate, and ferrous gluconate are better absorbed than ferric iron (Hoffman et al., 2000). The amount of iron available for absorption varies according to the supplement with ferrous fumarate having the highest amount of elemental iron available for absorption. As the dose of iron in the supplement increases, the proportion of iron absorbed decreases so that if iron

supplements are recommended they should be taken in two or three equally spaced doses.

About 10-15% of Northern European population is heterozygous for the common mutations in the *HFE* gene that predispose to iron overload (Merryweather-Clarke et al., 2000). Blanket routine supplementation may be harmful in women with this genetic predisposition (Zoller and Cox, 2005). Individually-tailored use of iron supplements according to blood indices for iron status may be recommended to avoid the potential harms (Milman, 2006b). However, this may not be cost-effective on a population level.

It has been postulated that iron supplements in ID women may increase the risk of oxidative damage and haemoconcentration (Lynch, 2011). Studying animal models suggests that glucose tolerance is reduced by iron supplementation (Dongiovanni et al., 2008). Links have been made between iron intake and the risk of type 2 diabetes and gestational diabetes (GDM) (Rajpathak et al., 2006, Qiu et al., 2011, Bowers et al., 2011). However, these are mainly observed for haem iron from meat sources and therefore the relationship could be between meat consumption and diabetes risk rather than iron *per se*. Evidence from a RCT in Iran showed that iron supplementation in women with Hb >13.2 g/dl in the second trimester is positively linked to gestational hypertension, as well as an increase in the risk of SGA birth (Ziaei et al., 2007). However, this association was not supported by another RCT which found no link between iron supplements in the second trimester and GDM (Chan et al., 2009). In other studies, multiple biomarkers of iron status were also elevated in pre-eclamptic compared to healthy pregnant women (Rayman et al., 2002).

2.2.8.4 The case for selective iron supplementation

Taking account of the above, some experts caution the administration of iron supplements to iron-replete women, especially those at risk of pregnancy complications such as pre-eclampsia and GDM (Weinberg, 2009), and those who are genetically predisposed to iron overload. It seems that any positive effects of iron supplementation on infant outcomes such as birth weight are enhanced the earlier iron supplements are taken in pregnancy (Agarwal et al., 1991). Therefore, individually-tailored use of iron supplements according to blood indices for iron status in early pregnancy such as ferritin levels can avoid any potential harms of mass supplementation to all pregnant women regardless of their iron status (Milman, 2006b).

One of the dilemmas of selective iron supplementation is the choice of a cut-off point beyond which iron supplements potentially have no benefit on health of the mother and baby. It has been suggested that pregnant women with sF >70 ug/l have no need for iron supplements (Milman et al., 2006). However, clinically significant cut-off points may vary according to stage of pregnancy. Also, selective supplementation may be considered unrealistic and not cost-effective for health services. There is a need for further research into the effectiveness, cost-effectiveness and feasibility of such selective supplementation policies in high and middle income countries. Routine iron supplementation to all pregnant women seems to be the most effective option in low income countries due to high prevalence of ID and anaemia.

2.3 Maternal iron in pregnancy and cardiovascular disease risk in the offspring

Fetal life is a period of rapid development. Inadequate or imbalanced maternal nutrition during this period can alter physiological structures and/or functions leading to an increased risk of chronic disease in later life (Hanson et al., 2009). Fetal growth and development is likely to be most sensitive to maternal dietary deficiencies during early pregnancy (Wu et al., 2004). Birth weight has been strongly linked to CVD morbidity and mortality (Barker, 2004). The initial Barker and colleagues finding 25 years ago of an association between weight at birth and mortality from ischaemic heart disease has resulted in extensive research into the developmental origins of health and disease (Lawlor and Davey Smith, 2005, Barker et al., 1989). However, the epidemiological evidence for the association between fetal growth during pregnancy and adult chronic disease risk still comes mainly from studies that have used birth weight as a proxy for fetal nutrition during pregnancy (Scientific Advisory Committee on Nutrition, 2011). There is a gap in the research evidence with regards to examining the relationship between maternal micronutrient status, including iron, during pregnancy and CVD outcomes and risk factors in the offspring. In this thesis, the markers of cardiovascular status used to assess outcomes are discussed in section 2.5.

2.3.1 Experimental studies

Maternal ID during pregnancy in animal models results in the development of offspring obesity, hypertension and other adverse cardiovascular outcomes in the long-term (Zhang et al., 2005, Gambling et al., 2003a, Lisle et al., 2003, Andersen et al., 2006,

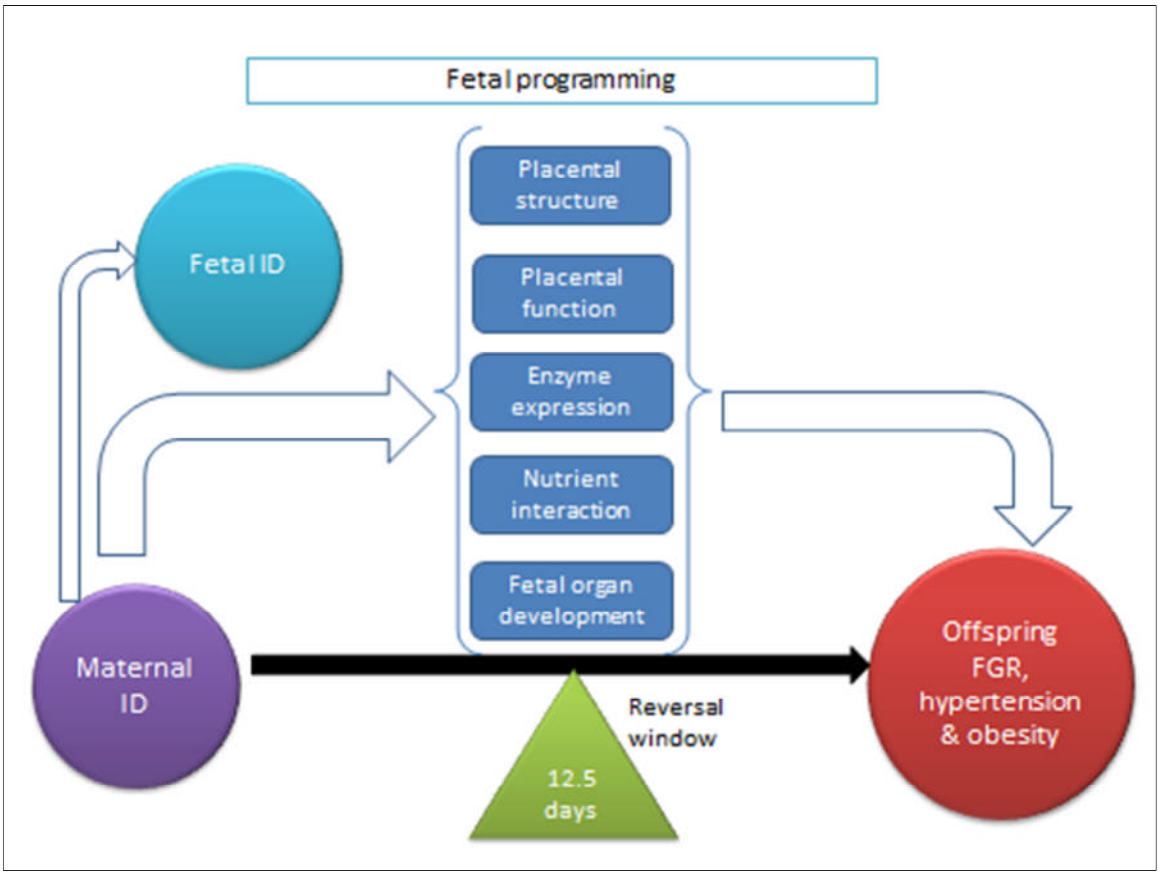
Gambling et al., 2004b, Gambling et al., 2003b, Crowe et al., 1995). This effect is observed even when the pups maintain normal iron levels throughout postnatal life (Gambling et al., 2003b, Gambling et al., 2003a, Crowe et al., 1995, Gambling et al., 2004b). The effect of maternal ID seems to be strongest when it is present in early pregnancy. In one study, diet-induced IDA in rhesus monkeys by the third trimester of pregnancy did not have an effect on growth or neurological development of the offspring (Golub et al., 2006).

Pups of rat models fed an iron-deficient diet prior to and throughout pregnancy have higher mortality rate, are born smaller, have larger hearts and smaller kidneys and spleens. Raised BP in males born to mothers in the intervention group was observed despite the offspring having a normal iron status (Gambling et al., 2003b). In another study, systolic BP was raised in both males and female offspring of iron-restricted dams at 3 months of age (Lewis et al., 2001). The postnatal rise in systolic BP associated with maternal anaemia during pregnancy was not related to the greater placental to birth weight ratio (Crowe et al., 1995). Iron supplementation during early, but not late, pregnancy reverses the effect of ID on birth size and the expression of iron metabolism genes (Gambling et al., 2004a).

2.3.2 Postulated biological mechanisms for the association between maternal ID and CVD risk in the offspring

Evidence from animal studies shows that birth weight is dependent on the mother's iron status and not that of the neonate (Gambling and McArdle, 2004). Thus, maternal ID may affect fetal development by indirect mechanisms (Figure 5). ID-induced changes in maternal metabolism may have downstream effects on placental structure

and function, enzyme expression, nutrient interactions and fetal organ development (Gambling and McArdle, 2004, McArdle et al., 2006). These potential biological pathways are reviewed below.



*FGR: fetal growth restriction

**Reversal window of adverse offspring outcomes if iron is administered by 12.5 days of rat gestation (Andersen et al., 2006)

Figure 5: Potential biological pathways for the observed effect of maternal iron deficiency on offspring CVD risk

2.3.2.1 Placental structure and function

The placenta is the principal organ that delivers nutrients to the developing fetus. Therefore, any stress that interferes with placental development or function is likely to have adverse consequences for the developing fetus. The placental structure is altered in maternal anaemia. Changes due to maternal IDA include reduced capillary length

and surface area and increased placental vascularisation at term (Lewis et al., 2001, Gambling et al., 2003a). The surface area of capillaries involved in gas exchange is strongly and inversely related to maternal sF concentrations (Steer et al., 1995). Maternal anaemia has also been shown to be associated with increased placental weight and the ratio of fetal weight to placental weight (Godfrey et al., 1991, Ronnenberg et al., 2004). The relationship between maternal ID with placental size and birth weight exists across the normal range for these measures and is not just restricted to severely anaemic mothers. This increase in placental weight has been interpreted as compensatory placental hypertrophy.

In terms of placental function, increased cytokine, leptin and tumour necrosis factor levels in the placenta have been associated with ID. Maternal ID has also been shown to cause fetal plasma amino acid and cholesterol and triacylglycerol levels to be decreased, suggesting decreased placental transport of amino acid and non-esterified fatty acids to the fetus (Lewis et al., 2001).

2.3.2.2 Enzyme expression

ID may reduce the activity of the enzymes that use iron as a cofactor, for example enzymes involved in neurotransmitter synthesis and neuronal energy (Gambling and McArdle, 2004). There is evidence that maternal ID leads to a reduction in γ-aminobutyric acid metabolism that is not reversible by postnatal iron supplementation (Cockell et al., 2009). In addition, areas of the brain that are involved in higher cognitive functions have lower cytochrome *c* oxidase activity in neonatal rats born to ID mothers (Vijayaraghaven, 2004)

2.3.2.3 Nutrient interactions

The effect of altered iron status on the metabolism of other metals, such as copper, and mediators of cell function during pregnancy have been observed (Gambling et al., 2003a). Generally, ID results in increased copper levels in the liver and rise in serum ceruloplasmin concentrations (Gambling and McArdle, 2004). In pregnancy, maternal ID has a differential effect on copper metabolism in the mother and fetus (Gambling et al., 2004b). In the maternal liver, copper levels are inversely correlated with those of iron, while in the fetus both iron and copper levels are reduced. A similar differential effect between mother and fetus is also seen in vitamin A metabolism. Maternal liver retinol levels are reduced in maternal ID, while in the fetus the opposite is seen, as the level of iron decreases the levels of retinol in the fetal liver increase (Gambling et al., 2001). This restriction in nutrient supply may have an impact on fetal development.

2.3.2.4 Fetal organ development

ID may also interfere with normal fetal kidney development by reducing nephron number (Gambling and McArdle, 2004). This may result in the observed association between maternal ID and high BP in the offspring as kidney nephron number is an important determinant of BP. Nephron number is established during kidney development, beyond which point the number cannot be increased (Golub et al., 2006). Low nephron number reduces the surface area available for filtration, and therefore limits the ability of the kidney to excrete sodium and maintain normal extracellular fluid volume and BP (Brenner et al., 1988). Expanding on their earlier work with the Wistar ID model, Hales and colleagues (Lisle et al., 2003) have investigated the effect of maternal ID on the renal morphology of the adult offspring in

rats. Their results show a reduction in the number of glomeruli in the kidney of offspring born to ID mothers. Offspring from both control and ID mothers also show an inverse relationship between glomerular number and BP.

2.3.3 Epidemiological studies

There is some indirect epidemiological evidence supporting the associations observed in animal studies described above. Godfrey et al. found that maternal ID during pregnancy in humans is associated with a high ratio of placental weight to birth weight, which is considered a predictor of adult hypertension (Godfrey et al., 1991). The relationship between maternal iron status in pregnancy and children's BP has been directly investigated in few studies. All of them have used maternal Hb as a proxy for maternal iron status in pregnancy, with two of them including iron intake from diet and supplements as an additional marker for maternal iron status (Belfort et al., 2008, Brion et al., 2008)**.** Table 2 summarises the characteristics of studies that have investigated the associations of indicators of maternal iron status in pregnancy with long-term health outcomes in the offspring.

Brion et al. analysed data from the ALSPAC cohort with a sample size of 1255 women in Bristol, UK with Hb information. In this study, there was an association between maternal anaemia with lower offspring systolic BP at 7 years only in women who did not take iron supplements during pregnancy (Brion et al., 2008). This is a direction of association opposite to what is expected from animal study findings. This study will be further discussed in chapters 5 and 7, in comparison with analysis included in this thesis using data from the same cohort.

In another study with a sample size of 1167 pregnant American women, there was no association between first and second trimester maternal Hb and anaemia with offspring BP at 3 years. However, offspring BP was positively associated with first trimester iron intake, again in contrast to animal studies findings. No relationship was observed in this study between second trimester iron intake and offspring BP (Belfort et al., 2008).

In a follow-up of a calcium supplementation trial in pregnancy in Argentina, Bergel et al. found a positive association between maternal Hb during pregnancy and offspring systolic BP at 5-9 years (Bergel et al., 2000). In contrast, Law et al. found an association between maternal anaemia in pregnancy (<10 g/dl) and higher offspring systolic BP at 4 years of 405 British children (Law et al., 1991). Godfrey et al. also found a negative association between systolic BP of 77 Jamaican children with an average age of 11 years and lowest maternal Hb during pregnancy (Godfrey et al., 1994). Whincup et al. found no evidence of association between lowest maternal Hb and change in mean corpuscular volume (MCV) during pregnancy with BP at 9-11 years of 662 children (Whincup et al., 1994).

To summarise, the direction of association between maternal Hb and offspring BP during childhood in two of these studies was against that expected from the results of animal studies as discussed in section 2.3.1 (Brion et al., 2008, Bergel et al., 2000). Two studies supported the direction of association observed in animal studies, i.e. maternal anaemia associated with higher offspring BP (Law et al., 1991, Godfrey et al., 1994), while the remaining two found no association (Belfort et al., 2008, Whincup et al., 1994). However, it is important to note that all of these studies did not use a direct

biomarker of iron status such as sF, and only two of them assessed maternal iron intake as an exposure ((Belfort et al., 2008, Brion et al., 2008).

In terms of other offspring health outcomes, data from the ALSPAC study on maternal *HFE, TF* and *TMPRSS6* genotypes were used as instrumental variables to investigate the association of maternal iron status and offspring's IQ at 8 years using a Mendelian randomization (MR) design (Lewis et al., 2014). The study found strong associations between maternal Hb levels in pregnancy and the selected genotypes. However, there was no evidence of association between offspring's cognitive function at 8 with either maternal genotype or Hb levels in pregnancy.

Study ID	Design	Study size (n)	Exposure assessment	Main outcome	Main finding
Belfort 2008	Prospective cohort	1,167	Semi-quantitative FFQ* in the first second trimesters, Hb & MCV** extracted from electronic laboratory database	Offspring BP# at 3 years	Maternal iron intake positively associated with Offspring BP
Brion 2008	Prospective cohort	1,255 (Hb) 7,484 (supplement) 7,130 (diet)	FFQ at 32 weeks Questionnaires at 18 and 32 weeks for supplements Hb extracted from medical records	Offspring BP at 7 years	Maternal anaemia associated with lower offspring BP in women not taking supplements
Law 1991	Historical cohort	405	Lowest Hb during pregnancy extracted from medical records	Offspring BP at 4 years	Maternal anaemia associated with higher offspring BP
Godfrey 1994	Prospective cohort	77	Lowest Hb out of up to 6 measurements throughout pregnancy	Offspring BP at 10-12 years	Negative association
Whincup 1994	Historical cohort	662	Lowest Hb in pregnancy and change in MCV extracted from medical records	Offspring BP at 9-11 years	No association
Bergel 2000	Prospective RCT# follow-up	518	Lowest Hb during pregnancy recorded in the trial	Offspring BP at 5-9 years	Positive association
Lewis 2014	Prospective cohort, Mendelian randomization	11,696	Maternal genotype at single nucleotide polymorphisms in the *HFE, TF* and *TMPRSS6* genes as instrumental variables	Offspring cognitive function measured by a shortened version Of The Wechsler Intelligence Scale For Children (WISC-III)	No association

* Food frequency questionnaire
** Mean corpuscular volume
#Blood pressure #Randomised controlled trial

Table 2: Characteristics of epidemiological studies investigating the association of maternal iron status and long term offspring health outcomes

2.4 Measures of iron status

The importance of iron status in pregnancy has been discussed earlier in this chapter in section 2.2. There are several biomarkers used to assess iron status. A nutritional biomarker is a biological specimen that acts as an indicator of nutritional status with respect to either intake and/or metabolism of dietary constituents (Potischman and Freudenheim, 2003). Below is a review of iron status biomarkers used in this thesis and their pros and cons.

2.4.1 Haemoglobin

The use of functional indices as biomarkers is useful when intake and status are sufficiently compromised to cause physiological and biochemical disturbances (Hambidge, 2003). An example is the use of Hb as an indicator of IDA (Zimmermann, 2008, Zimmermann and Hurrell, 2007). Hb is easy to measure and relatively cheap. Monitoring it is part of routine antenatal care for all pregnant women in most countries. However, this measure lacks sufficient sensitivity and specificity as there are other causes of anaemia that may not be related to ID or may coincide with it, including other nutritional deficiencies such as folic acid and vitamin B12, chronic inflammation, infections and hereditary causes (Rasmussen, 2001). It is also possible to be iron-deficient but not to the level of causing anaemia as 20-30% of body iron could be lost before Hb falls below the specified cut-off for the diagnosis of anaemia (Cook, 2005) (Figure 2).

2.4.2 Serum ferritin

The use of body stores as a measure of nutrient status is appropriate to use with iron, as it is stored when intake in generous and released when intake is less adequate or demand is increased, as for example in pregnancy. Circulating sF levels have a strong positive correlation with tissue iron stores (Cook, 2005). sF is the most widely used biomarker in the assessment of iron status. It is the second line of investigation of anaemia in pregnancy. Levels under 12 ug/l indicate absent iron stores i.e. iron depletion. In the presence of anaemia, such a level is diagnostic of IDA (Zimmermann, 2008).

However, ferritin is considered an acute phase protein and can be elevated in inflammatory conditions independent of body iron stores, thus affecting test sensitivity. Levels between 12-100 ug/l are difficult to interpret because inflammation even in the presence of ID can cause elevation of sF (Worwood, 1997). It is considered unreliable as a test for iron status in conditions of malignancy, heavy alcohol intake, thyroid and liver disease (Zimmermann, 2008). In the diagnosis of IDA, a practical approach to minimize this problem is to use a screening test for inflammation such as C-reactive protein to try and exclude the false negatives (Cook et al., 2003). In pregnancy, ferritin is considered a marker of infection such as sepsis, which is associated with premature rupture of membranes leading to preterm birth. This may limit ferritin's use as an accurate marker of iron status to use in investigating the associations between maternal iron status and birth outcomes (Goldenberg et al., 1998, Scholl, 1998).

2.4.3 Serum transferrin receptor

Circulating sTfR is a glycoprotein which constitutes the soluble form of the membrane receptor produced by proteolytic cleavage (Baynes, 1996). It transfers circulating iron into developing red cells (Zimmermann, 2008). Both the expression of TfR on the cell surface and the concentration of the soluble TfR are inversely related to the supply of iron reaching the cell membranes and the level of intracellular iron (Baynes, 1996, Hambidge, 2003). The advantage of sTfR over sF is that it can distinguish IDA from anaemia of chronic inflammation and it can identify iron depletion and functional ID in patients with concurrent inflammation (Allen et al., 1998).

IDE is the most common cause of raised sTfR. Depleted iron stores without IDE is not associated with raised sTfR, and is best indicated by low sF level. As ID progresses beyond depletion of iron stores into negative iron status, with inadequate iron supply from erythropoiesis, sTfR levels begin to rise, and continue to rise as IDE progressively worsens prior to the development of anaemia (Skikne, 2008).

The use of sTfR as a measure of iron status is limited by the current availability of several commercial assays that yield different values. There is a pressing need to calibrate sTfR assays against international reference standards to provide comparability across studies.

2.4.4 Serum transferrin receptor to serum ferritin ratio (R/F)

R/F ratio quantifies the entire spectrum of iron status from positive iron stores through to negative iron balance, and is particularly useful in evaluating iron status in population studies (Skikne, 2008). In the absence of inflammation, it is the most

sensitive method to assess iron status as it combines the use of sF as a measure of iron stores and sTfR as a measure of tissue ID (Zimmermann, 2008, Akesson et al., 1998, Carriaga et al., 1991, Rusia et al., 1999).

A study set out to determine the diagnostic value of R/F ratio against bone marrow aspirate examination found that it had the best diagnostic efficiency with a sensitivity of 81% and a specificity of 97%. SF alone with a cut-off of 60 ug/l had the same specificity but lower sensitivity (76%) (Ruivard et al., 2000). A close linear relationship was demonstrated between the logarithm of the concentrations, in micrograms per litre, of R/F ratio with body iron as calculated form the Hb iron after correction for the absorption of dietary iron (Cook et al., 2003). However, the use of R/F ratio as a gold standard measure of iron status is still limited by the lack of standardisation of the different commercial sTfR assays.

2.5 Measures of cardiovascular disease risk

Earlier in this chapter, the evidence around the association between maternal iron status and offspring cardiovascular risk has been discussed (section 2.3). Several measures of CVD risk indicators are used as outcomes in the included studies in this thesis. Below is an evaluation of those indicators and the rationale behind using them.

2.5.1 Pulse wave velocity

Stiff arteries increase the velocity at which the pulse wave travels across the vascular tree, resulting in earlier return of the reflected wave from peripheral sites. This leads to suboptimal ventricular-arterial interaction and increased left ventricular afterload (Cheung, 2010). PWV provides a measure of arterial stiffness which is a convenient,

precise and reliable. It can be used as an integrated index of vascular pathology over the life-course (Cruickshank et al., 2009). PWV is the speed at which the forward pressure of flow wave is transmitted from the aorta through the arterial tree. Measuring PWV over an arterial segment assesses the stiffness along the length of it or regional stiffness (Cheung, 2010). PWV is inversely related to arterial distensibility. The stiffer the artery, the faster is the pulse wave. It is determined by dividing the distance of the pulse travel, between two arterial sites, by the transit time (Cheung, 2010). .

2.5.1.1 Pulse wave velocity versus pulse wave analysis

The augmentation index is a measure of central pulse wave analysis. It is the difference between the first and second peaks of the central arterial waveform, expressed as a percentage of pulse pressure. It is determined by aortic PWV, age, height and diastolic BP (Laurent et al., 2006). It is negative in healthy young adults but becomes increasingly positive as arteries stiffen (the lower the AI is the better). PWV, as a measure of arterial elasticity, discards information about the shape of the pulse wave. Pulse wave analysis on the other hand, gives information about arterial elasticity and wave reflection distal to the measurement site and pulse pressure proximal to it. Both approaches are complementary. PWV as a means of estimating arterial elasticity is based on a well-understood and tested theoretical model. It does not permit quantification of functional stiffness *per se* but provides information about wave reflection and pulse pressure (Greenwald, 2002).

2.5.1.2 Pulse wave velocity as a predictor of cardiovascular disease

Prediction of CVD morbidity and mortality can be realized through studying arterial stiffness. Structural vascular abnormalities, in particular increased arterial stiffness,

have been considered early markers of accelerated vascular aging. Arterial stiffness is a strong independent cardiovascular risk factor in adults (Meaume et al., 2001). Vascular dysfunction plays a key role in the pathophysiology of hypertension. Arterial stiffness, measured non-invasively by PWV in adults, have been associated with systemic hypertension and left ventricular hypertrophy (Ligi et al., 2010). Therefore, PWV has been widely used as a predictor of CVD in adults (Weber et al., 2008, Jadhav and Kadam, 2005), and is associated with higher CVD mortality, coronary heart disease, and stroke (Vlachopoulos et al., 2010, Sutton-Tyrrell et al., 2005). Age is a major determinant of PWV, and its influence should be taken into account when using PWV as a marker of cardiovascular risk (Tomiyama and Yamashina, 2004). However, it is still not determined whether PWV measured in infancy is a predictor of long term cardiovascular health.

2.5.1.3 Developmental origins of arterial stiffness

Increased arterial stiffness measured in children, adolescents and young adults, has been correlated with LBW, which points towards an influence of the fetal developmental journey on arterial wall structure and function later in life . However, the relationship seems to be complex, and there is considerable heterogeneity in the evidence according to which measure of arterial stiffness was used, at which age arterial stiffness was assessed, and the degree of prematurity and LBW considered.

Oren et al. found an inverse association between gestational age and carotid-femoral PWV measured in young adults, but a positive association with birth weight, which was attenuated when excluding offspring of diabetic mothers (Oren et al., 2003). Lurbe et al. used augmentation index as a measure of pulse wave reflection in 7-18 year old

children and adolescents and found it to be higher in those born with LBW (Lurbe et al., 2003). Cheung et al. found that among 8 year old children who were born preterm, only those with intrauterine growth retardation (IUGR) had increased brachio-radial PWV (Cheung et al., 2004). In another study, carotid-radial PWV in adolescents was higher in those born preterm compared to those born at term (Rossi et al., 2011). A negative correlation was observed between carotid artery stiffness measured by ultrasound in 9-year old children and birth weight, despite observing no differences in arterial stiffness index between the normal weight and LBW groups (Martin et al., 2000). On the other hand, Salvi et al. also found no evidence of a linear relationship between PWV at 16-19 years of age and birth weight, though there was a positive relationship with LBW (Salvi et al., 2012). However, in another study there was no association observed between carotid and aortic arterial stiffness measured by ultrasound in 7-12 year old children with preterm birth or SGA (Bonamy et al., 2008, Bonamy et al., 2005). Schack-Nielsen et al. also reported a null association between aorto-radial and aorto-femoral PWV in 10 year old Danish children and birth weight, but arterial stiffness was positively associated with duration of breastfeeding (Schack-Nielsen et al., 2005).

In adults, the evidence is also inconsistent. Some found no evidence of association between birth weight or LBW and arterial stiffness in adulthood with an age range of 25 years to middle age (Montgomery et al., 2000, Te Velde et al., 2004, Kumaran et al., 2000), while a study of 55 year olds found that arterial compliance was lower in those who had been small at birth (Martyn et al., 1995).

The mechanism of association between LBW and increased arterial stiffness in childhood and adulthood remains unclear. Endothelial dysfunction in preterm and SGA babies suggests that functional alterations in arterial tone may contribute to it (Cheung, 2010). Therefore, this relationship may not be apparent when using structural methods to assess arterial stiffness such as PWV. Another proposed mechanism is altered synthesis of elastin in the arterial wall (Cheung, 2010). Arterial stiffness could be caused by a deficiency of in elastin synthesis in the wall of large arteries (Martyn and Greenwald, 1997). Elastin normally accumulates in the late prenatal period and its synthesis falls rapidly after birth (Ligi et al., 2010). Decreased elastin content in the umbilical arteries, which are direct branches of fetal iliac arteries, was associated with increased arterial stiffness in one study (Burkhardt et al., 2009). Gestational age was observed to be correlated with elastin content. Reduction in the elastin content of umbilical cord arteries has been shown to be associated with both preterm and SGA infants (Ligi et al., 2010).

In summary, there is some evidence linking size at birth and prematurity with arterial stiffness later in life. However, the methods used to assess arterial stiffness in these studies vary significantly, as does the age at which arterial stiffness was assessed. These issues render the studies quite heterogeneous and therefore difficult to draw solid conclusions about the magnitude of the association and potential thresholds in birth weight or gestational age at which adverse programming is likely.

2.5.1.4 Association between arterial stiffness and other cardiovascular markers in children

In the Lifestyle of Our Kids Study, carotid-femoral PWV was positively correlated with BMI, percentage body fat and WC in children with an average age of 10 years (Sakuragi et al., 2009). Brachial-ankle PWV in healthy adolescents was found to be associated with BP, BMI, percent body fat, waist-to-height ratio, sex, homocysteine levels, and metabolic risk variables (triglycerides, high-density lipoprotein cholesterol, atherogenic index, glucose, insulin, and insulin resistance) (Miyai et al., 2009, Im et al., 2007). PWV was associated with physical activity and dietary fat energy percentage in 10 year old Danish children. (Schack-Nielsen et al., 2005). A study which measured carotid-radial PWV in 33 obese adolescents and 18 lean controls (average age 14 years) found lower PWV in the obese group which might reflect general vasodilatation (Dangardt et al., 2008).

2.5.1.5 Studies of arterial stiffness in infants

Few studies measured arterial stiffness in infants. Akira et al. assessed aortic stiffness in infants using an index measured by ultrasound to estimate the aortic systolic and diastolic diameters and their correlation with BP, and found that it increased with gestational age at birth (Akira and Yoshiyuki, 2006). Using pulse pressure measurement (distensibility coefficient, and whole body arterial compliance measured by ultrasound and recording of aortic pulse pressure), increased arterial stiffness was observed as early as the fifth day of life in very LBW premature infants, and persisted at least until the 7^{th} week of life (Tauzin et al., 2006). Skilton et al. observed an increased aortic wall thickness, a marker of atherosclerosis, in SGA infants (Skilton et

al., 2005). Sehgal et al. calculated arterial wall stiffness index in appropriate for gestational age (AGA) and SGA infants, and found an increase with very SGA (<3rd centile) (Sehgal et al., 2014).

Very few population studies to date have examined arterial stiffness using PWV in the first weeks of life. Koudsi et al. measured aortic PWV in 30 babies in Manchester within 3 days of birth. They found an inverse association between maternal systolic BP at 28 weeks gestation and neonatal PWV, contrary to their hypothesis of a positive association between the two. Neonatal PWV was positively correlated with birth weight, birth length and neonatal systolic BP (Koudsi et al., 2007).

2.5.1.6 Maternal/early life nutrition and offspring PWV

Few studies have examined the relationship between maternal nutritional exposures and arterial stiffness in the offspring. One study examined the association of maternal vitamin D status during late pregnancy and arterial compliance measured by PWV at 9 years of age and found no evidence of association (Gale et al., 2007). Brachial PWV and augmentation index were used to evaluate arterial structure and function in 8 year old children who had participated in a RCT of dietary n-3 and n-6 fatty acid modification over the first 5 years of life (Ayer et al., 2009). This study also found no evidence of association between the dietary intervention and arterial stiffness in children.

Another study investigated whether fish oil supplementation versus olive oil supplementation of lactating mothers could modify PWV in their children after 2.5 years and found no association (Larnkjaer et al., 2006). Adolescent children of women who have received daily balanced protein-calorie supplements during pregnancy as part of a community trial in South India had a more favourable lower augmentation

index as a measure of arterial stiffness than children of women who did not receive the supplements (Kinra et al., 2008).

However, to date there are no published comparable studies assessing the relationship between maternal dietary exposures or nutritional status during pregnancy and infant arterial stiffness within the first few weeks of life. In chapter 4 of this thesis, the first research study to address this important question is presented.

2.5.2 Blood pressure

BP is strongly and directly linked to the risk of coronary and cerebrovascular events (Psaty et al., 2001). Almost 30% of coronary heart events can be attributed to high BP (Wilson et al., 1998). From middle age onwards, BP is strongly and directly related to vascular and overall mortality, without any evidence of a threshold down to at least 115/75 mm Hg (Prospective Studies Collaboration 2002). Lowering population-wide diastolic BP by only 2 mm Hg can reduce the prevalence of hypertension by 17 %, the risk of coronary heart disease by 6 % and the risk of stroke/transient ischaemic attacks by 15 % (Cook et al., 1995).

2.5.2.1 Developmental origins of hypertension

Adult BP has been inversely linked to birth weight (Barker et al., 2007). However, changes that occur in utero during fetal development that contribute to the developmental origins of CVD, including high BP, do not necessarily result in fetal growth restriction or LBW. There is evidence that children from deprived backgrounds, those whose mothers experience pregnancy-induced hypertension, those whose mothers smoke throughout pregnancy, those with LBW, who are not

breast-fed, who have high sodium diets in infancy and who are obese in childhood or adolescence tend to have higher BP as adults (Lawlor and Davey Smith, 2005). Postulated mechanisms for the developmental origins of high BP include fetal exposure to increased glucocorticoids, attenuation in kidney structure and function by permanently reducing nephron number, reduced elastin content of arterial walls, and epigenetic mechanisms involving changes in telomere length and sympathetic over-activity (Adair and Dahly, 2005). Maternal nutritional status prior to conception and during pregnancy is likely to be one of the important contributors to the origins of offspring adult hypertension.

2.5.2.2 Maternal nutrition in pregnancy and offspring BP

In animal studies, there is strong evidence of association between hypertension in the offspring and sub-optimal maternal nutritional statuses, including general under-nutrition, low-protein, and vitamin deficient diets (Woodall et al., 1996, Langley and Jackson, 1994, Sinclair et al., 2007). Earlier in this chapter, the evidence generated by animal studies of the effect of a maternal diet deficient in iron on offspring's BP was reviewed (section 2.3.1).

In humans, there is observational evidence of association between protein-carbohydrate balance and fat intake during pregnancy with adult and adolescent offspring BP (Campbell et al., 1996, Adair et al., 2001). Natural experiments, such as the Dutch Hunger Winter, revealed links between starvation of pregnant women in the first trimester of pregnancy and offspring hypertension (Schulz, 2010). There is also evidence of effect on child BP from follow-up RCTs of supplementation during pregnancy, such as calcium (Belizán et al., 1997), prebiotics (Aaltonen et al., 2008) and

multiple micronutrient supplements (Vaidya et al., 2008). Epidemiological evidence linking maternal iron intake in pregnancy and offspring BP in childhood was explored earlier in this chapter in section 2.3.3.

2.5.3 Obesity

Obesity is an important cardiovascular risk indicator in the offspring when considering the influence of lifecourse exposures on this risk. The prevalence of obesity has been on the rise since the early 1980s. Worldwide, at least 2.6 million people died of causes attributable to obesity in the year 2000 (Ezzati M et al, 2004). Being obese or overweight can increase the risk of developing chronic diseases such as type 2 diabetes, hypertension, CVD, cancer and all-cause mortality (Pi-Sunyer, 2012). It can also have a major impact on the individual's self-esteem, quality of life and educational attainment. Tackling obesity is now the concern of most Western governments who accept its major public health significance. The Foresight report states that, by 2050 over half of the UK adult population could be obese and the NHS costs attributable to overweight and obesity are projected to double to £10 billion per year. The wider costs to society and business are estimated to reach £50 billion per year (Foresight Report, 2007).

2.5.3.1 Measures of adiposity

BMI is calculated by dividing the weight in kilograms by the height in metres squared. It is accepted by the WHO as the appropriate method of classifying overweight and obesity in adults with cut-offs of 25 kg/m^2 for overweight and 20 kg/m^2 for obese (WHO, 1995). However, it is argued that different populations need different cut-offs due to population-specific differences in disease risk. Indians have a marked tendency

for abdominal obesity and insulin resistance and perhaps the greatest amplification of risk as weight increases. Lower BMI cut-offs of 23 kg/m^2 in Asia and 24 kg/m^2 in Hispanic countries are conventionally used (Sanchez-Castillo et al., 2006). However, deriving population-specific BMI cut-off points is difficult as the increase in observed risk varies from 22 to 25 kg/m^2 in different populations. WHO therefore recommends that the current BMI cut-off points should be retained as the international classification and additional points are to be used for public health action reporting (WHO expert consultation, 2004).

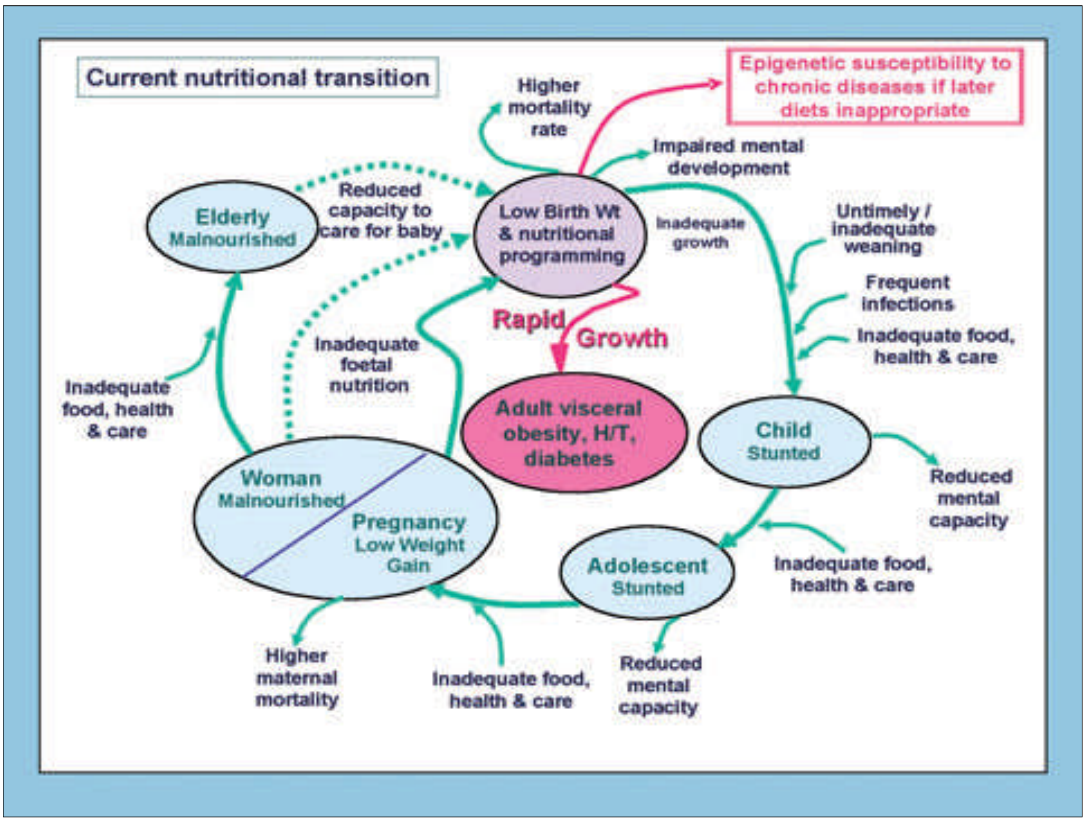
WC and waist-to-hip ratio are considered as measures of abdominal or central obesity. They have been linked to CVD incidence (de Koning et al., 2007). In the elderly, a U-shaped relationship was shown between WC and mortality (de Hollander et al., 2012). There are conflicting results regarding the association between this measure in adulthood and birth weight (Tian et al., 2006, Schooling et al., 2010). So far, the WHO has not specified cut-off points for WC and waist-to-hip ratio with regards to CVD risk prediction, as it did with BMI (Nishida et al., 2009). Some suggest a cut-off point for WC of ≥90 cm in men and ≥80 cm in women to mark increased CVD risk (Dobbelsteyn et al., 2001).

2.5.3.2 Developmental origins of obesity

There is now substantial evidence that early fetal and childhood 'programming' influences the development of obesity and its associated chronic diseases such as diabetes and hypertension in later life. This evidence started to emerge when Barker and colleagues observed that LBW was associated with a greater risk of developing abdominal obesity, diabetes and hypertension in late middle-age (Barker, 1998). The

life-course influences are now thought to be much more complex than the association with the crude measure of birth weight. Possible mechanisms could be through epigenetic and transcriptional regulation of key metabolic genes in response to nutritional stimuli during pregnancy that mediate persistent changes leading to an adult metabolic syndrome phenotype (Bruce and Hanson, 2010).

The concept of life-course influences on obesity and risk of chronic diseases and the complex interactions involved is illustrated in Figure 6.



Source: Ending malnutrition by 2020: an agenda for change in the millennium (James et al., 2000).

Figure 6: Life-course influences on obesity and chronic disease risk

2.5.3.3 Maternal nutrition in pregnancy and offspring obesity

Experimental evidence in animal studies supports a role of maternal nutritional quality in pregnancy on the development of obesity in the offspring (Howie et al., 2009, Bayol

et al., 2007, Remacle et al., 2011). The evidence specifically linking maternal ID with offspring adiposity in animal studies was reviewed earlier in this chapter in section 2.3.1.

Higher risk of obesity was observed among children of women who experienced the Dutch Famine (Painter et al., 2005), which extended to the second generation (Painter et al., 2008). However, despite the ample evidence from animal studies, epidemiological studies that characterise the relationship of maternal dietary patterns and components during pregnancy with offspring obesity later in life, as well the potential long-term effects of dietary interventions in the mother on offspring obesity, are almost lacking.

2.6 Significance of this thesis

2.6.1 How is this research scientifically novel?

This thesis is uniquely placed to contribute to the developmental origins of CVD research by analysing dietary exposures, biomarkers and clinical outcome data from large cohort studies (CARE, ALSPAC, UKWCS), as well as including a new study which utilises novel ways of exposure and outcome assessment (Baby VIP).

Data specifically collected for this thesis:

1. New data on the offspring of a sub-sample of the UKWCS
2. The Baby VIP study

Existing data analysed in this thesis come from the following cohorts:

1. The CARE study

2. ALSPAC study
3. *HFE* genotype data from the UKWCS

This programme of research is novel in several aspects:

- Maternal iron intake from the diet as well as supplements is assessed as a predictor of birth outcomes, including stratifying by type of iron (haem and non-haem) and testing for effect modification by vitamin C (chapters 3 & 5)
- The Baby VIP study (chapter 4) is the first study to examine the relationship between a measure of maternal nutritional status during pregnancy and infant PWV as a proxy for later cardiovascular risk.
- The best available measure of body iron status is used in the Baby VIP study: the R/F ratio.
- Infant PWV has not been assessed before in a population study with a sample size in hundreds. This is a novel measure which could provide a non-invasive predictor of later cardiovascular health that is easier to perform in infants than BP.
- Chapter 5 builds on the previous analysis performed in ALSPAC by Brion et al, by analysing additional offspring CVD markers as well as BP, including arterial stiffness, endothelial function and BMI. The sample included is bigger than the previous analysis, the outcome measurements are taken at a later offspring age, and include arterial stiffness and endothelial function and adiposity, which were not analysed in the Brion study.
- The UKWCS-IBPS element of the thesis (chapter 6) is the first study that uses a MR design to examine a transgenerational relationship between maternal micronutrient status and adult health indicators.

- All the above elements exploring the hypothesis of interest at different stages in the lifecourse are pulled together in this thesis for the first time

2.6.2 Relevance to public health

Any intervention that could result even in a modest reduction in BP, obesity and/or arterial stiffness at a population level, would have a significant public health impact on CVD morbidity and mortality. Maternal iron supplementation and/or optimising dietary iron intake during pregnancy could be such intervention. The outcome of this study will inform the debate on whether there is benefit to the offspring from recommending increased iron intake universally through diet and/or supplements during pregnancy, restricting this to mothers with IDA, or those who have ID even without anaemia. Positive results would suggest increasing iron intake during pregnancy is beneficial to the offspring's health in the long term. Negative results would suggest a different relationship between maternal iron status and offspring CVD risk in humans compared to that which has been observed in animal studies.

2.7 **Conclusion**

Maternal ID and IDA during pregnancy is a common problem if women start their pregnancy with inadequate iron stores. It is potentially associated with adverse consequences for the baby in the short term, including LBW, prematurity and infant IDA, particularly if the deficiency is present early in pregnancy. Methods to optimise iron intake during pregnancy include dietary optimisation of iron intake from natural sources, iron fortification of certain elements of the diet such as flour, and iron supplementation. The benefit of such interventions with regards to infant outcomes is not consistent, particularly in high income country settings.

Maternal ID in pregnancy has been shown to be causally associated with higher risk of offspring hypertension and obesity in animal studies. However, the available epidemiological evidence is insufficient to make any conclusions regarding the presence and direction of such associations. This thesis will test in humans the hypothesis generated by animal studies using epidemiological study designs. It will contribute to the evidence-base on whether a mother's iron intake and status during pregnancy are important predictors of later cardiovascular risk in her children. Each study included in this project addresses at least one aspect of the research question of whether maternal ID during pregnancy is associated with increased offspring's cardiovascular risk later in life.

3 Associations of dietary iron and supplement intake in pregnancy with birth outcomes in a cohort of British women: the CARE study

This chapter commences the investigation of the relationship between markers of maternal iron status during pregnancy, including intake from the diet and supplements, and Hb and MCV, with short-term birth outcomes including size at birth and duration of gestation.

Work from this chapter has formed the basis of two peer-reviewed papers (Alwan et al., 2011, Alwan et al., 2010), and three conference presentations.

3.1 Chapter summary

The aim of this study was to investigate the associations between dietary supplements and iron intake in pregnancy with size at birth and preterm birth using data from a prospective cohort of 1274 pregnant women aged 18-45 years in Leeds, UK, The Caffeine and Reproductive Health study (CARE). Dietary intake was reported in a 24-hour recall administered by a research midwife at 12-week gestation. Dietary supplement intake was ascertained using dietary recall and three questionnaires (first, second and third trimesters). Information on delivery details and antenatal pregnancy complications was obtained from the hospital maternity records.

Reported dietary supplement use declined from 82% of women in the first trimester of pregnancy to 22% in the second trimester and 33% in the third trimester. Folic acid was the most commonly reported supplement taken. 24%, 15% and 8% reported taking iron-containing supplements in the first, second and third trimesters respectively.

Eighty percent of women reported dietary iron intake below the UK RNI of 14.8 mg/day. Women with dietary iron intake >14.8 mg/day were more likely to be older, have a higher energy intake, have higher education and take daily supplements during the first trimester. They were less likely to be smokers, live in a deprived area, or report a chronic illness.

Vegetarian participants were less likely to have low dietary iron intake (OR=0.5, 95% CI 0.4, 0.8) and more likely to take iron-containing supplements during the first and second trimesters. Total iron intake, from food and supplements, was positively

associated with birth weight centile (adjusted change=2.5 centiles per 10 mg increase in iron, 95% CI 0.4, 4.6). Taking any type of daily supplement during any trimester was not significantly associated with size at birth taking into account known relevant confounders. Women taking MVM supplements in the third trimester were more likely to experience preterm birth (adjusted OR=3.4, 95% CI 1.2, 9.6, *P=0.02*). Hb at 28 weeks was associated with increased risk of SGA (adjusted OR per g/dl increase in Hb 1.4, 95% CI 1, 1.8, *P=0.03*).

3.2 Background

3.2.1 Dietary iron intake during pregnancy

Dietary recommendations and population levels of intake of iron from the diet during pregnancy have been reviewed earlier in chapter 2 (section 2.2.7). Results of studies investigating the relationship between dietary maternal iron intake during pregnancy and size at birth and/or gestational age are conflicting (Doyle et al., 1990, Godfrey et al., 1996, Scholl and Hediger, 1994, Mathews et al., 2004, Scholl et al., 1992, Lagiou et al., 2005, Al-Shoshan, 2007, Baker et al., 2009, Mitchell et al., 2004, Haste et al., 1991, Mathews et al., 1999). These have been reviewed in section 2.2.7.4 and summarised in Table 1. Many studies that assessed total iron intake did not model the relationships separately for iron from food and that from dietary supplements. Neither did they consider the potential differential effects of haem and non-haem iron. One study assessed the relationship between ascorbic acid and anaemia and well as vitamin C intake and iron status (Baker et al., 2009). However the potential interaction between iron intake and the vitamin C intake and other micronutrients has not been explored (Gibney et al., 2004).

3.2.2 Dietary supplement intake during pregnancy

Dietary supplements during pregnancy are becoming an attractive option considered by international agencies to improve the nutritional status of pregnant women in developing countries. Multivitamin-mineral supplements (MVM) supplements have been routinely recommended for pregnant women and those who might become pregnant in some developed countries such as the United States (Willett and Stampfer,

2001). They are not routinely recommended during pregnancy by the WHO, or in other developed countries such as the UK. These preparations are readily available over-the-counter, and heavily advertised and promoted to expectant mothers especially in Western countries. They are considered relatively cheap, feasible and have the potential to improve maternal nutrition when administered through national antenatal programmes. However, dietary supplements are not subject to the same rigorous safety and efficacy standards as prescription medications (Gardiner et al., 2008). Their proposed use during pregnancy is supported by findings from several RCTs in low and middle income country settings, where deficiency in micronutrients is more prevalent and more pronounced than high income countries.

3.2.2.1 Multivitamin-mineral supplements and birth outcomes

Multiple-micronutrient deficiency is common among pregnant women in low-income countries (Lumbiganon, 2007). However, pregnant women in developed countries are expected to have better baseline nutrient status compared to pregnant women in developing countries, and nutritional deficiencies are more likely to be restricted to specific micronutrients such as iron. Therefore, the effect of supplementation programmes in terms of birth outcomes may vary between the two settings.

3.2.2.1.1 Evidence from low and middle income countries

Studies in Nepal, India, Indonesia, Guinea-Bissau and Tanzania have shown positive effects on adverse birth outcomes such as infant mortality and LBW (Osrin et al., 2005, Gupta et al., 2007, The supplementation with Multiple Micronutrients Intervention Trial (SUMMIT) Study Group, 2008, Kaestel et al., 2005, Fawzi et al., 2007). However, other trials in Nepal, Mexico and Zimbabwe have failed to demonstrate a significant

effect on the incidence of LBW (Christian et al., 2003, Friis et al., 2004, Ramakrishnan et al., 2003, Christian et al., 2008), and some have even demonstrated an increased risk of adverse outcomes (Roberfroid et al., 2008, Christian et al., 2008) . In relation to the incidence of neural tube defects (NTD), a direct comparison of folate versus multivitamin supplementation indicated a significant reduction in the folate group suggesting that folate supplementation may be more useful that MVM considering this outcome (Lumley et al., 2002).

According to a Cochrane systematic review, there was a favourable effect of MVM supplementation on the incidence of LBW and SGA compared to none or placebo supplementation. However, there was insufficient evidence to suggest replacement of iron and folate supplementation with multiple micronutrient supplements. The review, which included 9 trials and around 15,000 women, recommended further research to quantify the degree of maternal or fetal benefit and to assess the risk of excess supplementation and the potential for adverse interactions between micronutrients. All the trials included in this review were conducted in low income countries (Haider and Bhutta, 2006).

An updated review by the same authors, including data from 17 studies all conducted in developing countries, showed a significant reduction of 9% in the risk of SGA compared to iron-folate supplementation (Haider et al., 2011). Also, a recent meta-analysis by Shah et al. of 13 RCTs on the effect of MVM supplements on infant outcomes showed a significant reduction in the risk of LBW in women who received these supplements during pregnancy compared to placebo (RR=0.8, 95% CI 0.7, 0.9). Mean birth weight was also higher as compared to women who took combined iron

and folic acid supplementation (Shah et al., 2009). There were no differences in the risk of preterm birth or SGA. One third of the women included were in the first trimester, half were in the second trimester and the rest in the third trimester during the trials. All, but one, of the included trials were conducted in a developing country. This review included a trial of HIV positive women, which the Cochrane review did not include.

3.2.2.1.2 Evidence from high income countries

Although multivitamin supplements have been recommended for women who might become pregnant in some high income countries such as the United States (Willett and Stampfer, 2001), there are few studies examining their effect on birth outcomes in developed countries, where there is likely to be a significant difference in women's baseline nutrient status compared to low income countries. A RCT in France showed significant positive effects for micronutrient supplementation versus placebo on the incidence of LBW (Hininger et al., 2004). However, this study had a relatively small sample size of 100 women and a very small number of babies born with LBW. The supplements given in this study were iron-free, and thus differ from currently available over the counter MVM preparations for pregnant women. There was no difference detected in the oxidative stress parameters measured in the study between supplemented and unsupplemented women.

The Camden study which examined the association of multivitamin supplementation intake in pregnancy with birth outcomes was conducted in a disadvantaged urban setting in the USA (Scholl et al., 1997). Reduced risks of both LBW and preterm delivery were associated with supplement use in the first and second trimester. Analysis was

restricted to data obtained by 28 weeks of pregnancy and did not report on the relationship between infant outcomes and supplement use in the third trimester of pregnancy.

In summary, MVM supplements are likely to improve infant outcomes in low income countries. There is a possibility in well-nourished women however of reduced bioavailability of micronutrients due to interactions, and there is no strong evidence of benefit of regular intake during pregnancy in high income country settings.

3.3 Hypothesis and objectives

It is hypothesised that women with adequate iron intake and those who take dietary supplements during pregnancy have, on average, bigger babies.

The objectives of this study were to:

1. Investigate the association between maternal iron intake during early pregnancy with both birth weight/SGA and preterm birth
2. Assess whether any relationships differ by source of iron (food versus dietary supplements) or by type of iron (haem versus non-haem)
3. Explore the role of vitamin C intake as an effect modifier in the association investigated by objective 1
4. Examine the relationship between supplement use during the first, second and third trimesters of pregnancy with birth weight and preterm delivery
5. Examine the relationship of maternal Hb during pregnancy with birth weight/SGA

3.4 Methods

3.4.1 Study design and participants

The CARE study is a prospective birth cohort in which low-risk pregnant women aged 18-45 years with singleton pregnancies were prospectively recruited at 8 to 12 weeks gestation from the Leeds Teaching Hospitals maternity units between 2003 and 2006 (CARE Study Group, 2008, Boylan et al., 2008). This was part of a multicentre study into maternal diet and birth outcomes. Women with concurrent medical disorders, psychiatric illness, HIV infection, or hepatitis B infection were excluded.

Eligible women were identified by screening their pre-booking maternity notes. They were contacted then sent detailed information about the study and were asked to return a reply slip to state whether they were willing to take part. A total of 4571 eligible women were approached in Leeds. Of these, 1374 consented. Those who agreed to participate were then interviewed. This interview was conducted either at the hospital, the participant's general practice, or her home by a research midwife. Demographic details were obtained using a self-reported questionnaire. Figure 7 illustrates the study's data collection points.

As part of the original study of caffeine and birth outcomes, it was planned to follow-up women several weeks after delivery to investigate how their caffeine metabolism had returned to normal, using a caffeine challenge. To reduce costs without introducing selection bias, follow up was aimed at all cases defined as women with LBW or SGA babies (< 2500 grams) but only a sample of controls, taken to be the two closest births either side of the case, following a nested case-control design. The

interviews for the third trimester data were performed retrospectively on this sub-sample of the cohort (n=425) with a ratio of 2 controls for every case. Almost all of the sampled women who were approached returned data for the third trimester of pregnancy.

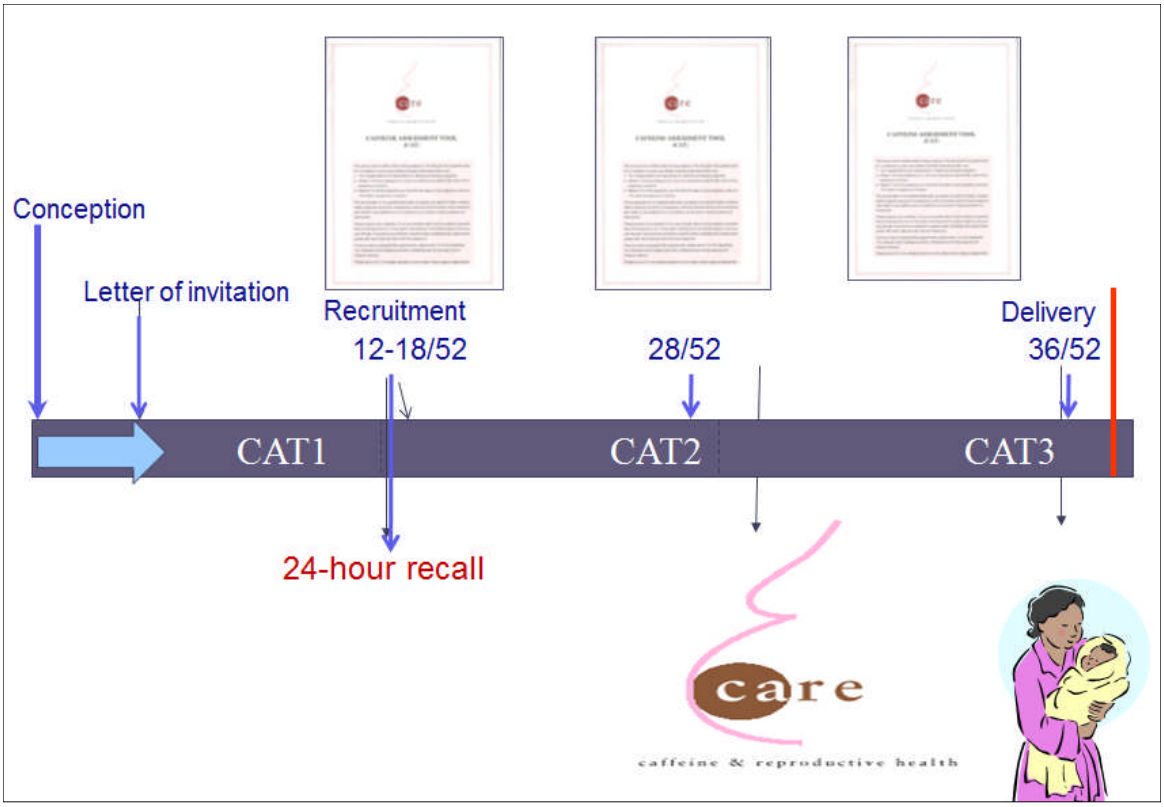


Figure 7: CARE study data collection points

3.4.2 Exposure assessment

3.4.2.1 Questionnaire assessment of supplement use

Supplement use was ascertained throughout pregnancy using questionnaires in the first, second and third trimesters. The questionnaires were interviewer-administered during the first and third trimesters and self-administered during the second trimester.

The respondents were asked to report the type/brand, frequency and the amount of all the dietary supplements they were using during each trimester. The questions were free text rather than multiple choice questions, asking participants to tick the type of supplements they were using to ensure all sources were covered. The supplements' types were then coded during data entry.

3.4.2.2 Recall dietary assessment

Dietary and supplement intake was reported through a 24-hour dietary recall administered by a research midwife at 8-12 weeks gestation (appendix 10.2). Values for the proportion of haem iron in each type of meat were used to derive haem values for each of the food codes. These values were derived by recording the meat content of each product, together with food tables values (McCance, 1990), to calculate a weighted mean meat content of each food item consumed. A literature search was carried out to arrive at 'haem factors' for different animal products that reflect the haem iron content of these foods. Values derived from the Schricker and modified Schricker methods, and the Hornsey method were used to calculate mean values for haem iron (Schricker and Stouffer, 1982, Hornsey, 1956). These values were then used to generate total iron values for each relevant food (O'Hara, 2004). The non-haem iron values were derived as the difference between total iron from food tables (McCance, 1990) and calculated haem values. Total iron was derived from adding dietary intake and supplement intake as reported in the recall. Iron content of each supplement reported was added to the dietary intake multiplied by total number of supplement tablets/capsules taken during the 24-hour recall. Vitamin C intake from the diet was

reported in the 24-hour recall and categorized into above or equal to/below the RNI of 50 mg/day in the analysis.

3.4.2.3 Haemoglobin

Data on Hb concentrations and MCV at 12 weeks (n=558) and 28 weeks (n=572) were extracted from the electronic antenatal laboratory records for a sub-sample of the cohort which was selected randomly from the main sample using study identification numbers.

3.4.3 Outcome assessment

The two primary outcome measures were birth weight and preterm birth. Birth weight and gestational age were extracted from the medical delivery notes. Birth weight was measured in grams, and as expressed as customised birth weight centile using charts which take into account gestational age, maternal height, weight, ethnicity and parity, and neonatal birth weight and sex (Gardosi, 2004). Duration of gestation was calculated from the date of the last menstrual period, and confirmed by ultrasound scans dating at around 12 and 20 weeks gestation. SGA was defined as $<10^{th}$ customised birth weight centile. Preterm birth was defined as delivery at <37 weeks (259 days) gestation. Information was obtained from the hospital maternity records on pregnancy and delivery complications.

3.4.4 Assessment of participants characteristics

Socioeconomic status was assessed using the Index of Multiple Deprivation (IMD) score. The IMD combines a number of indicators, chosen to cover a range of economic, social and housing issues into a single deprivation score for each small area

in England. This allows each area to be ranked relative to one another according to their level of deprivation (Department for Communities and Local Government, 2009). IMD however, is an area, not an individual, deprivation measure.

Mothers' educational level, smoking status, alcohol intake, parity, ethnicity, pre-pregnancy weight, past history of miscarriage, long-term chronic illness and vegetarian diet were self-reported in a first-trimester questionnaire. Salivary cotinine levels were measured using an enzyme-linked immunosorbent assay (ELISA) (Cozart Bioscience, Oxfordshire, UK). Participants were classified on the basis of these cotinine concentrations as active smokers (>5 ng/ml), passive/occasional smokers (1-5 ng/ml), or non-smokers (<1 ng/ml) (CARE Study Group, 2008).

3.4.5 Statistical power calculations

Comparing birth weights between mothers with dietary iron intake of >14.8 mg/day (the recommended UK RNI for women of childbearing age) to those with ≤ 14.8 mg/day during the first trimester of pregnancy, using the ratios of the low-intake to the high-intake group and the standard deviation (SD) for birth weight measured in this study (SD 577 g), this analysis had 85% power to detect a difference of 120 g in birth weight between the two groups for $P < 0.05$ and a two-sided test.

Comparing birth weights between supplement users and non-users within the first trimester, using the ratios of users to non-users and standard deviations identified in the study, this analysis had 80% power to detect a difference of 120 g and 90% power to detect a difference of 140 g, for $P<0.05$. Within trimester two, this study had 80% power to detect a difference of less than 115 g and 90% power to detect a difference

of 130 g. Within trimester 3, this study had 85% power to detect a doubling of the prevalence of SGA (from 13% to 26%), and to detect a tripling of the preterm birth rate (from 5% to 15%) for a two-sided test at *P<0.05*.

3.4.6 Statistical methods

Univariable comparisons were made using two-sample Student's t-test for continuous variables and chi-square test for categorical variables. Multiple linear regression using birth weight and customised birth weight centile as continuous outcomes, and unconditional logistic regression with preterm birth and SGA as binary outcomes were performed using STATA version 11 (College Station, TX, 2009).

Analysis was undertaken using dietary iron intake as a continuous variable and a binary variable using the UK RNI cut-off of 14.8 mg/day. Total iron from diet and supplements, assessed by the 24-hour recall, was analysed as a continuous variable. Intake of iron-containing supplements was analysed as a binary variable. With regards to supplement intake, analysis was performed using two groups; women who reported taking any type of daily supplements and those who specifically reported taking MVM supplements during pregnancy.

Maternal height, weight, ethnicity, parity, neonatal gestation at delivery and baby's sex were taken into account in the definition for customised birth centile, and were adjusted for in the model for birth weight. Statistical adjustment was also made for salivary cotinine levels, self-reported alcohol consumption, maternal age, maternal vegetarian diet, IMD, the mother having a university degree, past history of miscarriage and long-term chronic illness in all models.

Sensitivity analyses for the linear iron intake model were performed by excluding vegetarians from the model, and adding an interaction term for daily vitamin C intake in the model. Sensitivity analyses for the multivitamin models were performed taking into account clinical diagnosis of IUGR. Subgroup analysis using the multiple linear iron intake model was performed using type of dietary iron (haem versus non-haem). Multiple linear regression was also used to explore the association between Hb and MCV levels at 12 and 28 weeks of pregnancy with iron intake and birth weight/SGA.

3.4.7 Ethical approval

All women participating in the study gave informed written consent and the study was approved by the Leeds West Local Research Ethics Committee (reference number 03/054). All procedures were in accordance with the Helsinki Declaration of 1975 as revised in 1983.

3.4.8 Funding

The work included in this chapter was supported by the Wellcome Trust [Grant number WT87789 to N.A.A.] and the Food Standards Agency, United Kingdom [Grant number T01033]. The funders had no influence on the design or analysis of the study.

3.5 Results

3.5.1 Birth outcomes

There were 1259 babies with information on birth weight. Mean birth weight was 3439 g (SD 577 g) with 4.4% babies weighing less than 2500 g (n=55). 13% (n=166) were SGA (<10th centile), 8% (n=99) weighed less than the 5th centile, and 5% (n=65) less than the 3rd centile. 9% of babies (n=118) weighed more than the 90th centile. Out of the 1234 pregnancies with information on gestational age, 55 (4.5%) delivered their babies before 37 weeks gestation.

3.5.2 Dietary recall

Based on midwife-administered 24-hour recall dietary assessment at 8 to 12 weeks gestation, women in the CARE study had average dietary intakes from food above the RNI values for most vitamins and minerals (The Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy, 1991) except vitamin D, iron, folate, selenium and iodine.

Table 3 shows the mean intake in the cohort, the nutrient requirements for adult women plus the additional requirement recommended for consumption during pregnancy, and the proportion of the women with dietary intakes above the recommended RNI in pregnancy. The mean total energy intake was 2125 kcal/ day (95% CI 2084, 2166).

Micronutrient	Mean (SD)	RNI*	Increment**	% with intakes >pregnancy RNI (95% CI)
Thiamin (mg/d)	2.4 (7.7)	0.8	+0.1	85 (83,87)
Riboflavin (mg/d)	1.7 (0.8)	1.1	+0.3	58 (55,61)
Niacin (mg/d)	20 (10)	13	***	75 (72,77)
Vitamin B6 (mg/d)	2.1 (1.0)	1.2	***	85 (82,86)
Vitamin B12 (ug/d)	3.9 (3.7)	1.5	***	79 (77,82)
Folate (ug/d)	257 (119)	200	+100	32 (29,35)
Vitamin C (mg/d)	143 (129)	40	+10	75 (73,78)
Vitamin A (ug retinol equivalent/d)	803 (665)	600	+100	45 (42,48)
Vitamin D (ug/d)	2.5 (2.7)	-	10	2 (1,3)
Vitamin E (mg/d)	7.9 (5.4)	-	#	-
Calcium (mg/d)	938 (471)	700	***	65 (62,68)
Phosphorus (mg/d)	1344 (501)	550	***	98 (97,99)
Magnesium (mg/d)	283 (112)	270	***	49 (46,52)
Iron (mg/d)	11.5 (5.3)	14.8	***	20 (18,23)
Zinc (mg/d)	8.6 (4.3)	7	***	59 (56,62)
Copper (mg/d)	1.1 (0.6)	1.2	***	32 (29,35)
Selenium (ug/d)	58 (37)	60	***	40 (38,43)
Iodine (ug/d)	118 (82)	140	***	28 (24,29)

***** Reference nutrient intakes for women aged 19-50 years in the UK

**Recommended Increment to RNI during pregnancy ***No increment is recommended

#Safe intake – above 3mg/day for women (The Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy, 1991)

Table 3: Average daily intakes of vitamins and minerals (from diet alone) based on 24-hour dietary recall at 8-12 weeks of pregnancy in the CARE study (n=1257)

3.5.3 Iron intake

3.5.3.1 Iron intake from diet

1257 women had dietary recall information in the first trimester. The mean dietary iron intake from food was 11.5 mg/day (SD 5.3) with only 20% (n=257) of women reporting intake >14.8 mg/day (95% CI 18, 23%). 24% of women reported iron intake equal or less than the UK LRNI of 8 mg/day (95% CI 22, 27%). Only 4% reported a dietary iron intake of more than the US recommended intake during pregnancy of 27 mg/day (95% CI: 3, 5%). Mean haem iron intake was 0.6 mg/day (SD 0.8). This estimate for haem iron changed little after excluding the 114 reported vegetarian participants (with a haem iron intake of zero). Mean non-haem iron intake was 10.9 mg/day (SD 5.2) (Table 4).

	Mean (mg/day)	Standard deviation	Median (mg/day)	Interquartile range
Iron intake from food	11.5	5.3	10.5	8.1 , 13.7
Haem iron intake	0.6	0.8	0.3	0.1, 0.8
Non-haem iron	10.9	5.2	10	7.6, 13.0
Total iron from food and supplements	16.5	21.1	11.8	8.6, 19.1
Total iron from food and supplements excluding therapeutic iron preparations (≥ 65 mg/dose)	14.3	8.4	11.6	8.5, 18.6

Table 4: Average iron intake from food and dietary supplements as reported in first trimester 24-hour dietary recall in the CARE study (n=1257)

3.5.3.1.1 Characteristics of women with high versus low iron intake

Women with dietary iron intake >14.8 mg/day were more likely to be older, report a higher total energy intake, have a university degree, be vegetarian, and take daily supplements during the first trimester including iron-containing supplements. They

were less likely to be smokers, live in an area with the worst IMD quartile, or have a long-term illness (Table 5). Vegetarian participants were less likely to have dietary iron intake ≤14.8 mg/day (unadjusted odds ratio (OR) 0.5, 95% CI 0.4, 0.8, *P=0.004*). Vegetarians were also more likely to take iron-containing supplements during the first and second trimester (OR 2.9, 95% CI 2.0, 4.3, *P<0.0001* for the 1st trimester, OR 2.9, 95% CI 1.9, 4.4, *P<0.0001* for the 2nd trimester).

	>14.8 mg/day# (n=257)	**≤14.8 mg/day (n=1000)**	**P***
Dietary iron intake (mg/day) (mean, 95% CI)	19.6 (15.0, 31.7)	9.4 (4.5, 13.8)	-
Age of mother (years) (mean, 95% CI)	31 (30, 31)	30 (29, 30)	0.004
Pre-pregnancy weight (kg) (mean, 95% CI)	66 (64, 68)	68 (67, 68)	0.1
Total energy intake (kcal) (mean, 95% CI)	2777(2657,2897)	1958(1924,1991)	<0.0001
Active smoker at 12 weeks (%, 95% CI)	8 (5, 12)	20 (17, 23)	<0.0001
IMD most deprived quartile (%, 95% CI)**	25 (20, 31)	32 (29, 35)	0.03
Caucasian (%, 95% CI)	91 (87, 95)	94 (92, 95)	0.2
Higher education (%, 95% CI)	52 (49, 58)	35 (32, 39)	<0.0001
Vegetarian (ovo-lacto) (%, 95% CI)	13 (10, 18)	8 (6, 10)	0.004
Primigravida (%, 95% CI)	47 (41, 54)	46 (43, 49)	0.7
History of long term illness (%, 95% CI)	9 (6, 13)	14 (12, 16)	0.04
Average alcohol consumption more than 0.5 units/day throughout pregnancy (%, 95% CI)	30 (24, 36)	26 (23, 29)	0.2
Past history of miscarriage (%, 95% CI)	20 (16, 26)	25 (22, 27)	0.08
Report taking any form of daily supplements in the 1st trimester questionnaire (%, 95% CI)	87 (82, 91)	81 (78, 83)	0.01
Report taking daily iron-containing supplements in the first trimester (questionnaire) (%, 95% CI)	29 (23, 35)	23 (20, 25)	0.04

Reference nutrient intake (RNI) for iron for women aged 19-50 years in the UK
* P-value using two-sample t-test for continuous variables, chi-squared test for categorical variable
**Index of multiple deprivation

Table 5: Characteristics of women by dietary iron intake above versus equal to or below RNI during the first trimester as reported in a 24-hour dietary recall (n=1257)

3.5.3.2 Iron intake from supplements

20% of participants (95% CI 18, 22) reported taking iron-containing supplements in the recall compared to 24% (95% CI: 22, 26) in the first trimester questionnaire (Kappa agreement = 0.85). 15% (95% CI 13, 18) and 8% (95% CI 7, 10) reported taking iron-containing supplements in the second and third trimester questionnaires respectively. Median total iron intake from diet and supplements, as recorded in the recall, was 11.8 mg/day (IQR 8.6, 19.1). 34% (95% CI: 32, 37) of women had an iron intake >14.8mg/day from diet and supplements. Only 11 participants reported taking iron-only preparations in the recall, which were assumed to be the conventional therapeutic preparation with a dose of 65 mg iron/tablet, and 5 reported taking a preparation of iron and folic acid which contains 100 mg iron per dose. 8, 21 and 29 participants reported taking iron-only supplements in the first, second and third trimester questionnaires respectively. Median total iron excluding these 16 participants who reported taking iron-only supplements in the recall was 11.6 mg/day (IQR 8.5, 18.6).

3.5.3.3 Relationship between iron intake and birth weight

Dietary iron intake from food was significantly related to birth weight measured on the customised birth centile. The unadjusted change in birth weight centile per 10 mg/day increase in dietary iron intake during the first trimester was 5.2 centile points (95% CI 2.2, 8.2, *P=0.001*). Adjusting for maternal age, salivary cotinine levels and alcohol intake attenuated this relationship (adjusted change 3.1 centile points, 95% CI -0.2, 6.3, *P=0.07*) (Table 6). The estimate changed little when excluding vegetarians, or including calcium or zinc intake as interaction terms with iron intake (data not shown).

Considering birth weight in grams as an outcome, the unadjusted difference per 10 mg/day increase in dietary iron intake was 70 g (95% CI: 10, 130, *P=0.02*). When adjusting for maternal age, cotinine levels, alcohol intake, maternal weight, height, parity, ethnicity, gestational age and baby's sex, the difference was 34 g (95% CI: -13, 80, *P=0.2*).

There was no evidence of a relationship between haem iron intake and customised birth centile. Unadjusted difference per 1 mg/day increase in haem iron intake was -1.2 centile points (95% CI -3.3, 0.8, *P=0.2*). Birth weight centile was positively associated with non-haem iron intake (unadjusted change in birth weight centile per 1 mg/day increase in non-haem iron intake 0.6, 95% CI 0.3, 0.9, *P<0.0001*; adjusted change 0.3, 95% CI 0, 0.9, *P=0.05*).

There was a positive relationship between total iron intake, from food and supplements, with customised birth weight centile (unadjusted change in centile points per 10 mg/day increase in total iron intake 4.3, 95% CI 2.4, 6.3, *P<0.0001*, adjusted change 2.5, 95% CI 0.4, 4.6, *P=0.02*) (Table 6).

3.5.3.4 Relationship between iron intake and small for gestational age

Participants with dietary iron intake equal to or less than 14.8 mg/day were 1.6 times more likely to have a SGA baby (95% CI 1.0, 2.5, *P=0.05*). However, the adjusted relationship was not significant (1.4, 95% CI 0.9, 2.3, *P=0.2*). This association is similar for total iron intake from diet and supplements (Table 6).

3.5.3.5 Relationship between iron intake and preterm birth

There was no evidence of an association between iron intake from food only, or total iron intake from diet and supplements as recorded in the recall diary in the first trimester, with preterm birth (Table 6).

3.5.3.6 Role of vitamin C intake

The relationship between dietary iron intake from food and customised birth weight centile was significant in participants with vitamin C intake above the RNI of 50 mg/day (adjusted change in centile points per 10 mg/day increase in dietary iron intake 3.7, 95% CI 0.1, 7.3, *P= 0.04*), compared to -1.9 percentile units (95% CI -11.1, 7.5, *P=0.7*) for those with vitamin C intake ≤50 mg/day (n=253). However, the interaction between iron and vitamin C intakes on the outcome was not statistically significant (*P=0.3*). Similar relationships were observed for non-haem iron and total iron intake from diet and supplements using an interaction term between iron intake and vitamin C intake in the models (Table 6)

3.5.3.7 Relationship between intake of iron-containing supplements and birth outcomes

There was no association between daily intake of iron-containing supplements in the first and second trimester and customised birth centile. There was an inverse association between taking iron-containing supplements in the third trimester (73% of which as part of MVM preparations) and customised birth weight centile adjusted for salivary cotinine levels, alcohol intake and maternal age (adjusted difference -10.7, 95% CI -16.7, -4.8, *P <0.0001*). When looking at the relationship between taking any

iron-containing supplement (mainly as part of a MVM supplement) and preterm birth, there was an increased risk of preterm birth if taken in the third trimester (adjusted OR 3.0, 95% CI 1.2, 7.6, *P=0.02*).

Customised birth centile#						
	Unadjusted change	95% CI	P	Adjusted change*	95% CI	P
Dietary iron intake †	5.2	2.2, 8.2	0.001	3.1	-0.2,6.3	0.07
Dietary iron intake in participants with vitamin C intake > 50 mg/day †	5.3	1.9, 8.6	0.002	3.9	0.4, 7.5	0.03
Non-haem iron intake †	5.7	2.6, 8.8	<0.0001	3.4	0.0, 8.8	0.05
Non-haem iron intake in participants with vitamin C intake > 50 mg/day †	5.9	2.5, 9.3	0.001	4.4	0.7, 8.0	0.02
Haem iron intake ††	-1.2	-3.3, 0.8	0.2	-0.7	-2.8,1.4	0.6
Total iron intake *** †	4.3	2.4, 6.3	<0.0001	2.5	0.4, 4.6	0.02
Total iron intake *** in participants with vitamin C intake > 50 mg/day †	4.4	2.2, 6.5	<0.0001	3.0	0.7, 5.4	0.01
Small for gestational age (<10th centile)						
	Unadjusted OR **	95% CI	P	Adjusted OR*	95% CI	P
Dietary iron intake (≤14.8 mg/day)	1.6	1.0, 2.5	0.05	1.4	0.9, 2.3	0.2
Total iron intake *** (≤14.8 mg/day)	1.5	1.0, 2.1	0.04	1.2	0.8, 1.8	0.3
Preterm birth (<37 weeks gestation)						
	Unadjusted OR **	95% CI	P	Adjusted OR*	95% CI	P
Dietary iron intake (≤14.8 mg/day)	1.1	0.7, 2.3	0.7	1.0	0.5, 2.3	0.8
Total iron intake *** (≤14.8 mg/day)	1.5	0.8, 2.7	0.2	1.3	0.7, 2.5	0.4

#Takes into account: maternal pre-pregnancy weight, height, parity, ethnicity, gestation and baby's sex
*Adjusted for maternal age, salivary cotinine levels and alcohol intake in a multiple linear regression model, with an interaction term between iron and vitamin C intakes where the estimates are reported in the table to be for iron intake in the group with vitamin C intake >50 mg/day
**Odds ratio with dietary iron intake >14.8 mg/day as the reference group
†Percentage point change in birth weight centile per 10 mg/day increase in iron intake
†† Percentage point change in birth weight centile per 1 mg/day increase in haem iron intake
*** From food and supplements excluding therapeutic iron supplement takers (≥ 65mg/dose)

Table 6: The Relationship between maternal dietary iron intake (mg/day) during pregnancy and birth outcomes in the CARE study

3.5.4 Dietary supplement intake

All 1274 participants had information on supplement intake in the first and second trimester. 425 women had information on supplement intake in the third trimester. The proportion of pregnant women taking any form of daily supplements was 82%, 22% and 33% for the first, second and third trimester respectively (Table 8).

Out of the women who took daily supplements in the third trimester (n=139), 94% (n=131) also reported taking daily supplements in the first trimester of their pregnancy, and 66% (n=91) took daily supplements in their second trimester. Only five women, who reported taking daily supplements in the third trimester, had not taken supplements in the first or second trimester.

3.5.4.1 Characteristics of women in supplement-taking and non-supplement-taking groups

Women who reported taking supplements at any stage of pregnancy were more likely to have a university degree and be vegetarian, and less likely to be smokers. They were less likely to be living in an area with an IMD score in the most deprived quartile. Women who reported taking daily supplements in the first and second trimester were more likely to be primiparous. However, there was no difference between primiparous and multiparous women in their use of supplements in the third trimester. There were also no differences between women who reported taking daily supplements at any stage in pregnancy with those who did not with regards to pre-pregnancy weight, ethnic origin or history of long term illness (Table 7).

Characteristic	1st trimester			2nd trimester			3rd trimester		
Taking any daily supplements (n)	**Yes (1043)**	**No (231)**	**P***	**Yes (274)**	**No (1000)**	**P**	**Yes (139)**	**No (286)**	**P**
Age of mother (years) (m#, 95% CI**)	30 (30,31)	29 (28,29)	<0.001	31 (31,32)	30 (29,30)	<0.001	31 (30,32)	29 (28,30)	<0.001
Pre-pregnancy weight (kg)(m, 95% CI)	67 (66,68)	66 (65,68)	0.4	66 (65,68)	68 (67,68)	0.2	67 (64,69)	68 (66,70)	0.4
Total energy intake (kcal) (m, 95% CI)	2148 (2103, 2193)	2019 (1921, 2117)	0.02	2167 (2075, 2258)	2113 (2068, 2158)	0.3	2095 (1983, 2205)	2214 (2118, 2311)	0.1
Smoker at 12 weeks (%, 95% CI)	16 (14,18)	28 (21,37)	<0.001	11 (7,15)	19 (17,22)	0.002	15 (10,23)	24 (19,29)	0.04
IMD*** worst quartile (%, 95% CI)	28 (25,31)	41 (35,48)	<0.001	21 (16,26)	33 (30,36)	<0.001	18 (12,25)	34 (29,40)	0.002
European origin (%, 95% CI)	94 (92,95)	92 (88,95)	0.9	95 (91,97)	93 (91,95)	0.5	96 (91,98)	96 (93,98)	0.8
University degree (%, 95% CI)	43 (40,46)	20 (15,25)	<0.001	54 (48,60)	35 (32,38)	<0.001	51 (43,60)	31 (26,37)	<0.001
Vegetarian (%, 95% CI)	9 (7,11)	7 (4,12)	0.08	16 (12,21)	7 (5,9)	<0.001	15 (10,22)	5 (2,9)	0.003
Primigravida (%, 95% CI)	47 (44,50)	40 (34,47)	0.04	55 (49,61)	44 (41,47)	0.002	53 (45,62)	50 (44,56)	0.5
History of long term illness (%, 95% CI)	13 (11,15)	10 (6,15)	0.1	13 (9,18)	13 (11,15)	0.9	15 (10,22)	16 (12,21)	0.7
Average alcohol consumption >0.5 units/day (%, 95% CI)	28 (25,31)	20 (14,27)	0.03	28 (23,34)	27 (24,30)	0.7	27 (19,35)	25 (20,31)	0.09
Miscarriage history (%, 95% CI)	23 (21,33)	27 (21, 33)	0.3	27 (22,32)	23 (20,26)	0.2	21 (14,29)	23 (18,28)	0.8

#Mean * two-sample t-test for continuous variables, chi-squared test for categorical variables ** Confidence interval *** Index of Multiple Deprivation

Table 7: Characteristics of women by whether they have reported taking any daily supplements in the first, second and third trimester in the CARE study (n=1274 for first & second trimesters, n=425 for third trimester)

3.5.4.2 Type of supplements

Women reported taking 22 different types of supplements including folic acid, iron, combined folic acid-iron preparations, MVM preparations (6 brands), evening primrose, cod liver oil, omega 3, vitamin C, vitamin B, vitamin D, vitamin E, vitamin A, calcium, zinc, magnesium and selenium preparations (Table 8). Folic acid was the most frequently reported daily supplement in the first trimester. MVM supplements were the most frequently reported daily supplements in the third trimester.

Supplement	First trimester	Second trimester	Third trimester
Folic acid	845	51	2
Iron	8	21	29
Folic acid/iron	2	1	1
Multivitamin-mineral	293	177	79
Evening primrose	6	2	2
Cod liver oil	10	2	3
Omega 3 fish oil	11	12	9
Vitamin C	18	8	15
Vitamin B	7	0	2
Vitamin E	1	3	1
Vitamin A	0	0	1
Calcium	14	8	3
Zinc	7	1	1
Magnesium	2	0	0
Selenium	2	0	0

Table 8: Number of women taking different types of supplements during pregnancy

3.5.4.3 Relationship between supplement taking and birth weight

Using a multiple linear regression model, taking any type of daily supplement during the first, second or the third trimester of pregnancy was not associated with the customised birth weight centile as a measure of birth size (adjusted change in centile points 2.7, 95% CI -2.5, 7.8, *P=0.3* for the first trimester; 3.2, 95% CI -0.9, 7.4, *P=0.1* for the second trimester and 0.5, 95% CI -6.0, 7.0, *P=0.9* for the third trimester). These estimates are based on models adjusted for cotinine levels, self-reported alcohol intake, IMD group, having a university degree, mother being a vegetarian, history of long-term chronic illness and past history of miscarriage.

Using birth weight in grams as an outcome, and adjusting for the above factors as well as maternal age, height, ethnicity, pre-pregnancy weight, parity, gestational age and baby's sex, there was also no relationship between taking daily supplements at any stage in pregnancy and birth weight (adjusted change 6 g , 95% CI -70, 82, *P=0.9* for the first trimester, 24 g , 95% CI -36, 83, *P=0.4* for the second trimester, and -7 g , 95% CI -106, 91, *P=0.9* for the third trimester).

When looking at taking particular types of supplements, taking a daily MVM preparations at any stage in pregnancy was not associated with size at birth using the continuous outcomes of birth weight in grams and customised birth weight centile, as well as the binary outcome of SGA (adjusted OR 1.3, 95% CI 0.8, 1.9, *P=0.3* for the first trimester, 1.1, 95% CI 0.7, 1.9, *P=0.7* for the second trimester, 0.9, 95% CI 0.5, 1.7, *P=0.8* for the third trimester) (Table 9). It was not associated with having a baby weighing less than 3rd centile (adjusted OR 1.5, 95% CI 0.8, 2.7, *P=0.3* for the first trimester, 1.2, 95% CI 0.5, 2.6, *P=0.7* for the second trimester, 1.6, 95% CI 0.7, 3.7,

P=0.3 for the third trimester). There was no evidence of association observed between maternal MVM supplement intake with having a baby weighing less than 5th centile or more than 90th centile (data not shown).

3.5.4.4 Relationship between supplement use and preterm birth

A logistic regression model was used to examine the relationship between the risk of preterm birth and patterns of supplement taking during pregnancy adjusting for salivary cotinine levels, self-reported alcohol intake, vegetarian diet, ethnicity, maternal age, baby's sex, parity, IMD score, having a university degree, past history of miscarriage and long-term chronic illness. Any type of daily supplement-taking during the third trimester was associated with an increase in the risk of preterm birth (adjusted OR=3.0, 95% CI 1.2, 7.4, *P=0.02*). This relationship was not statistically significant for supplement-taking in the second trimester (adjusted OR 1.6, 95% CI 0.8, 3.2, *P=0.2*), and marginally significant in the first trimester though confidence intervals were wide (adjusted OR 4.3, 95% CI 1.0, 18.2, *P=0.05*).

Taking MVM supplement preparations during the third trimester was also associated with an increased risk of preterm birth (adjusted OR 3.4, 95% CI 1.2, 9.6, *P=0.02*). This relationship was not statistically significant in the first or second trimester (Table 9).

Birth weight (grams)				
Daily multivitamin supplements	*Unadjusted difference (95% CI[#])*	*P*	*Adjusted difference* (95% CI)*	*P*
1st trimester	30.0 (-45.7, 105.7)	0.5	16.9 (-42.3, 75.8)	0.7
2nd trimester	38.4 (-53.6, 130.5)	0.4	29.4 (-43.0, 101.5)	0.3
3rd trimester	-29.1 (-179.9, 121.6)	0.7	-50.4 (-168.7, 67.9)	0.4
Customised birth centile##				
	Unadjusted difference (95% CI)	*P*	*Adjusted difference** (95% CI)*	*P*
1st trimester	3.6 (-0.2, 7.5)	0.06	1.8 (-2.3, 5.9)	0.4
2nd trimester	5.1 (0.4, 9.7)	0.04	3.3 (-1.8, 8.3)	0.3
3rd trimester	1.2 (-6.5, 8.8)	0.8	-2.3 (-10.3, 5.7)	0.8
Small for gestational age (<10th centile)				
	Unadjusted odds ratio (OR) (95% CI)	*P*	*Adjusted OR ** (95% CI)*	*P*
1st trimester	1.0 (0.6, 1.4)	0.8	1.3 (0.8, 1.9)	0.3
2nd trimester	0.9 (0.5, 1.4)	0.6	1.1 (0.7, 1.9)	0.7
3rd trimester	0.7 (0.4, 1.3)	0.3	0.9 (0.5, 1.7)	0.8
Preterm birth (<37 weeks)				
	Unadjusted odds ratio (OR) (95% CI)	*P*	*Adjusted OR *** (95% CI)*	*P*
1st trimester	0.9 (0.5, 1.8)	0.8	1.3 (0.6, 2.7)	0.5
2nd trimester	1.0 (0.5, 2.2)	0.9	1.8 (0.8, 4.1)	0.2
3rd trimester	1.8 (0.8, 4.4)	0.2	3.4 (1.2, 9.6)	0.02

Confidence intervals

Takes into account: maternal pre-pregnancy weight, height, parity, ethnicity, gestation and baby's sex

***** Adjusted for gestational age, baby's sex, maternal age, height, pre-pregnancy weight, ethnicity, parity, salivary cotinine levels, self-reported alcohol intake, past history of miscarriage, long-term chronic illness, IMD score, educational attainment and maternal vegetarian diet in a multiple linear regression model

****** Adjusted for salivary cotinine levels, self-reported alcohol intake, past history of miscarriage, long-term chronic illness, IMD score, educational attainment and maternal vegetarian diet in a multiple linear regression model

******* Adjusted for salivary cotinine levels, self-reported alcohol intake, maternal age, maternal vegetarian diet, ethnicity, baby's sex, parity, IMD score, educational attainment, past history of miscarriage and long-term chronic illness in an unconditional logistic regression model

Table 9: The relationship between maternal multivitamin-mineral supplement use during pregnancy and birth outcomes in the CARE study

3.5.4.5 Sensitivity analyses

A sensitivity analysis adjusted for the clinical diagnosis of IUGR detected by ultrasound scan during pregnancy and documented in the maternity notes, in the relationship between taking a MVM supplement preparation and both birth weight and preterm birth. The risk of preterm birth when taking supplements in the third trimester (adjusted OR 3.5, 95% CI 1.2, 10.0, *P=0.02*) remained broadly unchanged.

To take into account the possibility that the pattern of multivitamin-mineral supplement use is influenced by previous adverse birth outcomes, the same analysis was performed separately by parity. In primiparous women, the adjusted OR for the relationship between taking MVM supplement in the third trimester and preterm birth was 5.4 (95% CI 1.3, 22.7, *P=0.02*). In multiparous women, the adjusted OR was 3.7 (95% CI 0.5, 29.4, *P=0.2*). However, numbers were small resulting in wide confidence intervals.

3.5.5 Haemoglobin and mean corpuscular volume

558 and 572 participants had information on Hb and MCV at 12 and 28 weeks gestation respectively. Mean Hb was 12.7 g/dl (SD 0.9 g/dl) at 12 weeks and 11.5 g/dl (SD 1.0 g/dl) at 28 weeks. The proportion of participants with Hb <11 g/dl was 3% at 12 week and 23% at 28 weeks. Mean MCV was 90 fl (SD 5.0 fl) at 12 weeks and 89 fl (SD 5.5 fl) at 28 weeks.

3.5.5.1 Relationship between blood indices and birth outcomes

There was no relationship between customised birth centile or birth weight in grams and Hb/MCV at 12 or 28 weeks pregnancy in this study. Hb at 28 weeks was associated

with SGA (unadjusted OR per g/dl increase in Hb =1.4, 95% CI 1.1, 1.8, *P=0.02*; OR adjusted for maternal age, salivary cotinine levels and alcohol intake 1.4, 95% CI 1, 1.8, *P=0.03*). Adjusting for dietary iron intake did not alter this relationship.

3.5.5.2 Relationship between blood indices and dietary iron intake

There was no relationship between maternal Hb/MCV at 12 or 28 weeks pregnancy with dietary iron intake in the first trimester. However, there was a positive relationship between taking iron-containing supplements as reported in the first trimester questionnaire and Hb at 12 and 28 weeks, and MCV at 28 weeks in univariable analyses. The relationship remained significant for Hb at 12 and 28 weeks after adjusting for maternal age, ethnicity, parity, educational attainment, vegetarian diet, and IMD score in multiple linear regression model. Taking iron-containing supplements in the second trimester was also positively associated with maternal Hb at 28 weeks with marginal statistical significance in the multivariable model (Table 10).

	Unadjusted change	95% CI[#]	P	Adjusted change*	95% CI	P value
Dietary iron intake = <14.8 mg/day in the first trimester						
Hb at 12 weeks (g/dl)	0.1	-0.1, 0.3	0.2	0.09	-0.1, 0.3	0.4
Hb at 28 weeks (g/dl)	-0.1	-0.3, 0.1	0.3	-0.1	-0.3, 0.1	0.4
MCV at 12 weeks (fl)**	0.2	-0.1, 1.2	0.7	0.3	-0.7, 1.3	0.6
MCV at 28 weeks (fl)	-0.9	-2.0, 0.2	0.1	-0.8	-1.9, 0.3	0.2
Daily intake of iron-containing supplements in the first trimester						
Hb at 12 weeks (g/dl)	0.3	0.1, 0.4	0.005	0.2	0.05, 0.4	0.01
Hb at 28 weeks (g/dl)	0.4	0.2, 0.6	<0.0001	0.3	0.2, 0.5	<0.0001
MCV at 12 weeks (fl)**	0.6	-0.4, 1.5	0.2	0.1	-0.8, 1.1	0.8
MCV at 28 weeks (fl)	1.3	0.4, 2.4	0.008	0.8	-0.2, 1.8	0.1
Daily intake of iron-containing supplements in the second trimester						
Hb at 28 weeks (g/dl)	0.3	0.1, 0.6	0.002	0.2	0.0, 0.5	0.05
MCV at 28 weeks (fl)	1.5	0.4, 2.8	0.01	0.7	-0.05, 2.0	0.3

#Confidence intervals

*Adjusted for: maternal age, ethnicity, chronic illness, Index of multiple deprivation score, educational attainment, parity and vegetarian diet in a linear regression model

Table 10: The Relationship between dietary and supplemental iron intake and maternal blood indices (Hb and MCV) during pregnancy in the CARE study

3.6 Discussion

3.6.1 Iron intake in pregnancy

This analysis shows a positive relationship between both total iron intake (from food and supplements) and non-haem iron intake, derived from 24-hour dietary recall in the first trimester of pregnancy, and birth weight. There was no association between maternal iron intake in pregnancy and preterm birth.

3.6.1.1 Confounding

In this dataset, non-haem, rather than haem iron, was positively related to size at birth. This raises the possibility that the observed relationship is due to residual confounding by an unmeasured factor associated with both non-haem iron intake and size at birth. Therefore a sensitivity analysis was carried out by excluding vegetarians as vegetarian status may be associated with a generally healthier diet & lifestyle. This did not change the regression estimates. It could be that participants with higher intake of haem iron are more likely to have adverse birth outcomes due to lifestyle and socioeconomic factors associated with high meat intake (Hulshof et al., 2003), thus counteracting any positive effect of haem iron. However, adjusting for educational status and IMD group did not change the results. Findings from the Motherwell cohort study suggest that a diet high in low-quality meat might itself reduce fetal growth, perhaps through stimulating a stress response in the mother (Herrick et al., 2003).

Adjustment for total energy intake is recommended if it is a confounder of the relationship being examined (Willett et al., 1997). However, we did not adjust for it here because it did not fulfil the definition of a “true” confounder. Confounding can

result if total energy intake is associated with both the exposure of interest and the main outcome (Pearl, 2000), which is not the case in this study as total energy intake was not associated with birth weight (data not shown).

3.6.1.2 Effect modification

Although effect modification was not statistically significant for vitamin C, the stronger association between iron intake and birth weight in participants whose vitamin C intake was more than 50 mg/day is of interest, as vitamin C is the best known enhancer of iron absorption (Gibney et al., 2004). We used a cut-off of the pregnancy RNI of 50 mg/day for vitamin C, but the threshold where daily vitamin C intake starts to have an effect on iron absorption in vivo is not exactly known.

3.6.1.3 Relationship of iron intake with blood indices

Hb and MCV were used as proxies for iron status to assess the extent of agreement with iron intake levels. However, there are major limitations for the use of Hb and MCV levels as indicators of iron status as they do not represent specific or sensitive measures of body iron stores (Milman, 2006a). These limitations were discussed earlier in section 2.4.1. There was no evidence of association between dietary iron intake and Hb or MCV levels. This is not a surprising finding as these blood indices are only affected when ID is pronounced. It is difficult to determine the direction of the relationship between iron-containing supplements and Hb. In this analysis, it was positive. However, anaemic participants are more likely to take iron-containing supplements. This is supported by the stronger positive relationship between taking iron-containing supplements in the first trimester and Hb at 28 weeks compared to that at 12 weeks gestation.

3.6.2 Supplement intake in pregnancy

This study has shown that taking daily MVM supplements (mostly iron-containing) during any stage in pregnancy was not associated with birth weight. However, taking MVM supplements, or any supplements, in the third trimester was associated with a three-fold increase in risk of preterm birth after adjustment for smoking, alcohol intake and other relevant maternal and socioeconomic factors. Preterm birth is a leading cause of perinatal morbidity and mortality with a frequency of about 12-13% of births is the US and 5-9% in many other developed countries (Goldenberg et al., 2008). The relationship between preterm birth and any supplement intake in the third trimester of pregnancy is likely to be driven by MVM supplements as they were the most common supplements to be taken in the CARE cohort (Table 8). This negative effect seems more pronounced in primiparous women. The mechanism for this is unclear and this study's findings need confirming by other cohorts and/or trials in high income countries, where pregnant women are predominantly micronutrient-replete.

3.6.2.1 Confounding

Although the number of supplement-taking women in the third trimester was considerably less than that for the first two trimesters, there was enough statistical power with the nested case-control design to detect an OR of 3 for the preterm birth outcome. However, this study is observational so causality cannot be inferred from the findings. As information on iatrogenic preterm birth was not available, it is possible that some women knew that they were at risk of preterm birth and that this knowledge initiated physician or patient-led supplementation. However, in this study, only 5 women who reported taking daily supplements only in the third trimester, did

not take supplements in the first and second trimester. None of these 5 women had a preterm birth. This means that most women have continued taking MVM supplements from the first or second trimester and have not stopped until the end of pregnancy.

Because this is not a RCT, there is a possibility that residual confounding may be contributing to this apparent association. There may be unmeasured or uncontrolled confounders resulting in the apparent negative relationship between multivitamin supplement taking in the third trimester and preterm birth. However, most factors known to confound this relationship were taken into account. The possibility that supplement use may be influenced by a woman knowing that the baby is not growing as it is expected to do is also taken into account by adjusting for the clinical diagnosis of IUGR as extracted from the pregnancy medical notes in a sensitivity analysis.

The potential for previous poor pregnancy outcomes influencing the mother's decision to take supplements in subsequent pregnancies was also considered. Therefore, past history of miscarriage was adjusted for in the main models, and a sensitivity analysis was performed separately for primiparous and multiparous women. The hypothesis is that women with previous adverse pregnancy outcomes would be more likely to take supplements as well as experience adverse outcomes in their subsequent pregnancies. This would confound the relationship between supplement-taking in the third trimester and preterm birth. However, in contrast to this hypothesis, this relationship was more pronounced in primiparous women. This means that the effect is not influenced by previous birth outcomes.

3.6.2.2 Interpretation of the observed association between maternal multivitamin-mineral supplement intake and preterm birth

The use of MVM supplements in the CARE cohort was restricted mainly to two pregnancy-specific brands. Both brands included folate and vitamin C exceeding the current recommended minimum during pregnancy. One of the brands had the additional components of B-carotene, vitamin K, selenium and iodine as well as higher doses of vitamins E, B1, B6, B12 and zinc (at least double) compared with the other main brand. Women in our cohort were having adequate amounts of these micronutrients from their diet alone as assessed by the 24 hour dietary recall (Table 3). This confirms the inverse supplement hypothesis that women who least need supplements are most likely to take them (Conner et al., 2003).

Some observational studies report a favourable effect of periconceptional use of MVM supplements on preterm birth and SGA (Catov et al., 2007, Catov et al., 2011). Other studies have suggested potential adverse effects of some supplements, specifically those containing antioxidant vitamins such as vitamins C and E, on pregnancy outcome when taken in women with adequate dietary micronutrient intake. Smedts et al., in a case control study of offspring with congenital heart disease, found that periconceptional use of vitamin E supplements with high dietary intake of the same vitamin was associated with up to nine-fold increase in the risk of congenital heart disease (Smedts et al., 2009). Another study found that use of vitamins C and E supplements was associated with an increased risk of premature rupture of membranes (Spinnato Ii et al., 2008). Unfortunately, this information was not recorded in the CARE study. In a RCT to assess the effect of vitamins E and C supplementation

during pregnancy on the incidence of pre-eclampsia, Poston et al. found that more low birth weight babies were born to women who took these antioxidants than to controls (Poston et al., 2006). A recent meta-analysis of seven studies concluded that combined vitamin C and E supplementation had no potential benefit in improvement of maternal and neonatal outcome and increased the risk of gestational hypertension in women at risk of pre-eclampsia (Rahimi et al., 2009).

It is well established that there are significant interactions between micronutrients and their metabolism. It has been shown in rats, for example, that copper deficiency during pregnancy can result in reduced iron status and vice versa, and that copper overload induces iron overload, by interfering with the iron regulatory mechanism (Fosset et al., 2009, Gambling et al., 2008). Others have demonstrated interactions between iron and zinc (Kelleher and Lönnerdal, 2006). During the third trimester, fetal growth is at its most rapid. The fetus not only needs minerals to sustain its growth, it is also a stage when the fetal liver builds up stores for the immediate post-natal period. A reduction in availability, by interactions between the nutrients in the maternal gut, liver or in the placenta itself may result in adverse outcomes for the baby.

The pattern of dietary supplement use in the CARE cohort, with most women taking supplements (mainly folic acid) in the first trimester, is expected as there is no national recommendation in England for routine supplement-taking during pregnancy apart from folic acid in the first trimester and vitamin D for pregnant women in “high-risk” groups (National Institute for Clinical Excellence (NICE), 2008). There is no national recommendation to take MVM supplements at any stage during pregnancy. However, they are readily available over-the-counter and are heavily promoted to expectant

mothers. Health value and susceptibility to illness are major predictors of supplement use by women, with dietary supplements acting as an insurance against possible ill health (Conner et al., 2001).

3.6.3 Strengths and limitations of the study

3.6.3.1 Study sample

This was a large prospective cohort study. Although a RCT is the gold standard study design to investigate causality, this design would be difficult to execute especially when the exposure is dietary intake. The response rate to take part in the study was 30% out of all the women who were invited. The percentage of LBW babies (<2500 g) in this study (4.4%) was less than the National UK (7.2%) and the Yorkshire & Humber region average (7.8%) for 2007 (Office for National Statistics). This raises the possibility that women who are more likely to have LBW babies were less likely to participate in this study.

3.6.3.2 Outcome measures

Customised birth weight centile, which takes into account gestational age, maternal height, weight, ethnicity and parity, and neonatal birth weight and sex was used in this chapter's analyses. However, it does not take into account paternal height, which has been shown to be related to birth weight (Morrison et al., 1991, Nahum and Stanislaw, 2003).

3.6.3.3 Exposure measures

Dietary iron intake was ascertained using 24-hour dietary recall recorded by a midwife-administered interview at around 12 weeks gestation. This method has been validated,

and found to be comparable to other dietary assessment methods such as FFQs and food diaries in estimating iron intake (Bingham et al., 1997). However, the 24-hour recall has its limitations such as failure to recall diet accurately and the chance of consuming non-typical diet during the day prior to the assessment. Whilst the study has a large sample size and hence good probable estimates of mean daily intake, these may be more widely dispersed than in reality due to the use of this dietary assessment method. It therefore may over-estimate the proportion of mothers with extremely high or low iron intakes, for example the proportion with daily iron intake <UK LRNI (24% in our sample). However, there is evidence, when validating 24-hour recalls against other methods of dietary assessment, that recall is prone to over-reporting low intakes and under-reporting high intakes (Gersovitz et al., 1978). The estimation of haem iron intake may have been subject to greater error than the estimation of non-haem intake, given that it constitutes a smaller proportion of total dietary iron.

The use of supplements was recorded both in the 24-hour recall and the interviewer-administered and self-reported questionnaires. The extent of agreement was high between the two methods in this study for reporting iron-containing supplements intake, however there is potential for measurement error using both methods. It is unlikely that women with adverse outcomes would have reported their supplement-use pattern or dietary intake differently to other women since it is a prospective study, therefore reducing the chance of differential bias. The supplements reported in the recall, rather than the questionnaire, were used to add up to the dietary iron in order to derive the total iron intake variable as they were both reported in the same recall i.e. came from the same source, hence less chance of reporting bias.

Lastly, iron intake is used in this study as a proxy for maternal iron status in pregnancy. Absorption of iron is influenced by several factors including the individual's iron status as discussed earlier in section 2.2. Therefore, maternal iron status in early pregnancy was assessed using biomarkers including sF and sTfR to investigate its association with birth outcomes in chapter 4 of this thesis.

3.6.4 Implications for research and practice

3.6.4.1 Iron intake in pregnancy

Further research is needed to explore the role of vitamin C intake in the relationship between dietary and supplementary iron intake and birth outcomes. A RCT of high dietary iron intake combined with vitamin C at mealtimes during early pregnancy can provide some important insights, as there are problems with just relying on supplement intake, as pointed out in this chapter, and earlier in sections 2.2.8.2 and 2.2.8.3. Public health messages about increasing iron intake during early pregnancy and ways to optimise its absorption need to be promoted.

3.6.4.2 Multivitamin-mineral intake in pregnancy

Most previous trials and observational studies in developed country settings have looked at the effect of taking multivitamin supplementation in early pregnancy on maternal and birth outcomes. More research is needed into the effect of taking MVM supplements in late pregnancy on birth outcomes in relatively well-nourished populations. Larger cohort studies are required to examine this association in detail and to validate the findings of this study. Results from our cohort also suggest that a

trial, in a high income country setting, is needed to weigh the possible benefits and harms of policies recommending supplementation or restriction of supplementation.

The study findings suggest that clinicians and midwives should be cautious when recommending over-the-counter MVM supplements to women in their late pregnancy. As in any clinical situation, they should weigh the potential risks and benefits when considering prescribing such supplements during late pregnancy. The type of supplement recommended or prescribed should be more focussed on the specific vitamin or mineral deficiency the woman has. Although the negative relationship between MVM in the third trimester and preterm birth needs to be investigated further, this study did not show any positive effect on birth weight and gestational age when these supplements are taken in any stage in pregnancy.

Conclusive evidence is provided solely for periconceptional folate supplementation in the prevention of neural tube defects. A Lancet review in 2005 recommended 16 interventions to improve neonatal survival, out of which two were supplementation programmes: folic acid to reduce neural tube defects incidence and calcium to reduce pre-eclampsia and eclampsia incidence (Darmstadt et al., 2005). MVM supplements may be beneficial in women with poor nutrition and multiple micronutrient deficiencies. Adverse effects seen in some studies associated with MVM supplementation may be due to the detrimental effects of certain components such as vitamins C and E, and/or to the interaction between the multiple ingredients of the preparation. In summary, there is much less evidence on the need for supplementation in general during pregnancy in high income compared to low income countries.

3.7 **Conclusion**

In this chapter, a positive association between total iron intake from food and supplements in the first trimester of pregnancy and customised birth weight centile was demonstrated. This association was stronger in the high vitamin C intake group, however effect modification was not significant. Although iron intake from food alone was not significantly associated with birth weight after adjustment, intake of non-haem iron was more strongly associated with birth weight than haem iron. Iron intake during the first trimester of pregnancy, both from diet and supplements, was higher in vegetarians and women with better socioeconomic profile.

In this study, the use of MVM supplement preparations during the third trimester in pregnancy was associated with an increased risk of preterm delivery, and was not associated with birth weight or SGA at any stage in pregnancy. These findings suggest that, at least in micronutrient-replete mothers, caution must be exercised when recommending MVM supplements in late pregnancy. This is an observational prospective study offering weaker causal evidence than a RCT. However, in the absence of a trial in a developed country setting, this study makes a useful contribution to the research evidence in this area. The findings generate a concern regarding multivitamin supplement use in late pregnancy that needs to be investigated by other studies.

In the next chapter, I extend the investigation commenced in this chapter by measuring iron status in pregnant mothers using sensitive and specific biomarkers, and including a measure of the vascular system of the baby, arterial stiffness, as an outcome, in addition to size at birth and preterm delivery.

4 Associations of maternal iron status in early pregnancy with birth outcomes and infant arterial stiffness: the Baby VIP study

In chapter 3, I investigated the association of maternal iron intake with size at birth and preterm delivery. In this chapter, I move a step forward by measuring iron status in mothers early in pregnancy and investigate its association with the same outcomes assessed in chapter 3 plus an additional innovative outcome measuring arterial stiffness in the infant: PWV.

The results presented in this chapter have been submitted as a peer-reviewed article for publication. This work has also formed the basis of three conference presentations (one already presented and two accepted abstracts).

4.1 Chapter summary

This chapter aims to examine the association between maternal iron status during the first trimester of pregnancy, and infant brachio-femoral PWV (bfPWV) at 2-6 weeks of age. Data from the Baby VIP study were used. This is a historical birth cohort that recruited 362 babies and their mothers after hospital delivery in Leeds, UK. sF and sTfR were measured in maternal samples previously obtained in the first trimester of pregnancy. Maternal Hb, birth weight, gestational age and other covariables used to derive customised birth weight centile were extracted from the medical records. Baby's bfPWV was measured during a home visit at 2-6 weeks.

The cohort included 33 (9%) preterm (<37 weeks gestation) and 64 (18%) SGA infants. Out of 348 pregnant women with information on sF in the first trimester, 79 (23%) had iron depletion (<15 ug/l). Prevalence of anaemia at ≤20 weeks (<11 g/dl) and >20 weeks gestation (<10.5 g/dl) was 5% (16/329) and 14% (48/337) respectively. Mean infant bfPWV was 6.7 m/s (SD=1.3, n=284). Maternal iron depletion in the first trimester was associated with a higher risk of a SGA baby (adjusted OR 2.2, 95% CI 1.1, 4.1). However, this relationship was attenuated when including early pregnancy Hb in the model (adjusted OR 1.6, 95% CI 0.8, 3.2). For every 1g/dl increase in maternal Hb level in the first half of pregnancy the risk of SGA was reduced by 30% (adjusted 95% CI 0-40%), with levels <11 g/dl associated with a 3-fold increase in the risk of SGA (95% 1.0, 9.0). There was no evidence of association between maternal iron status and preterm birth or gestational age.

Maternal anaemia at ≤20 weeks gestation was associated with a 1.0 m/s increase in infant PWV (adjusted 95% CI 0.1, 1.9, P=0.02). There was no evidence of association

between infant bfPWV and maternal sF analysed as a continuous variable (adjusted change in PWV in m/s per 10 ug/l change in sF = 0.02, 95% CI -0.01, 0.1, P=0.3), nor with maternal iron depletion (adjusted change in PWV in m/s = -0.2, 95% CI -0.6, 0.2, P=0.3). No evidence of association was observed between maternal sTfR or log R/F ratio with infant PWV.

In this chapter, depleted iron stores in early pregnancy were found to be associated with higher risk of having a SGA baby. However, this relationship seems to be mediated by maternal Hb levels. Increased arterial stiffness in the first few weeks of life was associated with maternal anaemia in the first half of pregnancy, but not with ID in the first trimester.

4.2 **Background**

The previous evidence on the association of maternal ID in pregnancy with birth outcomes was explored in sections 2.2.4 and 2.2.5. There are very few studies which have assessed iron status in the mother early in pregnancy and using multiple biomarkers, including sF and sTfR, in addition to Hb. Ferritin is the most widely used biomarker in the assessment of iron status in the general population. In women, levels under 15 ug/l indicate depleted iron stores (WHO, 2011). However, it is affected by inflammatory conditions and therefore may not be specific to distinguishing ID. Measuring STfR may provide more specific information, and it has the advantage over sF is that it can distinguish IDA from anaemia of chronic inflammation, as well as identify iron depletion and functional ID in patients with concurrent inflammation (Allen et al., 1998). The R/F ratio is considered the gold standard marker of iron status (Zimmermann, 2008), and has been used to assess iron status in pregnant populations such as the United States National Health and Nutrition Examination Survey (Mei et al., 2011). The advantages and disadvantages of these biomarkers have been explored further earlier in this thesis (section 2.4).

The clinical significance of arterial stiffness and its measurement method used in this chapter, PWV, was also explored in detail earlier in section 2.5.1. Few studies have examined the relationship between maternal nutritional exposures and arterial stiffness in the offspring (Larnkjaer et al., 2006, Gale et al., 2007, Kinra et al., 2008). So far, there have been no comparable studies published that have assessed the relationship between indicators of maternal nutritional status during pregnancy and neonatal or infant arterial stiffness.

4.3 Hypothesis and objectives

It is hypothesised that infants of women with ID in early pregnancy have less favourable cardiovascular risk profile indicated in this study by lower birth weight and stiffer arteries, expressed as increased PWV.

The objectives of this chapter are:

1. Examine the relationship of maternal iron status at the end of the first trimester of pregnancy with offspring's arterial stiffness measured by brachio-femoral PWV (bfPWV) within the first few weeks of life (2-6 weeks of age)
2. Examine the relationship of maternal iron status at the end of the first trimester of pregnancy with birth weight and preterm birth
3. Examine the relationship of birth weight/SGA and gestational age/preterm birth with offspring's arterial stiffness measured by bfPWV within the first few weeks of life (2-6 weeks of age)

4.4 Methods

4.4.1 Study design

Baby VIP study is a retrospective cohort study. The cohort comprises women aged 18 years or over who have given live birth at the Leeds Teaching Hospitals Trust Maternity Unit at a gestational age of 34 weeks or over in the period between February 2012 to January 2013. The participants were recruited from the postnatal wards of Leeds General Infirmary and St James's University Hospital after delivery. Upon consenting to taking part in the study, mothers were asked permission to access theirs' and their babies' medical notes from which clinical information relating to pregnancy and birth was extracted. They were also asked if the research team could contact them after they were discharged home to arrange a home visit within 6 weeks. The home visit was made by a research nurse after an appointment was arranged with the mother over the phone. During the visit, the nurse obtained mothers' consent for their babies to have PWV measured on them, and went on to perform the measurements as described below. Figure 8 illustrates the Baby VIP study participant flowchart. Appendices 10.3.1 and 10.3.2 include the study's consent forms and participant information sheet.

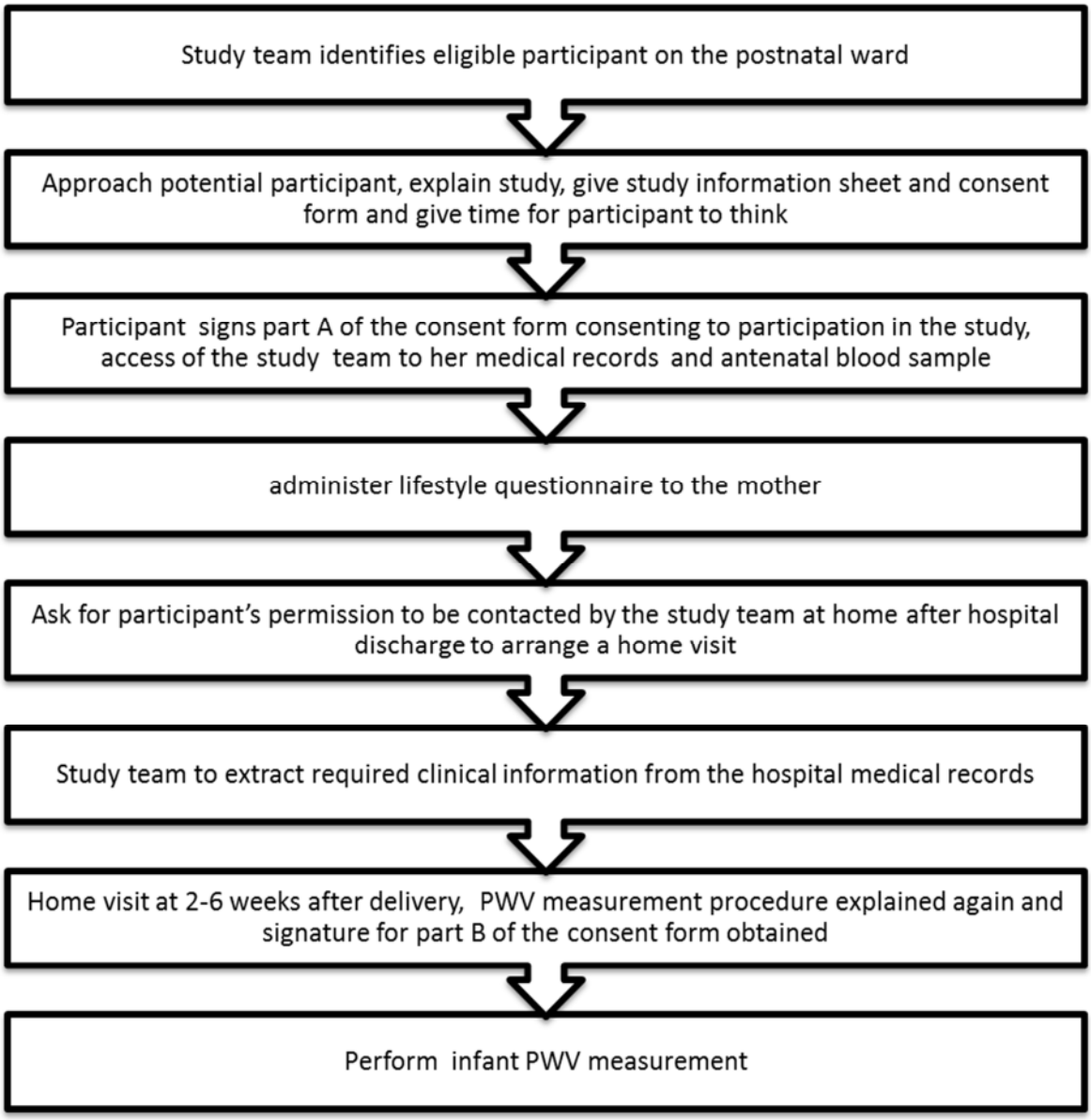


Figure 8: Baby VIP study participant flowchart

4.4.2 Exposure measurement

Maternal serum samples previously stored during the first trimester of pregnancy as part of routine antenatal care were analysed. sF was measured using ELISA (Demedeitic, Kiel, Germany) following the manufacturer's instructions. Briefly, 10 ul of plasma was treated in a sandwich ELISA method, using fluoremetric measurements and calibrated using standards supplied by the manufacturer. Quality controls were

included as appropriate. Data are expressed in ug/l. The WHO cut-off of 15 ug/l in sF was used to indicate depleted iron stores (WHO, 2011). STfR assays were performed using a commercially available kit based on a polyclonal antibody in a sandwich enzyme immunoassay (EIA) format (DTFR1; R&D Systems, Minneapolis, MN). This yielded sTfR levels in nmol/l units. The values were converted to ug/l using a molecular weight of sTfR of 75000 daltons (R&D technical data sheet). The R/F ratio was obtained by dividing sTfR over sF (ug/l: ug/l). This was logged to obtain a normal distribution.

In the UK, Hb is measured routinely in pregnancy at around 12 and 28 weeks gestation. Maternal Hb values were extracted from the antenatal care records and/or the hospital electronic results server. A cut-off of 11 g/dl in Hb was used to indicate anaemia at 20 weeks gestation or less, and 10.5 g/dl to indicate anaemia beyond 20 weeks gestation, following the NICE guidelines (National Institute for Clinical Excellence (NICE), 2008).

4.4.3 Outcome measurement

4.4.3.1 Pulse wave velocity

Brachio-femoral PWV was measured using the Vicorder device (Skidmore Medical), which uses an oscillometric technique to measure PWV (Figure 9). This kit provides a non-invasive and minimally-intrusive method of measuring PWV, and is thought to be less time consuming and less dependent on operator skills (van Leeuwen-Segarceanu et al., 2010). A standard operating procedure was developed and followed for each measurement (Appendix 10.3.3). Two infant-size cuffs were attached to the device. The arm cuff was wrapped around the bare skin of the baby's arm, with the mid cuff

(marked) being halfway between the shoulder and the elbow. The leg cuff was wrapped around the bare skin of the baby's ipsilateral thigh with the mid cuff point (marked) being halfway between the groin and the knee. Using a tape measure, the distance was measured in centimetres between the midpoint of the arm cuff to the midpoint of the left cuff in a straight line while keeping the baby's ipsilateral thigh straight, with the tape kept on the internal side of the arm alongside the trunk, not externally over the elbow and forearm. The pressure applied was 35 mmHg for both the arm and the leg. The pulse recording at the two arterial sites (brachial and femoral) was obtained simultaneously. Transit time was measured as the time delay between the feet of the proximal and the distal pulse waves. A minimum of two PWV readings was obtained from each baby. If they were more than 0.3 m/s different, a third reading was obtained. The average of all available readings for each baby was derived and used in the analyses. The baby's age and position at the time of PWV measurement (sleeping, feeding, lying down or in mother's arms) was recorded and taken into account in all the statistical models.

Figure 9: Vicorder kit (Skidmore Medical)

4.4.3.2 Size at birth

Birth weight in grams and gestational age in days were extracted from the medical notes. Customised birth weight centile was calculated using charts that take into account gestational age, maternal height, weight, ethnicity, parity, and neonatal sex (Gardosi, 2004). SGA was defined as less than the 10th centile. Duration of gestation was calculated from the date of the last menstrual period, and confirmed by ultrasound scans dating at around 12 and 20 weeks gestation. Preterm birth was defined as delivery at less than 37 weeks (259 days) gestation.

4.4.4 Covariable assessment

A basic demographic and lifestyle questionnaire was administered to the mother by the research team at the time of recruitment. IMD was derived from each participant's postcode using the GeoConvert tool utilising the 2001 UK census data (geoconvert.mimas.ac.uk). Parity, maternal height, weight, ethnicity, smoking, pregnancy complications (pre-eclampsia, GDM), BP measurements and intake of iron supplements were extracted from the mother and the baby's clinical records.

4.4.5 Statistical methods

Statistical analysis was performed using Stata version 11 (College Station, TX, 2009). Univariable analysis was performed using the independent samples t-test, one-way analysis of variance or Mann-Whitney test for continuous variables, and the chi-squared test for categorical variables. Multivariable linear regression was performed with PWV, customised birth weight centile, SGA, gestational age and preterm birth as outcomes, and indicators of maternal iron status including maternal sF, sTfR, Hb and R/F as predictors. When the outcome was PWV, the models were adjusted for baby covariables at birth (customised birth weight centile) and at measurement (age, position, measurement side and type of feeding), and maternal covariables including age, smoking status, the presence of GDM or pre-eclampsia, systolic and diastolic BP at booking and 36 weeks gestation, and IMD deprivation score. When the outcomes were birth weight centile/SGA, the models were adjusted for maternal age, smoking status, the presence of GDM or pre-eclampsia, and IMD deprivation score. When the outcomes were gestational age/preterm birth, the models were adjusted for maternal age, ethnicity, parity, pre-pregnancy BMI, smoking status, the presence of GDM or pre-

eclampsia, and IMD deprivation score. In all models, sensitivity analyses were performed taking into account the intake of iron supplements during pregnancy.

When examining the association between infant PWV and birth weight centile or SGA, multivariable models were adjusted for maternal factors including smoking, pre-eclampsia, GDM, systolic and diastolic BP at booking and 36 weeks, IMD deprivation score, and infant factors including type of feeding, baby's age, position and whether asleep or awake at the time of measurement.

4.4.6 Sample size calculation

For a difference of 0.3 m/s in neonatal aortic PWV between iron-deficient and non-iron deficient mothers, using a mean of 4.7 m/s, a SD of 0.6 m/s (Koudsi et al., 2007), and a prevalence of ID of 20% (Bergmann et al., 2002, Beard, 1994), a sample size of 265 mother-baby pairs was required to achieve a 90% power with *P=0.05.*

4.4.7 Ethical approval

Ethical approval was obtained from the South Yorkshire Research Ethics Committee of the NHS National Research Ethics Service (Reference number 11/YH/0064). All procedures were in accordance with the Helsinki Declaration of 1975 as revised in 1983. Mothers provided their written informed consent for themselves and their children to participate in this study.

4.4.8 Funding

The work included in this chapter was supported by the Wellcome Trust [Grant number WT87789 to N.A.A.] and the Scottish Government Rural and Environmental Services (RESAS) for the laboratory analysis of samples undertaken at the Rowett

Research Institute, University of Aberdeen, UK. The funders had no influence on the design or analysis of the study.

4.5 Results

4.5.1 Sample characteristics

In total, 362 women living in Leeds, UK and surrounding area were recruited for this study. 288 (80%) were of white ethnic origin. Mean maternal age was 31 years (SD 6), and mean maternal pre-pregnancy BMI was 26 kg/m^2 (SD 6). 47 women delivered by elective caesarean section (13%), and 68 (19%) by emergency section. 173 women were primiparous (40%). The average BP at booking was 112/67 mmHg, and at 36 weeks gestation 116/71 mmHg. 192 women said they never smoked (54%), compared to 49 (14%) smokers and 113 (32%) ex-smokers. 6 (2%) women had pre-eclampsia during pregnancy and a similar number had GDM. Half of the babies born to participants were male. 123 (43%) of those who were followed up at home were reported to be exclusively breast-fed, 110 (39%) bottle-fed and 53 (19%) received mixed breast and bottle feeding.

4.5.1.1 Infant pulse wave velocity

The total number of babies with PWV measurements performed at home was 284, out of a total of 362 mother-baby pairs recruited in hospital (79%). Out of these, 248 (87%) had a third measurement taken because the first two measurements differed by more than 0.3 m/s. Mean infant bfPWV was 6.7 m/s (SD 1.3). Mean baby age at the time of PWV measurement was 25 days (SD 6). 48 babies (16%) were asleep at the time of measurement.

Baby being asleep was, on average, associated with 0.9 m/s reduction in infant bfPWV (95% CI 1.3, 0.5, P<0.0001). The baby's feeding in his/her mother's arms at the time of

measurement was, on average, associated with a 0.7 m/s increase in infant bfPWV (95% CI 0.3, 1.0, *P<0.0001*), compared to being held in mother's arms without being fed. On the other hand, lying in a cot, on a sofa or on the floor at the time of PWV measurement was associated with a 0.4 m/s reduction in bfPWV (95% CI 0.1, 0.8, P=0.03) compared to being held in mother's arms without being fed. Baby's age at the time of PWV measurement, type of feeding (breast/bottle/mixed), and side of measurement (left or right arm and leg) were not associated with bfPWV in this study. Table 11 describes infant PWV in relation to measurement conditions and baby characteristics.

Mothers of infants who were followed up at home were likely to be older, primiparous and have taken multivitamin supplements during pregnancy compared to those whose infants did not have PWV measurements because a home visit was not possible to arrange or the mother decline the visit. Otherwise, there were no significant differences in baseline characteristics of the two groups.

Table 12 compares the characteristics of participants with PWV measurements (n=284) and those who were not followed up with a home visit (n=78).

Infant brachio-femoral pulse wave velocity (m/s)				
	n	**Mean**	**SD**	**P**
Sleeping status				<0.001*
asleep	48	5.9	1.1	
awake	239	6.8	1.3	
Position during measurement				<0.001**
In mother's arms	121	6.5	1.3	
Feeding in mother's arms	101	7.2	1.2	
In cot / on sofa or floor	61	6.1	1.1	
Measurement side				0.2*
Right	98	6.8	1.4	
Left	181	6.6	1.2	
Baby's age				0.7*
<28 days	206	6.6	1.3	
≥28 days	77	6.7	1.4	
Type of feeding				0.06**
Breast	122	6.9	1.4	
Bottled	109	6.5	1.3	
Mixed	53	6.4	1.2	

*Independent samples t-test

*** One-way analysis of variance

Table 11: Infant brachio-femoral pulse wave velocity (m/s) in relation to measurement conditions and infant characteristics in the Baby VIP study.

	With PWV measurements	N	With no PWV measurements	N	P#
Gestational age (days) (mean, sd*)	277 (14)	284	276 (14)	78	0.5
Birth weight (grams) (mean, sd)	3339 (636)	284	3293 (621)	78	0.6
Maternal age at antenatal booking (years) (mean, sd)	31.1 (5.5)	284	28.6 (6.0)	78	0.0007
Maternal body mass index at antenatal booking (Kg/m^2) (mean, sd)	26.5 (6.1)	281	25.3 (4.9)	77	0.1
Index of multiple deprivation (IMD) (mean, sd)	28.6 (19.1)	284	32.7 (19.6)	78	0.1
Maternal haemoglobin at ≤20 weeks gestation (g/dl) (mean, sd)	12.6 (1.0)	263	12.4 (1.2)	66	0.1
Maternal haemoglobin at >20 weeks gestation (g/dl) (mean, sd)	11.6 (1.0)	266	11.4 (1.1)	71	0.2
First trimester maternal serum ferritin (sF) (ug/l) (median, IQR)**	33.4 (17.4, 61.6)	273	28.6 (13.1, 68.7)	75	0.5
First trimester maternal serum transferrin receptor (sTfR) (nmol/l) (median, IQR)	13.1 (10.4, 16.1)	273	12.3 (10.1, 16.1)	75	0.6
First trimester maternal sF/STfR ratio (ug/l) (median, IQR)	27.5 (15.3, 61.9)	273	32.0 (14.0, 72.9)	75	0.6
Primiparous (n, %, 95% CI*)**	144, 51 (45, 57)	284	29, 37 (27, 49)	78	0.03
Male baby (n, %, 95% CI)	137, 48 (42, 54)	284	45, 58 (47, 69)	78	0.1
Maternal White ethnicity (n, %, 95% CI)	220, 78 (72, 82)	284	68, 87 (78, 94)	78	0.1
Maternal smoking at antenatal booking (n, %, 95% CI)	36, 13 (9, 18)	278	13, 17 (9, 28)	76	0.5
Gestational diabetes (n, %, 95% CI)	5, 2 (1, 4)	284	1, 1 (0, 7)	78	0.1
Pre-eclampsia (n, %, 95% CI)	3, 1.1 (0, 3)	284	3, 3.9 (1.0, 11)	78	0.1
Anaemia at ≤20 weeks gestation (<11 g/dl) (n, %, 95% CI)	10, 4 (2, 7)	263	6, 9 (3, 19)	66	0.1
Anaemia at >20 weeks gestation (<10.5 g/dl) (n, %, 95% CI)	35,13 (9, 2)	266	13, 18 (10, 29)	71	0.3
Taken iron supplements in pregnancy (n, %, 95% CI)	92, 32 (27, 38)	284	29, 38 (27,49)	77	0.4
Taken multivitamin supplements in pregnancy (n, %, 95% CI)	157, 55 (49, 61)	284	32, 42 (30, 53)	77	0.03

*Standard deviation **Inter quartile range ***Confidence interval
Independent samples t-test or Mann-Whitney test for continuous variables, and chi-square test for categorical variables

Table 12: Characteristics of Baby VIP study participants (n=362) by whether babies were followed-up by a home visits to measure pulse wave velocity (PWV)

4.5.1.2 Birth outcomes

In this study sample (n=362), mean birth weight was 3329 grams (SD 632), with 40 (11%) babies weighing less than 2500 grams. 358 women had information to derive customised birth weight centile. Mean customised birth weight centile was 41 (SD 29), with 64 (18%) babies weighing less than the 10th centile (SGA), and 29 (8%) babies weighing less than the 3rd centile. 20 (6%) babies weighed more than the 90th centile, of those, only 1 (5%) was born to a mother with GDM. Mean gestational age was 277 days (SD 14) i.e. 39.6 weeks. 33 (9%) babies were born preterm (between 34 and 37 weeks gestation).

Women with SGA babies were more likely to smoke, have lower early pregnancy Hb, be anaemic at ≤20 weeks gestation, be iron depleted in the first trimester (sF<15 ug/l) and have suffered from pre-eclampsia during pregnancy, compared to women with AGA babies. SGA babies were more likely to be born preterm (<37 weeks gestation), and to have lower bfPWV compared to AGA babies. Table 13 describes the characteristics of participants whose babies were born SGA compared to those with AGA babies.

	SGA$^{\alpha}$	N	AGA$^{\alpha\alpha}$	N	P$^{\#}$
Gestational age (days) (mean, sd*)	270 (15)	64	279 (13)	294	<0.0001
Birth weight (grams) (mean, sd)	2499 (446)	64	3510 (516)	294	<0.0001
Maternal age at antenatal booking (years) (mean, sd)	30.4 (6.0)	64	30.5 (5.7)	294	0.8
Maternal body mass index at antenatal booking (Kg/m^2) (mean, sd)	25.7 (5.8)	64	26.3 (5.8)	294	0.4
Index of multiple deprivation (IMD) (mean, sd)	30.6 (19.2)	64	29.2 (19.8)	294	0.6
Maternal haemoglobin at ≤20 weeks gestation (g/dl) (mean, sd)	12.3 (1.1)	54	12.7 (1.0)	272	0.02
Maternal haemoglobin at >20 weeks gestation (g/dl) (mean, sd)	11.6 (1.1)	55	11.6 (1.0)	279	0.9
First trimester maternal serum ferritin (sF) (ug/l) (median, IQR)**	33.5 (12.4, 52.5)	58	30.7 (17.9, 65.7)	286	0.8
First trimester maternal serum transferrin receptor (sTfR) (nmol/l) (median, IQR)	13.6 (10.0, 17.9)	58	12.5 (10.3, 16.0)	286	0.1
First trimester maternal sF/STfR ratio (ug/l) (median, IQR)	38.1 (16.4, 101.2)	58	28.0 (14.4, 61.2)	286	0.6
Infant brachio-femoral pulse wave velocity (m/s) (mean, sd)	6.3 (1.1)	48	6.7 (1.3)	233	0.04
Primiparous (n, %, 95% CI*)**	26, 41 (29, 54)	64	145, 49 (44, 55)	294	0.2
Male baby (n, %, 95% CI)	29, 45 (33, 58)	64	151, 51 (46, 57)	294	0.4
Preterm birth (<37 weeks gestation)	12, 19 (10,31)	64	21, 7 (5, 11)	294	0.004
Maternal White ethnicity (n, %, 95% CI)	48, 75 (63, 85)	64	237, 81 (76, 85)	294	0.3
Maternal smoking at antenatal booking (n, %, 95% CI)	13, 22 (13, 35)	58	36, 12 (9, 17)	292	0.04
Gestational diabetes (n, %, 95% CI)	2, 3 (0, 11)	62	4, 1 (0, 3)	294	0.3
Pre-eclampsia (n, %, 95% CI)	3, 5 (1, 13)	64	3, 1 (0, 3)	294	0.04
Anaemia at ≤20 weeks gestation (<11 g/dl) (n, %, 95% CI)	6, 11 (4, 23)	54	10, 4 (2, 7)	272	0.02
Anaemia at >20 weeks gestation (<10.5 g/dl) (n, %, 95% CI)	8,15 (6, 27)	55	40, 14 (10, 19)	279	0.9
Taken iron supplements in pregnancy (n, %, 95% CI)	20, 31 (20, 44)	64	100, 34 (29, 40)	293	0.7
Taken multivitamin supplements in pregnancy (n, %, 95% CI)	30, 47 (34, 60)	64	156, 53 (47, 59)	293	0.4

* Standard deviation **Inter quartile range ***Confidence interval α Small for gestational age (<10th birth weight centile) αα Appropriate for gestational age (≥ 10th birth weight centile) # Independent samples t-test or Mann-Whitney test for continuous variables, and chi-square test for categorical variables

Table 13: Characteristics of Baby VIP study participants (n=362) by size at birth

4.5.1.3 Biomarkers of maternal iron status

The first trimester serum samples of 348 mothers were accessed and analysed for sF and sTfR. Median sF was 13.7 ug/l (Interquartile range [IQR] 16.9, 62.4). 79 women (23%) had depleted iron stores by the end of the first trimester with sF <15 ug/l, and 278 women (80%) had sF levels < 70 ug/l. Median sTfR was 12.8 nmol/l (IQR 10.2, 16.1). According to the assay manufacturer (DTFR1; R&D Systems, Minneapolis, MN), the 2.5^{th} - 97.5^{th} percentile range of the reference distribution of sTfR concentration is 0.85 to 3.05 mg/l (n = 1,000) (Punnonen et al., 1997). In our pregnant study population (n=348), the range between the 2.5^{th} and 97.5^{th} percentiles was 0.6 to 2.0 mg/l after conversion to equivalent units. Median R/F was 28.4 ug/l (IQR 14.6, 65.4).

Mean maternal Hb was 12.6 g/dl and 11.6 g/dl (SD 1.0) in the first and second halves of pregnancy respectively. The prevalence of anaemia at ≤20 weeks (<11 g/dl) and >20 weeks gestation (<10.5 g/dl) was 5% (16/329) and 14% (48/337) respectively. Only half of anaemic women in the first half (n=8), and 45% of anaemic women in the second half of pregnancy (n=22), had a first trimester sF of less than 15 ug/l. However, 14 (89%) of anaemic women in the first half of pregnancy and 43 (90%) of anaemic women in the second half of pregnancy had sF of less than 70 ug/l.

4.5.1.4 Iron supplements

121 women (34%) took iron supplements at some stage during pregnancy. 8 (2%) started taking them in the first trimester, compared to 67 (18.6%) in the second trimester, and 46 (13%) in the third trimester. Out of those with iron depletion in the first trimester (sF<15 ug/l), only 46 (58%) had iron supplements during their

pregnancy, compared to 13 (81%) of anaemic women in the first half of pregnancy, and 40 (83%) of anaemic women in the second half of pregnancy.

4.5.2 Regression models

4.5.2.1 Maternal iron status and infant pulse wave velocity models

There was no evidence of association between infant bfPWV and maternal sF analysed as a continuous variable (adjusted change in PWV in m/s per 10 ug/l change in sF 0.02, 95% CI -0.01, 0.1, *P=0.3*), nor with maternal iron depletion (adjusted change in PWV in m/s -0.2, 95% CI -0.6, 0.2, *P=0.3*). No evidence of association was observed between maternal sTfR and log R/F with infant bfPWV. However, mothers who were anaemic in the first half of pregnancy (Hb<11 g/dl) had infants with higher PWV by 1.0 m/s on average (95% CI 0.1, 1.9, *P=0.2*) (Table 14). All the multivariable models adjusted for baby's age, PWV measurement circumstances (position, sleep, side, feeding), maternal age, smoking, GDM, pre-eclampsia, BP at booking and 36 weeks gestation, area deprivation score, and customised birth weight centile.

No association was observed between maternal intake of iron supplements at any stage in pregnancy and infant bfPWV (unadjusted change -0.1, 95% CI -0.5, 0.2). Adjusting for iron supplement intake in sensitivity analyses did not alter the results of the models examining the association between biomarkers of maternal iron status (sF, sTfR and R/F) with infant bfPWV. However, adjusting for maternal iron supplement intake strengthened the association between maternal anaemia in the first half of pregnancy and infant PWV. On average, there was 1.2 m/s increase in infant PWV if maternal Hb before 20 weeks gestation was less than 11 g/dl (95% CI 0.3, 2.1).

Predictor	Change in infant brachio-femoral pulse wave velocity (m/s)						
	Unadjusted	95% CI*	P	Adjusted**	Adjusted 95% CI	P	n (multivariable model)
Maternal serum ferritin (sF) at 12 weeks gest (per 10 ug/l change)	0.02	-0.01, 0.1	0.2	0.02	-0.01, 0.1	0.3	261
Maternal iron depletion at 12 w gest (sF<15 ug/l)	-0.2	-0.5, 0.3	0.4	-0.2	-0.6, 0.2	0.3	261
Maternal serum transferrin receptor (sTfR) at 12 weeks gestation (nmol/l)	0.03	-0.004, 0.1	0.1	0	-0.01, 0.04	0.3	261
Maternal log R/F[#] ratio at 12 weeks gestation (ug/l)	0	-0.1, 0.1	0.9	0	-0.2, 0.1	0.5	261
Maternal haemoglobin (Hb) at ≤20 weeks gestation (g/dl)	0.1	-0.1, 0.3	0.3	0.1	-0.1, 0.2	0.6	253
Maternal Hb at >20 weeks gestation (g/dl)	0.2	-0.001, 0.3	0.05	0.2	-0.004, 0.3	0.06	256
Maternal anaemia at ≤20 weeks gestation (<11 g/dl)	0.7	-0.1, 1.6	0.08	1.0	0.1, 1.9	0.02	253
Maternal anaemia at >20 weeks gestation (<10.5 g/dl)	-0.1	-0.5, 0.4	0.8	0.01	-0.5, 0.5	0.9	256

* Adjusted for baby's age, PWV measurement circumstances (position, feeding, asleep or awake, side), maternal age, smoking, gestational diabetes, pre-eclampsia, blood pressure at booking and 36 weeks gest, deprivation score and customised birth weight centile (takes into account maternal pre-pregnancy weight, height, ethnicity, parity, gestational age and baby's sex)

Table 14: Associations of infant brachio-femoral pulse wave velocity at 2-6 weeks (m/s) with indicators of iron status during pregnancy in the Baby VIP study

4.5.2.2 Maternal iron status and birth weight centile/SGA models

There was no evidence of association between maternal sF, sTfR, log R/F with birth weight centile. In univariable analysis, maternal anaemia in early pregnancy was associated with reduction of 15 centile points in birth weight (95% CI 1, 29, P=0.04). However, this association was attenuated when adjusting for maternal age, smoking, GDM, pre-eclampsia and IMD (adjusted change= -11 centile points, 95% CI -25, 3, *P=0.1*) (Table 15).

Maternal iron depletion in the first trimester (sF <15 ug/l) was associated with a higher risk of a SGA baby (adjusted OR 2.2, 95% CI 1.1, 4.1, P=0.02). Adjusting for maternal iron supplement intake in a sensitivity analysis did not alter this association (adjusted OR 2.3, 95% CI 1.2, 4.5, P=0.02). However, this relationship was attenuated when including early pregnancy Hb in the model (adjusted OR 1.6, 95% CI 0.8, 3.2, P=0.2). For every 1g/dl increase in maternal Hb level in the first half of pregnancy the risk of SGA was reduced by 30% (adjusted 95% CI 0-40%, P=0.03), with levels <11 g/dl associated with 3-fold increase in the risk of SGA (95% 1.0, 9.0, P=0.05) (Table 16). Maternal sTfR was also associated with SGA in the multivariable model adjusting for maternal age, smoking, GDM, pre-eclampsia and IMD (adjusted OR 1.1 for every 1 nmol/l increase in sTfR, 95% CI 1.0,1.1, P=0.04).

Predictor	Change in birth weight centile[#]						
	Unadjusted	95% CI*	P	Adjusted**	Adjusted 95% CI	P	n (multivariable model)
Maternal serum ferritin (sF) at 12 weeks gest (per 10 ug/l change)	-0.1	-1.0, 0.6	0.9	0.1	-0.6, 0.7	0.9	341
Maternal iron depletion at 12 w gest (sF<15 ug/l)	-4.2	-11.3, 2.3	0.3	-4.6	-11.7, 2.5	0.2	341
Maternal serum transferrin receptor (sTfR) at 12 weeks gestation (nmol/l)	-0.2	-0.7, 0.4	0.5	-0.3	-0.8, 0.3	0.3	341
Maternal log R/F ratio at 12 weeks gestation (ug/l)	-0.8	-3.4, 1.7	0.5	-1.2	-3.8, 1.3	0.3	341
Maternal haemoglobin (Hb) at ≤20 weeks gestation (g/dl)	1.4	-1.7, 4.5	0.4	1.0	-2.0, 4.1	0.6	326
Maternal Hb at >20 weeks gestation (g/dl)	-0.8	-3.7, 2.2	0.6	-1.2	-4.4, 1.4	0.3	334
Maternal anaemia at ≤20 weeks gestation (<11 g/dl)	-15.0	-29.2, -1.0	0.04	-11.2	-25.4, 3.1	0.1	326
Maternal anaemia at >20 weeks gestation (<10.5 g/dl)	-4.5	-13.1, 4.2	0.3	-1.4	-10.1, 7.3	0.8	334

*Confidence interval

** Adjusted for maternal age, smoking, gestational diabetes, pre-eclampsia, and area deprivation score (IMD)

Customised birth weight centile (takes into account maternal pre-pregnancy weight, height, ethnicity, parity, gestational age and baby's sex)

Table 15: Associations of customised birth weight centile with indicators of iron status during pregnancy in the Baby VIP study

Predictor	Odd ratio of SGA[#]						
	Unadjusted	95% CI*	P	Adjusted**	Adjusted 95% CI	P	n (multivariable model)
Maternal serum ferritin (sF) at 12 weeks gest (ug/l)	1.0	0.9, 1.0	0.8	1.0	0.9, 1.0	0.7	341
Maternal iron depletion at 12 w gest (sF<15 ug/l)	2.0	1.1, 3.7	0.02	2.2	1.1, 4.1	0.02	341
Maternal serum transferrin receptor (sTfR) at 12 weeks gestation (nmol/l)	1.0	1.0, 1.1	0.1	1.1	1.0, 1.1	0.04	341
Maternal log R/F ratio at 12 weeks gestation (ug/l)	1.1	0.9, 1.4	0.3	1.2	0.9, 1.5	0.1	341
Maternal haemoglobin (Hb) at ≤20 weeks gestation (g/dl)	0.7	0.6, 1.0	0.03	0.7	0.6, 1.0	0.03	326
Maternal Hb at >20 weeks gestation (g/dl)	1.0	0.7, 1.3	0.9	1.1	0.8, 1.4	0.7	334
Maternal anaemia at ≤20 weeks gestation (<11 g/dl)	3.3	1.1, 9.4	0.03	3.0	1.0, 9.0	0.05	326
Maternal anaemia at >20 weeks gestation (<10.5 g/dl)	1.0	0.5, 2.3	0.9	0.8	0.4, 2.0	0.7	334

*Confidence interval

** Adjusted for maternal age, smoking, gestational diabetes, pre-eclampsia, and area deprivation score (IMD)

<10th customised birth weight centile (takes into account maternal pre-pregnancy weight, height, ethnicity, parity, gestational age and baby's sex)

Table 16: Associations of being born small for gestational age with indicators of iron status during pregnancy in the Baby VIP study

4.5.2.3 Maternal iron status and gestational age/preterm birth models

There was no evidence of association between maternal iron status measured by sF, sTfR or log R/F with preterm birth or gestational age. However, there was an association observed between early pregnancy maternal Hb and gestational age in univariable analysis. For every 1 g/dl increase in early pregnancy Hb, there was an increase in gestational age by 2 days (95% CI 0.2, 3.0, P=0.03). This association was attenuated in the multivariable model adjusting for maternal age, ethnicity, parity, pre-pregnancy BMI, smoking, GDM, pre-eclampsia and IMD score. Mothers who were anaemic in the first half of pregnancy had on average a gestation shorter by 7 days (adjusted 95% CI 0, 14, *P=0.05*) compared to non-anaemic mothers. Association estimates are listed in Table 17 and Table 18.

Predictor	Change in gestational age (days)[#]						
	Unadjusted	95% CI*	P	Adjusted**	Adjusted 95% CI	P	n (multivariable model)
Maternal serum ferritin (sF) at 12 weeks gest (per 10 ug/l change)	0.1	-0.2, 0.4	0.6	-0.2	-0.4, 0.2	0.6	341
Maternal iron depletion at 12 w gest (sF<15 ug/l)	0.3	-3.4,4.0	0.9	0.02	-3.4, 3.4	0.9	341
Maternal serum transferrin receptor (sTfR) at 12 weeks gestation (nmol/l)	-0.1	-0.2, 0.2	0.5	-0.1	-0.3, 0.2	0.5	341
Maternal log R/F ratio at 12 weeks gestation (ug/l)	-0.6	-1.8, 0.6	0.3	0.01	-1.2, 1.3	0.9	341
Maternal haemoglobin (Hb) at ≤20 weeks gestation (g/dl)	1.6	0.2, 3.0	0.03	1.1	-0.4, 2.6	0.1	326
Maternal Hb at >20 weeks gestation (g/dl)	0.1	-1.3, 1.5	0.9	-0.5	-1.9, 0.9	0.5	334
Maternal anaemia at ≤20 weeks gestation (<11 g/dl)	-9.1	-15.8, -2.4	0.008	-6.8	-13.6, 0.1	0.05	326
Maternal anaemia at >20 weeks gestation (<10.5 g/dl)	1.6	-2.4, 5.6	0.4	3.7	-0.4, 7.8	0.07	334

*Confidence interval

** Adjusted for maternal age, pre=pregnancy body mass index, ethnicity, parity, smoking, gestational diabetes, pre-eclampsia, and area deprivation score (IMD)

Table 17: Associations of gestational age with indicators of iron status during pregnancy in the Baby VIP study

Predictor	OR of preterm birth (<37 weeks gestation)						
	Unadjusted	95% CI*	P	Adjusted**	Adjusted 95% CI	P	n (multivariable model)
Maternal serum ferritin (sF) at 12 weeks gest (ug/l)	1.0	0.9, 1.0	0.6	1.0	0.9, 1.0	0.6	341
Maternal iron depletion at 12 w gest (sF<15 ug/l)	1.6	0.7, 3.7	0.3	1.5	0.6, 3.8	0.4	341
Maternal serum transferrin receptor (sTfR) at 12 weeks gestation (nmol/l)	1.0	1.0, 1.1	0.5	1.0	1.0, 1.1	0.5	341
Maternal log R/F ratio at 12 weeks gestation (ug/l)	1.1	0.8, 1.4	0.8	1.0	0.7, 1.5	0.8	341
Maternal haemoglobin (Hb) at ≤20 weeks gestation (g/dl)	0.8	0.6, 1.1	0.2	0.9	0.6, 1.4	0.7	326
Maternal Hb at >20 weeks gestation (g/dl)	1.2	0.8, 1.7	0.5	1.3	0.9, 1.9	0.2	334
Maternal anaemia at ≤20 weeks gestation (<11 g/dl)	2.6	0.7, 9.5	0.2	1.3	0.3, 6.2	0.8	326
Maternal anaemia at >20 weeks gestation (<10.5 g/dl)	0.4	0.1, 1,9	0.3	0.2	0.04, 1.0	0.05	334

*Confidence interval

** Adjusted for maternal age, pre=pregnancy body mass index, ethnicity, parity, smoking, gestational diabetes, pre-eclampsia, and area deprivation score (IMD)

Table 18: Associations of being born preterm with indicators of iron status during pregnancy in the Baby VIP study

4.5.2.4 Infant pulse wave velocity and birth outcomes models

Infant bfPWV was inversely associated with SGA in a multivariable model that adjusted for baby's age, PWV measurement circumstances (position, sleep, side, feeding), maternal age, smoking, GDM, pre-eclampsia, BP at booking and 36 weeks gestation, and IMD score (adjusted change -0.5 m/s, 95% CI -1.0, -0.1, *P=0.01*). However, there was less evidence of this association for very SGA babies (<3rd birth weight centile). There was no evidence that infant PWV was associated with birth weight centile, gestational age or preterm birth (Table 19).

	Change in infant brachio-femoral pulse wave velocity (m/s)					
	Unadjusted	95% CI*	P	Adjusted**	Adjusted 95% CI	P
Birth weight centile# (per 10 points centile increase)	0.04	-0.01, 0.1	0.1	0.1	-0.01, 0.1	0.08
Small for gestational age##	-0.4	-0.9, 0	0.04	-0.5	-1.0, -0.1	0.01
Very small for gestational age###	-0.4	-0.9, 0.2	0.2	-0.4	-1.0, 0.2	0.2
Gestational age (per 10 days)	0.1	(-0.1, 0.2)	0.4	0.01	-0.1, 0.1	0.8
Preterm birth (<37 weeks gestation)	-0.5	-1.1, 0.1	0.1	-0.3	-0.9, 0.4	0.4

*Confidence interval

** Adjusted for baby's age, PWV measurement circumstances (position, sleep, side, feeding), maternal age, smoking, gestational diabetes, pre-eclampsia, blood pressure at booking and 36 weeks gestation, and deprivation score in case of birth weight centile and SGA as predictors, plus maternal parity, ethnicity and pre-pregnancy BMI in case of gestational age and preterm birth as predictors

Takes into account maternal pre-pregnancy weight, height, ethnicity, parity, gestational age and baby's sex

##<10th birth weight centile ### <3rd birth weight centile

Table 19: Associations of infant brachio-femoral pulse wave velocity with size at birth and gestational age in the Baby VIP study (n=267)

4.6 Discussion

PWV, a potential marker of cardiovascular health later in life, was ascertained in 284 babies aged 2-6 weeks at home in the Baby VIP study. There was no evidence of association between maternal iron status biomarkers in early pregnancy and infant PWV. However, maternal anaemia in the first half of pregnancy was associated with increased infant PWV, which indicates stiffer arteries. Maternal iron depletion in early pregnancy was associated with higher risk of SGA birth. This relationship seems to be mediated by early pregnancy maternal Hb, which was independently inversely associated with SGA.

4.6.1 Strengths and limitations

4.6.1.1 Exposure measures

This study assessed the exposure of interest prospectively, as the maternal serum samples were collected in the first trimester of pregnancy. Information on maternal Hb and iron supplements was ascertained objectively from the medical records, rather than by self-reporting. The best available measure, which utilised the ferritin and transferrin receptor biomarkers in a ratio that relates directly to total body iron stores, was used to assess maternal iron status (Zimmermann, 2008). A study set out to determine the diagnostic value of R/F ratio to determine body iron stores against bone marrow aspirate examination showed that R/F ratio had the best diagnostic efficiency with the sensitivity of 81% and a specificity of 97%. SF alone, with a cut-off of 60 ug/l, had the same specificity but lower sensitivity (76%) (Ruivard et al., 2000).

Deriving body iron stores estimates from R/F ratio is limited by the current availability of several commercial assays that yield different sTfR values. The calculation formula provided by Cook et al. used to deduct body iron stores values can only be used if sTfR assay commutability is established. Unfortunately, this is not the case in our study. It was not possible to convert values generated by the R&D assay into values that would be appropriate for the Cook formula which used another assay to measure sTfR (Cook, 2003). There is a pressing need to calibrate sTfR assays against international reference standards to provide comparability across studies.

4.6.1.2 Outcome measures

Birth weight was ascertained objectively from the medical birth records. Gestational age was calculated using information from a dating ultrasound scan at the end of the first trimester of pregnancy and extracted from the medical records. Therefore, these two outcome measures were not subject to measurement bias.

PWV, a new and innovative measure of cardiovascular health in neonates and young infants, was assessed in this study on a relatively large study population compared to other studies that have assessed PWV in this age group (Alhashemi et al., 2013,Koudsi et al., 2007). A potential source of error in measuring PWV in peripheral arteries is the use of the nearest superficial arterial site as a surrogate for inaccessible central arteries (Cheung, 2010). In this case, the brachial artery was used as a surrogate for the carotid. The study team have tried, at the pilot stage of Baby VIP, to measure carotid-femoral PWV, but have found that wrapping a cuff around the baby's neck caused distress to parents.

The estimation of the actual distance between the recording sites using surface measurements is another potential source of error. The shorter the distance, the - greater the absolute error in determining transit time (Laurent et al., 2006). However, the PWV data spread in this study, as reflected by the standard deviation, corresponds well with most other studies that measured PWV. Also any errors in measuring the distance between the arterial sites would be non-differential in terms of the exposure of interest (iron status) as the researcher who performed the measurements was blind to it. Confounders such as the baby being asleep or awake or position during the time of the measurement are known to potentially affect the PWV reading (Laurent et al., 2006). Therefore, these were adjusted for in all the statistical models.

Despite these limitations, PWV remains the most widely used technique for assessment of arterial stiffness (Cheung, 2010), and in babies, it causes less distress than measuring BP. It is difficult to compare studies using different measures used to assess arterial stiffness including PWV, central pressure and augmentation index. PWV is a direct measure while the other two are indirect surrogate measures but they provide additional information about wave reflection (Laurent et al., 2006).

4.6.2 Interpretation of results

4.6.2.1 Maternal iron status and infant pulse wave velocity

The elastic properties of the conduit arteries vary along the arterial tree; with more elastic proximal arteries and stiffer distal arteries. The amplitude of the pressure wave is higher in peripheral arteries than in central arteries. This is called the ‘amplification phenomenon’ (Laurent et al., 2006). Also, in younger subject the central arteries are

usually more elastic than the peripheral arteries (Laurent et al., 2006). This may explain the relatively higher PWV average that we got in our study (6.7 m/s) compared to the average in the other study which measured aortic PWV in infants (4.7 m/s) (Koudsi et al., 2007, Alhashemi et al., 2013). The mean brachio-ankle PWV in children with a mean age of 14 years is 10 m/s (Niboshi et al., 2006, Im et al., 2007). In adults, average brachio-ankle PWV was approximately 20% higher than carotid-femoral PWV (Tanaka et al., 2009). Given this, it is inaccurate to use brachial pressure as a surrogate for aortic or carotid pulse pressure, particularly in young subjects. Therefore, an association between maternal iron status in pregnancy and central arterial stiffness in babies cannot be excluded on the basis of this study.

There is very little research on the ability of PWV measured very early in life to predict later cardiovascular health. Although we did not find an association with maternal iron status early in pregnancy, this does not exclude the possibility that maternal iron status, as measured by R/F ratio, may be linked to offspring cardiovascular indicators in adulthood. To investigate this relationship, long term follow up of a birth cohort with information on maternal iron status in pregnancy is required.

In this study, there was an association between maternal anaemia early in pregnancy and infant arterial stiffness. Anaemia could reflect the extreme of the ID spectrum, and thus this association would support the hypothesis that ID early in pregnancy is linked to cardiovascular risk in the offspring. However, only about half of anaemic women in the study had ferritin values less than 15 ug/l. Analysis in chapter 5 from ALSPAC found no association between early pregnancy maternal Hb (<18 weeks gestation) and offspring PWV at 10 years of age (Alwan et al., 2014). In this chapter, this observed

association could be an expression of a true effect of early pregnancy maternal anaemia on arterial function in the baby, which may subside later in life. Alternatively, it could be due to residual confounding as other causes of poor health in the mother are likely to be associated with both the predictor and outcome.

4.6.2.2 Measures of maternal iron status and birth outcomes (birth weight and gestational age)

4.6.2.2.1 Maternal anaemia/Hb levels

In this study, anaemia in the first half of pregnancy was associated with a higher risk of having a baby who is SGA, and every 1 g/dl increase in early pregnancy maternal Hb was associated with a 30% reduction in the risk of SGA. This result supports the previous evidence of association between early maternal Hb and anaemia with the risk of LBW reviewed in section 2.2.4 of this thesis. Maternal Hb in the second half of pregnancy was not associated with SGA in Baby VIP , while in the analysis presented in chapter 3 of pregnant women participating in the CARE study, there was a 40% increase in the risk of SGA with every I g/dl increase in maternal Hb at 28 weeks gestation. The latter analysis was, however, performed on a bigger sample, so a type II error due to insufficient sample size in the Baby VIP sample is a possibility.

There was no evidence of association between the incidence of preterm birth and early pregnancy maternal Hb or anaemia in this study contrary to the findings of previous studies (Scanlon et al., 2000, Scholl et al., 1992). However, maternal anaemia in the first half of pregnancy was marginally associated with a reduction in gestational age when analysed as a continuous outcome, while maternal anaemia in the second

half of pregnancy was marginally associated with a reduction in the risk of preterm birth.

The evidence available in the literature of the association between maternal anaemia and birth outcomes suggests that it is U-shaped (Rasmussen, 2001). Causes of adverse birth outcomes may differ at the two extremes of the maternal Hb range. While low Hb in early pregnancy may reflect ID or other nutritional deficiencies such as vitamin B or folic acid, high Hb values later in pregnancy may reflect inadequate expansion of plasma volume. Rasmussen suggests that this U-shaped association is spurious due to the design of research evidence available, as it is more apparent in studies that use "lowest Hb" than in those that control for the stage of gestation or include data only from women very early in pregnancy, when changes in plasma volume are minimal (Rasmussen, 2001, Scanlon et al., 2000, Zhou et al., 1998).

4.6.2.2.2 Maternal ferritin and transferrin receptor levels

Participants in this study who were iron depleted at the beginning of pregnancy were twice as likely to have SGA babies. Maternal sTfR level measured in the first trimester of pregnancy, which increases in ID, was also marginally associated with higher risk of SGA. These results are in line with findings from previous studies (Ribot et al., 2012). The relationship between maternal iron depletion in the first trimester and SGA was tested for mediation by maternal Hb, as it was an independent predictor of SGA. Including maternal Hb in the model attenuated the relationship between maternal iron depletion and SGA. This may point to the possibility that the mechanism through which inadequate body iron could potentially result in small size at birth is through the efficiency of carrying oxygen to the placenta which is reduced by a reduction in

maternal Hb. IDA increases oxidative stress levels in the liver, heart, kidney and placenta as well as resulting in hypoxia and inflammation in placenta (Allen, 2001).

4.6.2.3 Infant pulse wave velocity and size at birth

In this study, SGA (<10th birth weight centile) was associated with 0.5 m/s lower infant PWV on average, while no association was observed in those who were born very SGA (<3rd birth weight centile). The evidence linking size at birth with PWV later in life was discussed earlier in section 2.5.1.3 of this thesis. There is some evidence that newborn arterial stiffness, measured by ultrasound and pulse BP measurement techniques, is associated with very SGA (section 2.5.1.5). Cheung et al. found that among 8 year old children who were born preterm, only those with IUGR had increased brachio-radial PWV (Cheung et al., 2004). Also, using pulse pressure measurement (distensibility coefficient and whole body arterial compliance by ultrasound recording or aortic pulse pressure), increased arterial stiffness was observed as early as the fifth day of life in very low gestational age infants, and persisted at least until the 7th week of life (Tauzin et al., 2006). Sehgal et al. calculated arterial wall stiffness index and found an increase with very SGA (<3rd centile) (Sehgal et al., 2014). Akira et al. found decreased arterial distensibility in SGA (<5th centile) infants using the stiffness index, however, this index also increased with gestational age at birth (Akira and Yoshiyuki, 2006).

The association observed between SGA and lower PWV is surprising. However, before interpreting it as contradictory to previous evidence of association and the mechanical basis for this relationship, there are a few considerations. First, It is cannot be assumed that PWV at this early age predicts PWV later in life, as there is no evidence to support such an assumption. Therefore, lower PWV in infancy does not necessarily mean more

favourable arterial stiffness profile in adulthood. Secondly, the observed association disappeared when examining it in very SGA babies, who are more likely to be small due to IUGR. Thirdly, the method of measuring PWV is relevant as the evidence of association between SGA and increased PWV comes from studies that have used different methods to assess arterial stiffness in newborns. The mechanism of association between LBW and increased arterial stiffness in childhood and adulthood remains unclear. One potential mechanism is altered synthesis of elastin in the arterial wall, while another is endothelial dysfunction in preterm and SGA babies leading to functional alterations in arterial tone (Cheung, 2010). Therefore, this relationship may not be apparent when using structural methods to assess arterial stiffness such as PWV.

4.7 **Conclusion**

To my knowledge, no previous studies have measured PWV in the first weeks of life to examine its association with maternal iron status in pregnancy. Also, this is the largest population study published to date which assessed PWV as a measure of arterial stiffness in infants. There was no evidence of association detected between maternal iron status early in pregnancy and bfPWV in babies aged 2-6 weeks. This study demonstrates that infant arterial stiffness can be feasibly assessed using non-invasive techniques of measuring PWV in population studies. Further research is needed to validate PWV measured early in life as a potential indicator of cardiovascular risk later in life and to investigate the relationship between biomarkers of maternal nutritional status during pregnancy and PWV in the child and adult offspring using prospective study design. This would help inform the understanding of potential pathways underlying the developmental origins of CVD.

In the next chapter, I move one step further in the life course and investigate the association of PWV and other cardiovascular indicators measured in 10 year old children in the ALSPAC cohort with indicators of maternal iron status including Hb and iron intake from diet and supplements.

5 Associations of maternal iron intake and haemoglobin in pregnancy with offspring vascular phenotypes and adiposity at age 10 in the ALSPAC study

In chapters 3 and 4 I have explored the relationship between maternal iron intake and status during pregnancy and short-term offspring outcomes at birth and in infancy. Following on from that, this chapter evaluates these relationships with more long-term outcomes through analysis of data from the ALSPAC cohort. This includes offspring indicators of cardiovascular risk measured at age 10.

Work from this chapter has formed the basis of a peer-reviewed paper (Alwan et al., 2014) and a conference presentation.

5.1 Chapter summary

The aim of this chapter is to examine the relationship between maternal pregnancy dietary and supplement iron intake and Hb, with offspring's arterial stiffness measured by carotid-radial PWV, endothelial function measured by brachial artery flow mediated dilatation (FMD), BP, and adiposity (measured by BMI), test for mediation by cord ferritin, birth weight, gestational age, and child dietary iron intake, and for effect modification by maternal vitamin C intake and offspring sex.

Prospective data from 2958 mothers and children pairs at 10 years of age enrolled in an English birth cohort, the ALSPAC study in Bristol, was analysed. In this cohort, 2639 (89%) mothers reported dietary iron intake in pregnancy below the UK reference RNI of 14.8 mg/day. 1328 (45%) reported taking iron supplements, and 129 (4%) were anaemic by 18 weeks gestation. No evidence of association was observed in this analysis between indicators of maternal iron status including dietary iron intake in pregnancy and maternal Hb concentration (which is less likely to be biased by subjective reporting) and markers of cardiovascular risk in the offspring, apart from a modest association between taking iron supplements in pregnancy with lower offspring systolic BP at 10 years (-0.8 mmHg, 99% CI -1.7 to 0, *P=0.01* in the sample with all relevant data observed, and -0.7 mmHg, 99% CI -1.3 to 0, *P=0.008* in the sample with missing data imputed).

5.2 Background

5.2.1 Previous studies

Although evidence from animal models suggests that maternal ID during pregnancy can result in the development of obesity and hypertension in the offspring (Gambling et al., 2003b, Gambling et al., 2003a, Crowe et al., 1995, Gambling et al., 2004b), evidence in humans regarding the effect of maternal ID on cardiovascular risk indicators in childhood remains inconclusive. Few studies have investigated the association of indicators of maternal iron status in pregnancy and BP in childhood. These are referred to briefly here, and their findings discussed earlier in section 2.3.3 of this thesis.

In a previous analysis of data from the same birth cohort analysed here, the ALSPAC cohort, Brion et al. reported an association between maternal anaemia and BP at 7 years only in women who did not take iron supplements during pregnancy (Brion et al., 2008). In another study, Belfort et al, with a sample size of 1167 pregnant American women, there was no association between first and second trimester maternal anaemia with offspring BP at 3 years. However, offspring BP was positively associated with first trimester iron intake, in contrast to animal studies findings (Gambling et al., 2003b, Gambling et al., 2003a, Crowe et al., 1995, Gambling et al., 2004b), while no relationship was observed for second trimester iron intake (Belfort et al., 2008). Other studies examining the association between maternal anaemia in pregnancy and offspring childhood BP reported conflicting findings ranging from positive to negative, and including null associations (Whincup et al., 1994, Bergel et al., 2000, Law et al.,

1991, Godfrey et al., 1994). The characteristics of these studies are summarised in Table 2.

5.2.2 Mediation in the relationship between iron intake in pregnancy and offspring cardiovascular health

It has been shown in animal studies that offspring outcomes such as birth weight are dependent on mother's iron status and not that of the neonate (Gambling et al., 2002). However, umbilical cord ferritin is strongly associated with maternal iron status (Agarwal et al., 1983, Kaneshige, 1981, Singla et al., 1996). There is also evidence of an association between cord ferritin levels and health outcomes such as mental and psychomotor development in children (Tamura et al., 2002). Therefore, cord ferritin could be a potential mediator in the relationships between maternal iron measures in pregnancy and offspring cardiovascular risk outcomes.

Other potential mediators include child's dietary iron intake, child's birth weight, and gestational age. As noted above, maternal iron levels in pregnancy are related to lower birth weight and preterm delivery; birth weight and gestational age are in turn inversely associated with CVD (Barker, 2004). Maternal diet is related to child's diet (Brion et al., 2010), and therefore it is likely that maternal iron intake in general, including during pregnancy, will be related to offspring dietary iron intake and this in turn may influence the child's vascular phenotypes and hence mediate the association.

5.2.3 Effect modification in the relationship between iron intake in pregnancy and offspring cardiovascular health

Animal studies suggest that the inverse association of maternal iron status with later offspring cardiovascular outcomes differs by offspring sex, being stronger in males (Gambling et al., 2003b, Lisle et al., 2003). Furthermore, since vitamin C is a key enhancer of iron absorption (Gibney et al., 2004, Collings et al., 2013), the relationship between maternal iron intake and perinatal or longer term outcomes may be stronger with adequate intake of vitamin C, as has been presented in chapter 3 of this thesis (Alwan et al., 2011). Therefore, both offspring sex and maternal vitamin C intake in pregnancy were considered as potential effect modifiers in the relationships under investigation.

5.2.4 Vascular markers in the offspring

Prediction of CVD morbidity and mortality can be realized through studying endothelial function and arterial stiffness in adults. FMD of the arm arteries is the most common technique to measure endothelium-dependent vasodilator function. The technique measures the ability of the arteries to respond with endothelial nitric oxide release during reactive hyperaemia (flow mediated) after a 5-minute occlusion of the brachial artery with a BP cuff (Flammer et al., 2012). It is diminished in patients with atherosclerosis and with coronary risk factors, and improves with risk-reduction therapy (Moens et al., 2005). There is evidence that endothelial function is prognostic of cardiovascular risk (Halcox et al., 2009, Halcox et al., 2002). Endothelial dysfunction in children, measured by brachial artery FMD, has also been linked to LBW (Leeson et al., 1997).

PWV, a measure of arterial stiffness, measures the time taken for the systolic pressure wave to travel a known distance. It is considered a convenient, precise, reliable, and integrated index of vascular pathology over the lifecourse (Cruickshank et al., 2009). It has been widely used as a predictor of CVD in adults (Weber et al., 2008, Jadhav and Kadam, 2005), and is associated with higher CVD mortality, coronary heart disease, and stroke (Vlachopoulos et al., 2010, Sutton-Tyrrell et al., 2005). The use of PWV as a lifecourse marker in investigating the developmental origins of disease has been explored earlier in this thesis in section 2.5.1.

5.3 Hypothesis and objectives

It is hypothesised that women with adequate iron intake and those who are not anaemic during pregnancy have, on average, children with healthier cardiovascular risk profile.

This chapter builds on the work of Brion et al. utilising ALSPAC data (Brion et al., 2008) using offspring's BP at a later age than the previous analysis, and utilising other indicators of offspring cardiovascular health as outcomes including PWV, FMD and BMI, all measured at around 10 years of age.

The objectives of this study are:

1. Examine the associations between indicators of maternal iron status in pregnancy (maternal iron intake and Hb concentrations) and indicators of child's circulatory health (BP, adiposity as assessed by BMI, endothelial function as assessed by brachial artery FMD, and arterial stiffness as assessed by carotid-radial PWV).
2. Examine whether any observed associations were mediated by cord blood ferritin levels, gestational age, offspring's birth weight, and dietary iron intake
3. Explore whether offspring sex and maternal vitamin C intake moderate any of these associations as effect modifiers.

5.4 Methods

5.4.1 Study design and participants

ALSPAC is a longitudinal population-based birth cohort study investigating influences on health and development across the life course, which recruited pregnant women between 1990 and 1992 and followed-up their children (n=14541) in the South West region of England (Fraser et al., 2012, Boyd et al., 2013). All pregnant women resident in a defined area (the Bristol area) were eligible for recruitment. The women who have been recruited and have not dropped-out of the study, have completed up to 20 questionnaires, and have had detailed data abstracted from their medical records, plus a detailed biobank of stored DNA, serum and plasma. Follow-up for the children included 59 questionnaires and 9 clinical assessment visits to date. The study website contains details of all the available data through a fully searchable data dictionary (www.bris.ac.uk/alspac/researchers/data-access/data-dictionary).

Figure 10 shows participants flow-chart for the complete case analysis performed for this study.

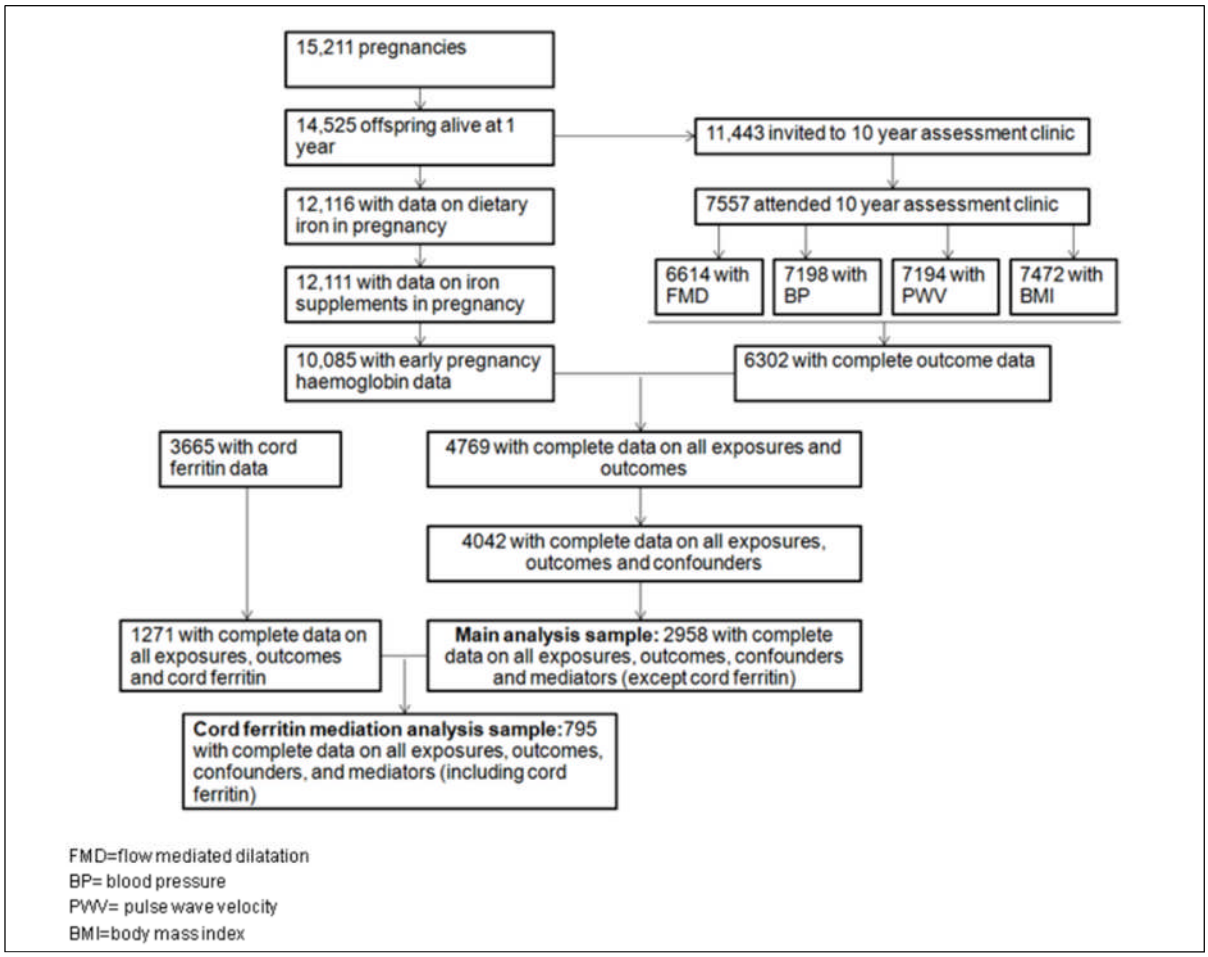


Figure 10: ALSPAC participant flowchart for the study samples used to investigate the associations of maternal iron and offspring vascular phenotypes

5.4.2 Exposure assessment

5.4.2.1 Maternal dietary iron intake

Iron intake from food was assessed using a FFQ sent to mothers at 32 weeks gestation covering all the main foods consumed in Britain. Mothers were asked how often they were currently consuming each of 43 food groups (Rogers and Emmett, 1998). The questionnaire administered to mothers including the food frequency questions can be retrieved from the ALSPAC study website (www.bristol.ac.uk/alspac/researchers/resources-available/data-details/questionnaires). Intakes for a range of nutrients, including iron, were derived using nutrient information on standard-sized portions based on the 5th edition of McCance and Widdowson's Composition of Food tables (Holland et al., 1991). The FFQ was used to calculate an approximate daily nutrient intake for each woman by multiplying the weekly frequency of consumption of a food by the nutrient content. The nutrient values obtained were then divided by seven to convert this to a daily intake including energy, protein, total fat, saturates, monounsaturates and polyunsaturates, total sugar, non-milk extrinsic sugar, dietary fibre, nine vitamins and five minerals (Rogers and Emmett, 1998).

5.4.2.2 Maternal iron supplements intake

Maternal iron supplement use was obtained from questionnaires sent at 18 weeks gestation (relating to anytime during pregnancy before the questionnaire date), and 32 weeks gestation (relating to 3 months of pregnancy between the first and second questionnaire). Mothers were asked whether they had taken iron supplements, vitamins, or any other supplements. In a separate question, women were also asked to list all pills,

medicines, and ointments they used, with a reminder to include iron tablets, vitamins, herbal medicines, etc. Responses at 18 and 32 weeks regarding iron supplements were combined to generate a binary variable (yes/no) for 'iron supplement used anytime during pregnancy up to 32 weeks gestation'.

5.4.2.3 Maternal haemoglobin

Maternal Hb concentrations were extracted from antenatal medical records of study participants as it was measured routinely in all pregnant women. An 'Early pregnancy haemoglobin' variable was derived, defined as the first measurement of Hb before 18 weeks. A mother was classified as having 'early pregnancy anaemia' if her Hb measurement was less than 11 g/dl, the threshold used to define pregnancy anaemia according to WHO guidelines (WHO, 2001).

5.4.3 Outcome assessment

BP, PWV, FMD and BMI were measured in child clinics at ages 10-11 years by six trained research technicians/fieldworkers over a two year study period (Donald et al., 2010). Carotid-radial pulse wave velocity was measured transcutaneously using a high-fidelity micromanometer (SPC-301, Millar Instruments, Houston, TX, USA). Integral software processed the data to calculate the mean time difference between R-wave and pressure wave on a beat-to-beat basis over 10 seconds, and the PWV was then calculated using the mean time difference and arterial path length between the two recording points (SphygmoCor version 7.1, Scanmed, UK) (Donald et al., 2010).

The right brachial artery was imaged, 5–10 cm above the antecubital fossa, using high-resolution ultrasound (ALOKA 5500) with the probe held in a stereotactic clamp that allowed micrometre positional adjustment. Brachial artery FMD was induced by a 5 min inflation of a pneumatic cuff to 200 mmHg, around the forearm immediately below the medial epicondyle, followed by rapid deflation using an automatic air regulator (Logan Research, UK). The diameter of the brachial artery was measured using edge detection software (Brachial Tools, MIA, IA, USA) from ECG-triggered ultrasound images captured at 3 second intervals throughout the 11 min recording protocol. FMD was expressed as the maximum percentage change in vessel diameter from baseline. The magnitude of the flow stimulus was recorded continuously by pulse wave Doppler and expressed as per cent reactive hyperaemia, derived from the maximum change in flow within 15 seconds of deflation of the pneumatic cuff, relative to the baseline flow. The coefficients of variation between technicians for FMD and PWV were 10.5% and 4.6% respectively at the beginning of the study, and reached 7.7% and 4.1% at the end of the study (Donald et al., 2010).

Weight to the nearest 0.1 kg was measured in light clothing and without shoes using SECA scales. Height to the nearest 0.1 cm was measured using a Leicester height meter. From these, BMI was calculated (weight in kg/height in metres^2).

5.4.4 Covariable assessment

5.4.4.1 Confounders

Current age of the child was recorded in months at the time of the assessment clinic. Child sex was recorded at birth from the obstetric records. We considered the following

covariables to be potential confounders (associated with both exposures and outcomes); maternal age, pre-pregnancy BMI, educational level (as a marker of socioeconomic status), smoking in pregnancy, parity, and maternal total energy intake as assessed by FFQ at 32 weeks.

Highest maternal educational qualification was self-reported at 32 weeks gestation, and was categorized as university degree, A-level or equivalent (A-level is Advanced-level and indicates a qualification usually taken around 18 years of age by individuals who have remained in school beyond the legal minimum age at which they can leave (16 years) and are likely to go on to higher or further education or train for a semi-skilled job), and less than A-level. Maternal smoking was self-reported at the 18 and 32 weeks gestation questionnaire. A variable was generated for any smoking during pregnancy reported at either or both of these time points.

5.4.4.2 Mediators and effect modifiers

On the basis of it being plausible that maternal iron status would affect them and that they could plausibly causally affect the offspring's cardiovascular profile (Tamura et al., 2002, Brion et al., 2010), we considered the following potential mediators of the association; cord ferritin, gestational age, offspring birth weight and offspring dietary iron intake. Gestational age at delivery and birth weight were obtained from the obstetric records. Ferritin was measured in cord heparin plasma at the ALSPAC laboratory using the DELFIA time resolved fluoroimmunoassay system. Ferritin assays were duplicated where possible and a coefficient of variation of approximately 4% was obtained. Offspring dietary iron intake was assessed by a FFQ administered at 3, 4, 7 and 9 years of age (Boyd et al., 2013). We used

the mean iron intake of these 4 assessments. Maternal vitamin C intake (which we considered a potential effect modifier of maternal iron status on offspring outcomes) was calculated from the FFQ as described above for iron intake. A binary variable for dietary vitamin C intake was created using the UK RNI cut-off of 50 mg/day and used to test for interaction (Food Standards Agency, 2003). Baby's sex (also a potential effect modifier) was obtained from the birth records.

5.4.5 Statistical methods

Analysis was performed using Stata version 11 (StataCorp LP, College Station, TX, USA). Characteristics of women with iron intake above or equal to the RNI were compared to those with intake below the RN using two-sample t-test for continuous variables and chi-squared test for categorical variables.

Linear regression was used to examine the association of maternal dietary iron intake (assessed as a continuous variable per 10mg/day and also as a binary variable < versus ≥ 14.8mg/day), whether or not the mother took iron supplements in pregnancy and early pregnancy Hb (assessed as a continuous 1g/dl and as a binary variable < versus ≥11g/dl) with offspring PWV, FMD, BP and BMI all measured at mean age 10 years. Initially, univariable (no adjustment for covariables) analyses were undertaken followed by multivariable models that adjusted first for potential confounding factors (maternal age, pre-pregnancy BMI, educational level, smoking in pregnancy, parity, and maternal total energy intake for analyses involving dietary iron intake as the exposure), then for potential mediation by birth weight, gestational age and offspring postnatal dietary iron intake.

Finally, in the subgroup with data on cord ferritin levels, its role as a mediator was examined.

The possibilities that offspring sex or maternal adequate intake of vitamin C (≤ versus > 50 mg/day) modified the association of maternal exposures with offspring outcomes were assessed by examining stratified (by sex and maternal adequate vitamin C intake) analyses and by including an interaction term in the confounder adjusted models.

A statistical significance level of 1%, with 99% confidence intervals, was used in the regression models to reduce the risk of type I error due to multiple statistical testing. Other strategies for correction for multiple testing, such as the Bonferroni correction, are over-conservative, and this is a more pragmatic approach that more easily facilitates the use of confidence intervals.

5.4.5.1 Sensitivity analyses

Sensitivity analysis which aimed at exploring whether missing data might have led to biased estimates was undertaken. To do this, multivariable multiple imputation was performed in Stata as described by Royston (Royston, 2004) to impute missing values for variables included in the main analysis models for any ALSPAC participant in the 12116 sample with dietary iron intake data. Twenty imputation datasets were generated in which missing variables values were imputed by chained equations including exposures, outcomes and covariables as used in the main confounder-adjusted models. The sensitivity analysis results are obtained by averaging over the results from each of the 20 datasets using Rubin's rules (Royston, 2004).

We also carried out another sensitivity analysis, adding maternal Hb to the models exploring the associations of maternal iron supplement intake with childhood outcomes. This was to account for potential reverse causality (the reason for taking the iron supplements is the mother's awareness of being anaemic).

5.4.6 Ethical approval

Ethical approval was obtained from the ALSPAC Law and Ethics Committee and the local research ethics committees, and procedures were in accordance with the Helsinki Declaration of 1975 as revised in 1983. Participants provided their written informed consent to participate in this study.

5.4.7 Funding

The work included in this chapter was supported by the Wellcome Trust [Grant number WT87789 to N.A.A.]. Core support for ALSPAC is provided by the UK Medical Research Council, the Wellcome Trust and the University of Bristol. Funding for vascular measurements used in this study was obtained from the British Heart Foundation. The funders had no influence on the design, analysis or reporting of the study.

5.5 Results

5.5.1 Study sample descriptives

The ALSPAC live births sample was 14597. Mean birth weight was 3382 grams (SD 581) with mean gestational age of 38.4 weeks (SD 5.4). The main analysis sample for this chapter was 2958 with complete data on all exposures, outcomes, confounders and mediators (except for cord ferritin). A description of the study sample characteristics, compared with the total ALSPAC sample with available maternal dietary data is shown in Table 20.

Mean maternal pregnancy iron intake was 10.7 mg/day (SD 3.2). The mean age of the child at the time of the focus clinic was 119 months (9.9 years, SD 0.3). Mean child iron intake was 6.8 mg/day (SD=2.2), 6.8 mg/day (SD=2), 8.8 mg/day (SD 2.5) and 9.2 mg/day (SD 2.6) at 3, 4, 7 and 9 years of age respectively.

Table 21 summarized participants' characteristics by dietary maternal iron intake. Women with dietary iron intake equal to or more than the UK RNI for iron were more likely to be older, vegetarian, with higher educational qualification, report higher total energy and vitamin C intake, have lower pre-pregnancy BMI, and were less likely to smoke during pregnancy.

	Complete case sample	Whole ALSPAC sample with dietary data
Dietary iron intake[#] (mg/day) (m, sd*)	10.7 (3.2)	10.4 (3.4)
Dietary iron intake <UK RNI[##] (%, 95% CI**)	89.2 (88.0, 90.3)	90.3 (89.7, 90.8)
Dietary iron intake <UK LRNI[###] (%, 95% CI)	18.0 (16.7, 19.5)	25.0 (24.2, 25.8)
Dietary vitamin C intake[##] (mg/day) (m, sd)	85.9 (34.5)	80.1 (35.3)
Age of mother (yrs) (m, sd)	29.5 (4.2)	28.5 (4.8)
Pre-pregnancy BMI (kg/m^2) (m, sd)	22.8 (3.5)	22.9 (3.6)
Total energy intake (kj) (m, sd)	7,483 (1,908)	7,415 (2,081)
Smoking in pregnancy (%, 95% CI**)	15.4 (14.1, 16.7)	25.2 (24.4, 26.0)
Caucasian (%, 95% CI)	98.8 (98.4, 99.2)	97.5 (97.2, 97.8)
University degree (%, 95% CI)	18.6 (17.2, 20.0)	13.8 (13.1, 14.4)
Vegetarian (%, 95% CI)	6.1 (5.3, 7.0)	5.2 (4.8, 5.6)
Primigravida (%, 95% CI)	48.8 (47.0, 50.6)	44.8 (43.9, 45.7)
Early pregnancy maternal anaemia (<11 g/dl) (%, 95% CI)	4.4 (3.7, 5.1)	5.1 (4.6, 5.5)
Report taking iron supplements Before 32 wks gestation (%, 95% CI)	44.9 (43.1, 46.7)	47.5 (46.6, 48.4)
Child gender (male) (%, 95% CI)	50.8 (49.0, 52.6)	51.5 (50.6, 52.4)
Birth weight (g) (m, sd)	3,448 (499)	3,411 (544)
Gestational age (wks) (m, sd)	39.6 (1.6)	39.5 (1.8)

#Food frequency questionnaire at 32 weeks gestation

UK Reference Nutrient Intake (14.8 mg/day)

###UK Lower Reference Nutrient Intake (8 mg/day)

*Mean, standard deviation

**95% confidence intervals

Table 20: Study sample characteristics (complete cases for exposures, outcomes, confounders and mediators n=2958) and ALSPAC sample characteristics (with dietary iron intake data n=12116)

	Dietary iron intake		
	≥14.8 mg/day[#] (n=319)	**<14.8 mg/day** (n=2,639)	**P value***
Dietary iron intake[##] (mg/day) (m, sd**)	16.9 (1.9)	10.1 (2.5)	-
Dietary vitamin C intake[##] (mg/day) (m, sd)	119.3 (35.6)	81.8 (32.1)	<0.001
Age of mother (yrs) (m, sd)	30.0 (4.2)	29.5 (4.2)	0.02
Pre-pregnancy BMI (kg/m^2) (m, sd)	21.7 (2.5)	22.9 (3.6)	<0.001
Total energy intake (kj) (m, sd)	10,100 (1,921)	7,167 (1,645)	<0.001
Smoking in pregnancy (%, 95% CI***)	10.7 (7.5, 14.6)	15.0 (14.6, 14.4)	0.001
Caucasian (%, 95% CI)	98.1 (96.0, 99.3)	99.0 (98.4, 99.3)	0.2
University degree (%, 95% CI)	27.0 (22.2, 32.2)	17.6 (16.2, 19.1)	<0.001
Vegetarian (%, 95% CI)	11.9 (8.3, 16.6)	5.5 (4.6, 6.4)	<0.001
Primigravida (%, 95% CI)	48.0 (42.4, 53.6)	51.0 (47.0, 50.9)	0.8
Early pregnancy maternal anaemia (<11 g/dl) (%, 95% CI)	6.6 (4.1, 9.9)	4.1 (3.4, 4.9)	0.04
Report taking iron supplements Before 32 wks gestation (%, 95% CI)	48.3 (42.7, 53.9)	44.5 (42.6, 46.4)	0.2
Cord ferritin (ug/l) (m, sd)	151.9 (n=-83) (104.8)	165.4 (n=712) (123.6)	0.3
Child systolic blood pressure (mmHg) (m, sd)	103.9 (8.7)	103.6 (8.8)	0.5
Child diastolic blood pressure (mmHg) (m, sd)	60.3 (7.3)	59.5 (7.8)	0.08
Child pulse wave velocity (m/s) (m, sd)	7.7 (1.3)	7.5 (1.2)	0.1
Child flow mediated dilatation (%) (m, sd)	8.3 (3.5)	8.0 (3.4)	0.1
Child body mass index (kg/m^2) (m, sd)	17.9 (2.7)	18.1 (3.0)	0.2
Child gender (male) (%, 95% CI)	55.2 (49.5, 60.7)	50.3 (48.3, 52.2)	0.1
Birth weight (g) (m, sd)	3,439 (486)	3,450 (500)	0.7
Gestational age (wks) (m, sd)	39.5 (1.6)	39.6 (1.6)	0.3

Reference nutrient intake (RNI) for iron for women aged 19-50 years in the UK ##Food frequency questionnaire at 32 weeks gestation
* P-value using two-sample t-test for continuous variables, chi-squared test for categorical variables
Mean, standard deviation * 95% confidence intervals

Table 21: Sample characteristics by dietary iron intake (n=2958 for all, except where cord ferritin data is used: n=795)

5.5.2 Complete case regression analyses

In unadjusted analyses, maternal dietary iron intake was associated with offspring BMI, and maternal pregnancy supplement intake was associated with offspring systolic BP. Child dietary iron intake was associated with maternal iron intake in pregnancy , from both diet and supplements, and early pregnancy Hb. Birth weight was associated with both maternal iron intake from supplements and early pregnancy Hb (Table 22).

With adjustment for confounding characteristics, there were no associations between the primary exposures and outcomes of interest apart from the inverse association between maternal iron supplement intake and offspring systolic BP which remained largely unchanged, with marginal statistical significance at the 1% significance level (0.8 mmHg lower with reporting taking iron supplements, 99% CI 0 to 1.7, P=0.01). This association was not markedly affected by adjustment for mediators (birth weight, gestational age, and offspring dietary iron intake), but was attenuated to the null in the smaller sub-sample with further adjustment for cord ferritin as a fourth mediator. The results from the unadjusted and the three adjusted models (confounders, confounders plus 3 mediators, and confounders plus 4 mediators) are illustrated in table 23 for maternal iron intake and Table 24 for maternal Hb and anaemia.

	Outcomes					Mediators			
Exposures	**Offspring pulse wave velocity (m/s)**	**Offspring flow mediated dilatation (%)**	**Offspring systolic blood pressure (mmHg)**	**Offspring diastolic blood pressure (mmHg)**	**Offspring body mass index (kg/m$^{2)}$**	**Cord ferritin (ug/l)**	**Birth weight (g)**	**Gestational age (wks)**	**Child dietary iron intake (mg/d)***
Maternal pregnancy dietary iron intake (continuous) (per 10mg/d)	0.2 (0, 0.3)	0.2 (-0.3, 0.5)	-0.1 (-1.6, 0.4)	0.4 (-0.5, 1.3)	-0.8 (-1.2, -0.5)	15.7 (-11.8, 43.1)	22.5 (-33.8, 78.8)	0 (-0.2, 0.2)	1.7 (1.6, 1.9)
Maternal iron intake < 14.8 mg/d	-0.1 (-0.3, 0)	-0.3 (-0.7, 0.1)	0.3 (-1.3, 0.7)	-0.8 (-1.7, 0.1)	0.2 (0.1, 0.6)	13.5 (-14.2, 41.3)	10.7 (-47.3, 68.8)	0.1 (-0.1, 0.3)	-1.1 (-1.3, -0.9)
Maternal pregnancy iron supplement use	0.1 (0, 0.2)	0.2 (0, 0.5)	-1.0 (-1.6, -0.3)	-0.4 (-1.0, 0.2)	-0.1 (-0.3, 0.1)	9.5 (-7.5, 26.6)	93.0 (56.9, 129.0)	0 (-0.1, 0.1)	0.1 (0, 0.2)
Maternal early pregnancy haemoglobin (g/dl)	-0.1 (-0.1, 0)	-0.1 (-0.2, 0.1)	0.4 (0, 0.7)	0 (-0.3, 0.3)	0.1 (0., 0.2)	0.7 (-8.9, 10.3)	-29.5 (-49.7, -9.3)	0 (-0.1, 0.5)	-0.1 (-0.1, 0)
Maternal early pregnancy anaemia (<11 g/dl)	0 (-0.2, 0.2)	-0.2 (-0.8, 0.4)	-1.5 (-3.1, 0.1)	-0.4 (-1.8,0.9)	-0.1 (-0.7, 0.4)	-18.3 (-55.6, 17.7)	69.1 (-19.0, 157.2)	-0.2 (-0.4, 0.1)	0.1 (-0.2, 0.4)

Regression coefficients are reported with 95% confidence intervals between brackets

Greyed cell indicate statistical significance at the 5% level

*Average of intake reported over the 4 assessment points (3, 4, 7 and 9 years)

Table 22: Univariable linear regression estimates of exposure-outcome and exposure-mediator relationships (n=2,958 for all, except where cord ferritin data is used: n=795)

	Offspring pulse wave velocity (m/s)			Offspring flow mediated dilatation (%)			Offspring systolic blood pressure (mmHg)			Offspring diastolic blood pressure (mmHg)			Offspring body mass index (kg/m^2)		
	B[#]	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P
Maternal pregnancy dietary iron intake (continuous) (per 10mg/d)															
Unadjusted	0.2	0, 0.4	0.02	0.2	-0.4, 0.6	0.5	-0.6	-1.9, 0.7	0.3	0.4	-0.8, 1.5	0.4	-0.8	-1.3, -0.4	<0.001
Model 1*	0.1	-0.2, 0.4	0.5	0.4	-0.3, 1.2	0.1	-0.3	-2.2, 1.7	0.7	0.7	-1.1, 2.4	0.3	0.1	-.0.5, 0.7	0.7
Model 2**	0.1	-0.2, 0.4	0.3	0.5	-0.3, 1.2	0.1	-0.1	-2.1, 2.0	0.9	1.0	-0.8, 2.8	0.2	0.1	-0.6, 0.7	0.8
Model 3***	0.5	-0.1, 1.1	0.04	0.3	-1.2, 1.9	0.6	0.7	-3.5, 4.9	0.7	1.2	-2.4, 4.8	0.4	0.2	-1.1, 1.5	0.7
Maternal iron intake < 14.8 mg/d															
Unadjusted	-0.1	-0.3, 0.1	0.1	-0.3	-0.8, 0.2	0.1	-0.3	-1.7, 1.0	0.5	-0.8	-2.0, 0.4	0.08	0.2	-0.2, 0.7	0.2
Model 1*	0	-0.3, -0.2	0.7	-0.5	-1.1, 0.1	0.05	-0.7	-2.3, 0.8	0.3	-0.9	-2.3, 0.4	0.08	-0.4	-0.9, 0.1	0.03
Model 2**	0	-0.3, 0.2	0.7	-0.5	-1.1, 0.1	0.04	-0.8	-2.3, 0.8	0.2	-1.0	-2.4, 0.3	0.05	-0.4	-0.9, 0.1	0.03
Model 3***	-0.1	-0.6, 0.3	0.5	0.1	-1.0, 1.2	0.8	-0.8	-3.9, 2.2	0.5	-1.2	-3.9, 1.5	0.3	-0.5	-1.5, 0.4	0.2
Maternal pregnancy iron supplement use															
Unadjusted	-0.1	-0.1, 0.2	0.07	0.2	-0.1, 0.5	0.1	-1.0	-1.8, -0.1	0.003	-0.4	-1.1, 0.3	0.2	-0.1	-0.4, 0.2	0.3
Model 1*	0.1	-0.1, 0.2	0.2	0.2	-0.1, 0.6	0.07	-0.8	-1.7, 0	0.01	-0.3	-1.1, 0.4	0.3	0	-0.2, 0.3	0.7
Model 2**	0.1	-0.1, 0.2	0.1	0.2	-0.1, 0.6	0.06	-0.8	-1.6, 0.1	0.02	-0.2	-1.0, 0.5	0.5	0	-0.3, 0.3	0.9
Model 3***	0	-0.2, 0.3	0.7	0.1	-0.6, 0.7	0.8	-0.9	-2.6, 0.8	0.2	0.1	-1.4, 1.5	0.9	-0.2	-0.7, 0.3	0.4

Change per 1 outcome unit

* Adjusting for confounders: maternal age, pre-pregnancy body mass index, smoking in pregnancy, educational qualification (as a proxy for socioeconomic status), parity (and maternal total energy intake in the models with dietary iron intake as exposure)

** Adjusting for confounders and three mediators: birth weight, gestational age, offspring dietary iron intake

*** Adjusting for confounders and four mediators: birth weight, gestational age, offspring dietary iron intake, cord ferritin

Table 23: Linear regression estimates for associations between maternal iron intake in pregnancy with offspring vascular indicators and body mass index (n=2958 for all, except where cord ferritin data is used: n=795)

	Offspring pulse wave velocity (m/s)			Offspring flow mediated dilatation (%)			Offspring systolic blood pressure (mmHg)			Offspring diastolic blood pressure (mmHg)			Offspring body mass index (kg/m^2)		
	B[#]	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P
Maternal early pregnancy haemoglobin (g/dl)															
Unadjusted	-0.1	-0.1, 0	0.07	-0.1	-0.2, 0.1	0.5	0.4	-0.1, 0.8	0.05	0	-0.4, 0.4	0.8	0.1	0, 0.3	0.06
Model 1*	0	-0.1, 0	0.09	-0.1	-0.3, 0.1	0.3	0.2	-0.3, 0.7	0.2	0	-0.5, 0.4	0.8	0	-0.2, 0.1	0.7
Model 2**	-0.1	-0.1, 0	0.08	-0.1	-0.3, 0.1	0.3	0.2	-0.3, 0.7	0.3	-0.1	-0.5, 0.4	0.7	0	-0.2, 0.2	0.9
Model 3***	-0.1	-0.2, 0.1	0.3	0	-0.4, 0.3	0.8	0.3	-0.6, 1.3	0.4	-0.1	-0.9, 0.7	0.7	0.1	-0.2, 0.4	0.6
Maternal early pregnancy anaemia (<11 g/dl)															
Unadjusted	0	-0.3, 0.3	0.8	-0.2	-1.0, 0.6	0.5	-1.5	-3.6, 0.6	0.06	0.4	-2.2, 1.4	0.5	-0.1	-0.8, 0.6	0.6
Model 1*	0	-0.3, 0.3	0.8	-0.2	-1.0, 0.6	0.6	-1.3	-3.3, 0.8	0.1	-0.3	-2.1, 1.5	0.6	0.1	-0.5, 0.8	0.7
Model 2**	0	-0.3, 0.3	0.8	-.0.2	-0.9, 0.6	0.6	-1.2	-3.2, 0.9	0.1	-0.2	-2.0, 1.6	0.7	0	-0.6, 0.7	0.8
Model 3***	0	-0.7, 0.3	0.2	-0.1	-1.4, 1.3	0.9	-1.0	-4.6, 2.6	0.5	-0.3	-3.4, 2.8	0.8	0.1	-1.0, 1.2	0.7

Change per 1 outcome unit

* Adjusting for confounders: maternal age, pre-pregnancy body mass index, smoking in pregnancy, educational qualification (as a proxy for socioeconomic status), parity (and maternal total energy intake in the models with dietary iron intake as exposure)

** Adjusting for confounders and three mediators: birth weight, gestational age, offspring dietary iron intake

*** Adjusting for confounders and four mediators: birth weight, gestational age, offspring dietary iron intake, cord ferritin

Table 24: Linear regression estimates for associations between maternal haemoglobin and anaemia in pregnancy with offspring vascular indicators and body mass index (n=2958 for all, except where cord ferritin data is used: n=795)

5.5.3 Multiply imputed data regression analyses

The main adjusted associations were largely similar when conducted using the multiple imputation databases (Table 25 & Table 26), compared to those conducted on complete data presented as primary analyses in this paper (Table 23 & Table 24). However, adjusting for cord ferritin as a mediator in the inverse association between maternal iron supplement use and offspring systolic BP in the imputed dataset had less impact than in the complete dataset (Table 25).

Adjusting for early pregnancy maternal Hb in the association between maternal iron supplement use and offspring systolic BP as sensitivity analysis did not change the magnitude of association (-0.8 mmHg, 99% CI 0.1 to -1.7, P=0.03 in the main complete dataset, and -0.6 mmHg, 99% CI 0 to -1.3, P=0.01 in the imputed dataset).

5.5.4 Interaction and mediation

There was also no evidence of effect modification by maternal vitamin C intake or child sex on any of the relationships (Table 27 & Table 28). There was also no association between cord ferritin and either maternal dietary iron intake or maternal Hb.

	Offspring pulse wave velocity (m/s)			Offspring flow mediated dilatation (%)			Offspring systolic blood pressure (mmHg)			Offspring diastolic blood pressure (mmHg)			Offspring body mass index (kg/m^2)		
	B	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P
Maternal pregnancy dietary iron intake (continuous) (per 10mg/d)															
Unadjusted	0	-0.1, 0.1	0.3	0.1	-0.3, 0.4	0.6	-1.0	-1.9, -0.1	0.004	-0.5	-1.3, 0.3	0.1	-0.7	-1.1, -0.4	<0.001
Model 1*	-0.1	-0.2, 0.1	0.5	0.5	-0.1, 1.0	0.03	0.2	-1.9, 1.4	0.7	-0.3	-1.6, 1.0	0.6	0.2	-0.3, 0.7	0.3
Model 2**	0	-0.4, 0.3	0.5	0.5	-0.1, 1.0	0.02	0	-1.6, 1.6	0.9	0	-1.3, 1.4	0.9	0.1	-0.4, 0.6	0.6
Model 3***	0	-0.2, 0.1	0.5	-0.2	-0.1, 1.1	0.6	0.1	-1.7,1.6	0.9	0	-1.4, 1.4	0.9	0	-0.4, 0.6	0.6
Maternal iron intake < 14.8 mg/d															
Unadjusted	-0.1	-0.2, 0	0.06	-0.2	-0.6, 0.2	0.1	0.6	-0.4, 1.6	0.1	0	-0.9, 0.9	0.9	0.3	0.1, 0.6	0.002
Model 1*	-0.1	-0.2, 0.1	0.3	-0.4	-0.8, 0.1	0.03	0	-1.3, 1.3	0.9	-0.3	-1.3, 0.7	0.5	-0.2	-0.5, 0.1	0.06
Model 2**	-0.1	-0.2, 0.1	0.2	-0.4	-0.8, 0.1	0.03	-0.1	-1.3, 1.2	0.9	-0.4	-1.4, 0.6	0.3	-0.2	-0.5, 0.1	0.08
Model 3***	-0.1	-0.2, 0.1	0.2	-0.4	-0.8, 0.1	0.02	-0.1	-1.3, 1.2	0.9	-0.4	-1.4, 0.6	0.3	-0.2	-0.5, 0.1	0.08
Maternal pregnancy iron supplement use															
Unadjusted	0.1	0, 0.1	0.2	0.1	-0.1, 0.4	0.2	-0.8	-1.4, -0.2	0.001	-0.3	-0.8, 0.2	0.2	-0.2	-0.4, 0	0.002
Model 1*	0	-0.1, 0.1	0.3	0.1	-0.1, 0.4	0.1	-0.7	-1.3, 0	0.008	-0.2	-0.7, 0.4	0.4	0	-0.2, 0.2	0.9
Model 2**	0	-0.1, 0.1	0.2	0.2	-0.1, 0.4	0.08	-0.6	-1.2, 0	0.01	-0.1	-0.7, 0.4	0.5	-0.1	-0.2, 0.1	0.5
Model 3***	0	0, 0.1	0.2	0.2	-0.1, 0.4	0.08	-0.6	-1.3, 0	0.01	-0.2	-0.7, 0.4	0.4	0	-0.2, 0.1	0.5

Change per 1 outcome unit

* Adjusting for confounders: maternal age, pre-pregnancy body mass index, smoking in pregnancy, educational qualification (as a proxy for socioeconomic status), parity (and maternal total energy intake in the models with dietary iron intake as exposure)

** Adjusting for confounders and three mediators: birth weight, gestational age, offspring dietary iron intake

***Adjusting for confounders and four mediators: birth weight, gestational age, offspring dietary iron intake, cord ferritin

Table 25: Linear regression estimates for associations of maternal iron intake in pregnancy with offspring vascular indicators and adiposity using multiple imputation dataset based on the sample with dietary iron intake data (n=12116)

	Offspring pulse wave velocity (m/s)			Offspring flow mediated dilatation (%)			Offspring systolic blood pressure (mmHg)			Offspring diastolic blood pressure (mmHg)			Offspring body mass index (kg/m^2)		
	B[#]	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P	B	99% CI	P
Maternal early pregnancy haemoglobin (g/dl)															
Unadjusted	0	-0.1, 0	0.1	0	-0.1, 0.1	0.8	0.3	-0.1, 0.7	0.05	0.1	-0.3, 0.4	0.6	0.1	0, 0.3	0.02
Model 1*	0	-0.1, 0	0.3	0	-0.1, 0.1	0.9	0.2	-0.2, 0.5	0.3	0	-0.3, 0.3	0.8	-0.1	-0.2, 0.1	0.2
Model 2**	0	-0.1, 0	0.3	0	-0.1, 0.1	0.9	0.1	-0.2, 0.5	0.3	0	-0.4, 0.3	0.8	0	-0.2, 0.1	0.3
Model 3***	0	-0.1, 0	0.3	0	-0.1, 0.1	0.9	0.1	-0.2, 0.5	0.3	0	-0.3, 0.3	0.8	-0.1	-0.2, 0.1	0.3
Maternal early pregnancy anaemia (<11 g/dl)															
Unadjusted	0	-0.3, 0.2	0.6	0	-0.6, 0.6	0.9	-0.1	-1.5, 1.2	0.8	0	-1.4, 1.2	0.9	0.1	-0.5, 0.6	0.8
Model 1*	-0.1	-0.3, 0.2	0.6	0	-0.6, 0.6	0.9	0.1	-1.2, 1.5	0.8	0.2	-1.2, 1.6	0.8	0.4	-0.2, 0.9	0.07
Model 2**	-0.1	-0.3, 0.2	0.5	0	-0.6, 0.6	0.9	0.1	-1.2, 1.5	0.8	0.1	-1.3, 1.5	0.8	0.4	-0.2, 0.9	0.08
Model 3***	-0.1	-0.3, 0.2	0.5	0	-0.6, 0.6	0.9	0.1	-1.2, 1.5	0.8	0.2	-1.2, 1.6	0.7	0.4	-0.2, 0.9	0.07

Change per 1 outcome unit

* Adjusting for confounders: maternal age, pre-pregnancy body mass index, smoking in pregnancy, educational qualification (as a proxy for socioeconomic status), parity (and maternal total energy intake in the models with dietary iron intake as exposure)

** Adjusting for confounders and three mediators: birth weight, gestational age, offspring dietary iron intake

***Adjusting for confounders and four mediators: birth weight, gestational age, offspring dietary iron intake, cord ferritin

Table 26: Linear regression estimates for associations of maternal haemoglobin and anaemia in pregnancy with offspring vascular indicators and adiposity using multiple imputation dataset based on the sample with dietary iron intake data (n=12116)

	Offspring pulse wave velocity (m/s)			Offspring flow mediated dilatation (%)			Offspring systolic blood pressure (mmHg)			Offspring diastolic blood pressure (mmHg)			Offspring body mass index (kg/m$^{2)}$		
	B#	99% CI	P*	B#	99% CI	P*	B#	99% CI	P*	B#	99% CI	P*	B#	99% CI	P*
Maternal pregnancy dietary iron intake (continuous) (per 10mg/d)															
In participants with vitamin C intake> 50 mg/day	0	-0.3, 0.3	0.5	0.6	-0.2, 1.5	0.5	-0.3	-2.4, 1.9	0.7	0.8	-1.1, 2.7	0.8	0	-.0.7, 0.6	0.3
In participants with vitamin C intake≤ 50 mg/day	0.2	-0.7, 1.1		-0.3	-2.7, 2.1		-1.8	-7.3, 3.7		1.2	-4.0, 6.4		1.2	-1.1, 3.5	
Maternal pregnancy iron supplement use															
In participants with vitamin C intake> 50 mg/day	0	-0.1, 0.2	0.2	0.3	0, 0.7	0.1	-0.8	-1.7, 0.1	0.9	-0.4	-1.2, 0.5	0.9	0.1	-0.2, 0.3	0.9
In participants with vitamin C intake≤ 50 mg/day	0.2	-0.1, 0.5		-0.3	-1.1, 0.6		-0.8	-2.7, 1.2		-0.1	-2.0, 1.8		0	-0.8, 0.8	

#Change per 1 unit in outcome adjusting for potential confounding characteristics: maternal age, pre-pregnancy BMI, smoking in pregnancy, educational qualification (as a proxy for socioeconomic status), parity, and maternal total energy intake in the models with dietary iron intake as exposure)
*Interaction P value, testing the null hypotheses that associations do not differ by maternal vitamin C level

Table 27: Multivariable linear regression estimates from stratified analyses for associations between maternal iron intake in pregnancy with offspring vascular indicators and body mass index with testing for effect modification by maternal vitamin C intake during pregnancy (n=2958)

	Offspring pulse wave velocity (m/s)			Offspring flow mediated dilatation (%)			Offspring systolic blood pressure (mmHg)			Offspring diastolic blood pressure (mmHg)			Offspring body mass index (kg/m^2)		
	B#	99% CI	P*	B#	99% CI	P*	B#	99% CI	P*	B#	99% CI	P*	B#	99% CI	P*
Maternal pregnancy dietary iron intake (continuous) (per 10mg/d)															
Males	0.2	-0.2, 0.6	0.4	0.6	-0.4, 1.6	0.2	0.4	-2.3, 3.1	0.9	0.9	-1.5, 3.2	0.7	0.2	-0.7, 1.0	0.4
Females	0	-0.4, 0.4		0.5	-0.7, 1.6		-0.9	-3.8, 2.0		0.7	-1.9, 3.3		0.1	-0.9, 1.0	
Maternal pregnancy iron supplement use															
Males	0.1	-0.1, 0.2	0.8	0.2	-0.2, 0.6	0.9	-0.9	-2.1, 0.3	0.7	-0.9	-1.9, 0.1	0.06	-0.1	-0.4, 0.3	0.5
Females	0.1	-0.1, 0.2		0.3	-0.3, 0.7		-0.7	-1.9, 0.5		0.2	-0.8, 1.3		0.1	-0.3, 0.5	
Maternal early pregnancy anaemia (<11 g/dl)															
Males	0	-0.4, 0.4	0.9	0.2	-0.9, 1.2	0.3	-1.3	-4.1, 1.4	0.8	0.3	-2.1, 2.6	0.4	0.4	-0.5, 1.2	0.2
Females	-0.1	-0.5, 0.4		-0.5	-1.7, 0.7		-1.0	-4.1, 2.1		-0.9	-3.7, 1.8		-0.2	-1.2, 0.8	

#Change per 1 unit in outcome adjusting for potential confounding characteristics: maternal age, pre-pregnancy BMI, smoking in pregnancy, educational qualification (as a proxy for socioeconomic status), parity, and maternal total energy intake in the models with dietary iron intake as exposure)
*Interaction P value, testing the null hypotheses that associations do not differ by offspring sex

Table 28: Multivariable linear regression estimates from stratified analyses for associations between maternal iron intake and haemoglobin in pregnancy with offspring vascular indicators and body mass index with testing for effect modification by child sex (n=2958)

5.6 Discussion

In this study, associations of maternal pregnancy iron intake and early-pregnancy Hb with several markers of offspring cardiovascular health at 10 years were examined. Of the 25 main associations examined, only one was observed that was statistically significant, that of an inverse association of maternal report of taking a supplement that contained iron before 32 weeks gestation with offspring systolic BP at 10 years of age. However, although the direction of the latter association is consistent with results from previous animal studies that support a causal relationship between maternal ID in pregnancy and raised offspring BP (Gambling et al., 2003b, Gambling et al., 2003a, Crowe et al., 1995, Gambling et al., 2004b), its magnitude was modest (1 mmHg lower offspring systolic BP on average in children whose mothers reported taking supplements compared to those who did not).

To take some account of multiple testing, a p-value threshold of 0.01 was used as a statistical significance level in this analysis, giving the association observed between maternal iron supplement intake and offspring BP only marginal statistical significance in the main complete dataset. Therefore, this result should be treated with caution as a potential chance finding.

5.6.1 Residual confounding

The association between maternal iron supplement intake and offspring BP could be heavily confounded by the detection of anaemia or ID in the mother. Mothers who are aware or suspect that they are anaemic are more likely to consume iron supplements during their pregnancy. The potential confounding of maternal Hb was taken into

account in a sensitivity analysis which did not change this result much. However, the detection of ID without anaemia during antenatal care through measuring sF concentrations could still constitute unmeasured confounding, as mothers may opt to take iron supplements if they become aware that they are iron deficient. There is also the possibility that these mothers may change their diet to contain more iron towards the end of their pregnancies.

5.6.2 Comparison with previous ALSPAC findings

The previous analysis in ALSPAC by Brion et al. reported an association between maternal anaemia and offspring BP at 7 years only in women who did not take iron supplements during pregnancy. The total sample size with data on maternal Hb (before stratification by supplement intake) was considerably smaller (n=1255) than this analysis (n=2958) (Brion et al., 2008). Also the definition of early anaemia differed in that analysis in that it included both the first and second trimester, while here Hb concentrations before 18 weeks gestation were used to define early pregnancy Hb. In this analysis, using a bigger sample, stratification by intake of iron supplements did not change the null result of the association between maternal anaemia with offspring BP. Analysis presented in chapter 4 found no evidence of association between intake of iron supplements in pregnancy and infant PWV, however adjusting for iron supplement intake strengthened the association observed between early pregnancy maternal anaemia and infant PWV (section 4.5.2.1).

5.6.3 Comparison with Baby VIP findings (chapter 4)

In contrast to the association between maternal anaemia in early pregnancy and increase infant PWV observed in Baby VIP, this study found no such association with PWV measured 10 years later in life. One possible explanation for this difference in findings is that PWV in the first few weeks of life may not reflect later cardiovascular risk and may not correlate strongly with PWV later in life. Also, central PWV was assessed in ALSPAC (carotid-radial) in comparison with peripheral PWV in Baby VIP (brachio-femoral), which may explain the difference in findings. Another possibility is a true effect of IDA on offspring cardiovascular profile as reflected by PWV in the short term, which is potentially modifiable by other environmental factors during the first years of life, leading to attenuating this relationship later in life.

5.6.4 Strengths & limitations of the study

5.6.4.1 Study sample

This is a relatively large study and the first to examine the association of maternal iron intake and maternal Hb in pregnancy with measures of offspring arterial stiffness, endothelial function and adiposity, as well as with BP. This sample size allowed the assessment of mediation and effect modification based on existing evidence to explain the mechanisms underlying any observed associations. However, the study sample analysed to test mediation by cord ferritin was considerably smaller. Therefore, when examining the association between maternal iron supplement intake and offspring systolic BP, lack of statistical power may explain the difference in the results between the complete data and multiple imputation models (Table 25 & Table 26).

5.6.4.2 Exposure measures

Maternal Hb in early pregnancy was objectively measured as part of routine antenatal care, and extracted for cohort participants from medical records, making bias due to selection and measurement unlikely. Although anaemia does not represent a specific or sensitive measure of body iron stores, it is likely to reflect ID when it is pronounced, particularly in the first trimester (Milman, 2006a).

An important limitation of this study is that a biomarker of maternal iron status during pregnancy, such as sF, or sTfR was not available in this study. Although there are reservations about the use of sF as a sole measure of iron status as it is an acute inflammatory marker (Zimmermann, 2008), it remains a better indicator compared to self-reported iron intake or Hb concentration (Zimmermann, 2008). Dietary iron was assessed by self-report, using a FFQ, which may provide less accuracy than more detailed methods of dietary assessment such as weighed food diaries. Furthermore, the long term outcomes of interest are cardiovascular events, which we are unable to assess in this cohort due to their young age.

It is also known that haem iron is absorbed better than non-haem iron. Although vegetarians often take iron supplements, these may not be as effective as the haem iron that is missing from their diets, and this may have a bearing on the interpretation of our results. However, results based on Hb concentrations in the blood were broadly consistent with those based on dietary intake, suggesting that the source of the iron was not relevant here.

Iron supplement intake was self-reported and the nature of the questions used in this study mean that we do not know the amount of supplementation or whether the iron

supplementation was in the form of iron only or as part of a multivitamin preparation. Therefore the association we observed between maternal reported iron supplementation and offspring systolic BP could be attributable to other micronutrient supplements that the mother was taking as well or our null associations may be masked since iron taken as part of a multivitamin preparation may be less readily absorbed compared to when taken on its own (Gambling et al., 2008, Gambling et al., 2003a, Kelleher and Lönnerdal, 2006).

It is difficult to conduct analyses in a British cohort study of iron-only supplements as the exposure. Firstly because, in the UK, these are not recommended routinely in pregnancy, therefore a small proportion of pregnant women would be expected to use them. Secondly, those who take iron-only supplements are likely to be taking the prescribed high-dose as opposed to the low-dose recommended routinely during pregnancy in other countries.

5.6.4.3 Outcome measures

Offspring cardiovascular and adiposity indicators (PWV, FMD, BP and BMI) were measured by trained skilled staff using standardised methods in this study. However, definite correlation between cardiovascular measures during childhood and cardiovascular end-points in adulthood, which are the main interest in the hypothesis underlying these investigations, is yet to be established. Therefore, re-testing of these associations using adult indicators of cardiovascular risk remains desired.

5.6.5 Implications for research and practice

There is a need to examine the main relationship of interest in this study utilising valid biomarkers of iron status in the mother such as R/F ratio. However, the results of this analysis are still relevant to the current debate regarding the long-term benefit of routine iron supplementation in pregnancy (Alwan and Cade, 2011). Some international and national guidelines recommend routine iron supplements during pregnancy including the World Health Organization and the US Centers for Disease Control and Prevention (CDC) (CDC, 1998, WHO, 2006). However, iron supplements can be associated with side-effects such as nausea, vomiting and constipation (British Medical Association and Royal Pharmaceutical Society of Great Britain, 2010). In the UK, routine iron supplementation during pregnancy is not recommended (National Institute for Clinical Excellence (NICE), 2008), although there is no recommendation for detection/intervention in pregnant women who are iron-deficient but not anaemic.

5.7 **Conclusion**

The findings in this chapter suggest that maternal dietary iron intake and Hb concentrations during pregnancy are unlikely to be related to childhood indicators of cardiovascular health at 10 years. However, they do not exclude a relationship between maternal iron status in pregnancy and cardiovascular indicators that could become apparent later in the offspring's lives.

In conclusion, this study suggests that maternal anaemia during early pregnancy is not an important determinant of future offspring cardiovascular health, using childhood vascular and adiposity indicators at 10 years. No associations were observed between maternal iron intake in pregnancy with offspring's vascular markers and adiposity except for a modest inverse association between self-reported maternal iron supplement intake during pregnancy and offspring systolic BP.

In the next chapter, offspring cardiovascular indicators are assessed in adulthood and their association with maternal iron status is examined using a Mendelian randomisation study design utilising IV techniques.

6 Exploring the association of maternal iron status with adult offspring's blood pressure and adiposity using Mendelian randomization: the UKWCS-IBPS

In the previous chapters, I have investigated the association between indicators of maternal iron status in pregnancy and offspring cardiovascular risk indicators in infancy and childhood. This chapter explores the relationship of maternal iron status with adult offspring health indicators including BP and adiposity, which are known to be strong predictors of CVD morbidity and mortality.

Work from this chapter has formed the basis of one peer-reviewed paper (Alwan et al., 2012b), and one conference presentation with a published peer-reviewed abstract (Alwan et al., 2012a).

6.1 Chapter summary

An IV analysis, using maternal C282Y as an instrument for mother's iron status, was undertaken to examine its association with offspring BP, WC and BMI, and the results were compared to that of ordinary least squares (OLS) regression. Offspring of a sub-cohort of mothers from the UK Women Cohort Study were recruited in 2009-10 (n=348, mean age=41 years). Their BP, height and weight were measured at their local general medical practice, and they were asked to self-measure their WC. About half were offspring of C282Y carriers. Maternal ferritin was used as a biomarker of maternal iron status.

Maternal C282Y was strongly associated with maternal ferritin (mean difference per allele=84 ug/l, 95% CI 31, 137, *P=0.002*). Using IV analyses, maternal ferritin was not linked to offspring's BP, BMI or WC. The first stage F statistic for the strength of the instrument was 10 (Kleibergen-Paap *rk* LM P-value=0.009). Maternal ferritin was linked to offspring diastolic BP, WC and BMI in univariable, but not in multivariable OLS analysis. There was no difference between the OLS and the IV models coefficients for any of the outcomes considered.

There was no evidence of association between maternal iron status and adult offspring's BP and adiposity using both multivariable OLS and IV modelling. This is the first study examining this relationship using offspring outcomes in adulthood. Further exploration in larger studies that have genetic variation assessed in both mother and offspring should be considered.

6.2 Background

6.2.1 Mendelian randomization

Association studies of specific nutrient effects with health outcomes in humans are difficult because of the likelihood of important confounding by other nutrients or dietary characteristics, as well as other lifestyle factors such as smoking and alcohol consumption, and socioeconomic factors potentially affecting these associations. Most of these studies are observational, as randomised controlled trials are often not feasible or ethical to study such relationships. One way to try and tackle this limitation is the use of the random assortment of genes from parents to offspring which provides one method for assessing the causal nature of some environmental exposures (Lawlor et al., 2004). This approach, known as Mendelian randomization (MR), uses genetic variants as instrumental variables for the environmental exposures of interest (Davey Smith, 2003).

Genetic variants are randomised at birth and are not influenced by the many lifestyle and environmental characteristics with which risk factors, such as ID, are associated. Since these genetic variants are allocated at conception, they cannot be influenced by either the later occurrence of disease processes or treatment (Davey Smith et al., 2007). Therefore associations derived from MR analysis are less likely to be affected by confounding and reverse causality that can bias established multivariable approaches to epidemiological association studies (Lawlor et al., 2008a).

In the context of CVD research, MR can be used to investigate causation of risk factors such as obesity. MR can also provide a valuable tool in the study of the developmental

origins of obesity by using paternal/maternal genes to control for the effect of offspring genes, and hence to separate 'developmental' effects during fetal life and early childhood from genetic inherited effects. For example, Lawlor et al. examined the effect of maternal adiposity measured by BMI on offspring fat mass. Using maternal FTO genotype as an IV in the analysis, and hence controlling for the offspring's FTO genotype, which would directly affect their fat mass, there was no evidence of association between maternal BMI and offspring fat mass (Lawlor et al., 2008b).

It is important to note that MR has important caveats. These include confounding by polymorphisms in linkage disequilibrium with the genetic variant under study, the fact that variants may have several phenotypic effects associated with the outcome of interest, canalisation which is the buffering of genetic effects during development, and the absence of a suitable polymorphism for studying the exposure of interest (Davey Smith, 2003).

6.2.1.1 Instrumental variable analysis

IV methods are commonly used for statistical analysis in econometrics. This approach is generally recommended for data analysis in MR studies (Lawlor et al., 2008a). An IV is a variable associated with the outcome only through its robust association with an intermediary variable, which is the exposure of interest in the case of MR (Figure 11).

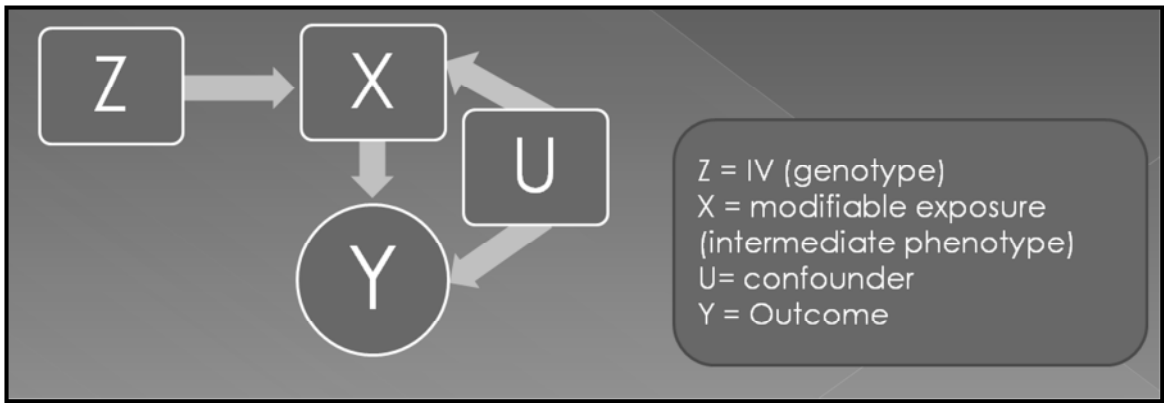


Figure 11: Illustration of IV analysis

There are conditions for IV analysis (Lawlor et al., 2008a). These are:

1. Z is robustly associated with X
2. Z is not associated with U (this can be tested in the study data)
3. Z is related to Y only through X
4. All associations are linear and unaffected by statistical interaction

In this study IV analysis uses the proportion of the variation in maternal iron status that is explained by the C282Y mutation in the *HFE* gene to provide an un-confounded estimate of the relationship with adult offspring's BP and obesity.

6.2.2 Genetic susceptibility to iron overload

Women who carry a C282Y mutation in the *HFE* gene are more likely, in the homozygous state, to suffer from haemochromatosis, a condition which is characterised by iron overload in the liver (Rhodes et al., 1997, Willis et al., 1999). C282Y homozygotes (aa) have significantly higher sF, TS and serum iron levels compared to wild type individuals (gg) (Cade et al., 2005).

About 12-20% of Northern Europeans are heterozygotes for this mutation (ag) (Rhodes et al., 1997). These *HFE* gene mutation carriers are usually asymptomatic, however there is evidence that C282Y heterozygotes have higher total body iron stores reflected by higher TS levels (Chan et al., 2005, Cade et al., 2005, Beutler et al., 2002, Jackson et al., 2001), and reflected by lower R/F ratio (Chan et al., 2005). In a survey in Denmark, Pedersen and Milman found that, among C282Y homozygotes, 89% had elevated TS (>=50%) and 94% had elevated sF (>300 mcg/L). Among C282/wild type (ag) heterozygotes, 9% had elevated TS, 9% had elevated sF, and 1.2% had elevated both markers (n=2550) (Pedersen and Milman, 2009) compared to wild type.

6.3 **Hypothesis and aim**

It is hypothesised that women who carry the C282Y mutation in the *HFE* gene are at lower risk of ID during pregnancy, and therefore their adult offspring will have on average lower BP and less adiposity, as indicated by BMI and WC, than the offspring of wild-type mothers.

The aim of this study is to use the concept of Mendelian randomisation study design to examine the relationship between maternal iron status, using maternal sF as the environmental measure of exposure (modifiable risk factor) and maternal C282Y genotype as the IV, with offspring BP and adiposity measures (BMI and WC) in adulthood, utilising both ordinary multivariable regression and IV regression methods and comparing between their results.

6.4 Methods

6.4.1 The cohort: the UK Women's Cohort Study

The UK Women's Cohort Study (UKWCS), which includes 35,372 women aged 35-69 at recruitment across England, Wales, and Scotland, was established in 1994 to examine the association of diet with cancer. Subjects in the cohort were selected to ensure that there was a wide range of dietary patterns represented. The cohort was constructed to have similar, large numbers of subjects in three main groups: vegetarian, fish-eaters and meat-eaters.

The cohort was taken from responders to the World Cancer Research Fund's (WCRF) direct mail survey. This included people living in England, Wales and Scotland and used direct mail lists, targeted towards females, with an overall response rate of 17%. Using this approach, about 16 000 self-reported vegetarians and a similar number of other non red-meat-eaters aged 35–69 years were identified, out of a total of 500 000 responders. Eighty-five per cent of the responders were women, and 75% indicated that they would be willing to participate in a more detailed survey. These women formed the population to be contacted to become part of the UK Women's Cohort. All of the vegetarians and the non red-meat-eaters were invited to take part in the study. A comparison group was selected from the remaining eligible women by selecting, for each vegetarian, the next non-vegetarian in the list aged within 10 years of the vegetarian. Further women were recruited from responders to the baseline data collection, who were asked to identify friends and relatives of a similar age group who were vegetarians and meat-eaters.

One hundred and seventy-four local research ethic committees were contacted and permission to carry out the study was obtained. Baseline data were collected between 1995 and 1998 via a postal questionnaire to each subject (Cade et al., 2004).

6.4.2 Exposure assessment in the second phase of UKWCS

Approximately 15,000 women from UKWCS were contacted for a second time between 1999 and 2002 to acquire more detail on diet. They were sent 2 cytology brushes and asked to provide cheek cell samples for DNA extraction (phase II data collection). These samples were returned by mail and refrigerated until DNA extraction could be undertaken.

A protocol for high throughput screening was developed for the *HFE* mutations associated with haemochromatosis. This involved a simple DNA extraction method that uses sodium hydroxide cell lysis for cheek cells, adapted from the method of Rudbeck and Dissing (Rudbeck and Dissing, 1998). This was followed by a highly sensitive fluorescent Amplification Refractory Mutation System (ARMS) technique. DNA from the buccal cells was of insufficient quality to use a traditional restriction digest for detection of the 2 mutations, C282Y and H63D (Worwood et al., 1997). The ARMS technique is a sensitive analysis that can be used for any point mutation (Newton et al., 1989, Baty et al., 1998).

All subjects who were found to be homozygous or heterozygous for the C282Y gene mutation were also asked to provide a blood sample, to confirm the result from the cheek cell DNA and to measure markers of iron status including sF. This was measured with a 2-site chemiluminometric (sandwich) immunoassay (Bayer, Newbury, United

Kingdom) performed in the Department of Clinical Biochemistry and Immunology. Methods were validated by participation in recognized quality-assurance schemes. In addition, 3000 women were randomly selected and asked to provide a blood sample for both measurement of iron storage markers and DNA to act as a control group (Cade et al., 2005).

C282Y homozygotes were found to have significantly higher sF, transferring saturation and serum iron levels compared to wild type women. C282Y heterozygotes had significantly higher TS levels compared to wild type women. Cade et al. describe the full results of this analysis (Cade et al., 2005) . The genotype assessed in this study was used as a proxy (IV) for maternal iron status in our sub-cohort as described below.

6.4.3 The sub-cohort: the UK Women's Cohort Study - Iron and Blood Pressure sub-cohort

For this analysis, 1416 mothers, identified as reporting having at least one live child in the first UKWCS questionnaire, were contacted between 2008 and 2010, of whom 716 were C282Y allele carriers (aa or ag), and 700 were wild type *HFE* genotype (gg). H63D carriers were not included in the sampling as this mutation on its own is not clinically-important unless it occurs in combination with the C282Y mutation (compound heterozygotes) (Waheed et al., 1997). The non-exposed in this sample were randomly selected from the pool of women with one or more children who were tested and found to have a wild type genotype. The total number of offspring for women contacted in this study was 3376, of whom 1686 were children to C282Y carrier mothers and 1690 to wild type mothers (Figure 12)

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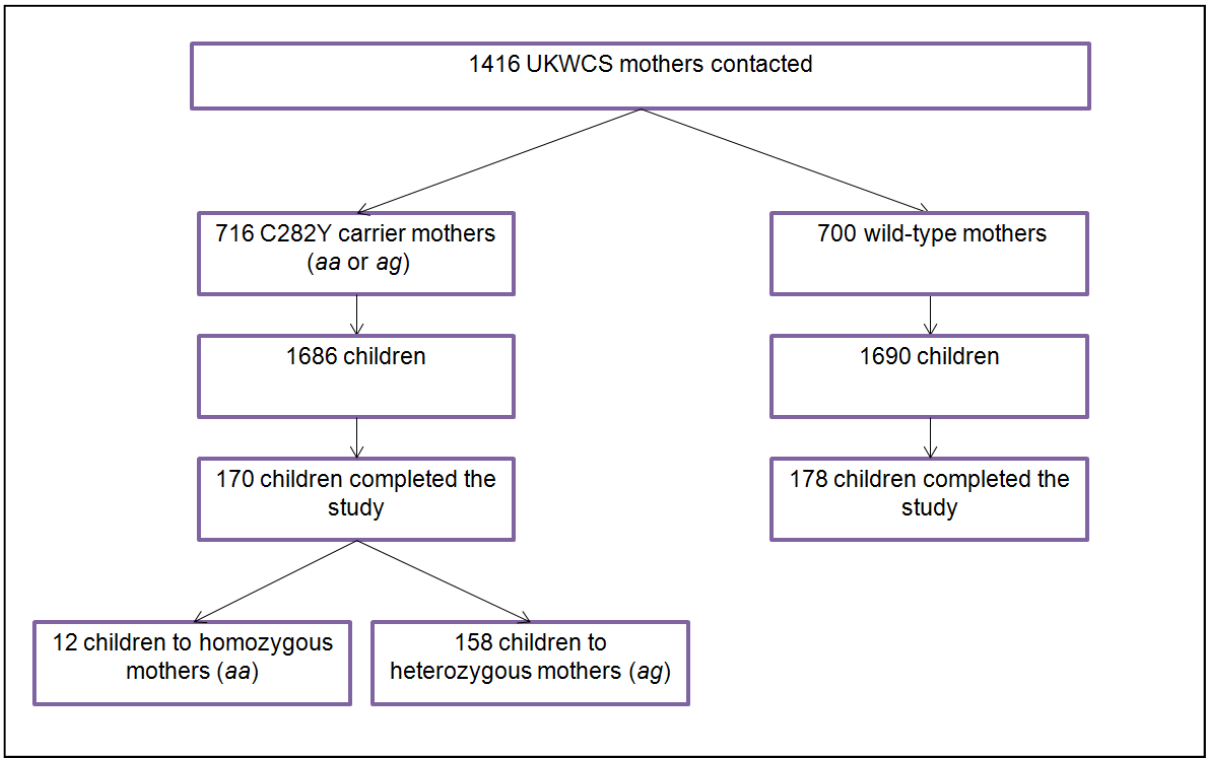


Figure 12: UKWCS- IBPS participant flowchart

6.4.3.1 Recruitment stages

Recruitment started in December 2008 and finished in October 2010.

Stage 1 – Study sample was identified as a sub-cohort of the UKWCS (as described above) and mailed in batches

Stage 2 –A study pack was sent to the mothers, containing a study invitation, a study information sheet, a pack for them to pass on to each of their offspring over the age of 18 with a stamped envelope for each of these packs. The offspring pack contained the offspring's study invitation letter, an offspring's information sheet, a study consent form to fill in, sign and send back to the study team with their contact details and a stamped, addressed envelope for the consent forms.

Stage 3 – Offspring who have replied wanting to take part in the study were sent a study pack including a tape measure with instructions on how to measure waist and hip circumference, a form to write down them down, a lifestyle and diet questionnaire to complete, a GP/practice nurse pack and an addressed freepost envelope for the participant to return the two measurement forms and the completed lifestyle questionnaire to the study team. The GP pack contained a GP/practice nurse information letter, BP, height and weight standard operating procedure (SOP), a measurement form, and a stamped envelope for the practice to claim any charges for the measurements (up to £15 per participant). Participants were asked to make an appointment with their practice requesting to have the measurements done as part of the study and to take the measurement form with them for the GP/nurse to fill-in and return to the study team.

Stage 4 – Reminder letters were sent to the mothers whose children had not replied. Also letters were sent to these mothers' alternative contacts to find out if the mothers' addresses were valid and obtain new addresses if appropriate.

Stage 5 – Second reminders were sent to the mothers with prepaid reply slips to let the study team know if they have passed the study packs to their children or if they do not wish to be contacted further in relation to this specific study.

Stage 6 – Reminders were sent to the children who have consented but not completed the study.

Stage 7 – Second reminders to the children who have consented but not completed the study were sent by e-mail. A telephone contact was made for participants who have not supplied a valid e-mail address.

All the study documents described above are included in appendix 10.5.

6.4.4 Outcome measurement

6.4.4.1 Blood pressure, height and weight

Height, weight and BP were measured at each participant's general practice. A standard operating procedure for the measurement of height, weight and BP sheet was attached to the GP/practice nurse's letter, and practice staff were asked to follow it when taking the measurements. We asked for two BP measurements at least 1 minute apart. If the first and second measurement differed by more than 5 mmHg for either the systolic BP or diastolic BP, a third measurement was requested at least 1 minute after the second measurement. The mean of the 2 or 3 readings was used in all analyses. The type of the BP machine, brand name if automatic, position (sitting/standing), left or right arm, and cuff size were asked to be recorded on the measurement form.

6.4.4.2 Waist circumference

The offspring who consented to take part in the study were asked to self-measure their WC following specific instructions using a tape measure supplied in the study pack. Two measurements were requested (in inches or centimetres), and their mean used in all analyses.

6.4.5 Covariable measurement

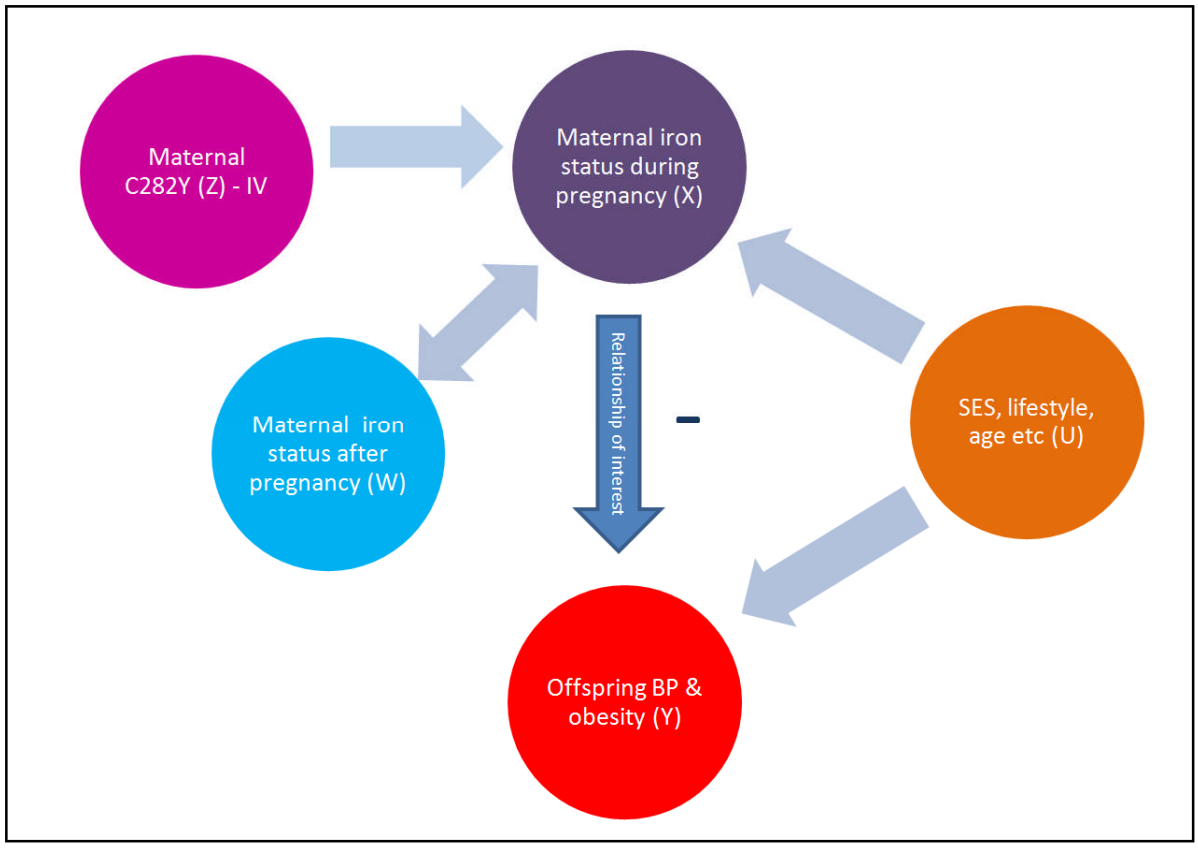
Each participant was asked to fill in a diet and lifestyle questionnaire. This included information on birth weight, siblings, educational attainment, employment, ethnic background, marital status, smoking, alcohol and caffeinated drinks use, physical activity, medical conditions, and medications consumed. The questionnaire included a 123-item FFQ as well as questions about other dietary habits including vegetarian status and salt intake.

6.4.6 The causal model

Figure 13 demonstrates the hypothesised causal relationships underlying the models tested in this study.

6.4.7 Sample size calculation

The SD for diastolic BP from the Health Survey for England 2003 is 11.6 mmHg for men aged 35-44 years and 11.0 mmHg for women of the same age group (Craig and Mindell, 2003). Using a SD of 11.3 mmHg (average), the sample size required to achieve adequate power for a range of effect sizes for a two-sample t-test at the 5% significance level to detect a difference in diastolic BP between offspring of women with the C282Y allele and offspring of women with the wild type allele was calculated using different levels of response rate (Table 29).



* The minus sign indicate a hypothesised inverse relationship

Figure 13: Causal diagram for the relationship between maternal iron status in pregnancy and BP/obesity in the offspring using IV analysis in the UKWCS – IBPS

Response rate	Number per group (n)	Minimum difference in diastolic BP in mmHg to detect at 80% power	Minimum difference in diastolic BP in mmHg to detect at 90% power
100%	1675	1.1	1.3
50%	840	1.6	1.8
30%	500	2.0	2.3
10%	160	3.6	4.1

Table 29: Sample size calculations for the UKWCS-IBPS

6.4.8 Statistical analysis

Analysis was performed using Stata, version 11 (Stata Corporation, College Station, TX). The relationship of interest was examined using both OLS regression and IV regression modelling using the 'ivreg2' command in Stata. The two modelling methods used (OLS and IV regression) are examining the same association of maternal iron status and offspring adiposity and BP outcomes, using the difference in offspring systolic BP, diastolic BP, WC and BMI per 10 ug/l greater maternal sF. Neither is testing the direct link between the maternal C282Y genotype and offspring outcomes.

Clustering of siblings (where there was more than one sibling per mum included in the study) was adjusted for using the 'robust cluster' command in Stata which computes a cluster-robust standard error of the difference in both the OLS and the IV analyses.

6.4.8.1 IV regression

We used two-stage least square (2SLS) as a method of estimation for IV regression, with maternal C282Y genotype (aa, ag or gg) as the instrument, under an additive model, and maternal ferritin as the modifiable risk factor of interest. We included systolic BP, diastolic BP, WC, and BMI, as outcomes. In the first stage of the 2SLS model, the regression of maternal ferritin on C282Y (instrument) is fitted. In the second stage, the outcome is regressed on the predicted values of maternal ferritin, the coefficient of which is the estimate of the causal effect. The standard errors of the second stage parameters are appropriately corrected to account for the uncertainty in the predicted values of the exposure from the first stage.

The first stage F-statistic is reported to provide an indicator of the instrument strength. Values of 10 and over are taken to be sufficient to exclude weak identification using the specified instrument (maternal C282Y) (Staiger and Stock, 1997). The weak identification F statistic we used for the IV regression in the case of specification of 'robust cluster' in the model is based on the Kleibergen–Paap *rk* statistic, the null hypothesis being that the estimator is weakly identified by the instrument (Baum et al., 2007). Rejecting the null hypothesis indicates the instrument has sufficient strength (Baum et al., 2007).

6.4.8.2 OLS regression

The exposure in these analyses was maternal sF and the main outcomes were offspring BP, MI and WC. Child age, maternal age (at phase II of the UKWCS) and child gender were considered confounders and adjusted for in the multivariable OLS models. In addition, maternal BMI (at phase II) and maternal social class (as classified according to the three category National Statistics Socio-economic Classification) were considered confounders and adjusted for in the multivariable OLS models with offspring BMI and WC as outcomes. Other potential confounding factors were tested for association with the exposure and offspring's BP and adiposity, and were included in the multivariable OLS model if they showed a statistically significant relationship with both.

6.4.8.3 The difference between the IV and the OLS estimates

The standard errors of the differences between the IV and the multivariable OLS estimates were estimated using 10,000 bootstrapped replications (Schluchter, 2008). P-values were then calculated from these standard errors based on a normal

approximation for the sampling distribution of the mean differences. The Stata code for this analysis is attached in appendix 10.5.3.

6.4.9 Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the NHS North West Research Ethical Committee (REC reference number 07/H1010/68). Written informed consent was obtained from all participants.

6.4.10 Funding

The work included in this chapter was supported by the Wellcome Trust [Grant number WT87789 to N.A.A.] The mailing and GP reimbursement costs were covered by grants from the Rural Environmental Scientific and Analytical Service (RESAS) of the Scottish Government and the European Union (EARNEST) to Professor Harry J McArdle at the Rowett Institute of Nutrition and Health, University of Aberdeen.

6.5 Results

6.5.1 Response rate

Of the original 3376 offspring, 517 (17%) consented to take part, and 348 offspring of 277 mothers completed the study (10% of the invited sample, 61% of those who consented). 170 (49%) were children of C282Y mutation genotype mothers, of whom 12 were children of 11 homozygotes mothers (aa), and 158 were children of heterozygous mothers (ag).

6.5.2 Sample characteristics

Of those 348 who completed the study, 247 (71%) were females and 101 (29%) were males. The mean age was 41 years for women (95% CI 40, 41) and 40 years (95% CI 38, 42) for men. All participants who reported ethnicity information (n=335) described their ethnic group as white. 212 offspring had no other siblings, 117 had one sibling included in the study, 15 had 2 siblings and 4 had 3 siblings included. Four percent (n=13) had systolic, and 2% (n=7) had diastolic hypertension, according to the WHO cut-off points of 140/90 mmHg (Whitworth, 2003). Around 10% of participants had BMI equal to or more than 30 kg/m^2. This is lower than the Health Survey for obesity estimate of 25% (Health Survey for England (HSE), 2010). Fifty eight percent of participants reported having professional or senior management jobs. The mean BMI was 24, and the mean BP was 117/73 mmHg.

The characteristics and measurements of the study participants are described in table 30 and table 31.

	Number	Percentage	95% CI
Body mass index (kg/m^2) (n=335)			
<25	214	63.9	58.5, 69.0
25-29	88	26.3	21.6, 31.3
≥30	33	9.9	6.9, 13.6
Smoking (n=340)			
Current smoker	33	9.7	6.8, 1.3
Ex-smoker	106	31.2	26.3, 36.4
Never smoked	201	59.1	53.7, 64.4
Alcohol intake (n=339)			
Regular	207	61.1	55.6, 66.3
Once/week	46	13.6	10.1, 17.7
Occasional / never	86	25.4	20.8, 30.4
Marital status (n=333)			
Married	105	55.6	50.0, 60.9
Single	115	34.5	29.4, 39.9
Divorced/separated/widowed	33	9.9	6.9, 13.6
Vegetarian (self-reported) (n=332)	44	13.3	10.0, 17.4
Hypertension (self-reported, n=340)	21	6.2	3.9, 9.3
Systolic blood pressure >140 mmHg (n=336)	13	3.9	2.1, 6.5
Diastolic blood pressure >90 mmHg (n=336)	7	2.1	0.8, 4.2
Any exercise during the week			
Vigorous (n=332)	197	59.5	53.8, 64.7
Moderate (n=337)	259	76.6	72.0, 81.3
Light (n=339)	315	92.9	89.6, 95.4
Self-reported occupation (n=286)			
Professional/senior managers	165	57.7	51.7, 63.5
Intermediate/technical	54	18.9	14.5, 24.1
Routine/semi-routine manual	15	5.2	3.0, 8.5
Self-employed	41	14.3	10.5, 18.9
Student	9	3.2	1.4, 5.9
Unemployed	2	0.7	0.08, 2.5
Self-reported low birth weight (<2500g) (n=230)	15	6.5	3.7, 10.5

Table 30: UKWCS-IBPS offspring's characteristics by sex

	Mean	SD	95% CI	Median
Height (cm) (n=335)	169	9.8	168, 170	168
F	165	7.0	164, 166	165
M	180	8.1	178, 181	179
Weight (kg) (n=338)	70	14.3	69, 72	67
F	66	12.1	78, 84	64
M	81	13.3	64, 67	80
BMI (kg/m$^{2)}$ (n=335)	24.3	4.2	23.9, 24.8	23.6
F	24.0	4.3	23.5, 24.6	23.3
M	25.0	3.9	24.2, 25.8	24.6
Systolic BP (mmHg) (n=336)	117	13.6	115, 118	116
F	114	12.6	112, 115	113
M	124	13.5	121, 126	123
Diastolic BP (mmHg) (n=336)	73	9.2	72, 74	72
F	71	8.7	70, 73	71
M	76	9.7	74, 78	76
Waist circumference (cm) (n=341)	83	12.4	82, 85	81
F	81	11.3	79, 82	78
M	90	12.7	87, 92	90
Hip circumference (cm) (n=342)	99	10.1	98, 100	99
F	99	10.1	98, 100	98
M	99	10.1	97, 101	100
Waist/hip ratio (n=341)	0.84	0.09	0.83, 0.85	0.83
F	0.81	0.07	0.81, 0.82	0.81
M	0.91	0.11	0.89, 0.93	0.91

Table 31: UKWCS-IBPS offspring's measurements

6.5.3 Differences between genotype groups

There were no statistically significant differences between offspring of C282Y mutation mothers (aa & ag) and those of wild-type (gg) mothers in age, gender, smoking, marital status, reported LBW, vegetarian status, frequency of takeaway foods, frequency of exercise, occupational classification (as a measure of socioeconomic status), systolic and diastolic hypertension, systolic BP, BMI category, BMI, and WC (adjusting for clustering of siblings within families). Figure 14 shows the average offspring BP, WC and BMI per each maternal genotype. There was a positive relationship between diastolic BP in females and maternal C282Y genotype (change 2.5 mmHg, 95% CI 0.3, 4.8, P=0.03). There was a statistically significant interaction between gender and maternal genotype on offspring's diastolic BP (P=0.05).

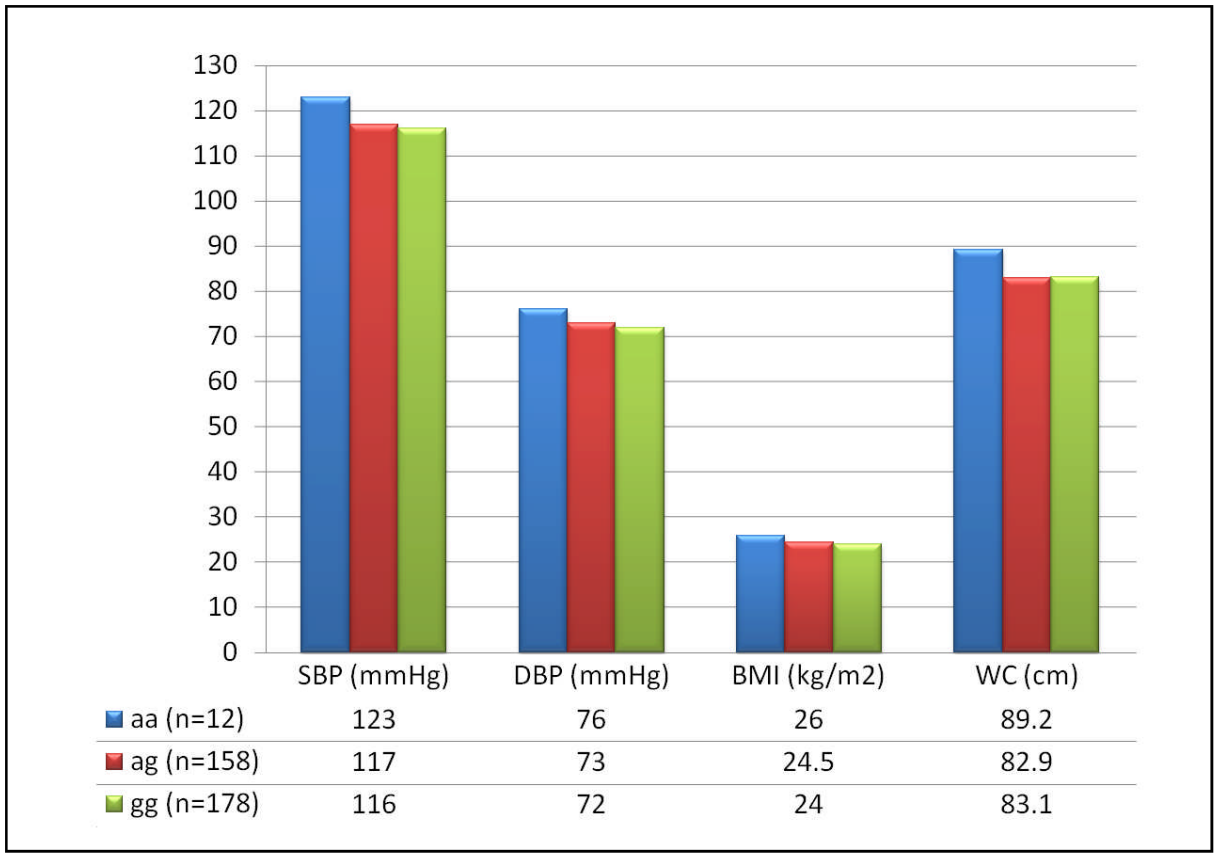


Figure 14: Mean offspring systolic and diastolic blood pressure (SBP & DBP), body mass index (BMI) and waist circumference (WC) per maternal genotype

6.5.4 Associations of the modifiable risk factor (maternal ferritin) versus the IV (maternal C282Y) with potential confounders

Maternal sF was associated with maternal age, offspring age, maternal and offspring vegetarian status, whereas there was no statistical evidence that maternal C282Y genotype was associated with any of these factors (Table 32). Maternal vegetarian status was significantly linked to both maternal ferritin and offspring DBP, and therefore was additionally entered in the multivariable OLS model with DBP as the outcome.

	Maternal Ferritin	Maternal C282Y
Maternal age	<0.0001	0.9
Offspring age	<0.0001	0.9
Offspring gender	0.3	0.4
Maternal vegetarian status	<0.0001	0.8
Offspring vegetarian status	0.003	0.9
Maternal smoking	0.1	0.1
Maternal social class	0.5	0.9
Maternal body mass index	0.2	0.8

Table 32: Univariable P values of the association between maternal serum ferritin and maternal C282Y with potential confounders in the relationship between maternal iron status and offspring outcomes

6.5.5 Associations of the modifiable risk factor (maternal ferritin) with the IV (maternal C282Y) and study outcomes

Maternal C282Y was strongly associated with maternal sF, with a mean difference per each additional allele of 84 ug/l (95% CI 31,137, *P=0.002*).

In univariable analysis, maternal ferritin was linked to offspring's diastolic BP (0.1mmHg for every 10 ug/l increase in maternal sF, 95% C 0-.03, 0.3, *P=0.02*), offspring WC (0.2 cm for every 10 ug/l increase in maternal sF, 95% CI 0.06, 0.3, *P=0.006*), and offspring's BMI (0.05 kg/m^2 for every 10 ug/l increase in maternal sF, 95% CI 0.002, 0.1, *P=0.04*). However, these associations did not persist in the multivariable OLS model. Table 33 shows the results of associations of maternal sF with each offspring outcome from both the confounder-adjusted multivariable analyses and the IV analyses using maternal C282Y as the instrument. The first stage F statistic for the strength of the instrument in the IV analysis was 10, and the Kleibergen-Paap *rk* LM P-value = 0.009 for all outcomes considered. There was no relationship between maternal sF and offspring BP, WC or BMI using both regression methods. There was also no significant difference between the OLS and the IV analyses regression coefficients for any of the outcomes considered.

Outcome	OLS* mean difference in outcome per 10 ug/l greater maternal ferritin (95% CI)	IV** regression mean difference in outcome per 10 ug/l greater maternal ferritin (95% CI)	P value*** For the difference in regression coefficients
Systolic BP (mmHg)	0.03 (-0.1, 0.1)	0.2 (-0.2, 0.6)	0.5
Diastolic BP (mmHg)	0.04 (-0.07, 0.2)	0.2 (-0.07, 0.5)	0.6
Body mass index (kg/m^2)	0.02 (-0.02, 0.07)	0.1 (-0.03, 0.2)	0.5
Waist circumference (cm)	0.1 (0, 0.2)	0.07 (-0.3, 0.5)	0.9

*Multivariable ordinary least squares models adjusted for maternal age, child age and gender in all models, and additionally for maternal vegetarian status in the diastolic BP model, and maternal BMI and social class (NS-SEC) in the BMI and WC models.

**Instrumental variable two stage least squares (2SLS), first stage F-statistic =10

*** Derived using 10,000 bootstrapped replications to estimate the standard errors of the differences between the IV and multivariable OLS regression estimates

Table 33: Association of maternal iron status with offspring blood pressure and adiposity measures using maternal C282Y status as an instrumental variable

6.6 Discussion

In this analysis, the association of maternal iron status with adult offspring's BP, BMI and WC was examined. Two methods were compared; confounder-adjusted multivariable analyses, and analyses where a genetic mutation (C282Y) in the *HFE* gene was used as an IV for maternal iron status. The study shows that maternal iron status was not associated with the three offspring outcomes considered in this study using either method. There was also no statistical evidence that the associations differed between these two statistical approaches. In this sample, maternal C282Y genotype was associated with ferritin level. This supports the apriori hypothesis that *HFE* C282Y could be used as an IV for iron status.

6.6.1 Previous studies

There are no previous studies examining the relationship of maternal iron status with adult offspring BP and adiposity using either approach (OLS or MR). Previous studies have examined the relationship of maternal iron intake and Hb levels in pregnancy with children's BP (Brion et al., 2008, Belfort et al., 2008). Belfort et al. reported a positive, rather than an inverse, association between maternal iron intake in pregnancy and child systolic BP at 3 years (Belfort et al., 2008), while Brion et al. found no association (Brion et al., 2008). The findings of these studies are discussed in more detail earlier in this thesis in section 2.3.3.

6.6.2 Study strengths

A key strength of this study is that it is the first to examine the association of maternal iron status with adult offspring BP and adiposity in a human population. Thus, the

observations from previous animal work (Gambling et al., 2003b, Gambling et al., 2003a, Crowe et al., 1995, Gambling et al., 2004b) were tested in a human population. Maternal genotype was robustly measured and ascertained, and the main outcomes measures of offspring BP and BMI were objectively measured by the general practitioner or the practice nurse following a standard operating procedure.

Maternal sF was used as a measure of iron status, as there is evidence that ferritin alone provides a good approximation of total body iron reserves, as validated by R/F ratio in people with un-elevated C-reactive protein (Yang et al., 2008). Although there is a possibility this approximation may vary in pregnant women.

I have tried to examine the association using two statistical approaches with different underlying assumptions for assessing causality that complement each other. The multivariable approach assumes that potential confounding factors are accounted for, correctly measured and modelled and that there is no possibility of reverse causality. In this case, reverse causality is unlikely (i.e. we cannot see how offspring outcomes could influence the mothers iron status). However, residual confounding is still possible with this approach.

6.6.3 Study limitations

6.6.3.1 Measurement of maternal iron status

This study did not have information on maternal iron intake during pregnancy. We have also used maternal sF concentrations assessed many years after they were pregnant with the offspring, and are therefore assuming in these analyses that this is a reasonable proxy for maternal iron status in pregnancy. Any potential measurement

error because of this is likely to be non-differential with respect to the offspring outcomes that we have assessed here, and therefore the expectation would be that results would be biased towards the null. Thus, this measurement error might explain the null multivariable observations.

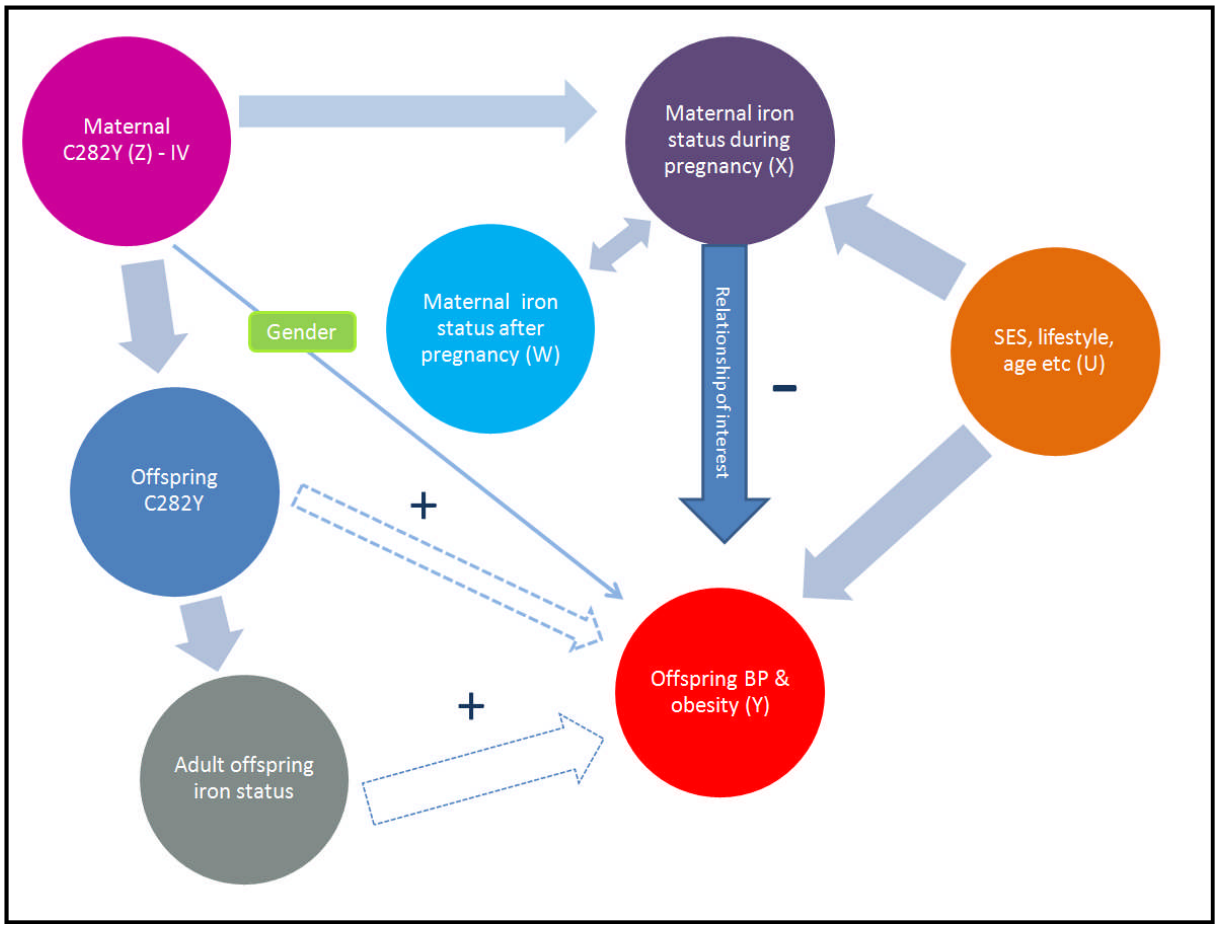
For the IV analyses, using maternal iron concentrations outside of pregnancy is not a major limitation as the genetic variant will have resulted in subtle differences in iron status throughout the woman's lifecourse including during pregnancy. This approach is also less influenced by residual confounding. However, the IV analysis assumes that the instrument is associated with the risk factor of interest, which we know is the case from previous studies and which we have demonstrated here, though the first stage F-statistic of 10, suggests that the instrument's strength to identify the estimator (maternal ferritin) is borderline (Staiger and Stock, 1997). This would potentially bias the IV analysis towards the null results seen in the multivariable analysis. Using another measure of iron stores during pregnancy which is not affected by inflammation, such as R/F ratio, could result in strengthening the genetic instrument.

6.6.3.2 Violation of IV analysis assumptions

It is further assumed that the IV (here C282Y mutation in the *HFE* gene) is not associated with any measured or unmeasured confounding i.e. is naturally randomised in the population. This is confirmed by testing the association between maternal C282Y and selected measured factors in our data as shown in table 32. However, one of the conditions of IV analysis is that the instrument should not be related to the outcome except through the exposure of interest (Z related to Y only through X). The fact that maternal genotype is linked to offspring genotype may constitute a violation to this

assumption. Figure 15 shows the causal diagram for the relationships in this study with this possibility taken into account. Such violation would be the case in all trans-generational studies that attempt to use a MR design. Statistical simulation work may be needed to simulate the genotype possibilities for the offspring and take them into account in the analysis. The ideal way to tackle this problem would be to measure offspring genotype and adjust for it. Hence, one of the main limitations in this study is that offspring genotype was not measured.

There is some evidence linking C282Y heterozygotes with an increased risk of CVD (Tuomainen et al., 1999, Rasmussen et al., 2001). . However, other studies show no association (Gunn et al., 2004, Ellervik et al., 2005). If there is a true positive relationship between offspring genotype and their BP, this may counteract an inverse relationship between maternal genotype (used as a proxy for maternal iron status here) and offspring's BP, potentially leading to a spurious non-association in the relationship of interest. However, it could be argued that it is the iron status at the time of gestation that is the contributing factor to cardiovascular risk, and not the phenotype of the fetus.



The minus sign indicate a hypothesised inverse relationship, and the plus sign indicate a hypothesised positive relationship

Figure 15: Causal diagram with the role of offspring genotype and iron status included - the UKWCS - IBPS

6.6.3.3 Response rate

The study's response rate of 10% meant that the study had a smaller sample size than initially planned. MR studies require sample sizes larger than classical epidemiological studies due to reduced precision of the estimates as genotype usually only explains a proportion of the variation in the environmental exposure of interest. Also over half of the offspring sample reported having professional/senior manager occupations, suggesting potential selection bias with responders being more likely to be health conscious with a lower prevalence of cardiovascular risk compared to the general

population. This is supported by the low prevalence of obesity in the study sample. Therefore, the results may not be generalisable to the general UK population.

The relatively poor response rate may be due to the indirect recruitment method used, as mothers were asked to pass on the study information to their children in paper format, which potentially involved posting it to them. Therefore, loss to follow up occurred at two levels in this study: mother and child. Some of the mothers were not contactable through their postal address, and the UKWCS database did not include a comprehensive list of participants' electronic contact addresses. However, every effort was made to achieve a good response rate by sending reminder letters to mothers whose children have not replied, as well as attempting to obtain the most recent addresses by sending letters to mothers' alternative contacts. Reminders were also sent to the children who have consented but not completed the study by post, e-mail or telephone contact.

Future MR studies should consider recruitment methods where the offspring are directly contacted and invited to take part. However, careful consideration to ethical issues implied in potentially sharing confidential maternal information (genotype) should be given. Also consent must be sought from both mother and child regarding mothers passing on contact information for their children to the research team.

The task of visiting the GP for the offspring which was involved in the study is likely to have also played a part in reducing the study's response rate, as over one third of the offspring who consented to take part, did not send their measurements. Some of these participants contacted the study team and apologised for not completing the study

due to being too busy to obtain a suitable time and date for a GP appointment due to work commitments.

6.7 **Conclusion**

In this chapter, there was no evidence of association between maternal iron status and adult offspring's BP and adiposity using both multivariable OLS regression and IV modelling with maternal C282Y mutation in the *HFE* gene as the instrument. However, the relatively small study sample for a MR design and the lack of measurement of the offspring C282Y genotype in order to adjust for any possible effect on the relationship of interest are likely to push the study results towards accepting the null hypothesis. Therefore, replication of findings in MR studies that avoid these limitations should be considered.

7 General discussion

7.1 Chapter summary

Chapters 3, 4, 5 and 6 contain discussion sections for the individual studies included in this thesis which include strengths, weaknesses and implications of findings for each study. However, in this chapter I attempt to pull all the previous chapters' findings together and further discuss their implications. I start with summarising the results across the four studies by type of exposure and exposure-outcome associations. This is followed by reviewing the main strengths and limitations of this thesis as a whole. I then discuss how the results from this thesis compare with findings of experimental animal studies described earlier in section 2.3.1. The findings relating to maternal Hb and iron intake from diet and supplements during pregnancy are further commented on in this chapter in relation to possible underlying mechanisms and relevance to practice. This leads to the general conclusion of this thesis and recommendations for further research and practice.

7.2 Summary of thesis findings

7.2.1 Maternal iron status indicators in pregnancy

I have shown in section 5.5.1, with data in the 1990s from ALSPAC based in the South West of England, that 90% of women had dietary iron intake below the UK RNI (14.8 mg/day) and a quarter below the UK LRNI (8 mg/day). A decade later in the CARE study in Leeds (section 3.5.3.1), this was much the same despite using different dietary assessment methods in the two studies, with 80% of women having daily intakes in early pregnancy below the UK RNI for iron, and nearly a quarter below the UK LRNI.

A considerable proportion of pregnant women reported taking iron supplements during pregnancy despite the lack of a national recommendation for routine iron supplements in the UK. In ALSPAC, 45% of women reported taking iron supplements before 32 weeks gestation. In the CARE study, 24% of women reported taking iron-containing supplements in the first trimester, 15% in the second trimester and 8% in the third trimester. In Baby VIP, conducted 10 years after CARE and 20 years after ALSPAC with supplement data extracted from the medical notes rather than self-reported, only 2% of women took iron supplements in the first trimester, 19% in the second trimester, and 13% in the third trimester. Out of those with iron depletion in the first trimester, only 58% took iron supplements at any stage in their pregnancy.

In the Baby VIP study, 23% of women had iron depletion in the first trimester as defined by WHO cut-off (sF <15 ug/l), and 80% had a sF of less than 70 ug/l, which is considered by some experts as the threshold above which there is no benefit of recommending iron supplements (Milman, 2006b). In this study, 5% of women were

anaemic (Hb <11 g/dl) in the first half of pregnancy, while 14% were anaemic (Hb <10.5 g/l) in the second half. In the CARE study, 3% of women were anaemic at 12 weeks, compared to 23% at 28 weeks gestation. In ALSPAC, 4% were anaemic by 18 weeks gestation.

7.2.2 Exposure-outcome associations

It is important to note that all the research studies included in this thesis are of observational study design, therefore causality cannot be inferred from any of the associations observed. However, this type of study design may be the best available for most associations examined in this thesis. Conducting RCTs to test the hypothesis of interest can be considered unethical particularly concerning the exposures of maternal dietary iron intake and ID in pregnancy.

In the CARE study, total iron intake from diet and supplements in early pregnancy was positively associated with birth weight centile, though the intake of the well-absorbed form of dietary iron, haem iron, was not. The potential explanation for this lack of association was discussed in sections 3.6.1.1 and 3.6.3.3. In this study, maternal dietary supplement intake at any stage in pregnancy was not associated with birth weight centile/SGA. However, MVM supplements intake in the third trimester of pregnancy, most of which contained iron, was associated with around a 3-fold increase in the risk of preterm birth. This finding can be due to residual confounding as discussed in section 3.6.2.1. Residual confounding may have also played a part in the observed association between maternal iron supplement intake before 32 weeks gestation and lower offspring systolic BP at 10 years in ALSPAC, as discussed in section 5.6.1. 1. In this study, maternal dietary iron intake assessed at 32 weeks gestation was

not associated with offspring cardiovascular indicators at 10 years (PWV, FMD. BP and BMI).

In the Baby VIP study, maternal anaemia in the first half of pregnancy was associated with increased infant bfPWV at 2-6 weeks. However, no association was observed between infant arterial stiffness and maternal iron depletion in early pregnancy. The implications of these findings were further discussed in section 4.6.2.1. Maternal ID was associated with increased risk of having a SGA baby, so was maternal anaemia (Hb <11 g/dl) in the first half of pregnancy. In contrast, Hb at 28 weeks gestation was positively associated with increased risk of SGA in the CARE study. However, associations between maternal Hb/anaemia during pregnancy were not observed later in life with offspring cardiovascular indicators at 10 years (PWV, FMD, BP and BMI). There was no evidence of association in this thesis between maternal iron status/depletion in early pregnancy (measured by sF and sTfR) and preterm birth/gestational age/birth weight centile. However, there are other studies which have found such associations as discussed earlier in section 2.2.5.

The association between maternal iron status and CVD risk indicators in the offspring was investigated in the UKWCS-IBPS element of this thesis. There was no evidence of association between Maternal sF and adult offspring BP/BMI/WC both using OLS and IV modelling with C282Y genotype. This genotype was shown to be associated with maternal sF in this dataset, justifying its utilisation as an IV. This was the first study to my knowledge examining the association of maternal iron status with adult offspring BP and adiposity in a human population using a MR design. The design of this study is innovative and the offspring outcomes are measured in adulthood, however

awareness of its limitations is essential before making solid conclusions about the lack of association between the exposure and outcomes of interest. These limitations with suggestions to improve the design in future studies were discussed in detail in section 6.6.3.

7.3 General strengths and limitations

7.3.1 Study design

All the four studies included in this thesis are of observational cohort study design. All the exposures were assessed before the occurrence of outcomes, therefore minimising the risk of bias incurred when the exposures are assessed after the assessment of outcomes. Three of the studies were of prospective cohort design (ALSPAC, CARE and UKWCS), while the Baby VIP study was of historical cohort design, where maternal iron status was assessed from samples collected in the first trimester of pregnancy and retrieved after recruitment at birth.

It is unethical to deprive participants of iron to induce ID before or during pregnancy. It can also be considered difficult to randomise them not to have supplements if they are routinely recommended in pregnancy as is the case in most countries, and following international guidelines such as the WHO's. However, in the UK routine iron supplementation in pregnancy is not recommended, therefore the potential is there for a RCT and a follow-up of a RCT to examine the effect of iron supplements on long term offspring cardiovascular risk indicators. Also RCT design could be implemented to compare the benefit of supplements versus dietary interventions in maximising iron intake and absorption during pregnancy.

7.3.2 Study sample

The collective sample size for all the studies investigated in this thesis was 4942 participants (CARE=1274, Baby VIP=362, ALSPAC=2958, UKWCS=348). Statistical power calculations were performed based on clinically-important associations as reported in the chapters' methods sections. However, for novel outcome measures such as infant PWV used in the Baby VIP study, a clinically or epidemiologically important effect size is still unknown. Therefore, if smaller differences are clinically important, in terms of predicting long-term health outcomes, then the current study will be underpowered to detect such associations and a larger study could be justified. Also, the response rate to recruitment and rate of study completion was relatively poor in some of the studies e.g. 20% in the CARE study and 10% in the UKWCS-IBPS. This limitation and its potential reasons is discussed further in section 6.6.3.3.

7.3.3 Exposure measures

In the UKWCS-IBPS, maternal genotype was robustly measured and ascertained. Maternal iron status in pregnancy was also assessed objectively using biomarkers including sF, sTfR and Hb. The best measure of body iron, R/F ratio, was used to assess maternal iron status in the Baby VIP study (Zimmermann, 2008). However, this was not feasible in all of the included studies. Although data on Hb levels was available in CARE, ALSPAC and Baby VIP, the limitations of using Hb as a marker of iron status were discussed earlier in section 2.4.1.

Supplement intake was assessed via ascertainment from the medical notes in Baby VIP, a researcher-administered interview in CARE and a self-reported questionnaire in ALSPAC, raising the possibility of misreporting bias. The limitations of the methods of

assessment of supplement intake are discussed further in sections 3.6.3.3 and 5.6.4.2. Dietary iron intake was reported using commonly-used dietary assessment methods including FFQ and 24-hour dietary recall. These have their own limitations as discussed in the corresponding chapters (sections 3.6.3.3 and 5.6.4.2), mainly the potential for considerable measurement bias. This point is also discussed further below in section 7.6.

7.3.4 Outcome measures

One of the main strengths of this thesis is the inclusion of objectively-assessed outcome measures including birth weight, gestational age, PWV, FMD, BP and BMI. None of the outcomes were self-reported apart from WC in the UKWCS-IBPS, and attempts were made to minimise the chance of bias in assessing them. These included rigorously training the researchers performing the measurements and carefully following standard operating procedures if measurements were made by people outside the research team, such as GPs and practice nurses. In case of vascular and adiposity outcomes in infanthood and childhood explored in Baby VIP and ALSPAC, there is no solid evidence that these measures predict cardiovascular end-points in adulthood. Therefore, examining these associations using adult indicators of cardiovascular risk is required.

7.3.5 Using multiple epidemiological studies at different time points in the lifecourse to address the hypothesis of interest

The hypothesis of interest addressed in this thesis would ideally be addressed with one longitudinal study that spans from pregnancy to offspring's old age, with valid and

reliable exposure and outcome assessment. Such data are unavailable at present. Therefore, a pragmatic approach was adopted here to examine different aspects of the hypothesis using multiple studies which assessed the outcomes at different time points in the offspring's lifecourse. This approach has its limitations as the studies are different in their design, exposure and outcome assessment methods, study population and size. All these factors play a big role in formulating the findings and influence the generalizability of the results. Synthesising the results together, as I attempt to do in this chapter, is difficult because of the significant heterogeneity between the studies. Therefore, each chapter has been written with a stand-alone specific hypothesis which it proceeds to test. Recommendations for future research studies that would potentially advance the science relating to the topic in this thesis are presented in the next chapter (section 8.3).

7.4 How do the findings compare with the evidence from experimental animal studies?

In this thesis, the clear-cut relationship generated by animal research between maternal iron status in pregnancy and offspring cardiovascular outcomes was not established. Neither has it been established in other research investigating this relationship in population studies (Brion et al., 2008, Belfort et al., 2008), which was reviewed in sections 2.3.3 and 5.6.2. Although there was an association in the expected direction between maternal anaemia in early pregnancy and infant PWV in the Baby VIP study, only half of the anaemic women in this sample were iron deficient. This raises the possibility that maternal anaemia is potentially related to infant outcomes through pathways other than ID such as chronic tissue hypoxia and

heightened maternal and fetal stress responses (Allen, 2001). Although infant PWV was unexpectedly inversely associated with SGA, but not very SGA (<3rd birth weight centile), the results of chapter 4 back Koudsi et al. finding of a positive association between infant aortic PWV and birth weight (Koudsi et al., 2007).

The other significant relationship observed in this thesis is from the ALSPAC analysis where there was a modest inverse association between the mother taking iron supplements in pregnancy any time before 32 weeks gestation and child systolic BP at 10 years. However, the possibility of residual confounding is high in this relationship, as intake of iron supplements may not be directly dependent on iron status, as shown in findings from the Baby VIP study (section 4.5.1.4). Rather it may be an indication of health beliefs in the mothers, which are usually reflected in other lifestyle factors (Conner et al., 2001).

There are some questions generated by comparing the findings in this thesis which were derived from population studies, to those shown in experimental animal studies. These are discussed with potential explanations below.

7.4.1 Is maternal iron more important to short term compared to long term offspring outcomes?

The findings of this thesis suggest that maternal iron intake and status has a strong association with immediate offspring birth outcomes such as size at birth. Other evidence also suggest an association with other outcomes in the first few months in life such as infant ID as discussed in section 2.2.6. However, such an association may start to attenuate the further the outcomes are assessed down the offspring's

lifecourse. Therefore, the effect of ID could be real but potentially modifiable by later diet and environment exposures throughout childhood and adolescence. This is hard to examine with the available data. In ALSPAC although maternal iron intake in pregnancy was associated with child offspring dietary iron intake in childhood, testing for mediation by child dietary iron intake did not make a difference in the exposure-outcome relationships. A long term follow up of a birth cohort with detailed dietary and biomarker assessment of iron status during, and preferably before pregnancy, and measurement of a combination of offspring cardiovascular markers from birth to later in life could potentially answer this question. A long-term follow up of a RCT of iron supplements during pregnancy might also shed light on these relationships.

7.4.2 Is the range of variation in iron status/intake too narrow in humans to show an association?

Extreme ID in animals cannot be replicated in observational studies in high income countries where most participants are nutrient-replete. This can only be explored further in low income countries where pregnant populations have more severe ID spanning a long period of time before conception, or in natural human experiments such as the Dutch hunger winter (Lumey et al., 2007) and the siege of Leningrad (Stanner and Yudkin, 2001).

The offspring adverse changes observed in animal studies could be due to severe ID in the mother rather than moderate or mild. Therefore, generalizability of study results should be carefully considered when considering evidence from different parts of the world, particularly concerning the effect of iron supplements. Most supplement trials are conducted in low or middle income countries (section 2.2.8.2), while most

observational studies that assess dietary intake in detail are conducted in high income countries.

7.4.3 Are the findings masked by measurement bias in population studies?

Animal studies usually involve accurate exposure assessment as they are usually controlled intervention studies. This compares to the big potential measurement bias involved in observational population studies, particularly when it comes to dietary assessment methods from which iron intake is derived. Measurement error is profoundly associated with all of the commonly-used dietary assessment methods in population health research such as FFQs, food diaries, and interviewer-administered dietary recalls. Innovative population-level dietary assessment methods such as methods based on information and communication technology are urgently needed that combine accuracy and reliability with feasibility, acceptability and low cost to assess nutritional exposures and deliver dietary interventions on a large population scale (Alwan and Cade, 2013). Such tools are starting to be developed and tested for effectiveness both as a method of dietary exposure assessment and as a dietary intervention (Carter et al., 2012, Carter et al.,2013a, Carter et al., 2013b). These could be implemented to assess dietary intake including iron at multiple stages during pregnancy in a birth cohort.

7.5 Maternal haemoglobin in pregnancy and birth outcomes

In this thesis, low Hb in early pregnancy was associated with adverse birth outcomes, including SGA and increased infant PWV. In contrast, low Hb in the second half of pregnancy was associated with favourable SGA risk. These findings go in line with the U-shaped relationship between maternal Hb and birth outcomes reported in other studies. This relationship was further discussed earlier in sections 2.2.4 and 4.6.2.2.1 of this thesis. In a recent meta-analysis of 12 observational studies, SGA was associated with moderate to severe (<9 g/dl), but not mild anaemia during pregnancy (<11 g/dl or <10 g/dl) (Kozuki et al., 2012).

A major problem in interpreting Hb values and how they relate to maternal iron status is the physiological process of plasma volume expansion in pregnancy. This leads to a fall in Hb level which obscures the typical relationship between ID and Hb. Not only that, it also makes it difficult to interpret plasma-based indicators of ID including sF due to plasma dilution. Also, the point at which Hb is assessed is of utmost importance as plasma volume and cell mass change in the different stages of pregnancy (Rasmussen, 2001). Assessing Hb in early pregnancy, before the materialisation of plasma expansion, reflects iron status better than later in pregnancy. However, many studies did not take account of these important physiological changes and used 'lowest Hb in pregnancy as a main exposure in relation to birth outcomes, without indicating when it was obtained, or adjusting for gestational age (section 2.3.3).

Although in the Baby VIP study, it appeared that the association between maternal ID and SGA was mediated by maternal Hb level, there could be another pathway of association between anaemia in pregnancy and adverse offspring outcomes other than ID, such as other nutrient deficiencies. Also, low Hb levels restrict oxygen circulation in the body, creating an environment of oxidative stress or chronic hypoxia, which could then cause fetal growth restriction (Kozuki et al., 2012).

7.6 Dietary iron intake during pregnancy

It has been shown in this thesis, including relatively large samples of British women from Leeds and Bristol, that iron intake in pregnancy in the UK is considerably lower than recommended. 80-90% of women had intakes less than the UK RNI and 25% less than the UK LRNI based on the CARE and the ALSPAC study samples. Vegetarians were less likely to report inadequate intakes of iron in their diet, but their net iron absorption could be less than meat eaters as discussed earlier in section 2.2.7.1. There is no focus in current UK antenatal care on providing specific dietary advice to maximize iron intake from food during pregnancy. Research into providing antenatal advice of how to ensure adequate iron intake during pregnancy using a public health approach is needed.

7.6.1 The meat paradox

There is some evidence that high red meat intake in relation to carbohydrate intake during late pregnancy has a possible negative influence from the developmental origins of disease angle, including higher offspring BP and cortisol levels (Herrick et al., 2003, Shiell et al., 2001, Godfrey et al., 1994). However, red meat also provides in

abundance the well-absorbed type of iron, haem iron. Therefore, advice to pregnant women about consumption needs to be tailored to this specific group of population according to a risk assessment based on the available evidence. Although the UK Scientific Advisory Committee on Nutrition (SACN) advise limiting red meat intake in adults in general to reduce the risk of colorectal cancer (Scientific Advisory Committee on Nutrition, 2010), recommending meat as the source of haem iron during the limited span of pregnancy is unlikely to have adverse effects in relation to lifetime risk of colorectal cancer given the available research evidence. Since iron intake seems to be of most importance in early pregnancy, meat intake can specifically be encouraged in moderate amounts during that period. Alternatively, small amounts of meat can be recommended to consume with sources of non-haem iron to aid its absorption (Skikne and Baynes, 1994).

7.7 Supplement intake during pregnancy

7.7.1 Multivitamin-mineral supplements

Analysis included in this thesis in chapter 3 demonstrates no evidence of association between taking daily MVM supplements during any stage in pregnancy and birth weight, SGA, or large for gestational age at any stage in pregnancy. However, taking MVM supplements in the third trimester was associated with an increased risk of preterm birth, with this effect being more pronounced in primiparous women.

Interactions between micronutrients in the same supplement or in different supplements may provide a possible explanation to any adverse associations such as the one observed in the CARE study in relation to preterm birth. Significant interaction

may decrease the bioavailability of micronutrients and their transfer across the placenta. For example, copper overload induces iron overload, by interfering with the iron regulatory mechanism, and iron interacts with zinc affecting absorption (Fosset et al., 2009, Gambling et al., 2008, Kelleher and Lönnerdal, 2006). A reduction in availability of micronutrients to the fetus, by interactions between the nutrients at maternal gut, liver or in the placenta itself, may result in adverse outcomes for the baby, or ineffective interventions at best. There is very limited evidence regarding the ideal doses of the micronutrients in the supplements that would prevent such undesirable effects. Preparations used in different studies are heterogeneous in type, ingredients and dosage. Another possible explanation for the conflicting evidence is the heterogeneity in the period of administration of MVM during pregnancy, as need and utilization of MVM may be substantially different in late compared to early pregnancy.

Clinicians and midwives in countries where gross multiple micronutrient deficiencies are not common should be cautious when recommending over-the-counter MVM supplements to nutrient-replete women. As in any clinical situation, they should weigh the potential risks and benefits when considering prescribing such supplements. It may be more effective for the type of supplement recommended/prescribed to be more focused on the specific vitamin/mineral deficiency the woman has. However, this raises the question whether screening for micronutrient deficiencies during pregnancy is a feasible and cost-effective option and there is little research examining this question.

Rigorous research is still needed to assess if routine MVM supplementation during pregnancy is required. There is no solid evidence that MVM supplementations have additional benefits over iron and folate alone in relation to the risk of infant mortality, though there is evidence of reducing the risk of LBW/SGA (Haider et al., 2011). There is no solid evidence to support the routine use of MVM supplements in developed country settings to improve infant outcomes. The WHO recommends further studies evaluating the effect of different combinations and dosages of the different micronutrients in the supplements (Lumbiganon, 2007).

7.7.2 Iron supplements

In the Baby VIP study, out of those with iron depletion in the first trimester (sF <15 ug/l), only 58% had iron supplements during their pregnancy, compared to 81% of anaemic women in the first half of pregnancy, and 83% of anaemic women in the second half of pregnancy. Data from relatively big national samples of pregnant women in the UK are needed to confirm these findings and provide national estimates of the prevalence of ID in pregnancy. If they are confirmed then selective iron supplementation during early pregnancy based on sF levels could be an option to consider for routine UK antenatal policy. However, this would be considered as an antenatal screening programme for ID and will need to be assessed for clinical and cost effectiveness as well as meeting the criteria of the UK National Screening Committee (www.screening.nhs.uk/criteria).

7.7.2.1 Why are some iron depleted women not receiving iron supplements?

In the UK, all pregnant women are screened for anaemia at their antenatal booking appointment at the end of the first trimester. However unless the woman is anaemic or the community midwife decides to test for ID by measuring sF, ID is not detected in routine antenatal check-up at this stage in pregnancy. Some practices with high proportion of registered population from ethnic minorities screen for ID at this stage, however this is not routine practice, and the evidence shows that ID is still commonly prevalent in women of Caucasian origin (Pavord et al., 2011). Pregnant women may benefit from screening for ID earlier in their pregnancy at around 8-10 weeks gestation, as the critical window of intervention to reverse adverse offspring effects has been identified in the first trimester by animal studies (McArdle et al., 2006).

The other possible explanation for the fact that ID women are not replenished with supplements is that iron supplements could be recommended and started in these women but not tolerated, especially in high doses, due to side effects. Constipation, nausea, vomiting and diarrhoea are commonly experienced side effects of iron supplements. Some have suggested that intermittent rather than daily supplementation may tackle this problem (Beaton and McCabe, 1999). Another option is trying a tailored dietary intervention to increase iron intake and absorption from the diet which could potentially help increase iron intake and absorption on its own, or in addition to tolerable doses of supplements. This needs to be tested using a RCT study design.

8 Conclusion

8.1 What is new?

This thesis is very relevant to the hypothesis of the developmental origins of health and disease, particularly the role of maternal diet and nutritional status during pregnancy in influencing CVD risk in the offspring later in life. Although, the importance of fetal nutrition on lifelong health was clearly emphasised in a British Medical Association report published in 2009, the gap in the current evidence around the effect of specific nutritional deficiencies on long term health outcomes was pointed out (Hanson et al., 2009). This thesis specifically attempts to address this gap in relation to maternal iron. Promoting a healthy diet during pregnancy to optimise short and long term birth outcomes is essential to good antenatal care, and this can only be achieved by conducting well designed investigations which would inform clinical and public health guidance in this field.

The novelty of the epidemiological research methodology used in this thesis is considered in detail earlier in section 2.6.1 of the background chapter. Each of the four studies included in this thesis stand-alone independently, as well as collectively addressing a common hypothesis generated from experimental animal studies which is very relevant to clinical and public health care globally. In relation to other population studies in the field of the developmental origins of health and disease, this thesis takes an innovative angle of looking at a single micronutrient level during pregnancy at a population level to assess its association with long-term offspring indicators, as well as the conventional measures of immediate birth outcomes such as birth weight. The

measurement of arterial stiffness in infancy and childhood using the non-invasive technique of PWV used in chapters 4 and 5 of this thesis could potentially be a promising new advancement in measuring predictors of CVD risk early in life in relation to prenatal exposures.

In terms of research findings, this thesis is the first to link infant arterial stiffness with maternal anaemia in early pregnancy. Only some of these anaemic mothers were iron deficient. This required further investigation to understand the potential biological mechanisms involved. This thesis is also the first to investigate and show no evidence of association between maternal iron status and offspring CVD indicators in adulthood in a population sample using Mendelian randomisation study design.

8.2 Implications for practice

This thesis falls in the realm of aetiological epidemiology research. Therefore, no direct recommendations for practice can be drawn from it without taking the findings further and using them to inform the design and testing of a healthcare and/or public health intervention using RCT study design. However, a couple of discussion points relevant to clinical and public health practice are considered below.

Currently in the UK, routine iron supplementation in pregnancy is not recommended. Pregnant woman are only screened for anaemia which is at the extreme end of the ID spectrum. Some argue for testing sF levels in the first trimester of pregnancy, preferably at the first antenatal booking visit (Milman, 2006b). If sF is low at that point in pregnancy indicating ID based on international cut-off values, then iron supplementation may be recommended plus dietary advice to optimise intake from

diet and maximise absorption of both non-haem iron and supplements. However, a screening approach of routine testing and selective supplementation requires solid evidence of clinical and cost effectiveness on a population level, taking into account country-specific prevalence rates of SGA and ID in women of childbearing age before it can be implemented. .

It is also important to address the issue of iron supplements' side effects and optimisation of their absorption in women who are deemed in need of them, particularly those who are anaemic in early pregnancy. Delayed-release iron preparations and intermittent oral iron supplementation are likely to lead to better overall compliance and reduction in side effects (Beard, 2000). Administering iron to the body through alternative routes such as the intravenous route has also been assessed and found to be more effective in treating maternal anaemia than the oral route (Al et al., 2005).

8.3 Implications for further research

There is much further epidemiological and interventional research needed to follow on from this thesis, based on the findings as well as evidence gaps identified in the process of designing and conducting the included studies.

Recommendations for further research studies include:

1. A systematic review and meta-analysis of observational studies to investigate the association of maternal dietary iron intake in pregnancy with birth outcomes

2. A systematic review and meta-analysis of observational studies to investigate the association between arterial stiffness at different points in the lifecourse with size at birth and preterm birth
3. A systematic review and meta-analysis of trials of dietary interventions (excluding trials of supplements alone) during pregnancy to improve birth outcomes
4. A birth cohort with valid and reliable dietary assessment at multiple points in pregnancy, preferably using easy to use and accessible innovative methods utilising information and communication technology which generate individual nutrient data in addition to dietary patterns, measuring offspring cardiovascular indicators including arterial stiffness and adiposity measures among other conventionally measured birth outcomes
5. A study aimed at standardising the different sTfR laboratory assays and providing a reliable and transferrable method to derive total body iron in pregnancy
6. A RCT to compare the effects of iron supplements alone against a dietary intervention aimed at optimisation of iron intake and absorption from the diet, with and without low dose supplements, measuring birth outcomes with follow up to assess CVD risk factors in the offspring. The intervention can be delivered using information and communication technology tools such as online websites and smartphone apps.

8.4 **Summary**

There are several important findings emerging from this thesis which examined the role of maternal iron in the developmental origins of CVD in the offspring. Total maternal iron intake in early, but not late, pregnancy was positively associated with birth weight centile. There was no evidence of association between taking iron-containing supplements in pregnancy with size at birth. However taking MVM supplements, which usually contain iron, in late pregnancy was associated with an increased risk of preterm birth. Also taking iron supplements up to 32 weeks gestation was associated with an average of 1 mmHg lower offspring systolic BP at 10 years.

Maternal anaemia and ID, defined as sF<15 ug/l, in early pregnancy was associated with an increased risk of giving birth to a SGA baby. Infant bfPWV measured at 2-6 weeks of age was found to be higher in babies of women who were anaemic in early pregnancy, but not in those who were only iron deficient without anaemia. Finally, using a Mendelian randomisation design, maternal iron status measured by sF with C282Y mutation as an IV was not found to be associated with adult offspring BP and adiposity.

9 Bibliography

AALTONEN, J., OJALA, T., LAITINEN, K., PIIRAINEN, T. J., POUSSA, T. A. & ISOLAURI, E. 2008. Evidence of infant blood pressure programming by maternal nutrition during pregnancy: a prospective randomized controlled intervention study. *J Pediatr,* 152**,** 79-84, 84 e1-2.

ADAIR, L. & DAHLY, D. 2005. Developmental determinants of blood pressure in adults. *Annu. Rev. Nutr.,* 25**,** 407-434.

ADAIR, L. S., KUZAWA, C. W. & BORJA, J. 2001. Maternal energy stores and diet composition during pregnancy program adolescent blood pressure. *Circulation,* 104**,** 1034-1039.

AGARWAL, K., AGARWAL, D. & MISHRA, K. 1991. Impact of anaemia prophylaxis in pregnancy on maternal haemoglobin, serum ferritin & birth weight. *Indian J Med Res,* 94**,** 277 - 280.

AGRAWAL, R. M. D., TRIPATHI, A. M. & AGARWAL, K. N. 1983. Cord blood haemoglobin, iron and ferritin status in maternal anaemia. *Acta Pædiatrica,* 72**,** 545-548.

AKESSON, A., BJELLERUP, P., BERGLUND, M., BREMME, K. & VAHTER, M. 1998. Serum transferrin receptor: a specific marker of iron deficiency in pregnancy. *Am J Clin Nutr,* 68**,** 1241-6.

AKIRA, M. & YOSHIYUKI, S. 2006. Placental Circulation, Fetal Growth, and Stiffness of the Abdominal Aorta in Newborn Infants. *The Journal of Pediatrics,* 148**,** 49-53.

AL, R. A., UNLUBILGIN, E., KANDEMIR, O., YALVAC, S., CAKIR, L., & HABERAL, A. 2005. Intravenous versus oral iron for treatment of anemia in pregnancy: a randomized trial. *Obstetrics & Gynecology*, 106, 1335-1340.

AL-SHOSHAN, A. A. 2007. Diet history and birth weight relationship among Saudi pregnant women. *Pakistan Journal of Medical Sciences,* 23**,** 176.

ALHASHEMI, H., COLEN, T., HIROSE, A., ALRAJAA, N., JAIN, V., SAVARD, W., STICKLAND, M., DAVIDGE, S. & HORNBERGER, L. 2013. Exploring the Relationship Between Increased Arterial Stiffness and Myocardial Hypertrophy in Infants of Diabetic Mothers. *Canadian Journal of Cardiology,* 29**,** S96-S97.

ALLEN, J., BACKSTROM, K. R., COOPER, J. A., COOPER, M. C., DETWILER, T. C., ESSEX, D. W., FRITZ, R. P., MEANS, R. T., MEIER, P. B. & PEARLMAN, S. R. 1998. Measurement of soluble transferrin receptor in serum of healthy adults. *Clinical chemistry,* 44**,** 35-39.

ALLEN, L. H. 2000. Anemia and iron deficiency: effects on pregnancy outcome. *American Journal of Clinical Nutrition,* 71**,** 1280S-4S.

ALLEN, L. H. 2001. Biological mechanisms that might underlie iron's effects on fetal growth and preterm birth. *Journal of Nutrition,* 131**,** 581S-589S.

ALWAN, N. & CADE, J. 2011. Routine iron supplementation in pregnancy: Why is the UK different? *Perspectives in Public Health,* 131**,** 207-208.

ALWAN, N., LAWLOR, D., MCARDLE, H., GREENWOOD, D. & CADE, J. 2012a. OP61 Is Maternal Iron Status Associated with offspring's Blood Pressure and Adiposity? A Mendelian Randomization Study. *Journal of Epidemiology and Community Health,* 66**,** A24.

ALWAN, N. A., CADE, J. E., GREENWOOD, D. C., DEANFIELD, J. & LAWLOR, D. A. 2014. Associations of maternal iron intake and hemoglobin in pregnancy with offspring vascular phenotypes and adiposity at age 10: Findings from the Avon Longitudinal Study of Parents and Children. *PLoS ONE,* 9(1)**,** e84684.

ALWAN, N. A., GREENWOOD, D. C., SIMPSON, N. A. B., MCARDLE, H. J. & CADE, J. E. 2010. The relationship between dietary supplement use in late pregnancy and birth outcomes: a cohort study in British women. *BJOG: An International Journal of Obstetrics & Gynaecology,* 117**,** 821-829.

ALWAN, N. A., GREENWOOD, D. C., SIMPSON, N. A. B., MCARDLE, H. J., GODFREY, K. M. & CADE, J. E. 2011. Dietary iron intake during early pregnancy and birth outcomes in a cohort of British women. *Human Reproduction,* 26**,** 911-919.

ALWAN, N. A., LAWLOR, D. A., MCARDLE, H. J., GREENWOOD, D. C. & CADE, J. E. 2012b. Exploring the relationship between maternal iron status and offspring's blood pressure and adiposity: a Mendelian randomization study. *Clinical Epidemiology,* 4**,** 193.

ALWAN N. & CADE J. 2013. How can 21st century public health research overcome the long-standing frustration associated with dietary assessment? *Perspectives in Public Health,*133, 242-43.

ANDERSEN, H. S., GAMBLING, L., HOLTROP, G. & MCARDLE, H. J. 2006. Maternal iron deficiency identifies critical windows for growth and cardiovascular development in the rat postimplantation embryo. *Journal of Nutrition,* 136**,** 1171.

ANDERSEN, N., FAGT, S., GROTH, M., HARTKOPP, H., MOLLER, A., OVESEN, L. & WARMING, D. 1995. Dietary habits in Denmark. Main Results. Copenhagen: National Food Agency of Denmark.

ARANDA, N., RIBOT, B., GARCIA, E., VITERI, F. E. & ARIJA, V. 2011. Pre-pregnancy iron reserves, iron supplementation during pregnancy, and birth weight. *Early Human Development*.

ARMAH, S. M., CARRIQUIRY, A., SULLIVAN, D., COOK, J. D. & REDDY, M. B. 2013. A Complete Diet-Based Algorithm for Predicting Nonheme Iron Absorption in Adults. *The Journal of Nutrition,* 143**,** 1136-1140.

AYER, J. G., HARMER, J. A., XUAN, W., TOELLE, B., WEBB, K., ALMQVIST, C., MARKS, G. B. & CELERMAJER, D. S. 2009. Dietary supplementation with n-3 polyunsaturated fatty acids in early childhood: effects on blood pressure and arterial structure and function at age 8 y. *Am J Clin Nutr,* 90**,** 438-446.

BAKER, P. N., WHEELER, S. J., SANDERS, T. A., THOMAS, J. E., HUTCHINSON, C. J., CLARKE, K., BERRY, J. L., JONES, R. L., SEED, P. T. & POSTON, L. 2009. A prospective study of micronutrient status in adolescent pregnancy. *American Journal of Clinical Nutrition,* 89**,** 1114.

BARKER, D. J. 1995. Fetal origins of coronary heart disease. *BMJ,* 311**,** 171-4.

BARKER, D. J. 1998. *Mothers, babies and health in later life* Edinburgh, Harcourt Brace & Co Ltd.

BARKER, D. J., OSMOND, C., WINTER, P., MARGETTS, B. & SIMMONDS, S. 1989. Weight in infancy and death from ischaemic heart disease. *The Lancet,* 334**,** 577-580.

BARKER, D. J. P. 2004. The developmental origins of chronic adult disease. *Acta Paediatrica,* 93**,** 26-33.

BARKER, D. J. P., OSMOND, C., FORSEN, T. J., KAJANTIE, E. & ERIKSSON, J. G. 2007. Maternal and Social Origins of Hypertension. *Hypertension,* 50**,** 565-571.

BARRETT, J. F. R., WHITTAKER, P. G., WILLIAMS, J. G. & LIND, T. 1994. Absorption of non-haem iron from food during normal pregnancy. *British Medical Journal,* 309**,** 79.

BATY, D., TERRON KWIATKOWSKI, A., MECHAN, D., HARRIS, A., PIPPARD, M. J. & GOUDIE, D. 1998. Development of a multiplex ARMS test for mutations in the HFE gene associated with hereditary haemochromatosis. *British Medical Journal,* 51**,** 73.

BAUM, C. F., SCHAFFER, M. E. & STILLMAN, S. 2007. Enhanced routines for instrumental variables/generalized method of moments estimation and testing. *Stata Journal,* 7**,** 465-506.

BAYNES, R. D. 1996. Assessment of iron status. *Clinical biochemistry,* 29**,** 209-215.

BAYOL, S. A., FARRINGTON, S. J. & STICKLAND, N. C. 2007. A maternal 'junk food'diet in pregnancy and lactation promotes an exacerbated taste for 'junk food'and a greater propensity for obesity in rat offspring. *British Journal of Nutrition,* 98**,** 843-851.

BEARD, J. L. 1994. Iron deficiency: assessment during pregnancy and its importance in pregnant adolescents. *American Journal of Clinical Nutrition,* 59**,** 502S.

BEARD, J. L. 2008. Why iron deficiency is important in infant development. *Journal of Nutrition,* 138**,** 2534-6.

BEARD, J. L. 2000. Effectiveness and strategies of iron supplementation during pregnancy. *The American Journal of Clinical Nutrition*, 71, 1288s-1294S.

BEATON, G. H. & MCCABE, G. P. 1999. Efficacy of intermittent iron supplementation in the control of iron deficiency anaemia in developing countries. An analysis of experience: final report to the Micronutrient Initiative.

BELFORT, M. B., RIFAS-SHIMAN, S. L., RICH-EDWARDS, J. W., KLEINMAN, K. P., OKEN, E. & GILLMAN, M. W. 2008. Maternal iron intake and iron status during pregnancy and child blood pressure at age 3 years. *International Journal of Epidemiology,* 37**,** 301-8.

BELIZÁN, J. M., VILLAR, J., BERGEL, E., PINO, A. D., FULVIO, S. D., GALLIANO, S. V. & KATTAN, C. 1997. Long term effect of calcium supplementation during pregnancy on the blood pressure of offspring: follow up of a randomised controlled trial. *BMJ,* 315**,** 281-285.

BERGEL, E., HAELTERMAN, E., BELIZAN, J., VILLAR, J. & CARROLI, G. 2000. Perinatal factors associated with blood pressure during childhood. *American Journal of Epidemiology,* 151**,** 594.

BERGMANN, R. L., GRAVENS-MÜLLER, L., HERTWIG, K., HINKEL, J., ANDRES, B., BERGMANN, K. E. & DUDENHAUSEN, J. W. 2002. Iron deficiency is prevalent in a

sample of pregnant women at delivery in Germany. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 102**,** 155-160.

BEUTLER, E., FELITTI, V. J., KOZIOL, J. A., HO, N. J. & GELBART, T. 2002. Penetrance of 845G--> A (C282Y) HFE hereditary haemochromatosis mutation in the USA. *The Lancet,* 359**,** 211-218.

BINGHAM, S. A., GILL, C., WELCH, A., CASSIDY, A., RUNSWICK, S. A., OAKES, S., LUBIN, R., THURNHAM, D. I., KEY, T. J. & ROE, L. 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**,** S137.

BONAMY, A.-K. E., ANDOLF, E., MARTIN, H. & NORMAN, M. 2008. Preterm birth and carotid diameter and stiffness in childhood. *Acta Pædiatrica,* 97**,** 434-437.

BONAMY, A.-K. E., BENDITO, A. N. A., MARTIN, H., ANDOLF, E., SEDIN, G. & NORMAN, M. 2005. Preterm Birth Contributes to Increased Vascular Resistance and Higher Blood Pressure in Adolescent Girls. *Pediatric Research,* 58**,** 845-849.

BOTHWELL, T. H. 2000. Iron requirements in pregnancy and strategies to meet them. *American Journal of Clinical Nutrition,* 72**,** 257S-264S.

BOWERS, K., YEUNG, E., WILLIAMS, M. A., QI, L., TOBIAS, D. K., HU, F. B. & ZHANG, C. 2011. A Prospective Study of Prepregnancy Dietary Iron Intake and Risk for Gestational Diabetes Mellitus. *Diabetes Care,* 34**,** 1557-1563.

BOYD, A., GOLDING, J., MACLEOD, J., LAWLOR, D. A., FRASER, A., HENDERSON, J., MOLLOY, L., NESS, A., RING, S. & DAVEY SMITH, G. 2013. Cohort Profile: The 'Children of the 90s'—the index offspring of the Avon Longitudinal Study of Parents and Children. *International Journal of Epidemiology,* 42**,** 111-127.

BOYLAN, S. M., CADE, J. E., KIRK, S. F. L., GREENWOOD, D. C., WHITE, K. L. M., SHIRES, S., SIMPSON, N. A. B., WILD, C. P. & HAY, A. W. M. 2008. Assessing caffeine exposure in pregnant women. *British Journal of Nutrition,* 100**,** 875-882.

BRENNER, B. M., GARCIA, D. L. & ANDERSON, S. 1988. Glomeruli and blood pressure Less of one, more the other? *American Journal of Hypertension,* 1**,** 335-347.

BRION, M., LEARY, S., DAVEY SMITH, G., MCARDLE, H. & NESS, A. 2008. Maternal anemia, iron intake in pregnancy, and offspring blood pressure in the Avon Longitudinal Study of Parents and Children. *Am J Clin Nutr,* 88**,** 1126-33.

BRION, M. J. A., NESS, A. R., ROGERS, I., EMMETT, P., CRIBB, V., DAVEY SMITH, G. & LAWLOR, D. A. 2010. Maternal macronutrient and energy intakes in pregnancy and offspring intake at 10 y: exploring parental comparisons and prenatal effects. *American Journal of Clinical Nutrition,* 91**,** 748.

BRISE, H. 1962. Influence of meals on iron absorption in oral iron therapy. *Acta Med Scand,* 376 (Suppl 171)**,** 31-45.

BRITISH MEDICAL ASSOCIATION & ROYAL PHARMACEUTICAL SOCIETY OF GREAT BRITAIN 2010. *British National Formulary.,* London.

BRUCE, K. D. & HANSON, M. A. 2010. The developmental origins, mechanisms, and implications of metabolic syndrome. *The Journal of Nutrition,* 140**,** 648-652.

BRUSSAARD, J., BRANTS, H., BOUMAN, M. & LÖWIK, M. 1997. Iron intake and iron status among adults in the Netherlands. *European journal of clinical nutrition,* 51**,** S51.

BURKHARDT, T., MATTER, C. M., LOHMANN, C., CAI, H., LÜSCHER, T. F., ZISCH, A. H. & BEINDER, E. 2009. Decreased Umbilical Artery Compliance and IGF-I Plasma Levels in Infants with Intrauterine Growth Restriction - Implications for Fetal Programming of Hypertension. *Placenta,* 30**,** 136-141.

CADE, J. E., BURLEY, V. J. & GREENWOOD, D. C. 2004. The UK Women's Cohort Study: comparison of vegetarians, fish-eaters and meat-eaters. *Public Health Nutrition,* 7**,** 871-878.

CADE, J. E., MORETON, J. A., O'HARA, B., GREENWOOD, D. C., MOOR, J., BURLEY, V. J., KUKALIZCH, K., BISHOP, D. T. & WORWOOD, M. 2005. Diet and genetic factors associated with iron status in middle-aged women. *American Journal of Clinical Nutrition,* 82**,** 813.

CAMPBELL, D., HALL, M., BARKER, D., CROSS, J., SHIELL, A. & GODFREY, K. 1996. Diet in pregnancy and the offspring's blood pressure 40 years later. *BJOG: An International Journal of Obstetrics & Gynaecology,* 103**,** 273-280.

CARE STUDY GROUP 2008. Maternal caffeine intake during pregnancy and risk of fetal growth restriction: a large prospective observational study. *BMJ,* 337**,** a2332.

CARRIAGA, M., SKIKNE, B., FINLEY, B., CUTLER, B. & COOK, J. 1991. Serum transferrin receptor for the detection of iron deficiency in pregnancy. *Am J Clin Nutr,* 54**,** 1077-81

CARTER, M., ALWAN, N. A., BURLEY, V., WARK, P. A., EVANS, C. E., GREENWOOD, D. C., HARDIE, L. J., FROST, G. & CADE, J. E. 2012. Use of information and communication technology to improve dietary assessment and tackle obesity. *The Lancet,* 380**,** S29.

CARTER, M. C., BURLEY, V. J., NYKJAER, C., & CADE, J. E. 2013a. 'My Meal Mate'(MMM): validation of the diet measures captured on a smartphone application to facilitate weight loss. *British Journal of Nutrition*, 109, 539-546.

CARTER, M. C., BURLEY, V. J., NYKJAER, C., & CADE, J. E. 2013b. Adherence to a smartphone application for weight loss compared to website and paper diary: pilot randomized controlled trial. *Journal of medical Internet research*, 15, 4.

CASANUEVA, E., VITERI, F., MARES-GALINDO, M., MEZA-CAMACHO, C., LORIA, A. & SCHNAAS, L. & VALDÉS-RAMOS, R. 2006. Weekly iron as a safe alternative to daily supplementation for nonanemic pregnant women. *Archives of Medical Research,* 37**,** 674-82.

CASANUEVA, E. & VITERI, F. E. 2003. Iron and oxidative stress in pregnancy. *Journal of Nutrition,* 133**,** 1700S-1708S.

CATOV, J. M., BODNAR, L. M., NESS, R. B., MARKOVIC, N. & ROBERTS, J. M. 2007. Association of periconceptional multivitamin use and risk of preterm or small-for-gestational-age births. *American journal of epidemiology,* 166**,** 296-303.

CATOV, J. M., BODNAR, L. M., OLSEN, J., OLSEN, S. & NOHR, E. A. 2011. Periconceptional multivitamin use and risk of preterm or small-for-gestational-age births in the Danish National Birth Cohort. *The American Journal of Clinical Nutrition,* 94**,** 906-912.

CDC 1998. Centers for Disease Control and Prevention: Recommendations to prevent and control iron deficiency in the United States. . *Morbidity and Mortality Weekly Report,* 77**,** 1-29.

CETIN, I., BERTI, C. & CALABRESE, S. 2009. Role of micronutrients in the periconceptional period. *Human Reproduction Update*.

CHAN, A. T., MA, J., TRANAH, G. J., GIOVANNUCCI, E. L., RIFAI, N., HUNTER, D. J. & FUCHS, C. S. 2005. Hemochromatosis gene mutations, body iron stores, dietary iron,

and risk of colorectal adenoma in women. *Journal of the National Cancer Institute,* 97**,** 917.

CHAN, K. K. L., CHAN, B. C. P., LAM, K. F., TAM, S. & LAO, T. T. 2009. Iron supplement in pregnancy and development of gestational diabetes—a randomised placebo-controlled trial. *BJOG: An International Journal of Obstetrics & Gynaecology,* 116**,** 789-798.

CHANG, S.-C., O'BRIEN, K. O., NATHANSON, M. S., MANCINI, J. & WITTER, F. R. 2003. Hemoglobin Concentrations Influence Birth Outcomes in Pregnant African-American Adolescents. *The Journal of Nutrition,* 133**,** 2348-2355.

CHEUNG, Y.-F. 2010. Arterial stiffness in the young: assessment, determinants, and implications. *Korean circulation journal,* 40**,** 153-162.

CHEUNG, Y., WONG, K., LAM, B. C. & TSOI, N. 2004. Relation of arterial stiffness with gestational age and birth weight. *Archives of Disease in Childhood,* 89**,** 217-221.

CHRISTIAN, P., DARMSTADT, G., WU, L., KHATRY, S., LECLERQ, S., KATZ, J., WEST, K. J. & ADHIKARI, R. K. 2008. The effect of maternal micronutrient supplementation on early neonatal morbidity in rural Nepal: a randomised, controlled, community trial. *Arch Dis Child.,* 93**,** 660-4.

CHRISTIAN, P., KHATRY, S. K., KATZ, J., PRADHAN, E. K., LECLERQ, S. C., RAM SHRESTHA, S., ADHIKARI, R. K., SOMMER, A. & WEST, K. P. 2003. Effects of alternative maternal micronutrient supplements on low birth weight in rural Nepal: double blind randomised community trial supplements *BMJ,* 326**,** 571-.

COCKELL, K. A., MILLER, D. C. & LOWELL, H. 2009. Application of the Dietary Reference Intakes in developing a recommendation for pregnancy iron supplements in Canada. *The American Journal of Clinical Nutrition,* 90**,** 1023-10, M., PARVANTA, I., ICKES, L., YIP, R. & BRITTENHAM, G. 2003. Iron supplementation during pregnancy, anemia, and birth weight: a randomized controlled trial. *Am J Clin Nutr,* 78**,** 773 - 781.

COLLINGS, R., HARVEY, L. J., HOOPER, L., HURST, R., BROWN, T. J., ANSETT, J., KING, M. & FAIRWEATHER-TAIT, S. J. 2013. The absorption of iron from whole diets: a systematic review. *The American Journal of Clinical Nutrition,* 98**,** 65-81.

COMMISSION OF THE EUROPEAN COMMUNITIES 1993. Nutrient and energy intakes for the European Community. Reports of the Scientific Committee for Food. *Directorate-General Industry.*

CONNER, M., KIRK, S., CADE, J. & BARRETT, J. 2003. Environmental influences: factors influencing the decision to use dietary supplements in women. *J Nutrition* 133**,** 1978S-1982S.

CONNER, M., KIRK, S. F. L., CADE, J. E. & BARRETT, J. H. 2001. Why do women use dietary supplements? The use of the theory of planned behaviour to explore beliefs about their use. *Social Science & Medicine,* 52**,** 621-633.

COOK, J. D. 2005. Diagnosis and management of iron-deficiency anaemia. *Best Practice & Research Clinical Haematology,* 18**,** 319-332.

COOK, J. D., FLOWERS, C. H. & SKIKNE, B. S. 2003. The quantitative assessment of body iron. *Blood,* 101**,** 3359.

COOK, N., COHEN, J., HERBERT, P., TAYLOR, J. & HENNEKENS, C. 1995. Implications of small reductions in diastolic blood pressure for primary prevention. *Arch Intern Med,* 155**,** 701-709.

CRAIG, R. & MINDELL, J. 2003. Health Survey for England The Information Centre.

CROWE, C., DANDEKAR, P., FOX, M., DHINGRA, K., BENNET, L. & HANSON, M. 1995. The effects of anaemia on heart, placenta and body weight, and blood pressure in fetal and neonatal rats. *J. Physiol.,* 488**,** 515-519.

CROZIER, S., ROBINSON, S., GODFREY, K., COOPER, C. & INSKIP, H. 2009. Dietary patterns change little from before to during pregnancy. *Journal of Nutrition,* 139.

CRUICKSHANK, J. K., REZAILASHKAJANI, M. & GOUDOT, G. 2009. Arterial Stiffness, Fatness, and Physical Fitness: Ready for Intervention in Childhood and Across the Life Course? *Hypertension,* 53**,** 602.

DANGARDT, F., OSIKA, W., VOLKMANN, R., GAN, L.-M. & FRIBERG, P. 2008. Obese children show increased intimal wall thickness and decreased pulse wave velocity. *Clinical Physiology and Functional Imaging,* 28**,** 287-293.

DARMSTADT, G. L., BHUTTA, Z. A., COUSENS, S., ADAM, T., WALKER, N. & DE BERNIS, L. 2005. Evidence-based, cost-effective interventions: how many newborn babies can we save? *The Lancet,* 365**,** 977-988.

DAVEY SMITH, G. 2003. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *International Journal of Epidemiology,* 32**,** 1.

DAVEY SMITH, G., LAWLOR, D. A., HARBORD, R., TIMPSON, N., DAY, I. & EBRAHIM, S. 2007. Clustered environments and randomized genes: a fundamental distinction between conventional and genetic epidemiology. *PLoS Medicine,* 4**,** e352.DE

HOLLANDER, E. L., BEMELMANS, W. J., BOSHUIZEN, H. C., FRIEDRICH, N., WALLASCHOFSKI, H., GUALLAR-CASTILLÓN, P., WALTER, S., ZILLIKENS, M. C., ROSENGREN, A., LISSNER, L., BASSETT, J. K., GILES, G. G., HEIM, N., VISSER, M., DE GROOT, L. C. & COLLABORATORS, F. T. W. E. 2012. The association between waist circumference and risk of mortality considering body mass index in 65- to 74-year-olds: a meta-analysis of 29 cohorts involving more than 58 000 elderly persons. *International Journal of Epidemiology*.

DE KONING, L., MERCHANT, A. T., POGUE, J. & ANAND, S. S. 2007. Waist circumference and waist-to-hip ratio as predictors of cardiovascular events: meta-regression analysis of prospective studies. *European Heart Journal,* 28**,** 850-856.

DE UNGRIA, M., RAO, R., WOBKEN, J. D., LUCIANA, M., NELSON, C. A. & GEORGIEFF, M. K. 2000. Perinatal iron deficiency decreases cytochrome c oxidase (CytOx) activity in selected regions of neonatal rat brain. *Pediatric Research,* 48**,** 169-176.

DEPARTMENT FOR COMMUNITIES AND LOCAL GOVERNMENT. 2009. *Indices of deprivation* [Online]. London. Available: www.communities.gov.uk [Accessed 29 July 2009].

DEPARTMENT OF HEALTH 1991. Dietary Reference Values for food energy and nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy. London: Her Majesty's Stationery Office.

DOBBELSTEYN, C., JOFFRES, M., MACLEAN, D. & FLOWERDEW, G. 2001. A comparative evaluation of waist circumference, waist-to-hip ratio and body mass index as indicators of cardiovascular risk factors. The Canadian Heart Health Surveys. *International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity,* 25**,** 652.

DOMELLÖF, M., THORSDOTTIR, I. & THORSTENSEN, K. 2013. Health effects of different dietary iron intakes: A systematic literature review for the 5th Nordic Nutrition Recommendations.

DONALD, A. E., CHARAKIDA, M., FALASCHETTI, E., LAWLOR, D. A., HALCOX, J. P., GOLDING, J., HINGORANI, A. D., DAVEY SMITH, G. & DEANFIELD, J. E. 2010. Determinants of vascular phenotype in a large childhood population: the Avon Longitudinal Study of Parents and Children (ALSPAC). *European Heart Journal,* 31**,** 1502.

DONGIOVANNI, P., VALENTI, L., LUDOVICA FRACANZANI, A., GATTI, S., CAIRO, G. & FARGION, S. 2008. Iron Depletion by Deferoxamine Up-Regulates Glucose Uptake and Insulin Signaling in Hepatoma Cells and in Rat Liver. *Am J Pathol,* 172**,** 738-747.

DOWDLE, W. 1989. CDC Criteria for anemia in children and childbearing-aged women. *Morb Mortal Wkly Rep,* 38**,** 400-404.

DOYLE, W., CRAWFORD, M. A., WYNN, A. H. A. & WYNN, S. W. 1990. The association between maternal diet and birth dimensions. *Journal of Nutritional and Environmental Medicine,* 1**,** 9-17.

ELLERVIK, C., TYBJÆRG-HANSEN, A., GRANDE, P., APPLEYARD, M. & NORDESTGAARD, B. G. 2005. Hereditary Hemochromatosis and Risk of Ischemic Heart Disease. *Circulation,* 112**,** 185-193.

EZZATI, M., LOPEZ, A. D., RODGERS, A. & MURRAY, C. J. L. 2004. *Comparative quantification of health risks : Global and regional burden of disease attributable to selected major risk factors,* Geneva, World Health Organization.

FAWZI, W. W., MSAMANGA, G. I., URASSA, W., HERTZMARK, E., PETRARO, P., WILLETT, W. C. & SPIEGELMAN, D. 2007. Vitamins and Perinatal Outcomes among HIV-Negative Women in Tanzania. *N Eng J Med,* 356**,** 1423-1431.

FLAMMER, A. J., ANDERSON, T., CELERMAJER, D. S., CREAGER, M. A., DEANFIELD, J., GANZ, P., HAMBURG, N. M., LÜSCHER, T. F., SHECHTER, M. & TADDEI, S. 2012. The Assessment of Endothelial Function From Research Into Clinical Practice. *Circulation,* 126**,** 753-767.

FOOD STANDARDS AGENCY 2003. National Diet and Nutrition Survey: Adults aged 19 to 64, Volume 3 London: Food Standards Agency.

FORESIGHT REPORT 2007. *Tackling Obesity: Future Choices,* London, Government Office for Science.

FORSÉN, T., ERIKSSON, G. J., TUOMILEHTO, J., OSMOND, C., BARKER, D. J. P. & 1999. Growth in utero and during childhood among women who develop coronary heart disease: longitudinal study *BMJ,* 319**,** 1403-1407.

FOSSET, C., DANZEISEN, R., GAMBLING, L., MCGAW, B. A. & MCARDLE, H. J. 2009. Cu loading alters expression of non-IRE regulated, but not IRE regulated, Fe dependent proteins in HepG2 cells. *J Inorg Biochem,* 103**,** 709-16.

FRASER, A., MACDONALD-WALLIS, C., TILLING, K., BOYD, A., GOLDING, J., DAVEY SMITH, G., HENDERSON, J., MACLEOD, J., MOLLOY, L., NESS, A., RING, S., NELSON, S. M. & LAWLOR, D. A. 2012. Cohort Profile: The Avon Longitudinal Study of Parents and Children: ALSPAC mothers cohort. *International Journal of Epidemiology*.

FRIIS, H., GOMO, E., NYAZEMA, N., NDHLOVU, P., KRARUP, H., KAESTEL, P. & MICHAELSEN, K. F. 2004. Effect of multimicronutrient supplementation on gestational length and birth size: a randomized, placebo-controlled, double-blind effectiveness trial in Zimbabwe. *American Journal of Clinical Nutrition,* 80**,** 178-184.

GALE, C. R., ROBINSON, S. M., HARVEY, N. C., JAVAID, M. K., JIANG, B., MARTYN, C. N., GODFREY, K. M. & COOPER, C. 2007. Maternal vitamin D status during pregnancy and child outcomes. *Eur J Clin Nutr,* 62**,** 68-77.

GAMBLING, L., ANDERSEN, H. & MCARDLE, H. 2008. Iron and copper, and their interactions during development. *Biochemical Society Transactions,* 36**,** 1258-1261.

GAMBLING, L., ANDERSEN, H. S., CZOPEK, A., WOJCIAK, R., KREJPCIO, Z. & MCARDLE, H. J. 2004a. Effect of timing of iron supplementation on maternal and neonatal growth and iron status of iron-deficient pregnant rats. *The Journal of Physiology,* 561**,** 195.

GAMBLING, L., CHARANIA, Z., HANNAH, L., ANTIPATIS, C., LEA, R. G. & MCARDLE, H. J. 2002. Effect of iron deficiency on placental cytokine expression and fetal growth in the pregnant rat. *Biology of reproduction,* 66**,** 516.

GAMBLING, L., DANZEISEN, R., FOSSET, C., ANDERSEN, H. S., DUNFORD, S., SRAI, S. K. S. & MCARDLE, H. J. 2003a. Iron and copper interactions in development and the effect on pregnancy outcome. *The Journal of Nutrition,* 133**,** 1554S-1556S.

GAMBLING, L., DANZEISEN, R., GAIR, S., LEA, R. G., CHARANIA, Z., SOLANKY, N., JOORY, K. D., SRAI, S. K. & MCARDLE, H. J. 2001. Effect of iron deficiency on placental transfer of iron and expression of iron transport proteins in vivo and in vitro. *Biochemical Journal,* 356**,** 883.

GAMBLING, L., DUNFORD, S. & MCARDLE, H. J. 2004b. Iron deficiency in the pregnant rat has differential effects on maternal and fetal copper levels. *The Journal of Nutritional Biochemistry,* 15**,** 366-372.

GAMBLING, L., DUNFORD, S., WALLACE, D., ZUUR, G., SOLANKY, N., KAILA, S. & MCARDLE, H. 2003b. Iron deficiency during pregnancy affects postnatal blood pressure in the rat. *J. Physiol.,* 552**,** 603-610.

GAMBLING, L., LANG, C. & MCARDLE, H. J. 2011. Fetal regulation of iron transport during pregnancy. *The American Journal of Clinical Nutrition*.

GAMBLING, L. & MCARDLE, H. J. 2004. Iron, copper and fetal development. *Proceedings of the Nutrition Society,* 63**,** 553-562.

GARDINER, P. M., NELSON, L., SHELLHAAS, C. S., DUNLOP, A. L., LONG, R., ANDRIST, S. & JACK, B. W. 2008. The clinical content of preconception care: nutrition and dietary supplements. *American Journal of Obstetrics and Gynecology,* 199**,** S345-S356.

GARDOSI, J. 2004. Customised fetal growth standards: rationale and clinical application. *Semin Perinatol,* 28**,** 33-40.

GERSOVITZ, M., MADDEN, J. P. & SMICIKLAS-WRIGHT, H. 1978. Validity of the 24-hr. dietary recall and seven-day record for group comparisons. *Journal of the American Dietetic Association,* 73**,** 48.

GIBNEY, M. J., MARGETTS, B. M., KEARNEY, J. M. & ARAB, L. 2004. *Public Health Nutrition,* Oxford, Blackwell Publishing

GLUCKMAN, P. & HANSON, M. (eds.) 2006. *Developmental origins of health and disease,* Cambridge: University Press.

GODFREY, K., REDMAN, C., BARKER, D. & OSMOND, C. 1991. The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight. *British Journal of Obstetrics and Gynaecology,* 98**,** 886-891.

GODFREY, K., ROBINSON, S., BARKER, D. J. P., OSMOND, C. & COX, V. 1996. Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. *British Medical Journal,* 312**,** 410.

GODFREY, K. M., FORRESTER, T., BARKER, D., JACKSON, A. A., LANDMAN, J., HALL, J. S. E., COX, V. & OSMOND, C. 1994. Maternal nutritional status in pregnancy and blood pressure in childhood. *BJOG: An International Journal of Obstetrics & Gynaecology,* 101**,** 398-403.

GOLDENBERG, R. L., CULHANE, J. F., IAMS, J. D. & ROMERO, R. 2008. Epidemiology and causes of preterm birth. *The lancet,* 371**,** 75-84.

GOLDENBERG, R. L., MERCER, B. M., MIODOVNIK, M., THURNAU, G. R., MEIS, P. J., MOAWAD, A., PAUL, R. H., BOTTOMS, S. F., DAS, A. & ROBERTS, J. M. 1998. Plasma ferritin, premature rupture of membranes, and pregnancy outcome. *American journal of obstetrics and gynecology,* 179**,** 1599-1604.

GOLDENBERG, R. L., TAMURA, T., DUBARD, M., JOHNSTON, K. E., COPPER, R. L. & NEGGERS, Y. 1996. Plasma ferritin and pregnancy outcome. *American journal of obstetrics and gynecology,* 175**,** 1356-1359.

GOLUB, M. S., HOGREFE, C. E., TARANTAL, A. F., GERMANN, S. L., BEARD, J. L., GEORGIEFF, M. K., CALATRONI, A. & LOZOFF, B. 2006. Diet-induced iron deficiency anemia and pregnancy outcome in rhesus monkeys. *The American Journal of Clinical Nutrition,* 83**,** 647-656.

GRANTHAM-MCGREGOR, S. & ANI, C. 2001. A review of studies on the effect of iron deficiency on cognitive development in children. *The Journal of Nutrition,* 131**,** 649S-668S.

GREENWALD, S. E. 2002. Pulse pressure and arterial elasticity. *QJM,* 95**,** 107.

GUNN, I., MAXWELL, F., GAFFNEY, D., MCMAHON, A. & PACKARD, C. 2004. Haemochromatosis gene mutations and risk of coronary heart disease: a west of Scotland coronary prevention study (WOSCOPS) substudy. *Heart,* 90**,** 304.

GUPTA, P., RAY, M., DUA, T., RADHAKRISHNAN, G., KUMAR, R. & SACHDEV, H. P. S. 2007. Multimicronutrient Supplementation for Undernourished Pregnant Women and the Birth Size of Their Offspring: A Double-blind, Randomized, Placebo-Controlled Trial. *Arch Pediatr Adolesc Med.,* 161**,** 58-64.

HAIDER, B. & BHUTTA, Z. 2006. Multiple-micronutrient supplementation for women during pregnancy. *Cochrane Database Syst Rev. ,* 2006(4).

HAIDER, B., YAKOOB, M. & BHUTTA, Z. 2011. Effect of multiple micronutrient supplementation during pregnancy on maternal and birth outcomes. *BMC Public Health,* 11**,** S19.

HAIDER, B. A., OLOFIN, I., WANG, M., SPIEGELMAN, D., EZZATI, M., & FAWZI, W. W. 2013. Anaemia, prenatal iron use, and risk of adverse pregnancy outcomes: systematic review and meta-analysis. *BMJ: British Medical Journal*, 346.

HALCOX, J. P. J., DONALD, A. E., ELLINS, E., WITTE, D. R., SHIPLEY, M. J., BRUNNER, E. J., MARMOT, M. G. & DEANFIELD, J. E. 2009. Endothelial function predicts progression of carotid intima-media thickness. *Circulation,* 119**,** 1005-1012.

HALCOX, J. P. J., SCHENKE, W. H., ZALOS, G., MINCEMOYER, R., PRASAD, A., WACLAWIW, M. A., NOUR, K. R. A. & QUYYUMI, A. A. 2002. Prognostic value of coronary vascular endothelial dysfunction. *Circulation,* 106**,** 653-658.

HALLBERG, L. 2002. Advantages and disadvantages of an iron-rich diet. *European journal of clinical nutrition,* 56**,** 12-18.

HAMBIDGE, M. 2003. Biomarkers of trace mineral intake and status. *Journal of Nutrition,* 133**,** 948S.

HANSON, M., FALL, C., ROBINSON, S. & BAIRD, J. 2009. Early life nutrition and lifelong health. London: British Medical Association.

HARAM, K., NILSEN, S. & ULVIK, R. 2001. Iron supplementation in pregnancy - evidence and controversies. *Acta Obstet Gynecol Scad,* 80**,** 683-688.

HASTE, F. M., BROOKE, O. G., ANDERSON, H. R. & BLAND, J. M. 1991. The effect of nutritional intake on outcome of pregnancy in smokers and non-smokers. *British Journal of Nutrition,* 65**,** 347-354.

HEALTH SURVEY FOR ENGLAND (HSE) 2010. Statistics on obesity, physical activity and diet: England, 2010. London: NHS Information Centre.

HEATH, A. L. M. & FAIRWEATHER-TAIT, S. J. 2002. Clinical implications of changes in the modern diet: iron intake, absorption and status. *Best Practice & Research Clinical Haematology,* 15**,** 225-241.

HEATH, A. L. M., SKEAFF, C. M., WILLIAMS, S. & GIBSON, R. S. 2007. The role of blood loss and diet in the aetiology of mild iron deficiency in premenopausal adult New Zealand women. *Public Health Nutrition,* 4**,** 197-206.

HERRICK, K., PHILLIPS, D. I. W., HASELDEN, S., SHIELL, A. W., CAMPBELL-BROWN, M. & GODFREY, K. M. 2003. Maternal consumption of a high-meat, low-carbohydrate diet in late pregnancy: relation to adult cortisol concentrations in the offspring. *Journal of Clinical Endocrinology & Metabolism,* 88**,** 3554.

HININGER, I., FAVIER, M., ARNAUD, J., FAURE, H., THOULON, J. M., HARIVEAU, E., FAVIER, A. & ROUSSEL, A. M. 2004. Effects of a combined micronutrient supplementation on maternal biological status and newborn anthropometrics

measurements: a randomized double-blind, placebo-controlled trial in apparently healthy pregnant women. *Eur J Clin Nutr,* 58**,** 52-59.

HOFFMAN, R., BENZ, E., SHATTIL, S., FURIE, B., COHEN, H., SILBERSTEIN, L. & MCGLAVE, P. 2000. Disorders of Iron Metabolism: Iron deficiency and overload. *Hematology: Basic Principles and Practice.* 3 ed. New York: Churchill Livingstone.

HOLLAND, B., WELCH, A., UNWIN, I., BUSS, D., PAUL, A. & SOUTHGATE, D. 1991. *McCance and Widdowson's the composition of food,* Cambridge, The Royal Society of Chemistry.

HORNSEY, H. 1956. The colour of cooked cured pork. 1. Estimation of the nitric oxide-haem pigments. *Journal of the science of food and agriculture* 7**,** 541.

HOWIE, G., SLOBODA, D., KAMAL, T. & VICKERS, M. 2009. Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. *The Journal of Physiology,* 587**,** 905-915.

HULSHOF, K. F. A. M., BRUSSAARD, J. H., KRUIZINGA, A. G., TELMAN, J. & LOWIK, M. R. H. 2003. Socio-economic status, dietary intake and 10[emsp14]y trends: the Dutch National Food Consumption Survey. *Eur J Clin Nutr,* 57**,** 128-137.

HUR, J., KIM, H., HA, E.-H., PARK, H., HA, M., KIM, Y., HONG, Y.-C. & CHANG, N. 2013. Birth Weight of Korean Infants Is Affected by the Interaction of Maternal Iron Intake and GSTM1 Polymorphism. *The Journal of Nutrition,* 143**,** 67-73.

HURRELL, R. F. 1997. Preventing iron deficiency through food fortification. *Nutrition Reviews,* 55**,** 210-222.

HYPPÖNEN, E., LEON, D., M G KENWARD, M. & LITHELL, H. 2001. Prenatal growth and risk of occlusive and haemorrhagic stroke in Swedish men and women born 191529: historical cohort study. *BMJ,* 2001**,** 1033-1034.

IM, J.-A., LEE, J.-W., SHIM, J.-Y., LEE, H.-R. & LEE, D.-C. 2007. Association between brachial-ankle pulse wave velocity and cardiovascular risk factors in healthy adolescents. *The Journal of pediatrics,* 150**,** 247-251.

IOM 2001. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington DC: Institute of Medicine, National Academy Press.

JACKSON, H., CARTER, K., DARKE, C., GUTTRIDGE, M., RAVINE, D., HUTTON, R., NAPIER, J. & WORWOOD, M. 2001. HFE mutations, iron deficiency and overload in 10 500 blood donors. *British journal of haematology,* 114**,** 474-484.

JADHAV, U. & KADAM, N. 2005. Non-invasive assessment of arterial stiffness by pulse-wave velocity correlates with endothelial dysfunction. *Indian Heart J. ,* 57**,** 226-32.

JAMES, W., NORUM, K., SMITASIRI, S., SWAMINATHAN, M., TAGWIREYI, J. & UAUY, R. 2000. Ending Malnutrition by 2020: an Agenda for Change in the Millennium: Final Report to the ACC/SCN by the Commission on the Nutrition Challenges of the 21st Century. Supplement to the Food and Nutrition Bulletin. 2000 September. *Food & Nutrition Bulletin,* 21.

KAESTEL, P., MICHAELSEN, K. F., AABY, P. & FRIIS, H. 2005. Effects of prenatal multimicronutrient supplements on birth weight and perinatal mortality: a randomised, controlled trial in Guinea-Bissau. *Eur J Clin Nutr,* 59**,** 1081-1089.

KANESHIGE, E. 1981. Serum ferritin as an assessment of iron stores and other hematologic parameters during pregnancy. *Obstetrics & Gynecology,* 57**,** 238.

KELLEHER, S. L. 2006. Effects of age and mineral intake on the regulation of iron absorption in infants. *The Journal of pediatrics,* 149**,** S69-S73.

KELLEHER, S. L. & LÖNNERDAL, B. 2006. Zinc supplementation reduces iron absorption through age-dependent changes in small intestine iron transporter expression in suckling rat pups. *The Journal of Nutrition,* 136**,** 1185-1191.

KEY, T. J., APPLEBY, P. N., SPENCER, E. A., TRAVIS, R. C., RODDAM, A. W. & ALLEN, N. E. 2009. Cancer incidence in vegetarians: results from the European Prospective Investigation into Cancer and Nutrition (EPIC-Oxford). *The American Journal of Clinical Nutrition,* 89**,** 1620S-1626S.

KINRA, S., SARMA, K., GHAGOORUNISSA, MENDU, V., RAVIKUMAR, R., MOHAN, V., WILKINSON, I., COCKROFT, J., DAVEY SMITH, G. & BEN-SHLOMO, Y. 2008. Effect of integration of supplemental nutrition with public health programmes in pregnancy and early childhood on cardiovascular risk in rural Indian adolescents: long term follow-up of Hyderabad nutritional trial. *BMJ,* 337**,** a605.

KOUDSI, A., OLDROYD, J., MCELDUFF, P., BANERJEE, M., VYAS, A. & CRUICKSHANK, J. K. 2007. Maternal and Neonatal Influences on, and Reproducibility of, Neonatal Aortic Pulse Wave Velocity. *Hypertension,* 49**,** 225-231.

KOZUKI, N., LEE, A. C. & KATZ, J. 2012. Moderate to Severe, but Not Mild, Maternal Anemia Is Associated with Increased Risk of Small-for-Gestational-Age Outcomes. *The Journal of Nutrition,* 142**,** 358-362.

KUMARAN, K., FALL, C. H., MARTYN, C. N., VIJAYAKUMAR, M., STEIN, C. & SHIER, R. 2000. Blood pressure, arterial compliance, and left ventricular mass: no relation to small size at birth in south Indian adults. *Heart,* 83**,** 272-7.

LAGIOU, P., MUCCI, L., TAMIMI, R., KUPER, H., LAGIOU, A., HSIEH, C. C. & TRICHOPOULOS, D. 2005. Micronutrient intake during pregnancy in relation to birth size. *European Journal of Nutrition,* 44**,** 52-59.

LANGLEY-EVANS, S. 2009. *Nutrition: a lifespan approach*, Wiley. com.

LAO, T., CHAN, P. & TAM, K. 2001. Gestational diabetes mellitus in the last trimester - a feature of maternal iron excess? *Diabetic Medicine,* 18**,** 218-223.

LARNKJAER, A., CHRISTENSEN, J. H., MICHAELSEN, K. F. & LAURITZEN, L. 2006. Maternal Fish Oil Supplementation during Lactation Does Not Affect Blood Pressure, Pulse Wave Velocity, or Heart Rate Variability in 2.5-y-old Children. *J. Nutr.,* 136**,** 1539-1544.

LAURENT, S., COCKCROFT, J., VAN BORTEL, L., BOUTOUYRIE, P., GIANNATTASIO, C., HAYOZ, D., PANNIER, B., VLACHOPOULOS, C., WILKINSON, I. & STRUIJKER-BOUDIER, H. 2006. Expert consensus document on arterial stiffness: methodological issues and clinical applications. *European Heart Journal,* 27**,** 2588-2605.

LAW, C. M., BARKER, D. J., BULL, A. R. & OSMOND, C. 1991. Maternal and fetal influences on blood pressure. *British Medical Journal,* 66**,** 1291.

LAWLOR, D. A., DAVEY, S. G., KUNDU, D., BRUCKDORFER, K. R. & EBRAHIM, S. 2004. Those confounded vitamins: what can we learn from the differences between observational versus randomised trial evidence? *Lancet,* 363**,** 1724.

LAWLOR, D. A., HARBORD, R. M., STERNE, J. A., TIMPSON, N. & DAVEY SMITH, G. 2008a. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. *Statistics in Medicine,* 27**,** 1133-1163.

LAWLOR, D. A. & DAVEY SMITH, G. 2005. Early life determinants of adult blood pressure. *Current opinion in nephrology and hypertension,* 14**,** 259-264.

LAWLOR, D. A., TIMPSON, N. J., HARBORD, R. M., LEARY, S., NESS, A., MCCARTHY, M. I., FRAYLING, T. M., HATTERSLEY, A. T. & DAVEY SMITH, G. 2008b. Exploring the

Developmental Overnutrition Hypothesis Using Parental-Offspring Associations and FTO as an Instrumental Variable. *PLoS Med,* 5**,** e33.

LAWN, J. E., COUSENS, S., BHUTTA, Z. A., DARMSTADT, G. L., MARTINES, J., PAUL, V., KNIPPENBERG, R., FOGSTADT, H., SHETTY, P. & HORTON, R. Why are 4 million newborn babies dying each year? *The Lancet,* 364**,** 399-401.

LEESON, C., WHINCUP, P., COOK, D., DONALD, A., PAPACOSTA, O., LUCAS, A. & DEANFIELD, J. 1997. Flow-Mediated Dilation in 9-to 11-Year-Old Children The Influence of Intrauterine and Childhood Factors. *Circulation,* 96**,** 2233-2238.

LEWIS, R., PETRY, C., OZANNE, S. & HALES, C. 2001. Effects of maternal iron restriction in the rat on blood pressure, glucose tolerance, and serum lipids in the 3-month-old offspring. *Metabolism,* 50**,** 562-7.

LEWIS, S., BONILLA, C., BRION, M., LAWLOR, D., GUNNELL, D., BEN-SHLOMO, Y., NESS, A. & DAVEY SMITH, G. 2014. Maternal iron levels early in pregnancy are not associated with offspring IQ score at age 8, findings from a Mendelian randomization study. *European journal of clinical nutrition*.

LIGI, I., GRANDVUILLEMIN, I., ANDRES, V., DIGNAT-GEORGE, F. & SIMEONI, U. Low birth weight infants and the developmental programming of hypertension: a focus on vascular factors. Seminars in perinatology, 2010. Elsevier, 188-192.

LISLE, S., LEWIS, R., PETRY, C., OZANNE, S., HALES, C. & FORHEAD, A. 2003. Effect of maternal iron restriction during pregnancy on renal morphology in the adult rat offspring. *British Journal of Nutrition,* 90**,** 33-9.

LOZOFF, B. 2000. Perinatal iron deficiency and the developing brain. *Pediatric Research,* 48**,** 137.

LUMBIGANON, P. 2007. Multiple-micronutrient supplementation for women udirng pregnancy: RHL commentary [Online]. Geneva: World Health Organization. Available: http://apps.who.int/rhl/pregnancy_childbirth/antenatal_care/nutrition/focom/en/index.html [Accessed 27 July 2011].

LUMEY, L., STEIN, A. D., KAHN, H. S., VAN DER PAL-DE, K. M., BLAUW, G., ZYBERT, P. A. & SUSSER, E. S. 2007. Cohort profile: the Dutch Hunger Winter families study. *International Journal of Epidemiology,* 36**,** 1196-1204.

LUMLEY, J., WATSON, L., WATSON, M. & BOWER, C. 2002. Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects (Review). *Cochrane Database of Systematic Reviews*.

LURBE, E., TORRO, M. I., CARVAJAL, E., ALVAREZ, V. & REDÓN, J. 2003. Birth weight impacts on wave reflections in children and adolescents. *Hypertension,* 41**,** 646-650.

LYNCH, S. 2011. Improving the assessment of iron status. *The American Journal of Clinical Nutrition,* 93**,** 1188-1189.

MARTIN, H., HU, J., GENNSER, G. & NORMAN, M. 2000. Impaired Endothelial Function and Increased Carotid Stiffness in 9-Year-Old Children With Low Birthweight. *Circulation,* 102**,** 2739-2744.

MARTYN, C. N., BARKER, D. J., JESPERSEN, S., GREENWALD, S., OSMOND, C. & BERRY, C. 1995. Growth in utero, adult blood pressure, and arterial compliance. *Br Heart J,* 73**,** 116-21.

MARTYN, C. N. & GREENWALD, S. E. 1997. Impaired synthesis of elastin in walls of aorta and large conduit arteries during early development as an initiating event in pathogenesis of systemic hypertension. *Lancet,* 350**,** 953-955.

MATHEWS, F., YOUNGMAN, L. & NEIL, A. 2004. Maternal circulating nutrient concentrations in pregnancy: implications for birth and placental weights of term infants. *The American Journal of Clinical Nutrition,* 79**,** 103-110.

MATHEWS, F., YUDKIN, P. & NEIL, A. 1999. Influence of maternal nutrition on outcome of pregnancy: prospective cohort study. *British Medical Journal,* 319**,** 339.

MCARDLE, H. J., ANDERSEN, H. S., JONES, H. & GAMBLING, L. 2006. Fetal programming: Causes and consequences as revealed by studies of dietary manipulation in rats-A review. *Placenta,* 27**,** 56-60.

MCARDLE, H. J., GAMBLING, L. & KENNEDY, C. 2013. Iron deficiency during pregnancy: the consequences for placental function and fetal outcome. *The Proceedings of the Nutrition Society***,** 1-7.

MCCANCE, R. A. 1990. *Composition of foods*, Royal Society of Chemistry.

MCMAHON, L. P. 2010. Iron deficiency in pregnancy. *Obstet Med,* 3**,** 17-24.

MEAUME, S., RUDNICHI, A., LYNCH, A., BUSSY, C., SEBBAN, C., BENETOS, A. & SAFAR, M. E. 2001. Aortic pulse wave velocity as a marker of cardiovascular disease in subjects over 70 years old. *Journal of Hypertension,* 19**,** 871-877.

MEI, Z., COGSWELL, M. E., LOOKER, A. C., PFEIFFER, C. M., CUSICK, S. E., LACHER, D. A. & GRUMMER-STRAWN, L. M. 2011. Assessment of iron status in US pregnant women from the National Health and Nutrition Examination Survey (NHANES), 1999–2006. *The American journal of clinical nutrition,* 93**,** 1312-1320.

MERRYWEATHER-CLARKE, A., POINTON, J., JOUANOLLE, A., ROCHETTE, J. & ROBSON, K. 2000. Geography of HFE C282Y and H63D mutations. *Genet Test,* 4**,** 183-98.

MILMAN, N. 2006a. Iron and pregnancy--a delicate balance. *Annals of Hematology,* 85**,** 559-65.

MILMAN, N. 2006b. Iron prophylaxis in pregnancy--general or individual and in which dose? *Annals of Hematology,* 85**,** 821-8.

MILMAN, N. 2008. Prepartum anaemia: prevention and treatment. *Annals of Hematology,* 87**,** 949-959.

MILMAN, N., BERGHOLT, T., ERIKSEN, L., BYG, K. E., GRAUDAL, N., PEDERSEN, P. & HERTZ, J. 2005. Iron prophylaxis during pregnancy-How much iron is needed? A randomized dose-response study of 20-80 mg ferrous iron daily in pregnant women. *Acta Obstetricia et Gynecologica Scandinavica,* 84**,** 238-247.

MILMAN, N., BYG, K. E., BERGHOLT, T., ERIKSEN, L. & HVAS, A. M. 2006. Body iron and individual iron prophylaxis in pregnancy—should the iron dose be adjusted according to serum ferritin? *Annals of Hematology,* 85**,** 567-573.

MILMAN, N., BYG, K. E., OVESEN, L., KIRCHHOFF, M. & JURGENSEN, K. S. L. 2003. Iron status in Danish women, 1984-1994: a cohort comparison of changes in iron stores and the prevalence of iron deficiency and iron overload. *European Journal of Haematology,* 71**,** 51-61.

MILMAN, N., CLAUSEN, J. & BYG, K. E. 1998. Iron status in 268 Danish women aged 18–30 years: influence of menstruation, contraceptive method, and iron supplementation. *Annals of Hematology,* 77**,** 13-19.

MISHRA, V., THAPA, S., RETHERFORD, R. D. & DAI, X. 2005. Effect of iron supplementation during pregnancy on birthweight: evidence from Zimbabwe. *Food & Nutrition Bulletin,* 26**,** 338-347.

MITCHELL, E., ROBINSON, E., CLARK, P., BECROFT, D., GLAVISH, N., PATTISON, N., PRYOR, J., THOMPSON, J. & WILD, C. 2004. Maternal nutritional risk factors for small

for gestational age babies in a developed country: a case-control study. *Archives of Disease in Childhood Fetal and Neonatal Edition,* 89**,** F431.

MIYAI, N., ARITA, M., MIYASHITA, K., MORIOKA, I. & TAKEDA, S. 2009. The influence of obesity and metabolic risk variables on brachial-ankle pulse wave velocity in healthy adolescents. *J Hum Hypertens,* 23**,** 444-50.

MOENS, A. L., GOOVAERTS, I., CLAEYS, M. J. & VRINTS, C. J. 2005. Flow-Mediated Vasodilation. A Diagnostic Instrument, or an Experimental Tool? *Chest,* 127**,** 2254-2263.

MONTGOMERY, A. A., BEN-SHLOMO, Y., MCCARTHY, A., DAVIES, D., ELWOOD, P. & DAVEY SMITH, G. 2000. Birth size and arterial compliance in young adults. *Lancet,* 355**,** 2136-7.

MORRISON, J., WILLIAMS, G. M., NAJMAN, J. M. & ANDERSEN, M. J. 1991. The Influence of Paternal Height and Weight on Birth weight. *Australian and New Zealand Journal of Obstetrics and Gynaecology,* 31**,** 114-116.

NAHUM, G. G. & STANISLAW, H. 2003. Relationship of paternal factors to birth weight. *Journal of reproductive medicine,* 48**,** 963-968.

NATIONAL CENTER FOR HEALTH STATISTICS 1994. Plan and Operation of the Third National Health and Nutrition Examination Survey, 1988–94. Washington, DC: U.S. Dept. of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention.

NATIONAL INSTITUTE FOR CLINICAL EXCELLENCE (NICE) 2008. Antenatal care: Routine care for the healthy pregnant woman. London.

NATIONAL IRON+ INITIATIVE 2013. Guidelines for Control of Iron Deficiency Anaemia. India: Ministry of Health.

NEWTON, C. R., GRAHAM, A., HEPTINSTALL, L. E., POWELL, S. J., SUMMERS, C., KALSHEKER, N., SMITH, J. C. & MARKHAM, A. F. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Research,* 17**,** 2503.

NIBOSHI, A., HAMAOKA, K., SAKATA, K. & INOUE, F. 2006. Characteristics of brachial–ankle pulse wave velocity in Japanese children. *European journal of pediatrics,* 165**,** 625-629.

NISHIDA, C., KO, G. T. & KUMANYIKA, S. 2009. Body fat distribution and noncommunicable diseases in populations: overview of the 2008 WHO Expert Consultation on Waist Circumference and Waist-Hip Ratio. *Eur J Clin Nutr,* 64**,** 2-5.

NORDIC COUNCIL OF MINISTERS 2004. Nordic Nutrition Recommendations (NNR 2004). Copenhagen

O'BRIEN, K. O., ZAVALETA, N., ABRAMS, S. A. & CAULFIELD, L. E. 2003. Maternal iron status influences iron transfer to the fetus during the third trimester of pregnancy. *American Journal of Clinical Nutrition,* 77**,** 924.

O'BRIEN, K. O., ZAVALETA, N., CAULFIELD, L. E., WEN, J. & ABRAMS, S. A. 2000. Prenatal iron supplements impair zinc absorption in pregnant Peruvian women. *Journal of Nutrition,* 130**,** 2251.

O'BRIEN, K. O., ZAVALETA, N., CAULFIELD, L. E., YANG, D. X. & ABRAMS, S. A. 1999. Influence of prenatal iron and zinc supplements on supplemental iron absorption, red blood cell iron incorporation, and iron status in pregnant Peruvian women. *American Journal of Clinical Nutrition,* 69**,** 509.

O'HARA, B. A. 2004. *The joint effects of genotype and nutrient intake on iron status in women (PhD thesis).* PhD, University of Leeds.

OFFICE FOR NATIONAL STATISTICS. *Low birth weight live births 2007* [Online]. London. Available: http://www.data4nr.net/resources/524 [Accessed 27 July 2010].

OREN, A., VOS, L. E., BOS, W.-J. W., SAFAR, M. E., UITERWAAL, C. S., GORISSEN, W. H., GROBBEE, D. E. & BOTS, M. L. 2003. Gestational age and birth weight in relation to aortic stiffness in healthy young adults: two separate mechanisms? &ast. *American Journal of Hypertension,* 16**,** 76-79.

OSRIN, D., VAIDYA, A., SHRESTHA, Y., BANIYA, R. B., MANANDHAR, D. S., ADHIKARI, R. K., FILTEAU, S., TOMKINS, A. & DE L COSTELLO, A. M. 2005. Effects of antenatal multiple micronutrient supplementation on birthweight and gestational duration in Nepal: double-blind, randomised controlled trial. *The Lancet,* 365**,** 955-962.

PAINTER, R., ROSEBOOM, T. & OP, B. 2005. Prenatal exposure to the Dutch famine and disease in later life: an overview. *Reprod Toxicol.,* 20**,** 345-52.

PAINTER, R. C., OSMOND, C., GLUCKMAN, P., HANSON, M., PHILLIPS, D. I. W. & ROSEBOOM, T. J. 2008. Transgenerational effects of prenatal exposure to the Dutch

famine on neonatal adiposity and health in later life. *BJOG: An International Journal of Obstetrics & Gynaecology,* 115**,** 1243-1249.

PAVORD, S., MYERS, B., ROBINSON, S., ALLARD, S., STRONG, J. & OPPENHEIMER, C. 2011. UK guidelines on the management of iron deficiency in pregnancy. London: British Committee for Standards in Haematology.

PEARL, J. 2000. Why there is no statistical test for confounding, why many think there is, and why they are almost right. *Causality: models, reasoning, and inference.* Cambridge: Cambridge University Press.

PEDERSEN, P. & MILMAN, N. 2009. Genetic screening for HFE hemochromatosis in 6,020 Danish men: penetrance of C282Y, H63D, and S65C variants. *Annals of Hematology,* 88**,** 775-784.

PENA-ROSAS, J. P. & VITERI, F. E. 2009. Effects and safety of preventive oral iron or iron+folic acid supplementation for women during pregnancy. *Cochrane Database of Systematic Reviews***,** CD004736.

PI-SUNYER, F. X. 2012. The obesity epidemic: pathophysiology and consequences of obesity. *Obesity research,* 10**,** 97S-104S.

POSTON, L., BRILEY, A. L., SEED, P. T., KELLY, F. J. & SHENNAN, A. H. 2006. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *The Lancet,* 367**,** 1145-1154.

POTISCHMAN, N. & FREUDENHEIM, J. L. 2003. Biomarkers of nutritional exposure and nutritional status: an overview. *Journal of Nutrition,* 133**,** 873S.

POULTER, N. R., CHANG, C. L., MACGREGOR, A. J., SNIEDER, H. & SPECTOR, T. D. 1999. Association between birth weight and adult blood pressure in twins: historical cohort study. *BMJ,* 319**,** 1330-1333.

PROSPECTIVE STUDIES COLLABORATION 2002. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet,* 360**,** 1903-1913.

PSATY, B. M., FURBERG, C. D., KULLER, L. H., CUSHMAN, M., SAVAGE, P. J., LEVINE, D., O'LEARY, D. H., BRYAN, R. N., ANDERSON, M. & LUMLEY, T. 2001. Association between blood pressure level and the risk of myocardial infarction, stroke, and total mortality: the cardiovascular health study. *Archives of Internal Medicine,* 161**,** 1183-1192.

PUNNONEN, K., IRJALA, K. & RAJAMAKI, A. 1997. Serum Transferrin Receptor and Its Ratio to Serum Ferritin in the Diagnosis of Iron Deficiency. *Blood,* 89**,** 1052-1057.

QIU, C., ZHANG, C., GELAYE, B., ENQUOBAHRIE, D. A., FREDERICK, I. O. & WILLIAMS, M. A. 2011. Gestational Diabetes Mellitus in Relation to Maternal Dietary Heme Iron and Nonheme Iron Intake. *Diabetes Care,* 34**,** 1564-1569.

RAHIMI, R., NIKFAR, S., REZAIE, A. & ABDOLLAHI, M. 2009. A meta-analysis on the efficacy and safety of combined vitamin C and E supplementation in preeclamptic women. *Hypertens Pregnancy,* 28**,** 417-34.

RAJPATHAK, S., MA, J., MANSON, J., WILLETT, W. C. & HU, F. B. 2006. Iron Intake and the Risk of Type 2 Diabetes in Women. *Diabetes Care,* 29**,** 1370-1376.

RAMAKRISHNAN, U., GONZALEZ-COSSIO, T., NEUFELD, L. M., RIVERA, J. & MARTORELL, R. 2003. Multiple micronutrient supplementation during pregnancy does not lead to greater infant birth size than does iron-only supplementation: a randomized controlled trial in a semirural community in Mexico. *Am J Clin Nutr,* 77**,** 720-725.

RAMSAY, M. M., JAMES, D., STEER, P. J., GONIK, B. & WEINER, C. P. 2000. *Normal values in pregnancy,* London, Bailliere Tindall.

RASMUSSEN, K. M. 2001. Is There a Causal Relationship between Iron Deficiency or Iron-Deficiency Anemia and Weight at Birth, Length of Gestation and Perinatal Mortality? *J Nutr.,* 131**,** 590S-603.

RASMUSSEN, M. L., FOLSOM, A. R., CATELLIER, D. J., TSAI, M. Y., GARG, U. & ECKFELDT, J. H. 2001. A prospective study of coronary heart disease and the hemochromatosis gene (HFE) C282Y mutation: the Atherosclerosis Risk in Communities (ARIC) study. *Atherosclerosis,* 154**,** 739-746.

RAYMAN, M. P., BARLIS, J., EVANS, R. W., REDMAN, C. W. G. & KING, L. J. 2002. Abnormal iron parameters in the pregnancy syndrome preeclampsia. *American journal of obstetrics and gynecology,* 187**,** 412-418.

REILLY, J. J., ARMSTRONG, J., DOROSTY, A. R., EMMETT, P. M., NESS, A., ROGERS, I., STEER, C., SHERRIFF, A. & TEAM, F. T. A. L. S. O. P. A. C. S. 2005. Early life risk factors for obesity in childhood: cohort study. *BMJ,* 330**,** 1357-.

REMACLE, C., BIESWAL, F., BOL, V. & REUSENS, B. 2011. Developmental programming of adult obesity and cardiovascular disease in rodents by maternal nutrition imbalance. *The American Journal of Clinical Nutrition,* 94**,** 1846S-1852S.

RHODES, D., RAHA-CHOWDHURY, R., COX, T. & TROWSDALE, J. 1997. Homozygosity for the predominant Cys282Tyr mutation and absence of disease expression in hereditary haemochromatosis. *J Med Genet,* 34**,** 761-4.

RIBOT, B., ARANDA, N., VITERI, F., HERNÁNDEZ-MARTÍNEZ, C., CANALS, J. & ARIJA, V. 2012. Depleted iron stores without anaemia early in pregnancy carries increased risk of lower birthweight even when supplemented daily with moderate iron. *Human Reproduction,* 27**,** 1260-1266.

RICH-EDWARDS, J. W., KLEINMAN, K., MICHELS, K. B., STAMPFER, M. J., MANSON, J. E., REXRODE, K. M., HIBERT, E. N. & WILLETT, W. C. 2005. Longitudinal study of birth weight and adult body mass index in predicting risk of coronary heart disease and stroke in women. *BMJ,* 330**,** 1115-.

RICHARDSON, D. K., PHIBBS, C. S., GRAY, J. E., MCCORMICK, M. C., WORKMAN-DANIELS, K. & GOLDMANN, D. A. 1993. Birth weight and illness severity: independent predictors of neonatal mortality. *Pediatrics,* 91**,** 969.

ROBERFROID, D., HUYBREGTS, L., LANOU, H., HENRY, M.-C., MEDA, N., MENTEN, J., KOLSTEREN, P. & FOR THE, M. S. G. 2008. Effects of maternal multiple micronutrient supplementation on fetal growth: a double-blind randomized controlled trial in rural Burkina Faso. *Am J Clin Nutr,* 88**,** 1330-1340.

ROBINSON, S., GODFREY, K., DENNE, J. & COX, V. 1998. The determinants of iron status in early pregnancy. *British Journal of Nutrition,* 79**,** 249-255.

ROGERS, I. & EMMETT, P. 1998. Diet during pregnancy in a population of pregnant women in South West England. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. *European journal of clinical nutrition,* 52**,** 246.

RONNENBERG, A. G., WOOD, R. J., WANG, X., XING, H., CHEN, C., CHEN, D., GUANG, W., HUANG, A., WANG, L. & XU, X. 2004. Preconception hemoglobin and ferritin concentrations are associated with pregnancy outcome in a prospective cohort of Chinese women. *The Journal of Nutrition,* 134**,** 2586-2591.

ROSSANDER-HULTEN, L., BRUNE, M., SANDSTROM, B., LONNERDAL, B. & HALLBERG, L. 1991. Competitive inhibition of iron absorption by manganese and zinc in humans. *American Journal of Clinical Nutrition,* 54**,** 152.

ROSSI, P., TAUZIN, L., MARCHAND, E., BOUSSUGES, A., GAUDART, J. & FRANCES, Y. 2011. Respective Roles of Preterm Birth and Fetal Growth Restriction in Blood Pressure

and Arterial Stiffness in Adolescence. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine,* 48**,** 520-522.

ROUGHEAD, Z. K. & HUNT, J. R. 2000. Adaptation in iron absorption: iron supplementation reduces nonheme-iron but not heme-iron absorption from food. *American Journal of Clinical Nutrition,* 72**,** 982.

ROYSTON, P. 2004. Multiple imputation of missing values. *Stata Journal,* 4**,** 227-241.

RUDBECK, L. & DISSING, J. 1998. Rapid, simple alkaline extraction of human genomic DNA from whole blood, buccal epithelial cells, semen and forensic stains for PCR. *Biotechniques,* 25**,** 588.

RUIVARD, M., BOURSIAC, M., MAREYNAT, G., SAPIN, A., GERBAUD, L., DERUMEAUX, H., FOUILHOUX, A., TRAVADE, P. & PHILIPPE, P. 2000. [Diagnosis of iron deficiency: evaluation of the" soluble transferrin receptor/transferrin" ratio]. *La Revue de medecine interne/fondee... par la Societe nationale francaise de medecine interne,* 21**,** 837-843.

RUSIA, U., DFLOWERS, C., MADAN, N., AGARWAL, N., SOOD, S. & SIKKA, M. 1999. Serum transferrin receptors in detection of iron deficiency in pregnancy. *Ann Hematol,* 78**,** 358-63.

SAKURAGI, S., ABHAYARATNA, K., GRAVENMAKER, K. J., O'REILLY, C., SRIKUSALANUKUL, W., BUDGE, M. M., TELFORD, R. D. & ABHAYARATNA, W. P. 2009. Influence of Adiposity and Physical Activity on Arterial Stiffness in Healthy Children: The Lifestyle of Our Kids Study. *Hypertension,* 53**,** 611-616.

SALVI, P., REVERA, M., JOLY, L., REUSZ, G., IAIA, M., BENKHEDDA, S., CHIBANE, A., PARATI, G., BENETOS, A. & TEMMAR, M. 2012. Role of Birth Weight and Postnatal Growth on Pulse Wave Velocity in Teenagers. *Journal of Adolescent Health*.

SANCHEZ-CASTILLO, C., BERBER, A., LARA, A. & JAMES, W. 2006. The optimum BMI and waist circumferences of Hispanics. *Obes Rev,* 7(Suppl. 2)**,** 23 (IS0096).

SCANLON, K. S., YIP, R., SCHIEVE, L. A. & COGSWELL, M. E. 2000. High and low hemoglobin levels during pregnancy: differential risks for preterm birth and small for gestational age. *Obstetrics & Gynecology,* 96**,** 741.

SCHACK-NIELSEN, L., MØLGAARD, C., LARSEN, D., MARTYN, C. & MICHAELSEN, K. F. 2005. Arterial stiffness in 10-year-old children: current and early determinants. *British Journal of Nutrition,* 94**,** 1004-1011.

SCHLUCHTER, M. D. 2008. Flexible approaches to computing mediated effects in generalized linear models: Generalized estimating equations and bootstrapping. *Multivariate Behavioral Research,* 43**,** 268-288.

SCHOLL, T. O. 1998. High Third-Trimester Ferritin Concentration: Associations With Very Preterm Delivery, Infection, and Maternal Nutritional Status. *Obstetrics & Gynecology,* 92**,** 161-166.

SCHOLL, T. O. 2005. Iron status during pregnancy: Setting the stage for mother and infant. *American Journal of Clinical Nutrition,* 81 (suppl)**,** 1218S-22S.

SCHOLL, T. O. & HEDIGER, M. L. 1994. Anemia and iron-deficiency anemia: compilation of data on pregnancy outcome. *Am J Clin Nutr,* 59**,** 492S-500.

SCHOLL, T. O., HEDIGER, M. L., BENDICH, A., SCHALL, J. I., SMITH, W. K. & KRUEGER, P. M. 1997. Use of Multivitamin/Mineral Prenatal Supplements: Influence on the Outcome of Pregnancy. *Am. J. Epidemiol.,* 146**,** 134-141.

SCHOLL, T. O., HEDIGER, M. L., FISCHER, R. L. & SHEARER, J. W. 1992. Anemia vs iron deficiency: increased risk of preterm delivery in a prospective study. *Am J Clin Nutr,* 55**,** 985-988.

SCHOLL, T. O. & REILLY, T. 2000. Anemia, Iron and Pregnancy Outcome. *J Nutr.,* 130**,** 443-.

SCHOOLING, C., JIANG, C., LAM, T., COWLING, B., AU YEUNG, S., ZHANG, W., CHENG, K. & LEUNG, G. 2010. Estimated birth weight and adult cardiovascular risk factors in a developing southern Chinese population: a cross sectional study. *BMC Public Health,* 10**,** 270.

SCHRICKER, B. R. & STOUFFER, D. 1982. Measurement and content of nonheme and total iron in muscle. *Journal of Food Science,* 47**,** 740-743.

SCHULZ, L. C. 2010. The Dutch Hunger Winter and the developmental origins of health and disease. *Proceedings of the National Academy of Sciences,* 107**,** 16757-16758.

SCIENTIFIC ADVISORY COMMITTEE ON NUTRITION 2008. *The nutritional health of the British population,* London, The Stationary Office.

SCIENTIFIC ADVISORY COMMITTEE ON NUTRITION 2010. Iron and Health. London: Department of Health.

SCIENTIFIC ADVISORY COMMITTEE ON NUTRITION 2011. *The influence of maternal, tonen*

fetal and child nutrition on the development of chronic disease in later life, London, The Stationary Office.

SEHGAL, A., DOCTOR, T. & MENAHEM, S. 2014. Cardiac function and arterial indices in infants born small for gestational age: analysis by speckle tracking. *Acta Paediatrica,* 103**,** e49-e54.

SHAH, P. S., OHLSSON, A., KNOWLEDGE SYNTHESIS GROUP ON DETERMINANTS OF LOW BIRTH, W. & PRETERM, B. 2009. Effects of prenatal multimicronutrient supplementation on pregnancy outcomes: a meta-analysis. *CMAJ Canadian Medical Association Journal,* 180**,** E99-108.

SHIELL, A. W., CAMPBELL-BROWN, M., HASELDEN, S., ROBINSON, S., GODFREY, K. M. & BARKER, D. J. 2001. High-meat, low-carbohydrate diet in pregnancy relation to adult blood pressure in the offspring. *Hypertension,* 38**,** 1282-1288.

SINGLA, P. N., TYAGI, M., SHANKAR, R., DASH, D. & KUMAR, A. 1996. Fetal iron status in maternal anemia. *Acta Paediatrica,* 85**,** 1327-1330.

SKIKNE, B. & BAYNES, R. D. 1994. Iron absorption. *In:* BROCK, J., HALLIDAY, J., PIPPARD, M. & POWELL, L. (eds.) *Iron metabolism in health and disease.* London: Saunders.

SKIKNE, B. S. 2008. Serum transferrin receptor. *American journal of hematology,* 83**,** 872-875.

SKILTON, M. R., EVANS, N., GRIFFITHS, K. A., HARMER, J. A. & CELERMAJER, D. S. 2005. Aortic wall thickness in newborns with intrauterine growth restriction. *Lancet,* 365**,** 1484-1486.

SMEDTS, H. P. M., VRIES, J. H. D., RAKHSHANDEHROO, M., WILDHAGEN, M. F., VERKLEIJ-HAGOORT, A. C., STEEGERS, E. A. & STEEGERS-THEUNISSEN, R. P. M. 2009. High maternal vitamin E intake by diet or supplements is associated with congenital heart defects in the offspring. *BJOG: An International Journal of Obstetrics & Gynaecology,* 116**,** 416-423.

SPINNATO II, J. A., FREIRE, S., PINTO E SILVA, J. L., RUDGE, M. V. C., MARTINS-COSTA, S., KOCH, M. A., GOCO, N., DE BARROS SANTOS, C., CECATTI, J. G., COSTA, R., RAMOS, J. G., MOSS, N. & SIBAI, B. M. 2008. Antioxidant supplementation and premature

rupture of the membranes: a planned secondary analysis. *American Journal of Obstetrics and Gynecology,* 199**,** 433.e1-433.e8.

STAIGER, D. & STOCK, J. 1997. Instrumental variables estimation with weak instruments. *Econometrica,* 65**,** 393-415.

STANNER, S. A. & YUDKIN, J. S. 2001. Fetal Programming and the Leningrad Siege Study. *Twin Research and Human Genetics,* 4**,** 287-292.

STEER, P., ALAM, M. A., WADSWORTH, J. & WELCH, A. 1995. Relation between maternal haemoglobin concentration and birth weight in different ethnic groups. *BMJ: British Medical Journal,* 310**,** 489-491.

STENE, L. C., MAGNUS, P., LIE, R. T., SØVIK, O., JONER, G. & GROUP, T. N. C. D. S. 2001. Birth weight and childhood onset type 1 diabetes: population based cohort study. *BMJ,* 322**,** 889-892.

SUTTON-TYRRELL, K., NAJJAR, S. S., BOUDREAU, R. M., VENKITACHALAM, L., KUPELIAN, V., SIMONSICK, E. M., HAVLIK, R., LAKATTA, E. G., SPURGEON, H., KRITCHEVSKY, S., PAHOR, M., BAUER, D., NEWMAN, A. & FOR THE HEALTH, A. B. C. S. 2005. Elevated Aortic Pulse Wave Velocity, a Marker of Arterial Stiffness, Predicts Cardiovascular Events in Well-Functioning Older Adults. *Circulation,* 111**,** 3384-3390.

TABRIZI, F. M. & SARASWATHI, G. 2011. Maternal Nutrient Intake and Maternal Serum Micronutrients and Their Relation to Birth Weight-A longitudinal study. *Journal of Collaborative Research on Internal Medicine & Public Health,* 3**,** 617-632.

TAMURA, T., GOLDENBERG, R. L., HOU, J., JOHNSTON, K. E., CLIVER, S. P., RAMEY, S. L. & NELSON, K. G. 2002. Cord serum ferritin concentrations and mental and psychomotor development of children at five years of age. *Obstetrical & Gynecological Survey,* 57**,** 493.

TANAKA, H., MUNAKATA, M., KAWANO, Y., OHISHI, M., SHOJI, T., SUGAWARA, J., TOMIYAMA, H., YAMASHINA, A., YASUDA, H. & SAWAYAMA, T. 2009. Comparison between carotid-femoral and brachial-ankle pulse wave velocity as measures of arterial stiffness. *Journal of Hypertension,* 27**,** 2022-2027.

TAUZIN, L., ROSSI, P., GIUSANO, B., GAUDART, J., BOUSSUGES, A., FRAISSE, A. & SIMEONI, U. 2006. Characteristics of arterial stiffness in very low birth weight premature infants. *Pediatric Research,* 60**,** 592-596.

TE VELDE, S. J., FERREIRA, I., TWISK, J. W., STEHOUWER, C. D., VAN MECHELEN, W. & KEMPER, H. C. 2004. Birthweight and arterial stiffness and blood pressure in adulthood—results from the Amsterdam Growth and Health Longitudinal Study. *International Journal of Epidemiology,* 33**,** 154-161.

THE PANEL ON DIETARY REFERENCE VALUES OF THE COMMITTEE ON MEDICAL ASPECTS OF FOOD POLICY 1991. Dietary Reference Values for food eneregy and nutrients for the United Kingdom Department of Health.

THE SUPPLEMENTATION WITH MULTIPLE MICRONUTRIENTS INTERVENTION TRIAL (SUMMIT) STUDY GROUP 2008. Effect of maternal multiple micronutrient supplementation on fetal loss and infant death in Indonesia: a double-blind cluster-randomised trial. *Lancet,* 371**,** 215-227.

TIAN, J.-Y., CHENG, Q., SONG, X.-M., LI, G., JIANG, G.-X., GU, Y.-Y. & LUO, M. 2006. Birth weight and risk of type 2 diabetes, abdominal obesity and hypertension among Chinese adults. *European Journal of Endocrinology,* 155**,** 601-607.

TOMIYAMA, H. & YAMASHINA, A. 2004. Pulse Wave Velocity. *Rinsho Byori. ,* 52**,** 669-75.

TRYGG, K., LUND-LARSEN, K., SANDSTAD, B., HOFFMAN, H. J., JACOBSEN, G. & BAKKETEIG, L. S. 1995. Do pregnant smokers eat differently from pregnant non-smokers. *Paediatr Perinat Epidemiol,* 9**,** 307-19.

TUOMAINEN, T. P., KONTULA, K., NYYSSONEN, K., LAKKA, T. A., HELIO, T. & SALONEN, J. T. 1999. Increased risk of acute myocardial infarction in carriers of the hemochromatosis gene Cys282Tyr mutation: a prospective cohort study in men in eastern Finland. *Circulation,* 100**,** 1274.

TZOULAKI, I., BROWN, I., CHAN, Q., VAN HORN, L., UESHIMA, H., ZHAO, L., STAMLER, J. & ELLIOTT, P. 2008. Relation of iron and red meat intake to blood pressure: cross sectional epidemiological study. *BMJ,* 337**,** a258.VAIDYA, A., SAVILLE, N., SHRESTHA, B. P., DE L COSTELLO, A. M., MANANDHAR, D. S. & OSRIN, D. 2008. Effects of antenatal multiple micronutrient supplementation on children's weight and size at 2 years of age in Nepal: follow-up of a double-blind randomised controlled trial. *The Lancet,* 371**,** 492-499.

VAN LEEUWEN-SEGARCEANU, E. M., TROMP, W. F., BOS, W. J. W., VOGELS, O. J. M., GROOTHOFF, J. W. & VAN DER LEE, J. H. 2010. Comparison of two instruments

measuring carotid-femoral pulse wave velocity: Vicorder versus SphygmoCor. *Journal of Hypertension,* 28**,** 1687.

VIJAYARAGHAVEN, K. 2004. Iron-deficiency anemias. *In:* GIBNEY, M., MARGETTS, B., KEARNEY, J. & ARAB, L. (eds.) *Public Health Nutrition.* Oxford: Blackwell Publishing.

VLACHOPOULOS, C., AZNAOURIDIS, K. & STEFANADIS, C. 2010. Prediction of Cardiovascular Events and All-Cause Mortality With Arterial Stiffness:: A Systematic Review and Meta-Analysis. *Journal of the American College of Cardiology,* 55**,** 1318-1327.

WAHEED, A., PARKKILA, S., ZHOU, X. Y., TOMATSU, S., TSUCHIHASHI, Z., FEDER, J. N., SCHATZMAN, R. C., BRITTON, R. S., BACON, B. R. & SLY, W. S. 1997. Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with 2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 12384.

WALTER, T., OLIVARES, M., PIZZARO, F. & HERTRAMPF, E. 2001. Fortification *In:* RAMAKRISHNAN, U. (ed.) *Nutritional anemias.* Boca Raton: CRC Press.

WEBER, T., O'ROURKE, M. F., AMMER, M., KVAS, E., PUNZENGRUBER, C. & EBER, B. 2008. Arterial Stiffness and Arterial Wave Reflections Are Associated With Systolic and Diastolic Function in Patients With Normal Ejection Fraction. *Am J Hypertens,* 21**,** 1194-1202.

WEINBERG, E. D. 2009. Are iron supplements appropriate for iron replete pregnant women? *Medical hypotheses,* 73**,** 714-715.

WHINCUP, P., COOK, D., PAPACOSTA, O., WALKER, M. & PERRY, I. 1994. Maternal factors and development of cardiovascular risk: evidence from a study of blood pressure in children. *Journal of human hypertension,* 8**,** 337-344.

WHITTAKER, P. G., LIND, T. & WILLIAMS, J. G. 1991. Iron absorption during normal human pregnancy: a study using stable isotopes. *Br J Nutr,* 65**,** 457-463.

WHITWORTH, J. 2003. 2003 World Health Organization (WHO)/International Society of Hypertension (ISH) statement on management of hypertension. *Journal of Hypertension,* 21**,** 1983.

WHO 1995. *Physical Status: the Use and Interpretation of Anthropometry. Technical Report. Series 854,* Geneva, World Health Organization, .

WHO 2001. Iron deficiency anemia assessment prevention and control: a guide for program managers. Geneva: World Health Organization.

WHO 2006. Iron and Folate Supplementation. Standards for Maternal and Neonatal Care. *Integrated Management of Pregnancy and Childbirth (IMPAC).* Geneva: World Health Organization. Department of Making Pregnancy Safer (MPS).

WHO. 2010. *Micronutrient deficiencies* [Online]. Geneva: World Health Organisation. Available: http://www.who.int/nutrition/topics/ida/en/index.html.

WHO 2011. Serum ferritin concentrations for the assessment of iron status and iron deficiency in populations. Geneva.

WHO EXPERT CONSULTATION 2004. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *The Lancet***,** 157-163.

WILLETT, W. C., HOWE, G. R. & KUSHI, L. H. 1997. Adjustment for total energy intake in epidemiologic studies. *American Journal of Clinical Nutrition,* 65**,** 1220S.

WILLETT, W. C. & STAMPFER, M. J. 2001. What Vitamins Should I Be Taking, Doctor? *N Engl J Med,* 345**,** 1819-1824.

WILLIS, G., WIMPERIS, J., SMITH, K., FELLOWS, I. & JENNINGS, B. 1999. Haemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet,* 354**,** 221-2.

WILSON, P. W., D'AGOSTINO, R. B., LEVY, D., BELANGER, A. M., SILBERSHATZ, H. & KANNEL, W. B. 1998. Prediction of coronary heart disease using risk factor categories. *Circulation,* 97**,** 1837-1847.

WOODALL, S., JOHNSTON, B., BREIER, B. & GLUCKMAN, P. 1996. Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. *Pediatric Research,* 40**,** 438-443.

WORWOOD, M. 1997. The laboratory assessment of iron status—an update. *Clinica Chimica Acta,* 259**,** 3-23.

WORWOOD, M., SHEARMAN, J. D., WALLACE, D. F., DOOLEY, J. S., MERRYWEATHER-CLARKE, A. T., POINTON, J. J., ROSENBERG, W. M. C., BOWEN, D. J., BURNETT, A. K. & JACKSON, H. A. 1997. A simple genetic test identifies 90% of UK patients with haemochromatosis. *Gut,* 41**,** 841-844.

WU, G., BAZER, F. W., CUDD, T. A., MEININGER, C. J. & SPENCER, T. E. 2004. Maternal nutrition and fetal development. *The Journal of Nutrition,* 134**,** 2169-2172.

WYNN, S. W., WYNN, A. H. A., DOYLE, W. & CRAWFORD, M. A. 1994. The association of maternal social class with maternal diet and the dimensions of babies in a population of London women. *Nutrition and Health,* 9**,** 303-316.

YANG, Z., DEWEY, K. G., LONNERDAL, B., HERNELL, O., CHAPARRO, C., ADU-AFARWUAH, S., MCLEAN, E. D., COHEN, R. J., DOMELLOF, M. & ALLEN, L. H. 2008. Comparison of plasma ferritin concentration with the ratio of plasma transferrin receptor to ferritin in estimating body iron stores: results of 4 intervention trials. *American Journal of Clinical Nutrition,* 87**,** 1892.

YANG, Z. & HUFFMAN, S. L. 2011. Review of fortified food and beverage products for pregnant and lactating women and their impact on nutritional status. *Maternal & Child Nutrition,* 7**,** 19-43.

YURDAKÖK, K., TEMIZ, F., YALÇIN, S. S. & GÜMRÜK, F. 2004. Efficacy of daily and weekly iron supplementation on iron status in exclusively breast-fed infants. *Journal of Pediatric Hematology/Oncology,* 26**,** 284.

ZHANG, J., LEWIS, R., WANG, C., HALES, N. & BYRNE, C. 2005. Maternal dietary iron restriction modulates hepatic lipid metabolism in the fetuses. *Am J Physiol Regul Integr Comp Physiol. ,* 288**,** R104-11.

ZHOU, L.-M., YANG, W.-W., HUA, J.-Z., DENG, C.-Q., TAO, X. & STOLTZFUS, R. J. 1998. Relation of Hemoglobin Measured at Different Times in Pregnancy to Preterm Birth and Low Birth Weight in Shanghai, China. *American Journal of Epidemiology,* 148**,** 998-1006.

ZIAEI, S., NORROZI, M., FAGHIHZADEH, S. & JAFARBEGLOO, E. 2007. A randomised placebo-controlled trial to determine the effect of iron supplementation on pregnancy outcome in pregnant women with haemoglobin ≥ 13.2 g/dl. *BJOG: An International Journal of Obstetrics & Gynaecology,* 114**,** 684-688.

ZIMMERMANN, M. 2008. Methods to assess iron and iodine status. *British Journal of Nutrition,* 99**,** S2-S9.

ZIMMERMANN, M. B. & HURRELL, R. F. 2007. Nutritional iron deficiency. *The Lancet,* 370**,** 511-520.

ZOLLER, H. & COX, T. M. 2005. Hemochromatosis: genetic testing and clinical practice. *Clinical Gastroenterology and Hepatology,* 3**,** 945-958.

10 Appendices

10.1 Electronic search strategy for section 2.2.7.4

Ovid MEDLINE(R) (1950 to February Week 1 2013), EMBASE Classic + EMBASE (1947 to 2010 February 10), and Maternity and Infant Care (1971 to December 2009) databases were searched using the following terms:

#1 pregnan*
#2 gestation*
#3 iron
#4 iron intake
#5 diet
#6 birth weight
#7 birth weight
#8 preterm
#9 small for gestation*
#10 an?emia
#11 birth outcome*
#12 postnatal
#13 1 or 2
#14 3 or 4
#15 6 or 7 or 8 or 9 or 10 or 11 or 12
#19 13 and 14 and 15 and 5

10.2 CARE study Dietary recall form

10.3 Baby VIP study documents

10.3.1 Consent forms

10.3.2 Participant information sheet

10.3.3 Standard Operating Procedure for PWV measurement

10.3.4 Medical information sheet

10.3.5 Lifestyle questionnaire

10.4 Stata code for multiple imputation analysis in ALSPAC

```
misstable patterns, frequency
mi set mlong
mi register imputed pwv fmdp dp sp DVoffspringbmi
mi register regular DVcironRNI
mi set M = 20
mi impute mvn pwv fmdp dp DVoffspringbmi = ciron DVcironRNI, force
replace
mi describe

set level 99
**MI UV analysis**
mi estimate: regress pwv ciron
mi estimate: regress fmdp ciron
mi estimate: regress sp ciron
mi estimate: regress dp ciron
mi estimate: regress DVoffspringbmi ciron
mi estimate: regress cordferr ciron
mi estimate: regress kz030 ciron
mi estimate: regress GA ciron
mi estimate: regress  DVavchildiron ciron

mi estimate: regress pwv  DVcironRNI
mi estimate: regress fmdp  DVcironRNI
mi estimate: regress sp DVcironRNI
mi estimate: regress dp DVcironRNI
mi estimate: regress DVoffspringbmi DVcironRNI
mi estimate: regress cordferr DVcironRNI
mi estimate: regress kz030 DVcironRNI
mi estimate: regress GA DVcironRNI
mi estimate: regress DVavchildiron DVcironRNI

mi estimate: regress DVavchildiron  DVironsupppreg
mi estimate: regress GA  DVironsupppreg
mi estimate: regress kz030  DVironsupppreg
mi estimate: regress cordferr  DVironsupppreg
mi estimate: regress DVoffspringbmi DVironsupppreg
mi estimate: regress sp DVironsupppreg
mi estimate: regress dp DVironsupppreg
mi estimate: regress fmdp DVironsupppreg
mi estimate: regress pwv DVironsupppreg

mi estimate: regress pwv firsthaem
mi estimate: regress fmdp firsthaem
mi estimate: regress sp firsthaem
mi estimate: regress dp firsthaem
mi estimate: regress DVoffspringbmi firsthaem
mi estimate: regress cordferr firsthaem
mi estimate:regress kz030 firsthaem
mi estimate: regress GA firsthaem
mi estimate: regress DVavchildiron firsthaem

mi estimate: regress pwv DVmat_anaemia
mi estimate: regress fmdp DVmat_anaemia
```

```
mi estimate: regress sp DVmat_anaemia
mi estimate: regress dp DVmat_anaemia
mi estimate: regress DVoffspringbmi DVmat_anaemia
mi estimate: regress cordferr DVmat_anaemia
mi estimate: regress kz030 DVmat_anaemia
mi estimate: regress GA DVmat_anaemia
mi estimate: regress DVavchildiron DVmat_anaemia

mi estimate: regress pwv cordferr
mi estimate: regress fmdp cordferr
mi estimate: regress sp cordferr
mi estimate: regress dp cordferr
mi estimate: regress DVoffspringbmi cordferr

mi estimate: regress pwv kz030
mi estimate: regress fmdp kz030
mi estimate: regress sp kz030
mi estimate: regress dp kz030
mi estimate: regress DVoffspringbmi kz030

mi estimate: regress pwv GA
mi estimate: regress fmdp GA
mi estimate: regress sp GA
mi estimate: regress dp GA
mi estimate: regress DVoffspringbmi GA

mi estimate: regress pwv DVavchildiron
mi estimate: regress fmdp DVavchildiron
mi estimate: regress sp DVavchildiron
mi estimate: regress dp DVavchildiron
mi estimate: regress DVoffspringbmi DVavchildiron

**MI MV analysis**
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy ciron
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy ciron
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy ciron
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy ciron
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 cenergy ciron


mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy DVcironRNI
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy DVcironRNI
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy DVcironRNI
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy DVcironRNI
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 cenergy DVcironRNI

****add firsthaem as sensitivity analyses****
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVironsupppreg
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVironsupppreg
```

```
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVironsupppreg
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVironsupppreg
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 DVironsupppreg

mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
firsthaem DVironsupppreg
********
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
firsthaem
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
firsthaem
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
firsthaem
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
firsthaem
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 firsthaem

mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVmat_anaemia
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVmat_anaemia
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVmat_anaemia
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
DVmat_anaemia
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 DVmat_anaemia


**with 3 mediators**
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron ciron
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron ciron
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron ciron
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron ciron
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 cenergy kz030 GA DVavchildiron ciron


mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron DVcironRNI
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron DVcironRNI
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron DVcironRNI
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron DVcironRNI
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 cenergy kz030 GA DVavchildiron DVcironRNI

****add firsthaem as sensitivity analyses****
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVironsupppreg
```

```
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVironsupppreg
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVironsupppreg
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVironsupppreg
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 kz030 GA DVavchildiron DVironsupppreg

mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
firsthaem kz030 GA DVavchildiron DVironsupppreg
********
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron firsthaem
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron firsthaem
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron firsthaem
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron firsthaem
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 kz030 GA DVavchildiron firsthaem

mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVmat_anaemia
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVmat_anaemia
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVmat_anaemia
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron DVmat_anaemia
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 kz030 GA DVavchildiron DVmat_anaemia


**with 4 mediators**
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr ciron
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr ciron
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr ciron
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr ciron
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 cenergy kz030 GA DVavchildiron cordferr ciron


mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr DVcironRNI
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr DVcironRNI
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr DVcironRNI
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
cenergy kz030 GA DVavchildiron cordferr DVcironRNI
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 cenergy kz030 GA DVavchildiron cordferr DVcironRNI

****add firsthaem as sensitivity analyses****
```

```
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVironsupppreg
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVironsupppreg
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVironsupppreg
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVironsupppreg
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 kz030 GA DVavchildiron cordferr DVironsupppreg

mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
firsthaem kz030 GA DVavchildiron cordferr DVironsupppreg
********
mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr firsthaem
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr firsthaem
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kz030 GA DVavchildiron cordferr firsthaem
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 kz030 GA DVavchildiron cordferr firsthaem

mi estimate: regress pwv e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVmat_anaemia
mi estimate: regress fmdp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVmat_anaemia
mi estimate: regress sp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVmat_anaemia
mi estimate: regress dp e695 DVppbmi DVmatsmoke DVmatcatqual b032
kz030 GA DVavchildiron cordferr DVmat_anaemia
mi estimate: regress DVoffspringbmi e695 DVppbmi DVmatsmoke
DVmatcatqual b032 kz030 GA DVavchildiron cordferr DVmat_anaemia
```

10.5 UKWCS-IBPS documents

10.5.1 Letters

10.5.1.1 Letter to mother

10.5.1.2 Letters to participant

10.5.1.3 Participant measurement instructions

10.5.1.4 Letter to GP/practice nurse

10.5.1.5 Standard operating procedure for GP

10.5.2 Measurement forms

10.5.2.1 GP measurement form

10.5.2.2 Self-measurement form

10.5.3 Stata code for the differences between the instrumental variable (IV) and the ordinary least square (OLS) estimates

```
capture program drop comparison
program define comparison, eclass

ivreg2 av_sbp (ferritin = matgeno), robust cluster(Motherid)
local iv_ferritin=_b[ferritin]

xi:regress av_sbp age childage i.childgender ferritin, robust
cluster(Motherid)
local ols_ferritin=_b[ferritin]
ereturn scalar iv_ferritin=`iv_ferritin'
ereturn scalar ols_ferritin=`ols_ferritin'
end

use "C:\Nisreen Laptop\c drive\mednal\My Documents\HH
Study\databases\HHstudy_Sept2011.dta" , clear

bootstrap diff=(e(ols_ferritin)-e(iv_ferritin)) e(ols_ferritin)
e(iv_ferritin), reps(10000) cluster(Motherid) saving("C:\Nisreen
Laptop\c drive\mednal\My Documents\HH Study\databases\ols versus iv
comparison_final.dta", replace every (10)):comparison


ivreg2 av_sbp (ferritin = matgeno), first robust cluster(Motherid)

*************

capture program drop comparison
program define comparison, eclass

ivreg2 av_dbp (ferritin = matgeno), robust cluster(Motherid)
local iv_ferritin=_b[ferritin]

xi:regress av_dbp age childage i.vege i.childgender ferritin, robust
cluster(Motherid)
local ols_ferritin=_b[ferritin]
ereturn scalar iv_ferritin=`iv_ferritin'
ereturn scalar ols_ferritin=`ols_ferritin'
end

use "C:\Nisreen Laptop\c drive\mednal\My Documents\HH
Study\databases\HHstudy_Sept2011.dta" , clear

bootstrap diff=(e(ols_ferritin)-e(iv_ferritin)) e(ols_ferritin)
e(iv_ferritin), reps(10000) cluster(Motherid) saving("C:\Nisreen
Laptop\c drive\mednal\My Documents\HH Study\databases\ols versus iv
comparison_final.dta", replace every (10)):comparison


ivreg2 av_dbp (ferritin = matgeno), first robust cluster(Motherid)

*************
```

```
capture program drop comparison
program define comparison, eclass

ivreg2 av_waist_all (ferritin = matgeno), robust cluster(Motherid)
local iv_ferritin=_b[ferritin]

xi:regress av_waist_all age childage p2bmi i.newclass i.childgender
ferritin, robust cluster(Motherid)
local ols_ferritin=_b[ferritin]
ereturn scalar iv_ferritin=`iv_ferritin'
ereturn scalar ols_ferritin=`ols_ferritin'
end

use "C:\Nisreen Laptop\c drive\mednal\My Documents\HH
Study\databases\HHstudy_Sept2011.dta" , clear

bootstrap diff=(e(ols_ferritin)-e(iv_ferritin)) e(ols_ferritin)
e(iv_ferritin), reps(10000) cluster(Motherid) saving("C:\Nisreen
Laptop\c drive\mednal\My Documents\HH Study\databases\ols versus iv
comparison_final.dta", replace every (10)):comparison


ivreg2 av_waist_all (ferritin = matgeno), first robust
cluster(Motherid)

*************

capture program drop comparison
program define comparison, eclass

ivreg2 bmi (ferritin = matgeno), robust cluster(Motherid)
local iv_ferritin=_b[ferritin]

xi:regress bmi age childage p2bmi i.newclass i.childgender ferritin,
robust cluster(Motherid)
local ols_ferritin=_b[ferritin]
ereturn scalar iv_ferritin=`iv_ferritin'
ereturn scalar ols_ferritin=`ols_ferritin'
end

use "C:\Nisreen Laptop\c drive\mednal\My Documents\HH
Study\databases\HHstudy_Sept2011.dta" , clear

bootstrap diff=(e(ols_ferritin)-e(iv_ferritin)) e(ols_ferritin)
e(iv_ferritin), reps(10000) cluster(Motherid) saving("C:\Nisreen
Laptop\c drive\mednal\My Documents\HH Study\databases\ols versus iv
comparison_final.dta", replace every (10)):comparison


ivreg2 bmi (ferritin = matgeno), first robust cluster(Motherid)
```