UNIVERSITAT ROVIRA I VIRGILI

EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS AND CHILD NEURODEVELOPMENT

Lucía Iglesias Vázquez

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EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS AND CHILD NEURODEVELOPMENT

LUCÍA IGLESIAS VÁZQUEZ

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EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS AND CHILD NEURODEVELOPMENT

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ROVIRA i VIRGILI

FAIG CONSTAR que aquest treball, titulat "EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS AND CHILD NEURODEVELOPMENT", que presenta Lucía Iglesias Vázquez per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Ciències Mèdiques Bàsiques d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS AND CHILD NEURODEVELOPMENT", que presenta Lucía Iglesias Vázquez para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Ciencias Médicas Básicas de esta universidad.

I STATE that the present study, entitled "EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS AND CHILD NEURODEVELOPMENT", presented by Lucía Iglesias Vázquez for the award of the degree of Doctor, has been carried out under my supervision at the Department of Basic Medical Sciences of this university.

Reus, 20 Juliol 2023/ Reus, 20 Julio 2023 / Reus, July 20th 2023

El/s director/s de la tesi doctoral
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ABSTRACT **13**

INTRODUCTION **17**

IRON 19

Iron functions in the body 19
Iron metabolism 20
Iron status in pregnancy 22
Iron requirements 22
Deficit and excess: causes and consequences 23
Iron status biomarkers 26
Prevalence of different iron states 27
Factors associated with maternal iron status 28
Biological factors 28
Genetic factors 29
Sociodemographic factors 30
Lifestyle factors 31

CHILD NEURODEVELOPMENT 33

Stages and milestones 33
Prenatal and perinatal periods 33
Infancy 34
Toddlerhood 35
Early childhood 36
Neurodevelopmental disabilities 36
Scales used for neurodevelopmental assessment 38
Scales of neurodevelopment in infancy 39
Scales of intelligence and neuropsychological functions in early childhood 41
Behavioural and emotional problems in early childhood 43

PRENATAL IRON SUPPLEMENTATION 48

Effects on maternal iron status 48
Effects on child neurodevelopment 50
Routine vs. individualized prenatal iron supplementation 50

HYPOTHESIS AND OBJECTIVES **53**

METHODOLOGY **59**

ECLIPSES AND ECLIPSES-NEN STUDIES 61
MOBA COHORT 69

RESULTS **71**

CHAPTER 1. WHAT IS KNOWN ABOUT THE EFFECT OF PRENATAL IRON STATUS ON CHILD NEURODEVELOPMENT? 73

Effects of prenatal iron status on child neurodevelopment and behavior: A systematic review 75

CHAPTER 2. DETERMINANTS OF MATERNAL IRON STATUS 89

Maternal factors associated with iron deficiency without anaemia in early pregnancy: ECLIPSES study 91

Iron status in mid-pregnancy and associations with interpregnancy interval, hormonal contraceptive use, dietary factors, and supplement use: A prospective pregnancy cohort study 101

CHAPTER 3. EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS 115

Effectiveness of different doses of iron supplementation and prenatal determinants of maternal iron status in Spanish women: ECLIPSES Study 117

CHAPTER 4. EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON CHILD NEURODEVELOPMENT 139

Adapting prenatal iron supplementation to maternal needs results in optimal child neurodevelopment: a follow-up of the ECLIPSES study 141

Importance of maternal iron status on the improvement of cognitive function in children after prenatal iron supplementation 157

Prenatal iron supplementation adjusted to maternal iron stores in early pregnancy improves behaviour in children at 4 years of age: ECLIPSES study 171

SUMMARY OF THE RESULTS **205**

GENERAL DISCUSSION **211**

Effect of adjusting prenatal iron supplementation on maternal iron status 214
Effect of adjusting prenatal iron supplementation on child neurodevelopment 219
Strengths and limitations 223

CONCLUSIONS **227**

REFERENCES **233**

ANNEXES **259**

ANNEX 1. OTHER SCIENTIFIC CONTRIBUTIONS 261
ANNEX 2. INTERNATIONAL RESEARCH COLLABORATIONS 263

Abstract

Justification: Both prenatal iron deficiency (ID) and excess have been associated with negative health consequences for mothers and children, such as reduced cognitive, language and motor skills, as well as behavioural problems in childhood. Prenatal iron supplementation has proven beneficial for cognitive and behavioural development in developing countries and anaemic women. However, routine supplementation in well-nourished women without ID may not provide clear benefits and could potentially harm child neurodevelopment. The debate between routine and personalized iron supplementation for non-anaemic women during early pregnancy continues, with limited research on the topic.

Objective: The main aim of this thesis was to evaluate the effect of adjusting prenatal iron supplementation to individual needs on iron status at the end of gestation and children's neurodevelopment, by involving data from the ECLIPSES and ECLIPSES-NEN studies (Spain). A secondary objective was to determine the potential predictors of maternal iron status in early- and mid-pregnancy, which was assessed using data from the ECLIPSES study and the MoBa Cohort (Norway).

Main conclusions: The results indicate that adjusting iron supplementation improved maternal iron status, reducing both deficiency and excess in late pregnancy. This approach also enhanced cognitive functioning and reduced behavioural and psychological problems in children at age 4. Factors such as body mass index, parity, smoking, and diet were found to be associated with iron deficiency without anaemia during pregnancy, emphasizing the need to manage iron status in specific subgroups. Assessing serum ferritin levels and, sometimes, genetic conditions would help determine appropriate iron supplementation doses. In summary, the thesis highlights the importance of personalised iron supplementation during pregnancy considering individual factors when prescribing iron supplements and shows the long-term benefits on infant neurodevelopment.

Introduction

IRON

Iron functions in the body

Iron is an essential mineral that plays a critical role in various functions of the human body. It is required for the synthesis of haemoglobin, a protein found in red blood cells (RBCs) that carries oxygen from the lungs to other parts of the body (Camaschella et al., 2022; Singh, 2018), and myoglobin, which is a protein that helps muscles store and use oxygen (Vanek & Kohli, 2022). In addition to its role in oxygen transport, iron is necessary for DNA synthesis, as it is involved in the production of deoxyribonucleotides, the building blocks of DNA (Puig et al., 2017; C. Zhang, 2014). Iron is also crucial for the proper functioning of the immune system as it is needed for the growth and differentiation of immune cells and regulates the production of cytokines, which are signalling molecules involved in the immune response (Gombart et al., 2020; Ni et al., 2022). Additionally, iron is involved in the production of adenosine triphosphate, the primary source of energy for the body's cells (Paul et al., 2017; S. Zhang et al., 2022), as well as in the synthesis of collagen, a protein that provides structure and support to the skin, bones, muscles, and other tissues in the body (O'Dell, 1981; Wright et al., 2014).

Iron is also essential for brain development and functioning (McCann et al., 2020). First, iron is involved in the production of myelin, a fatty substance that covers nerve fibres, which helps in the transmission of nerve impulses. The development of myelin is critical for the proper functioning of the brain and nervous system (Cheli et al., 2020; Santiago González et al., 2019). Furthermore, iron plays a crucial role in the formation of neurotransmitters such as dopamine, norepinephrine, and serotonin, which are the chemical messengers that allow nerve cells to communicate with each other. Proper communication between nerve cells is essential for the healthy functioning of the brain and the body (Ferreira et al., 2019; Kim & Wessling-Resnick, 2014).

Iron metabolism

Iron is an essential mineral required for many important biological processes in the body. However, because excess iron can be toxic, the body has a highly regulated system for iron metabolism to maintain the proper balance of iron levels (Anderson & Frazer, 2017; Fisher & Nemeth, 2017).

Iron metabolism begins with iron ingestion. Dietary iron exists in two primary forms: heme iron and non-heme iron, with the former being predominant in animal-based foods and the latter in plant-based foods. Although heme iron is less abundant in Western diets than non-heme iron, it is more bioavailable, so the body can absorb and utilize it more efficiently. Non-heme iron bioavailability is affected by several factors, such as the presence of other dietary components in the meal that can enhance or inhibit its absorption. Factors that can enhance non-heme iron absorption include vitamin C, meat, fish, and poultry. Vitamin C, for example, can form a complex with non-heme iron, increasing its solubility and bioavailability. Meat, fish, and poultry contain heme iron, which can enhance the absorption of non-heme iron through a mechanism known as the "meat factor". On the other hand, factors that can inhibit non-heme iron absorption include phytates, polyphenols, calcium, and tannins. Phytates, which are found in grains, legumes, and nuts, can bind to non-heme iron, forming an insoluble complex that is not easily absorbed by the body (Hooda et al., 2014; Piskin et al., 2022).

Iron absorption takes place in the small intestine, following which it is transported to the liver and stored in a protein called ferritin. When the body requires more iron, it mobilizes stored iron from ferritin and releases it into the bloodstream. Iron exists in the bloodstream in two main forms: bound to transferrin and contained within RBCs. Transferrin, synthesized in the liver, is a protein that binds to iron and delivers it to cells throughout the body, where it is needed for various metabolic processes, including the production of new RBCs. Once the iron is delivered to the cells, it is incorporated into various proteins, including haemoglobin. Haemoglobin is an essential protein found in RBCs that binds to oxygen and transports it throughout the body. In fact, approximately two-thirds of the iron in the body is found in RBC because they need it to produce haemoglobin. The lifespan of RBCs is approximately 120 days, after which they are broken down, and their components, including iron, are recycled. The iron from the broken-down RBCs can be reused to make new RBCs or stored in the body for later use (Abbaspour et al., 2014; Fisher & Nemeth, 2017).

The regulation of iron metabolism is a complex process that involves multiple proteins and pathways. One key regulator protein is hepcidin, which is produced by the liver in response to various signals, including iron levels and inflammation. Hepcidin functions by inhibiting iron absorption in the proximal small intestine and the cellular efflux of iron, helping to maintain the proper balance of iron in the body (Collins et al., 2008; Liu et al., 2016; Silva & Faustino, 2015). Hepcidin expression is tightly regulated, with iron deprivation and increased erythropoietic demand leading to down-regulation and increased body iron stores, infection, or inflammation leading to up-regulation (Collins et al., 2008). Hepcidin regulation is carried out by a complex of interacting proteins including the haemochromatosis (HFE) protein, which is encoded in the *HFE* gene (Barton et al., 2015; Hollerer et al., 2017). Mutations in the *HFE* gene result in a deficit in hepcidin release or activity and, ultimately, excessive iron absorption. Iron excess accumulates in the organs resulting in tissue damage and increasing the risk of various conditions such as cirrhosis, diabetes, and heart disease (Barton et al., 2015; Hanson et al., 2001; Silva & Faustino, 2015).

Overall, disruptions in iron metabolism can lead to various health conditions, including iron deficiency and iron excess, which can have adverse effects on overall health and well-being (Abbaspour et al., 2014; Dev & Babitt, 2017).

Iron status in pregnancy

Iron requirements

During pregnancy, iron requirements increase substantially to support the growing foetus and the mother's changing physiology (Fisher & Nemeth, 2017). The amount of iron required during pregnancy depends on various factors, such as the mother's pre-pregnancy iron status, the number of foetuses, and the duration of pregnancy (Fisher & Nemeth, 2017). The recommended dietary allowance (RDA) for iron during pregnancy is 27 mg/day, which is almost double the RDA for non-pregnant women (National Institutes of Health, 2022). However, dietary iron intake is generally insufficient to meet the recommendations during pregnancy, making it necessary to use iron supplements in most cases (Brannon & Taylor, 2017).

As mentioned earlier, iron requirements increase significantly from the first to the third trimester of pregnancy. During the first trimester, iron requirements decrease because of the cessation of menstruation. However, the obligatory losses of iron from the body via the gut, skin, and urine still need to be met during this period. In contrast, iron requirements increase substantially during the second and third trimesters due to the rapid foetal growth and the expansion of maternal red blood cell mass. In total, it has been estimated that the body iron requirement for an average pregnancy is approximately 1,000 mg (Bothwell, 2000; Fisher & Nemeth, 2017).

The increased iron requirement during pregnancy is met by both increased absorption and mobilization of iron from the maternal stores. Nevertheless, women with sufficient body iron reserves have lower absorption than those with depleted reserves, since increased absorption is partly due to progressive iron depletion (Piskin et al., 2022). Therefore, it is crucial for women to enter pregnancy with sufficient iron stores to support foetal growth and avoid iron deficiency anaemia (Bothwell, 2000; Fisher & Nemeth, 2017).

Iron supplementation is often routinely recommended during pregnancy to meet increased iron requirements and prevent pregnant women from iron deficiency. The World Health Organization recommends daily iron and folic acid supplementation for pregnant women to prevent anaemia and improve a wide range of maternal and child health outcomes (Fisher & Nemeth, 2017; World Health Organization et al., 2012).

Deficit and excess: causes and consequences

Iron deficiency is a condition that results from a decrease in the body's iron stores, leading to insufficient iron to meet physiological needs (Camaschella, 2019). Iron deficiency can occur due to various factors such as inadequate dietary intake, malabsorption, or increased iron loss from menstrual bleeding (Camaschella, 2019). In the early stages of iron deficiency, iron stores in the liver, spleen, and bone marrow are depleted, leading to a decrease in circulating iron (Abbaspour et al., 2014). As iron stores continue to be depleted, the body's ability to produce haemoglobin is compromised, which can ultimately result in a decrease in RBC and iron deficiency anaemia, the most frequent form of anaemia (M. Wang et al., 2022). While iron deficiency is the main cause of anaemia, accounting for about 50% of cases, it can also occur without anaemia. In such cases, there is insufficient iron to meet the body's physiological needs, but haemoglobin levels remain within the normal range (M. Wang et al., 2022). Iron deficiency without anaemia can also have severe consequences, especially in vulnerable populations such as pregnant women and developing foetuses (Al-Naseem et al., 2021; Balarajan et al., 2011).

Extensive research has been conducted on iron deficiency during pregnancy, and its negative impact on both maternal and child health has been well-documented (Benson et al., 2022). Iron deficiency and iron deficiency anaemia can cause mild complications like fatigue and weakness, but also serious events such as preeclampsia, preterm delivery, miscarriage, and postpartum depression (Azami et al., 2019; Smith et al., 2019). Maternal iron deficiency can also lead to physical developmental issues such as preterm birth, low birth weight, and small-for-gestational-age infants, which can have long-term effects on growth and development (Means, 2020; Quezada-Pinedo et al., 2021). Additionally, impaired immune function in infants and children has been linked to maternal iron deficiency (Quezada-Pinedo et al., 2021). Furthermore, although research on the effects of maternal iron status on child neurodevelopment is still limited and inconclusive compared to other child health outcomes (McCann et al., 2020), the evidence indicates that prenatal exposure to iron deficiency is associated with impaired cognitive and motor function, lower intelligence quotient (IQ) scores, reduced attention span, and more psychological problems (Doom & Georgieff, 2014; Janbek et al., 2019; McWilliams et al., 2022; Radlowski & Johnson, 2013). Furthermore, the timing of iron deficiency during pregnancy is a critical factor in determining its effects on neurodevelopment. Different alterations are observed depending on whether iron deficiency occurs in early or late pregnancy (Doom & Georgieff, 2014; Radlowski & Johnson, 2013).

On the other hand, haemoconcentration (or iron excess) is a condition in which the concentration of blood components, including RBC and plasma proteins, increases without a corresponding increase in plasma volume. This occurs naturally in the third trimester of pregnancy due to increased demand for oxygen and nutrients by the growing foetus, resulting in a relative decrease in plasma volume and an increase in the concentration of blood components. Haemoconcentration is a normal physiological adaptation to pregnancy, but excessive haemoconcentration can lead to complications (Hans, 2016).

Although not as extensively studied as iron deficiency, the effects of excess iron during pregnancy have recently begun to gain prominence (Quezada-Pinedo et al., 2021). Some studies have shown that prenatal haemoconcentration can lead to adverse events similar to those of iron deficiency, such as pre-eclampsia, preterm birth and low birth weight (Dewey & Oaks, 2017; Quezada-Pinedo et al., 2021). Additionally, high haemoglobin levels during pregnancy can results in gestational diabetes mellitus and high blood pressure, according to recent research (Sissala et al., 2022). Emerging evidence suggests that haemoconcentration can also be associated with impaired child cognition, motor development and behavioural problems in the offspring (Quezada-Pinedo et al., 2021). The proposed mechanisms underlying these effects are as follows: firstly, excess iron can cause an increase in maternal blood viscosity, which can hinder the delivery of nutrients and oxygen to the developing foetus. This can compromise foetal development, including foetal hypoxia and growth restriction. In addition, it has been observed that excess iron can cause oxidative stress and inflammation in the developing brain, which can damage brain cells and tissues, leading to impaired neurodevelopment (Dewey & Oaks, 2017; Quezada-Pinedo et al., 2021).

Overall, there is emerging evidence on a U-shaped curve for the risk of adverse pregnancy and birth outcomes with maternal iron status during pregnancy. That is, both iron deficiency and excess during pregnancy can lead to adverse maternal and infant health outcomes, with sometimes wide-ranging and long-lasting consequences (Dewey & Oaks, 2017; Quezada-Pinedo et al., 2021).

Iron status biomarkers

Iron status biomarkers are essential laboratory tests that evaluate the body's iron stores and metabolism, crucial in diagnosing and managing iron deficiency and excess. The most frequently used biomarkers include serum ferritin, transferrin saturation, and haemoglobin (Pfeiffer & Looker, 2017).

Serum ferritin levels are generally considered the most reliable indicator of iron stores. For pregnant women, the World Health Organization defines iron deficiency as serum ferritin levels below 15 ng/mL in the first trimester (World Health Organization, 2020). Given that plasma volume expansion occurs from the second trimester onwards, a threshold of 12 ng/mL is more appropriate for the second and third trimesters of gestation (Daru et al., 2017; World Health Organization, 2020).

Transferrin saturation assesses the proportion of transferrin that is saturated with iron. Levels above 45% may suggest iron excess, while levels below 20% indicate iron deficiency. Nonetheless, transferrin saturation levels can also be impacted by inflammation and other factors, emphasizing the need to consider the patient's overall clinical picture (Elsayed et al., 2016).

However, both serum ferritin concentration and transferrin saturation can be influenced by inflammation, making it crucial to interpret the results in the context of the patient's clinical history (Gosdin et al., 2022; Pfeiffer & Looker, 2017). Since serum ferritin is an acute-phase reactant, its levels may increase during inflammation even in the presence of iron deficiency, which can result in a misdiagnosis of iron excess (Pfeiffer & Looker, 2017). Similarly, transferrin saturation may decrease due to inflammation, leading to an underestimation of iron status (Pfeiffer & Looker, 2017). To address these potential confounding factors, measuring C-reactive protein can be useful for accurately interpreting biomarkers of iron status. C-reactive protein is an acute-phase protein synthesized by the liver in response to inflammation (Sproston &

Ashworth, 2018). Elevated levels of C-reactive protein indicate inflammation and taking them into account can help correct serum ferritin and transferrin saturation values, providing a more accurate assessment of iron status (Pfeiffer & Looker, 2017).

Finally, haemoglobin levels are commonly used to diagnose and monitor iron deficiency anaemia (Benoist et al., 2008). For pregnant women, the World Health Organization has defined anaemia as haemoglobin levels below 110 g/L, while the cut-off point for iron haemoconcentration at the end of pregnancy is set at 130 g/L (Benoist et al., 2008). However, it is important to note that factors other than iron deficiency can also affect haemoglobin levels, such as inflammation, chronic diseases, and genetic conditions. Therefore, haemoglobin levels must always be evaluated in conjunction with the patient's medical history, symptoms, and other laboratory findings. In addition, screening for *HFE* gene mutations in cases of elevated haemoglobin levels in early pregnancy may also help to detect women at increased risk of haemoconcentration.

Prevalence of different iron states

Anaemia is a major public health concern globally, affecting 30% of reproductive-age women and 38% of pregnant women worldwide, according to the most updated estimates (Stevens et al., 2013). Pregnant women are particularly vulnerable to this condition due to the increased physiological demand for iron during pregnancy, as aforementioned. In Europe, the prevalence of anaemia among pregnant women varies by region and country, with an overall estimate of around 25%, predominantly caused by iron deficiency (World Health Organization, 2015).

Iron deficiency without anaemia is also a common issue among pregnant women (Al-Naseem et al., 2021). Unfortunately, this condition often goes undiagnosed and untreated due to the need for further testing beyond routine haemoglobin measurements (Soppi, 2018). Consequently, obtaining updated estimates regarding the prevalence of iron deficiency without anaemia is challenging. However, it has been reported that 10-32% of pregnant women in Europe experience iron deficiency (Milman et al., 2017).

Regarding haemoconcentration, while there is no specific prevalence data available in pregnancy in Europe, estimates ranging from 8.7% to 42% have been reported in different studies (Arija et al., 2013; Peña-Rosas & Viteri, 2009). The variation in prevalence may be due to differences in study populations and diagnostic criteria. Nonetheless, it highlights the importance of regular monitoring of haemoconcentration levels in pregnant women to ensure the safety of both the mother and the developing foetus.

Factors associated with maternal iron status

Several factors can affect maternal iron status during pregnancy, including biological and genetic conditions, sociodemographic characteristics, and lifestyle. While some of these factors tend to be common among populations around the world, others are very specific to each population.

Biological factors

Biological factors such as the mother's age, body mass index (BMI), and parity have been found to be associated with iron status. Younger mothers (da Costa et al., 2016; Loy et al., 2019) and those who have had multiple pregnancies (Al-Farsi et al., 2011; Imai, 2020) are at increased risk of iron deficiency. As for BMI, a high BMI (defined as a BMI of 25 or greater) is associated with an increased risk of iron deficiency due to several reasons. Adipose tissue produces hepcidin, a hormone that regulates iron absorption and metabolism. Higher levels of adipose tissue can lead to increased production of hepcidin, which can decrease iron absorption and contribute to iron deficiency (Cepeda-Lopez & Baye, 2020). Additionally, obesity-related inflammation can interfere with iron metabolism and contribute to iron deficiency (Aigner et al., 2014). On the other hand, a low BMI (defined as a BMI of less than 18.5) is also associated with an increased risk of iron deficiency. Low BMI can be indicative of poor nutritional status, including inadequate iron intake. Additionally, low BMI can be a symptom of underlying medical conditions, such as celiac disease, that can interfere with iron absorption and contribute to iron deficiency (Mayasari et al., 2021; Tan et al., 2018).

Genetic factors

Genetic alterations affecting iron status, particularly those associated with iron excess, have become an increasingly recognized area of interest in the field of haematology and have shed light on the complex interplay between genetic and environmental factors in regulating iron metabolism. As mentioned above, one such genetic condition is mutation in the *HFE* gene, which encode a protein involved in regulating the hepcidin expression (Barton et al., 2015; Hollerer et al., 2017; Silva & Faustino, 2015). Mutations in the *HFE* gene have been identified, therefore, as a risk factor for hereditary hemochromatosis, a disorder characterized by reduced hepcidin production and, eventually, excessive iron absorption and storage, which result in iron deposits and tissue damage (Barton et al., 2015; Hanson et al., 2001). The most common *HFE* mutations are C282Y and H63D (Bacon & Britton, 2008; Pedersen & Milman, 2009). Homozygosity for C282Y allele accounts for 80-90% of cases of hereditary haemoconcentration in European populations (Crownover & Covey, 2013; Petitti, 2009). However, the C282Y mutation has a frequency of approximately 5% among Europeans, with a decreasing gradient from north to south. In contrast, the H63D variant in *HFE* is more prevalent than C282Y, with an allele frequency of between 10 and 20% among Europeans (Merryweather-Clarke et al., 2000). It has a broader geographical distribution and is present in varying frequencies in most populations worldwide (Lucotte & Dieterlen, 2003; Merryweather-Clarke et al., 2000). Although generally not considered pathogenic, as most homozygous individuals do not exhibit iron overload, compound heterozygosity for C282Y and H63D can lead to increased haemoglobin levels (Walsh et al., 2006). In general, an elevated haemoglobin concentration can lead to an increased risk of blood clots and decreased blood flow, which can result in complications such as stroke, myocardial infarction, or pulmonary embolism (Folsom et al., 2020; Hultcrantz et al., 2020). These conditions become particularly important during pregnancy because of the implication of excess iron for their maintenance and ultimately for maternal and infant health outcomes, as explained above (Dewey & Oaks, 2017; Quezada-Pinedo et al., 2021; Sissala et al., 2022). In the context of pregnancy, an additional concern arises regarding iron supplementation in women carrying *HFE* mutations. Given that these women may be at increased risk of iron excess, the evidence suggests that routine iron supplementation should be avoided. Instead, their iron status should be carefully monitored during pregnancy, allowing iron supplementation to be planned according to their actual needs (Milman, 2021; Shamas, 2023).

Sociodemographic factors

Sociodemographic factors such as socioeconomic status, education level, and ethnicity have also been found to be associated with maternal iron status. Research has shown that women living in low-income countries or even in low-income neighbourhoods from developed countries are at higher risk of iron deficiency during pregnancy, compared to those living in non-low-income areas (Oyelese et al., 2021; Quezada-Pinedo et al., 2022; Sunuwar et al., 2020; VanderMeulen et al., 2021). Similarly, women with lower levels of education may also be at increased risk of iron deficiency during pregnancy. This may be due to the lack of knowledge about proper nutrition during pregnancy, limited access to nutritious foods or prenatal supplements containing iron, and limited access to healthcare services (Teichman et al., 2021). A high prevalence of other risk factors such as infections and parasitic diseases can also contribute to iron deficiency in developing world (Steketee, 2003).

Certain ethnic groups, including African American and Hispanic women, have also been identified as having higher risk of iron deficiency during pregnancy. These populations may have genetic or cultural factors that contribute to a higher risk of iron deficiency and other socioeconomic factors that increase the risk (Kang et al., 2021; Quezada-Pinedo et al., 2022).

Lifestyle factors

Lifestyle factors such as dietary intake and physical activity levels can also affect maternal iron status. Iron-rich foods such as red meat, poultry, and fish can help maintain adequate iron levels, while consuming foods that inhibit iron absorption, such as tea and coffee, can lead to iron deficiency (Piskin et al., 2022). Physical activity levels during pregnancy can also impact iron status, with sedentary behaviour associated with a higher risk of iron deficiency (Crouter et al., 2012). While exercise stimulates the release of the hormone erythropoietin, increasing iron absorption to improve the oxygen transport capacity (Mairbäurl, 2013), sedentary behaviour is associated with obesity and obesity-related comorbidities, including inflammation which can result in decreased iron absorption and utilization (Burini et al., 2020).

Toxic lifestyle habits, such as smoking and excessive alcohol consumption, can also affect iron status leading to either iron deficiency or iron excess. As for smoking, the mechanism underlying this effect involves a combination of factors, including the direct toxicity of cigarette smoke and the indirect effects of smoking on iron metabolism. First, there is wide evidence that smoking induces lung and systemic iron dysregulation (Lee et al., 2016; Malenica et al., 2017; W. Zhang et al., 2020).

Additionally, smoking can cause inflammation, which can further reduce the absorption and utilization of iron (Elisia et al., 2020). On the other hand, smoking reduces the expression of hepcidin, leading to increased iron absorption and storage over time (Chełchowska et al., 2016). Chronic alcohol consumption can lead to iron deficiency by affecting the absorption and utilization of iron in the body. Firstly, alcohol can damage the cells lining the stomach and intestines, which can reduce the absorption of iron from the diet (Bishehsari et al., 2017; Koop, 2006). Additionally, alcohol can interfere with the metabolism of iron in the liver and, like smoking, lead to hepcidin down-regulation (Mehta et al., 2016; Ohtake et al., 2007). This can result in a decreased amount of iron available for the formation of new RBC, leading to anaemia (Collins et al., 2008; Koenig et al., 2014). Otherwise, excessive alcohol consumption can result in an accumulation of iron in the liver cells, leading to iron overload and damaging the tissues (Ioannou et al., 2004; Whitfield et al., 2001).

CHILD NEURODEVELOPMENT

Stages and milestones

Child neurodevelopment refers to the complex process by which a child's brain and nervous system grow and develop, beginning *in utero* and continuing throughout childhood and adolescence. However, the period more important for the foundations of neurodevelopment across an individual's lifespan is the first 1000 days of life. During this period, the brain undergoes a rapid and dynamic process of growth and maturation, laying the foundation for lifelong cognitive, social, and emotional functioning (Cusick & Georgieff, 2016).

Prenatal and perinatal periods

During the prenatal and perinatal periods, the foundation for a child's neurological and cognitive development is laid down. These are critical periods when the brain develops rapidly as most of the neurons are formed. Neuronal differentiation, synapse production and myelination start early in foetal life to become very active in the last trimester of gestation and the first year of age. Thereafter, these processes continue at a slower pace (Hadders-Algra, 2022). Foetal brain development is a complex process that can be influenced by a variety of environmental factors, including maternal stress, malnutrition, and exposure to toxins. These factors can have a profound impact on foetal programming, which can lead to alterations in the normal neurodevelopmental process of the child (Faa et al., 2016; Vasistha & Khodosevich, 2021). Prenatal and perinatal insults, such as prematurity, hypoxia, infection, gestational diabetes mellitus, or nutritional deficiencies, can therefore disrupt this process and have important irreversible short- and long-term consequences (Mwaniki et al., 2012). These adverse environmental factors together with a genetic risk predispose to the appearance of psychopathological conditions that emerge early in life and persist throughout adulthood: neurodevelopmental disorders (Chen et al., 2021; Cortés-Albornoz et al., 2021; Makris et al., 2023).

Infancy

Infancy typically refers to the first year of a child's life, from birth to 12 months old. In addition to the rapid growth of the brain and the establishment of neural networks, infancy is a critical period for the development of sensory perception (Hadders-Algra, 2022). Infants are born with all the neurons they will ever have, but the connections between those neurons are not yet fully formed. During infancy, the brain makes millions of new connections every second as it responds to sensory input from the environment (Hadders-Algra, 2022). This period is also associated to synapse elimination through a process called synaptic pruning, which helps to refine and strengthen the remaining neural networks that will underpin all future cognitive, social, and emotional functioning (Neniskyte & Gross, 2017).

Basic cognitive functions such as attention, memory, and language are also rapidly developing during infancy. Infants can recognize and respond to familiar faces and voices, track moving objects with their eyes, and begin to differentiate between different sounds and tones of voice. They also begin to babble and imitate sounds, laying the foundation for later language development (G. D. Reynolds & Romano, 2016).

Infants undergo rapid changes in motor development during this period (Hadders-Algra, 2022), which makes them able to explore their environment more actively and gain greater independence. Motor development is an important manifestation of the integrity and functionality of the central nervous system which makes it important not only for physical activity but also for cognitive and social development (Adolph & Hoch, 2019).

Toddlerhood

Toddlerhood usually refers to the period between 12 months and 36 months of age. During this time, the brain continues to undergo rapid growth and development, particularly in the areas of language and social-emotional functioning. Toddlers begin to develop a sense of self-awareness and self-identity, which is reflected in their increasing desire for independence and autonomy (US National Research Council & US Institute of Medicine, 2000a). They also begin to develop a better understanding of the emotions and perspectives of others, which is essential for developing positive social relationships and empathy (Zubler et al., 2022).

Language development progresses rapidly during toddlerhood, with toddlers expanding their vocabulary and developing more complex grammatical structures (US National Research Council & US Institute of Medicine, 2000b). They begin to use simple sentences to express their wants and needs and engage in basic conversations with others. They also begin to develop the ability to understand and follow simple instructions (Zubler et al., 2022).

In addition to language and social-emotional development, early childhood is a critical period for the development of executive functions, which are higher-order cognitive processes that enable goal-directed behaviour and self-regulation (Zubler et al., 2022). These functions mature until early adulthood and include working memory, inhibitory control, and cognitive flexibility, all of which are essential for academic and social success. Working memory allows children to hold information in their minds while completing a task, inhibitory control enables them to resist impulsive behaviours, and cognitive flexibility enables them to shift their attention and adapt to changing situations (Diamond, 2013; Hughes et al., 2020).

Early childhood

Early childhood usually refers to the period between 3 and 6 years of age and is a time of important behavioural development and psychological growth in addition to cognitive development (Zubler et al., 2022). Children learn how to interact with others and develop important social skills (Development, 2000; US National Research Council & US Institute of Medicine, 2000a). They also begin to establish their own identity and develop a sense of self. One important aspect of behavioural development in childhood is the emergence of emotional regulation. Children learn how to manage their emotions and express them appropriately, which is essential for building healthy relationships and coping with stress (Zubler et al., 2022). However, some children may struggle with emotional regulation, which can lead to behavioural problems such as aggressiveness and emotional problems such as anxiety (Ogundele, 2018).

Neurodevelopmental disabilities

From a diagnostic point of view, both the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) from the American Psychiatric Association (American Psychiatric Association, 2013) and the International Classification of Diseases 11th Revision (ICD-11) from the World Health Organization (World Health Organization, 2022) define neurodevelopmental disorders as a group of conditions with onset in the developmental period, inducing persistent deficits that produce impairments of functioning. Neurodevelopmental disorders result in difficulties in personal, social, academic, and occupational functioning, ultimately affecting the overall quality of life (Löytömäki et al., 2022).

Neurodevelopmental disorders include a wide range of diagnoses, the most common being intellectual disability, autism spectrum disorder (ASD), communicative disorders, attention deficit hyperactivity disorder, and specific learning disabilities (American Psychiatric Association, 2013). Moreover, it is not uncommon for individuals with neurodevelopmental disorders to present comorbidity with other neurodevelopmental disabilities as well as a higher risk for suffering psychopathological problems (Cainelli & Bisiacchi, 2022; David et al., 2022). These aspects, together with the variability in the clinical spectrum, make it difficult the fulfilment of diagnostic criteria of a neurodevelopmental disorder according to current international classifications such as the DSM-5 or the ICD-11. Regardless of the diagnosis, however, minor difficulties in several areas of neurodevelopment are common in children, and this leads to differences in one or more aspects of development, such as language, social interactions, cognitive skills, and behavior, which may also have some impact on their academic, social and emotional functioning. These dysfunctions are part of the neurodiversity in the population and may also be the result of the interaction of risk and protective factors at the prenatal and perinatal, and postnatal levels (Diamond, 2013; Löytömäki et al., 2022). They may also experience difficulties with executive functioning including sensory processing, attention, and self-regulation in a large variability of severity levels (Crisci et al., 2021). Cognitive and behaviour dysfunctions associated to neurodevelopmental vulnerabilities often change or evolve as a child grows older and may present differently in each individual (Morris-Rosendahl & Crocq, 2020).

Although the aetiology of the neurodevelopmental disorders or disturbances is still fully unknown, these conditions are thought to result from a combination of genetic, environmental, and neurological factors that impact brain development during foetal life, infancy, and childhood (De Felice et al., 2015; Reichard & Zimmer-Bensch, 2021). As already mentioned, the prenatal stage is a critical period, as adverse events affecting the neurodevelopmental process at that time may have irreversible consequences later in life (Faa et al., 2016).

In the present thesis, diagnoses of neurodevelopmental disorders have not been made. However, the degree of development of neuropsychological functions, IQ, as well as the symptoms of risk of Attention-Deficit/Hyperactivity Disorder (ADHD), ASD, and behavioural and emotional problems have been assessed.

Scales used for neurodevelopmental assessment

Assessing a child's neurodevelopment, cognitive abilities, behaviour, and psychological problems in infancy and childhood presents unique challenges due to the developmental changes that occur during these stages, which require age-appropriate measures. Assessing neurodevelopment in newborns presents additional challenges, as they have limited ability to engage in any behavioural or cognitive tasks. However, some measures have been developed to assess the neurological functioning of newborns, such as the assessment of reflexes and motor activity. As newborns grow and develop, they acquire new skills and abilities, and the assessment methods must adapt to their changing needs. For example, as infants develop more complex motor, language, and social skills, assessment methods must be adapted to measure these domains of development (Hadders-Algra, 2021).

Different scales exist to assess neurodevelopment in infancy and early childhood, as well as cognitive development, behaviour, and psychological problems at preschool age. These scales have evolved over time, with some very robust scales developed decades ago and updated over the years, and newer scales being developed as the need for new, more specific instruments or simplified options arises. In the following sections, the scales used in this thesis for the assessment of neurodevelopment at each stage are explained.

Scales of neurodevelopment in infancy

The earliest scale for evaluating neurodevelopment is The Neonatal Behavioral Assessment Scale - Fourth edition (NBAS-IV), also known as the Brazelton Scale (Brazelton & Nugent, 1995, 2011). It employs a qualitative assessment approach, focusing on the infant's interactive capacity, to detect deficits and identify emerging abilities in newborns. Several other scales utilize a psychometric model to assess neurodevelopment in different age ranges. For instance, the Psychomotor Development Scale of Brunet-Lézine is suitable for children aged 2-30 months (Josse, 1997), while the Battelle Developmental Inventory, second edition (BDI-2), covers birth to 8 years of age (Newborg, 2005). The Merrill-Palmer-Revised (MP-R) developmental scale (Roid & Sampers, 2004) is applicable from 1 to 78 months and is commonly used for screening and diagnosing developmental disorders. However, the Bayley Scale of Infant and Toddler Development (BSID) (Bayley, 2006) holds the distinction of being the most widely used scale internationally for both clinical and research purposes. All these scales count with Spanish adaptation and validation.

In the present thesis, child neurodevelopment was assessed at 40 days of age by the BSID-Third edition (BSID-III) (Bayley, 2006), specifically the Spanish Adaptation of BSID-III (Bayley, 2015), a standardized assessment tool used to evaluate the development of infants and toddlers from birth to 42 months of age. It is a widely used instrument worldwide (Abdoola et al., 2023; McLester-Davis et al., 2021; Morgan et al., 2023; Toh et al., 2023), recognized as a gold standard for measuring developmental outcomes in young children given its high reliability and validity (Klein-Radukic & Zmyj, 2023; McLester-Davis et al., 2021; Ranjitkar et al., 2018). The BSID-III is administered by trained professionals, such as psychologists, developmental specialists, and occupational therapists, who are knowledgeable in child development and assessment. The assessments are conducted in a standardized manner, ensuring that all children are evaluated under the same conditions.

The BSID-III measures multiple areas of development, including cognitive, language, and motor skills, and provides scores in several domains, including the Mental Development Index (MDI) and the Psychomotor Development Index (PDI).

The MDI assesses a child's cognitive and language development and is calculated based on the child's performance on tasks such as problem-solving, memory, and understanding and using language.

There are two main types of language scales:

- Expressive language: it refers to the ability to use language to express oneself, and convey ideas, thoughts, feelings, and information to others. This involves using words, gestures, facial expressions, and tone of voice to communicate effectively with others.
- Receptive language: it refers to the ability to understand and comprehend language that is being spoken or presented in written form. This involves listening, paying attention, and processing the meaning of the words, sentences, or phrases that are being conveyed to the individual. Receptive language skills are essential for effective communication, reading comprehension, and learning in general.

The PDI assesses a child's gross and fine motor skills, including their ability to control their body movements, coordinate their movements, and manipulate objects. There are two main categories of motor skills:

- Gross motor skills: they involve large movements and include actions such as rolling over, crawling, walking, running, and jumping.
- Fine motor skills: they involve small and precise movements, and include actions such as grasping, reaching for objects, writing, and buttoning a shirt.

Both the MDI and PDI are components of the BSID-III assessment and are used to evaluate a child's developmental level and identify areas of potential concern or delay. The results can help guide interventions and support the child's development.

Scales of intelligence and neuropsychological functions in early childhood

Within the field of psychological assessment, there are various scales that have been specifically designed and validated to accurately and reliably measure both IQ and neurocognitive abilities in preschool children. Among the most prominent scales are the Wechsler Preschool and Primary Scale of Intelligence - Fourth Edition (WPPSI-IV) (Wechsler, 2014), the British Ability Scales - Second Edition (BAS-II) (Elliott et al., 1996, 1997) applicable between the ages 3-17 years, the Reynolds Intellectual Assessment Scales (RIAS) (C. R. Reynolds & Kamphaus, 2003, 2015b), and the McCarthy Scales of Children's Abilities (McCarthy, 1970)which is suitable for children aged 2 years 6 months to 8 years 4 months. These tools have demonstrated their validity and reliability in assessing IQ in preschool children, providing comprehensive information about their strengths and areas for improvement in the cognitive domain. As for neuropsychological domain, various scales designed specifically for the preschool population are used to evaluate these functions. Some of the most commonly used and recognized scales in this field include the Neuropsychological Assessment Battery - Second Edition (NEPSY-II) (Korkman et al., 2007), the Luria Neuropsychological Battery - Initial (LURIA-INICIAL) (Ramos & Manga, 2009), and the Child Neuropsychological Maturity Questionnaire - Second Edition (CUMANIN-2) for children aged 3-17 years (Portellano et al., 2021). The use of these scales enables a comprehensive understanding of the neurocognitive abilities, including attention, memory, language, visual perception, and executive skills in preschool children. All these scales count with Spanish adaptation and validation.

Among all the available instruments, the WPPSI-IV (Wechsler, 2014) and NEPSY-II (Korkman et al., 2007) were chosen in the present thesis for assessing the neurocognitive development of children at 4 years of age. These are two standardized assessment tools used to evaluate cognitive and neuropsychological development in children. Both the WPPSI-IV and NEPSY-II are widely used in clinical and research settings to assess cognitive and neuropsychological development in children (Espinosa da Silva et al., 2022; Guxens et al., 2022; Irvine et al., 2023; Kvestad et al., 2022; Sato et al., 2022; Spittle et al., 2022; Tong et al., 2023; Ulak et al., 2022; H. Wang et al., 2023) given their high reliability and validity (Davis & Matthews, 2010; Syeda & Climie, 2014). Both tests must be administered by trained professionals, such as psychologists, neuropsychologists, and educational specialists, in a standardized manner, ensuring that all children are evaluated under the same conditions.

The WPPSI-IV is a comprehensive measure of cognitive abilities in young children aged 2-7 years. The test provides scores in several domains, including the following:

- Verbal Comprehension Index: it measures a child's ability to understand and use language to express their thoughts and ideas. It assesses skills such as vocabulary, verbal reasoning, and comprehension.
- Visual Spatial Index: it measures a child's ability to perceive and manipulate visual information. It assesses skills such as visual-motor integration, spatial reasoning, and visual memory.
- Fluid Reasoning Index: it measures a child's ability to reason, problem-solve, and think abstractly. It assesses skills such as identifying patterns and relationships, making inferences, and drawing conclusions.
- Working Memory Index: it measures a child's ability to hold and manipulate information in their working memory. It assesses skills such as auditory attention and memory, visual working memory, and processing speed.

- Processing Speed Index: it measures a child's ability to process information quickly and accurately. It assesses skills such as visual scanning, discrimination, and decision-making.

The WPPSI-IV also provides a composite score, the Full-Scale IQ (FSIQ), which is a measure of overall cognitive ability. The FSIQ is derived from the child's performance on all the domains mentioned above.

The NEPSY-II is a neuropsychological assessment tool designed to evaluate a wide range of cognitive and behavioural functions in children aged 3-16 years. The NEPSY-II assesses various domains of cognitive functioning, including attention and executive functioning, language, sensorimotor function, visuospatial processing, memory and learning, and social perception. In the present thesis, the following subtests were considered to complement the WPPSI-IV scores:

- Verbal Fluency: it assesses a child's ability to generate words or names belonging to a specific category (e.g., animals, fruits) or beginning with a specific letter. The subtest measures the child's ability to switch between categories or letters and generate words quickly and efficiently.
- Visual-Motor Precision: it assesses a child's ability to integrate visual and motor skills. The subtest requires the child to copy increasingly complex designs and shapes using a pencil and paper.
- Emotion Recognition: assesses a child's ability to recognize and interpret emotional expressions from faces, voices, and situational cues. The subtest measures the child's ability to accurately identify and differentiate between various emotions, such as happiness, sadness, anger, and fear.

Behavioural and emotional problems in early childhood

Various scales have been specifically designed to assess behavioural characteristics and difficulties in preschool age. Among the notable scales are the

Child and Adolescent Assessment System (SENA) (Fernández-Pinto et al., 2015), the Strengths and Difficulties Questionnaire (SDQ) (Goodman, 1997), and the Behaviour Assessment System for Children - 3rd Edition (BASC-3) (C. R. Reynolds & Kamphaus, 2015a). These scales are suitable for children and adolescents, covering the age range from 2 to 21 years. They have been developed and validated to comprehensively evaluate a wide range of behavioural domains, including emotional functioning, social skills, hyperactivity, and conduct problems. All of them count with Spanish adaptation and validation.

However, the most internationally used scales are the Child Behavior Checklist for Ages 1½-5 (CBCL1½-5) (Achenbach & Rescorla, 2000) and the Teacher Report Form for Ages 1½-5 (TRF1½-5) (Achenbach & Rescorla, 2000), which are the ones chosen in this thesis together with the Behavior Rating Inventory of Executive Function-Preschool Version (BRIEF-P) (Gioia et al., 2016) for assessing behavioural development of children and the presence of psychological problems at 4 years of age. All three assessments are standardized and have strong psychometric properties, including high reliability and validity (Achenbach, 2019; Achenbach et al., 2017; Rescorla et al., 2011, 2019). They are widely used in clinical and research settings to assess behavioural and emotional functioning in young children and to guide intervention planning (Bishop et al., 2023; Boone et al., 2022; Conradt et al., 2023; Z. Li et al., 2023; Siersbaek et al., 2022; Voltas et al., 2022; Xie et al., 2022; Q. Zhang et al., 2023).

Both CBCL1½-5 and TRF1½-5 are standardized assessment tools used to evaluate emotional and behavioural problems in young children aged 18 months to 5 years old. The CBCL1½-5 is a parent-report questionnaire, while the TRF1½-5 is completed by teachers and caregivers. By using both the CBCL1½-5 and the TRF1½-5, professionals can compare the child's behaviour in different settings, such as home and school. This can help provide a more comprehensive understanding of the child's emotional and behavioural functioning.

In the present thesis, the following domains of the CBCL 1½-5 and TRF 1½-5 were assessed:

- Internalizing problems, which assess a child's emotional functioning, including anxiety, depression, and withdrawn behaviour.
- Externalizing problems, which assess a child's behaviour problems, including aggression, defiance, and hyperactivity.

Additionally, while the CBCL 1½-5 and TRF 1½-5 are not diagnostic tools on their own, the information they provide can be used in conjunction with clinical interviews and other assessments to help diagnose mental health disorders according to DSM-5 criteria. The following were evaluated in the present thesis:

- Depressive disorders, which are characterized by feelings of sadness, hopelessness, and/or a loss of interest or pleasure in activities that were previously enjoyed. The severity and duration can vary and include a wide range of symptoms.
- Anxiety disorders, which are characterized by excessive and persistent fear, worry, and/or anxiety that can interfere with an individual's daily functioning.
- Autism Spectrum Disorders (ASD), which are characterized by persistent deficits in social communication and social interaction across multiple contexts, as well as restricted, repetitive patterns of behaviour, interests, or activities. The symptoms must be present in the early developmental period but may not become fully manifest until social demands exceed limited capacities or may be masked by learned strategies in later life.
- Attention-Deficit/Hyperactivity Disorder (ADHD), which is characterized by a persistent pattern of inattention and/or hyperactivity-impulsivity that

interferes with functioning or development. Inattention symptoms include difficulty focusing, lack of attention to details, and avoidance of mentally challenging tasks. Hyperactivity-impulsivity symptoms include restlessness, excessively talking, interrupting others, and engaging in risky behaviour without thinking about the consequences.

- Oppositional Defiant Disorder, which is characterized by a persistent pattern of negative, defiant, disobedient, and hostile behaviour toward authority figures, such as parents, teachers, or other adults. This behaviour, which is not typical of the child's developmental stage, often co-occurs with other mental health disorders, such as ADHD, anxiety disorders, or depression and causes significant impairment in their social, academic, and occupational functioning.

The BRIEF-P are the only behavioural scales to assess executive functioning according to parents or teachers. This is therefore a widely used parent-report questionnaire that assesses executive functioning in preschool-aged children (ages 2-5 years). This test assesses executive function skills in several domains, from which the following have been considered in the present thesis:

- Inhibition: the ability to control impulses and stop oneself from engaging in inappropriate behaviours.
- Flexibility: the ability to switch between tasks or adapt to changes in the environment.
- Emotional control: the ability to regulate emotions and respond appropriately to different situations.
- Working memory: the ability to hold and manipulate information in the mind over a short period of time.
- Planning/Organization: the ability to plan and organize actions to achieve a goal.

The BRIEF-P also includes several summary scales that provide an overall picture of a child's executive function skills, based on the responses to the individual items and subscales. Some of them, included in the present thesis, are as follows:

- Behavioural Regulation Index: it measures a child's ability to regulate his or her behaviour in response to environmental demands and includes the Emotional Control, Shift, and Initiation subscales.
- Flexibility Index: it measures a child's ability to adapt to changing situations and includes the Shifting and Flexibility subscales.
- Metacognition Index: it measures a child's awareness of his or her own thinking processes and includes the Working Memory and Plan/Organization subscales.
- Global Executive Index: it provides an overall measure of executive function, combining scores from all the subscales and summary scales.

PRENATAL IRON SUPPLEMENTATION

So far, we have seen that iron plays a critical role during pregnancy, especially in proper foetal development and being of particular relevance to brain development. We have also seen that it is during the prenatal stage that the foundations of the child's cognitive and behavioural development are laid, and an adverse *in utero* environment can lead to long-lasting neurodevelopmental disorders. Thus, maternal iron status and its impact on children's neurodevelopment has long been a topic of interest. Adequate prenatal care, with a focus on maintaining appropriate nutrition and maternal iron levels, is, therefore, crucial to promote optimal neurodevelopment and preventing the occurrence of neurodevelopmental disorders, setting children up for long-term success (Quezada-Pinedo et al., 2021). To this end, prenatal iron supplementation has long been implemented in several countries around the world as a public health measure to prevent iron deficiency anaemia and its associated negative health outcomes in pregnant women and their offspring (World Health Organization et al., 2012). However, evidence on the effect of routine prenatal iron supplementation on clinical outcomes other than haematological parameters is still scarce (Peña-Rosas et al., 2015). In the following sections, the available evidence on its effects on maternal iron status and child neurodevelopment is described.

Effects on maternal iron status

Prenatal iron supplementation is a widely recognized preventive action given its successful results in preventing iron deficiency anaemia and improving maternal iron status, especially in low- and middle-income countries. The evidence indicates that women receiving iron supplements are more likely to have higher haemoglobin and serum ferritin concentrations at the end of pregnancy and in the postpartum period. However, excessive iron supplementation for non-anaemic pregnant women may lead to iron excess and negative consequences for both the mother and the developing foetus (Fisher & Nemeth, 2017; Georgieff et al., 2019; Peña-Rosas et al., 2015).

Women who are already anaemic or have low iron stores are at a higher risk of developing anaemia during pregnancy, which can have detrimental effects on both maternal and foetal health. For this reason, studies have highlighted the significance of early initiation of prenatal iron supplementation in improving maternal iron status. Given the high cost of iron in pregnancy, it is essential to ensure that women enter pregnancy with adequate iron stores (Brannon & Taylor, 2017; Fisher & Nemeth, 2017). One way to achieve this is through preconception care, which involves optimizing a woman's health and nutritional status before conception. This includes assessing and addressing any nutritional deficiencies, including iron deficiency, before getting pregnant. Starting iron supplementation before pregnancy can help ensure that women enter pregnancy with adequate iron stores, reducing the risk of iron deficiency and associated adverse outcomes (Ali et al., 2023; World Health Organization, 2013).

Furthermore, the effectiveness of iron supplementation may vary depending on the individual's iron status. Studies suggest that individuals with low iron stores are more responsive to iron supplementation than those with normal iron stores. This is due to the limited capacity of the body to absorb additional iron in individuals with normal iron stores, leading to insignificant improvements in iron status or ferritin levels with iron supplementation (Georgieff et al., 2019; Koenig et al., 2014). Therefore, assessing an individual's iron status before prescribing iron supplementation is crucial in determining the potential benefits and optimizing treatment outcomes.

Effects on child neurodevelopment

While the effectiveness of prenatal iron supplementation in improving maternal iron status is well-established, its impact on child neurodevelopment remains uncertain and the available evidence is controversial, which has raised questions about the potential benefits and risks of prenatal iron supplementation (Jayasinghe et al., 2018; Quezada-Pinedo et al., 2021).

As iron deficiency during pregnancy has been linked to poor cognitive and motor development in children (Quezada-Pinedo et al., 2021), it would be logical to assume that prenatal iron supplementation may have positive effects on child neurodevelopment. However, the systematic reviews addressing this issue have consistently failed to support this idea (Chmielewska et al., 2019; Jayasinghe et al., 2018; Szajewska et al., 2010). These works shed light on the limited research focused on assessing the effect of prenatal iron supplementation on child neurodevelopment, most of them evaluating short-term outcomes only. Furthermore, if the findings indicate that universal prenatal iron supplementation has no significant effect on child neurodevelopment, or that the effect is minor, it challenges logical thinking and prompts questions about the role of timing, dosage, or maternal iron stores in determining the efficacy of supplementation. This creates an opportunity to explore and debate the advantages and drawbacks of providing pregnant women with iron in a generalized manner versus tailoring iron supplementation to meet individual needs.

Routine vs. individualized prenatal iron supplementation

Historically, routine iron supplementation has been recommended, often without first clinically monitoring iron biomarkers (World Health Organization et al., 2012). Universal iron supplementation is characterized by prescribing all pregnant women a common iron dose, which is 40 mg of elemental iron per day in Spain (Arija et al., 2014). However, this practice has been questioned as emerging evidence suggests that the use of standard dosages of iron supplements may not be appropriate for all pregnant women (Quezada-Pinedo et al., 2021). The potential problems associated with routine iron supplementation include the risk of iron excess, especially in high-income countries, and the consequent adverse effects on maternal and foetal health (Brannon & Taylor, 2017). Iron excess can occur in non-anaemic women who receive high-dose iron supplementation or those carriers of *HFE* gene mutations, who may require lower doses or none (Milman, 2021; Shamas, 2023). This emphasizes the importance of proper dosing of iron supplementation during pregnancy, making the use of routine vs individualized iron supplementation during pregnancy a topic of discussion in recent years (Georgieff et al., 2019; Peña-Rosas et al., 2015).

Recent studies suggest that personalizing iron supplementation during pregnancy, based on factors such as pre-pregnancy iron stores, dietary habits, and genetic determinants, can help ensure women receive the appropriate amount of iron. This approach can prevent inadequate or excessive intake, which improves maternal iron status and supports optimal pregnancy outcomes and offspring development (Friedrisch & Friedrisch, 2017; Georgieff et al., 2019; Peña-Rosas et al., 2015). However, there is currently no research evaluating the efficacy of individualized prenatal iron supplementation on child neurodevelopment, cognitive functioning, and psychological well-being.

Hypothesis and objectives

Hypotheses

The main hypothesis of this thesis proposes that adjusting prenatal iron supplementation to the actual needs of each woman would enhance the neurodevelopment of their children, with the underlying mechanism being an improvement in maternal iron status.

The secondary hypothesis posits that several factors, including biological, sociodemographic, and lifestyle aspects, can exert an influence on maternal iron status during pregnancy. By gaining a deeper understanding of how these factors impact maternal iron levels, public health strategies can be effectively developed to improve them.

Objectives

The main objective of this thesis was to assess the effect of adjusting prenatal iron supplementation to the actual needs of women on their iron status at the end of gestation, as well as on the neurodevelopment of their children.

Specific objectives included the following:

State-of the-art:

- To update the state-of-the-art about the association between maternal iron status, prenatal iron supplementation, and child neurodevelopment.

Maternal iron status:

- To assess the effect of adjusting prenatal iron supplementation to the actual needs of women on maternal iron status at the end of gestation.
- To assess the influence of maternal environmental factors on iron levels during pregnancy in two European populations.

Child neurodevelopment:

- To assess the effect of adjusting prenatal iron supplementation to the actual needs of women on mental, language, and motor development at 40 days of age.
- To assess the effect of adjusting prenatal iron supplementation to the actual needs of women on cognitive development and executive functioning at 4 years of age.
- To assess the effect of adjusting prenatal iron supplementation to the actual needs of women on behavioural development and psychological problems at 4 years of age.

Methodology

This section explains the methods of the empirical studies from which data have been used in this thesis.

ECLIPSES AND ECLIPSES-NEN STUDIES

The foundation of this thesis rests on a research strand focused on assessing maternal health status across various domains (including nutrition, psychology, and environment) and its impact on the offspring. This research is composed of two consecutive projects, funded by FIS-ISCIII grants: the study called ECLIPSES (Effectiveness of adapting the dose of iron supplementation in pregnancy on maternal and child health. Randomised Clinical Trial, PI12/02777) and the study called ECLIPSES-NEN (Effect of maternal prenatal factors on child neurodevelopment, PI17/01754). In particular, this thesis aims to determine the optimal dosage of iron supplementation during gestation for promoting the health of both the mother and her offspring, with assessments conducted up to the age of 4 years. The analysis of results includes multivariate techniques to effectively control potential confounding factors associated with the investigated relationship.

Study design

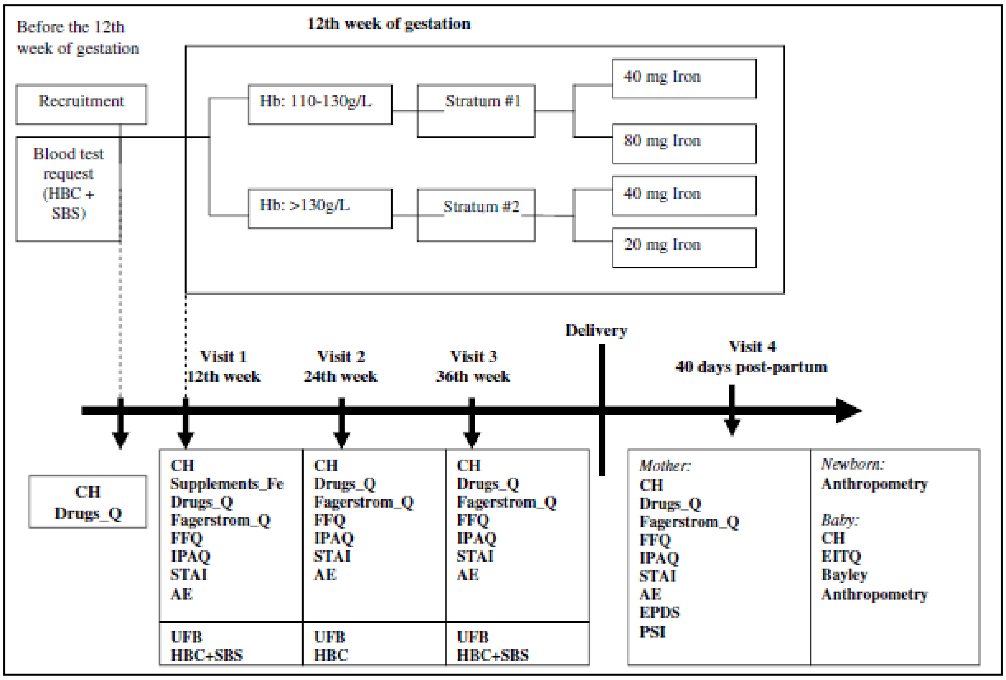
The ECLIPSES study was a comprehensive clinical trial conducted in the province of Tarragona (Catalonia, Spain) from 2013 to 2017. It was a multi-centred, parallel-group, controlled, triple-blind, randomized clinical trial designed to investigate the effectiveness of prenatal iron supplementation at different doses, tailored to the maternal haemoglobin concentration at the start of pregnancy. The study aimed to assess the impact of these supplementation regimens on maternal iron status at the end of gestation and the health of the newborns (Arija et al., 2014).

The study population consisted of healthy, non-anaemic pregnant women who were recruited before reaching 12 weeks of gestation. Recruitment took place during their first routine pregnancy visit with the midwives at ten Primary Care Centres affiliated with the Catalan Sexual and Reproductive Health Service of the Catalan Institute of Health. To be eligible for participation, the women had to be 18 years or older, within the first 12 weeks of gestation, not anaemic (with a haemoglobin concentration above 110 g/L), capable of understanding the official state languages (Spanish or Catalan), and willing to sign the informed consent form. Conversely, the exclusion criteria included multiple pregnancies, iron intake exceeding 10 mg during the three months prior to the 12th week of gestation, hypersensitivity to egg protein (as the iron prescription formula contained ovalbumin), previous serious illnesses (e.g., immunosuppressed status, heart disease, endocrinopathy), or chronic illnesses that could impact nutritional status (e.g., cancer, malabsorption, type 1 or 2 diabetes), alcoholism, liver diseases (such as chronic hepatitis or cirrhosis), morbid obesity, maternal infection, and adverse obstetric history (including previous preeclampsia, uterine malformation, uterine surgery, suspected foetal malformation, and perinatal recurrent death).

The ECLIPSES study involved various stages, including a recruitment visit before the 12th week of gestation, one visit per trimester (at the 12th, 24th, and 36th weeks of gestation), and a final visit 40 days postpartum. Any adverse events since the previous visit were recorded at each visit, and participants who met any exclusion criteria were excluded from the study.

During the recruitment visit, a blood sample was taken from each participant for standard biochemical analyses, including haemoglobin, which was sent to a centralized laboratory for processing. Based on the baseline haemoglobin concentration, the participants were assigned to one of two groups: Group 1 if their haemoglobin concentration was between 110 and 130 g/L, and Group 2 if their haemoglobin concentration was above 130 g/L. Within each group, women were randomly assigned different doses of iron supplements. Group 1 participants received daily doses of either 80 or 40 mg of iron, while Group 2 participants received daily doses of either 20 or 40 mg of iron. The supplementation continued until delivery, and the degree of compliance was recorded during the follow-up period.

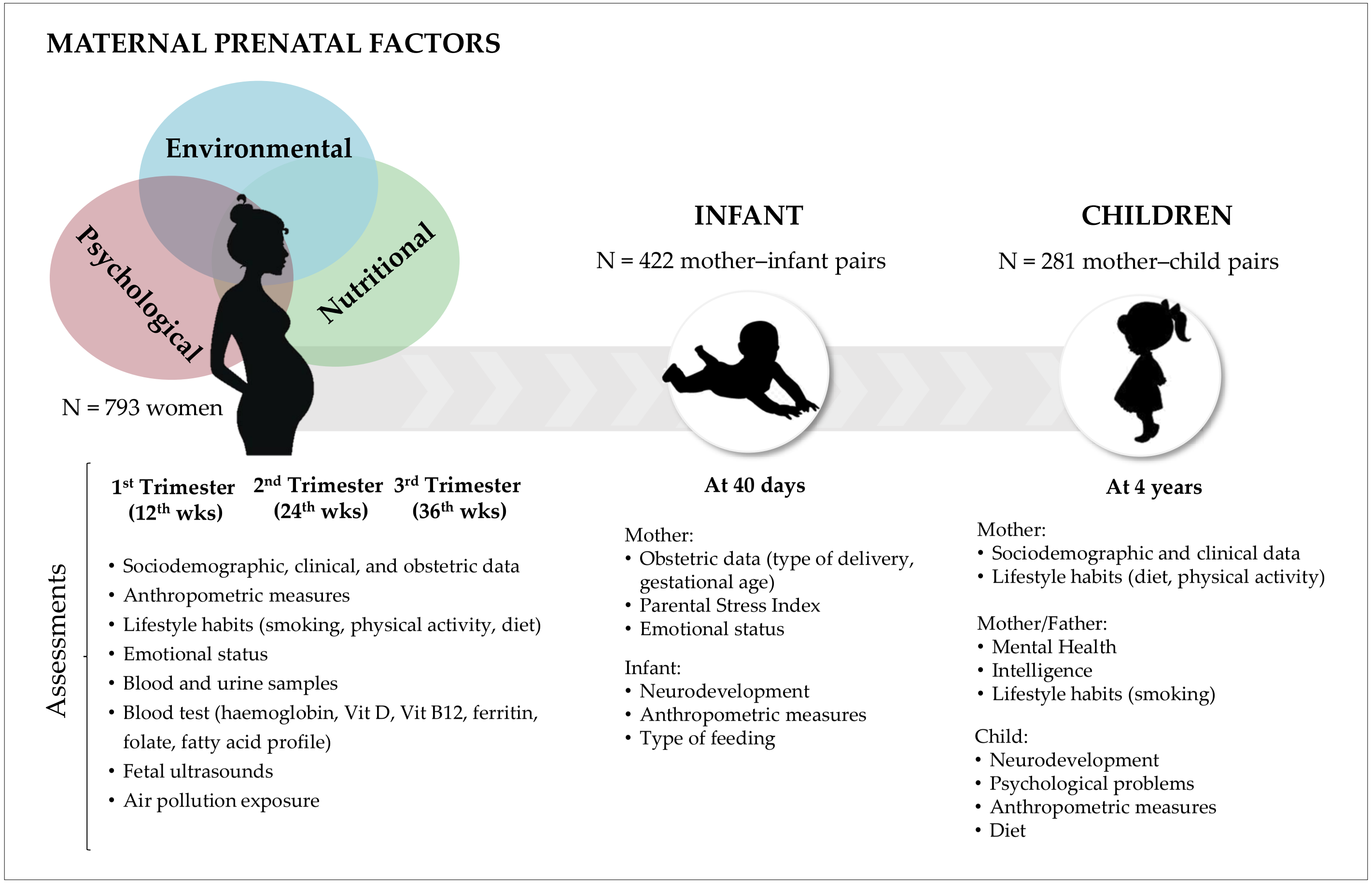
The overall design of the ECLIPSES study is summarized as follows:



In this study, ferrimannitol ovalbumin was used as the iron formula instead of the more commonly commercialized ferrous sulphate. The ferrimannitol ovalbumin formula was chosen due to its reduced gastrointestinal side effects, which contributed to improved compliance. The doses of daily 20 mg, 40 mg, and 80 mg of elemental iron correspond to 150 mg, 300 mg, and 600 mg ferrimannitol ovalbumin. The supplements were provided by an external laboratory (Meiji Pharma Spain ES, Madrid) under a codification system to ensure the blinding of the researchers, midwives, and participants regarding the assigned doses. Meiji Pharma Spain ES manufactured and distributed all the iron doses for the study free of charge.

The clinical trial (protocol code: IJG-FER-2012) underwent rigorous control and approval by the Spanish Agency for Medicines and Medical Devices (AEMPS), an entity operating under the Ministry of Health responsible for ensuring the quality, safety, efficacy, and accurate information regarding medicines and medical devices provided to society. The study was classified as a Phase IV trial, as it compared the standard dose typically prescribed for pregnant women (40 mg/day) as a control group, with experimental doses of 80 mg/day and 20 mg/day. The AEMPS closely monitored adherence to the study methodology, encompassing participant selection, inclusion and exclusion criteria, medication administration, compliance assessment, blinding and randomization procedures, potential unblinding in serious cases, study withdrawal protocols, and weekly participant monitoring using an Electronic Data Register administered by a certified external auditor. Safety analysis and pharmacovigilance measures were implemented throughout the follow-up period. Upon completion of the study fieldwork, prior to unblinding the identification of the iron dose received by participants, the proposed statistical analyses (intention-to-treat and per protocol efficacy) were thoroughly reviewed by the AEMPS. Following approval, participants were informed of their respective prescribed doses.

The ECLIPSES-NEN study is an extension of the ECLIPSES study and involves a follow-up of the participants' children until they reach the age of 4 years. The goal is to assess the long-term impact of different doses of prenatal iron supplementation, adjusted to the maternal haemoglobin concentration at the start of pregnancy, on the neurodevelopment of the children. The image below illustrates the complete timeline and follow-up of both studies.



MATERNAL PRENATAL FACTORS

N = 793 women

1st Trimester (12th wks) **2nd Trimester (24th wks)** **3rd Trimester (36th wks)**

- Sociodemographic, clinical, and obstetric data
- Anthropometric measures
- Lifestyle habits (smoking, physical activity, diet)
- Emotional status
- Blood and urine samples
- Blood test (haemoglobin, Vit D, Vit B12, ferritin, folate, fatty acid profile)
- Fetal ultrasounds
- Air pollution exposure

INFANT

N = 422 mother–infant pairs

At 40 days

Mother:
- Obstetric data (type of delivery, gestational age)
- Parental Stress Index
- Emotional status

Infant:
- Neurodevelopment
- Anthropometric measures
- Type of feeding

CHILDREN

N = 281 mother–child pairs

At 4 years

Mother:
- Sociodemographic and clinical data
- Lifestyle habits (diet, physical activity)

Mother/Father:
- Mental Health
- Intelligence
- Lifestyle habits (smoking)

Child:
- Neurodevelopment
- Psychological problems
- Anthropometric measures
- Diet

Outcomes

Maternal iron status in late pregnancy

The primary outcome concerning the mothers was their iron status at the end of pregnancy, which was evaluated by measuring haemoglobin and serum ferritin concentrations. Serum ferritin was measured by immunochemiluminescence.

Iron stores were categorized as follows: iron deficiency (serum ferritin below 15 µg/L), normal or optimal iron stores (serum ferritin between 15 and 65 µg/L), and normal-high iron stores (serum ferritin 65 µg/L or above). Haemoglobin concentration was used to determine anaemia (haemoglobin below 110 g/L in the first and third trimesters, and below 105 g/L in the second trimester of gestation) or haemoconcentration (haemoglobin above 130 g/L in the third trimester of gestation). Iron deficiency anaemia was defined as haemoglobin below 110 g/L and serum ferritin below 15 µg/L at the same time.

Child neurodevelopment

Within the ECLIPSES study, child neurodevelopment was evaluated at 40 days and 4 years of age.

At 40 days, the assessment was conducted using the BSID-III (Bayley, 2006, 2015). Additionally, the temperament of the children was assessed at this age utilizing the Early Infancy Temperament Questionnaire (Carey & McDevitt, 1995), offering valuable insights into their behavioural characteristics during the initial stages of life.

At 4 years, various aspects of neurodevelopment were assessed. Cognitive functions were evaluated using the WPPSI-IV (Wechsler, 2014) and the NEPSY-II (Korkman et al., 2007). Furthermore, children's behavioural development and psychological problems were assessed through parent information using the BRIEF-P (Gioia et al.,

2016) (executive functions) and the CBCL1½-5 (Achenbach & Rescorla, 2000) (psychological problems), and through teacher information using the TRF1½-5 (Achenbach & Rescorla, 2000) (psychological problems). Extensive information on these assessment instruments can be found in the Introduction section under the heading "Scales used for neurodevelopmental assessment". Finally, the Childhood Asperger Syndrome Test (CAST) (Morales-Hidalgo et al., 2017; Scott et al., 2002) was used for evaluating ASD risk symptoms in children.

Covariates

During the recruitment visit, the participants' clinical history was recorded, and questionnaires were administered to gather information on the use of iron-containing supplements or multivitamins, other treatments, and, for smokers, the Fagerstrom test for tobacco dependency (Fagerström, 1978).

At the visits of the 12th, 24th, and 36th weeks of gestation, women were provided with questionnaires to complete at home. The self-reported information was reviewed during subsequent visits. These questionnaires included inquiries about supplement use or other treatments, a self-reported food frequency questionnaire for dietary intake assessment (Trinidad Rodríguez et al., 2008), the International Physical Activity Questionnaire (Craig et al., 2003), the State-Trait Anxiety Inventory to assess maternal anxiety (Spielberger CD, Gorsuch RL, 1997), and the Fagerstrom questionnaire to evaluate tobacco dependence (Fagerström, 1978). The same questionnaires were filled out at 40 days postpartum, along with two additional questionnaires: the Parenting Stress Index (Abidin, 1995), and the Edinburgh Postnatal Depression Scale (Cox et al., 1987; Garcia-Esteve et al., 2003) to evaluate the risk of postpartum depression.

Blood samples were collected at each visit for various analyses, including biochemical measurements such as haemoglobin, serum ferritin, C-reactive protein (measured by immunoturbidimetry determination), and cortisol (measured by immunochemiluminescence). Additionally, genetic screening for mutations in the *HFE* gene was conducted in the first blood sample.

Detailed data pertaining to the birth and clinical history of the infants were meticulously recorded, encompassing essential information such as Apgar test scores, as well as anthropometric measurements including weight, length, and head circumference. These same anthropometric measurements were subsequently measured again when the infants reached 40 days of age. Concurrently, the women were also queried about the feeding methods employed for their children, specifically whether breastfeeding or bottle-feeding was utilized.

When the children reached the age of 4 years, their weight and height were measured to assess their ongoing physical development. Furthermore, a dietary assessment was conducted for both the children and their mothers, employing the same self-reported food frequency questionnaire that the women had previously filled out during pregnancy. Additionally, the parents were asked about their mental health through the General Health Questionnaire (Goldberg & Blackwell, 1970), and an approximation of their IQ was derived by averaging the results obtained from both the mother and father on The Wechsler Adult Intelligence Scale, Fourth Edition (WAIS-IV) (Wechsler, 2008), an intelligence test based on logical reasoning.

MOBA COHORT

Study design

The Norwegian Mother, Father, and Child Cohort study (MoBa) is an ongoing prospective population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health (Magnus et al., 2006, 2016). The MoBa study incorporates data from The Medical Birth Registry of Norway, which provides comprehensive information on pregnancy, delivery, and maternal and neonatal health for all births in Norway (Irgens, 2000). This thesis focuses on data collected from 2990 women who were pregnant between 2002 and 2008, with available iron status measurements obtained from The Norwegian Environmental Biobank (Rønningen et al., 2006).

The establishment and data collection procedures of the MoBa study were previously authorized by the Norwegian Data Protection Agency and approved by The Regional Committee for Medical Research Ethics. Presently, the study adheres to regulations outlined in the Norwegian Health Registry Act. The current study has received approval from The Regional Committee for Medical Research Ethics Southeast Norway (2015/2393).

Outcome

The aim of this analysis, which focused on a subset of participants within the MoBa cohort called MoBa-ETox, was to describe the iron status of women during mid-pregnancy and the prevalence of iron deficiency. Additionally, the study aimed to identify potential determinants of maternal iron status.

For assessing iron status, blood samples were collected at gestational week 18 and were sent to the Finnish Institute for Health and Welfare in Helsinki (Finland) for biochemical analysis. Non-fasting plasma ferritin (P-Fe) levels were measured as an indicator of iron stores, using a chemiluminescent microparticle immunoassay. Depleted iron stores were defined as P-Fe concentrations below 15 μg/L, while low iron stores were defined as P-Fe concentrations below 30 μg/L. Maternal haemoglobin measurements were also collected from the maternity record. The lowest haemoglobin measurement was considered the most clinically relevant indicator for assessing low iron status in this study. Accounting for possible inflammation, C-reactive protein was measured using immunoturbidimetric determination, which has high sensitivity and is suitable for measuring low-range values.

As for the assessment of potential predictors of maternal iron status, information regarding age, year of participation, parity, and time since the previous pregnancy (for multiparous women) was obtained through linkage with the Medical Birth Registry of Norway. The interpregnancy interval (IPI) was calculated as the time between the birth date of the previous child and the conception date of the current pregnancy. The first questionnaire in MoBa, completed during gestational week 15, provided data on medical history, use of hormonal contraceptives, regularity of menstrual cycles, sociodemographic factors, and lifestyle. Chronic disease was defined as self-reported asthma, diabetes, inflammatory bowel disease, rheumatic disease, epilepsy, multiple sclerosis, or cancer, reported either before or during pregnancy. Dietary intake and supplement usage were assessed using a semi-quantitative food frequency questionnaire completed during mid-pregnancy.

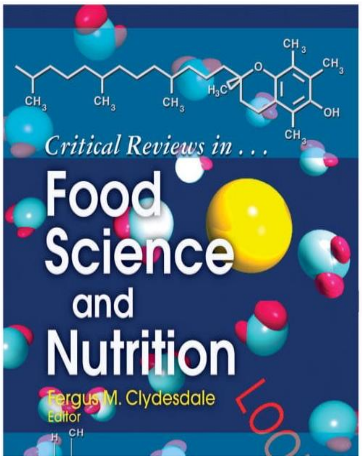
Results

CHAPTER 1.

WHAT IS KNOWN ABOUT THE EFFECT OF PRENATAL IRON STATUS ON CHILD NEURODEVELOPMENT?

Effects of prenatal iron status on child neurodevelopment and behavior: A systematic review

Lucía Iglesias-Vázquez, Josefa Canals, Victoria Arija



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Effects of prenatal iron status on child neurodevelopment and behavior: A systematic review

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ABSTRACT
Iron deficiency and iron-deficiency anemia are the main worldwide nutritional disorders. A good level of prenatal iron is essential for the correct child neurodevelopment but this association has been poorly investigated. To gather the scientific evidence on the relation between prenatal iron status and child neurodevelopment. To emphasize the importance of personalize the dose and type of supplementation. Wide search strategy was performed in electronic databases for English language articles with no limitations as regards the language or date of publication. Additional studies were selected by hand search. The inclusion criteria were pregnant women without high-risk pregnancy and their children as study population and neurodevelopment as the main outcome. Six RCTs and 13 observational studies were included. The majority concluded that deficit or excess iron during pregnancy injures the mental and psychomotor development of child. Other authors found no association of low iron level with troubles in neurodevelopment, recommended multi-micronutrients instead of iron alone and/or showed inconsistent results. Both iron deficiency as its excess are harmful for the child neurodevelopment. The prenatal iron supplementation should be adjusted for each woman, taking into account the iron stores, some genetic mutation and other health habits.

KEYWORDS
Neurodevelopment; mental; psychomotor; supplementation; iron; children

Introduction

Iron deficiency is the most common and widespread nutritional disorder in the world, and the main cause of anemia. Currently, it has been estimated that anemia affects around 800 million children and women world-wide; in the 50% of women and in the 42% of children it is caused by iron deficiency (ID) (World Health Organization, 2015). The low-income areas show a high prevalence of anemia (Stevens et al., 2013) but it is also present in developed countries, being the only significantly prevalent nutrient deficiency in industrialized countries (Miller, 2013; CDC, 2002). Infants, children, adolescents, and women of child-bearing age, especially pregnant women, are the main risk population because of their high requirements of iron (Harvey et al., 2013) although women are also susceptible of suffer anemia in the postpartum (Organization, 2016). Apart of these, there are other factors may also contribute to the disorder, including overall nutritional status and genetic conditions (Harvey et al., 2013). Several countries have launched supplementation programs as health policy to prevent iron deficiency (Stevens et al., 2013), most of them generalized and routine, which may in general be positive. However, in this regard it is also important to consider that in some cases the iron supplementation may contribute to hemoconcentration if women have a genetic disorder in the "hemochromatosis" (HFE) gene (Arija et al., 2014).

While the maternal nutrient status and micronutrient supplementation can influence fetal development, including birth weight and infant physical development, the evidence for an association between gestational nutrition and brain development has been particularly strong for iron, n-3 fatty acids, and folate. The role of iron in signal controlling in some neurotransmitters and their involvement in the myelination process makes iron necessary for brain development and maturation during the fetal period and infancy (CDC, 2002). However, knowledge about this relationship is very limited and there is some controversy regarding the issue of supplementation (Leung et al., 2011).

In this systematic review, we compile evidence that a good prenatal iron status improves the child's mental and psychomotor neurodevelopment, and of the damage caused by either a deficit or excess.

Methods

Search strategy

The search strategy selected randomized controlled trials (RCTs) and observational studies in humans, with no limitations as regards the language or date of publication. The Medline/PubMed, Cochrane Library and Scopus electronic literature databases were searched on December 21, 2014. Our search strategy was as follows: ("iron" OR "iron supplementation" OR "hemoglobin" OR "haemoglobin" OR "anemia" OR "anaemia" OR ("Ferritins"[Mesh]) OR ("Hemoglobins"[Mesh])

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OR ("Anemia"[Mesh])) AND ("pregnancy" OR "pregnant" OR gesta*) AND ("neurobehavioural" OR "neurobehavioral" OR "neurodevelopmental" OR" behaviour" OR "behavior" OR "birth outcomes" OR "pregnancy outcomes" OR "cognition" OR "offspring outcomes" OR "neurodevelopment" OR "psychomotor development" OR "cognitive" OR "mental" OR "newborn behavioral assessment scale" OR "brazelton" OR "neurophysiological"). Additionally, we identified more studies throughout hand search of references from previous reviews. An updated search was conducted on April 18, 2016 to look for articles published since the first search.

The registration number is CRD42015016541.

Inclusion criteria

RCTs and observational studies that investigated the effect of prenatal iron status on child neurodevelopment (including mental, psychomotor and behavioral domain) were included. The study population of these investigations was pregnant women without high-risk pregnancy and their offspring. Studies conducted in populations with a disease and/or investigations that reported effects of prenatal iron on pregnancy, birth outcomes, and physical development or growth were excluded.

Data extraction and quality assessment

The information from the studies was summarized in separate tables for RCTs and observational studies, including subject and intervention/observation characteristics, outcomes of interest and psychological tests used. The data are not comparable between different types of study, and as such the results were discussed separately according whether they came from RCTs or from observational studies.

The data were extracted independently and in the case of Lewis et al. (2014), we contacted the authors to obtain a better understanding of their statistical methods, but they did not respond to our request.

The quality assessment for RCTs and observational studies was carried out using the revised CONSORT checklist (Schulz et al., 2010) and the STROBE checklist (von Elm et al. 2008), respectively. The articles were rated qualified as follows: "good" if the score was $\geq$17 items ($>$80% of the checklist), "average" if the score was 13–16 items (60–79% of the checklist) and "poor" if the score was $\leq$12 items ($<$50% of the checklist). The Cochrane Collaboration tool (Higgins and Green 2011) was used to evaluate the risk of bias in each study, and the overall percentage of each type of risk.

Results

We identified a total of 1,806 articles from the electronic databases search and three more from handsearching, of which 106 were selected for careful reading of the abstract. Finally, 19 studies met the inclusion criteria (6 RCTs and 13 observational studies) and were considered in this systematic review. 87 articles were excluded according to the exclusion criteria (Figure 1). The studies reviewed were published between 1986 and 2015 and varied widely in size, location and intervention

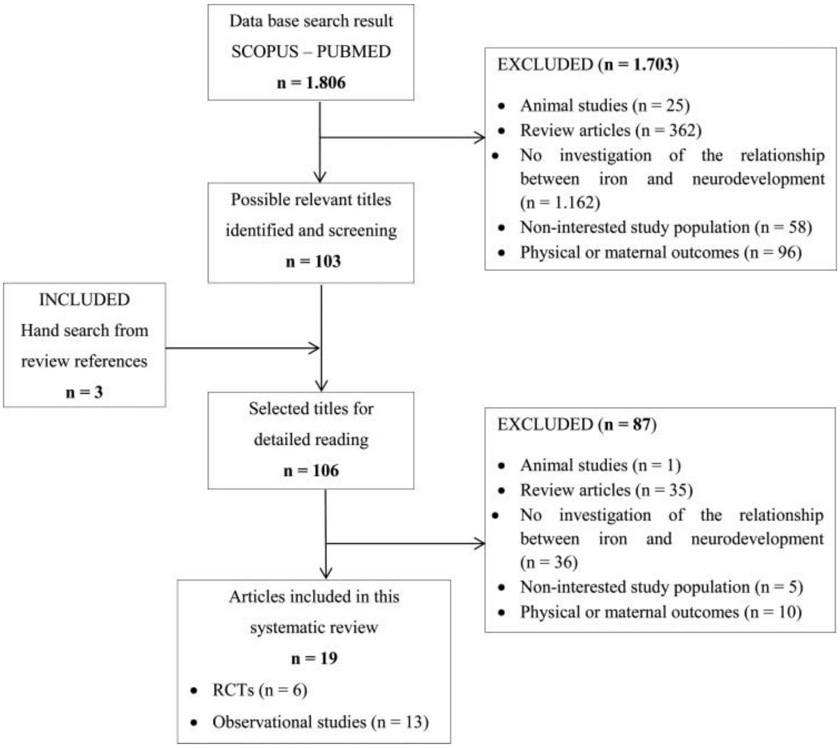

Figure 1. Flowchart of selection process of included articles in systematic review.

and parameters of observation, but they all focused on some feature of child neurodevelopment.

Tables 1 presents the characteristics of the RCTs, which were located as follows: two were conducted in Australia (Zhou et al., 2006; Parsons et al., 2008), a developed country, while the others were conducted in developing countries; two in rural China (Li et al., 2009; Chang et al., 2013), one in Indonesia (Prado et al., 2012) and other in rural Vietnam (Hanieh et al., 2013). For the supplementation, most of the researchers recruited volunteers at between 14 and 20 weeks of gestation, and they administered the micronutrients until delivery. Zhou et al. (2006) randomly assigned the pregnant women into two groups, which received 20 mg of iron or placebo daily. Two years later, Parsons et al. (2008) continued the observation of outcomes, leading to another report. Other researchers, Li et al. (2009) and Chang et al. (2013), allocated the women into three supplementation groups: folic acid (FA) (400 μg/d), folic acid plus iron (IFA) (with 60 mg/d of Fe) and multimicronutrients (MMN) (with 30 mg/d of Fe). Hanieh et al. (2013) wanted to assess if the effect was different with daily IFA (with 60 mg of Fe), intermittent IFA or intermittent MMN supplementation (both including 60 mg of Fe twice a week). Prado et al. (2012) did not assess the effect of iron in isolation, but their intervention groups were IFA or MMN (including iron), with 30 mg/d of Fe in both cases.

Table 2 presents the characteristics of the observational studies. Five were conducted in developing areas: Vietnam (Tran et al., 2013; Tran et al., 2014), a poor state of the United States (Vaughn et al., 1986), rural China (Yang et al., 2010) and the Republic of Benin (Mireku et al. 2015). The rest were reported in the United States (Tamura et al., 2002; Schmidt et al., 2014; Wehby and Murray, 2008), the United Kingdom (Lewis et al., 2014), Finland (Fararouei et al., 2010), Canada (Rioux et al., 2011) and Spain (Hernández-Martínez et al., 2010; Cucó et al., 2005).These investigations focus on maternal iron status throughout pregnancy. Some reports specify whether the women received supplements supplemented and the doses of supplementation, while others lack this information and work with biochemical data.

As regards the point at which the effect of supplementation was evaluated, in the majority of RCTs and observational studies this took place in toddlers and school-age children while in six of them (Li et al., 2009; Hanieh et al., 2013; Tran et al., 2013, 2014; Vaughn et al., 1986; Rioux et al., 2011) the age of the group was less than 1 year old, while in two others (Cucó et al., 2005; Hernández-Martínez et al., 2010) the assessment was conducted at 2–3 days old and another one (Fararouei et al., 2010) evaluated the effect over 30 years.

Quality of reporting

The total scores on the CONSORT and STROBE checklist ranged from 14 to 20, with a mean score of 18; as a result, thirteen studies had a "good" quality and six were rated "average." The quality of the studies included was also assessed by using Cochrane tool mentioned above (Higgins and Green, 2011). Figure 2 shows the percentage of each type of bias among the studies. In general, the risk of bias is low, most of the researchers present the complete outcome data and they do not incur any reporting bias, but several studies lack information about the blinding of the outcome assessment, so the risk is not clear in these cases. The observational studies have a high probability of selection bias because the sequence is not usually generated from the general population and exposure cannot be controlled by the researchers. The blinding of participants is another factor that is usually uncontrolled in studies of this type, which gives them a high probability of risk of performance bias.

Maternal iron assessment

There are several indicators that complement each other when evaluating the body's iron level. Iron stores are usually measured based on the Serum Ferritin (SF) concentration while the best measure of circulating iron is transferrin saturation (TS). Researchers assess the maternal iron status at various points in the pregnancy and most consider iron deficiency (ID) to be if SF < 15 μg/L (Tran et al., 2014; Hanieh et al., 2013; Tamura et al., 2002) but SF < 12 μg/L may also be found in the literature (Hernández-Martínez et al., 2010; Zhou et al., 2006; Parsons et al., 2008). TS $< 16\%$ is another indicator of ID but only Hernández-Martínez et al. (2010) used it in our review. A generic diagnosis of anemia requires a hemoglobin (Hb) concentration lower than 110 g/L (Chang et al., 2013; Prado et al., 2012; Vaughn et al., 1986; Yang et al., 2010; Fararouei et al., 2010) but iron-deficiency anemia (IDA) requires one or more ID factors and Hb < 110 g/L (Zhou et al., 2006; Parsons et al., 2008; Tran et al., 2013; Hanieh et al., 2013). Furthermore, Yang et al. (2010) take into account high values of Hb ($\geq$124 g/L) when discussing possible adverse effects on maternal and fetal health.

Schmidt et al. (2014), Li et al. (2009), and Lewis et al. (2014) did not take into account maternal iron status, but they established conclusions based on extensive scientific evidence that maternal iron supplementation improves prenatal iron status.

Child neurodevelopment assessment

Neurodevelopment is a broad concept that includes the maturation of the central nervous system, which may be assessed at early stages using psychological tests of mental and psychomotor development, and behavioral assessment measures. Later, in childhood, neuropsychological and intellectual ability (Intellectual Quotient, IQ) tests and other behavioral-emotional assessment tests may be used.

Mental development (MD) assessment

To assess infant MD, six studies were included—three RCTs (Hanieh et al., 2013; Li et al., 2009; Chang et al., 2013) and three observational studies (Tran et al. 2014, 2013; Rioux et al., 2011)—using Bayley Scales (in the first (Bayley, 1969), second (Bayley 1993) and third edition (Bayley, 2006)), which include many aspects of mental development such as language and cognition. Rioux et al (2011) also used the Brunet-Lézine Scale of Psychomotor Development of Early Childhood (Josse, 1997) on 6-month-old infants. At the pre-school and school age, the Stanford–Binet Intelligence Scale (Becker, 2003) and Wechsler Intelligence Scales for Children (Wechsler 1974, 2002),

Table 1. Characteristics of randomized controlled trials included in the review.

Reference	Location	n	Supplementation	Duration	Parameters	Child age	Outcomes	Neurodevelopment tests
Zhou, S (2006)	Australia	430	20 mg/d Fe vs placebo	From 20 weeks to delivery	Hb	4 years	IQ	Stanford-Binet Intelligence Scale
	Adelaide				SF		Behavior	Strength and Difficulties Questionnaire (SDQ)
Parsons, A (2008)	Australia	264	20 mg/d Fe vs placebo	From 20 weeks to delivery	Hb	6–8 years	Temperament	Short Temperament Scale for Children
	Adelaide				SF		Behavior	Strength and Difficulties Questionnaire (SDQ)
Li, Q (2009)	China	1.305	AF vs	From 14 weeks to delivery	—	3, 6, 12 months	Mental and Psychomotor Development	BSID–I
			IFA (60 g/d Fe) vs MMN (30 g/d Fe)					
Prado, E (2012)	Indonesia *Lombok*	487	IFA (30 mg/d Fe) vs	From pregnancy to 3 months after delivery	Hb	3.5 years	Psychomotor Development	BSID-II
			MMN (30 mg/d Fe)					Ages and Stages Questionnaire
							Language Ability	MacArthur-Bates Communicative Development Inventory–Level III
								The Picture Vocabulary Scale
							Visuospatial Ability	British Ability Scale
								Wechsler Preschool and Primary Scale of Intelligence–III
							Visual Attention	NEPSY Developmental Neuropsychological Assessment
							Executive	Snack Delay Test
							Function	Windows Test
							Socioemotional Development	Brief Infant–Toddler Social and Emotional Assessment
Hanieh, S (2013)	Vietnam	1.258	IFA (60 mg Fe/d) vs	From 16 weeks to 6 months after delivery	Hb	6 months	Mental and Psychomotor Development	BSID–III
	Ha Nam		IFA (60 mg Fe/2 per wk) vs MMN (60 mg Fe/2 per wk)		SF			
Chang, S (2013)	China	850	AF vs IFA (60 g/d Fe) vs MMN (30 g/d Fe)	—	Hb	2 years	Mental and Psychomotor Development	BSID–II

Fe: iron; Hb: hemoglobin; SF: serum ferritin; FA: folic acid; IFA: folic acid plus iron or iron-folic acid; MMN: multimicronutrients.

Table 2. Characteristics of observational studies included in the review.

Reference	Location	n	Duration	Parameters	Offspring age	Outcomes	Neurodevelopment tests
Vaughn, J (1986)	USA *New Orleans*	115	—	Hb SF Serum Iron Iron-binding capacity	3 days 5 months	Behavior	Brazelton Neonatal Behavior Assessment Scale BSID–I
Tamura, T (2002)	USA *Alabama*	278	—	SF	5 years	IQ Auditory comprehension Fine and Gross Motor Skills Attention	Wechsler Intelligence Scale for Children–R Test for Auditory Comprehension of Language Peabody Developmental Motor Scales Yale Children's Inventory for attention and tractability
Cucó, G (2005)	Spain *Reus*	66	Before pregnancy to 38 weeks	—	3 days	Behavior Psychomotor Development	Brazelton Neonatal Behavior Assessment Scale
Wehby, GL (2008)	USA *Iowa*	6.774	Before pregnancy and/or during the first 12 weeks	—	3 years	Fine and Gross Motor Skills Language Ability Personal-social Contact	The Denver Developmental Screening Test
Yang, L (2010)	China	3.609	—	Hb	4–6 years	IQ	Chinese-Wechsler Intelligence Scale for Children
Fararouei, M (2010)	Finland	11.656	From 24 to 28 weeks to 31 years after delivery	Hb	14 years 16 years 31 years	Educational Achievement	*School cores*: Self-reported questionnaires School reports Self-reported questionnaires Details of university degree
Hernández-Martínez, C (2011)	Spain *Reus*	216	From 13 weeks to delivery	SF TS	2–3 days	Behavior	Brazelton Neonatal Behavior Assessment Scale
Rioux, FM (2011)	Canada	63	—	Blood iron Hb	6 months	Cognition	Brunet-Lézine Scale of Psychomotor Development of Early Childhood BSID-III (Mental Scale)
Tran, T (2013)	Vietnam *Ha Nam*	378	From 12 to 20 weeks to 6 months after delivery	Hb SF	6 months	Mental Development	BSID–III (Mental Scale)
Tran, T (2014)	Vietnam *Ha Nam*	418	From 12 to 20 weeks to 6 months after delivery	Hb SF	6 months	Psychomotor Development	BSID–III (Motor Scale)
Lewis, SJ (2014)	United Kingdom *Bristol*	11.696	—	Hb HFE, TMPRSS6 and TF genotype	8 years	IQ	Wechsler Intelligence Scale for Children-III
Schmidt, R (2014)	USA *California*	866	From 3 months before pregnancy to breastfeeding	—	2–5 years	ASD Cognition Adaptive function	Autism Diagnostic Interview–Revised Autism Diagnostic Observation Schedule–Generic (ADOS) Social Communication Questionnaire Mullen Scales of Early Learning Vineland Adaptive Behavior Scales
Mireku, MO (2015)	Republic of Benin *Allanda*	636	Before 29 weeks of pregnancy to delivery	Hb	1 year	Cognition Psychomotor Development	Mullen Scales of Early Learning

Hb: hemoglobin; SF: serum ferritin; IQ: intelligence quotient; TS: transferrin saturation; BSID: Bayley Scales of Infant and Toddler Development; ASD: Autistic Spectrum Disorders.

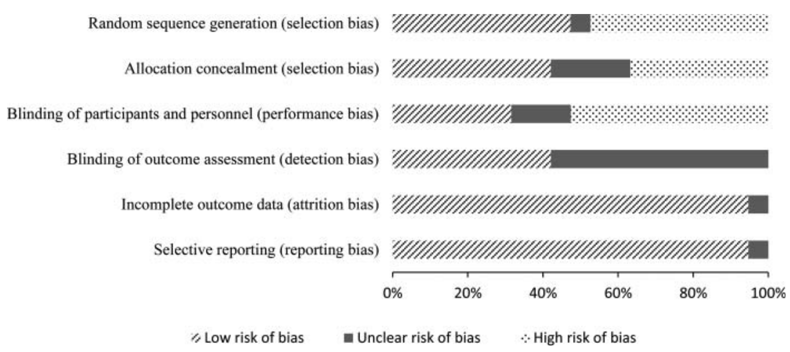

Figure 2. Percentage of bias types among studies.

respectively were used to determine IQ (Zhou et al., 2006; Tamura et al., 2002; Yang et al., 2010; Lewis et al., 2014). The Test for Auditory Comprehension of Language (Carow-Woolfolk 1998) provided information on the children's auditory acuity in Tamura's research (2002) and the Yale Children's Inventory for Attention and Tractability (Sally et al., 1988) was used to determine if children suffered from attention deficits and learning disabilities. To assess educational achievement over 30 years, Fararouei et al. (2010) used school scores at 14 and 16 years old and the highest level of education at 31 years old. Prado et al. (2012) adapted the following tests to evaluate several cognitive domains in Indonesian children aged 42 months: the Picture Vocabulary Scale (Dunn et al., 1997) and the MacArthur–Bates Communicative Development Inventory–Level III (Fenson et al., 2007) were used to assess language abilities; the Block Design Test of British Ability Scale (Elliot, 1996) and the Wechsler Preschool and Primary Scale of Intelligence–III (Wechsler, 2002) for assessing visuospatial ability; the Visual Search Test of NEPSY Developmental Neuropsychological Assessment (Korkman et al., 1998) for assessing visual attention and the Snack Delay Test (Spinrad et al., 2007) and the Windows Test (Russell et al., 1991) were used to determine executive function.

In one study, symptoms of Autistic Spectrum Disorders (ASD) were identified using the Social Communication Questionnaire (Rutter et al., 2003) and ASD were diagnosed using the Autism Diagnostic Interview–Revised (Lord et al., 1994) and the Autism Diagnostic Observation Schedule–Generic (ADOS) (Lord et al., 2000). Schmidt et al. (2014) assessed cognitive function using the Mullen Scales of Early Learning (Mullen 1995), like Mireku et al. (2015) 1 year later, and adaptive function was established using the Vineland Adaptive Behavior Scales (Sparrow et al., 1984).

Behavior assessment

The Bayley Scales also evaluate the socio-emotional dimension and adaptive behavior of children, and were the measure used by Vaughn et al. (1986) in addition to the Brazelton Neonatal Behavior Assessment Scale (NBAS) (Als et al., 1977). Other researchers (Cucó et al., 2005; Hernández-Martínez et al., 2010) also used the NBAS to evaluate children's behavior because it enables observation of the best capacities of newborn children. Prado et al. (2012) used the Brief Infant–Toddler Social and Emotional Assessment (Briggs-Gowan et al., 2002) to evaluate the emotional development of children, while another test, the Strength and Difficulties Questionnaire (SDQ) (Goodman, 2006) was useful due to being able to report possible emotional and behavioral problems in children (4–17 years) based on information from parents and teachers (Zhou et al., 2006; Parsons et al., 2008). Children's temperament also was evaluated in one study (Parsons et al., 2008) using the Short Temperament Scale for Children (Prior et al., 2000).

Psychomotor development (PD) assessment

Seven of the studies also evaluated children's psychomotor development. Most of the researchers used the motor score on the Bayley Scale (Li et al., 2009; Chang et al., 2013; Hanieh et al., 2013; Tran et al., 2014; Prado et al., 2012) in addition to the mental domain of BSID. Meanwhile, Tamura et al. (2002) used the Peabody Developmental Motor Scales (Folio and Fewell, 2000) to assess the fine and gross motor capacity of children at 5 years old, and Wehby and Murray (2008) used the Denver Developmental Screening Test (Frankenburg and Dodds, 1967) for the same parameter at 3 years of age. Cucó et al. (2005) used the Motor cluster of NBAS, Prado et al. (2012) applied the Ages and Stages Questionnaire (Schaefer and DiGeronimo, 2000) and Mireku et al. (2015) used the Mullen Scales of Early Learning (Mullen 1995) to assess PD in children.

Discussion

Maternal iron status

When discussing maternal iron status during pregnancy and its importance for the mother and child's health is necessary to take into account the metabolism of iron and the different factors that may influence it. Apart from the fact that iron absorption depends on several dietary conditions which are even more pronounced in supplemented pregnant women, the storage levels of iron, overall nutritional status, genetic mutations and specific physiological states such as pregnancy and infancy also may modify its absorption (Forrellar et al., 2000; García et al., 2010; Olivares et al., 2010; Harvey et al., 2013). During pregnancy, iron needs increase from 0.8 mg/d in the first trimester to 7.5 mg/d in the third trimester (FAO and WHO, 2001; Cao and O'Brien, 2013) because the child's development depends on the transfer of iron from the mother. The increased ability to absorb nutrients is directly related to nutritional requirements (Andrews, 1999; Hallberg, 2001) but the absorption of iron from the diet is known to be insufficient to cover increased iron needs during pregnancy, meaning that the iron balance is dependent on the amount of stored iron. It is therefore important to have good nutritional health before getting pregnant and there is also a need to monitor possible genetic mutations in the HFE gene that causes iron overload.

Maternal iron deficit and mental development

The first author to investigate the relationship between maternal iron status and child mental development was Vaughn et al. (1986), who concluded that no maternal biochemical or hematological parameters were related with BSID scores in a study with 115 pregnant women and their babies. He may have reached that conclusion because at that time only the first edition of the BSID had been published, which was not as specific as the following versions, but it also may be due to the sample size being insufficiently representative for an observational study. Later, Tamura et al. (2002) assessed children's auditory comprehension of language and their ability to obey rules and follow general decorum orders, and they concluded that a low cord serum ferritin concentration correlated with worse scores in all tests. In 2008, Wehby and Murray (2008) analyzed the effects of some micronutrient supplementation at least 3 days a week during the 3 months prior to becoming aware of the pregnancy, and/or during the following 3 months, on the language skills of children aged 3; they concluded that MMN use was associated with a reduced odds ratio for moderate risk to language but not supplementation with iron alone. Li et al. (2009) subsequently tested the effect of iron in 1,305 women, both alone and in combination with FA, versus MMN supplementation on the MD of children, and found additional effects in the IFA group compared with FA alone; however, the BSID scores were better in the mental development index (MDI) in the MMN group than in the others, consistent with the findings of Wehby and Murray (2008) and Prado et al. (2012), who conducted a similar study with 487 pregnant women. Two more recent investigations have been conducted in low income countries, and this may be the reason for the results obtained, because the requirements of micronutrients were so high in malnourished women that iron alone is not sufficient to ameliorate the mother and the child's health. Another case with negative results was the study by Rioux et al. (2011), who evaluated maternal iron status and infants' cognitive performance in 63 Canadian mother–infant dyads; they did not observe any relation between these two variables, which may be due to the small sample size. Fararouei et al. (2010) conducted a cohort study in Finland on 11,656 pregnant women and their offspring at 14, 16, and 31 years old; they aimed to assess the educational achievement of the offspring as related to the maternal Hb level during pregnancy and unlike the authors above, they concluded that there was a direct association between these two parameters, especially in the final stages of pregnancy. These data therefore suggest long-term influences of the prenatal iron status on the cognitive development of offspring, although further research is needed to replicate these results. More recently, Chang et al. (2013) and Hanieh et al. (2013) conducted their respective RCTs in Southeast Asia and both concluded that prenatal iron supplementation led to increased BSID scores in the cognition domain. When Hanieh carried out his study of in 1,258 women, comparing daily and intermittent (twice a week) iron supplementation, he recommended the second option for non-anemic women as well as women of child-bearing age to prevent beginning pregnancy with low iron reserves. However, this recommendation should not be extrapolated to populations with a high prevalence of anemia because intermittent supplementation might be insufficient if women have poor iron reserves. Hanieh's result is supported by the findings of Chang et al., who observed that supplementation is more effective in women beginning pregnancy without ID, and recommend beginning the supplementation before becoming pregnant. Likewise, Tran et al. (2013) used the third edition of the BSID to evaluate the 378 children in their observational study conducted in Vietnam, and the results showed that ID during the gestational period leads to diminished cognitive abilities. Mireku et al. (2015) were more neutral in their conclusions from a study conducted on 636 Beninese children, in which they found no relation between prenatal Hb concentration and the cognitive abilities of infants at 1 year of age.

Maternal iron deficit and behavior

For children's behavioral development, Vaughn et al. (1986) concluded that the mother's iron-binding capacity was significantly related to irritability in children at 3 days of age, according to the NBAS and at 5 months, according to the Bayley Scale. He therefore observed that mothers of more irritable infants had lower levels of circulating iron than mothers of less irritable infants. Zhou et al. (2006) concluded from their RCT of 430 women and their children that SDQ mean scores in behavioral and emotional difficulties did not differ significantly between the supplemented and control group; this was perhaps because the dose of supplementation was too low to notice any significant clinical effect. Two years later, Parsons et al. (2008) continued with observations of 264 children in the study in Australia, and according to their results a better maternal iron status during pregnancy is related with an absence of behavior problems at 4, 6, and 8 years of age. They also evaluated the temperament of children aged 6 and 8, and found no difference between the Fe and placebo groups, so they concluded that routine iron supplementation in pregnancy in a well-nourished population has no clearly beneficial effect on parental reports of child temperament. These observations may be due to the fact that the Australian population had a good baseline nutritional status, but also may be because the iron dose was insufficient. In contrast, when Wehby and Murray (2008) conducted a large study ($n = 6.774$) in the American population, they found that iron use reduced the risk (OR 0.5 [0.35–0.72]) on the personal-social scale of the Denver Test (Frankenburg and Dodds, 1967), even when the dose of supplementation was ignored. A Spanish longitudinal study carried out by Hernández-Martínez et al. (2010) was also based on well-nourished pregnant women who were supplemented with 40 mg/d of Fe, and the researchers concluded that ID affects children's neurodevelopment differently depending on when it occurs. In specific terms, the autonomous response of the neonate may be altered if ID occurs early in pregnancy, consistent with a worse response of the NBAS. However, when iron levels were assessed in terms of intake and not at the biochemical level, Cucó et al. (2005) found no differences in any behavioral cluster.

Recently, in the United States, Schmidt et al. (2014) conducted an observational study, knowing whether women had received supplements and the amount of dietary iron intake, to assess the effect of these on the risk of ASD of children. They monitored the psychiatric clinical evolution of 866 children and found initial evidence for an association between increased maternal Fe supplementation and a reduced risk of ASD.

Maternal iron deficit and IQ

Tamura et al. (2002) evaluated the IQ score of 278 children aged 5 in an observational study conducted in Alabama, and concluded that there is an association between both the highest and lowest quartile of cord serum ferritin concentration and lower scores on intelligence tests, while the middle two quartiles are associated with better scores. Albeit weakly, maternal and cord ferritin concentration correlates significantly (Rusia et al. 1995; Nemet et al. 1986) which highlights the importance of a good maternal state of iron and demonstrates that low iron status impairs children's neural development during pregnancy. As in the case of behavior, Zhou et al. (2006) concluded that routine iron supplementation is not sufficient for favorable effects on the IQ of children and similarly, the explanation may be that 20 mg/d of iron may be too low for the most of the pregnant women. Likewise, Prado et al. (2012) observed in Indonesia that maternal MMN intake compared with IFA supplementation can improve the cognitive abilities of children at 3.5 years old, especially when the women were undernourished or anemic during pregnancy because the absorption is incremented. As in the other aspects of neurodevelopment, this result may due to the fact that the nutritional requirements involve more than iron in low income areas. In line with previous investigations, Lewis et al. (2014) used a population-based cohort (ALSPAC) in the United Kingdom to assess the effect of the iron status of 11,696 pregnant women on the cognition of their children, and found no evidence that low iron levels in pregnancy have a detrimental effect on the brain of the developing fetus and therefore on IQ in childhood. This observation is inconsistent with most findings, and may be because in the United Kingdom health carers prescribe iron supplements at a very early stage in pregnancy for women with low iron stores, meaning that the researchers were unable to study the effects of low iron levels in late period of pregnancy in this cohort.

Maternal iron deficit and psychomotor development

Hernández-Martínez et al. (2010) found in Spain that the third trimester of pregnancy is a critical period in neonatal motor performance. They demonstrated that ID or anemia in this period may delay the children neuromotor development. Accordingly, the observational study also conducted by Cucó et al. (2005) in Spain concluded that an appropriate supply of iron, mainly in the final weeks of gestation, contributes to the maturation of the infant neuromotor system, as reflected in improved NBAS test scores for newborns after 3 days of life. These results reinforce the conclusion of Tamura et al. (2002), who some years previously asserted that children aged 5 whose cord serum ferritin concentration was low obtained worse scores in motor tests than children with intermediate values of ferritin. The conclusion of Tamura's team has recently been confirmed by the results of Mireku et al. (2015) from an African cohort; their study revealed that there was an inverted U-shaped relationship between prenatal Hb during the first half of pregnancy and infant gross motor function at 1 year of age. This shows that low Hb concentrations may be detrimental to the early motor skills of children. Similarly, Li et al. (2009) aimed to determine what kind of supplementation may benefit infant psychomotor development, and they conducted an interventional study comparing the effect of MMN (with 30 g/d of Fe) and IFA (with 60 g/d of Fe). They found further evidence that micronutrient supplementation, but not IFA in this case, improves PD at 1 year of age. Various micronutrients play a role in children's neural development, which is more evident in regions with low recourses such as rural China, where the effect of IFA seems to be insufficient to improve infants' health, although the dose of Fe is high in this group. Prado et al. (2012) arrived at the same conclusion in Indonesia, due to the similar nutritional status of the population and the equivalent nature of the intervention, and therefore recommended MMN supplementation for undernourished pregnant women to improve the PD of children at 3.5 years old. However, Hanieh et al. (2013), who counterpoised daily IFA, intermittent IFA and intermittent MMN supplementation, concluded that at 6 months of age there was no difference between the twice-weekly supplement groups and the daily group in an area of Southeast Asia with low anemia prevalence. This result was perhaps due to the fact that in a well-nourished population, the effect of the variability of total dose of iron or the combination with other micronutrients is less evident than in needy regions. Several years after the study by Tamura et al. (2002), and following Hernández-Martínez et al. (2010), Chang et al. (2013) investigated 850 mother–child pairs in rural China, and they also found that anemia in the third trimester was related with poor development of motor skills among children aged 2. The recent results of Tran et al. (2014) are consistent with these data, and indicate that anemia (Hb $<$ 110 g/L) in late pregnancy is associated with poor infant PD at 6 months of age, but this association with maternal iron deficiency was not evident (SF $<$ 15 μg/L). Like the authors mentioned above, Tran et al. also concluded that even if ID and IDA in early pregnancy do not have a direct effect on BSID-Motor scores, both conditions indirectly affected the outcomes, increasing the risk of anemia in late pregnancy. As a result, they suggest that interventions to promote infant development should be performed in early pregnancy and explicitly addressed at these antenatal factors.

Excessive maternal iron and neurodevelopment

As discussed in the paragraph "*Maternal iron assessment*," some gene mutations can affect iron homeostasis. The HFE gene mutation causes ineffective hepcidin production (Andrews, 1999), and a low hepcidin plasma concentration leads to a lack of downregulation of FPN, allowing increased iron uptake (Lewis et al., 2014; Fleming and Ponka, 2012; Crownover and Covey, 2013; Muñoz et al., 2011) which causes hemoconcentration, especially in pregnant women receiving iron supplements (Casanueva et al., 2006; Aranda et al., 2013; Arija et al., 2013). Excess iron damages cells primarily by catalyzing the production of reactive oxygen species (ROS) in quantities that the cellular antioxidant system is unable to delete (Fleming and Ponka, 2012). During gestation, the iron overload may increase blood pressure, the risk of eclampsia (Ziaei et al., 2007) and blood viscosity, which can affect the blood flow between the uterus and placenta, diminishing the perfusion of nutrients and increasing the risk of placenta infarction (Aranda et al., 2013). Fetal development may therefore be harmed because the amount of nutrients reaching the fetus is not enough to meet its needs (Yip, 2000).

According to the literature, there is some evidence that an elevated concentration of iron may be harmful to children's neurodevelopment. Tamura et al. (2002) were the first to observe this effect and they concluded that a high Hb concentration is related with a lower IQ in children, which shows that a high iron status impairs child neural development in pregnancy, to the same extent as a deficit. Later, Yang et al. (2010) strengthened this hypothesis, showing the adverse effects on cognitive development produced by excess iron in early pregnancy. Parsons et al. (2008) also contributed by evaluating children's behavior and found that that significantly more children in the Fe group had an abnormal teacher-rated peer problems score than in the placebo group, which suggests that routine Fe supplementation may even have a negative influence on behavior if the mother does not have ID. Further evidence to that effect comes from Hanieh's research (2013), which compared daily and intermittent supplementation with iron and specifically concluded that 40% of women in the daily IFA group had ferritin levels higher than 41 μg/L at 32 weeks of gestation, although this was not associated with hemoconcentration (Hb $>$ 130 g/L). It therefore follows that daily IFA supplementation may lead to an excessive increase in maternal SF, which causes adverse outcomes for mother–child health during pregnancy, such as those discussed above. The final evidence for the detrimental effect of excess iron on children's neurodevelopment was provided by Mireku et al. (2015), who noticed that both high and low prenatal Hb concentrations were harmful to children's gross motor development. According to their data, optimal range of maternal Hb seems to be 90–110 g/L.

In addition to the body's iron stores, physiological state or general nutritional situation, during pregnancy the iron levels may therefore also depend on genetic individualities, meaning that the same iron intake may be beneficial for one woman while for another it may be excessive and lead to a harmful iron overload. For this reason, the scientific evidence points to the need to personalize the supplementation dose in accordance with all these individual characteristics.

Strengths and limitations

We used a wide search strategy, which included observational studies and RCTs, which were selected according to the aim of the study, i.e., children's neurodevelopment after iron supplementation in pregnancy, as well as their iron profile. The year of publication was not a filter in the selection process of the articles included, and as such our systematic review gathers widespread scientific evidence about the effect of a low prenatal iron status and the benefits, but sometimes also the damage, of iron supplementation during pregnancy for children's neurodevelopment. The risk of bias was assessed to take into account the quality of the studies included and to analyze the results more precisely. As mentioned above, the inclusion of observational studies in the review provided more knowledge than if we had only used intervention trials but the high risk of selection and performance bias is nevertheless a limitation. However, we have discussed the results of the studies considering the bias of each one in order to extract wide-ranging and objective knowledge.

Conclusion

Although the research on child neurodevelopment related to prenatal iron levels is very limited, the scientific evidence shows an association between ID or IDA during pregnancy and problems in MD, behavior and cognitive abilities. PD is also improved by a good maternal iron status during gestation, which is endorsed by all the authors who investigate this outcome. Some research has found evidence that excess iron is also harmful to brain formation and maturation in the fetus and therefore the child's development in the mental and motor domains. However, further investigation is necessary for a better understanding of how a deficit or excessive maternal iron may damage the child's neural development and how to personalize iron supplementation to fit the individual characteristics of each woman in order to avoid ID, IDA and hemoconcentration in pregnancy.

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Conflict of interest

The authors declare that they do not have conflict of interest.

Authors' contributions

Lucía Iglesias: searched the articles, conducted its selection process, interpreted them and wrote the manuscript. Josefa Canals: contributed in the search of articles, interpreted its information, contributed to the discussion and review the manuscript. Victoria Arija: planned the systematic review, interpreted its information, contributed to the discussion and reviewed the manuscript. All authors read and approved the final manuscript.

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CHAPTER 2.

DETERMINANTS OF MATERNAL IRON STATUS

Maternal factors associated with iron deficiency without anaemia in early pregnancy: ECLIPSES study

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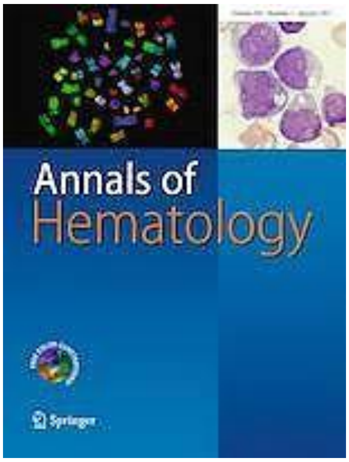

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ORIGINAL ARTICLE

Maternal factors associated with iron deficiency without anaemia in early pregnancy: ECLIPSES study

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Abstract

Several population-specific genetic, sociodemographic, and maternal lifestyle factors are related to iron status in early pregnancy, and their identification would allow preventive actions to be taken. The study aimed to identify maternal factors associated with iron deficiency (ID) in early pregnancy in non-anaemic pregnant women from a European Mediterranean country. Cross-sectional study using the initial population of the ECLIPSES study performed in non-anaemic pregnant women before gestational week 12. Serum ferritin (SF) and haemoglobin concentrations were measured to evaluate iron status, and ID was defined as SF < 15 µg/L. Several sociodemographic and lifestyle data were recorded and used as covariates in the multivariate-adjusted regression models. Out of the 791 participants, 13.9% had ID in early pregnancy. Underweight (OR 3.70, 95%CI 1.22, 15.53) and parity (1 child: OR 2.03, 95%CI 1.06, 3.88; ≥ 2 children: OR 6.96, 95%CI 3.09, 15.69) increased the odds of ID, while a high intake of total meat (≥ 108.57 g/day: OR 0.37, 95%CI 0.15, 0.87), red/processed meat (≥ 74.29 g/day: OR 0.70, 95%CI 0.35, 0.98), protein (≥ 65.05 g/day: OR 0.85, 95%CI 0.30, 0.99), and dietary iron (≥ 8.58 mg/day: OR 0.58, 95%CI 0.35, 0.94) protected against it. Smoking was also associated with a reduction in ID odds (OR 0.34, 95%CI 0.12, 0.99). Baseline BMI, parity, smoking, and diet are associated with ID in early pregnancy in non-anaemic women. Pregnancy planning policies should focus on women at higher risk of ID, such as those who are underweight, multiparous, or following vegetarian diets. This clinical trial was registered at www.clinicaltrialsregister.eu as EudraCT number 2012–005,480-28 and at www.clinicaltrials.gov with identification number NCT03196882.

Keywords Prenatal · Maternal · Iron status · Iron deficiency · Haemoglobin · Serum ferritin

Introduction

Maternal iron status during pregnancy is a public health concern given the high prevalence of anaemia and iron deficiency (ID) during the gestational period. Estimates indicate that around 25% of pregnant women worldwide suffer from anaemia, mainly caused by ID [1]. Anaemia poses significant problems for maternal and child health [2–4], so haemoglobin levels are routinely monitored to detect it preventively. However, there is a high percentage of pregnant women presenting ID without anaemia who are not diagnosed and monitored in daily clinical practice, as serum ferritin (SF) is not routinely measured [5, 6]. In Europe, 10–33% of pregnant women have ID [7], such as in our study where we have previously observed 14% of participants with ID in early pregnancy [8]. It is therefore of great importance among childbearing women to maintain an adequate iron balance, given the role of iron in multiple physiological

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processes that take place during pregnancy. Wide evidence supports that prenatal iron imbalances also in absence of anaemia can be detrimental to mother and child. In this regard, previous studies have found that not only anaemia but also ID during the gestational period is associated with preeclampsia, premature births, and even miscarriages [4, 9], as well as physical and cognitive developmental delays in children in postnatal life [3, 10–13].

It is worth mentioning that maternal iron levels in early pregnancy by themselves strongly influence the progression of iron stores during gestation and a woman's iron status at the end of pregnancy. Indeed, in the ECLIPSES study, we already found a positive association of iron-related biomarker concentrations between the first and third trimester of gestation [8]. Previous studies reached similar findings when assessing the correlation of iron status between early and late pregnancy [14, 15].

Several studies in developed countries have identified some maternal factors that influence the initial iron status of pregnant women, such as sociodemographic, genetic, and lifestyle characteristics [16–19]. However, not all factors that are related have always been analysed together. Many of the risk factors for ID are population-specific, and it is therefore important to disentangle which maternal characteristics place women at risk for ID in each population group. In this regard, few studies have assessed factors associated with iron status in non-anaemic pregnant women.

This study aimed to identify maternal factors associated with ID in early pregnancy in a sample of non-anaemic pregnant women, although with a moderate prevalence of ID, from a European Mediterranean country.

Material and methods

Study design and population

The present work included the baseline population of the ECLIPSES study, a community-based randomized controlled trial (RCT) conducted in the province of Tarragona (Catalonia, Spain) [20], before starting the intervention. Briefly, the participants were recruited by midwives in their primary care centres during the routine obstetrical visits prior to gestational week (GW) 12. The main inclusion criteria were over 18 years old and not having anaemia (haemoglobin [Hb] $\geq$ 110 g/L). Women who had taken > 10 mg iron daily during the 3 months prior to GW12 were excluded.

Outcome

The study outcome was the iron status of women in early pregnancy and its associated factors. For this, concentrations of iron-related biomarkers (Hb and SF) were measured. Since having anaemia was an exclusion criterion, only ID defined as SF < 15 μg/L according to the WHO guidelines was considered [21].

Data collection

The research staff recorded the sociodemographic and lifestyle data of the participants during a personal interview using specific questionnaires, including maternal age, baseline body mass index (BMI), smoking habit, ethnicity, parity, pregnancy planning, and use of hormonal contraceptives. The educational level and occupational status of women and their partners were also registered. The family's socioeconomic status (SES) was calculated from the sociodemographic data of participants and their partners, including educational level and occupational status. Dietary assessment was done using a short food frequency questionnaire (FFQ) validated in our population [22]. Food groups assessed included total meat, red and processed meat, fish, fruits, vegetables, legumes, and dairy products as grams per day (g/day). From this information, energy intake (kcal/day) and nutrients (g/day or mg/day) were calculated using the REGAL (Répertoire Général des Aliments) food composition table [23], complemented by a Spanish food composition table [24]. As for the nutrient intake, protein, fibre, vitamin C, calcium, and dietary iron were assessed. Detailed information is available in Aparicio et al. [25]. Information from the FFQ allowed us to calculate the percentage of adherence to the Mediterranean diet, considered a high–quality dietary pattern [25, 26]. Extended information on data collection can be found elsewhere [8, 20].

Blood samples were taken on GW12 to perform blood and genetic tests. Haematological parameters (Hb and MCV), some specific biochemical markers (SF and C-reactive protein [CRP]), and genetic mutations of the *HFE* gene (C282Y, H63D and S65C) were performed.

Statistical analyses

Continuous variables (mean and SD) were described using Student's *t*-test and ANOVA test, while the chi-squared test was used for categorical variables (percentages). Natural logarithm (Ln) transformation was applied to normalize the distribution of SF, increasing the validity of analyses, and using the median and interquartile ranges (IQR). Multivariate regression models (multiple linear regressions and logistic regressions) were used to assess the effect of different prenatal predictors on maternal iron status in early pregnancy. The regression models were adjusted for a wide range of potential confounders, described in the bivariate analyses, including age (< 25 years, 25–35 years, and > 35 years), baseline BMI (underweight, BMI < 18.5; normal weight, BMI 18.5–24.9; overweight, BMI 25–29.9; and obesity, BMI ≥ 30), smoking habit (yes or not), SES (low or middle-high), ethnicity (Caucasian, Latin American, Arab, and Black), parity (primiparous, 1 child, or ≥ 2 children), pregnancy planning (yes or no), use of hormonal contraceptives (yes or no), *HFE* genotype (WT/WT, C282Y/WT, H63D carrier, and S65C carrier), and dietary intake expressed as quartiles. Daily dietary consumption of food groups (total meat, red and processed meat, fish, fruits, vegetables, legumes, and dairy products) and nutrients (protein, fibre, vitamin C, calcium, and iron) were separately included in the regression models to avoid over-adjustment. Daily energy intake as kcal was included in both models. Given that SF can raise in infectious or inflammatory processes, the regression model for SF concentration was additionally adjusted for CRP levels. All statistical analyses were performed using SPSS (version 25.0 for Windows; SPSS Inc., Chicago, IL, USA) and statistical significance was set at $p < 0.05$.

Results

The study included 791 pregnant women, with a median age of 31 (17–46) years and a median gestational age of 12 weeks at the assessment. Sociodemographic and lifestyle characteristics were presented in Table 1. Regarding the body mass index (BMI), near of 60% of participants had normal weight and more than 40% had excess weight, including overweight and obesity. Most of them were Caucasian (80.4%) and belonged to a middle SES (67%), almost 18% were smokers at the recruitment, and 18.3% reported having used hormonal contraceptives before getting pregnant. For 50.4% of the participants, this was their first pregnancy, and 80% had planned to become pregnant. As for the *HFE* genotype, 33.1% had some mutation, the H63D/WT (26.1%) and C282Y/WT (3.5%) being the most represented genotypes. The less represented

Table 1 Baseline characteristics of the population in the study ($n = 791$)

Age, years	31 [17–46]	Maternal ethnic origin	
< 25	14.7	Caucasian	80.4
25–35	63.3	Latin American	10.7
> 35	22.0	Arab	6.3
Gestational age, weeks	12 [8–12]	Black	2.0
Baseline BMI	25.05 (4.50)	Asian	0.6
Underweight	1.6	Education	
Normal weight	57.8	Unfinished primary school	4.6
Overweight	26.4	Primary school	28.4
Obesity	14.2	Secondary school	38.3
Smoking habit (yes)	17.8	Higher/vocational education	28.7
Use of hormonal contraceptives (yes)	18.3	Familiar SES	
Parity		Low	16.2
Primiparous	50.4	Middle	67.0
1 child	37.6	High	16.8
≥ 2 children	11.9	Food intake	
Pregnancy planning (yes)	80.0	Total meat (g/d)	92 [0–314]
HFE genotype		Red and processed meat (g/d)	56 [0–239]
WT/WT	66.9	Fish (g/d)	45 [0–179]
H63D/WT	26.1	Fruits (g/d)	244 [0–929]
C282Y/WT	3.5	Vegetables (g/d)	78 [0–441]
S65C/WT	1.3	Legumes (g/d)	15 [0–60]
C282Y/H63D	1.0	Dairy products (g/d)	294 [0–1530]
H63D/H63D	1.0	Energy (kcal/d)	1788 [853–4290]
H63D/S65C	0.2	Nutrient intake	
S65C/S65C	0.2	Protein (g/d)	56 [10–167]
C282Y/ C282Y	0.0	Fibre (g/d)	13 [3–35]
C282Y/ S65C	0.0	Vitamin C (mg/d)	77 [3–280]
		Calcium (mg/d)	663 [55–2123]
		Iron (mg/d)	8 [2–24]

Data are expressed as mean (SD), median [min–max] and %

BMI body mass index, *WT* wild type, *SES* socioeconomic status genotypes were considered together, leaving the following categories: "WT/WT," "C282Y/WT," "H63D carrier" (including C282Y/H63D, H63D/ H63D), and "S65C carrier" (including H63D/S65C, S65C/S65C). In relation to educational level, almost 30% accounted for higher or vocational education, while less than 5% reported having unfinished primary school. Median dietary iron intake was 8 mg/day, and adherence to the Mediterranean diet was high in almost all the participants in the study. A strong statistically significant correlation (Spearman rho 0.915, $p < 0.001$) was found between energy (kcal) and iron intake. Compared to women of normal weight, underweight women reported lower intakes of energy (1827.96 and 1724.57 kcal/day, respectively) and iron (7.97 and 7.40 mg/day, respectively), although the difference was not statistically significant (data not shown).

Concentrations of SF and Hb, as well as the percentage of women with ID in early pregnancy, were described according to genetic, sociodemographic and lifestyle characteristics (Supplementary Table 1) and by quartiles of dietary intake (Supplementary Table 2). From the overall sample, 13.9% had ID in early pregnancy. Both SF and Hb concentrations increased across increasing BMI categories. In addition, smokers and primiparous women showed higher SF concentrations and a lower percentage of ID than their counterparts early in pregnancy. As for dietary intake, SF concentrations increased progressively across quartiles of total meat and iron intake, whereas a U-shaped distribution was observed for red and processed meat intake, and an inverse U-shaped for calcium intake by quartiles.

Multivariate adjusted analyses showed the effect of prenatal sociodemographic and lifestyle factors on SF and Hb concentrations, and ID at GW12 (Table 2). Parity and being underweight were negatively associated with maternal Hb and SF concentrations, whereas a high intake of total meat (≥ 108.57 g/day), and red and processed meat (≥ 74.29 g/day) increased them in early pregnancy. Additionally, young maternal age (< 25 years) reduced and smoking increased SF concentrations at GW12 but did not show any effect on Hb levels. In relation to ID, smoking, and a high intake of total meat, and red and processed meat, reduced its odds by 66%, 63% and 30%, respectively. Consistent with the observed effect on SF levels, underweight and non-parous women were more likely to suffer from ID in early pregnancy compared to their peers. As for dietary intake, complementarily, when the models were adjusted for daily nutrient intake instead of food groups consumption, a high intake of protein (≥ 65.05 g/day) and iron (≥ 8.58 mg/day) was positively associated with Hb and SF concentrations in early pregnancy, as well as with a reduction in the percentage of ID.

Discussion

This study contributes to the identification of some maternal sociodemographic and lifestyle factors associated with the risk of developing ID in early pregnancy in a sample of non-anaemic pregnant women from northeastern Spain, with the aim of preventing this deficiency, which is so common during pregnancy, and to avoid its negative consequences.

From all the analysed maternal biological, sociodemographic, and lifestyle conditions, the most relevant predictor of iron status in early pregnancy identified in this work was multiparity, showing almost 7 times higher odds of ID than primiparous women. That has already been repeatedly reported from around the world [16, 17, 27, 28] and the main explanation is that the high iron cost of pregnancy puts multiparous women at risk of not recovering iron stores from one pregnancy to the next one [29]. Another important predictor of ID in early pregnancy was being underweight, in accordance with previous findings [16, 18, 30, 31]. This association would be indirectly reflecting the effect of poor nutritional status, with low food and nutrient intake. Additionally, underweight women in our study reported lower energy and iron intake than those of normal weight, reinforcing the proposed argument. Other of the studied maternal factors showed a minor impact on the women's iron status. This is the case of age which, although previous studies have associated younger age with a higher risk of ID [17, 32], being a young mother led to a decrease in SF concentration but did not influence the likelihood of ID in our case. As for the smoking habit, smokers usually show higher levels of SF than non-smokers [19] and, therefore, apparently decrease the odds of ID, as has been found in the present study. According to scientific evidence, cigarette smoking disrupts iron homeostasis inducing a systemic accumulation [19, 33, 34], which leads to the detection of an increase in iron reserves.

This study also assessed dietary intake and its relationship with iron levels. Thus, it was expected that a higher daily intake of total, red and processed meat, as well as protein and iron, would increase both Hb and SF concentrations in early pregnancy, protecting against ID. However, a concern arising from our results is the low iron intake that women reported (median: 8 mg/day), with only 8 participants (1%) meeting the DRI (16 mg/day), and 35.5% not even reaching the EAR (7 mg/day) that EFSA indicates for pregnant women [35]. However, according to a recent review [36], that is not an isolated problem, but the dietary iron intake of most pregnant women in Europe is well below the recommendations. Finally, it has to be stated that, contrary to expected, no effect was found of

Table 2 Associations between early pregnancy concentrations of haemoglobin and serum ferritin and iron deficiency, and predictor variables

	Haemoglobin (g/L) β (95%CI)	Serum ferritin (μg/L)	ID (SF < 15 μg/L) OR (95%CI)
Age (years)[a]			
< 25	−0.28 (−2.68, 2.11)	−0.24 (−0.45, −0.03)*	1.71 (0.70, 4.18)
25–35	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
> 35	1.62 (−0.16, 3.40)	−0.14 (−0.30, 0.02)	1.06 (0.56, 2.03)
Smoking			
No	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
Yes	1.17 (−0.94, 3.27)	0.08 (0.10, 0.27)*	0.34 (0.12, 0.99)*
Baseline BMI (kg/m^2)			
< 18.5	−0.70 (−2.59, −0.05)*	−0.35 (−0.90, −0.20)*	3.70 (1.22, 15.53)*
18.5–24.9	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
25–29.9	0.89 (−0.92, 2.71)	−0.04 (−0.21, 0.12)	0.85 (0.43, 1.67)
≥ 30	1.81 (−0.44, 4.06)	0.02 (−0.19, 0.22)	0.80 (0.33, 1.91)
Parity			
Primiparous	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
1 child	−2.33 (−3.99, −0.68)*	−0.17 (−0.31, −0.02)*	2.03 (1.06, 3.88)*
≥ 2 children	−2.76 (−4.78, −0.46)*	−0.48 (−0.71, −0.25)*	6.96 (3.09, 15.69)*
Total meat intake (g/d)			
< 68.57	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
68.57–91.73	0.66 (−1.65, 2.97)	0.12 (−0.08, 0.31)	0.53 (0.25, 1.12)
91.74–108.56	0.06 (−2.14, 2.26)	0.12 (−0.07, 0.30)	0.47 (0.22, 1.02)
≥ 108.57	2.48 (0.25, 4.72)*	0.20 (0.15, 0.33)*	0.37 (0.15, 0.87)*
Red and processed meat (g/d)			
< 37.14	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
37.14–55.99	0.75 (−1.62, 3.13)	0.04 (−0.17, 0.24)	0.42 (0.14, 1.28)
56.00–74.28	0.30 (−2.62, 2.03)	0.01 (−0.19, 0.21)	0.55 (0.24, 1.29)
≥ 74.29	1.02 (0.65, 3.91)*	0.16 (0.08, 0.41)*	0.70 (0.35, 0.98)*
Age (years)[b]			
< 25	−0.28 (−2.23, 2.78)	−0.24 (−0.45, −0.03)*	1.62 (0.66, 4.01)
25–35	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
> 35	0.86 (−1.00, 2.72)	−0.14 (−0.30, 0.02)	1.11 (0.57, 2.14)
Smoking			
No	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
Yes	0.82 (−1.37, 3.02)	0.08 (0.10, 0.27)*	0.33 (0.11, 0.98)*
Parity			
Primiparous	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
1 child	−2.30 (−4.03, −0.58)*	−0.17 (−0.31, −0.02)*	2.20 (1.13, 4.28)*
≥ 2 children	−1.23 (−3.96, 1.50)	−0.48 (−0.71, −0.25)*	7.39 (3.10, 17.59)*
Protein (g/d)			
< 48.10	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
48.10–55.92	0.34 (−0.85, 1.07)	0.12 (−0.09, 0.33)	0.84 (0.31, 2.27)
55.93–65.04	0.85 (0.52, 2.01)*	0.19 (0.01, 0.37)*	0.70 (0.22, 0.96)*
≥ 65.05	1.06 (0.87, 2.65)*	0.25 (0.10, 0.31)*	0.85 (0.20, 0.99)*
Iron (mg/d)			
< 6.31	0.00 (Ref.)	0.00 (Ref.)	1.00 (Ref.)
6.31–7.67	0.28 (−0.45, 1.65)	0.06 (−0.20, 0.15)	0.89 (0.64, 1.98)
7.68–8.57	0.94 (−0.67, 1,96)	0.26 (−0.39, 0.42)	0.62 (0.50, 1.32)
≥ 8.58	1.13 (0.21, 2.98)*	0.30 (0.12, 0.73)*	0.58 (0.35, 0.94)*

ID iron deficiency, *SF* serum ferritin, *BMI* body mass index

*Statistically significant differences compared to the reference group

[a] Adjusted for age (< 25, 25–35, > 35), baseline BMI (< 18.5, 18.5–24.9, 25–29.9, ≥ 30), smoking habit

Table 2 (continued)

(yes/no), SES (low, middle-high), ethnicity (Caucasian, Latin American, Arab, Black), parity (primiparous, 1 child, ≥ 2 children), pregnancy planning (yes/no), use of hormonal contraceptives (yes/no), *HFE* genotype (WT/WT, C282Y/WT, H63D carrier, S65C carrier), and daily dietary intake (kcal, total meat, red and processed meat, fish, fruits, vegetables, legumes, dairy products)

[b] Adjusted for age (< 25, 25–35, > 35), baseline BMI (< 18.5, 18.5–24.9, 25–29.9, ≥ 30), smoking habit (yes/no), SES (low, middle-high), ethnicity (Caucasian, Latin American, Arab, Black), parity (primiparous, 1 child, ≥ 2 children), pregnancy planning (yes/no), use of hormonal contraceptives (yes/no), *HFE* genotype (WT/WT, C282Y/WT, H63D carrier, S65C carrier), and daily dietary intake (kcal, protein, fibre, vitamin C, calcium, iron)

mutated *HFE* genotypes on iron-related biomarker concentrations or ID in early pregnancy. Despite *HFE* mutations being common in the European population, they are less frequent in Southern Europe [37, 38]. Especially, the variant with high clinical penetrance, *HFE* C282Y homozygous [39, 40], is absent in our study population which may preclude observing some influence of *HFE* mutations on women's iron status.

A high percentage of women with ID in early pregnancy show no signs of anaemia and their low iron stores go undetected and untreated since Hb is often the only biomarker measured for assessing iron status in routine practice [5, 6]. It is worth mentioning that Hb is the biomarker that is altered the latest when iron status is assessed, as it is only altered when iron stores are already nil and erythropoietic synthesis is unable to synthesise Hb in the required amount. We must therefore consider Hb to be an ineffective biomarker for the prevention of ID during pregnancy, where a decrease in iron levels as pregnancy progresses is the norm unless preventive iron supplementation is carried out. Therefore, some women, even if they have normal Hb levels, may have a high chance of developing ID and anaemia later in pregnancy, with negative consequences for their health and that of their baby. Otherwise, measurement of SF concentrations, which is not common in clinical practice, would provide valuable information on iron stores, including incipient iron deficiency states. The concentration of SF is internationally recognized as a very robust biomarker for assessing iron reserves; when low, there is no possibility of false positives for ID and, although it is true that it may increase in presence of infectious/inflammatory processes, the analyses in our study were adjusted for CRP concentration to account for acute infections.

It must be said that deciphering which factors are associated with ID in early pregnancy does not mean that iron stores should not continue to be monitored throughout pregnancy. However, it has been observed that early iron status is highly correlated with iron status during and at the end of gestation. Therefore, it is in the early stages of pregnancy, and even periconceptionally, that effective preventive actions can be considered, such as increased promotion of healthier lifestyles from pregnancy planning services and increased monitoring of non-modifiable characteristics where appropriate. Thus, knowing which maternal conditions or characteristics may affect maternal iron stores would allow obstetricians and midwives to focus public health actions on the target population for early prevention of ID, helping women to achieve the best possible iron status.

The strengths of the present work include (1) the extensive data collection carried out, which covered many sociodemographic and lifestyle characteristics of the participants, including diet and toxic habits; (2) SF levels were adjusted for CRP concentrations in multivariate analyses allowing control for acute inflammatory processes at the time of measurements that could bias the results. However, some limitations must be considered when interpreting these findings. First, the observational design of the study may influence the external validity of the results. On the assumption that the sample includes only non-anaemic women in early pregnancy, the current findings cannot be extrapolated to other populations. Second, information on recent blood donations, which would reduce iron reserves, was not available. Third, dietary assessment using questionnaires is susceptible to misreporting bias; however, given that women with low BMI in our study did not over-report food intake, as is often the case, we believe that potential misreporting bias would not greatly influence our results. Finally, information about interpregnancy interval was not available, which could have allowed further interpretation of parity as a predictor of iron status.

Conclusion

Since Hb concentration is very often the only biomarker of iron status used in clinical practice, pregnant women with anaemia are treated with iron supplementation, but those with ID without anaemia remain under-diagnosed and untreated, so estimating SF concentration in early pregnancy and its associated factors is of great importance for early prevention of ID. Multiparity and being underweight are strong predisposing factors of maternal ID in early pregnancy in non-anaemic women. A diet high in meat, protein, and iron reduce the likelihood of starting pregnancy with ID. Midwives and obstetricians should pay special attention to the iron status of pregnant women at high risk of ID, such as those who are underweight, multiparous or on vegetarian diets.

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Data Availability The datasets analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval The study was designed in agreement with the Declaration of Helsinki/Tokyo. All procedures involving human subjects were approved by Clinical Research Ethics Committee of the Jordi Gol University Institute for Primary Care Research (Institut d' Investigació en Atenció Primària; IDIAP), the Pere Virgili Health Research Institute (Institut d'Investigació Sanitària Pere Virgili; IISPV), and of the Spanish Agency for Medicines and Medical Devices (Agencia Española del Medicamento y Productos Sanitarios; AEMPS).

Informed consent Written informed consent was obtained from all women participating in the study.

Conflict of interest The authors declare no competing interests.

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Iron status in mid-pregnancy and associations with interpregnancy interval, hormonal contraceptive use, dietary factors, and supplement use: A prospective pregnancy cohort study

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Iron status in mid-pregnancy and associations with interpregnancy interval, hormonal contraceptives, dietary factors and supplement use

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Abstract

Adequate iron supply in pregnancy is important for both the woman and the fetus, but iron status is often assessed late in first trimester, if assessed at all. Therefore, identification of factors associated with iron status is important to target vulnerable groups with increased risk of deficiency. Our objectives were to (1) describe iron status in mid-pregnancy and (2) identify sociodemographic and lifestyle predictors of pregnancy iron status. This cross-sectional study uses data from The Norwegian Mother, Father and Child Cohort Study (collected 2002–2008) and The Medical Birth Registry of Norway. Iron status was measured as non-fasting plasma ferritin (P-Fe) and transferrin in gestational week (GW) 18 (*n* 2990), and by lowest reported Hb in GW 0–30 (*n* 39 322). We explored predictors of iron status with elastic net, linear and log-binomial regression models. Median P-Fe was 33 μg/l, and 14 % had depleted iron stores (P-Fe <15 μg/l). P-Fe below 30 μg/l was associated with reduced Hb. We identified eleven predictors, with interpregnancy interval (IPI) and parity among the most important. Depleted iron stores was more common among women with IPI < 6 months (56 %) and 6–11 months (33 %) than among those with IPI 24–59 months (19 %) and among nulliparous women (5 %). Positively associated factors with iron status included hormonal contraceptives, age, BMI, smoking, meat consumption and multi-supplement use. Our results highlight the importance of ferritin measurements in women of childbearing age, especially among women not using hormonal contraceptives and women with previous and recent childbirths.

Key words: Iron deficiency: Ferritin: Transferrin: Pregnancy: Interpregnancy interval

Inadequate iron status during pregnancy may lead to unwanted effects for both the woman and the developing fetus(1,2), including increased risk of preterm birth and low birth weight(3–5), as well as adverse effects on child neurodevelopment(6,7). According to WHO, iron deficiency (ID) contributes to about half of all anaemia cases globally, which affects about 25–35 % of women of reproductive age(8). Although supplementation initiated in pregnancy can correct a maternal deficiency, it is not necessarily sufficient to reverse or prevent adverse effects on child health(9,10).

Women of childbearing age may be at risk of ID resulting from inadequate dietary iron intake, blood loss from menstruation and after childbirth due to depletion of maternal reserves(2). In pregnancy, iron demands increase progressively to support placental and fetal growth(11) and to meet the increase in maternal erythrocyte count(12). It has been suggested that a serum ferritin concentration of at least 70 μg/l is required at the time of conception to avoid developing ID or ID anaemia during a normal pregnancy(13). The depletion of maternal iron stores during pregnancy and lactation can therefore have consequences for a subsequent pregnancy if maternal reserves are not sufficiently replaced during the interpregnancy period(2).

Iron supplementation has for many decades been universally recommended for all pregnant women in many countries(14), but not all(15). In Norway, iron supplementation has historically been recommended at moderate doses for women with ID(16). However, assessment of iron status (ferritin) was not included in the antenatal guidelines between 2005 and 2018. In this period, iron supplements were recommended based on anaemia screening (low Hb)(17), although ID may also exist in the absence of anaemia(18). After revision of the Norwegian guidelines in

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Abbreviations: CRP, C-reactive protein; GW, gestational week; ID, iron deficiency; IPI, interpregnancy interval; MoBa, The Norwegian Mother, Father and Child Cohort Study; P-Fe, plasma ferritin.

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2018, ferritin is now again assessed for all pregnant women before gestational week (GW) 16 and moderate doses of iron supplement intake (40–60 mg/d) are indicated at ferritin < 70 μg/l[16].

Given the relatively high prevalence of ID in the Norwegian population[19], efforts should be made to secure an adequate iron status in women not only in the last half of pregnancy but also prior to conception.[20] Therefore, identification of factors associated with iron status is important to target vulnerable groups with increased risk of ID. The aims of this study were therefore, in a group of 2990 pregnant women, (1) to describe iron status in mid-pregnancy and (2) to identify sociodemographic and lifestyle predictors of pregnancy iron status.

Materials and methods

Study population

This study is based on The Norwegian Mother, Father and Child Cohort study (MoBa, www.fhi.no/moba), a prospective population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health[21]. MoBa participants were recruited from all over Norway during 1999–2008, and the participation rate was 40·6 %. MoBa data also include information from The Medical Birth Registry of Norway, which comprises information about pregnancy, delivery and health of the mother and the neonate for all births in Norway[22]. In MoBa, blood samples were collected in GW 18[23] and biomarkers have been measured in a subsample as part of the Norwegian Environmental Biobank[24]. The main analysis in the current study includes 2990 women who were pregnant in 2002–2008, with available iron status measurements from Norwegian Environmental Biobank (see online Supplementary material, Supplementary Fig. S1). In a secondary analysis, we included all participants in MoBa with singleton pregnancies, available birth records from the Medical Birth Registry and available self-registered pregnancy Hb measurement and determinant variables in MoBa (*n* 39 322). This study is based on version 11 of the quality-assured MoBa data files released for research in 2018.

Ethics approval

The establishment and data collection in MoBa were previously based on a license from the Norwegian Data Protection Agency and approval from The Regional Committee for Medical Research Ethics, and it is now based on regulations related to the Norwegian Health Registry Act. The current study has been approved by The Regional Committee for Medical Research Ethics South East Norway (2015/2393).

Assessment of potential predictors from registry data and questionnaires

Definitions of all potential predictor variables are included in online Supplementary Table S1. Information about age, participation year, parity and time since previous pregnancy (for multiparae women) were obtained from the MoBa linkage to Medical Birth Registry of Norway[22]. Interpregnancy interval (IPI) was calculated as time from date of birth of the previous child to date of conception of the current pregnancy, rounded down to whole months. From the first questionnaire in MoBa (GW 15), we collected information on medical history, hormonal contraceptives use, regularity of menstrual cycle, sociodemographic factors and lifestyle. Chronic disease was defined as any self-reported asthma, diabetes, inflammatory bowel disease, rheumatic disease, epilepsy, multiple sclerosis or cancer, before or during pregnancy.

Diet and dietary supplement use were assessed by a semi-quantitative FFQ answered in mid-pregnancy. The FFQ was designed to capture dietary habits and use of supplements during the first half of pregnancy and has been described previously[25,26]. We converted food frequencies to food and nutrient intakes based on standard Norwegian portion sizes and using FoodCalc[27] and the Norwegian food composition table. We aimed to include food groups (milk, meat, tea, coffee) and food components (fibre, vitamin C) which are relevant for iron status, according to the literature. The nutrient intake from supplements was estimated using a database with nutrient content of more than 1000 different supplement brands collected from suppliers[28]. Participating women recorded the frequency and quantity, as well as the name and manufacturer of supplement(s) used.

Assessment of iron status and biomarkers from blood samples

Biochemical analyses were performed at the Finnish Institute for Health and Welfare (THL) in Helsinki, Finland. Non-fasting plasma ferritin (P-Fe) indicates the size of iron stores in the absence of concurrent infection[29]. Concentrations <15 μg/l are generally considered to be indicative of depleted iron stores for individuals above 5 years of age[29]; however, no cut-offs for ID are established for pregnancy[15]. In this study, we defined depleted iron stores as P-Fe concentrations <15 μg/l and low iron stores as P-Fe <30 μg/l. P-Fe was analysed by a chemiluminescent microparticle immunoassay (ARCHITECT Ferritin assay; Abbott Laboratories). The CV of control samples was 2·7–3·7 %. Plasma transferrin was analysed by an immunoturbidimetric procedure (Architect Transferrin assay; Abbott Laboratories). The CV of control samples was 1·8–1·9 %. As an indicator of inflammation, C-reactive protein (CRP) was measured by the Multigent CRP Vario assay, which is suitable for measuring CRP at variable assay ranges, including the low range requiring high sensitivity. The quantification limit was 0·10 mg/l. The CV of control samples was 1·5–4·2 %. The laboratory participated in an external quality assessment scheme for ferritin, transferrin and CRP was organised by Labquality (Finland). From a questionnaire answered around GW 30, participants transferred Hb measurements results from their maternity record: lowest, highest and latest measurement in pregnancy, with corresponding GW. In this study, we considered lowest Hb as the most clinically relevant indicator when studying low iron status.

Statistical analyses

We used a three-step exploratory approach to identify main predictors of iron status. First, we report descriptive statistics of iron status and prevalence of iron depletion across potentially relevant predictors from literature.

Second, we used elastic net regression to select variables associated with iron status, with natural log-transformed (ln-) P-Fe as the dependent variable. Elastic net is a regularised regression method and a useful variable selection strategy in case of multicollinearity between predictor variables(30). To determine the penalty parameter (α) and the amount of penalisation (λ), we minimised the root-mean-squared error of prediction by 10-fold cross-validation. We used λ_{1se} (largest value of lambda that gives an error within 1 SE of the minimum), which gives a more parsimonious model than λ_{min} (gives the minimum mean cross-validated error). Before running elastic net regression, we imputed missing values in independent variables up to the full sample of 2990 with multiple imputation by chained equations. Variable selection by elastic net was then repeated on each of 100 imputed data sets, and variables that were selected in more than half of the models were included in further analysis(31).

In the third step, the variables selected by elastic net regression were included as independent variables in a linear model with ln P-Fe as a dependent variable and in log-binomial models with P-Fe <15 or <30 μg/l as a dependent variable. Continuous independent variables were scaled. All models were adjusted for chronic illness, recent cold, CRP and gestational age at the time of blood sampling (mean 18·5 (SD 1·3) weeks) to account for variation in P-Fe not related to iron status. Effect estimates are reported as relative differences (in %) and risk ratios with 95 % CI. All predictors were included in the regression model and therefore mutually adjusted for each other. Linear and log-binomial models were run on pooled imputed data sets. This third step was repeated in the large study sample (*n* 39 322) with lowest Hb value in pregnancy as dependent variable in a linear model, to investigate associations between lowest Hb and the main predictor variables selected by elastic net regression with P-Fe.

Associations were examined for non-linearity by non-parametric generalised additive models, using thin plate regression splines as smoothers (see online Supplementary material, Supplementary Fig. S2).

In a secondary analysis, we used plasma transferrin as an alternative measure of iron status and repeated the variable selection by elastic net regression, followed by linear regression models with transferrin as the dependent variable. The variables selected by the elastic net regression to predict transferrin were similar to the variables selected for ferritin; however, age and education were not among selected predictors for transferrin. The transferrin results are presented in online Supplementary Table S3. Statistical analyses were performed using R(32) and packages *mice*(33), *mgcv*(34) and *glmnet*(35).

Results

Median P-Fe concentration was 33 μg/l, ranging from 3·2 to 304 μg/l (interquartile range 20–56 μg/l). In total, 84 % had a P-Fe concentration below 70 μg/l, 44 % below 30 μg/l, 14 % below 15 μg/l (Table 1) and 9 % had P-Fe below 12 μg/l. P-Fe concentrations and use of single iron supplement across the study participation years are shown in online Supplementary Table S3. P-Fe was associated with reported lowest Hb measurement, and the reduction in Hb was evident at P-Fe concentrations lower than 30 μg/l (Fig. 1). For the subset with P-Fe< 30 μg/l, Hb increased with a mean difference of 2·8 (95 % CI 1·1, 4·5) g/l per doubling in P-Fe concentration, while no clear association was seen for higher P-Fe concentrations (mean difference 0·6 (95 % CI –0·4, 1·6) g/l per doubling in P-Fe). Among those with P-Fe below 30 μg/l, 17 % reported an Hb measurement lower than 105 g/l. Conversely, among those with an Hb measurement below 105 g/l, 55 % had P-Fe below 30 μg/l.

Geometric mean and median P-Fe concentrations suggested a crude positive association with pre-pregnancy BMI (Table 1). P-Fe was lower among non-smokers and non-consumers of alcohol during pregnancy. Median concentrations decreased with increasing parity (40 μg/l for primiparae women to 15 μg/l for women with ≥4 children) and with shorter IPI (31 μg/l for ≥60 months to 14 μg/l for <6 months). Users of hormonal contraceptives, either non-oral or oral, had higher median P-Fe than non-users, and P-Fe increased with longer duration of oral contraceptives use. Moreover, women reporting anaemia before pregnancy (3 %) had lower P-Fe concentrations (median 23 μg/l) than those not reporting anaemia (33 μg/l).

Median intake of iron from the diet (excluding supplements) was 10·8 (interquartile range 8·9–13·2) mg/d, and P-Fe tended to increase with meat intake (Table 2). P-Fe concentrations were lower among consumers of milk and slightly lower for consumers of coffee. Median values of P-Fe did not substantially differ across categories of black tea, herbal tea, vitamin C or fibre intake.

Use of iron-containing supplements during the first half of pregnancy was reported by 52 %, and 59 % reported to have used iron supplements between 29 weeks before conception and 28 weeks of gestation. P-Fe was lower for those with iron supplement intake (Table 2), for example, women with high-dose (30–50 mg/d) supplementary iron intakes had lower median P-Fe (30 μg/l), than those taking low dose (≤15 mg/d, 34 μg/l) and those with no iron supplement intake (35 μg/l). The negative association between iron supplement use and P-Fe appeared to be most profound among women who initiated iron supplement use after becoming pregnant. Moreover, P-Fe increased with longer duration of single iron supplement use in the period 8 weeks before conception to GW 20: 23 μg/l for 1–120 d of use *v.* 29 μg/l for 121–210 d of single iron supplement use. Regarding multi-supplements, women with supplemental iron intake only from multi-supplements (i.e. non-users of single supplements) had higher P-Fe than others. Also, users of multi-supplements *without* iron had higher P-Fe than users of iron-containing multi-supplements and those not using multi-supplements at all, Table 2.

Eleven variables were selected by the elastic net regression model and subsequently included in linear and log-binomial models while mutually adjusting for each other (Table 3). Parity and IPI were strongly associated with P-Fe; for parous women, an IPI < 6 months was associated with a −50·5 (95 % CI –64·6, –31·0) % reduction in P-Fe compared with 24–59 months. Further, an IPI < 12 months was associated with higher risk of depleted iron stores (adjusted risk ratio 2·40 (95 % CI 1·53, 3·73) for P-Fe < 15 μg/l), compared with

Table 1. Plasma ferritin (P-Fe) concentrations by sociodemographic and lifestyle factors
(Numbers and percentages; mean values and standard deviations; medians and interquartile ranges (IQR))

	P-Fe (μg/l)						P-Fe (μg/l) grouped							
							<15		≥15 to <30		≥30 to <70		≥70	
	n	%	Geometric mean	SD	Median	IQR	*n*	%	*n*	%	*n*	%	*n*	%
All	2990	100	33	2·1	33	20–56	431	14	897	30	1166	40	496	16
Subset with CRP ≤ 10 mg/l	2517 (86)*		32	2·1	32	20–53	373	14	779	31	979	39	386	16
Subset with CRP ≤ 5 mg/l	1622 (54)*		32	2·1	31	20–53	233	14	529	33	607	37	253	16
Age (years)														
≤25	383	13	33	2·1	36	22–53	55	14	100	26	166	43	62	16
26–30	1222	41	35	2·0	34	21–58	142	12	368	30	502	41	210	17
31–35	1056	35	32	2·1	32	19–53	176	17	323	31	384	36	173	16
>35	329	11	30	2·2	30	18–52	58	18	106	32	114	35	51	16
Education														
<12 years	134	4	28	2·1	28	17–47	31	23	37	28	49	37	17	13
Upper secondary	749	25	33	2·1	34	20–56	114	15	208	28	294	39	133	18
Bachelor	1371	46	34	2·0	34	20–56	178	13	420	31	560	41	213	16
Master	673	23	33	2·1	32	20–56	96	14	213	32	248	37	116	17
Missing	63	2	36	2·3	30	19–72	12	19	19	30	15	24	17	27
Pre-pregnancy BMI (kg/m^2)														
<18·5	95	3	23	1·9	23	15–36	25	26	38	40	25	26	7	7
18·5–24·9	1918	64	32	2·1	32	20–53	285	15	592	31	756	39	285	15
25–29·9	689	23	37	2·1	38	23–63	84	12	174	25	286	42	145	21
≥30	230	8	37	2·2	38	21–68	27	12	65	28	84	37	54	23
Missing	58	2	26	2·0	26	17–44	10	17	28	48	15	26	5	9
Parity														
Primipara	1535	51	40	2·0	40	25–65	120	8	406	26	661	43	348	23
1 child	992	33	28	2·0	28	16–44	206	21	333	34	353	36	100	10
2 children	379	13	28	2·1	28	17–48	78	21	126	33	130	34	45	12
3 children	65	2	24	1·9	24	15–35	17	26	26	40	19	29	3	5
≥4 children	19	1	17	1·8	15	12–24	10	53	6	32	3	16	0	0
Interpregnancy interval†														
<6 months	16	1	14	2·1	14	8–23	9	56	4	25	3	19	0	0
6–11 months	109	8	21	1·9	21	13–32	36	33	41	38	28	26	4	4
12–17 months	225	16	24	1·9	25	16–37	50	22	84	37	83	37	8	4
18–23 months	210	14	26	1·9	25	16–38	47	22	78	37	68	32	17	8
24–59 months	630	43	29	2·0	30	17–50	117	19	204	32	237	38	72	11
≥60 months	230	16	32	2·2	31	18–56	40	17	71	31	79	34	40	17
Missing	35	2	39	2·5	34	22–71	12	33	9	25	7	19	8	22
Smoking during pregnancy														
No	2756	92	33	2·1	33	20–55	403	15	837	30	1071	39	445	16
Yes	174	6	39	2·2	41	23–67	19	11	47	27	66	38	42	24
Missing	60	2	35	2·1	37	25–60	9	15	13	22	29	48	9	15
Alcohol during pregnancy														
No	2649	89	33	2·1	33	20–55	387	15	805	30	1028	39	429	16
<2 units/month	287	10	36	2·0	36	22–59	36	13	82	29	116	40	53	18
≥2 units/month	54	2	40	2·4	43	25–71	8	15	10	19	22	41	14	26
Non-oral hormonal contraceptives (IUD)														
No	2680	90	33	2·1	33	20–55	391	15	809	30	1055	39	425	16
Yes	129	4	41	2·2	42	25–73	14	11	30	23	51	40	34	26
Missing	181	6	33	2·2	31	19–54	26	14	58	32	60	33	37	20
Oral hormonal contraceptive use														
Never	323	11	26	2·1	25	16–41	74	23	115	36	103	32	31	10
Recent use (≤12 months)	1293	43	35	2·1	35	22–57	147	11	390	30	525	41	231	18
Past use (>12 months)	1058	35	33	2·1	34	20–56	156	15	305	29	424	40	173	16
Missing	316	11	33	2·2	32	18–56	54	17	87	28	114	36	61	19

Table 1. (*Continued*)

			P-Fe (µg/l)				P-Fe (µg/l) grouped							
							<15		≥15 to <30		≥30 to <70		≥70	
	n	%	Geometric mean	SD	Median	IQR	*n*	%	*n*	%	*n*	%	*n*	%
Oral hormonal contraceptives, duration of use														
Never	323	11	26	2·1	25	16–41	74	23	115	36	103	32	31	10
<1 years	212	7	29	2·0	27	17–45	40	19	75	35	72	34	25	12
1–3 years	516	17	32	2·0	32	21–50	73	14	159	31	210	41	74	14
4–6 years	654	22	33	2·1	33	20–56	93	14	197	30	257	39	107	16
7–9 years	600	20	35	2·0	38	22–58	71	12	167	28	254	42	108	18
≥10 years	490	16	42	2·1	43	25–67	39	8	134	27	197	40	120	24
Missing	195	7	30	2·1	31	17–54	41	21	50	26	73	37	31	16
Regular menstruation cycle														
No	660	22	33	2·1	34	19–55	106	16	175	27	271	41	108	16
Yes	2316	77	33	2·1	33	20–56	323	14	716	31	890	38	387	17
Missing	14	1	27	2·2	23	15–47	2	14	6	43	5	36	1	7
Anaemia before pregnancy														
No	2886	97	34	2·1	33	20–56	402	14	863	30	1142	40	479	17
Yes	104	3	25	2·4	23	14–48	29	28	34	33	24	23	17	16

CRP, C-reactive protein; IUD, intrauterine device.
* Percentage of full sample (*n* 2990).
† Interpregnancy interval is shown for parous women only (*n* 1456, 49 % of the total sample).

24–59 months. Notably, P-Fe was no longer negatively associated with age in the regression analysis, rather, regression analysis controlling for other variables showed increased P-Fe with increasing age (Table 3 and see online Supplementary material, Supplementary Fig. S2). The regression analysis showed lower P-Fe among underweight women compared with normal weight. Also, overweight and obesity were associated with higher P-Fe compared with normal weight. Further, smoking during pregnancy and use of hormonal contraceptives were also selected as predictors of P-Fe; smokers had 19·2 (95 % CI 7·4, 32·4) % higher P-Fe, while non-oral hormonal contraceptive use was associated with a 45·8 (95 % CI 29·6, 64·0) % increase in P-Fe.

Dietary variables were also associated with P-Fe in the regression analysis. A meat intake in the highest quartile (>156 g/d) was associated with a 9·5 (95 % CI 2·3, 17·3) % increase in P-Fe compared with being in the lowest quartile (<113 g/d). Initiation of iron-containing supplement in the period before pregnancy or during pregnancy was associated with lower P-Fe compared with no use, and the negative association between supplement use and P-Fe was stronger when the use was initiated after becoming pregnant (–20·6 (95 % CI −25·6, −15·3) % for initiation in GW 9–20, compared with no use). The opposite trend was seen for those with supplementary iron intake from multi-supplements only, which was associated with 20·3 % increased P-Fe concentrations.

The alternative model, using lowest Hb as an outcome, agreed with the P-Fe results for education, pre-pregnancy BMI, use of hormonal contraceptives, meat intake and duration and use of iron-containing supplements, but did not show the same strong association with IPI and parity. Associations were of opposite directions for age and smoking, which were positively associated with P-Fe, but negatively associated with Hb (see online Supplementary material, Supplementary Table S4).

Discussion

A main finding of this study was that a substantial number of women had low iron stores in mid-pregnancy: 14 % had P-Fe below 15 µg/l and 44 % below 30 µg/l. Further, 84 % had P-Fe below 70 µg/l, which is the cut-off for recommending supplements after GW 18–20 in the updated Norwegian antenatal guidelines(16). Our results suggested that a P-Fe concentration below approximately 30 µg/l was associated with reduced Hb in pregnancy (as reported in GW 30). Only 17 % of women with P-Fe below 30 µg/l reported an Hb measurement lower than 105 g/l, suggesting that Hb measurements may not be a sensitive indicator of low iron status in pregnancy. In a larger study in MoBa(28), median intake of iron from diet was about 11 mg/d (similar to this study) and half of the pregnant women had an iron intake below the recommendation of 15 mg/d for women(36). Median ferritin concentrations and prevalence of ID in this group of pregnant women were within the same range as in European women of reproductive age, as summarised by Milman *et al.*(15). Data from >15 European countries showed an average serum ferritin concentration at 26–38 µg/l, and about 40–55 % had low or depleted iron stores (P-Fe < 30 µg/l).

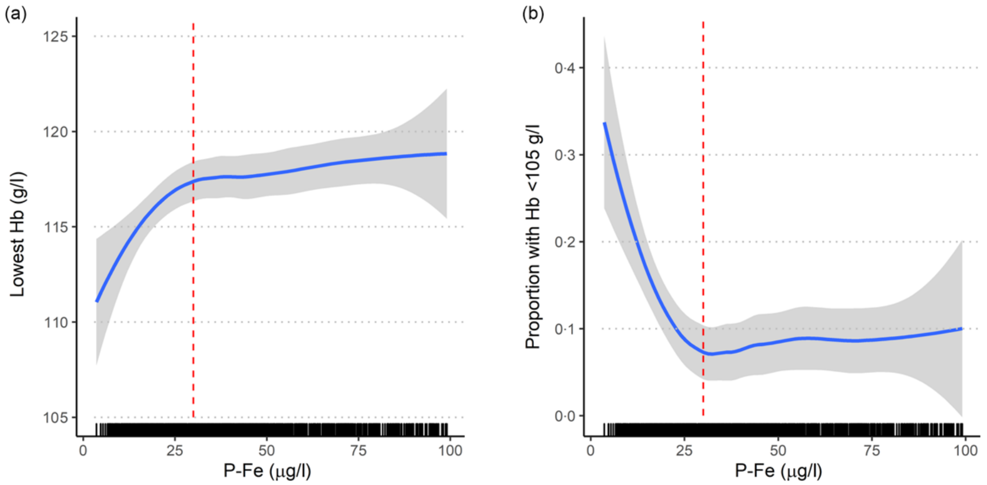

Fig. 1. Crude association between ferritin (P-Fe, µg/l) measured in mid-pregnancy (mean 18·5 (SD 1·2) gestational weeks) and (a) lowest Hb (g/l) during pregnancy; (b) proportion with lowest Hb < 105 g/l (measured in mean 23·0 (SD 6·2) gestational weeks), shown for a subset (*n* 1086) with P-Fe < 100 µg/l. Red dashed vertical line indicates a P-Fe concentration of 30 µg/l. The association is estimated with 95 % CI using local regression (loess) as smoother.

Another main finding was the identification of factors associated with increased risk of ID among pregnant women. Using an exploratory approach, we identified eleven sociodemographic, reproductive and lifestyle variables as predictors of low iron stores, including short IPI, increasing parity and low BMI. Moreover, prolonged pre-pregnancy use of hormonal contraceptives, particularly non-oral, was associated with higher iron status, together with increasing age and high meat intake. Early initiation of an iron-containing supplement before or early in pregnancy was associated with higher P-Fe compared with initiation after pregnancy was known (GW 9–20). Women who were taking supplementary iron from multi-supplements only (i.e. not from prescribed single high-dose supplements) had higher P-Fe compared with others.

In contrast, users of high-dose iron supplements had lower median P-Fe than non-users in this group of women; however, among those who did take single iron, prolonged use was associated with increasing P-Fe. This finding may reflect that single iron supplements were used mainly by women with known ID, according to prevailing guidelines in the study period. Also, high-dose iron supplements may potentially decrease iron absorption through increased hepcidin(37). The increase in P-Fe with iron-containing multi-supplement use and prolonged use of high-dose iron supplement suggests a beneficial effect of supplements on iron status, although the direction of causality could not be assessed in this study.

We found a positive association with average meat consumption as reported by the FFQ, and meat consumption was among the selected predictors. Average intakes of other specific foods or beverages were not selected as important predictors. However, median P-Fe was slightly lower among those with high average intake of milk, black tea, coffee and fibre, and slightly higher among those with high vitamin C intake. These foods and beverages are known in the literature to affect the bioavailability of iron in the diet when consumed in the same meal(36,38,39).

We found that short IPI was associated with lower ferritin concentrations and increased risk of small or depleted iron stores, suggesting insufficient repletion of iron stores after a previous pregnancy. Our findings thus support the recommendation from WHO of at least 24 months between pregnancies in order to reduce risk of adverse maternal, perinatal and infant outcomes(40,41); however, a reduction in iron stores was found for all multiparae women compared with primiparae. Indeed, short IPI has been linked to adverse maternal or child outcomes(40,42,43). Micronutrient depletion of both iron and folic acid has been suggested to play a role(44), as these stores often remain low for several months after delivery(45). Our results suggest that maternal iron depletion may be a potential mediator of the adverse health outcomes associated with short IPI.

The positive association between use of hormonal contraceptives and iron status may be explained by the reduced menstrual flow quantity caused by modern low-dose hormonal contraceptives(46,47). Oral hormonal contraceptive use has been shown to increase serum ferritin levels especially in women with low iron stores (<10 µg/l)(46).

Pre-pregnancy BMI was positively associated with P-Fe for underweight, normal-weight and overweight women, but the direction of the association was unclear for obese women, online Supplementary Fig. S2. Low iron status has been related to low BMI(48), but more often with high BMI(49,50), although with inconsistent evidence when assessed as serum ferritin(51). The low-grade inflammation related to obesity has been shown to increase secretion of hepcidin, which in turn decreases iron absorption and thus leads to low iron status(52,53).

Smokers tend to have higher ferritin levels than non-smokers(54), which we also observed in this study. There is substantial evidence that cigarette smoking leads to iron dysregulation,

Table 2. Plasma ferritin (P-Fe) concentrations by dietary intake from food and supplements
(Numbers and percentages; mean values and standard deviations; medians and interquartile ranges (IQR))

	P-Fe (μg/l)						P-Fe (μg/l) grouped							
							<15		≥15 to <30		≥30 to <70		≥70	
	n	%	Geometric mean	SD	Median	IQR	*n*	%	*n*	%	*n*	%	*n*	%
Iron intake from diet (mg/d)														
<8·9	747	25	33	2·1	32·9	20–54	108	14	225	30	290	39	124	17
9·0–10·8	747	25	33	2·1	33·6	21–56	107	14	220	29	303	41	117	16
10·9–13·1	747	25	33	2·1	32·0	20–56	103	14	242	32	276	37	126	17
≥13·2	748	25	33	2·1	33·4	20–56	113	15	210	28	297	40	128	17
Meat intake (g/d)														
<113	463	25	33	2·0	32·0	19–50	105	14	242	33	279	38	106	14
113–134	475	25	33	2·0	31·2	21–51	109	15	227	30	295	39	116	16
135–154	475	25	32	2·1	31·2	20–53	114	15	237	32	274	37	122	16
>154	475	25	37	2·0	37·8	24–62	94	13	188	25	315	42	151	20
Milk (g/d)														
No	122	4	38	2·2	38·1	23–70	14	11	32	26	46	38	30	25
≤200	1033	35	34	2·1	33·5	21–54	131	13	312	30	422	41	168	16
201–500	1264	42	33	2·1	32·4	20–57	201	16	363	29	486	38	214	17
>500	571	19	32	2·1	31·4	19–53	85	15	190	33	212	37	84	15
Tea, black (g/d)														
No	599	20	34	2·1	35·4	20–58	82	14	160	27	249	42	108	18
≤100	1169	39	33	2·1	32·5	20–57	172	15	351	30	455	39	191	16
>100	1222	41	33	2·1	32·2	20–53	177	14	386	32	462	38	197	16
Tea, herbal (g/d)														
No	1592	53	33	2·2	32·6	19–56	262	16	453	28	605	38	272	17
≤100	935	31	34	2·0	33·3	21–56	110	12	295	32	383	41	147	16
>100	463	15	34	2·0	33·6	20–55	59	13	149	32	178	38	77	17
Coffee (g/d)														
No	1076	36	34	2·1	34·1	20–57	159	15	297	28	423	39	197	18
≤100	1056	35	34	2·0	33·4	21–56	143	14	321	30	422	40	170	16
>100	858	29	32	2·1	30·9	19–53	129	15	279	33	321	37	129	15
Total vitamin C intake (mg/d)														
≤141	998	33	33	2·1	32·9	20–54	149	15	302	30	384	38	163	16
142–218	997	33	33	2·1	33·0	21–56	151	15	289	29	390	39	167	17
>218	994	33	34	2·1	33·4	20–56	131	13	306	31	392	39	165	17
Fibre (g/d)														
≤25·7	996	33	34	2·1	34·2	20–57	139	14	292	29	388	39	177	18
25·8–33·4	996	33	33	2·1	33·0	20–57	145	15	300	30	386	39	165	17
≥33·5	997	33	32	2·1	32·0	20–53	147	15	305	31	392	39	153	15
Iron intake from supplements (mg/d)*														
No iron from supplements	1442	48	35	2·1	35·1	21–58	201	14	403	28	580	40	258	18
≤15	886	30	34	2·1	34·1	21–55	120	14	259	29	351	40	156	18
15–30	345	11	31	2·0	30·5	19–50	55	16	110	32	138	40	42	12
30–50	105	4	33	2·1	29·6	21–50	9	9	44	42	22	31	19	18
>50	212	7	27	2·0	25·1	16–43	46	22	81	38	64	30	21	10
Iron from supplements, initiation†														
No reported use	1209	40	35	2·1	35·6	21–59	165	14	332	27	488	40	224	19
26–9 weeks before conception	364	12	35	2·1	34·6	21–60	46	13	105	29	147	40	66	18
8–0 weeks before conception	153	5	31	1·9	29·8	20–46	21	14	56	37	58	38	18	12
GW 0–4	201	7	36	2·0	37·0	24–56	19	9	56	28	93	46	33	16
GW 5–8	218	8	30	2·0	29·0	20–46	32	15	82	38	79	36	25	11
GW 9–12	131	4	29	2·1	28·2	17–47	29	22	39	30	44	34	19	15
GW 13–16	320	11	29	2·2	28·1	16–50	68	21	104	33	103	32	45	14
GW 17–20	70	2	27	1·8	27·2	16–41	9	13	27	39	29	41	5	7
Missing	324	11	34	2·1	34·2	20–56	42	13	96	30	125	39	61	19
Iron supplement, number of days used‡														
Not reported	2607	87	35	2·1	35·9	21–58	344	13	751	29	1044	40	468	18
1–120	262	9	24	2·0	22·9	15–37	70	27	99	38	72	27	21	8
121–210	121	4	29	1·8	29·3	19–44	17	14	47	39	50	41	7	6
Multi-supplement														
No use	467	16	32	2·2	31·9	19–54	85	18	130	28	179	38	73	16
Yes, multi-supplement with iron	1507	50	32	2·0	31·8	20–52	215	14	482	32	576	38	234	16
Yes, multi-supplement without iron	1016	34	36	2·1	36·1	21–60	131	13	285	28	411	40	189	19
Iron from multi-supplement only														
No	2110	71	32	2·1	31·4	19–53	343	16	654	31	800	38	313	15
Yes	880	29	37	2·0	36·2	23–63	88	10	243	28	366	42	183	21

GW, gestational week.

* Estimated intake of iron from supplements (single and multi).

† Based on reported time period of single iron supplement use from 26 weeks before conception until GW 28.

‡ Based on reported time period and frequency of single iron supplement use from 8 weeks before conception until GW 20.

Table 3. Associations between plasma ferritin (P-Fe) and selected (by elastic net regression) predictor variables, with regression coefficients (adjusted relative difference and risk ratios (RR) with 95 % confidence intervals) from linear and log-binomial models*†
(Numbers and percentages; risk ratios and 95 % confidence intervals, *n* 2990)

	P-Fe		P-Fe < 15 *v.* ≥15 µg/l				P-Fe < 30 *v.* ≥30 µg/l			
	Relative difference		<15 µg/l				<30 µg/l			
	%	95 % CI	*n*	%	RR	95 % CI	*n*	%	RR	95 % CI
Age (1 SD, 4·2 years)	2·1	–0·8, 5·1	431	14	0·97	0·85, 1·10	1328	44	0·99	0·90, 1·09
Education										
<12 years	–15·8	–25·4, –5·1	32	23	2·03	1·25, 3·23	69	50	1·36	0·92, 2·02
Upper secondary	–0·5	–6·4, 5·7	117	15	1·17	0·88, 1·54	331	43	0·96	0·78, 1·17
Bachelor	0·0	Reference	181	13	1·00	Reference	609	44	1·00	Reference
Master	–2·3	–8·2, 4·0	101	15	1·14	0·85, 1·51	319	46	1·06	0·86, 1·29
Pre-pregnancy BMI (kg/m^2)										
<18·5	–23·8	–33·6, –12·4	25	26	2·00	1·17, 3·32	63	66	2·30	1·45, 3·69
18·5–24·9	0·0	Reference	285	15	1·00	Reference	880	46	1·00	Reference
25–29·9	7·0	1·0, 13·4	91	12	0·85	0·64, 1·11	286	39	0·84	0·69, 1·01
≥30	7·5	–2·0, 17·9	30	12	0·85	0·54, 1·31	99	41	0·96	0·70, 1·30
Interpregnancy interval and parity										
<6 months	–50·5	–64·6, –31·0	9	56			13	81		
6–11 months‡	–23·7	–33·4, –12·5	37	33	2·40	1·53, 3·73	79	71	2·26	1·46, 3·57
12–17 months	–12·1	–20·6, –2·6	53	23	1·23	0·83, 1·80	138	60	1·24	0·90, 1·72
18–23 months	–10·6	–19·3, –0·9	48	22	1·28	0·86, 1·88	129	59	1·41	1·02, 1·97
24–59 months	0·0	Reference	121	19	1·00	Reference	329	51	1·00	Reference
≥60 months	5·6	–4·7, 17·0	42	18	1·00	0·65, 1·52	113	48	0·94	0·68, 1·30
Primiparae	40·9	31·8, 50·7	121	8	0·35	0·26, 0·48	527	34	0·44	0·35, 0·55
Smoking										
No	0·0	Reference	412	15	1·00	Reference	1262	45	1·00	Reference
Sometimes or daily	19·2	7·4, 32·4	19	11	0·61	0·34, 1·01	66	38	0·73	0·51, 1·03
Non-oral hormonal contraceptives										
No	0·0	Reference	416	15	1·00	Reference	1281	45	1·00	Reference
Yes	45·8	29·6, 64·0	15	11	0·46	0·25, 0·80	47	34	0·41	0·28, 0·60
Oral hormonal contraceptives, duration of use										
No use	0·0	Reference	87	23	1·00	Reference	215	57	1·00	Reference
<1 years	12·8	1·0, 26·1	44	19	0·72	0·46, 1·12	126	55	0·82	0·57, 1·17
1–3 years	16·5	6·6, 27·3	80	14	0·64	0·44, 0·92	245	44	0·64	0·48, 0·85
4–6 years	14·6	5·2, 24·9	101	15	0·73	0·52, 1·04	309	45	0·72	0·54, 0·94
7–9 years	21·2	11·0, 32·2	76	12	0·58	0·40, 0·83	252	40	0·58	0·43, 0·77
≥10 years	38·2	26·0, 51·6	43	8	0·42	0·27, 0·64	181	36	0·50	0·37, 0·68
Meat intake (g/d)										
<113	0·0	Reference	105	14	1·00	Reference	347	47	1·00	Reference
113–134	2·6	–4·1, 9·8	109	15	1·02	0·75, 1·38	336	45	0·89	0·72, 1·11
135–156	1·4	–5·3, 8·6	114	15	1·07	0·79, 1·46	351	47	0·98	0·79, 1·22
>156	9·5	2·3, 17·3	94	13	0·89	0·65, 1·23	282	38	0·68	0·55, 0·86
Iron from supplements, time of initiation										
No reported use	0·0	Reference	170	13	1·00	Reference	516	41	1·00	Reference
26–9 weeks before	–8·7	–15·9, –0·9	47	12	1·12	0·75, 1·64	159	40	1·29	0·99, 1·69
8–0 weeks before	–14·8	–23·9, –4·6	21	13	1·18	0·68, 1·97	78	47	1·67	1·16, 2·41
GW 0–8	–19·2	–25·2, –12·8	62	13	1·37	0·96, 1·95	218	45	1·82	1·42, 2·34
GW 9–20	–20·6	–25·6, –15·3	131	19	1·70	1·29, 2·25	357	52	1·91	1·55, 2·36
Supplementary iron from multi-supplements only										
No	0·0	Reference	343	16	1·00	Reference	997	47	1·00	Reference
Yes	20·3	13·2, 27·9	88	10	0·57	0·42, 0·75	331	38	0·57	0·47, 0·70

GW, gestational week.
* Models are adjusted for chronic illness, reported recent infections, C-reactive protein and gestational age at the time of blood sampling in addition to mutual adjustment for all variables listed in the table.
† The following variables were included in the elastic net regression, but not selected: Intake of coffee, herbal tea, black tea, milk, fibre, vitamin C intake, total intake of iron, duration of single iron supplement use, cumulative use of single iron supplement (frequency × duration), use of iron-containing multi-supplements, regularity of menstruation cycle, recent use of oral contraceptives (last 12 months, yes/no) and previous smoking.
‡ For log-binomial models, <6 months was collapsed with 6–11 months due to low *n*.

resulting in accumulation of iron both in the lung and systemically(55). The imbalance in iron homoeostasis caused by smoking has been suggested to increase oxidative stress and play a role in pathogenesis, for example, of respiratory diseases(54,56).

Ferritin has limitations as indicator of iron status, especially during pregnancy due to physiological haemodilution, which also introduces additional inter-individual variation. Moreover, the normal decrease in iron status throughout pregnancy is accompanied with increased intestinal iron absorption(57). As women with depleted reserves have higher iron absorption than those with adequate iron status(57,58), this may introduce bias when studying dietary intake as a predictor. However, the increase in iron demands is largest in the second half of pregnancy(13), and we assume that the distribution of P-Fe in week 18 is representative of that earlier in pregnancy. Although most women in this study donated blood for ferritin assessment (around GW 18) prior to filling in the FFQ (around GW 22), studies show that dietary patterns are fairly consistent between the first and second trimester(59,60). Therefore, we consider this to have minimal influence on the findings. Although we adjusted ferritin for CRP and included transferrin as a second iron status indicator in a sensitivity analysis, additional indicators of iron status, such as transferrin saturation, would have strengthened our study(29).

Second, this study was observational with limitations to external validity. Predictors of iron status vary between populations(61), and important predictors in Norwegian pregnant women will likely differ from those in universally supplemented populations. Also, iron status was measured in a sample of women who had completed all the first six questionnaires in MoBa, possibly introducing selection bias to our study. Still, we expect that important predictors of iron status found in this study are generalisable to the general pregnant population in Norway. Furthermore, ethnic minorities are not well represented in MoBa. Low iron stores have been shown to be more common among pregnant women in certain minority groups in Norway(62). We had no information of recent blood donations prior to pregnancy, which reduce iron stores(48,63).

A third limitation of this study relates to the estimation of iron intake from food and supplements based on questionnaires, which are, as all dietary assessments, prone to bias due to misreporting. Dietary iron intake is strongly correlated with energy intake (Pearson correlation coefficient, $r = 0{\cdot}8$ in this study), and the estimated iron intake in this study will thus be biased by under- or overreporting in the FFQ(26). Also, we had no information on meal composition, only on frequency of food consumption, which limits the assessment of dietary intakes as predictors of iron status.

Two main strengths of this study were (i) the large number of women with available ferritin measurements in mid-pregnancy (n 2990) and (ii) the extensive data collection in MoBa, which allows studying a wide range of variables related to sociodemographic factors, medical history, lifestyle including diet and supplement use. Moreover, coinciding CRP measurements enabled control for on-going inflammation in the analysis.

Conclusions

Mid-pregnancy P-Fe in this study suggested that a considerable group of Norwegian women may have low or depleted iron stores. The potential health consequences for mother and child of low ferritin, also at stages where Hb is within a range considered normal for pregnancy, should be elucidated in further research. Main predictors of P-Fe status were related to reproductive factors as IPI, parity and use of hormonal contraceptives in the past. Lifestyle factors, including diet, were of less importance. The presence of depleted iron stores in mid-pregnancy in an assumed well-nourished population like the Norwegian underlines the importance of ferritin measurements in women of childbearing age, and particularly in women with previous and recent childbirths, and among those not using hormonal contraceptives.

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I. H. C. was responsible for the conception of the article, statistical analyses and revision of the manuscript. L. I. V. drafted the manuscript and assisted in interpretation of results. M. H. A. and A. L. B. assisted in conception of the article, interpretation of results and revising the manuscript. V. A. assisted in interpretation of results and revising the manuscript. I. E. was responsible for chemical analysis and assisted with interpretation of results and revising the manuscript. H. M. M. supervised the acquisition of data and assisted with conception of the article, interpretation of results and revision of the manuscript. All authors have approved to publication of the article and accept responsibility for the content of the paper.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114521000295

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CHAPTER 3.

EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON MATERNAL IRON STATUS

Effectiveness of different doses of iron supplementation and prenatal determinants of maternal iron status in Spanish women: ECLIPSES Study

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The Effectiveness of Different Doses of Iron Supplementation and the Prenatal Determinants of Maternal Iron Status in Pregnant Spanish Women: ECLIPSES Study

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Abstract: Iron deficiency (ID), anemia, iron deficiency anemia (IDA) and excess iron (hemoconcentration) harm maternal–fetal health. We evaluated the effectiveness of different doses of iron supplementation adjusted for the initial levels of hemoglobin (Hb) on maternal iron status and described some associated prenatal determinants. The ECLIPSES study included 791 women, randomized into two groups: Stratum 1 (Hb = 110–130g/L, received 40 or 80mg iron daily) and Stratum 2 (Hb > 130g/L, received 20 or 40mg iron daily). Clinical, biochemical, and genetic information was collected during pregnancy, as were lifestyle and sociodemographic characteristics. In Stratum 1, using 80 mg/d instead of 40 mg/d protected against ID on week 36. Only women with ID on week 12 benefited from the protection against anemia and IDA by increasing Hb levels. In Stratum 2, using 20 mg/d instead of 40 mg/d reduced the risk of hemoconcentration in women with initial serum ferritin (SF) ≥ 15 µg/L, while 40 mg/d improved SF levels on week 36 in women with ID in early pregnancy. Mutations in the *HFE* gene increased the risk of hemoconcentration. Iron supplementation should be adjusted to early pregnancy levels of Hb and iron stores. Mutations of the *HFE* gene should be evaluated in women with high Hb levels in early pregnancy.

Keywords: iron supplementation; pregnancy; randomized controlled trial; serum ferritin; hemoglobin; iron status; iron stores; *HFE* gene

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1. Introduction

Iron requirements increase during pregnancy. Since dietary sources cannot always prevent iron deficit, iron supplements are usually prescribed to women who plan to become pregnant. However, there is no consensus on the ideal iron dosage during pregnancy. Anemia is the most common and widespread nutritional disorder globally and a significant public health problem [1,2]. Anemia is attributed to iron deficiency (ID) in half of the cases in the general population [1,3] and in up to 90% of cases of pregnant women [4]. Studies show that an inadequate iron status during pregnancy can lead to adverse mother–child outcomes. In the mother, iron deficiency, anemia, and iron deficiency anemia (IDA) have been associated with preeclampsia, preterm delivery, and even miscarriage, and in the child with fetal growth restriction, low birth weight and impaired cognitive development [5–10]. Furthermore, some studies have underscored the importance of timing of ID and IDA, since some long–term consequences, especially regarding the development and functioning of the child's brain, are irreversible, even after correcting iron levels [11,12]. As a result, it is essential to maintain good nutritional care even before getting pregnant, as well as throughout the whole gestation, to ensure an optimal health status for mother and baby.

In addition to participating as enzymatic cofactor in a wide range of metabolic reactions, iron is indispensable for the synthesis of hemoglobin (Hb), the synthesis and methylation of DNA, and oxygen transport [13,14]. The increase in blood volume and the formation of new tissue during pregnancy are the main mechanisms underlying the increased iron requirements [15–17]. Crucially, iron has a key role in neuronal proliferation, myelination, and the synthesis of several neurotransmitters during the development of the fetal brain [11,18]. Despite concerns about the state of prenatal iron, which caused the launch of public health policies to address iron deficiency [19], it is estimated that in Europe, around 25% of pregnant women become anemic during pregnancy [2,3,19]. The prevalence of ID is greater than the prevalence of IDA, and it often develops during the later months of pregnancy, even in women with sufficient iron stores at the start of the pregnancy [20]. In addition, while diet and supplementation are the main sources of iron, the maternal iron status is influenced by many other biological, lifestyle, and even social factors. According to published research, genetic alterations, ethnicity, obstetric history, toxic habits (i.e., smoking or alcohol), and socioeconomic status (SES) could have a defining role [21–25].

On the other hand, unnecessary or excessive iron supplementation might generate high levels of Hb, also known as the risk of hemoconcentration, in the second and third trimesters of pregnancy. This condition, which affects between 8.7% and 42% of pregnancies in industrialized countries [26,27], increases oxidative stress and blood viscosity, causing placental infarction and hindering the perfusion of oxygen and nutrients to the fetus [28–31]. Although hemoconcentration can be as harmful as iron deficiency for maternal health and children's health, in clinical practice, iron supplementation is usually not adjusted to fit iron status.

The primary aim of this study was to evaluate the effectiveness of iron supplements during pregnancy in different doses adjusted to the Hb levels of the first trimester. As secondary outcomes, we described the percentage of ID, anemia, IDA, and risk of hemoconcentration in a large sample of pregnant Spanish women and the prenatal factors associated with maternal iron status at the end of pregnancy.

2. Materials and Methods

2.1. Study Design

The ECLIPSES study [32] was a community randomized controlled trial (RCT) conducted in the province of Tarragona (Catalonia, Spain) between 2013 and 2017. The 791 participants were contacted in their primary care centers during the first routine visit with midwives and were included in the trial according to the following inclusion criteria: over 18 years of age, gestation time ≤12 weeks, no lab indication of anemia (Hb ≥ 110 g/L on week 12), ability to understand the official State languages

(Spanish or Catalan), and the ability to understand the characteristics of the study. Women with multiple pregnancy, adverse obstetric history, those who had taken >10 mg iron daily during the three months prior to week 12 of gestation, and those who reported a previous severe illness (immunosuppression) or chronic disease that could affect their nutritional status (cancer, diabetes, malabsorption, or liver disease) were excluded. A signed informed consent was obtained from all participants.

The participants were allocated into two strata according their initial Hb levels on week 12 of pregnancy, as follows:

(1) Stratum 1: women with initial Hb levels between 110 and 130 g/L were prescribed 40 or 80 mg/d of iron supplementation.

(2) Stratum 2: women with initial Hb levels > 130 g/L were prescribed 40 or 20 mg/d iron supplementation. Although in clinical practice, only plasma Hb and serum ferritin (SF) levels are measured, we suspected that women with initial Hb > 130 g/L could have some alteration in the *HFE* gene which would predispose them to iron overload.

In addition to the recruitment visit before the 12th week of gestation, the study consisted of three visits throughout the pregnancy: at the 12th, 24th, and 36th weeks of gestation. Separately, the women attended routine pregnancy visits with their midwives and obstetricians.

During the first visit (12th week), the midwives delivered the supplements to the participants according to the intervention group to which they had been assigned. The prescription of each dose of supplements within the groups was randomized and triple blinded. The laboratories Tedec–Meiji made the same box for all different doses of supplements, so that the laboratory technicians, the clinical staff and the researchers did not know the dose of iron received by each woman until the study ended. Women were advised to take one pill per day until the next visit, at which time they had to return any left-over pills to evaluate adherence. An independent investigator compared the number of pills left over with the compliance reported by the participants. Good compliance was considered for women who reported having forgotten to take the supplement less than twice per week at every visit of the study. When they reported forgetting two or more times per week in any of the visits, compliance was considered low.

If women developed anemia in the middle of pregnancy (24th week), they received the usual treatment for anemia.

The sample size was calculated according to previous data from our research group [9,33], taking into account the risk of IDA and hemoconcentration during the third trimester of pregnancy as principal variables [32]. The study was designed in agreement with the Declaration of Helsinki/Tokyo. All procedures involving human subjects were approved by Clinical Research Ethics Committee of the Jordi Gol University Institute for Primary Care Research (*Institut d' Investigació en Atenció Primària; IDIAP*), the Pere Virgili Health Research Institute (*Institut d'Investigació Sanitària Pere Virgili; IISPV*), and the Spanish Agency for Medicines and Medical Devices (*Agencia Española del Medicamento y Productos Sanitarios; AEMPS*). Signed, informed consent was obtained from all women participating in the study. This clinical trial was registered at www.clinicaltrialsregister.eu as EudraCT number 2012-005480-28 and at www.clinicaltrials.gov with identification number NCT03196882.

2.2. Data Collection

2.2.1. Baseline Data (on Week 12 of Gestation)

Midwives and researchers of the study (dietitians) compiled clinical and obstetrical data from participants during the first visit. They obtained the following information during the personal interview and from specific questionnaires: date of birth, weight, height, blood pressure, parity (yes/no), number of previous children, planned pregnancy (yes/no), previous use of contraceptives (yes/no), and type of contraceptives. Medical and surgical history and obstetric data were also recorded.

Maternal age was classified as <25 years, 25–34 years, and ≥35 years. Each maternal pre–pregnancy body mass index (BMI, Kg/cm^2) was categorized as underweight (BMI < 18.5), normal weight (BMI 18.5–24.9), overweight (BMI 25–29.9), or obese (BMI ≥ 30).

The dietary assessment was obtained using a short food frequency questionnaire (FFQ) validated in our population [34] and filled by participants at each visit of the study. From this information, we were able to calculate the percentage of adherence to the Mediterranean diet [35], considered a high–quality dietary pattern. In addition, women were asked about their use of multivitamin supplements, including >10mg iron, which constituted an exclusion criteria for this study.

Lifestyle habits before conception were also recorded, including alcohol intake and smoking. To assess smoking, we used the Fagerström test [36] and women were classified as smokers and non-smokers at the first visit of the study. The International Physical Activity Questionnaire (IPAQ) [37] was used to record the physical activity (PA) of participants. They reported the time spent doing exercise of different intensity (vigorous, moderate or a walk lasting at least 10 minutes) during the previous week; the information was recorded as “days per week” in which physical activity of each intensity was performed, and the “hours” and “minutes” dedicated in each of those days. Women also reported amount of time spent sitting during a typical day. We used these data to calculate the metabolic equivalents of task.

Sociodemographic data of participants and their partners were also recorded. The educational level was classified into four groups: unfinished primary school (<12 years old), primary school (up to 12 years old), secondary school (up to 18 years old) and higher education, which included university and vocational studies. Regarding occupational status, women were classified as students, employed or unemployed. Women in employment were asked about their profession, which was classified following the Catalan Classification of Occupations (CCO-2011) [38]. All this information was used to calculate the family’s socioeconomic status (SES).

Regarding ethnicity, five categories were used: Caucasian, Latin American, Asian, Arab, and Black.

Blood samples were taken on week 12 of gestation to perform blood and genetics tests. Hematological parameters (Hb, mean corpuscular volume (MCV), and hematocrit) and some specific biochemical markers (serum ferritin (SF) and C–reactive protein (CRP)) were measured, and genetic mutations of the *HFE* gene (C282Y, H63D, and S65C) were checked for. The samples were stored in the BioBank for future use.

2.2.2. Data Recorded during Scheduled Study Visits

Diet and physical activity were also evaluated at 24th and 36th weeks of gestation. In addition, blood was collected during both visits to analyze routine blood parameters, including Hb levels. On week 36, SF levels were also measured.

Any adverse effect from the supplementation was recorded and included in the statistical analyses.

2.2.3. Definition of Iron Status

Anemia was defined as Hb < 110 g/L at 12th and 36th weeks and Hb < 105 g/L at 24th week of gestation. ID was defined as SF < 15 µg/L and IDA as anemia and one of the following criteria: SF < 15 µg/L or MCV < 70 fL. SF levels ≥ 15 µg/L was considered as non–deficient or normal iron stores.

2.3. Statistical Analysis

All statistical analyses were performed for the population by intention to treat (ITT) and per-protocol. The population by ITT considered all the participants that were initially included in the study; the per-protocol population, however, consisted only of those participants who complied with the protocol of the study. In the latter, therefore, we excluded women who developed anemia on visit 2, at 24 weeks of gestation.

All analyses were performed separating the sample by stratum; i.e., according to the Hb levels in the first visit of the study. Student’s *t*-test and ANOVA were used to describe continuous variables (mean and SD), and the chi-squared test for categorical variables (percentages). Natural logarithm (Ln) transformation was applied to normalize the distribution of SF, increasing the validity of analyses, and using the median and interquartile ranges (IQR).

Multivariate regression models (multiple linear regressions and logistic regressions) were used to assess the effect of different doses of iron supplementation, along with other prenatal predictors, on maternal iron status on week 36 of pregnancy. The models were adjusted for the following variables: maternal age, parity, socioeconomic status, use of hormonal contraception prior to getting pregnant, planned pregnancy, smoking habit, alcohol intake, pre–pregnancy maternal BMI, gestational weight gain, Hb on week 12 of gestation, SF on week 12 of gestation, CRP on week 12 of gestation, *HFE* gene genotypes, maternal ethnic origin, physical activity as weekly mean of metabolic equivalent of task (METs), and adherence to Mediterranean diet.

Furthermore, adjusted multivariate regression models were performed for each stratum, separating women with and without ID in the first trimester in order to explore whether iron supplementation acted differently according to iron reserves at the beginning of pregnancy. They were adjusted for the same variables previously mentioned, except for SF on week 12 of gestation. To avoid information overload, the tables only show the statistically significant regression models.

SPSS (version 25.0 for Windows; SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Statistical significance was set at $p < 0.05$.

3. Results

Of the total of 791 pregnant women included in the study at week 12 of pregnancy (529 from Stratum 1 and 262 from Stratum 2), the data shown in this article are based on the population by ITT, which consisted of of 534 women with data on week 36 (354 from Stratum 1 and 180 from Stratum 2). Attrition was due to: voluntary abandonment (22.75%); miscarriage (1.64%); emergence of exclusion criteria during pregnancy (5.82%), including serious or chronic illness that could affect the nutritional development (e.g., cancer, diabetes, and malabsorption); and participants lost to follow up (2.28%). Attrition was proportional in both Strata, as shown in the Flowchart (Figure 1). In the supplementary materials, we also show the analyses for the per-protocol population, which excluded anemic women at 24th week of gestation (11.7% in Stratum 1 and 2.7% in Stratum 2).

Table 1 shows the biological, lifestyle, and sociodemographic characteristics of participants at baseline. Compared with Stratum 1, women from Stratum 2 had a statistically significant higher baseline weight (64.83 and 67.17 kg, respectively, $p = 0.017$) and pre–pregnancy BMI (24.66 and 25.82, respectively, $p = 0.001$), and had gained significantly less weight during gestation (11.11 and 9.69 kg, respectively, $p = 0.030$). These differences did not translate into a significant effect on maternal iron status in the multivariate analyses. Table 1 also shows a trend ($p = 0.075$) toward a higher percentage of women with previous pregnancies in Stratum 1 (62.3%) than in Stratum 2 (55.7%). No significant differences in baseline characteristics were detected between women who dropped out of the study and women who reached the end of the intervention (Table S1).

We excluded the S65C mutation in the *HFE* gene from the multivariate analyses because of its low prevalence in our sample. For the same reason, subjects who were homozygous and heterozygous for H63D, together with the combined heterozygote H63D/C282Y, were grouped as "carrier of the H63D mutation." We compared, therefore, three categories of mutation of the *HFE* gene in the multivariate analyses: wild type (WT/WT), heterozygous for C282Y/WT, and carrier of the H63D mutation. A similar situation occurred with maternal ethnic origin: we excluded Asian and Black subjects from subsequent analyses due to the low representation in the studied population, and only three final categories were considered: Caucasian, Arab, and Latin American.

Since diet is expected to influence iron status, adherence to the Mediterranean diet was compared among the different study groups (Figure 2), but no significant differences were found.

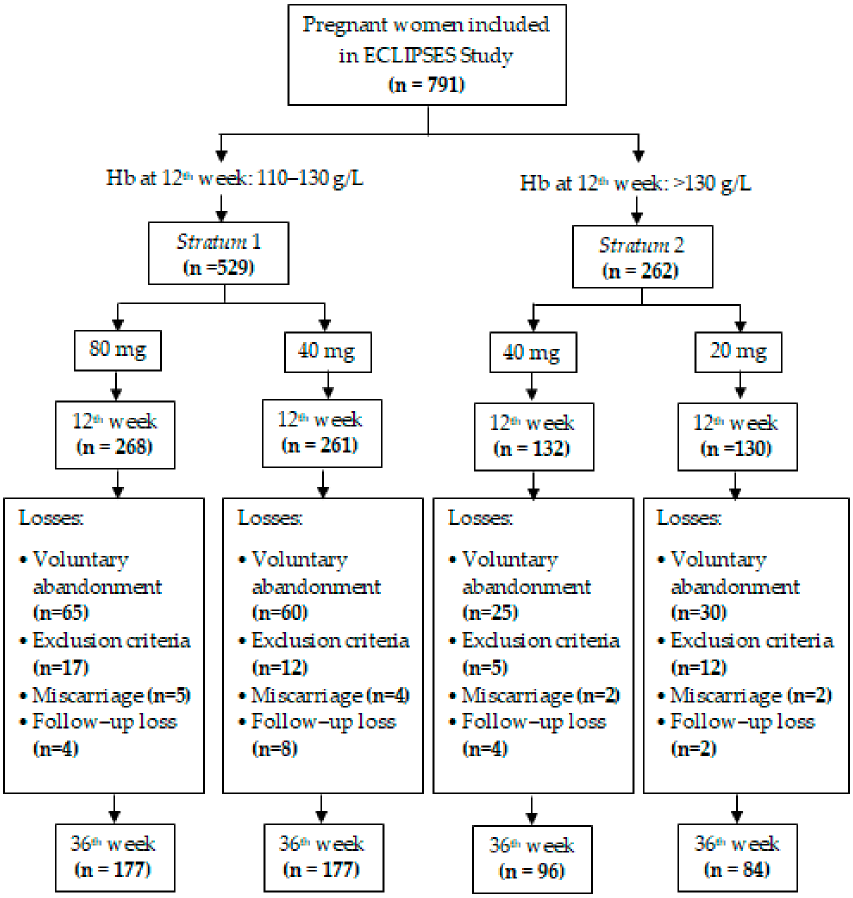

Figure 1. Flowchart of the study.

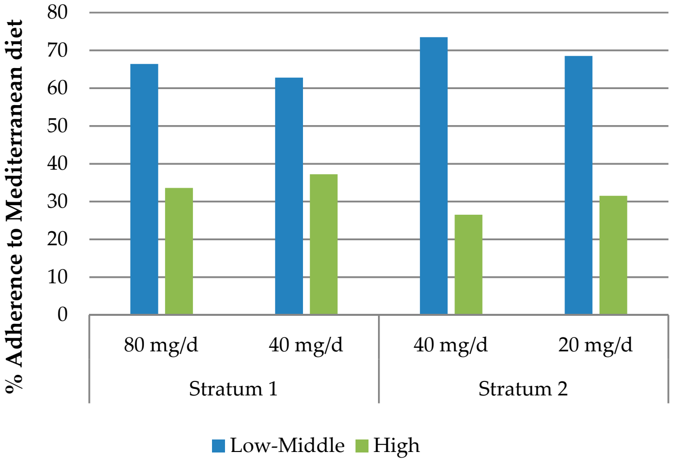

Figure 2. Adherence to the Mediterranean diet in the different groups of iron supplementation.

Table 1. Baseline characteristics of the study population.

	***Stratum* 1 (*n* = 529)**		***Stratum* 2 (*n* = 262)**		***p***
	Mean	**SD**	**Mean**	**SD**	
Age, years	30.40	5.07	30.21	5.28	0.613
Weight, Kg	64.83	11.31	67.17	13.60	0.017
Pre–pregnancy BMI, Kg/m^2	24.66	4.13	25.82	5.08	0.001
Gestational weight gain, Kg	11.11	8.17	9.69	9.47	0.030
	% (*n*)		% (*n*)		
Smoking	16.6 (88)		20.2 (53)		0.214
Parity	62.3 (329)		55.7 (146)		0.075
Planned pregnancy	79.8 (422)		80.5 (211)		0.801
Use of hormonal contraception	18.3 (97)		18.1 (47)		0.929
Pre–pregnancy BMI					
Underweight	1.3 (7)		2.3 (6)		0.314
Normal weight	60.9 (322)		51.5 (135)		0.012
Overweight	25.7 (136)		27.9 (73)		0.518
Obesity	12.1 (64)		18.3 (48)		0.018
HFE gene mutation	31.7 (130)		32.53 (68)		0.834
HFE genotype					
WT/WT	67.3 (280)		66.2 (141)		0.779
C282Y/WT	3.8 (16)		2.8 (6)		0.506
Carrier of H63D mutation	27.4 (114)		29.1 (62)		0.652
Carrier of S65C mutation	1.4 (6)		1.9 (4)		0.679
Family socioeconomic status					
Low	16.4 (87)		15.6 (41)		0.774
Middle	66.7 (353)		67.6 (177)		0.816
High	16.8 (89)		16.8 (44)		0.991
Maternal ethnic origin					
Caucasian	82.8 (405)		82.9 (203)		0.991
Asian	0.2 (1)		0.8 (2)		0.221
Arab	7.8 (38)		8.2 (20)		0.853
Black	1.8 (9)		0.4 (1)		0.114
Latin American	7.4 (36)		7.7 (19)		0.849
Adherence to Mediterranean diet					
Low–Middle	64.7 (342)		71.0 (186)		0.075
High	35.3 (187)		29.0 (76)		0.075

BMI: body mass index; WT: wild type. Sample size *HFE* genotype = 629; sample size maternal ethnic origin = 734.

We also performed a bivariate analysis comparing the percentage of women with and without risk of hemoconcentration on week 36 of gestation based on their initial Hb levels and *HFE* genotypes. As shown in Figure 3, we found that the H63D mutation in the *HFE* gene was significantly more prevalent among women from Stratum 2 (initial Hb levels > 130 g/L) who developed iron overload, compared with women who completed the pregnancy without risk of hemoconcentration (41.4% and 19.8%, respectively, $p = 0.045$). Similar results were obtained regarding the S65C mutation, which was observed in 6.9% of women who showed risk of hemoconcentration at the end of gestation, compared to 0.8% of women with normal Hb levels in the last trimester ($p = 0.031$). On the other hand, women with wild type (WT) genotype, i.e., without mutations in the *HFE* gene, were significantly more prevalent in the group from Stratum 2 who finished the pregnancy without risk of excess iron, than among women with Hb levels above 130 g/L on week 36 of gestation (74.6% and 51.7%, respectively, $p = 0.015$).

In Table 2 we describe and compare the blood tests results of women on weeks 12 and 36 of gestation among the intervention groups; a significant difference ($p = 0.042$) was observed in SF levels at week 36 between 80 and 40 mg/d iron in Stratum 1 (median: 17.19, IQR: 11.53, and median: 14.70, IQR: 9.37, respectively) in the non–adjusted bivariate analyses. Table 2 also shows that the prevalence of ID on week 36 was significantly higher ($p = 0.012$) in the group receiving 40 mg iron per day (51%)

than in women receiving 80 mg daily (38.2%). No other significant differences were observed between groups regarding prevalence of various iron states, although the risk of hemoconcentration in the third trimester of pregnancy showed a tendency to be higher among women who received 40 mg daily of iron (24%) than those receiving 20 mg of iron per day (13.1%). The same results were obtained in the per–protocol population (Table S2).

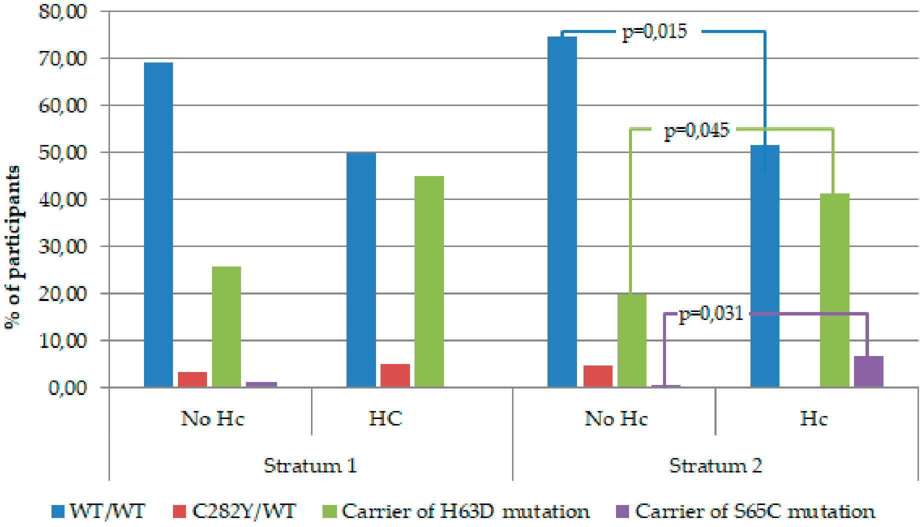

Figure 3. Percentage of women with and without risk of hemoconcentration (Hc) on week 36 of pregnancy, according to their initial hemoglobin (Hb) levels and *HFE* genotypes.

Multivariate analyses were performed to explore the effectiveness of iron dosages evaluated in Stratum 1 (80 mg/d and 40 mg/d) and Stratum 2 (40 mg/d and 20 mg/d), as well as the impact of several possible prenatal determinant factors. The results of the adjusted multivariate analyses for Stratum 1, summarized in Table 3, show that taking an iron supplement of 40 mg/d instead of 80 mg/d significantly reduced SF levels ($p = 0.026$) and doubled the risk of ID ($p = 0.022$) at the end of pregnancy. In contrast, the intervention with different doses of iron did not significantly change Hb levels ($p = 0.718$), the risk of anemia ($p = 0.166$), or IDA ($p = 0.299$). SF levels in early pregnancy were positively associated with Hb levels (β: 1.70; SE: 0.66; $p = 0.010$) and SF levels (β: 0.60, SE: 0.04, $p < 0.001$) in the third trimester. Additionally, maternal age 35 years and above increased SF in week 36 of pregnancy (β: 0.21; SE: 0.07; $p = 0.002$). Increasing early pregnancy levels of SF showed a protective effect against ID (OR: 0.29; 95%CI: 0.19–0.45; $p < 0.001$), anemia (OR: 0.54; 95%CI: 0.32–0.90; $p = 0.018$), and IDA (OR: 0.32; 95%CI: 0.17–0.59; $p < 0.001$). No differences were observed between the iron dosages evaluated in Stratum 1 in relation to the risk of hemoconcentration at week 36 of gestation after adjusting for possible confounders ($p = 0.481$). The adjusted multiple linear regression model for the risk of hemoconcentration was not statistically significant ($p = 0.071$). Moreover, in Stratum 1, when the regression models were performed separating women with and without ID on week 12 (Table 4), we observed that only in women with ID, the dose of 80 mg/d instead of 40 mg/d increased Hb levels in the third trimester (β: 8.81; SE: 2.40; $p = 0.001$), protecting women against anemia and IDA (OR: 0.03; 95%CI: 0.01–0.60; $p = 0.021$, for both cases).

Table 2. Blood tests results of participants on week 36 of gestation according to supplementation dose.

	***Stratum* 1**			***Stratum* 2**		
	80 g/d	**40 g/d**	***p***	**40 g/d**	**20 g/d**	***p***
		12th week				
Hemoglobin (g/L)	123.26 (5.32)	123.44 (4.77)	0.689	135.68 (4.59)	136.61 (4.44)	0.098
Serum ferritin (μg/L)	38.95 (26.10)	38.20 (25.05)	0.740	38.50 (28.98)	40.75 (30.00)	0.965
Mean corpuscular volume (fL)	87.08 (6.36)	87.30 (6.63)	0.696	88.53 (3.43)	88.54 (3.74)	0.980
C–reactive protein (mg/L)	0.73 (0.62)	0.74 (0.72)	0.815	0.72 (0.54)	0.70 (0.53)	0.779
Iron deficiency (%)	14.2 (38)	14.2 (37)	0.999	14.4 (19)	12.3 (16)	0.620
		36th week				
Hemoglobin (g/L)	117.63 (7.55)	117.21 (8.35)	0.622	123.07 (10.19)	121.04 (8.85)	0.157
Serum ferritin (μg/L)	17.19 (11.53)	14.70 (9.38)	0.042	11.10 (8.10)	11.00 (6.80)	0.798
Mean corpuscular volume (fL)	89.31 (6.87)	88.19 (12.06)	0.261	90.23 (4.19)	89.61 (4.11)	0.299
C–reactive protein (mg/L)	0.76 (0.74)	0.71 (0.64)	0.470	0.70 (0.69)	0.75 (0.56)	0.593
Iron deficiency (%)	38.2 (71)	51 (98)	0.012	66 (68)	69.7 (62)	0.590
Iron deficiency anemia (%)	8.5 (15)	9.6 (17)	0.711	7.3 (7)	11.9 (10)	0.291
Anemia (%)	11.9 (21)	13 (23)	0.747	8.3 (8)	11.9 (10)	0.426
Hemoconcentration (%)	6.8 (12)	7.9 (14)	0.684	24 (23)	13.1 (11)	0.063

Continuous variables expressed as means (SD), except for serum ferritin, which is expressed as median (interquartile range). Categorical variables expressed in percentages (*n*).

Table 3. The effects of the intervention with iron supplementation (40 or 80 mg/day) throughout pregnancy on hemoglobin and serum ferritin levels and on the risk of iron deficiency (ID), anemia, iron deficiency anemia (IDA), and hemoconcentration on the third trimester in women from Stratum 1.

Hemoglobin levels				
Independent variables	**β**	**SE**	***p***	**Model**
[a] Intervention (0:80 mg/d, 1:40 mg/d)	−0.42	0.85	0.622	$R^2 = -0.002$ $p = 0.622$
[b] Intervention (0:80 mg/d, 1:40 mg/d)	0.33	0.92	0.718	$R^2 = 0.031$ $p = 0.050$
Hemoglobin on week 12 of pregnancy	0.25	0.10	0.015	
Serum ferritin on week 12 of pregnancy	1.70	0.66	0.010	
Serum ferritin levels				
Independent variables	**β**	**SE**	***p***	**Model**
[a] Intervention (0:80 mg/d, 1:40 mg/d)	−0.10	0.06	0.085	$R^2 = 0.004$ $p = 0.085$
[c] Intervention (0:80 mg/d, 1:40 mg/d)	−0.12	0.05	0.026	$R^2 = 0.436$ $p < 0.001$
Serum ferritin on week 12 of pregnancy	0.60	0.04	<0.001	
Maternal age (0:25–34 years, 1:<25 years)	0.05	0.08	0.559	
Maternal age (0:25–34 years, 1:≥35 years)	0.21	0.07	0.002	
Iron deficiency (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	***p***	**Model**
[a] Intervention (0:80 mg/d, 1:40 mg/d)	1.69	1.12–2.54	0.012	R^2 Nagelkerke = 0.022 $p = 0.012$
[c] Intervention (0:80 mg/d, 1:40 mg/d)	1.82	1.09–3.03	0.022	R^2 Nagelkerke = 0.241 $p < 0.001$
Serum ferritin on week 12 of pregnancy	0.29	0.19–0.45	<0.001	
Anemia (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	***p***	**Model**
[a] Intervention (0:80 mg/d, 1:40 mg/d)	1.11	0.59–2.09	0.747	R^2 Nagelkerke = 0.001 $p = 0.747$
[b] Intervention (0:80 mg/d, 1:40 mg/d)	1.70	0.80–3.61	0.166	R^2 Nagelkerke = 0.146 $p = 0.027$
Planned pregnancy (0:no, 1:yes)	3.57	1.00–12.80	0.050	
Serum ferritin on week 12 of pregnancy	0.54	0.32–0.90	0.018	

Table 3. *Cont.*

Iron–deficiency anemia (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	***p***	**Model**
[a] Intervention (0:80 mg/d, 1:40 mg/d)	1.15	0.55–2.38	0.711	R^2 Nagelkerke = 0.001 $p = 0.711$
[b] Intervention (0:80 mg/d, 1:40 mg/d)	1.58	0.67–3.71	0.299	R^2 Nagelkerke = 0.19 $p = 0.004$
Serum ferritin on week 12 of pregnancy	0.32	0.17–0.59	<0.001	
Hemoconcentration (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	***p***	**Model**
[a] Intervention (0:80 mg/d, 1:40 mg/d)	1.18	0.53–2.63	0.684	R^2 Nagelkerke = 0.001 $p = 0.684$
[b] Intervention (0:80 mg/d, 1:40 mg/d)	1.44	0.52–3.97	0.481	R^2 Nagelkerke = 0.166 $p = 0.071$
Genotype *HFE* (0:WT/WT, 1: carrier of H63D)	3.28	0.09–6.68	0.026	
Genotype *HFE* (0:WT/WT, 1: C282Y/WT)	1.93	0.21–18.02	0.566	
Parity (0:no, 1:yes)	0.26	0.09–0.76	0.014	

a Crude model. b Adjusted for: iron supplementation dosage, maternal age, use of hormonal contraception, pre–pregnancy maternal body mass index, gestational weight gain, *HFE* genotypes, maternal ethnic origin, hemoglobin on week 12, serum ferritin on week 12, c–reactive protein on week 12, socioeconomic status, weekly mean METS on week 12, smoking habit, alcohol intake, planned pregnancy, parity, mean caloric intake during pregnancy, and adherence to a Mediterranean diet. c Adjusted for: model b, except for hemoglobin on week 12.

Table 4. The effect of the intervention with iron supplementation in Stratum 1 (0:80 mg/d, 1:40 mg/d) throughout pregnancy on maternal iron status on the third trimester, according to their initial iron stores.

SF < 15 µg/L				
Hemoglobin levels	**β**	**SE**	***p***	**Model**
Crude model	− 7.06	2.43	0.006	$R^2 = 0.129$ $p = 0.006$
[a] Adjusted model	− 8.81	2.40	0.001	$R^2 = 0.32$ $p = 0.003$
Iron deficiency (0:no, 1:yes)	**OR**	**95% CI**	***p***	**Model**
Crude model	3.10	0.93–10.39	0.066	R^2 Nagelkerke = 0.091 $p = 0.060$
[b] Adjusted model	4.51	0.78–26.08	0.092	R^2 Nagelkerke = 0.429 $p = 0.013$
Anemia (0:no, 1:yes)	**OR**	**95% CI**	***p***	**Model**
Crude model	5.50	1.05–28.75	0.043	R^2 Nagelkerke = 0.145 $p = 0.025$
[a] Adjusted model	29.14	1.67–508.56	0.021	R^2 Nagelkerke = 0.596 $p = 0.020$
Iron–deficiency anemia (0:no, 1:yes)	**OR**	**95% CI**	***p***	**Model**
Crude model	5.50	1.05–28.75	0.043	R^2 Nagelkerke = 0.145 $p = 0.025$
[a] Adjusted model	29.14	1.67–508.56	0.021	R^2 Nagelkerke = 0.596 $p = 0.020$
SF≥15 µg/L				
Hemoglobin levels	**β**	**SE**	***p***	**Model**
Crude model	0.75	0.88	0.395	$R^2 = -0.001$ $p = 0.395$
[a] Adjusted model	0.42	0.96	0.664	$R^2 = 0.035$ $p = 0.031$

a Adjusted for: iron supplementation dosage, maternal age, use of hormonal contraception, pre–pregnancy maternal body mass index, gestational weight gain, *HFE* gene genotypes, maternal ethnic origin, hemoglobin on week 12, c–reactive protein on week 12, socioeconomic status, weekly mean of METS on week 12, smoking habit, alcohol intake, planned pregnancy, parity, mean caloric intake during pregnancy, and adherence to Mediterranean diet. b Adjusted for: model a, except for hemoglobin on week 12.

Similarly, Table 5 shows the results of multivariate analyses performed after selecting women from Stratum 2. Adjusting for possible confounding factors, we found that a daily iron supplementation of 20 mg as opposed to 40 mg during pregnancy reduced the risk of hemoconcentration by 69% ($p = 0.035$) without increasing the risk of any iron deficit states studied at the end of pregnancy. Similarly to Stratum 1, higher SF levels on week 12 of gestation were positively correlated with SF levels (β: 0.42; SD: 0.06; $p < 0.001$) in the last months. Increasing SF levels in early pregnancy protected, therefore, against ID (OR: 0.36; 95%CI: 0.19–0.68; $p = 0.002$), anemia and IDA (OR: 0.26; 95%CI: 0.08–0.66; $p = 0.023$, for both cases). Furthermore, the analyses showed the effect of maternal age on iron status on week 36, with women under 25 years presenting reduced SF levels (β: –0.28; SE: 0.11; $p = 0.013$), and women 35 years and older at lower risk of ID (OR: 0.37; 95%CI: 0.16–0.91; $p = 0.029$) than women between 25 and 34 years of age. It was also found that the middle–high SES, compared with low SES, protected against anemia and IDA (OR: 0.06; 95%CI: 0.01–0.40; $p = 0.003$, for both cases) in women who started pregnancy with Hb levels above 130 g/L. Regarding iron overload, in addition to the aforementioned effect of the low iron dose, higher Hb levels early in pregnancy and being a carrier of the H63D mutation significantly increased Hb levels on week 36 (β: 0.72; SE: 0.16, and β: 3.93; SE: 1.74, respectively) and the risk of hemoconcentration (OR: 1.20; 95%CI: 1.08–1.33, and OR: 3.09; 95%CI: 1.10–8.71, respectively). When the multivariate analyses were applied to the sample of women from Stratum 2, categorized according their initial iron stores, we found that compared to 20 mg, 40 mg of iron per day increased SF on week 36 (β: 0.39; SE: 0.15; $p = 0.014$) only in women with iron deficiency, while 20 mg/d reduced the risk of hemoconcentration (OR: 0.25; 95%CI: 0.07–0.85; $p = 0.027$) in women with initial iron stores within the normal range (Table 6).

Table 5. The effects of the intervention with iron supplementation (40 or 20 mg/day) throughout pregnancy on hemoglobin and serum ferritin levels and on the risk of ID, anemia, IDA, and hemoconcentration on the third trimester in women from Stratum 2.

Hemoglobin levels				
Independent variables	**β**	**SE**	***p***	**Model**
[a] Intervention (0:40 mg/d, 1:20 mg/d)	−1.91	1.44	0.188	$R^2 = 0.004$ $p = 0.188$
[b] Intervention (0:40 mg/d, 1:20 mg/d)	−2.50	1.47	0.092	$R^2 = 0.116$ $p = 0.003$
Genotype *HFE* (0:WT/WT, 1: carrier of H63D)	3.93	1.74	0.025	
Genotype *HFE* (0:WT/WT, 1: C282Y/WT)	1.34	3.70	0.718	
Hemoglobin on week 12 of pregnancy	0.72	0.16	<0.001	
Serum ferritin levels				
Independent variables	**β**	**SE**	***p***	**Model**
[a] Intervention (0:40 mg/d, 1:20 mg/d)	0.02	0.07	0.734	$R^2 = -0.003$ $p = 0.734$
[c] Intervention (0:40 mg/d, 1:20 mg/d)	0.01	0.08	0.954	$R^2 = 0.218$ $p < 0.001$
Maternal age (0:25–34 years, 1:<25 years)	−0.28	0.11	0.013	
Maternal age (0:25–34 years, 1:≥35 years)	0.12	0.10	0.221	
Serum ferritin on week 12 of pregnancy	0.42	0.06	<0.001	
Low iron stores (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	***p***	**Model**
[a] Intervention (0:40 mg/d, 1:20 mg/d)	1.18	0.64–2.17	0.590	R^2 Nagelkerke = 0.002 $p = 0.590$
[c] Intervention (0:40 mg/d, 1:20 mg/d)	1.45	0.69–3.02	0.326	R^2 Nagelkerke = 0.229 $p = 0.003$
Maternal age (0:25–34 years, 1:<25 years)	3.07	0.78–12.12	0.109	
Maternal age (0:25–34 years, 1:≥35 years)	0.37	0.16–0.91	0.029	
Serum ferritin on week 12 of pregnancy	0.36	0.19–0.68	0.002	

Table 5. *Cont.*

Anemia (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	***p***	**Model**
[a] Intervention (0:40 mg/d, 1:20 mg/d)	1.48	0.56–3.96	0.428	R^2 Nagelkerke = 0.007 p = 0.426
[b] Intervention (0:40 mg/d, 1:20 mg/d)	2.01	0.44–9.09	0.364	R^2 Nagelkerke = 0.468 p = 0.002
SES (0:low; 1:middle + high)	0.06	0.01–0.40	0.003	
Serum ferritin on week 12 of pregnancy	0.26	0.08–0.66	0.023	
Iron–deficiency anemia (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	**p**	**Model**
[a] Intervention (0:40 mg/d, 1:20 mg/d)	1.78	0.62–4.74	0.295	R^2 Nagelkerke = 0.013 p = 0.291
[b] Intervention (0:40 mg/d, 1:20 mg/d)	2.01	0.44–9.09	0.364	R^2 Nagelkerke = 0.468 p = 0.002
SES (0:low; 1:middle + high)	0.06	0.01–0.40	0.003	
Serum ferritin on week 12 of pregnancy	0.26	0.08–0.66	0.023	
Hemoconcentration (0:no, 1:yes)				
Independent variables	**OR**	**95% CI**	***p***	**Model**
[a] Intervention (0:40 mg/d, 1:20 mg/d)	0.48	0.22–1.05	0.067	R^2 Nagelkerke = 0.031 p = 0.060
[b] Intervention (0:40 mg/d, 1:20 mg/d)	0.31	0.11–0.92	0.035	R^2 Nagelkerke = 0.282 p = 0.004
Hemoglobin on week 12 of pregnancy	1.20	1.08–1.33	0.001	
Genotype *HFE* (0:WT/WT, 1: carrier of H63D)	3.09	1.10–8.71	0.033	
Genotype *HFE* (0:WT/WT, 1: C282Y/WT)	0.00	.	0.999	

a Crude model. b Adjusted for: iron supplementation dosage, maternal age, use of hormonal contraception, pre–pregnancy maternal body mass index, gestational weight gain, *HFE* gene genotypes, maternal ethnic origin, hemoglobin on week 12, serum ferritin on week 12, c–reactive protein on week 12, socioeconomic status, weekly mean of METS on week 12, smoking habit, alcohol intake, planned pregnancy, parity, mean caloric intake during pregnancy, and adherence to a Mediterranean diet. c Adjusted for: model b, except for hemoglobin on week 12.

Table 6. The effect of the intervention with iron supplementation on Stratum 2 (0:40 mg/d, 1:20 mg/d) throughout pregnancy regarding maternal iron status in the third trimester, according to initial iron stores.

SF<15 μg/L				
Serum ferritin levels	**β**	**SE**	**p**	**Model**
Crude model	−0.24	0.14	0.083	R^2 = 0.061 p = 0.083
[b] Adjusted model	−0.39	0.15	0.014	R^2 = 0.344 p = 0.021
Iron deficiency (0:no, 1:yes)	**OR**	**95% CI**	***p***	**Model**
Crude model	6.11	0.60–62.23	0.126	R^2 Nagelkerke = 0.163 p = 0.085
[b] Adjusted model	42.09	0.00–50.00	0.614	R^2 Nagelkerke = 0.924 p = 0.001
SF≥15 μg/L				
Hemoglobin levels	**β**	**SE**	***p***	**Model**
Crude model	–1.75	1.54	0.260	R^2 = 0.002 p = 0.260
[a] Adjusted model	–2.00	1.56	0.200	R^2 = 0.184 p < 0.001

Table 6. *Cont.*

Serum ferritin levels	**β**	**SE**	***p***	**Model**
Crude model	0.06	0.08	0.455	$R^2 = -0.002$ $p = 0.455$
[b] Adjusted model	0.08	0.09	0.368	$R^2 = 0.077$ $p = 0.008$
Anemia (0:no, 1:yes)	**OR**	**95% CI**	***p***	**Model**
Crude model	1.34	0.43–4.20	0.611	R^2 Nagelkerke = 0.004 $p = 0.611$
[a] Adjusted model	1.02	0.20–5.10	0.984	R^2 Nagelkerke = 0.383 $p = 0.007$
Iron–deficiency anemia (0:no, 1:yes)	**OR**	**95% CI**	***p***	**Model**
Crude model	1.63	0.50–5.39	0.421	R^2 Nagelkerke = 0.010 $p = 0.417$
[a] Adjusted model	1.02	0.20–5.10	0.984	R^2 Nagelkerke = 0.383 $p = 0.007$
Hemoconcentration (0:no, 1:yes)	**OR**	**95% CI**	***p***	**Model**
Crude model	0.46	0.20–1.06	0.068	R^2 Nagelkerke = 0.035 $p = 0.061$
[a] Adjusted model	0.25	0.07–0.85	0.027	R^2 Nagelkerke = 0.261 $p = 0.001$

a Adjusted for: iron supplementation dosage, maternal age, use of hormonal contraception, pre–pregnancy maternal body mass index, gestational weight gain, *HFE* gene genotypes, maternal ethnic origin, hemoglobin on week 12, C–reactive protein on week 12, socioeconomic status, weekly mean of METS on week 12, smoking habit, alcohol intake, planned pregnancy, parity, mean caloric intake during pregnancy, and adherence to a Mediterranean diet. b Adjusted for: model a, except for hemoglobin on week 12.

In the multivariate analyses of Stratum 2, the results for the per–protocol and for the ITT populations were the same (Table S4); for Stratum 1, the regression models for Hb levels, anemia and IDA lost statistical significance when women who were anemic at mid–pregnancy were removed from the sample. However, the results about the effects on SF levels and ID were the same as for the ITT population (Table S3).

4. Discussion

Despite the wealth of research on prenatal iron supplementation, there is a lack of consensus on the optimal iron dosage in relation to the characteristics of each woman. Consequently, we were determined to investigate the effectiveness of different doses of iron supplementation on preventing iron deficiency and excess iron in the last trimester of gestation. To our knowledge, few publications address the interplay of early maternal iron status and the effect of prenatal iron supplementation [39].

Firstly, we observed that the prevalence of ID found in both strata of our study population (38.2%–69.70%) was in the range of the European estimates for pregnant women published in the most recent reports [2,3]; regarding the prevalence of anemia (8.3%–13%) and IDA (7.3%–11.9%), our results were considerably lower than the estimates of the same reports (24.5% and 35%, respectively). In relation to the risk of hemoconcentration, we observed that its prevalence (~13%) was similar to previous reports from Spain by Arija et al. [27] and within the wide range reported in European countries (8.7% to 42%) [26]. We should underscore that most research focuses on iron deficiency, and only few studies have described the prevalence of excess iron; consequently, the estimates on iron overload are less updated and not as established. As expected, we observed a significantly higher prevalence of risk of hemoconcentration in Stratum 2 (13.1% for 20 mg/d and 24% for 40 mg/d) than in Stratum 1 (6.8% for 80 mg/d and 7.9% for 40 mg/d) at the end of pregnancy. This difference supports our hypothesis that women with normal–high initial Hb levels were at greater risk of iron overload, possibly due to the persistent effect that genetic alterations in the *HFE* gene exert on iron levels [40,41]. Our results also show a higher prevalence of *HFE* gene mutations in women from Stratum 2 at risk of hemoconcentration on week 36, as opposed to the higher prevalence of the wild type genotype in women who finished the pregnancy without that risk (see Figure 2). This highlights the influence of the genetic alteration in the *HFE* gene on the risk of iron overload in women with initial Hb levels > 130 g/L. Moreover, within Stratum 2, we found that the percentage of women at risk of hemoconcentration on week 36 in the group of 20 mg of iron per day was fifty percent less than in the group receiving 40 mg daily (13.1% and 24%, respectively, $p = 0.063$), confirming our hypothesis that low iron doses are the best option in this case.

To clarify the effectiveness of different doses of prenatal iron supplementation on maternal iron status, the multivariate analyses were adjusted for several associated variables, including obstetric, biological, and socioeconomic conditions, as well as *HFE* gene genotype and iron–related blood parameters. In this regard, in women from Stratum 1 who began the gestation with Hb levels between 110 and 130 g/L, we observed that a daily dosage of 80 mg iron, as opposed to 40 mg, improved SF levels (b: 0.12, $p = 0.026$) and protected against ID (OR: 0.55, $p = 0.022$) at the end of pregnancy. Furthermore, when we explored the effect of iron supplementation in women within Stratum 1 according their initial iron reserves, we found that the higher dose of iron (80 mg/d) reduced the risk of anemia and IDA (OR: 0.03 and $p = 0.021$, for both cases) during the last months of gestation in women with iron–deficiency (SF < 15 µg/L, 14.2%) at the start of the pregnancy. In contrast, no significant effect was observed in women with SF ≥ 15 µg/L on week 12. These results respond to the physiological regulation of intestinal iron absorption in accordance with iron reserves, by which the body strongly regulates iron absorption when stores are sufficient [42,43]. On the contrary, and in agreement with Milman et al. [44], we did not find additional effects of high doses of iron in women with correct iron reserves at the beginning of the study. We can conclude that the usual prescribed dose of 40 mg daily would be effective in women with optimal initial iron reserves, but not in women with iron deficiency in early pregnancy.

On the other hand, in Stratum 2 (initial Hb levels >130 g/L), women who received a daily dosage of 20 mg iron, compared with the group that received 40 mg, reduced the risk of hemoconcentration in the third trimester (OR: 0.31, $p = 0.035$), without increasing the risk of iron deficit. In this case, we should underscore that the risk of iron overload trebles in carriers of the H63D mutation of the *HFE* gene (OR: 3.09, $p = 0.033$). Accordingly, we would advise to prescribe low doses of iron to women with normal–high Hb (>130 g/L) levels in early pregnancy. Interestingly, the baseline prevalence of ID was higher than expected in this group (13.4%); similarly to *Stratum* 1, the different doses produced different results regarding iron status, which varied in accordance with the initial iron stores. The protective effect of 20 mg iron per day against the risk of hemoconcentration (OR: 0.25, $p = 0.027$) was only observed in women with sufficient iron reserves in early pregnancy (SF ≥ 15 µg/L).

Based on these findings, we emphasize that iron supplementation during pregnancy should be adapted to the initial iron status of each woman, assessed not only by Hb levels but also by SF levels, to prevent both iron deficiency and iron overload at the end of gestation. These conclusions are in agreement with the valuable contributions of Milman et al. [45,46], Casanueva et al. [47], and Peña-Rosas and Viteri [26], who advocate adapting prenatal iron supplementation in view that both iron deficit and hemoconcentration have been associated with negative effects on maternal–child health [5–10,28–30].

Generally, in clinical practice only Hb levels are measured to monitor maternal iron status during pregnancy. However, while detecting anemia, Hb levels fail to diagnose ID. Our results show that the effects of iron supplementation vary as a function of initial iron reserves, indicating the importance of detecting ID at the beginning of the gestation. We advocate for the routine measurement of SF levels during antenatal checks. We also underscore that mutations in the *HFE* gene should be studied in women with normal–high Hb levels at the beginning of pregnancy to avoid excessive iron supply.

Indeed, in relation to this, it is known that there is a racial difference in the prevalence of alterations in the *HFE* gene, being greater in the populations of northern Europe than in the Mediterranean countries [24]. This adds even more weight to the premise that it is necessary to evaluate the individual characteristics of women to prescribe the most efficient prenatal iron supplementation in each case.

In this study, the multivariate analyses have also revealed some prenatal determinants of maternal iron status at the end of pregnancy. For instance, high SF levels on week 12 were associated with the increase of Hb and SF levels on week 36 in both strata, reducing the risk of all iron deficiency states: 71% and 64% lower risk of ID, 68% and 74% lower risk of IDA, and 46% and 74% lower risk of anemia for Stratum 1 and Stratum 2, respectively (see Tables 3 and 4). The results show that SF levels on week 36 increased with maternal age, and in Stratum 2, maternal age was also linked to the risk of ID in the third trimester of gestation, although to our knowledge, the underlying mechanism of this association is not yet elucidated. We found a protective role of middle–high SES against anemia and IDA (OR: 0.06, $p = 0.003$, for both cases), specifically in women from Stratum 2. This finding coincides with previous reports that conclude that low–income status is a risk factor for iron deficiency, presenting as ID, anemia and IDA, especially in developing countries [23,48,49]. This observation stresses that a low SES might be associated with less healthy lifestyles and under-attendance to antenatal care [50,51]. Also in agreement with other studies [40,52,53], we found that the H63D mutation in the *HFE* gene increased Hb levels (b: 3.93, $p = 0.025$) and trebled the risk of hemoconcentration (OR: 3.09, $p = 0.033$) on week 36. It is well established that mutations in the *HFE* gene are highly prevalent in Caucasian populations and that they are linked to iron overload [3,54]. It has been suggested that *HFE* gene mutations increase intestinal iron absorption [41,55]. In our study, therefore, the results suggest that the presence of some mutation in the *HFE* gene would increase iron absorption in women with initial Hb levels >130mg/L. Unexpectedly, maternal iron status was not significantly associated with diet in the multivariate analyses in any strata. Similarly, comparative analyses, including adherence to the Mediterranean diet failed to show significant differences between different supplementation groups. This result suggests that the diet was very similar among all the women in the study. Finally, the trend for a higher percentage of parity in Stratum 1 (62.3%) than in Stratum 2 (55.7%) suggests that previous births could weaken the iron status of women at the beginning of pregnancy. Interestingly, in the multivariate analyses parity seemed to reduce by 74% the risk of hemoconcentration in women of Stratum 1, but the results in the regression model were not statistically significant ($p = 0.071$).

Understanding that the prenatal iron supplementation has a different effect on maternal iron status at the end of pregnancy according to initial levels of Hb and SF could contribute to improving public health policies and to adapting clinical practices to the population groups at risk. Taking into consideration other associated prenatal determinants of maternal iron status can also improve antenatal care. In view of the evidence presented in this study, we emphasize firstly, the importance of full iron reserves before pregnancy, in preparation for the high cost of iron during gestation; and secondly, we recommend that clinicians adapt iron supplementation to the initial levels of Hb and iron reserves (see Figure 4). To assess the presence of genetic mutations in the *HFE* gene in women with normal–high Hb levels and full iron reserves at the beginning of pregnancy can help to reduce the risk of hemoconcentration in this group.

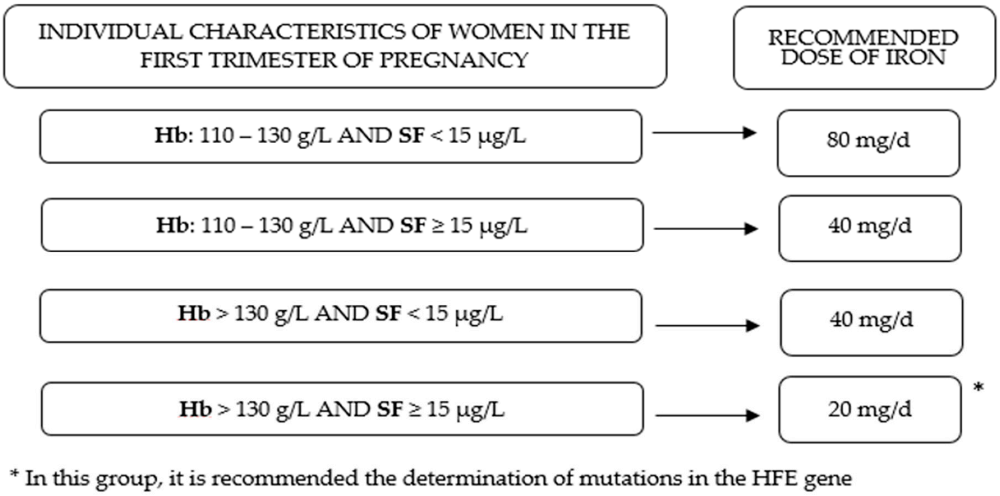

Figure 4. Adaptation of prenatal iron supplementation according the individual characteristics of women in the first trimester of pregnancy.

Strengths and Limitations

The main strengths of the current community RCT are the large sample size ($n = 791$) and the extensive data collection regarding sociodemographic conditions, clinical information, obstetric data, and lifestyle, including diet and physical activity. In addition, testing for *HFE* gene mutations has added valuable information on the effect of genetic variability on iron metabolism and on the possible impact of personalized iron supplementation. Methodologically, we were able to evaluate the progression of iron status by monitoring blood parameters at different stages of pregnancy. However, some limitations must be taken into account when interpreting the findings of this study. Firstly, the notable dropout rate, although this is not uncommon in community interventions such as ours, which require several visits. No woman dropped out due to gastrointestinal side effects, since we used ferrimanitol ovalbumin instead of ferrous sulfate in our study. Another limitation was the lack of SF measurements in the 24th week of pregnancy, which would have strengthened the results. Since women gave birth in hospitals, data on maternal iron status at delivery were not available for inclusion.

5. Conclusions

In conclusion, we advise routine monitoring of Hb and SF during antenatal check–ups. These tools can be used in clinical practice to prescribe the optimal dose of iron supplements, with the ultimate aim of achieving the best pregnancy outcomes. In addition, the study of mutations in the *HFE* gene in women with normal–high Hb levels at the beginning of pregnancy could reduce the risk of hemoconcentration. Further studies are needed to assess the effect of mutations in the *HFE* gene on the maternal iron status and its interplay with prenatal iron supplementation to determine if there is a real need to use supplements in these cases. Future studies should also assess whether, in addition to the benefits for pregnant women, the supplementation with different doses of iron have benefits for their children.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/10/2418/s1, Table S1: Baseline characteristics of the population lost trhought the study; Table S2: Biochemical characteristics of participants at 36th week of gestation according to dose of supplementation (by protocol); Table S3: Effect of the intervention with iron supplementation (40 or 80 mg/day) through pregnancy on hemoglobin and serum ferritin levels and on the risk of ID, anemia, IDA and hemoconcentration at third trimester in women from Stratum 1 (by protocol); Table S4: Effect of the intervention with iron supplementation (40 or 20 mg/day) through pregnancy on hemoglobin and serum ferritin levels and on the risk of ID, anemia, IDA and hemoconcentration at third trimester in women from Stratum 2 (by protocol).

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CHAPTER 4.

EFFECT OF ADJUSTING PRENATAL IRON SUPPLEMENTATION ON CHILD NEURODEVELOPMENT

Adapting prenatal iron supplementation to maternal needs results in optimal child neurodevelopment: a follow-up of the ECLIPSES study

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Adapting prenatal iron supplementation to maternal needs results in optimal child neurodevelopment: a follow-up of the ECLIPSES Study

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Abstract

Background: Prenatal prescription of standard iron supplements to prevent iron deficiency appears not to be appropriate for all women and their children, as some women may be at risk of iron deficiency and others at risk of iron excess early in pregnancy. The present study aimed to assess whether prenatal iron supplementation adapted to the needs of each pregnant woman affects their child's neurodevelopment.

Methods: Follow-up of a community-based RCT involving 503 mother–child pairs. Non-anaemic pregnant women recruited in Tarragona (Spain) early in pregnancy were prescribed a daily iron dose based on their initial haemoglobin levels: *Stratum* 1 (Hb = 110–130 g/L, 80 or 40 mg/d of iron) and *Stratum* 2 (Hb > 130 g/L, 40 or 20 mg/d of iron). Women receiving 40 mg/d were considered the control group in each *Strata*. The child's neurodevelopment was assessed at 40 days of age using the Bayley Scales of Infant Development-III (BSID-III). Adjusted multiple regression models were used.

Results: Multiple regression analyses showed no association between the intervention and control group within each *Strata* on the BSID-III scores on any of the developmental scales in children, including cognitive, language, and motor development: *Stratum* 1 (β 1.46, 95%CI -2.15, 5.07; β 1.30, 95%CI -1.99, 4.59; and β 2.04, 95%CI -3.88, 7.96, respectively) and *Stratum* 2 (β -4.04, 95%CI -7.27, 0.80; β -0.36, 95%CI -3.47, 2.75; and β -3.76, 95%CI -9.30, 1.78, respectively).

Conclusions: In non-anaemic women in early pregnancy, no differences were found in the cognitive, language and motor development of children at 40 days of age between the dose of iron tested in each case –adjusted to initial Hb levels– compared to the dose of the control group. Further studies are guaranteed to confirm our findings.

Trial registration: The ECLIPSES study was registered at www.clinicaltrialsregister.eu as EudraCT number 2012–005,480-28.

Keywords: Iron supplementation, Prenatal, Neurodevelopment, Cognitive development, Language development, Motor development

Background

Accumulating evidence indicates that maternal iron status during the gestational period is of great importance for mother–child health [1–7]. The wide variety of

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processes in which iron is involved means that prenatal iron deficiency might negatively affect the child's development and, especially, brain functioning [8–10]. However, current evidence has mostly originated from animal studies and observational studies in humans. Only a few randomized controlled trials (RCT) have been conducted to evaluate the association between maternal iron status and the child's cognitive and motor performance. Nevertheless, some important findings have arisen from these studies. In fact, since the harmful effects of prenatal suboptimal iron status have been observed in the short term [11], they could also persist even after correcting iron deficit [12–18]. Thus, iron supplements are usually prescribed to pregnant women [19–21], with good results in improving the serum iron-related biomarkers during gestation, [22–25] although they have not been always associated with better developmental outcomes for their children [26–28]. Also, it should be pointed out the risk of iron overload when an iron-replete woman or those with mutations in the *HFE* gene –which increases the intestinal iron absorption, especially in homozygotes but also in heterozygosis to a lesser extent– receives routine prenatal iron supplements [29–31], as well as the negative consequences that have also been associated with prenatal excess iron on the neuropsychological functions of the child [14, 22, 25, 32, 33]. In this regard, it is important to highlight that the overall prevalence of *HFE* gene alterations in our population is quite high [34]. After some authors have concluded that both iron deficit and excess may injure the child's cognitive and motor development [32, 35, 36], they advise that prenatal iron supplementation should be individualized considering maternal iron stores, as well as other lifestyle and biological conditions [37, 38]. We supported this advice after having shown by a community-based Randomized Clinical Trial (RCT) the effectiveness of adapting iron doses to maternal haemoglobin (Hb) levels early in pregnancy in preventing iron deficiency, anaemia, and haemoconcentration [39]. [34, 40]In this study, the obtained results were adjusted for iron stores and specific *HFE* genotypes, among other women's characteristics, demonstrating that Hb levels in early pregnancy (at gestational week 12) were related to serum ferritin concentrations and abnormalities in the *HFE* gene. Like ours, some authors advocated personalizing iron supplements used during pregnancy to provide the most appropriate supply of iron for each woman, which helps to prevent iron deficiency in some women and iron excess in others, improving the maternal iron status during pregnancy in all of them [22, 23, 25, 41]. However, little research has focused on the effects of this prenatal iron supplementation on the child's neurocognitive abilities, and the available studies show inconsistent results [33, 42–44].

We have already shown in our study population that adapting prenatal iron supplementation (80, 40, and 20 mg) to early pregnancy iron levels (normal, Hb 110–130 g/L, or normal-high Hb > 130 g/L) in non-anaemic women, prevents iron imbalances during pregnancy, both due to iron deficiency and excess [39]. Specifically, we found that in women at risk of iron deficiency (those with normal Hb levels at the beginning of pregnancy), a daily dose of 80 mg of iron reduced the risk of iron deficiency, compared to women in the control group receiving 40 mg iron daily, without increasing the risk of excess of iron that can be caused by supplementation with high doses. Similarly, in women at risk of haemoconcentration (those with basal normal-high Hb levels), the tested iron dose of 20 mg daily prevented haemoconcentration compared to the control group, without causing iron deficiency. As a step forward, the present study aimed to evaluate the association between different prenatal doses of iron and the child's neurodevelopment under the hypothesis that having corrected the women's iron status by adapting prenatal iron supplementation to their individual needs would also have beneficial effects on the child's neurodevelopment.

Methods

Study design and data collection

The present work is the follow-up of the ECLIPSES study, a community-based RCT that aimed to assess the effectiveness of different doses of prenatal iron supplementation on the maternal iron status at the end of gestation, and now, on the child's neurodevelopment. The ECLIPSES study was conducted in the province of Tarragona (Catalonia, Spain) on women recruited before gestational week 12 between 2013–2017 and allocated into two groups according to their Hb levels aiming to prevent an iron deficit in women from the *Stratum* 1 (initial Hb = 110–130 g/L) and risk of developing iron excess in those from the *Stratum* 2 (initial Hb > 130 g/L). We used Hb levels as a screening biomarker because they are usually related to iron stores and *HFE* gene mutations [34, 40]. Women in *Stratum* 1 were randomly prescribed a daily dose of 40 or 80 mg of iron supplements, while those in *Stratum* 2 received 40 or 20 mg of iron daily (Fig. 1). Since previous literature has already made it clear that prenatal iron supplementation is recommended and leads to better pregnancy outcomes than non-supplementation, we did not include a control group of non-supplemented women. Otherwise, women in each *Strata* receiving 40 mg/day, which is the commonly prescribed dose of iron, were considered the control group against which the higher and lower iron dose interventions were performed. The prescription was triple-blinded, so neither

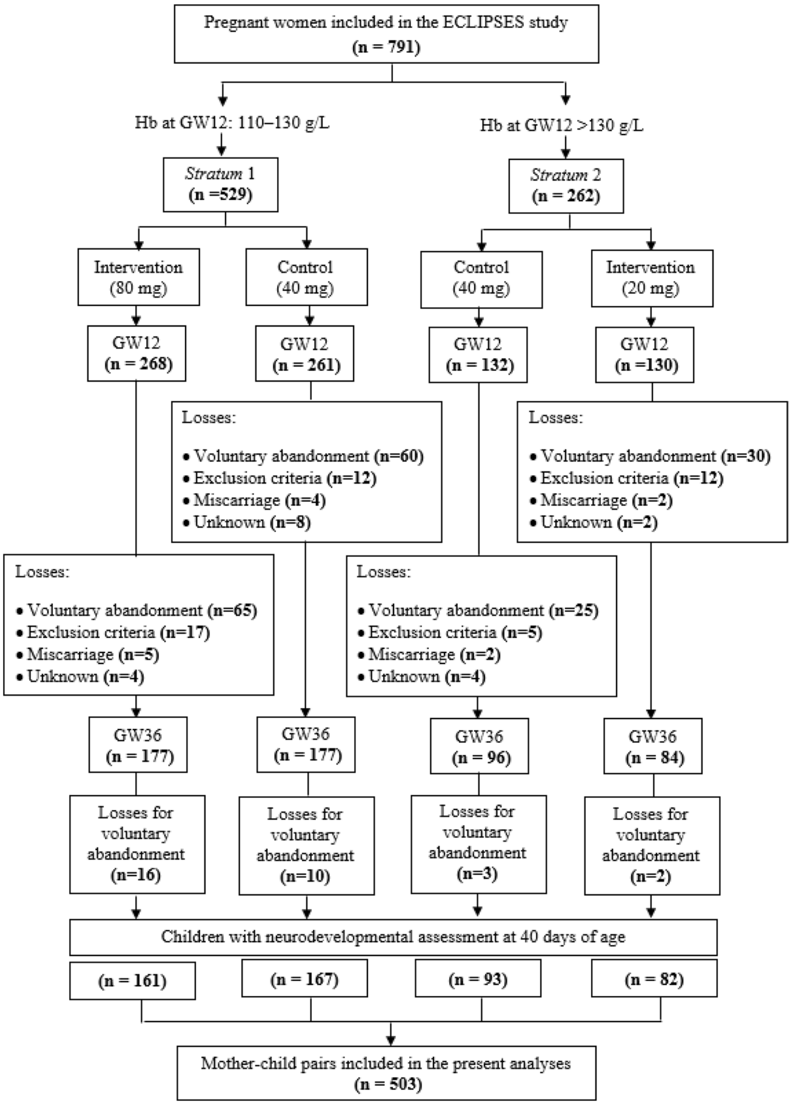

Fig. 1 Flowchart of the study. Hb, haemoglobin; GW, gestational week

the supplement providers, the health workers, nor the researchers knew what dose of iron each woman received until the end of the study. The women were instructed to take one pill a day. At the next study visit, they were to return any leftover pills to assess compliance. This was done by comparing the number of leftover pills with the participants' self-reported compliance. Compliance was considered good when women had forgotten to take the supplement less than twice a week while adherence was considered low when they had forgotten two or more times a week at any of the study visits.

The ECLIPSES study was registered at www.clinicaltrialsregister.eu as EudraCT number 2012–005,480-28 and its methodological details can be found extended elsewhere [39, 45].

Women were visited in the first, second, and third trimesters of pregnancy, and on average at 40 days post-partum. A summary of maternal information recorded by midwives from questionnaires was as follows:

- Clinical and obstetrical history: maternal age, parity, pregnancy planning.
- Anthropometric measurements: weight and height. Body mass index (BMI, kg/m^2) was calculated.
- Dietary assessment: self-administered food frequency questionnaire (FFQ) previously validated in our population [46]. Participants reported usual food consumption retrospectively at weeks 12, 24, and 36 of pregnancy and 40 days post-partum. The FFQ consisted of 45 items classified into 12 food groups: 1–read and processed meat, 2.–poultry, fish, and eggs, 3.–fruits, 4.–vegetables, 5.–dairy products, 6.–salted cereals (breakfast cereals, bread, pasta, and rice), 7.–sweet cereals (biscuits, pastries), 8.–legumes, 9.–nuts, 10.–sweets, 11.–sweetened beverages, 12.–alcoholic drinks. The FFQ data were reviewed and analysed by trained nutritionists, who calculated the intake of each food group in grams/day. Additionally, the women's degree of adherence to the Mediterranean diet was calculated using an rMED score based on the intake of 9 components of this diet. Each rMED component (apart from alcohol) was expressed in grams per 1000 kcal/day (to express intake as energy density) and was divided by terciles of dietary intake. Each tercile was assigned a value of 0, 1, and 2 points. Out of the 9 components of the rMED, 6 of them (fruit, vegetables, legumes, cereals, fresh fish and seafood, and olive oil) scored positively, while 2 scored negatively (total and processed meat, dairy products). Alcohol was scored as a dichotomous variable (0 for women who consumed alcohol, and 2 for women who did not drink alcohol). The score assigned to each pregnant woman thus ranged from 0 points indicating minimum adherence to 18 points indicating maximum adherence to the Mediterranean diet. The total rMED score was classified into three categories: 0–6 it was considered as "low", 7–10 as "medium", and 11–18 as "high". Extended information can be found in Jardí et al. [47].
- Lifestyle at the time of recruitment: use of prenatal supplements other than iron, smoking habit (using the Fagerström test [48]), and physical activity (using the short form of the International Physical Activity Questionnaire [IPAQ] [49]). The IPAQ assesses physical activity considering the following domains: leisure time, domestic activities, work-related physical activity, and transport-related physical activity. Within these domains, the IPAQ short form focus on walking, moderate-intensity activities, and vigorous-intensity activities. Total scores are computed based on the duration (in minutes) and frequency (in days) of each type of activity. The IPAQ offers the specific algorithms to obtain the classification of "low", "moderate", and "high" physical activity by combining the duration and frequency of different types of activities.Sociodemographic characteristics: ethnic origin, familiar socioeconomic status (SES) calculated from educational level and occupational status both from participants and their partners.
- Maternal anxiety status: State-Trait Anxiety Inventory (STAI) [50]. The STAI test assessed two separate concepts of anxiety, each with 20 items. On one hand, anxiety as a state assesses a transient emotional state, characterised by subjective, consciously perceived feelings of alertness and apprehension and by hyperactivity of the autonomic nervous system. On the other hand, anxiety as a trait indicates a relatively stable anxious propensity that characterises individuals with a tendency to perceive situations as threatening.Post-partum depression: Edinburgh Postnatal Depression Scale (EPDS) [51].

Blood samples were collected in 2 tubes of 7.5 ml, one containing EDTA as anticoagulant and the other without anticoagulant. The samples were transported to the BioBank for immediate analyses. Before processing, the samples in the EDTA tubes were inversion-mixed 10 times to ensure that the blood was mixed, then centrifuged at 4°C to separate plasma. The tube without anticoagulant was left without mixing for 30 minutes at room temperature to enable coagulation, then the serum was separated also by centrifugation. All the samples were stored at -80°C. DNA was extracted and stored as well at -80°C for subsequent genetic analyses. The stored samples in the BioBank were thawed at the end of the clinical study and processed simultaneously to minimize inter-batch variation. Biochemical determinations of Hb, serum ferritin (SF) and cortisol were done by immunochemiluminescence at each trimester of pregnancy. The serum concentration of C-reactive protein (CRP) was measured by immunoturbidimetry also at each trimester of pregnancy. Plasma polyunsaturated fatty acids (PUFA) concentrations were analysed by using a combination of gas chromatography–mass spectrometry (GC-MS) after their derivatization to methyl ester (FAMEs) due to their higher volatility. [52] Detailed information on laboratory procedures for PUFA measurements can be found in Aparicio et al. [53] Serum concentrations of vitamin D were quantified by an automated chemiluminescent immunoassay method as described in Díaz-López et al. [54] The rationale for including serum concentrations of vitamin D and fatty acids in the analyses is that these components are involved in brain development, so maternal levels during pregnancy may influence foetal neurodevelopment. [55–57] Folate and vitamin B_{12} measurements were also done by using a chemiluminescence immunoassay only in the first trimester of gestation. Then, RBC folate concentration was calculated as follows: (serum folate in haemolysed whole blood * dilution factor in haemolysis * 100)/haematocrit. [58] Genetic analyses to detect mutations in the *HFE* gene were done using polymerase chain reaction (PCR) and digestion with specific enzymes.

As for the infants' information, the following data were recorded at birth: sex, gestational age (calculated based on the time elapsed since the first day of the last self-reported menstrual period), Apgar test score, type of feeding, and anthropometric measurements including length, weight, and head circumference. At 40 days of age, children were visited again and information about the type of feeding as well as weight and height measurements were recorded that time.

Outcome

The individualized assessment of the child neurodevelopment was performed by two trained psychologists in the facilities of the health care centre participating in the study at the average age of 40 days using The Bayley Scales of Infant Development, 3rd edition (BSID-III) [59]. This test consists of three general scales (cognitive, language, and motor) obtaining a standardized IQ score (mean of 100 and a standard deviation of 15) and four subscales (expressive language, receptive language, fine motor, and gross motor) obtaining a standardized scalar score (mean of 10 and a standard deviation of 3). Higher scores represent better development.Continuous BSID-III scores for general scales were categorized as follows according to the test rates: low (scores < 85), middle (scores ≥ 85–115), and high (scores ≥ 115). Similarly, the classification was as follows for subscales: low (scores < 7), middle (scores ≥ 7–13), and high (scores ≥ 13). Given the low number of children in the "high" category in cognitive and language scales, they were merged with those in the "middle" category when performing logistic regression analysis. In the case of the motor scale, children in the "low" category were merged with those in the "middle" category for the same reason.

Statistical analyses

The analyses were considered as *per protocol* analyses and were stratified according to the design by Hb concentration category at baseline.. Bivariate analyses to describe the variables of interest were performed using the conventional statistical techniques: Student T and ANOVA tests for continuous variables using mean and standard deviation (SD) and Chi-square test of percentages for categorical ones. Natural logarithm (Ln) transformation was applied to normalize the distribution of SF, increasing the validity of analyses.

Linear and logistic regression models were used to assess the effect of different doses of prenatal iron supplementation (*Stratum* 1: 80 mg vs control, *Stratum* 2: 20 mg vs control) on the child's neurodevelopment. Crude and adjusted estimates are shown. Based on previous knowledge, the models were adjusted for those maternal and child variables that could affect the studied relationship as follows: maternal age at recruitment, parity (yes or no), pregnancy planning (yes or no), familiar socioeconomic status (low, middle, high), smoking at recruitment (yes or no), baseline maternal BMI (normal weight, overweight, obesity), gestational weight gain, maternal anxiety during pregnancy, post-partum depression, serum levels of Hb, ferritin, vitamin D and polyunsaturated fatty acids at the first and third trimester of pregnancy, serum levels of RBC folate and vitamin B_{12} at the first trimester of pregnancy, physical activity during pregnancy (low, moderate, high), adherence to the Mediterranean diet (low, middle, high) and daily energy intake at the first trimester of pregnancy, child's age at assessment, sex, gestational age, Apgar test scores (< 7 or ≥ 7 points), and head circumference at birth.

The statistical analyses were done using the SPSS software (version 27.0 for Windows; SPSS Inc., Chicago, IL, USA).

Ethical approval

The study was designed in agreement with the Declaration of Helsinki/Tokyo. All procedures involving human subjects were approved by the Clinical Research Ethics Committee of the Jordi Gol University Institute for Primary Care Research [Institut d'Investigació en Atenció Primària; IDIAP], the Pere Virgili Health Research Institute [Institut d'Investigació Sanitària Pere Virgili; IISPV] and of the Spanish Agency for Medicines and Medical Devices [Agencia Española del Medicamento y Productos Sanitarios; AEMPS]. Signed informed consent was obtained from all women participating in the study.

The quality of the present community-based RCT has been assessed by the Consolidated Standards of Reporting Trials (CONSORT).

Results

The current analyses consisted of 503 mother–child pairs from the ECLIPSES study, from which data on child neurodevelopment assessment at 40 days of age was available. Table 1 summarizes the maternal baseline characteristics, being remarkable that the median ± interquartile range maternal age was 31 ± 7 years, that near

Table 1 Maternal characteristics

	***Stratum* 1 (Hb 110–130 g/L)**		***Stratum* 2 (Hb > 130 g/L)**	
	Intervention (80 mg/d) (*n* = 161)	**Control (40 mg/d) (*n* = 167)**	**Intervention (20 mg/d) (*n* = 82)**	**Control (40 mg/d) (*n* = 93)**
Baseline				
Age, years	31 ± 7	31 ± 7	31 ± 7	32 ± 7
Parity, yes	55.9 [90]	58.7 [98]	47.6 [39]	54.8 [51]
Pregnancy planning, yes	78.9 [127]	81.4 [136]	86.6 [71]	80.6 [75]
Body mass index				
Underweight	1.2 [2]	1.2 [2]	2.4 [2]	2.2 [2]
Normal weight	57.8 [93]	64.1 [107]	62.2 [51]	50.5 [47]
Overweight	28.6 [46]	21.6 [36]	19.5 [16]	33.3 [31]
Obesity	12.4 [20]	13.2 [22]	15.9 [13]	14.0 [13]
Smoking, yes	15.6 [25]	12.1 [21]	15.9 [13]	15.1 [14]
Familiar socioeconomical status[a]				
High	16.8 [27]	22.2 [37]	15.9 [13]	20.4 [19]
Middle	65.8 [106]	67.7 [113]	68.3 [56]	71.0 [66]
Low	17.4 [28]	10.2 [17]	15.9 [13]	8.6 [8]
Ethnicity				
White	79.5 [128]	79.6 [133]	86.6 [71]	69.9 [65]
Asian	0.6 [1]	0 [0]	0 [0]	2.2 [2]
Black	2.5 [4]	3.0 [5]	1.2 [1]	0.0 [0]
Arab	6.8 [11]	4.2 [7]	2.4 [2]	8.6 [8]
Latin American	8.7 [14]	10.8 [18]	8.5 [7]	12.9 [12]
Whole pregnancy				
Adherence to the Mediterranean diet[a]				
Low-Middle	64.0 [103]	59.9 [100]	69.5 [57]	67.7 [63]
High	36.0 [58]	40.1 [67]	30.5 [25]	32.3 [30]
Physical activity[a]				
Low	14.9 [24]	18.0 [30]	18.3 [15]	26.9 [25]
Moderate	55.3 [89]	52.1 [87]	45.1 [37]	36.6 [34]
High	18.0 [29]	12.6 [21]	17.1 [14]	24.7 [23]
Anxiety assessment[b]				
Trait	18.04 (9.30)	15.19 (8.06)	16.82 (9.85)	14.43 (8.03)
State	18.07 (7.77)	15.41 (6.79)	16.99 (8.03)	15.84 (7.21)
After delivery				
Post-partum depression[c]	7.58 (5.22)	6.67 (4.78)	6.50 (5.46)	6.44 (4.36)

Data are expressed in mean (SD) for continuous normally distributed variables, median ± interquartile range for continuous non-normally distributed variables, and % [n] for categorical variables

[a] For an explanation of how categories were defined see the Methods section

[b] Measured by STAI questionnaire (score range: 0 to 60 points). Trait means a relatively stable, anxious propensity that characterises individuals with a tendency to perceive situations as threatening. State means a transient emotional state, characterised by subjective, consciously perceived feelings of attention and apprehension and by hyperactivity of the autonomic nervous system

[c] Measured by Edinburg questionnaire (score range: 0 to 30 points)

of 40% were overweight or obese 14.7% were smokers at the conceptional time, and most of them were White, had low or middle SES, and had planned the pregnancy. We also found that more than half of women (61.2%) showed low-middle adherence to the Mediterranean diet and moderate physical activity during pregnancy (66.9%). There was a high compliance to the intervention throughout pregnancy (around 94%). No association was found regarding sociodemographic characteristics and lifestyle between participants whose data were included or not included in the present analyses (Supplementary Table 1).

Maternal concentrations of iron-related biomarkers in the first and third trimesters are shown in Supplementary Table 2. As for the maternal iron status, since anaemia was an exclusion criterion for the recruitment and haemoconcentration is a condition associated with late pregnancy, only data for the third trimester of gestation are shown. The participants showed a low prevalence of iron-deficiency anaemia in all the iron groups (1 to 5%), without any association among them. As for haemoconcentration, the overall prevalence was 13.7% with the higher percentage being shown by women in *Stratum* 2. Additional information on maternal levels of vitamin B_{12} and RBC folate at the beginning of pregnancy are also shown in Supplementary Table 2.

The child's characteristics according to the intervention group are depicted in Table 2. The results showed that 49.5% of the participating children were girls, had a mean gestational age of 39.7 weeks, and a median age of 47 ± 14 days when neurodevelopment was assessed. Regarding the BSID-III scores, they were normally distributed in our study population and the mean scores obtained for all the children were in the normal ranges at each scale when they were analysed as a continuous variable. However, when the scores were categorized considering the clinical cut-off point for normality (85 or 7 points for main scales and subscales, respectively), a small percentage of children were under the normality for language development (8.1%, $n = 45$) and, specifically, for expressive language development (11.9%, $n = 65$). No association was found in the BSID-III scores or in the percentage of children below the cut-off point considered normal, between the different doses of iron in any *Strata*.

In multiple linear regression analysis, no association was found between prenatally prescribed iron doses in *Stratum* 1 or 2 about BSID-III scores on any of the developmental scales in children at 40 days of age, including cognitive, language, and motor development (Table 3, Fig. 2). Neither association was found between the doses of iron in any of the groups in the logistic regression analysis on the chances of moving from low to medium–high mental and language development, and from low-medium to high motor development (Table 3, Fig. 2).

Table 2 Characteristics of children according to the dose of maternal iron supplementation during pregnancy

	***Stratum* 1 (Hb 110–130 g/L)**		***Stratum* 2 (Hb > 130 g/L)**	
	Intervention (80 mg/d) (*n* = 161)	**Control (40 mg/d) (*n* = 167)**	**Intervention (20 mg/d) (*n* = 82)**	**Control (40 mg/d) (*n* = 93)**
Age at assessment, days	47 ± 15	47 ± 13	47 ± 14	47 ± 14
Sex, girl	51.1 [81]	51.6 [83]	45.9 [37]	45.5 [44]
Gestational age, weeks	39.62 (1.51)	39.74 (1.38)	39.61 (1.50)	39.85 (1.30)
Apgar test score ≥ 7 points, %	98.8 [159]	100 [167]	100 [82]	98.9 [92]
Breastfeeding, yes				
At birth	63.9 [98]	68.3 [114]	63.1 [50]	63.6 [60]
At assessment	54.4 [87]	63.5 [106]	51.2 [41]	56.5 [52]
Neurodevelopment				
Cognitive development[a]	100.81 (8.86)	101.79 (8.55)	101.28 (9.16)	103.28 (8.95)
Score < 85, %	3.1 [5]	2.4 [4]	3.7 [3]	1.1 [1]
Language development[a]	96.44 (8.34)	95.92 (8.61)	95.04 (7.35)	97.34 (8.23)
Score < 85, %	8.0 [13]	10.1 [17]	8.5 [7]	4.3 [4]
Expressive language[b]	8.04 (1.39)	8.16 (1.65)	7.88 (1.41)	8.09 (1.78)
Score < 7, %	9.3 [14]	12.5 [20]	11.0 [9]	16.1 [15]
Receptive language[b]	10.71 (2.12)	10.41 (2.17)	10.43 (2.04)	10.98 (2.04)
Score < 7, %	4.3 [7]	4.2 [7]	2.4 [2]	4.3 [4]
Motor development[a]	107.27 (12.74)	107.83 (10.27)	107.16 (10.02)	108.08 (11.35)
Score < 85, %	3.1 [5]	2.4 [4]	2.4 [2]	4.3 [4]
Fine motor[b]	11.46 (1.98)	11.53 (1.85)	11.39 (1.92)	11.43 (2.09)
Score < 7, %	0.6 [1]	0.6 [1]	1.2 [1]	2.2 [2]
Gross motor[b]	11.12 (2.34)	11.00 (2.26)	11.01 (2.34)	11.19 (2.46)
Score < 7, %	0 [0]	0.6 [1]	0 [0]	0 [0]

Data are expressed in median ± interquartile range, mean (SD) and % [n]

[a] The normal score range for BSID-III was 85–115

[b] The normal score range for BSID-III was 7–13

Table 3 Effect of iron supplementation on the neurodevelopment of children at around 40 days of life

	***Stratum* 1 (0: 80 mg/d, 1: 40 mg/d)**		***Stratum* 2 (0: 40 mg/d, 1: 20 mg/d)**	
	β (95%CI)	**OR (95%CI) ᵃ**	**β (95%CI)**	**OR (95%CI) ᵃ**
Cognitive development				
Crude model	0.98 (-0.82, 2.87)	1.31 (0.34, 4.95)	-2.00 (-4.71, 0.70)	0.29 (0.03, 2.81)
Adjusted model	1.46 (-2.15, 5.07)	0.68 (0.09, 5.29)	-4.04 (-7.27, 0.80)	0.85 (0.26, 2.68)
Language development				
Crude model	-0.53 (-2.37, 1.32)	0.78 (0.36, 1.65)	-2.31 (-4.65, 0.03)	0.48 (0.14, 1.71)
Adjusted model	1.30 (-1.99, 4.59)	0.54 (0.14, 2.05)	-0.36 (-3.47, 2.75)	1.35 (0.65, 5.79)
Receptive language				
Crude model	0.30 (-0.77, 0.17)	1.04 (0.36, 3.03)	-0.55 (-1.16, 0.06)	1.80 (0.32, 10.08)
Adjusted model	-0.14 (-1.04, 0.75)	0.72 (0.10, 5.35)	-0.52 (-1.32, 0.28)	0.94 (0.32, 2.68)
Expressive language				
Crude model	0.12 (-0.22, 0.45)	0.70 (0.34, 1.44)	-0.21 (-0.69, 0.27)	1.56 (0.64, 3.78)
Adjusted model	0.44 (-0.19, 1.06)	1.30 (0.30, 5.61)	0.18 (-0.36, 0.72)	2.64 (0.48, 14.58)
Motor development				
Crude model	0.56 (-1.96, 3.08)	1.03 (0.60, 1.78)	-0.92 (-4.13, 2.30)	0.68 (0.33, 1.42)
Adjusted model	2.04 (-3.88, 7.96)	1.19 (0.45, 3.15)	-3.76 (-9.30, 1.78)	0.91 (0.30, 2.73)
Fine motor				
Crude model	0.07 (-0.34, 0.49)	0.78 (0.41, 1.47)	-0.04 (-0.64, 0.56)	1.16 (0.47, 2.82)
Adjusted model	0.23 (-0.58, 1.03)	0.43 (0.13, 1.43)	-0.24 (-1.29, 0.81)	0.97 (0.26, 3.61)
Gross motor				
Crude model	-0.12 (-0.62, 0.38)	0.73 (0.40, 1.32)	-0.18 (-0.90, 0.53)	0.71 (0.32, 1.59)
Adjusted model	0.32 (-0.65, 1.30)	1.03 (0.34, 3.11)	-0.13 (-0.66, 0.41)	0.84 (0.31, 8.65)

Doses of iron: *Stratum* 1 (80 vs 40 mg/d) and *Stratum* 2 (40 vs 20 mg/d)

Models adjusted for maternal age at recruitment, parity, pregnancy planning, familiar socioeconomic status, smoking at recruitment, baseline maternal body mass index, gestational weight gain, maternal anxiety during pregnancy, postpartum depression, serum biomarker levels at the first and third trimester of pregnancy (haemoglobin, ferritin, vitamin D and polyunsaturated fatty acids), serum biomarker levels at the first trimester of pregnancy (red blood cell folate and vitamin $B_{12)}$, physical activity during pregnancy, adherence to Mediterranean diet and daily energy intake at the first trimester of pregnancy, child's age at assessment, child's sex, gestational age, Apgar test scores, head circumference at birth, and type of feeding at birth and assessment

[a] Odds ratios express the chance to go from low to middle-high cognitive development and language development, and from low-middle to high motor development

Discussion

The need for preventive prenatal iron supplementation to avoid iron deficiency is well known, although favourable results on the child's development have not always been observed. Routine preventive supplementation with standard doses of iron for all women could probably be insufficient for those who start pregnancy with low iron levels and too much for those with high initial iron concentrations. Thus, iron supplementation adapted to maternal needs would prevent both extremes and, in turn, improve child outcomes. This community-based study found that adapting prenatal iron supplementation in non-anaemic women to their individual needs to prevent iron deficit and excess, led to similar results in their children's cognitive, language, and motor development in all iron supplementation groups (daily 20 mg, 80 mg, and control group of 40 mg), resulting in a lack of association between the tested doses and the control group in each *Strata* for child's neurodevelopment.

Little research assessing the effect of prenatal iron supplementation on the child's neurodevelopment has been conducted on well-nourished non-anaemic women, and studies until now only evaluated the effect of taking or not prenatal iron supplements, without considering different doses according to the individual needs of each woman. Results from the AMBIT study [26, 27], conducted in non-anaemic Australian pregnant, indicated no effects of prenatal iron supplementation (20 mg daily) compared to placebo on the offspring's intelligence and behavioural skills at 4 and 6–8 years of age. Some years later, a study conducted in 9-months-old Chinese children assessing the effectiveness of antenatal (60 mg daily vs placebo) and infant iron supplementation found an improvement in the child's gross motor development, although it was not due to iron supplementation during pregnancy, but during infancy [28]. Despite the tested doses in these studies being around the usual doses in routine

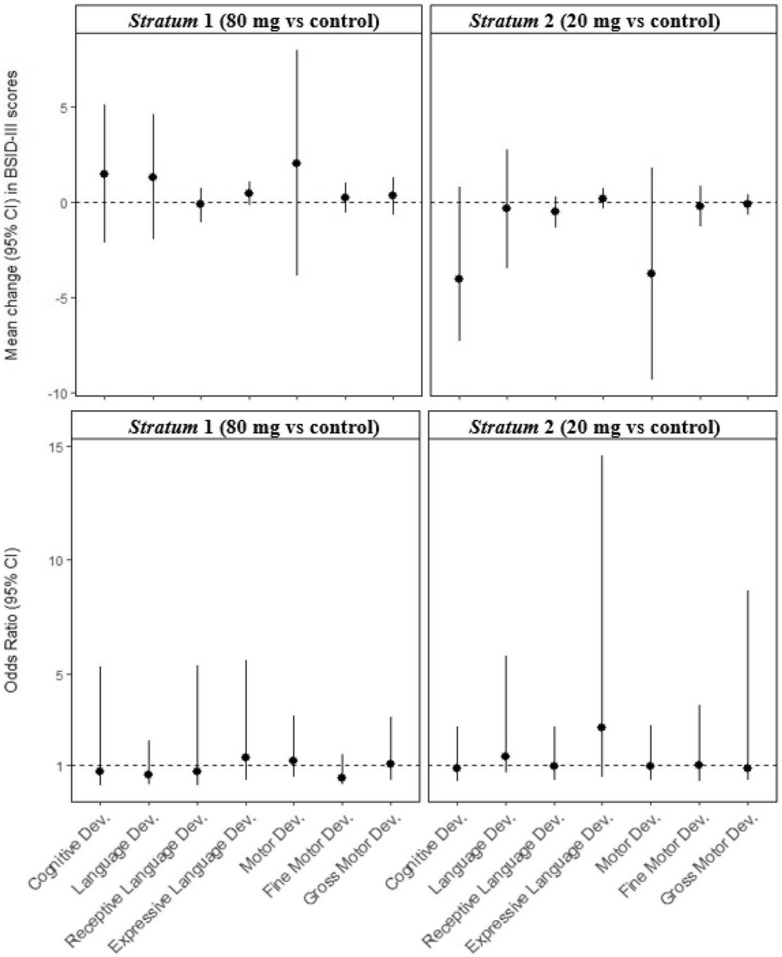

Fig. 2 Effect of iron supplementation on the neurodevelopment of children at around 40 days of life. The control group in each *Stratum* were women who received the commonly prescribed dose of 40 mg/d of iron. Models adjusted for maternal age at recruitment, parity, pregnancy planning, familiar socioeconomic status, smoking at recruitment, baseline maternal body mass index, gestational weight gain, maternal anxiety during pregnancy, postpartum depression, serum biomarker levels at the first and third trimester of pregnancy (haemoglobin, ferritin, vitamin D and polyunsaturated fatty acids), serum biomarker levels at the first trimester of pregnancy (red blood cell folate and vitamin $B_{12)}$, physical activity during pregnancy, adherence to Mediterranean diet and daily energy intake at the first trimester of pregnancy, child's age at assessment, child's sex, gestational age, Apgar test scores, head circumference at birth, and type of feeding at birth and assessment. Odds Ratios express the chance to go from low to middle-high cognitive development and language development, and from low-middle to high motor development

supplementation, considering that the participating women were not anaemic, they could have been inadequate depending on their iron stores and other factors. In this regard, women with homozygous mutations in the *HFE* gene, which increase iron absorption, may be at risk of iron overload if they receive iron supplements [29–31]. Despite the low prevalence of homozygosis for *HFE* gene mutations in our study population (only 4.8% for H63D genotype), the prevalence of having any *HFE* gene mutation is around 46% in the Mediterranean population [34, 60], which turn it into an important risk factor to consider when prescribing iron supplementation. There are some indications from observational studies that not only iron deficiency but also iron excess negatively affects the child's neurodevelopment [18, 32, 33, 35, 36] and, based on that, some main researchers in this field have proposed to adapt the prenatal iron supplementation to the individual's requirements to mitigate the potential damage from any maternal iron imbalance [22, 23, 25, 41]. However, few studies have tested this hypothesis, obtaining inconsistent results [37, 61]. We observed that our intervention successfully corrected maternal iron status when compared with the estimates of the prevalence of anaemia and haemoconcentration during pregnancy. While the prevalence of anaemia in Europe is around 25% in pregnant women [19, 62, 63], we found that only 3.3–5% of participants at risk developed it at the end of pregnancy. On the other hand, despite the estimates indicating that up to 42% of women suffer from haemoconcentration in industrialized countries [25, 64], we observed a prevalence of 15.6–25.6% among participants at risk of iron excess. We believe, therefore, that having provided the most appropriate amount of iron for each woman helping them to reach an optimal iron status in most cases has been the physiological mechanism underlying the lack of a remarkable association between different prenatal iron doses and child's neurodevelopment. We found that only 2.6%, 8.1%, and 3% of children obtained scores below the normal range for cognitive, language, and motor development scales, respectively. The high heterogeneity among the epidemiological studies assessing the effect of prenatal iron supplementation on the child's neurodevelopment makes it difficult to compare our findings. Nonetheless, some evidence from observational studies indicate that failure to prevent women from suffering from both iron deficiency and excess in pregnancy results in neurodevelopmental impairment in children. That is the case of two Spanish studies [11, 18] and others from Vietnam [65] and China [66] from which the authors concluded that iron deficiency or anaemia in late pregnancy, compared with having a correct iron status, may be associated with lower motor scores in young children. Similarly, maternal iron deficiency during pregnancy can result in poorer cognitive and language abilities in children, according to some of those studies [18, 67]. But, as previously discussed, that prenatal iron excess could entails harmful consequences for child neurodevelopment has also been stated in the literature, especially associated with cognitive function in this case [32, 36, 68]. And, going further, a couple of studies made sense of the present work, showing in their study population that the association between maternal iron status and child neurodevelopment is sometimes inverted U-shaped [69, 70]. Considering the available evidence, our results suggest that preventive prenatal supplementation with different doses of iron as long as they are in a range appropriate to each woman's needs, i.e. adapted to their initial Hb levels, lead to similar neurodevelopmental outcomes in infants at birth. The main strengths of the present work were the study design, which was a community-based triple-blinded community-based RCT and the extensive data collection regarding sociodemographic conditions, clinical and lifestyle information from both mothers and children. Also, It should be noted that women in our study were non-anaemic when they were recruited in the first trimester of pregnancy and started iron supplementation. This does not mean that they cannot become anaemic during pregnancy. However, by adjusting for maternal serum ferritin and Hb concentrations in both the first and third trimesters of pregnancy, we were able to rule out the possible effect of maternal iron status and estimate the true effect of prenatal iron supplementation on the child's neurodevelopment. However, some limitations should also be considered. First, despite being common in community-based intervention studies implying several visits and a long follow-up, the substantial drop-out that occurred could have reduced the statistical precision and may have led to biased estimates of intervention effects. Second, although the BSID-III is an internationally used and recognised tool for assessing the child's cognitive function, the neurodevelopmental assessment shows low stability in early childhood [71], which could have led to estimates of nullity. And finally, residual confounding, due to unmeasured or unknown risk factors that may occur even after adjustment for known potential confounders, could have been a limitation when interpreting our findings.

Conclusions

Our findings suggest that in non-anaemic women at the start of pregnancy, there were no differences in the cognitive, language and motor development of children at 40 days of age between the dose of iron tested in each case (80 or 20 mg/d) –adjusted to initial Hb levels– compared to the dose of the control group (40 mg/d), which is the dose commonly used in clinical practice in SpainThe research in this regard is still scarce and further studies are guaranteed to better understand the possible effects of different types of prenatal iron supplementation on the child's neurodevelopment, including the follow-up at older ages.

Abbreviations

RCT: Randomized clinical trial; Hb: Haemoglobin; BMI: Body mass index; FFQ: Food frequency questionnaire; SES: Socioeconomic status; STAI: State-Trait Anxiety Inventory; EPDS: Edinburgh Postnatal Depression Scale; SF: Serum ferritin; RBC: Red blood cell; BSID: Bayley Scales of Infant Development; SD: Standard deviation; Ln: Natural logarithm.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12884-022-05033-y.

Additional file 1. Supplementary Table 1. Maternal characteristics of participants included and non-included in theanalyses.

Additional file 2. Supplementary Table 2. Maternal concentrations of iron-related biomarkers, vitamin B12, and RBC folate.

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Authors' contributions
VA conceived and designed the work, obtained funding and resources, participated in the research, as well as in the administration and supervision of the project. JC contributed to the conception and design of the study and participated in the research and supervision of the work. PC and MG provided the iron supplements for the intervention and participated in the research. CH-M and NV conducted the neurodevelopmental assessment of the child and participated in the research process. LI-V performed the formal analyses, contributed to the research and data visualization, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was designed in agreement with the Declaration of Helsinki/Tokyo. All procedures involving human subjects were approved by the Clinical Research Ethics Committee of the Jordi Gol University Institute for Primary Care Research [Institut d'Investigació en Atenció Primària; IDIAP], the Pere Virgili Health Research Institute [Institut d'Investigació Sanitària Pere Virgili; IISPV] and of the Spanish Agency for Medicines and Medical Devices [Agencia Española del Medicamento y Productos Sanitarios; AEMPS]. Signed informed consent was obtained from all women participating in the study.

Consent for publication
Not applicable.

Competing interests
The authors state that they have no conflict of interest to declare.

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Importance of maternal iron status on the improvement of cognitive function in children after prenatal iron supplementation

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RESEARCH ARTICLE

Importance of Maternal Iron Status on the Improvement of Cognitive Function in Children After Prenatal Iron Supplementation

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Introduction: The effectiveness of prenatal iron supplementation improves maternal hematological outcomes, but little research has focused on child outcomes. The objective of this study was to assess whether prenatal iron supplementation adjusted to maternal needs improves children's cognitive functioning.

Methods: The analyses included a subsample of nonanemic pregnant women recruited in early pregnancy and their children aged 4 years (*n*=295). Data were collected between 2013 and 2017 in Tarragona (Spain). On the basis of hemoglobin levels before the 12th gestational week, women receive different iron doses: 80 vs 40 mg/d if hemoglobin is 110−130 g/L and 20 vs 40 mg/d if hemoglobin >130 g/L. Children's cognitive functioning was assessed using the Wechsler Preschool and Primary Scale of Intelligence-IV and Developmental Neuropsychological Assessment-II tests. The analyses were carried out in 2022 after the completion of the study. Multivariate regression models were performed for assessing the association between different doses of prenatal iron supplementation and children's cognitive functioning.

Results: Taking 80 mg/d of iron was positively associated with all the scales of the Wechsler Preschool and Primary Scale of Intelligence-IV and Neuropsychological Assessment-II when mothers had initial serum ferritin <15 μg/L, but it was negatively associated with Verbal Comprehension Index, Working Memory Index, Processing Speed Index, and Vocabulary Acquisition Index from Wechsler Preschool and Primary Scale of Intelligence-IV and verbal fluency index from Neuropsychological Assessment-II when mothers showed initial serum ferritin >65 μg/L. In the other group, taking 20 mg/d of iron was positively associated with Working Memory Index, Intelligence Quotient, verbal fluency, and emotion recognition indices when women had initial serum ferritin >65 μg/L.

Conclusions: Prenatal iron supplementation adjusted to the maternal hemoglobin levels and baseline iron stores improves cognitive functioning in children aged 4 years.

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INTRODUCTION

Cognitive development is based on brain formation and maturation, highly sensitive processes that begin at the fetal stage.[1] The prenatal environment, particularly maternal nutritional status, plays a key role in fetal brain development.[2] Maternal iron status during pregnancy is a major public health concern because it can have an impact on maternal and child health in many ways, including child neurodevelopment.[3] Several animal and human studies have shown that both iron deficiency (ID) and excess can impair cognitive abilities in the short and long term.[4–7] Whereas perinatal ID has been associated with alterations in brain energy and dopamine metabolism[8] and myelination in various brain regions,[9,10] iron excess can be toxic by forming deposits that lead to cellular damage, although there are still significant knowledge gaps.[11]

Prenatal iron supplementation has been successful in improving maternal iron status in late pregnancy, but it has not yet shown clear benefits for children's cognitive development.[12] Studies conducted to date have examined a single dose of iron compared with a placebo[13,14] or in combination with other micronutrients.[15–17] However, because several factors are related to maternal iron status and because women's iron requirements may vary accordingly,[18–20] some experts have long advocated adjusting prenatal iron supplementation to the needs of individual women, considering both their iron stores and other conditions.[21] Indeed, in the previously published results of the ECLIPSES study, it was found that adjusting prenatal iron supplementation in nonanemic women to their initial iron status was a good strategy to prevent ID and iron excess in women who are at risk.[22] However, in clinical practice, the same dose is still usually prescribed to all women, leaving many women at risk of iron imbalance. This can not only harm the mother's health but can also have lasting consequences on the baby's health. This study aimed to investigate whether the benefits of adjusting prenatal iron supplementation to mothers' needs previously observed in their iron status at the end of pregnancy extend to children's cognitive functions.

METHODS

Study Sample

The ECLIPSES study is a population-based RCT conducted in 2013–2017 in Tarragona, Spain, that aimed to evaluate the effectiveness of prescribing different doses of prenatal iron supplementation to nonanemic women in early pregnancy on their iron status at the end of gestation. Participating women were recruited before the 12th gestational week and allocated into two groups according to their hemoglobin (Hb) levels. Women in Stratum 1 (initial Hb=110–130 g/L) were randomly assigned to receive a daily dose of 40 mg or 80 mg of iron aiming to prevent an ID, whereas those in Stratum 2 (initial Hb>130 g/L) received 20 mg or 40 mg of iron daily aiming to prevent the risk of developing iron excess (Figure 1). Because the usually prescribed dose of iron is 40 mg daily, women in each stratum who received this dose were considered the control group on which the higher and lower iron doses were tested. The intervention was triple blinded, meaning that neither the researchers, the supplement providers, nor healthcare workers knew the dose of each woman's iron supplement until the end of the study. The adherence to the intervention was determined by comparing the number of leftover pills participants brought back at each visit with self-reported compliance and was rated good if women forgot to take the pill less than twice a week and low if they forgot to take it two or more times a week at any of the study visits. Later, the ECLIPSES-NEN study consisted of a follow-up of the women's children at age 4 years intending to assess the children's cognitive functioning. This analyses, carried out in 2022, were based on a subsample from the main study including children at age of 4 years and their mothers.

The study was designed in agreement with the Declaration of Helsinki/Tokyo. All procedures were approved by the Clinical Research Ethics Committee of the Jordi Gol University Institute for Primary Care Research (Institut d'Investigació en Atenció Primària), of the Pere Virgili Health Research Institute (Institut d'Investigació Sanitària Pere Virgili), and of the Spanish Agency for Medicines and Medical Devices (Agencia Española del Medicamento y Productos Sanitarios). Signed informed consent was obtained from all women participating in the study.

Measures

Individualized cognitive assessment of children aged 4 years was performed in a Primary Care Centre facility, by two trained psychologists, with parents present, using the Spanish version of The Wechsler Preschool and Primary Scale of Intelligence (WPPSI)-IV[23] and some parts of the NEPSY-II: A Developmental Neuropsychological Assessment.[24]

The WPPSI-IV assesses cognitive abilities using 15 subtests, from which 5 primary indices, 4 secondary indices, and the full-scale intelligence quotient (IQ) can be obtained. The following primary indices were obtained: Verbal Comprehension Index (VCI) (the ability to verbally reason, influenced by semantic knowledge), Fluid Reasoning Index (FRI) (the ability to think logically, identifying abstract relationships between pairs of words or images), Working Memory Index (WMI) (the ability to hold information temporarily and then process it), and Processing Speed Index (PSI) (the speed to understand information and begin to respond). As for visuospatial ability, the Block Design subtest was used, which measures an individual's ability to analyze, synthesize, and reproduce an abstract design. As secondary indices, the Vocabulary Acquisition Index (VAI) (the ability to acquire new vocabulary skills) was considered. Finally, the full-scale IQ provides a general measure of cognitive and intellectual performance. All indices have a mean of 100 and an SD of 15, whereas the subtests have a mean of 10 and an SD of 2.

The NEPSY-II is a comprehensive neuropsychological assessment tool. The verbal fluency subtest (language domain),

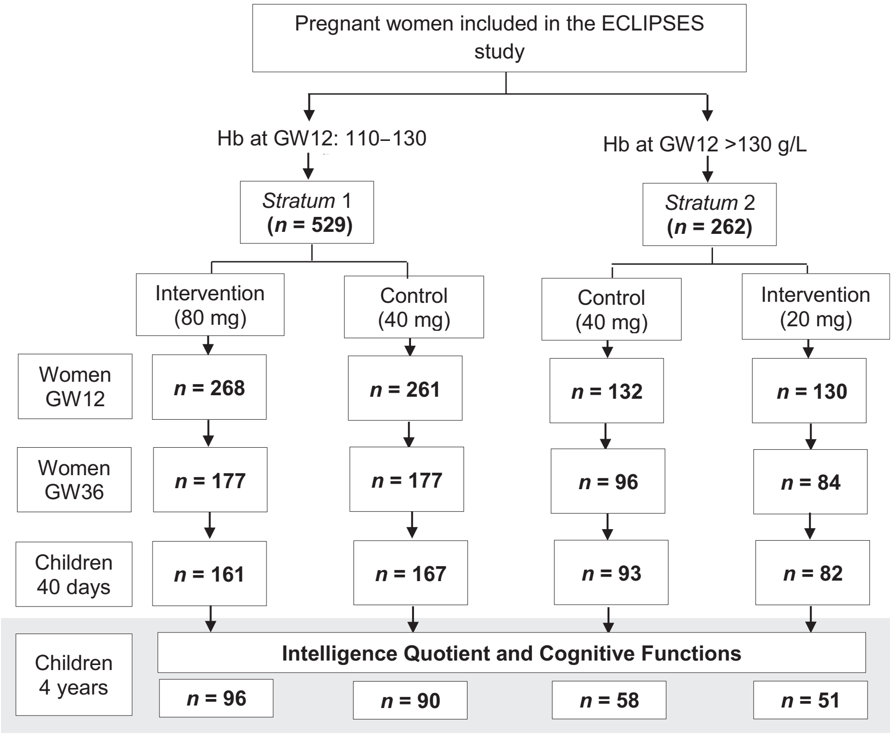

Figure 1. Flowchart of the study.

Hb, hemoglobin; GW, gestational week.

the visual-motor precision subtest (sensorimotor domain), and the emotion recognition subtest (social perception domain) were used to assess some cognitive functions that complement the WPPSI-IV results. The NEPSY-II subtests have a mean of 10 and an SD of 3.

Women were visited once in each trimester of pregnancy, and a wide range of information was recorded. Clinical and obstetric history was requested, including maternal age, parity, and pregnancy planning. Anthropometric measurements (weight and height) were taken, from which BMI was calculated. Dietary habits were assessed using a self-administered food frequency questionnaire (FFQ) previously validated in the study population.[25] In this process, participants retrospectively provided information on their usual food consumption at Weeks 12, 24, and 36 of pregnancy; 40 days after delivery; and at follow-up when the children were aged 4 years. The FFQ was reviewed and analyzed by trained dietitians who calculated the daily food intake, energy content, and various nutrients.[26] Maternal lifestyle information included smoking and physical activity. Family SES was calculated using participants' and partners' educational levels and occupational status.[22] The parental IQ approach was assessed using the Matrix Reasoning subscale of the Wechsler Adult Intelligence Scale, 4th edition.[27] Maternal anxiety status, measured by the State–Trait Anxiety Inventory, and postpartum depression, assessed by the Edinburgh Postnatal Depression Scale, provided information about women's emotional status during pregnancy and after delivery. The State–Trait Anxiety Inventory test assessed two separate concepts of anxiety: state and trait. State anxiety was included, which assesses a transient emotional state characterized by subjective, consciously perceived feelings of alertness and apprehension and autonomic-nervous-system hyperactivity. Detailed information can be found elsewhere.[22]

Blood samples were collected in each trimester of pregnancy. Biochemical determinations of Hb and serum ferritin (SF) were done by immunochemiluminescence while the serum concentration of C-reactive protein was measured by immunoturbidimetry. Plasma polyunsaturated fatty acids (PUFAs) and serum vitamin D concentrations were quantified because they are involved in brain development, so maternal levels during pregnancy may affect fetal neurodevelopment.[28,29] Then, PUFA concentrations were measured by gas chromatography-mass spectrometry,[30] and serum concentrations of vitamin D were quantified by an automated chemiluminescent immunoassay method.[31] Folate and vitamin B_{12} measurements were also performed in the first trimester of pregnancy. Red blood cell folate concentrations were then calculated using the following formula: (serum folate in hemolyzed whole blood × dilution factor in hemolysis × 100)/hematocrit. Genetic determinations of *HFE* gene mutations were performed.

As for information about the children, the following data were recorded. At birth, sex, gestational age (calculated from the time since the first day of the last menstrual period), and Apgar test score were obtained. Anthropometric measurements were recorded at birth and at age 40 days (length, head circumference, and weight) and repeated at age 4 years (height and weight). Similarly, the feeding mode was recorded at birth and age 40 days, and children's diet at age 4 years was reported by parents using the same FFQ as for participating women.[26] All children were schooled by the time of the assessment.

Statistical Analysis

Analyses were performed per protocol and stratified first according to baseline Hb concentrations following the study design and then within each stratum according to women's baseline iron stores, defined by their SF levels (SF<15 μg/L, ID; SF 15−65 μg/L, adequate iron stores; SF>65 μg/L, normal-high iron stores). The SF threshold for normal-high iron stores corresponded to the 85th percentile.

Descriptive analyses of the variables studied were performed using the Student's *t* test and the ANOVA test for continuous variables and the chi-square test for categorical variables. The natural logarithm transformation was applied to the SF concentration to normalize its distribution. For statistically significant differences, the effect size was assessed using Cohen's d. Multivariate regression models provided estimates of the effect of different doses of prenatal iron supplementation (Stratum 1:80 mg vs control, Stratum 2: 20 mg vs control) on the child's cognitive functioning. The models were adjusted a priori for those covariates that might influence this association. Maternal covariates include age, BMI in early pregnancy, parity (yes, meaning having previous children, or no, meaning having no previous children), pregnancy planning (yes or no), type of delivery (eutocic or dystocic), family SES (low, middle, high), smoking (yes or no), emotional status, postpartum depression, parental IQ approach, *HFE* gene mutations (yes or no), energy intake, dietary intake of iron and nutrients related to its metabolism (fiber, calcium, vitamin C), dietary intake of nutrients related to brain development (PUFAs, vitamin B_{12}, folate), serum concentrations of Hb, vitamin D, PUFA, and C-reactive protein at the first and third trimester of pregnancy and serum concentrations of red blood cell, folate, and vitamin B_{12} at the first trimester of pregnancy. As for the child's covariates, the following were included: gestational age, sex, head circumference at birth, and dietary intake at age 4 years (energy, iron, PUFAs, vitamin B_{12}, and folate). The statistical analyses were done using the SPSS software (Version 27.0 for Windows; SPSS Inc., Chicago, IL).

RESULTS

These analyses were based on a sample of 295 mother−child pairs (Figure 1). Table 1 shows the main characteristics of the participants included and not included in the present sample after losses of follow-up, with no differences between them. Maternal characteristics of the participants included in this analyses are described according to their baseline iron status and dose of iron supplementation in Appendix Table 1 (available online). The change in maternal iron status after prenatal iron supplementation is also shown in Appendix Table 2 (available online).

Scores obtained by children at age 4 years on the WPPSI-IV and NEPSY-II tests for the intervention (80 or 20 mg/d iron) and control (40 mg/d iron) group according to the mother's baseline iron stores in each stratum are shown in Table 2. In the unadjusted analyses, some differences were found between the control and intervention groups and in the effect of iron doses according to maternal iron stores at baseline. For the WPPSI-IV, children of women in Stratum 1 (baseline Hb of 110−130 g/L) intervention group (80 mg/d iron) scored higher on FRI than those in the control group when their mothers started pregnancy with SF levels below 15 μg/L, whereas they scored lower on VCI, WMI, PSI, and VAI scales when their mothers' baseline SF levels were above 65 μg/L. Regarding the NEPSY-II, children of women in Stratum 1 intervention group obtained higher scores than their control peers on verbal fluency, visual-motor precision, and emotion recognition when their mothers were iron deficient in early pregnancy. For the visual-motor precision scale, the intervention resulted in higher scores than in the control group when women started pregnancy with SF>65 μg/L. As for Stratum 2, the tested dose of 20 mg/d of iron daily during pregnancy led children to obtain better scores in emotion recognition than those whose mothers received 40 mg/d of iron prenatally daily.

Multivariate adjusted analyses provided estimates of the effect of different doses of prenatal iron supplementation on children's cognitive functioning. First, no difference was found in the WPPSI-IV and NEPSY-II scores when comparing the intervention and control group in each stratum by the mother's baseline Hb levels without considering their baseline iron stores (data not shown). However, the analyses stratified by the women's baseline iron stores, classified according to their SF concentrations, unveiled various associations between different doses of prenatal iron supplementation on the child's cognitive functions and IQ (Table 3). Regarding the WPPSI-IV scores, the tested iron dose (80 mg/d) in Stratum 1 compared with 40 mg/d was positively associated with all the scales and full IQ when mothers started pregnancy with SF<15 μg/L. By contrast, when mothers showed SF>65 μg/L at the beginning of gestation, a negative association was found with VCI (β= −9.02, 95% CI= −9.13, −8.91), WMI (β= −17.55, 95% CI= −19.53, −15.56), PSI (β= −3.48, 95% CI= −3.99, −2.97), and VAI (β= −7.69, 95% CI= −8.35, −7.02). In Stratum 2, the tested iron dose of 20 mg/d compared with 40 mg/d was positively associated with WMI (β=11.63, 95% CI=4.24, 22.83) and the full IQ (β=3.64, 95% CI=2.98, 4.31) when women started pregnancy with SF>65 μg/L. Similar results were found for the NEPSY-II scores. In Stratum 1, the tested iron dose was positively associated with all the indices compared with the control dose when women started pregnancy with ID. Conversely,

Table 1. Maternal Characteristics of Participants Included and Not Included in the Present Analyses

Maternal characteristics	Included (*n*=295)	Not included (*n*=496)	*p*-value
Baseline			
Age, years	32±6	31±7	0.098
Parity, yes	55.8 (164)	57.1 (283)	0.410
Pregnancy planning, yes	84.0 (248)	82.3 (408)	0.201
Body mass index			0.881
Underweight	1.9 (6)	1.7 (8)	
Normal weight	56.4 (166)	58.5 (290)	
Overweight	27.9 (82)	25.4 (126)	
Obesity	13.8 (41)	14.4 (72)	
Smoking, yes	16.9 (50)	18.4 (91)	0.770
Family socioeconomic status			0.235
High	22.9 (68)	18.7 (93)	
Middle	66.5 (196)	67.4 (334)	
Low	10.7 (31)	13.9 (69)	
HFE gene mutation, yes	32.3 (95)	33.6 (167)	0.732
Whole pregnancy			
Physical activity			0.419
Low	26.7 (79)	22.6 (112)	
Moderate	68.1 (200)	70.1 (348)	
High	5.2 (16)	7.3 (36)	
Anxiety assessment[a]			
Trait	14.49 (8.52)	13.55 (8.06)	0.065
State	15.15 (7.31)	14.94 (7.65)	0.158
Type of delivery			0.354
Eutocic	66.8 (198)	60.4 (300)	
Dystocic	32.9 (97)	39.6 (196)	
After delivery			
Postpartum depression[b]	6.82 (4.93)	6.87 (4.98)	0.925

Note: Data are expressed in mean (SD) for continuous normally distributed variables, median±IQR for continuous non-normally distributed variables, and % (*n*) for categorical variables.

[a] Measured by STAI, State-Trait Anxiety Inventory.

[b] Measured by EPDS Scale, Edinburgh Postnatal Depression Scale.

when initial SF levels were above 65 μg/L, taking 80 mg/d of iron instead of 40 mg/d was associated with lower verbal fluency scores and visual-motor precision scores. Regarding Stratum 2, the dose of 20 mg/d of iron compared with the control dose was positively associated with verbal fluency when women's SF levels were 15–65 μg/L and SF>65 μg/L in early pregnancy, and with emotion recognition only for the latest ones. On the contrary, the intervention resulted in a worse performance of visual-motor precision in children from women with baseline SF levels of 15–65 μg/L.

Although the scores obtained by most of the children on the WPPSI-IV indices were in the normal range, a percentage of them scored below the threshold for optimal cognitive functioning (<85 points), as follows: 5.1% for the VCI, 7.2% for the FRI, 16% for the WMI, 20.7% for the PSI, 5.8% for the full-scale IQ, and 13.1% for the VAI. Logistic regressions showed no statistically significant difference between the control and tested doses of iron in each stratum on the WPPSI-IV scores (data not shown).

DISCUSSION

The main finding of this study was that adapting prenatal iron supplementation in nonanemic women not only to their Hb concentrations but also to their iron reserves in early pregnancy improved neuropsychological functioning in children at age 4 years. As far as is known, this is the first study to assess the effects of different doses of prenatal iron, depending on maternal iron stores in early pregnancy on child cognitive development, which makes it difficult to compare the current results. In evaluating the effects of routine prenatal iron

Table 2. Scores From WPPSI-IV and NEPSY-II Tests by Prenatal Iron Supplementation According to Maternal Iron Stores

	SF<15 μg/L			SF 15−65 μg/L			SF>65 μg/L		
Stratum 1 (Hb 110−130 g/L)	**40 mg/d (*n*=16)**	**80 mg/d (*n*=13)**	***p*-value**	**40 mg/d (*n*=63)**	**80 mg/d (*n*=61)**	***p*-value**	**40 mg/d (*n*=11)**	**80 mg/d (*n*=18)**	***p*-value**
WPPSI-IV									
Verbal Comprehension Index	101.13 (12.55)	104.64 (15.03)	0.491	105.33 (12.04)	102.86 (15.18)	0.312	**106.73 (11.62)**	**102.94 (11.84)**	**0.015**[a]
Fluid Reasoning Index	**95.50 (13.42)**	**106.77 (9.64)**	**0.014**[b]	**103.81 (13.45)**	**100.16 (14.18)**	**0.044**[c]	106.64 (9.80)	106.94 (13.82)	0.949
Working Memory Index	98.63 (13.13)	102.08 (13.10)	0.674	98.35 (11.92)	96.05 (12.49)	0.765	**98.55 (10.74)**	**95.28 (7.76)**	**0.029**[a]
Processing Speed Index	89.19 (13.39)	98.85 (13.50)	0.065	**94.25 (13.07)**	**91.84 (12.52)**	**0.043**[c]	**97.15 (12.89)**	**95.17 (11.00)**	**0.046**[c]
Full intelligence quotient	97.44 (13.73)	105.77 (11.33)	0.091	102.49 (11.98)	100.54 (12.83)	0.384	102.45 (10.11)	102.17 (10.03)	0.658
Vocabulary Acquisition Index	90.50 (12.73)	97.86 (16.11)	0.174	99.42 (13.39)	94.88 (14.44)	0.061	**99.27 (12.19)**	**95.84 (15.66)**	**0.038**[c]
Block Design subtest	10.69 (10.69)	11.79 (1.93)	0.061	10.79 (2.18)	11.40 (2.22)	0.054	**12.08 (2.39)**	**11.00 (1.76)**	**0.018**[a]
NEPSY-II									
Verbal fluency	**8.33 (3.33)**	**9.69 (2.84)**	**0.034**[a]	9.16 (2.69)	8.34 (2.89)	0.116	10.73 (2.32)	8.44 (3.07)	0.060
Visual-motor precision	**9.44 (3.44)**	**11.85 (4.12)**	**0.028**[a]	10.11 (2.50)	9.64 (3.64)	0.407	**9.04 (2.42)**	**10.94 (2.86)**	**0.017**[a]
Emotion recognition	**8.44 (2.13)**	**9.69 (2.29)**	**0.038**[a]	**9.37 (2.68)**	**8.42 (2.71)**	**0.052**[a]	10.36 (1.21)	8.94 (2.07)	0.952
Stratum 2 (Hb>130 g/L)	**40 mg/d (*n*=8)**	**20 mg/d (*n*=7)**		**40 mg/d (*n*=39)**	**20 mg/d (*n*=38)**		**40 mg/d (*n*=9)**	**20 mg/d (*n*=12)**	
WPPSI-IV									
Verbal Comprehension Index	106.88 (10.97)	97.00 (5.34)	0.090	105.14 (12.21)	107.10 (14.25)	0.521	110.67 (9.63)	105.27 (13.86)	0.337
Fluid Reasoning Index	103.13 (13.89)	105.60 (14.03)	0.761	103.05 (12.97)	104.68 (10.58)	0.550	104.55 (12.87)	96.11 (16.21)	0.210
Working Memory Index	92.25 (12.15)	98.60 (6.99)	0.315	97.16 (12.15)	98.22 (13.07)	0.720	97.11 (11.90)	100.70 (11.55)	0.054
Processing Speed Index	95.13 (12.41)	94.20 (13.92)	0.903	97.78 (10.72)	97.08 (12.90)	0.800	99.70 (11.00)	99.22 (12.47)	0.930
Full intelligence quotient	101.88 (9.88)	96.60 (8.26)	0.342	102.35 (10.95)	103.08 (10.28)	0.767	103.10 (12.89)	104.33 (10.87)	0.300
Vocabulary Acquisition Index	101.00 (14.68)	91.00 (10.56)	0.184	99.37 (14.20)	99.31 (12.87)	0.984	99.70 (12.37)	97.60 (15.48)	0.741
Block Design subtest	10.75 (1.83)	9.43 (1.72)	0.175	11.31 (1.88)	11.51 (2.42)	0.675	11.18 (2.12)	11.82 (2.36)	0.353
NEPSY-II									
Verbal fluency	10.50 (1.69)	8.50 (2.43)	0.093	**8.69 (2.60)**	**9.50 (3.09)**	**0.041**[c]	9.50 (2.32)	10.27 (170)	0.065
Visual-motor precision	10.50 (3.82)	10.80 (3.90)	0.894	10.81 (3.67)	9.69 (2.46)	0.069	11.20 (2.47)	10.30 (3.27)	0.554
Emotion recognition	8.88 (2.95)	11.60 (1.52)	0.085	9.65 (2.35)	8.95 (2.58)	0.222	**9.00 (2.40)**	**10.00 (2.87)**	**0.020**[a]

Note: Boldface indicates statistical significance ($p<0.05$).
Data are expressed in mean (SD). Cohen's d for assessing effect size was indicated as described by the superscripted letters.
[a] Medium effect size (>0.3−0.8).
[b] Large effect size (>0.8).
[c] Low effect size (0.2−0.3).
NEPSY, Developmental Neuropsychological Assessment; SF, serum ferritin; WPPSI, Wechsler Preschool and Primary Scale of Intelligence.

Table 3. Adjusted Estimates of the Effect of Different Doses of Prenatal Iron Supplementation on Cognitive Functions at age 4 Years

	SF<15 μg/ L (*n*=29)			**SF 15–65 μg/ L (*n*=124)**			**SF>65 μg/ L (*n*=29)**		
Stratum 1 (0: 40 mg/d, 1: 80 mg/d)	***β***	**95% CI**	***p*-value**	***β***	**95% CI**	***p*-value**	***β***	**95% CI**	***p*-value**
WPPSI-IV									
Verbal Comprehension Index	**12.06**	**10.37, 21.75**	**0.007**	−2.98	−10.62, 4.66	0.426	**−9.02**	**−9.13, −8.91**	**0.001**
Fluid Reasoning Index	**35.90**	**35.66, 36.13**	**<0.001**	**−10.89**	**−18.28, −3.51**	**0.006**	−6.66	−15.96, 2.64	0.107
Working Memory Index	**24.39**	**18.35, 30.62**	**0.003**	−6.34	−17.27, 4.59	0.240	**−17.55**	**−19.53, −15.56**	**0.001**
Processing Speed Index	**26.50**	**19.76, 28.41**	**0.013**	**−10.31**	**−18.94, −1.68**	**0.022**	**−3.48**	**−3.99, −2.97**	**0.007**
Full intelligence quotient	**27.38**	**25.96, 28.80**	**0.003**	−7.79	−19.15, 3.56	0.168	−1.86	−2.72, 1.51	0.123
Vocabulary Acquisition Index	**24.73**	**23.37, 26.09**	**0.003**	−8.79	−19.15, 1.57	0.092	**−7.69**	**−8.35, −7.02**	**0.004**
Block Design subtest	**2.86**	**0.60, 5.13**	**0.023**	**2.14**	**0.37, 3.90**	**0.021**	**−4.26**	**−4.92, −3.60**	**0.008**
NEPSY-II									
Verbal fluency	**1.81**	**1.31, 2.31**	**0.001**	0.46	−1.89, 2.81	0.689	**−0.93**	**−1.16, −0.69**	**0.013**
Visual-motor precision	**3.17**	**3.08, 3.26**	**<0.001**	−1.04	−3.42, 1.34	0.370	**2.67**	**1.93, 4.41**	**0.012**
Emotion recognition	**2.56**	**2.36, 2.77**	**<0.001**	1.44	−3.68, 0.81	0.197	−0.75	−4.41, 2.91	0.599
Stratum 2 (0: 40 mg/d, 1: 20 mg/d)				**(*n*=77)**			**(*n*=21)**		
WPPSI-IV									
Verbal Comprehension Index	—	—	—	2.37	−5.90, 10.63	0.557	−3.83	−17.37, 9.71	0.434
Fluid Reasoning Index	—	—	—	5.36	−3.66, 14.38	0.230	−9.23	−24.64, 6.18	0.123
Working Memory Index	—	—	—	6.39	−4.20, 16.98	0.224	**11.63**	**4.24, 22.83**	**0.047**
Processing Speed Index	—	—	—	−3.65	−10.63, 3.33	0.289	9.51	−0.25, 16.59	0.095
Full IQ	—	—	—	4.72	−2.03, 11.47	0.160	**3.64**	**2.98, 4.31**	**0.009**
VAI	—	—	—	−4.94	−17.12, 7.23	0.405	−1.50	−12.68, 9.68	0.622
Block Design subtest	—	—	—	0.12	−0.77, 1.01	0.778	0.68	−5.67, 3.96	0.954
NEPSY-II									
Verbal fluency	—	—	—	**1.99**	**0.06, 3.92**	**0.044**	**1.82**	**1.16, 2.49**	**0.018**
Visual-motor precision	—	—	—	**−2.51**	**−4.57, −0.47**	**0.019**	−0.33	−1.21, 0.55	0.131
Emotion recognition	—	—	—	−1.89	−3.37, 0.42	0.104	**4.19**	**3.76, 4.62**	**0.005**

Note: Boldface indicates statistical significance ($p<0.05$).

Models adjusted by maternal age, BMI in early pregnancy, parity (yes/no), pregnancy planning (yes/no), type of delivery (eutocic or dystocic), family SES (low, middle, high), smoking at recruitment (yes/no), maternal emotional status during pregnancy, postpartum depression, parental IQ approximation, *HFE* gene mutations (yes/no), maternal diet (energy, fiber, iron, PUFAs, calcium, vitamin C, vitamin B_{12}, folate), serum concentrations of Hb, vitamin D, PUFAs, and CRP at the first and third trimester of pregnancy; RBC, folate, and vitamin B_{12} concentrations at the first trimester of pregnancy; SF concentrations at the third trimester of pregnancy; gestational age, child sex, head circumference at birth, and child diet at age 4 years (energy, iron, polyunsaturated fatty acids, vitamin B_{12}, folate).

CRP, C-reactive protein; Hb, hemoglobin; IQ, intelligence quotient; NEPSY, Developmental Neuropsychological Assessment; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SF, serum ferritin; VPI, Vocabulary Acquisition Index; WPPSI, Wechsler Preschool and Primary Scale of Intelligence.

supplementation, in which all women receive a single dose of iron, no clear evidence of benefit to offspring cognitive development has been found in previous studies.[12] At this point, it is worth noting the "U"-shaped risk for iron status, which suggests that both ID and iron excess have adverse health effects and would justify prescribing appropriate prenatal iron supplementation to avoid both situations.[32] This may be one of the reasons that studies examining the effect of a single iron dose compared with that of no supplementation found no significant differences[16,33] or even an adverse effect.[13] The dose tested may be inadequate or too high, depending on the case.

All women in this study started pregnancy without anemia because this was an exclusion criterion for eligibility, but some of them had ID. This has been referred to as ID without anemia, which has recently emerged and suggests that even intermediate ID states are potentially harmful to health and should receive more attention in clinical practice.[34] Indeed, the differences found in the effect of prenatal iron doses (the usual dose of 40 mg/d in Spain versus a higher and a lower dose of 80 and 20 mg/d, respectively) on the neuropsychological functions of the children depended not only on the initial maternal Hb levels (normal: 110–130 g/L or normal-high: >130 g/L) but also on their iron stores at the beginning of pregnancy. Specifically, prenatal administration of high-dose iron (80 mg/d) in Stratum 1 women with ID in early pregnancy improved all neuropsychological functions and IQ in their children at age 4 years. This finding highlights that optimal iron status from early pregnancy, which in this case compensates for low iron stores through adequate supplementation, is essential for the child's cognitive and neuropsychological functions assessed on the WPPSI-IV. By contrast, prenatal administration of the commonly prescribed iron dose (40 mg/d) in women from Stratum 1 who began pregnancy with adequate iron stores resulted in improved indices of executive function in their children, including WMI and PSI from the WPPSI-IV. This indicates that not only is the prevention of ID important, but also the prevention of possible iron excess leads to better neurocognitive development of the child.

In support of this point, the results in Stratum 2 women with SF$\geq$65 μg/L showed better performance in WMI and IQ in children whose mothers had received low-dose iron supplementation (20 mg/d) prenatally, again indicating the need for prevention of iron excess. Another notable finding is that the children of women from Stratum 2 with SF$\geq$65 μg/L at baseline who received low-dose iron prenatally showed significant improvement in the NEPSY-II emotion recognition index, an item related to some aspects of psychological problems such as autism spectrum disorders.[35] On the basis of this observation, it could be suggested that preventing iron excess by considering both women's initial Hb concentration and iron stores when adjusting the dose of iron supplements has positive effects on children.

It was expected that a high prenatal iron dose would have a positive effect on the development of the child if women started the pregnancy with ID. In contrast, the deleterious effect that high prenatal iron intake may have on the children of iron-repleted women has not been as clear. It can be hypothesized that this may be because women who had higher iron stores at the beginning of pregnancy are more likely to have excess iron, which in turn may impair fetal brain development by causing iron deposition in specific areas involved in some cognitive functions. Although this is a process that occurs naturally with aging and is usually associated with neurodegeneration, the results of some recent studies suggest that maternal iron overload during pregnancy may have a similar effect on the fetal brain, affecting its formation and development as well as the child's later cognitive functions.[36,37] Given the physiology of iron during pregnancy and its high requirement, this does not mean that women who have good iron stores at the beginning of pregnancy do not need supplemental iron but that low iron doses are sufficient to provide benefits for infant cognitive development, as shown by the present results. These findings are of great importance for clinical practice because if iron stores in early pregnancy are not assessed by determining concentrations at SF, crucial data are lacking to adjust the dose of prenatal iron supplementation to the actual needs of individual women. Overall, they underscore the importance of considering not only the presence of anemia, as is the common practice, but also maternal iron stores when prescribing prenatal iron supplementation. To this end, physicians and midwives should consider beginning routine measurement of SF concentration beyond Hb early in pregnancy and even earlier as part of pregnancy planning programs.

Limitations

This study has several strengths worth mentioning, including (1) the original study design, which was a triple-blinded, population-based, RCT; (2) the comprehensive data collection from mothers, including sociodemographic, clinical, emotional, and lifestyle information; and (3) the comprehensive and detailed assessment of children's neuropsychological functioning using internationally accepted and reliable tests. However, some limitations must also be considered when interpreting the results of this study. First, the high number of dropouts may have affected the statistical accuracy and impact of the intervention. Nonetheless, selection bias was accounted for by comparing the characteristics of participants included and not included in this analyses. Second, the sample size in the conducted analyses was small, which might weaken statistical power. Finally, there may be some residual confounding attributable to unmeasured or unknown risk factors after adjustment for known potential confounders.

CONCLUSIONS

Adjusting prenatal iron supplementation by considering maternal baseline Hb concentration and iron stores together, even in nonanemic women, can improve cognitive functioning in children aged 4 years. The best results in neuropsychological development of children aged 4 years were found in children of mothers who received a higher (80 mg/d) than usually prescribed iron dose when they had normal Hb concentrations at the beginning of pregnancy but ID, in infants of mothers who received the standard iron dose (40 mg/d) when they had normal Hb levels and no ID in early pregnancy, and in infants of mothers who received the lower than the usual iron dose (20 mg/d) when they had normal-high Hb concentrations and SF>65 μg/L at the beginning of pregnancy. The present results experimentally support that high-dose prenatal iron supplementation is beneficial for nonanemic women with low initial iron stores, whereas on the contrary, it seems to have a negative effect on women with full iron stores who would benefit from low doses. Given the great importance of ID even in intermediate phases, routine determination of SF concentration in addition to Hb level in early pregnancy would allow physicians to prescribe appropriate prenatal iron supplementation. Further studies with larger populations and greater statistical power would be useful to verify these findings.

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Lucía Iglesias-Vázquez: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. Núria Voltas: Investigation. Carmen Hernández-Martínez: Investigation. Josefa Canals: Conceptualization, Investigation, Methodology, Writing – review & editing. Pilar Coronel: Investigation, Resources. Mercedes Gimeno: Investigation, Resources. Victoria Arija: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing – review & editing.

SUPPLEMENTAL MATERIAL

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Prenatal iron supplementation adjusted to maternal iron stores in early pregnancy improves behaviour in children at 4 years of age: ECLIPSES study

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Prenatal iron supplementation adjusted to maternal iron stores reduces the risk of psychological problems in 4-y-old children

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Conflict of interest

The authors state that they have no conflict of interest to declare.

ABSTRACT

Background: Prenatal iron supplementation improves children's health and cognitive performance, but few studies explore behavioural development. We **asse**ssed the effects of adjusting prenatal iron supplementation to maternal iron stores in early pregnancy on children's behaviour and psychological problems.

Methods: Randomized controlled trial involving 230 non-anaemic pregnant women from Tarragona (Spain) and their 4-year-old children. Based on haemoglobin (Hb) levels before gestational week (GW) 12, women receive different iron doses: 80vs40 mg/d if Hb 110-130 g/L and 20vs40 mg/d if Hb>130 g/L. Iron stores at GW12 were classified using serum ferritin as low (<15 μg/L), normal (15-65 μg/L), and normal-high (>65 μg/L). Children's behaviour was assessed by parents using the Child Behaviour Checklist for ages 1.5-5 years and the Behaviour Rating Inventory of Executive Function-Preschool Version), and by teachers using the Teacher's Report Form for ages 1.5-5 years.

Results: Taking 80 mg/d of iron was associated with lower scores of any psychological problems when women had low iron stores but increased the risk of internalizing and externalizing problems when mothers had normal-high iron stores. Taking 20 mg/d of iron decreased the anxiety and autism spectrum problems risk only in those children whose mothers had SF>65 μg/L in early pregnancy. Additionally, executive functioning improved at high doses of prenatal iron when maternal baseline SF<15 μg/L and got worse when mothers had normal-high iron stores.

Conclusions: Adjusting prenatal iron supplementation to both maternal baseline Hb levels and iron stores improves behaviour and reduces psychological problems in 4-year-old children.

KEYWORDS: iron supplementation; pregnancy; psychological problems; child behaviour; executive functioning.

INTRODUCTION

Society is currently facing an epidemic of mental health problems, with a worrying increase in the prevalence of behavioural and psychological problems in childhood (Barican et al., 2022). Despite the multifactorial aetiology of psychopathological disorders, the intrauterine environment is one of the factors with which they are strongly associated (DiPietro et al., 2018; Feldman, 2008; Takegata et al., 2021; Tearne et al., 2015). On the one hand, behaviour has its physiological basis in the brain's structure, whose formation and maturation begin during the foetal phase (Cusick & Georgieff, 2016). Proper brain development requires iron as an essential element for many physiological events, so maternal iron status during pregnancy plays an important role in this process (Georgieff, 2007; McCann et al., 2020; Quezada-Pinedo et al., 2021).

Prenatal iron deficiency (ID) has been associated with poorer cognitive development, intelligence quotient (IQ), and executive functions in children, even in mild stages without signs of anaemia (Gaillard et al., 2014; Hernández-Martínez et al., 2011; McCarthy et al., 2021; Radlowski & Johnson, 2013). Initial evidence of an association between maternal iron status and children's behaviour has been also recently provided (Díaz-López et al., 2022; Hernández-Martínez et al., 2011; Iglesias et al., 2017). In a large Spanish cohort of mother-child pairs, high ferritin levels during pregnancy showed a protective effect against inattentive-type attention-deficit/hyperactivity disorder (ADHD) symptoms assessed at 4 years of age (Santa-Marina et al., 2020). Further, some studies have referred to specific periods during pregnancy of greater vulnerability to ID regarding child outcomes, although the evidence is inconsistent. On the one hand, a recent systematic review concluded that low iron, particularly in the third trimester of gestation, may be associated with adverse offspring neurodevelopment (Janbek et al., 2019). On the other hand, population registers data from the Stockholm Youth Cohort indicated that iron deficit early in pregnancy was associated with an increased risk of a diagnosis of autism spectrum disorders and ADHD in offspring (Wiegersma et al., 2019). Furthermore, neonatal ID assessed by umbilical cord serum ferritin concentrations, which correlate with maternal iron status, has been associated with lasting behavioural consequences at 5 years (McCarthy et al., 2021).

According to previous findings, prenatal iron supplementation is effective in improving maternal haematological outcomes (Iglesias Vázquez et al., 2019; Peña-Rosas et al., 2015), but very little effort has been devoted to assessing its effect on child neurodevelopment. In this regard, previous data from the ECLIPSES study have shown that adapting prenatal iron supplementation to maternal needs based on haemoglobin (Hb) and serum ferritin (SF) concentrations improves the child's cognitive functioning at 4 years of age (Iglesias-Vázquez et al., 2023). Other areas of neurodevelopment, such as behavioural development and the risk of psychological problems are still poorly investigated. As for the development of psychological problems in infancy, only one study appears to have addressed this question, without being able to provide clear evidence of the benefit of prenatal iron supplementation on the behaviour of children aged 4 (Zhou et al., 2006) and 6-8 years (Parsons et al., 2008). In contrast, other authors found a slightly higher percentage of children with abnormal behaviour at both ages among those whose mothers received iron compared to those who received a placebo (Szajewska et al., 2010). Since it is known that many biological and lifestyle conditions could influence maternal iron status (Hanson, Imperatore, & Burke, 2001; Quezada-Pinedo et al., 2022; Rasmussen et al., 2005), the present study aimed to assess the effect of prenatal iron supplementation in non-anaemic pregnant women by adjusting the dose to the mother's actual iron needs on their children's risk of psychological problems at 4 years of age.

METHODS

Study Sample

The ECLIPSES study was a population-based randomized controlled trial conducted in 2013-2017 in Tarragona, Spain, that aimed to evaluate the effectiveness of different doses of prenatal iron supplementation on maternal iron status at the end of pregnancy in non-anaemic women in early pregnancy (Iglesias Vázquez et al., 2019). Secondary objectives also included the assessment of the effectiveness of different doses of prenatal iron supplementation on the cognitive (Iglesias-Vázquez et al., 2023) and behavioural development of their children. The current analyses aimed to assess the effect of that supplementation on child behavioural development and their risk of having psychological problems at 4 years of age.

Participating women were recruited before the gestational week (GW) 12 and allocated into two groups according to their Hb levels at the time. Women in *Stratum* 1 (initial Hb=110-130 g/L) were randomly prescribed a daily dose of 40 or 80 mg of iron aiming to prevent an iron deficit, while those in *Stratum* 2 (initial Hb>130 g/L) received 40 or 20 mg of iron daily aiming to prevent the risk of developing iron excess (**Figure 1**). Since the usually prescribed dose of iron is daily 40 mg, women in each *Strata* receiving that dose were considered the control group against which the higher and lower iron dose interventions were tested. The intervention was triple-blind, meaning that neither the researchers, the supplement providers, nor the health workers knew each woman's dose of iron supplements until the end of the study. Compliance with the intervention was done by comparing the number of leftover pills that participants return in each visit with the self-reported compliance and was considered "good" when women forgot to take the pill less than twice a week, and "low" when they forgot it two or more times a week at any of the study visits. The ECLIPSES study was registered at www.clinicaltrialsregister.eu as EudraCT number

2012-005480-28 and its methodological details can be found extended elsewhere (Arija et al., 2014; Iglesias Vázquez et al., 2019).

Ethical Considerations

The study was designed in agreement with the Declaration of Helsinki/Tokyo. All procedures were approved by the Clinical Research Ethics Committee of the Jordi Gol University Institute for Primary Care Research [Institut d'Investigació en Atenció Primària; IDIAP], the Pere Virgili Health Research Institute [Institut d'Investigació Sanitària Pere Virgili; IISPV] and of the Spanish Agency for Medicines and Medical Devices [Agencia Española del Medicamento y Productos Sanitarios; AEMPS]. Signed informed consent was obtained from all women participating in the study.

Measures

Behavioural and emotional problems of children at age 4 were reported by parents using the Child Behaviour Checklist for ages 1.5 to 5 years (CBCL 1½-5) (Achenbach & Rescorla, 2000), and by teachers using the Teacher's Report Form for ages 1.5 to 5 years (TRF1½-5) (Achenbach & Rescorla, 2000). The CBCL1½-5 and TRF1½-5 are tests of 99 items (with 3 response options: not true; somewhat or sometimes true; very true or often true), that provide 6 empirically based syndrome scales (emotional reactivity, anxiety/depression, somatic complaints, withdrawal, attention problems, and aggressive behaviour) and DSM 5-oriented scales (depressive problems, anxiety problems, autism spectrum problems, attention-deficit/hyperactivity problems, oppositional defiant problems). The emotional reactivity, anxiety/depression, somatic complaints, and withdrawal scales constitute the scale of the internalizing problems, while the attention problems and aggressive behaviour scales constitute the scale of the externalizing problems. All the syndromic scales together constitute the total problem scale. T-scores for the Spanish version for all scales were used.

Scores <65 are within the normal range, scores between 65 and 69 are borderline and scores >69 are in the clinical range. Internal consistency of the Spanish version covered the range of moderate to good (de la Osa et al., 2016).

The Behaviour Rating Inventory of Executive Function-Preschool Version (BRIEF-P) (Gioia et al., 2016) is a test for the evaluation of daily behavioural and observable aspects of the executive functions of children between 2 and 5 years old. This test of 63 items was answered by the parents, who must indicate with a Likert scale (never, sometimes, frequently) the frequency of certain problematic behaviours. The BRIEF-P finally accounted for the following executive functions: inhibition, flexibility, emotional control, working memory, and plan/organize; and allow to obtain four indexes: behavioural regulation index, flexibility index, metacognition index, and global executive index. T-scores (mean 50, SD 10) were used, with higher scores being equivalent to higher levels of executive dysfunction. The Spanish-adapted version of the BRIEF-P was used, which showed good data of reliability (Cronbach alpha between 0.77 and 0.95 for the several scales) and validity (Gioia et al., 2016).

Women were visited once in each trimester of pregnancy, and a wide range of information was recorded. Clinical and obstetric history was requested, including maternal age, parity, and pregnancy planning. Anthropometric measurements (weight and height) were taken, and body mass index was calculated. Dietary habits were assessed using a self-administered food frequency questionnaire previously validated in our population (Trinidad Rodríguez et al., 2008). In this process, participants retrospectively provided information on their usual food consumption at weeks 12, 24, and 36 of pregnancy, 40 days after delivery, and at follow-up when the children were 4 years old. The food frequency questionnaire was reviewed and analysed by trained dietitians, who calculated the daily food intake, energy content, and various nutrients (Aparicio et al., 2020). Maternal lifestyle information included smoking and physical activity. Family socioeconomic status was calculated using the participants' and partners' educational level and occupational status (Iglesias Vázquez et al., 2019). The parental IQ approach was assessed using the Matrix Reasoning subscale of the Wechsler Adult Intelligence Scale-4th edition (WAIS-IV) (Wechsler, 1997). Maternal anxiety status, measured by the State-Trait Anxiety Inventory, and postpartum depression, assessed by the Edinburgh Postnatal Depression Scale, provided information about women's emotional status during pregnancy and after delivery. The State-Trait Anxiety Inventory test assessed two separate concepts of anxiety: "state" and "trait". We included state anxiety as a covariate, which assesses a transient emotional state characterized by subjective, consciously perceived feelings of alertness and apprehension and autonomic nervous system hyperactivity. Detailed information can be found elsewhere (Iglesias Vázquez et al., 2019).

In each trimester of pregnancy, blood samples were collected for biochemical determinations of Hb, SF, and C-reactive protein. Plasma polyunsaturated fatty acids (PUFAs) and serum vitamin D concentrations were quantified because they are involved in brain development, so maternal levels during pregnancy may affect foetal neurodevelopment (Voltas et al., 2020; Zou et al., 2021). Folate and vitamin B_{12} measurements were also performed in the first trimester of pregnancy. Red blood cell (RBC) folate concentrations were then calculated using the following formula: (serum folate in haemolysed whole blood*dilution factor in haemolysis*100)/haematocrit. Genetic determinations of *HFE* gene mutations were performed.

As for information about the children, the following data were recorded. At birth, sex, gestational age (calculated from the time since the first day of the last menstruation), and Apgar test score were obtained. Anthropometric measurements were recorded at birth and 40 days of age (length, weight, and head circumference) and repeated at 4 years of age (weight and height). Similarly, the feeding mode was recorded at birth and 40 days of age, and children's diet at 4 years of age was reported by parents using the same food frequency questionnaire as for participating women. Children's IQ was obtained at 4 years of age. All children were schooled by the time of the assessment.

Statistical analyses

Analyses were performed *per protocol* and stratified first according to baseline Hb concentrations following the study design, and then within each *Stratum* according to women's baseline iron stores, defined by their SF levels (SF<15 μg/L, iron deficiency; SF 15-65 μg/L, adequate iron stores; SF>65 μg/L, normal-high iron stores). The SF threshold for normal-high iron stores corresponded to the 85th percentile.

Descriptive analyses of the variables studied were performed using the Student T and the ANOVA tests for continuous variables and the Chi-square test for categorical variables. The natural logarithm (Ln) transformation was applied to the SF concentration to normalize its distribution. For statistically significant results, the effect size was assessed using Cohen's D. Due to the multiple comparisons, the Bonferroni correction was applied to control the increase in type I error. Multivariate regression models provided estimates of the effect of different doses of prenatal iron supplementation (S*tratum* 1: 80 mg vs control, *Stratum* 2: 20 mg vs control) on the child's behaviour and psychological problems. The models were adjusted *a priori* for those covariates that might influence this association. Maternal covariates include family socioeconomic status (low, middle, high), smoking (yes or no), emotional status, postpartum depression, parental IQ approach, energy intake, dietary intake of iron and nutrients related to its metabolism (fibre, calcium, vitamin C), dietary intake of nutrients related to brain development (PUFAs, vitamin B_{12}, folate), serum concentrations of Hb, vitamin D, PUFAs, and C-reactive protein at the first and third trimester of pregnancy, and serum concentrations of RBC folate and vitamin B_{12} at the first trimester of pregnancy. As for the child's covariates, the following were included: gestational age, sex, IQ, head circumference at birth, energy intake, and dietary intake of iron, PUFAs, vitamin B_{12}, and folate at 4 years of age.

The statistical analyses were done using the SPSS software (version 27.0 for Windows; SPSS Inc., Chicago, IL, USA).

RESULTS

The current analyses were based on a sub-sample of the ECLIPSES study of 230 mother-child pairs from which the assessment of children's behaviour and the risk of having psychological problems at 4 years of age was available (**Figure 1**).

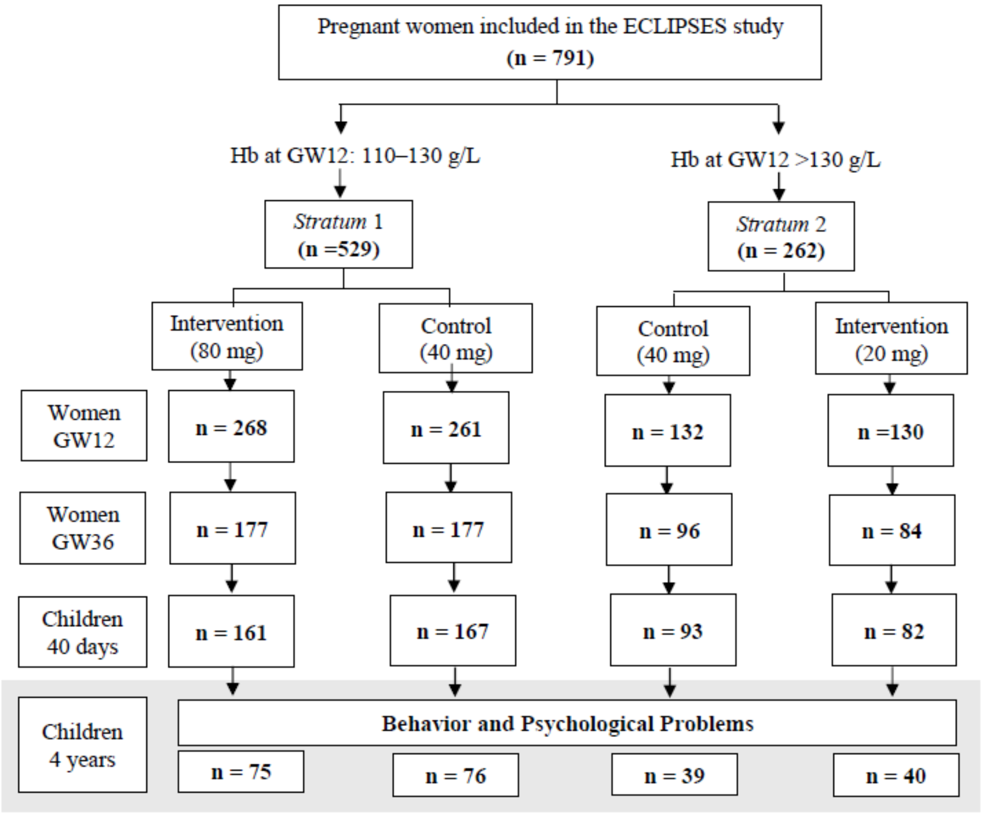

Figure 1. Flowchart of the study. Hb, haemoglobin; GW, gestational week

Table 1 shows the main characteristics of the participants included and not included in the present sample with no statistically significant differences between them. The maternal characteristics of the participants included in the present analyses were also described according to their baseline iron status and dose of iron supplementation in **Appendix Table S1**.

Table 1. Maternal characteristics of participants included and not included in the present analyses.

	Included (n=230)	**Not included (n=561)**	**p-value**
Baseline			
Age, years	32 ± 6	31 ± 7	0.098
Parity, yes	55.8	57.1	0.410
Pregnancy planning, yes	84.0	82.3	0.201
Body mass index			0.881
Underweight	1.9	1.7	
Normal weight	56.4	58.5	
Overweight	27.9	25.4	
Obesity	13.8	14.4	
Smoking, yes	16.9	18.4	0.770
Family socioeconomic status			0.235
High	22.9	18.7	
Middle	66.5	67.4	
Low	10.7	13.9	
HFE gene mutation, yes	32.3	33.6	0.732
Whole pregnancy			
Physical activity			0.419
Low	26.7	22.6	
Moderate	68.1	70.1	
High	5.2	7.3	
Anxiety[*]			
Trait	14.49 (8.52)	13.55 (8.06)	0.065
State	15.15 (7.31)	14.94 (7.65)	0.158
After delivery			
Postpartum depression[†]	6.82 (4.93)	6.87 (4.98)	0.925

Data are expressed in mean (SD) for continuous normally distributed variables, median ± interquartile range for continuous non-normally distributed variables, and % [n] for categorical variables.

[*] Measured by State-Trait Anxiety Inventory. The score ranges from 0 to 60 points.

[†] Measured by the Edinburgh Postnatal Depression Scale. The score ranges from 0 to 30 points.

Scores obtained by children aged 4 years on the CBCL 1½-5 and TRF1½-5 for the intervention (80 or 20 mg/d iron) and control group (40 mg/d iron) according to the mother's baseline iron stores in each *Stratum* were shown in **Appendix Table S2** and **Appendix Table S3**, respectively. In analyses, some differences were already found between the control and intervention groups, and in the effect of iron doses according to maternal iron stores at baseline. Multivariate analyses provided estimates of the effect of different doses of prenatal iron supplementation on children's behaviour and psychological problems. First, no difference was found in the CBCL1½-5, TRF, and BRIEF-P scores when comparing the intervention and control group in each *Stratum* by the mother's baseline Hb levels without considering their baseline iron stores (data not shown). However, the analyses stratified by the women's baseline iron stores, classified according to their SF concentrations, unveiled various associations between different doses of prenatal iron supplementation on the child's psychopathology. The tested iron dose (80 mg/d) in *Stratum* 1 compared to 40 mg/d was associated with lower scores in all the CBCL1½-5 scales, including internalizing, externalizing, and total problems as well as the DSM scales when women started pregnancy with ID, while it was associated with higher scores in most of the CBCL1½-5 scales except for depressive problems and attention/hyperactivity problems when women started pregnancy with SF>65 µg/L. In *Stratum* 2, the tested iron dose (20 mg/d) compared to 40 mg/d led to lower scores in some of the CBCL1½-5 scales only in the group of children whose mothers showed baseline SF>65 µg/L, as follows: externalizing problems (β: -9.26, 95%CI: -9.91, -8.61), total problems (β: -6.31, 95%CI: -6.35, -6.28), anxiety problems (β: -3.18, 95%CI: -5.88, -0.49), and autism spectrum problems (β: -5.72, 95%CI: -5.86, -5.58) (**Table 2**). Although the primary scale "internalizing problems" did not get affected by the intervention in *Stratum* 2, two items that compose that scale showed lower scores in children from mothers with initial SF>65 µg/L receiving 20 mg/d instead of 40 mg/d of iron: emotional reactivity (β: -4.70, 95%CI: -5.16, -4.25), and withdrawal (β: -3.43, 95%CI: -4.93, -1.92) (data not shown). The results obtained from the information reported by the teachers through the TRF1½-5, although less than that obtained from the parents as expected, reinforced those of the CBCL1½-5 (**Table 3**).

As for the executive functioning of children at 4 years of age, the tested iron dose (80 mg/d) in *Stratum* 1 compared to 40 mg/d was associated with lower scores in all the BRIEF-P items except for flexibility when women started pregnancy with SF<15 μg/L, while it was associated with higher scores in flexibility, emotional control, plan/organize, and global executive index when women started pregnancy with SF>65 μg/L. In *Stratum* 2, the tested iron dose (20 mg/d) compared to 40 mg/d led to lower scores in emotional control, working memory, and behavioural regulation index only in the group of children whose mothers showed baseline SF>65 μg/L (**Table 4**).

Table 2. Effect of prenatal iron supplementation by *Strata* and maternal iron stores in early pregnancy on psychological problems at 4 years of age, measured by CBCL1½-5.

***Stratum* 1 (0: 40 mg/d, 1: 80 mg/d)**	**SF<15 μg/L (n=21)**			**SF 15-65 μg/L (n=104)**			**SF>65 μg/L (n=26)**		
	β	95%CI	p	β	95%CI	p	β	95%CI	p
Internalizing Problems	**-9.61**	**-12.88, -2.34**	**0.001**	-1.02	-9.97, 7.94	0.810	**6.53**	**4.15, 10.83**	**0.010**
Externalizing Problems	**-3.44**	**-6.67, -1.80**	**0.008**	6.85	-1.11, 14.80	0.086	**1.61**	**0.74, 6.53**	**0.038**
Total Problems	**-6.30**	**-9.15, -3.46**	**0.009**	2.84	-4.51, 10.20	0.418	**1.10**	**0.15, 5.94**	**0.046**
DSM Scales									
Depressive Problems	**-6.54**	**-10.75, -3.58**	**0.029**	4.48	-0.24, 9.19	0.061	10.00	-15.10, 35.10	0.294
Anxiety Problems	**-3.25**	**-8.24, -1.13**	**0.015**	2.44	-3.63, 8.51	0.403	**5.35**	**2.20, 9.50**	**0.001**
Autism Spectrum Problems	**-5.26**	**-7.38, -1.04**	**0.045**	-0.19	-8.27, 7.89	0.961	**1.17**	**0.37, 15.97**	**0.047**
Attention-Deficit/Hyperactivity Problems	**-3.90**	**-8.51, -1.29**	**0.037**	**7.78**	**3.35, 10.22**	**<0.001**	9.67	-9.65, 28.99	0.210
Oppositional Defiant Problems	**-3.67**	**-9.40, -0.93**	**0.043**	**2.03**	**0.22, 3.84**	**0.030**	**2.69**	**0.11, 5.26**	**0.046**
***Stratum* 2 (0: 40 mg/d, 1: 20 mg/d)**	**SF<15 μg/L (n=10)**			**SF 15-65 μg/L (n=54)**			**SF>65 μg/L (n=15)**		
	β	95%CI	p	β	95%CI	p	β	95%CI	p
Internalizing Problems	3.51	-2.54, 5.49	0.548	0.09	-13.29, 13.47	0.989	-2.50	-12.78, 7.80	0.496
Externalizing Problems	1.06	-2.61, 4.26	0.749	3.64	-8.34, 15.61	0.527	**-9.26**	**-9.91, -8.61**	**0.003**
Total Problems	-4.16	-9.32, 8.54	0.641	-0.78	-13.12, 11.57	0.895	**-6.31**	**-6.35, -6.28**	**<0.001**
DSM Scales									
Depressive Problems	7.15	-3.25, 11.73	0.594	2.53	-5.66, 10.72	0.521	-4.14	-12.07, 3.78	0.195
Anxiety Problems	2.83	-5.17, 6.25	0.684	-5.58	-9.23, 8.06	0.888	**-3.18**	**-5.88, -0.49**	**0.037**
Autism Spectrum Problems	3.42	-4.84, 6.45	0.491	-4.21	-11.02, 2.59	0.207	**-5.72**	**-5.86, -5.58**	**0.001**
Attention-Deficit/Hyperactivity Problems	4.16	-6.15, 7.65	0.562	2.83	-7.35, 13.01	0.564	-1.76	-14.66, 11.15	0.694
Oppositional Defiant Problems	6.94	-2.96, 12.68	0.314	1.02	-6.71, 8.74	0.783	-2.99	-16.66, 10.67	0.536

CBCL, Child Behaviour Checklist; SF, serum ferritin. Statistically significant associations are highlighted in bold.

Models adjusted by family socioeconomic status (low, middle, high), smoking at recruitment (yes/no), maternal emotional status during pregnancy, postpartum depression, parental IQ approximation, maternal diet (energy, fibre, iron, polyunsaturated fatty acids, calcium, vitamin C, vitamin B_{12}, folate), serum concentrations of Hb, vitamin D, polyunsaturated fatty acids, and C-reactive protein at the first and third trimester of pregnancy, RBC folate and vitamin B_{12} concentrations at the first trimester of pregnancy, and SF concentrations at the third trimester of pregnancy, gestational age, child sex, children's IQ, head circumference at birth, and child diet at 4 years of age (energy, iron, polyunsaturated fatty acids, vitamin B_{12}, folate).

Table 3. Effect of prenatal iron supplementation by *Strata* and maternal iron stores in early pregnancy on psychological problems at 4 years of age, measured by TRF½-5.

***Stratum* 1 (0: 40 mg/d, 1: 80 mg/d)**	**SF<15 μg/L (n=18)**			**SF 15-65 μg/L (n=87)**			**SF>65 μg/L (n=18)**		
	β	**95%CI**	**p**	**β**	**95%CI**	**p**	**β**	**95%CI**	**p**
Internalizing Problems	-7.68	-18.67, 3.30	0.071	8.24	-0.27, 16.74	0.057	**9.31**	**6.95, 15.66**	**0.019**
Externalizing Problems	**-4.92**	**-18.57, -1.27**	**0.003**	-4.38	-12.86, 4.11	0.287	**5.95**	**3.54, 8.36**	**0.020**
Total Problems	-3.04	-18.72, 12.64	0.246	5.71	-1.81, 13.24	0.126	**9.46**	**7.07, 11.85**	**0.013**
DSM Scales									
Depressive Problems	-8.33	-31.28, 14.61	0.259	3.58	-1.20, 8.36	0.131	**3.02**	**2.51, 3.53**	**0.009**
Anxiety Problems	**-7.70**	**-13.57, -1.83**	**0.038**	4.29	-1.54, 10.11	0.138	4.67	-14.51, 23.84	0.495
Autism Spectrum Problems	1.33	-10.14, 12.81	0.667	3.61	-1.25, 8.47	0.130	2.67	-4.58, 9.91	0.326
Attention-Deficit/Hyperactivity Problems	3.51	-3.21, 10.24	0.095	-1.32	-6.19, 3.54	0.567	3.24	-0.65, 7.12	0.070
Oppositional Defiant Problems	**-7.54**	**-20.34, -2.74**	**0.038**	-2.40	-6.98, 2.19	0.281	-3.38	-8.06, 1.30	0.190
***Stratum* 2 (0: 40 mg/d, 1: 20 mg/d)**	**SF<15 μg/L (n=8)**			**SF 15-65 μg/L (n=47)**			**SF>65 μg/L (n=12)**		
	β	**95%CI**	**p**	**β**	**95%CI**	**p**	**β**	**95%CI**	**p**
Internalizing Problems	-4.12	-9.54, 7.52	0.549	-3.00	-8.33, 2.32	0.237	**-7.41**	**-10.02, -4.80**	**0.018**
Externalizing Problems	-8.41	-12.63, 0.97	0.080	-0.84	-5.98, 4.29	0.719	1.17	-26.04, 28.38	0.900
Total Problems	-6.87	-10.39, 2.64	0.105	-1.59	-8.97, 5.78	0.644	0.73	-7.11, 8.56	0.729
DSM Scales									
Depressive Problems	-1.59	-3.49, 2.17	0.491	-0.24	-5.76, 5.29	0.928	2.33	-23.69, 28.35	0.974
Anxiety Problems	0.51	-1.25, 2.16	0.812	0.60	-1.38, 2.57	0.512	**-8.79**	**-10.30, -7.27**	**0.009**
Autism Spectrum Problems	-1.54	-3.81, 2.98	0.694	-3.77	-10.88, 3.34	0.273	**-1.64**	**-1.72, -1.57**	**0.002**
Attention-Deficit/Hyperactivity Problems	-1.62	-3.62, 2.63	0.259	-4.57	-16.40, 7.26	0.421	1.50	-24.83, 27.83	0.868
Oppositional Defiant Problems	-3.45	-6.41, 3.10	0.463	-6.05	-8.63, -3.47	<0.001	**-3.80**	**-5.53, -2.07**	**0.023**

CBCL, Child Behaviour Checklist; SF, serum ferritin. Statistically significant associations are highlighted in bold.

Models adjusted by family socioeconomic status (low, middle, high), smoking at recruitment (yes/no), maternal emotional status during pregnancy, postpartum depression, parental IQ approximation, maternal diet (energy, fibre, iron, polyunsaturated fatty acids, calcium, vitamin C, vitamin B_{12}, folate), serum concentrations of Hb, vitamin D, polyunsaturated fatty acids, and C-reactive protein at the first and third trimester of pregnancy, RBC folate and vitamin B_{12} concentrations at the first trimester of pregnancy, and SF concentrations at the third trimester of pregnancy, gestational age, child sex, children's IQ, head circumference at birth, and child diet at 4 years of age (energy, iron, polyunsaturated fatty acids, vitamin B_{12}, folate).

Table 4. Effect of prenatal iron supplementation by *Strata* and maternal iron stores in early pregnancy on executive functions at 4 years of age, measured by the BRIEF-P.

***Stratum* 1 (0: 40 g/d, 1: 80 mg/d)**	**SF<15 μg/L (n=21)**			**SF 15-65 μg/L (n=104)**			**SF>65 μg/L (n=26)**		
	β	**95%CI**	**p**	**β**	**95%CI**	**p**	**β**	**95%CI**	**p**
Inhibition	**-6.26**	**-7.98, -4.54**	**0.014**	1.16	-4.17, 6.50	0.651	-0.46	-3.47, 2.56	0.583
Flexibility	-7.00	-59.34, 45.34	0.623	0.81	-7.90, 9.52	0.847	**6.50**	**1.54, 11.46**	**0.022**
Emotional Control	**-4.51**	**-7.18, -2.84**	**0.015**	**5.48**	**1.81, 9.15**	**0.006**	**9.64**	**13.40, 5.87**	**0.001**
Working Memory	**-5.40**	**-10.43, -3.37**	**<0.001**	2.24	-4.68, 9.15	0.504	4.25	-9.18, 17.68	0.429
Plan/Organize	**-6.59**	**-12.95, -3.01**	**0.030**	-0.17	-7.97, 7.64	0.964	**8.29**	**7.73, 8.86**	**0.003**
Indices									
Behavioural Regulation Index	**-7.11**	**-9.69, -4.54**	**0.018**	1.84	-2.68, 6.66	0.431	-1.00	-11.62, 9.62	0.807
Flexibility Index	-12.00	-54.38, 30.38	0.347	2.34	-4.16, 8.83	0.458	4.85	-0.08, 9.77	0.052
Metacognition Index	**-8.37**	**-14.23, -6.51**	**0.021**	1.22	-5.93, 8.36	0.723	1.25	-16.87, 19.37	0.857
Global Executive Index	**-7.51**	**-10.49, -2.53**	**0.012**	1.88	-3.67, 7.73	0.484	**6.21**	**3.12, 9.31**	**0.020**

***Stratum* 2 (0: 40 mg/d, 1: 20 mg/d)**	**SF<15 μg/L (n=10)**			**SF 15-65 μg/L (n=54)**			**SF>65 μg/L (15)**		
	β	**95%CI**	**p**	**β**	**95%CI**	**p**	**β**	**95%CI**	**p**
Inhibition	2.35	-4.85, 5.30	0.258	4.23	-4.47, 12.93	0.311	-2.31	-13.54, 8.92	0.599
Flexibility	1.71	-3.14, 2.99	0.146	0.36	-9.63, 10.34	0.940	1.10	-12.70, 14.90	0.846
Emotional Control	0.95	-2.51, 4.15	0.365	1.39	-8.60, 11.37	0.770	**-7.69**	**-15.73, -5.65**	**0.007**
Working Memory	-1.24	-4.20, 3.21	0.314	-0.19	-13.42, 13.03	0.975	**-8.90**	**-17.70, -0.10**	**0.048**
Plan/Organize	-2.36	-5.16, 1.78	0.152	4.96	-7.92, 17.83	0.425	2.80	-14.80, 20.40	0.699
Indices									
Behavioural Regulation Index	3.87	-1.94, 6.57	0.225	2.50	-8.05, 13.04	0.619	**-4.43**	**-6.57, -2.29**	**0.012**
Flexibility Index	1.60	-2.63, 5.40	0.394	-0.76	-12.21, 10.68	0.889	5.88	-2.02, 13.78	0.108
Metacognition Index	-3.28	-7.15, 2.38	0.184	1.97	-11.67, 15.62	0.762	-4.90	-16.35, 6.55	0.321
Global Executive Index	-0.15	-4.68, 3.95	0.471	0.32	-13.44, 14.08	0.961	-0.93	-4.26, 2.40	0.440

BRIEF-P, Behaviour Rating Inventory of Executive Function-Preschool version; SF, serum ferritin. Statistically significant associations are highlighted in bold.

Models adjusted by family socioeconomic status (low, middle, high), smoking at recruitment (yes/no), maternal emotional status during pregnancy, postpartum depression, parental IQ approximation, maternal diet (energy, fibre, iron, polyunsaturated fatty acids, calcium, vitamin C, vitamin B_{12}, folate), serum concentrations of Hb, vitamin D, polyunsaturated fatty acids, and C-reactive protein at the first and third trimester of pregnancy, RBC folate and vitamin B_{12} concentrations at the first trimester of pregnancy, and SF concentrations at the third trimester of pregnancy, gestational age, child sex, children's IQ, head circumference at birth, and child diet at 4 years of age (energy, iron, polyunsaturated fatty acids, vitamin B_{12}, folate).

DISCUSSION

The present study found that adapting prenatal iron supplementation in non-anaemic women according to their Hb levels and iron reserves in early pregnancy would improve behaviour and reduce psychological problems in children aged 4 years. We obtained information from both parents and teachers and observed that the relationship between iron supplementation adjusted to maternal baseline SF levels and children's psychological problems was more evident when considering information reported by parents. Some studies have previously shown differences between the data reported by different informants, with parents more frequently rating their children higher than teachers on behavioural problems and hyperactivity (Canals Sans et al., 2021) as well as on total psychological problems (Martinsone et al., 2022).

To the best of our knowledge, this is the first study that considers together Hb level and iron stores at the beginning of pregnancy to evaluate the effect of different doses of prenatal iron supplements on the child’s behavioural development and psychological problems, which makes it difficult to compare the current results. Previous studies evaluating the effects of routine prenatal iron supplementation with a single dose of iron failed to provide clear evidence of benefits. Since it is well known that both iron deficiency and excess during pregnancy can have long-term consequences on the child's behaviour and increase the risk of psychological problems by being detrimental to the development and maturation of the foetal brain, one could argue that having administered a single dose to all women in those studies may have been insufficient or excessive, depending on the case, to observe positive effects or even to report, by contrary, adverse effects after prenatal supplementation.

It is worth mentioning that since anaemia was an exclusion criterion for participation in the study, none of the participants was anaemic at the beginning of pregnancy, but some of them showed ID at that time. And this point is relevant in the present study because the differences found in the effect of prenatal iron doses on children's behaviour and psychological problems depended on women's iron stores at the beginning of pregnancy beyond their Hb levels. Specifically, prenatal administration of high-dose iron (80 mg/day) in women with normal Hb concentration improved, according to information reported by parents, most of the executive functions and reduced all the psychological problems in their 4-year-old children when the women had baseline ID, whereas it resulted in worse scores for some items about executive functioning, as well as for internalizing, externalizing and total problems, and most DSM scales, specifically autism spectrum problems in the children of those who began pregnancy with normal-high iron stores (SF≥65 μg/L). This is consistent with previous results from our own study regarding different scales of child cognitive development (Iglesias-Vázquez et al., 2023), including working memory which is a component of executive functioning and is associated with ADHD. This observation indicates that, in the first case, an iron dose higher than that usually prescribed in Spain (40 mg/d) compensated for the low iron stores that could have undermined the children's psychological development at 4 years of age, although the Hb levels did not indicate the presence of anaemia, which until now would not have justified the prescription of higher prenatal doses of iron in routine clinical practice. On the other hand, the results suggest that having received a high dose of iron could have led to an excess of iron in the second case when the women started the pregnancy with normal-high iron stores, which translated into poorer behavioural development of their children. These findings highlight that not only is the prevention of ID important, but the prevention of possible iron excess leads to better emotional and behavioural development of the child. The results in *Stratum* 2 supported this argument, with those with SF≥65 μg/L showing better performance in emotional control, working memory and behaviour regulation, externalizing and total problems, as well as the DSM scales anxiety and autism spectrum problems in children whose mothers had received low-dose iron supplementation (20 mg/d) prenatally, again indicating the need for prevention of iron excess when aiming to improve the child psychological development.

That a high prenatal iron dose improves the behaviour and executive functioning and reduces psychological problems of children when women entered the pregnancy with ID was an expected finding. Indeed, there is abundant evidence in the literature that low iron status during pregnancy is associated with an increased risk of a wide range of neurodevelopmental disorders in children (Gaillard et al., 2014; Georgieff, 2008; Hernández-Martínez et al., 2011; Iglesias et al., 2017; Radlowski & Johnson, 2013). Along the same line, higher maternal SF levels, the use of prenatal iron supplementation, and higher overall iron intake generally have shown a protective effect against such problems (Arija et al., 2019; Díaz-López et al., 2022; Santa-Marina et al., 2020; Schmidt et al., 2014). In contrast, although some studies reported no improvement in the outcomes studied in relation to child cognitive or behavioural development following prenatal iron supplementation (Jayasinghe et al., 2018; Parsons et al., 2008; Zhou et al., 2006), the detrimental effect of high-dose supplementation observed in the present study in children of mothers who started pregnancy with full iron stores was not so clear until now. One explanation for this finding is that giving high doses of prenatal iron to women who do not really need it could lead to excess iron, causing oxidative stress and even iron deposits that, in turn, could impair foetal brain development and lead to a poorer behavioural and psychological performance in infancy (Lavezzi et al., 2011; Zerem et al., 2021). Recent research has noted that epigenetics may also play a role in the association between maternal iron status and children's behavioural development by differential DNA methylation (Taeubert et al., 2022). As the authors explained, oxidative stress would be the key behind this physiological process as it leads to a reduction in enzymatic activity important during DNA demethylation (Niu et al., 2015).

Strengths and limitations

The main strengths of the present study were (1) the original study was a population-based triple-blind randomized controlled trial, which is a very robust design; (2) comprehensive data on sociodemographic, clinical, and lifestyle information of the mothers were recorded; and (3) the assessment of children's behaviour and psychological problems was conducted comprehensively using reliable and internationally recognized tests. However, some limitations should be kept in mind when interpreting our results. The main limitation was the small sample size of the subgroups analysed because of high losses to follow-up which, despite being common in population-based intervention studies and even more so when prolonged follow-up is required, could have weakened the statistical power of the analyses. In addition, unmeasured or unknown risk factors could have resulted in some residual confounding even after adjusting for known potential confounders.

CONCLUSIONS

Adjusting prenatal iron supplementation to maternal baseline iron stores even in non-anaemic women improves behaviour and reduces the risk of psychological problems in children aged 4 years. Briefly, taking 80 mg/d of iron was related to lower scores of any psychological problems when women had low iron stores but increased the risk of internalizing and externalizing problems when mothers had normal-high iron stores. On the other hand, taking 20 mg/d of iron decreased the anxiety and the risk of autism spectrum problems only in those children whose mothers had SF>65 μg/L in early pregnancy. As for executive functioning, it was improved in the group of children whose mothers who took high-dose prenatal iron had ID in early pregnancy but worsened if the mothers had normal-high iron stores. Therefore, our data experimentally support that prenatal high-dose iron supplementation is beneficial for women with ID without anaemia in early pregnancy and for their children, whereas it appears to lead to iron excess iron in those who enter gestation with full iron stores, which may lead to negative mental health outcomes in children later in life. The latter as well as their offspring would benefit from the use of prenatal iron supplements in low doses, as reflected in behavioural developmental outcomes and psychological problems in children. the present results provide valuable information for obstetricians and midwives in pregnancy planning services. Given that SF concentration is not commonly measured in clinical practice, key information is being lost in deciding the best dose of prenatal iron supplementation for each woman. For this reason, routine measuring of SF in addition to Hb concentration from early pregnancy would be advisable to be able to help women to reach and maintain good iron status along gestation which will result in better behavioural development and fewer psychological problems for their children. Further studies would be useful to replicate and verify the present findings.

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The quality of the present population-based randomized controlled trial has been assessed by the Consolidated Standards of Reporting Trials (CONSORT).

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Appendix Table 1. Maternal characteristics according to their baseline iron status and dose of iron supplementation.

	***Stratum* 1**					
	SF<15 µg/L		**SF 15-65 µg/L**		**SF>65 µg/L**	
	C (n=16)	**I (n=13)**	**C (n=63)**	**I (n=61)**	**C (n=11)**	**I (n=18)**
Baseline						
Age, years	32 ± 7	33 ± 5	32 ± 6	32 ± 6	30,5 ± 8	31 ± 6
Parity, yes	81,3 [13]	85,7 [11]	56,5 [36]	52,1 [32]	58,3 [6]	52,6 [9]
Pregnancy planning, yes	75,0 [12]	85,7 [11]	88,4 [56]	74,6 [46]	100 [11]	89,5 [16]
Body mass index						
Underweight	0 [0]	7,1 [1]	2,9 [2]	0 [0]	0 [0]	5,3 [1]
Normal weight	87,5 [14]	57,1 [7]	59,4 [37]	47,9 [29]	58,3 [6]	73,7 [13]
Overweight	6,3 [1]	14,3 [2]	21,7 [14]	39,4 [24]	41,7 [5]	10,5 [2]
Obesity	6,3 [1]	21,4 [3]	15,9 [10]	12,7 [8]	0 [0]	10,5 [2]
Smoking, yes	6,3 [1]	7,1 [1]	14,5 [9]	19,7 [12]	8,3 [1]	15,8 [3]
Family SES						
High	18,8 [3]	14,3 [2]	26,1 [16]	21,1 [13]	33,3 [3]	26,3 [5]
Middle	75,0 [12]	71,4 [9]	68,1 [43]	62 [38]	50,0 [6]	57,9 [10]
Low	6,3 [1]	14,3 [2]	5,8 [4]	16,9 [10]	16,7 [2]	15,8 [3]
HFE gene mutation, yes	50,0 [8]	50,0 [7]	50,0 [32]	50,0 [31]	36,4 [4]	31,3 [6]
Parental IQ approximation	8,40 (4,32)	9,62 (3,66)	9,78 (3,30)	8,45 (3,09)	9,83 (4,30)	8,67 (3,05)
Whole pregnancy						
Physical activity						
Low	26,7 [4]	23,1 [3]	27,6 [17]	22,2 [14]	20,0 [2]	25,1 [5]
Moderate	73,3 [12]	76,9 [10]	69 [43]	73,0 [45]	50,0 [6]	75,0 [13]
High	0 [0]	0 [0]	3,4 [3]	4,8 [3]	30,0 [3]	0 [0]
Anxiety*						
Trait	17,45 (8,84)	15,48 (10,57)	12,47 (7,54)	16,89 (8,74)	15,69 (7,70)	12,02 (8,95)
State	17,43 (7,80)	16,57 (7,24)	13,44 (7,06)	16,95 (7,79)	15,54 (5,95)	12,81 (6,99)
After delivery						
Postpartum depression†	5,67 (5,55)	6,67 (6,18)	5,81 (3,92)	8,51 (5,51)	6,20 (4,05)	6,40 (3,03)

Data are expressed in mean (SD) for continuous normally distributed variables, median ± interquartile range for continuous non-normally distributed variables, and % [n] for categorical variables.

C, control; I, intervention; SF, serum ferritin; SES, socioeconomic status.

The control group always received 40 mg/d of iron while the intervention group received 80 mg/d of iron in Stratum 1 and 20 mg/d of iron in Stratum 2.

*Measured by STAI questionnaire. The score ranges from 0 to 60 points.

†Measured by Edinburg questionnaire. The score ranges from 0 to 30 points.

Continue Appendix Table 1. Maternal characteristics according to their baseline iron status and dose of iron supplementation.

	***Stratum* 2**					
	SF<15 µg/L		**SF 15-65 µg/L**		**SF>65 µg/L**	
	C (n=8)	**I (n=5)**	**C (n=39)**	**I (n=38)**	**C (n=9)**	**I (n=10)**
Baseline						
Age, years	32,5 ± 11	32 ± 7	33 ± 8	33 ± 6	29 ± 4	29 ± 6
Parity, yes	87,5 [7]	60,0 [3]	47,5 [18,5]	56,1 [2]	36,4 [3]	27,3 [3]
Pregnancy planning, yes	87,5 [7]	100 [5]	77,5 [30]	90,2 [34]	100 [9]	72,7 [7]
Body mass index						
Underweight	12,5 [1]	0 [0]	0 [0]	0 [0]	0 [0]	9,1 [1]
Normal weight	50,0 [4]	40,0 [2]	47,5 [19]	61,0 [23]	36,4 [3]	54,5 [5]
Overweight	25,0 [2]	20,0 [1]	46,2 [18]	22,0 [9]	36,4 [3]	18,2 [2]
Obesity	12,5 [1]	40,0 [2]	5,0 [2]	17,1 [6]	36,4 [3]	18,2 [2]
Smoking, yes	12,5 [1]	20,0 [1]	25 [10]	17,1 [6]	27,3 [2]	18,2 [2]
Family SES						
High	25,0 [2]	40,0 [2]	27,5 [11]	14,6 [5]	18,2 [2]	27,3 [3]
Middle	75,0 [6]	60,0 [3]	70,0 [27]	70,7 [28]	66,7 [6]	63,6 [6]
Low	0 [0]	0 [0]	2,5 [1]	14,6 [5]	9,1 [1]	9,1 [1]
HFE gene mutation, yes	57,1 [5]	40,0 [2]	41,4 [16]	24,3 [9]	44,4 [4]	33,3 [3]
Parental IQ approximation	9,75 (5,39)	8,50 (3,42)	9,28 (3,39)	8,50 (3,73)	7,30 (2,95)	9,75 (5,63)
Whole pregnancy						
Physical activity						
Low	25,0 [2]	40,0 [2]	34,4 [13]	28,1 [11]	44,4 [4]	30,0 [3]
Moderate	62,5 [5]	60,0 [3]	59,4 [23]	65,6 [25]	55,5 [5]	70,0 [7]
High	12,5 [1]	0 [0]	6,3 [3]	6,3 [2]	0 [0]	0 [0]
Anxiety*						
Trait	9,72 (6,32)	15,15 (7,12)	14,40 (8,00)	13,62 (9,59)	13,11 (4,16)	16,55 (10,43)
State	11,78 (4,48)	18,85 (6,52)	15,63 (7,28)	13,41 (7,09)	15,14 (5,18)	17,50 (8,71)
After delivery						
Postpartum depression†	2,40 (1,95)	5,80 (2,49)	7,38 (4,64)	7,80 (6,42)	5,17 (5,31)	6,56 (4,25)

Data are expressed in mean (SD) for continuous normally distributed variables, median ± interquartile range for continuous non-normally distributed variables, and % [n] for categorical variables.

C, control; I, intervention; SF, serum ferritin; SES, socioeconomic status.

The control group always received 40 mg/d of iron while the intervention group received 80 mg/d of iron in Stratum 1 and 20 mg/d of iron in Stratum 2.

*Measured by STAI questionnaire. The score ranges from 0 to 60 points.

†Measured by Edinburg questionnaire. The score ranges from 0 to 30 points.

Appendix Table 2. CBCL1½-5 scores at 4y by daily doses of prenatal iron supplementation by *Strata* and maternal baseline iron stores (n=230).

	SF<15 µg/L					**SF 15-65 µg/L**					**SF>65 µg/L**				
	40 mg/d (n=11)		**80 mg/d (n=10)**		**p**	**40 mg/d (n=54)**		**80 mg/d (n=50)**		**p**	**40 mg/d (n=11)**		**80 mg/d (n=15)**		**p**
	Mean	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**	
***Stratum* 1 (Hb 110-130 g/L)**															
Internalizing Problems	**57,00**	**15,83**	**51,70**	**11,45**	**0,018**[a]	57,00	11,60	53,26	11,04	0,095	**55,53**	**11,36**	**60,67**	**13,60**	**0,033**[a]
Externalizing Problems	**55,18**	**13,78**	**51,50**	**7,25**	**0,045**[a]	52,37	9,63	55,26	10,80	0,152	52,67	13,66	54,87	9,97	0,253
Total Problems	56,91	17,12	51,30	9,89	0,076	52,76	10,59	57,00	12,10	0,060	56,60	12,66	57,89	17,11	0,134
DSM Scales															
Depressive Problems	**58,82**	**9,15**	**53,40**	**5,23**	**0,017**[b]	55,78	6,75	57,88	7,86	0,146	57,60	8,07	58,22	10,17	0,270
Anxiety Problems	**58,00**	**14,02**	**55,70**	**5,79**	**0,026**[a]	56,83	6,97	59,24	9,18	0,138	**56,93**	**6,93**	**59,22**	**7,95**	**0,046**[b]
Autism Spectrum Problems	59,36	9,23	55,60	6,92	0,068	59,24	7,28	56,46	7,23	0,054	58,67	8,15	59,78	10,18	0,171
Attention-Deficit/Hyperactivity Problems	59,45	9,41	53,80	5,45	0,057	55,98	5,55	58,5	8,58	0,082	56,78	9,71	57,67	6,03	0,783
Oppositional Defiant Problems	57,55	9,19	52,20	2,15	0,087	54,31	6,10	55,48	6,87	0,362	54,67	6,61	55,22	6,92	0,086
***Stratum* 2 (Hb>130 g/L)**	**40 mg/d (n=5)**		**20 mg/d (n=5)**		**p**	**40 mg/d (n=27)**		**20 mg/d (n=27)**		**p**	**40 mg/d (n=7)**		**20 mg/d (n=8)**		**p**
	Mean	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**	
Internalizing Problems	42,80	23,92	47,00	13,25	0,743	54,00	10,69	56	13,28	0,545	58,14	7,36	53,75	12,44	0,429
Externalizing Problems	40,20	20,04	42,40	7,40	0,824	52,52	12,08	54,7	10,23	0,477	57,38	16,57	53,86	10,81	0,095
Total Problems	47,00	14,78	42,80	9,04	0,603	55,63	9,59	54,96	13,70	0,837	57,14	9,03	54,75	14,91	0,833
DSM Scales															
Depressive Problems	43,20	23,56	51,60	3,05	0,472	57,19	7,91	57,93	7,25	0,721	58,38	7,23	56,57	7,16	0,636
Anxiety Problems	48,00	27,52	53,00	5,61	0,701	57,96	9,46	56,63	6,05	0,540	57,57	6,48	55,88	6,27	0,065
Autism Spectrum Problems	47,20	25,67	54,20	5,50	0,568	57,52	8,29	56,89	8,15	0,780	58,43	2,70	55,88	6,40	0,059
Attention-Deficit/Hyperactivity Problems	45,80	24,07	50,80	0,84	0,667	56,44	8,62	58,93	8,54	0,293	62,00	10,41	59,88	9,64	0,688
Oppositional Defiant Problems	43,00	22,20	50,80	0,45	0,476	54,37	7,55	55,44	6,71	0,583	58,50	9,52	54,29	4,92	0,297

CBCL, Child Behavior Checklist; SF, serum ferritin; Hb, haemoglobin. Statistically significant associations are highlighted in bold.

Cohen's D for assessing effect size was indicated as follows: [a]Large effect size (>0.8), [b]Medium effect size (>0.3-0.8), [c]Low effect size (0.2-0.3).

Appendix Table 3. TRF1½-5 scores at 4y by daily doses of prenatal iron supplementation by *Strata* and maternal baseline iron stores (n=190).

	SF<15 μg/L					**SF 15-65 μg/L**					**SF>65 μg/L**				
	40 mg/d (n=9)		**80 mg/d (n=9)**		**p**	**40 mg/d (n=42)**		**80 mg/d (n=45)**		**p**	**40 mg/d (n=11)**		**80 mg/d (n=7)**		**p**
	Mean	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**	
***Stratum* 1 (Hb 110-130 g/L)**															
Internalizing Problems	46.00	9.77	43.11	4.54	0.438	47.18	8.71	51.07	10.28	0.664	46.00	9.96	47.86	9.10	0.696
Externalizing Problems	45.56	5.98	44.67	8.43	0.800	50.26	8.50	49.91	10.52	0.864	48.57	6.50	51.45	6.96	0.393
Total Problems	44.89	8.51	43.67	5.07	0.716	47.11	10.56	49.45	9.66	0.538	48.14	6.54	49.91	7.91	0.629
DSM Scales															
Depressive Problems	53.11	6.86	51.22	3.67	0.477	52.82	4.36	54.79	7.56	0.146	53.64	5.56	55.29	6.29	0.567
Anxiety Problems	51.22	2.54	50.89	2.57	0.588	53.02	4.63	54.38	5.54	0.220	53.86	4.91	53.82	6.54	0.989
Autism Spectrum Problems	51.22	2.22	52.11	2.89	0.475	43.22	5.41	54.17	7.10	0.489	53.82	8.59	50.86	0.69	0.381
Attention-Deficit/Hyperactivity Problems	51.00	2.29	52.89	5.18	0.332	54.67	6.28	54.56	6.63	0.936	53.14	3.39	54.55	5.20	0.538
Oppositional Defiant Problems	51.00	2.65	45.32	2.12	1.000	53.71	5.59	52.93	4.12	0.458	53.00	2.89	52.64	2.84	0.796
***Stratum* 2 (Hb>130 g/L)**	**40 mg/d (n=3)**		**20 mg/d (n=5)**		**p**	**40 mg/d (n=25)**		**20 mg/d (n=22)**		**p**	**40 mg/d (n=6)**		**20 mg/d (n=6)**		**p**
	Mean	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**	
Internalizing Problems	47.33	11.93	43.60	10.41	0.657	50.44	10.14	48.55	7.85	0.482	48.83	10.36	45.83	13.14	0.670
Externalizing Problems	57.67	10.07	43.60	8.08	0.071	53.40	11.98	50.32	10.63	0.359	50.33	9.59	51.00	10.47	0.911
Total Problems	52.67	11.68	41.40	10.81	0.214	52.04	12.62	49.86	9.36	0.510	47.83	12.48	48.83	11.75	0.889
DSM Scales															
Depressive Problems	52.67	4.62	50.80	1.79	0.562	54.04	4.77	54.64	5.22	0.684	53.83	7.60	54.17	6.59	0.937
Anxiety Problems	52.67	4.62	52.80	6.26	0.974	54.08	5.48	54.50	4.87	0.784	55.33	4.63	53.17	5.00	0.454
Autism Spectrum Problems	53.67	6.35	51.40	2.61	0.606	55.00	7.64	53.36	4.49	0.370	53.33	3.67	53.17	5.85	0.954
Attention-Deficit/Hyperactivity Problems	55.33	8.39	51.40	2.61	0.505	56.72	10.75	56.18	7.41	0.845	55.17	6.04	55.17	7.14	1.000
Oppositional Defiant Problems	55.00	6.25	50.60	1.34	0.346	55.40	7.06	53.41	4.07	0.237	55.33	6.65	53.50	6.32	0.635

TRF, Teacher Reported Form; SF, serum ferritin; Hb, haemoglobin. Statistically significant associations are highlighted in bold.

Cohen's D for assessing effect size was indicated as follows: [a]Large effect size (>0.8), [b]Medium effect size (>0.3-0.8), [c]Low effect size (0.2-0.3).

Summary of the results

The following pages include a relation of the main results from each work that conforms to this thesis:

What is known about the effect of prenatal iron status on child neurodevelopment?

- **Effects of prenatal iron status on child neurodevelopment and behavior: A systematic review**

  The prevailing consensus suggests that an imbalance of iron levels during pregnancy, both deficit and excess, can adversely affect the mental and psychomotor development of a child. However, contrasting viewpoints have emerged from different authors, who have not found a significant correlation between low iron levels and neurodevelopmental issues. Moreover, very little research has considered excess iron during pregnancy and its effects on child neurodevelopment.

  In view of these findings, emerging evidence suggests that prenatal iron supplementation should be personalized for each woman, taking into consideration several factors that can modify their iron needs such as existing iron stores, specific genetic mutations, and other health habits.

Determinants of maternal iron status

- **Maternal factors associated with iron deficiency without anaemia in early pregnancy: ECLIPSES study**

  Among the 791 non-anaemic participants in the ECLIPSES study, 13.9% experienced iron deficiency in early pregnancy. Factors such as being underweight and multiparity increased the likelihood of iron deficiency, while higher consumption of total meat, red/processed meat, protein, and dietary iron had a protective role. Smoking was associated with fewer odds of iron deficiency.

Pregnancy planning policies should prioritize women who are at higher risk of developing iron deficiency, such as those who are underweight, have multiple children, or follow vegetarian diets.

- **Iron status in mid-pregnancy and associations with interpregnancy interval, hormonal contraceptive use, dietary factors, and supplement use: A prospective pregnancy cohort study**

Among the 2990 participants from The Norwegian Mother, Father, and Child Cohort Study (MoBa cohort), depleted iron stores were more common among women with interpregnancy interval (IPI) < 6 months (56 %) and 6-11 months (33 %) than among those with IPI 24-59 months (19 %) and among nulliparous women (5%). Positively associated factors with iron status included hormonal contraceptives, age, body mass index, smoking, meat consumption and multi-supplement use.

Our results highlight the importance of ferritin measurements in women of childbearing age, especially among women not using hormonal contraceptives and women with previous and recent childbirths.

Effect of adapting prenatal iron supplementation on maternal iron status

- **Effectiveness of different doses of iron supplementation and prenatal determinants of maternal iron status in Spanish women: ECLIPSES Study**

In women with initial haemoglobin levels between 110 and 130 g/L, the prenatal use of 80 mg/d instead of 40 mg/d of iron protected against iron deficiency on gestational week 36. Only women with iron deficiency in early pregnancy benefited from the protection against anaemia and iron deficiency anaemia by increasing haemoglobin levels.

In women with initial haemoglobin levels <130 g/L, the prenatal use of 20 mg/d instead of 40 mg/d of iron reduced the risk of haemoconcentration when they had good iron stores (serum ferritin ≥15 μg/L), while 40 mg/d of iron improved serum ferritin levels on gestational week 36 in those with iron deficiency in early pregnancy.

Mutations in the *HFE* gene increased the risk of haemoconcentration.

Prenatal iron supplementation should be adjusted to early pregnancy levels of haemoglobin and iron stores. Mutations of the *HFE* gene should be evaluated in women with normal-high haemoglobin levels in early pregnancy.

Effect of adapting prenatal iron supplementation on the child's neurodevelopment

- **Adapting prenatal iron supplementation to maternal needs results in optimal child neurodevelopment: a follow-up of the ECLIPSES Study**

  In pregnant women without anaemia in early pregnancy, no differences were found in the cognitive, language and motor development of children at 40 days of age between the dose of iron tested in each case (80 or 20 mg/d) -adjusted to initial haemoglobin levels- compared to the dose of the control group (40 mg/d).

- **Importance of maternal iron status on the improvement of cognitive function in children after prenatal iron supplementation.**

  In women with initial haemoglobin levels between 110 and 130 g/L, the prenatal use of 80 mg/d instead of 40 mg/d of iron was associated with a better cognitive performance of their children, measured by WPPSI-IV and NEPSY-II scales, when mothers had initial iron deficiency. However, it resulted in poorer results on Verbal Comprehension Index, Working Memory Index, Processing Speed Index, and Vocabulary Acquisition Index from WPPSI-IV and verbal fluency index from NEPSY-II when mothers showed initial serum ferritin above 65 μg/L.

In women with initial haemoglobin levels >130 g/L, the prenatal use of 20 mg/d instead of 40 mg/d of iron was associated with their children obtaining better results on Working Memory Index, IQ, verbal fluency, and emotion recognition indices when women had initial serum ferritin above 65 μg/L.

- **Prenatal iron supplementation adjusted to maternal iron stores in early pregnancy improves behaviour in children at 4 years of age**: **ECLIPSES study**

In women with initial haemoglobin levels between 110 and 130 g/L, the prenatal use of 80 mg/d instead of 40 mg/d of iron was associated with fewer behavioural and psychological problems in their children, measured by CBCL 1½–5 scale, when mothers had initial iron deficiency. However, it resulted in more behavioural and psychological problems for their children when mothers showed initial serum ferritin above 65 μg/L.

In women with initial haemoglobin levels >130 g/L, the prenatal use of 20 mg/d instead of 40 mg/d of iron reduced the behavioural and psychological problems of their children, when women had initial serum ferritin above 65 μg/L.

General discussion

Extensive research has been conducted on the adverse effects of prenatal iron deficiency and iron deficiency anaemia on pregnancy outcomes and maternal and child health (Benson et al., 2022). On the other hand, although it has been less studied, increasing evidence suggests that iron excess during pregnancy can also be associated with health risks (Brannon & Taylor, 2017; Quezada-Pinedo et al., 2021).

The systematic review that forms part of this thesis sheds light specifically on the effect of maternal iron status on child neurodevelopment. Among the included studies, prenatal iron deficiency was linked to worse scores on language development, diminished cognitive and motor abilities, and reduced educational achievement of the offspring (Chang et al., 2013; Fararouei et al., 2010; Hernández-Martínez et al., 2011; Mireku et al., 2015; Tamura et al., 2002; Tran et al., 2013, 2014). Low levels of circulating iron in mothers were also related to altered child behaviour, including infant irritability (Hernández-Martínez et al., 2011; Vaughn et al., 1986). On the other hand, high prenatal iron levels have been associated with impaired cognitive development and increased behavioural problems in children (Mireku et al., 2015; Tamura et al., 2002; Yang et al., 2010). Overall, agreeing with former studies (Brannon & Taylor, 2017; Dewey & Oaks, 2017), our findings suggest a U-shaped association between maternal iron status and child neurodevelopment, indicating that both iron deficiency and excess during pregnancy can impact foetal neurodevelopment and long-term health outcomes.

As an update since the completion of our systematic review, a recent systematic review consistently supports our findings, also highlighting controversial results (Quezada-Pinedo et al., 2021). Additionally, a prospective cohort study has recently found that high maternal ferritin levels are associated with poorer child cognitive abilities and smaller brain volume (Sammallahti et al., 2021).

As for prenatal iron supplementation, while it has been shown to improve child cognitive and behavioural development (Chang et al., 2013; Hanieh et al., 2013; Q. Li et al., 2009; Prado et al., 2012; Schmidt et al., 2014; Wehby & Murray, 2008) in some cases, most of these studies were conducted in developing countries or anaemic women. On the contrary, routine iron supplementation in well-nourished women without iron deficiency may have no clear benefit and could even have negative effects on child neurodevelopment (Parsons et al., 2008; Zhou et al., 2006), with an excessive increase in maternal iron status proposed as a mediator for these findings (Georgieff et al., 2019; Hanieh et al., 2013). Nowadays, there is still no consensus on guidelines for prenatal iron supplementation in non-anaemic women in early pregnancy. In this context, the debate about the risks and benefits of routine versus personalized iron supplementation is ongoing (Brannon & Taylor, 2017; Georgieff et al., 2019), but little intervention research has addressed this approach.

Effect of adjusting prenatal iron supplementation on maternal iron status

Due to the current absence of a consensus regarding the ideal iron dosage to promote maternal and child health during pregnancy, coupled with the limited information on the potential risks and benefits of iron supplementation in non-anaemic women, this thesis aimed to provide further insights into the effectiveness of different doses of prenatal iron supplementation on maternal iron status towards the end of gestation. In this regard, the goal of the ECLIPSES study was to prevent both iron deficiency and iron excess in the last trimester of pregnancy by means of the most accurate dose of iron for each woman, considering their baseline iron status and other conditions.

In order to put this discussion into context, the methodological design of the ECLIPSES study consisted of two groups according to the woman's haemoglobin level in early pregnancy (before gestational week 12): group 1, if haemoglobin levels were between 110 and 130 g/L (hereafter referred to as “normal haemoglobin levels”);

and group 2, if they were above 130 g/L (hereafter referred to as "normal-high haemoglobin levels"). Within each group women were randomly assigned to receive one or the other dose of iron: women in group 1 received 80 or 40 mg/d while those in group 2 received 20 or 40 mg/d. Because iron supplementation is *a priori* recommended during pregnancy to ensure proper development of the pregnancy and optimal health outcomes for both mother and child, it would be unethical to consider a control group without any supplementation. Thus, given that the commonly prescribed dose in Spain is 40 mg of iron daily, this was considered the control group in each case. Thus, the novelty of the ECLIPSES study compared to previous studies was to test different doses of prenatal iron supplementation according to maternal haemoglobin concentration at the start of pregnancy and, in addition, to analyse their effects according to the mother's initial iron stores. Maternal iron stores were defined as "iron deficient" when serum ferritin concentration was below 15 μg/L, and "normal iron stores" when it was above 15 μg/L.

First, since anaemia was an exclusion criterion for participating in the ECLIPSES study, none of the participants was anaemic at the beginning of pregnancy; however, a significant proportion (around 14%) showed iron deficit at that time. This recalls the concept of "iron deficiency without anaemia", which has gained prominence in recent times and implies that pre-anaemia iron deficiency states, based on iron stores, can be as detrimental to health as anaemia itself and should receive more attention in clinical practice (Al-Naseem et al., 2021).

In general terms, we observed that the intervention by adjusting prenatal iron supplementation to the initial maternal haemoglobin levels, successfully corrected maternal iron status when compared with the estimates of the prevalence of anaemia and haemoconcentration during pregnancy. While the prevalence of anaemia in Europe is around 25% in pregnant women (Milman et al., 2017; Stevens et al., 2013; WHO, 2008), we found that only 8.3-13% of participants at risk developed it at the end of pregnancy. On the other hand, despite the estimates indicating that up to 42% of women suffer from haemoconcentration in industrialized countries (Arija et al., 2013; Peña-Rosas & Viteri, 2009), we observed a prevalence of 6.8-24% among participants at risk of iron excess.

Moving to the specific effects of adjusted iron supplementation, the study found that a higher than commonly prescribed dose of iron, in this case daily 80 mg of iron, compared to a daily 40 mg, improved serum ferritin levels and protected against iron deficiency at the end of pregnancy in women who began the gestation with normal haemoglobin levels. Moreover, taking 80 mg/d of iron reduced the risk of anaemia and iron deficiency anaemia during the last months of gestation only in women with iron deficiency at the start of the pregnancy. In contrast, no significant effect was observed in women with good iron stores on gestational week 12. This finding is consistent with the physiological regulation of intestinal iron absorption in accordance with iron reserves, by which the body strongly regulates iron absorption when stores are sufficient. These results suggest that the usual prescribed dose of 40 mg daily would be effective in women with optimal initial iron reserves, but not in women with iron deficiency in early pregnancy.

On the other hand, our results showed that among women who began pregnancy with normal-high haemoglobin levels, those who received a daily dose of 20 mg of iron, as compared to 40 mg, experienced a reduction in the risk of haemoconcentration in the third trimester of gestation without increasing the risk of iron deficit, but only when they had sufficient baseline iron stores. These findings suggest that doses lower than the usual prescribed dose of 40 mg daily may be more appropriate for women with adequate iron status at the start of pregnancy.

Overall, iron supplementation was found to have variable effects on women's haematological parameters depending on their initial iron status. In relation to this, a wide range of maternal characteristics was explored in this thesis to unravel their potential impact in modifying women's iron status in early- and mid-pregnancy. Thus, based on data from the ECLIPSES study and the Norwegian Mother, Father, and Child Cohort Study (MoBa), many factors beyond supplement use have been described as potential determinants of baseline maternal iron status, including multiparity, short interpregnancy intervals, the use of contraceptives prior to pregnancy, dietary factors, pre-pregnancy body mass index, and smoking (see "Chapter 2. Determinants of iron status" for further details).

The role of *HFE* gene mutations in this context deserves special mention. It is worth mentioning that among the population of the study, there were no individuals homozygous for the C282Y allele, which has the highest clinical penetrance (Crownover & Covey, 2013; Grosse et al., 2017). The most prevalent genotype was the heterozygous H63D, which accounted for 26.1% of the study population. While the frequency of the C282Y allele has a decreasing gradient from north to south of Europe, the H63D allele has been found widely distributed (Lucotte & Dieterlen, 2003; Merryweather-Clarke et al., 2000). The H63D frequency observed in the ECLIPSES study was similar to those previously reported in Catalonia (24%), other Spanish regions such as Basque Country (30.4%), as well as other countries across Europe such as The Netherlands (29.5%) and Germany (22%) (Altes et al., 2004; Gottschalk et al., 1998; Merryweather-Clarke et al., 1997). The ECLIPSES study showed that pregnant women with normal-high haemoglobin levels in early pregnancy were more likely to develop haemoconcentration in the third trimester than those who started pregnancy with normal haemoglobin levels. The main hypothesis to explain this observation lies in the influence of a possible alteration in the *HFE* gene. Indeed, this was confirmed by the finding that, among women with normal-high haemoglobin levels, those who eventually showed haemoconcentration in the third trimester presented a greater frequency of the H63D allele than that of the wild-type genotype than their counterparts without haemoconcentration. Additionally, the risk of haemoconcentration was found to be around three times higher among carriers of the H63D allele compared to wild-type participants, regardless of the dose of iron that women received. Thus, although mutations in the *HFE* gene were not found to be associated with iron-related biomarker concentrations in early pregnancy, our results suggest that it was an important condition associated with maternal haemoconcentration in late pregnancy, which is extensively supported by previous literature (Bacon & Britton, 2008; Burke et al., 2000; Hanson et al., 2001; Pedersen & Milman, 2009). Gene regulation may explain why the effects of mutations in the *HFE* gene appear to be absent in early pregnancy and become evident as pregnancy progresses (Camaschella et al., 2020; Silva & Faustino, 2015). Hepcidin expression is tightly regulated by iron levels and requirements, among other mechanisms, in complex crosstalk involving several proteins, including the HFE protein (Rishi et al., 2015; Silva & Faustino, 2015). Briefly, in early pregnancy, when iron needs are relatively low, genes involved in iron metabolism, including the *HFE* gene, may remain less active. However, as pregnancy progresses, iron demands increase and systemic iron levels fall, the gene becomes more active to meet the increased needs. In women without mutations, this regulatory mechanism allows hepcidin to be properly regulated and the body's iron balance to be maintained. However, in women with *HFE* mutations, activation of the gene during pregnancy leads to an excessive increase in iron absorption, which can result in iron overload if the woman receives iron supplements (Barton et al., 2015; Hanson et al., 2001). Thus, even though the milder penetrance of the H63D allele, its moderate frequency in the Spanish population described above makes it important to consider *HFE* gene genotyping in women with normal-high haemoglobin levels in early pregnancy before prescribing iron supplements. This precaution may help to avoid excessive iron supply in women especially prone to develop haemoconcentration.

The findings presented in this study hold significant clinical and public health implications and can provide a foundation for the development of public health policies and clinical practices aimed at at-risk populations. Currently, clinical practice commonly relies on measuring only haemoglobin levels to assess the iron status of pregnant women. However, it is important to note that while haemoglobin concentration can detect anaemia, it does not identify iron deficiency. Based on our findings, it is strongly recommended to adjust prenatal iron supplementation according to women’s baseline iron status, considering not only haemoglobin levels but also iron stores. This approach is crucial to prevent both iron deficiency and iron overload during late pregnancy. Therefore, it is advisable to routinely measure serum ferritin levels during prenatal care, as this would contribute to better health outcomes for both the mother and the child. Furthermore, it would be also important to consider other factors such as sociodemographic, lifestyle, and genetic conditions that may affect the mother's iron status. By focusing efforts primarily on women who are most vulnerable to iron imbalance during pregnancy, healthcare professionals can effectively address iron-related issues and ensure optimal health for both mother and child.

Effect of adjusting prenatal iron supplementation on child neurodevelopment

The overall findings of the present thesis suggest, in relation to child neurodevelopment, that adjusting prenatal iron supplementation in non-anaemic women considering both their haemoglobin levels and iron stores at the beginning of pregnancy improves cognitive functions and reduces behavioural and psychological problems in children at 4 years of age, although no differences were observed in their cognitive, language, and motor development at 40 days of age. Little research assessing the effect of prenatal iron supplementation on the child's neurodevelopment has been conducted on well-nourished non-anaemic women, and studies until now only evaluated the effect of taking or not prenatal iron supplements, without considering different doses according to the individual needs of each woman. In this scenario, the results from the ECLIPSES and ECLIPSES-NEN studies provide valuable insights into the impact of adjusting prenatal iron supplementation on the child's neurodevelopment.

Previous studies evaluating the effects of routine prenatal supplementation with a single dose of iron failed to provide clear evidence of benefits on the child's cognitive development and IQ, nor on behavioural and psychological problems (Angulo-Barroso et al., 2016; Hanieh et al., 2013; Jayasinghe et al., 2018; Parsons et al., 2008). It could be argued in this regard that giving a single dose of prenatal iron to all women may be insufficient or excessive, depending on the case, leading some women to suffer from iron deficiency and others from haemoconcentration. In this regard, it should be mentioned that the risk of adverse outcomes depending on the iron status follows a U-shaped distribution, which indicates that both iron deficit and iron excess can result in detrimental health effects (Brannon & Taylor, 2017; Dewey & Oaks, 2017; Quezada-Pinedo et al., 2021). On the contrary, one of the main findings of the ECLIPSES-NEN study in relation to child neurodevelopment is that the variable effect of different doses of iron according to the mother's initial iron stores observed on haematological levels and maternal iron status at the end of pregnancy persists in cognitive performance and psychological functioning of children. This means that the effect of prenatal iron supplementation on the neurodevelopment of the child varies according to the mother's initial iron stores. As a brief reminder of the results, among women with normal haemoglobin levels at the beginning of pregnancy, the administration of a high-dose iron (80 mg) compared to the standard dose (40 mg) showed significant improvements in cognitive and executive functioning, as well as a reduction in psychological problems in their 4-year-old children. However, these positive effects were observed only in women who had iron deficiency during early pregnancy. On the contrary, when women started pregnancy with normal-high iron stores (serum ferritin above 65 μg/L), high-dose iron supplementation resulted in adverse outcomes in child cognitive functioning, including verbal development, working memory, and processing speed. It also contributed to increased internalizing, externalizing, and total problems, including ASD symptoms. Conversely, among women with adequate iron stores in early pregnancy, the standard dose of iron supplementation led to improved executive functioning in their children, including enhancements in working memory and processing speed. On the other hand, among women with normal-high haemoglobin levels at the beginning of pregnancy, low-dose iron supplementation (20 mg) compared to the standard dose demonstrated positive effects on working memory, IQ, verbal fluency, and emotion recognition index in their children, being the later a trait associated with ASD. However, these benefits were observed specifically when women had normal-high iron stores in early pregnancy. Based on our results, it becomes evident that high-dose prenatal iron supplementation is beneficial when women enter pregnancy with iron deficiency, as it supports positive neurodevelopmental outcomes in their children. However, caution is needed when administering high iron doses to iron-replete women, as it can lead to adverse cognitive, behavioural, and psychological outcomes in their offspring during childhood.

A beneficial effect on the child's neurodevelopment of providing a high dose of prenatal iron supplements when women start pregnancy with iron deficiency was an expected finding. Since maternal iron deficiency has been found negatively associated with child neurodevelopmental outcomes (Doom & Georgieff, 2014; Janbek et al., 2019; McWilliams et al., 2022; Quezada-Pinedo et al., 2021; Radlowski & Johnson, 2013; Zhu et al., 2023), a high iron dose would help women to face iron deficiency and prevent its detrimental effects. In contrast, this is the first time that the harmful impact of prenatal high-dose iron supplementation has on the offspring of iron-replete women is shown so clearly, as little research until now had experimentally addressed the possibility of iron excess following iron supplement use. These effects could be attributed to potential iron excess and its impact on foetal brain development. Iron deposition in specific areas crucial for cognitive functions may hinder brain formation and development, leading to poorer cognitive and neuropsychological outcomes in children. Additionally, oxidative stress and differential DNA methylation have been proposed as potential mechanisms linking maternal iron status to children's behavioural development. However, further research is necessary to validate these findings and better understand the underlying mechanisms.

Finally, regarding the absence of a significant association between different doses of prenatal iron supplementation and child neurodevelopment at 40 days of age, it is important to note that most children participating in the ECLIPSES study exhibited normal scores on the BSID-III scales for cognitive, language, and motor development at that age. Only a small percentage, 2.6%, 8.1%, and 3% respectively, scored below the normal range in these domains. However, when considering the subsequent findings at 4 years of age, it can be argued that the effects of prenatal iron supplementation on neurodevelopment manifest more prominently during childhood rather than early infancy. This could explain the lack of notable differences observed between the intervention groups at 40 days of age. Additionally, as mentioned earlier, the ECLIPSES intervention successfully reduced the prevalence of anaemia, iron deficiency, and haemoconcentration at the end of pregnancy compared to estimated rates. Consequently, it is plausible to hypothesize that the absence of significant differences between the prenatal iron dose groups could be attributed to the fact that most women in the study achieved an optimal iron status, which facilitated consistent and appropriate neurodevelopment across all groups in their children.

In summary, the study highlights the complex relationship between prenatal iron supplementation, maternal iron status, and children's neurodevelopment. The findings emphasize the significance of preventing both iron deficiency and excess during pregnancy. Achieving and maintaining an optimal iron status is crucial for ensuring positive neurodevelopmental outcomes in children. To achieve this, it is essential to adjust prenatal iron supplementation based on individual factors, considering not only the presence of anaemia but also the initial maternal iron stores. Routine measurement of serum ferritin concentration early in pregnancy, beyond monitoring haemoglobin levels, is critical in determining the appropriate iron supplementation dose for each woman. Unfortunately, the current clinical practice often overlooks this measurement, highlighting the need for improved assessment protocols and iron supplementation approaches to promote optimal neurodevelopmental outcomes in children.

Strengths and limitations

The studies presented in this thesis offer a comprehensive analysis of the effects of adjusting prenatal iron supplementation to the actual needs of each woman on their iron status and on children's neurodevelopment. The research has several key strengths that enhance the findings.

Firstly, the studies were designed with great robustness. The ECLIPSES study, for instance, was a triple-blinded community-based randomized controlled trial, while the MoBa cohort was an ongoing national birth cohort. These designs ensured that the research was conducted with high levels of scientific rigour, minimizing potential sources of bias. Specifically for the ECLIPSES study, the use of ferrimannitol ovalbumin instead of ferrous sulphate for prenatal iron supplementation was an innovative approach that aimed to reduce gastrointestinal side effects associated with iron supplementation. Furthermore, the data collected were extensive, including information on sociodemographic characteristics, biological conditions, and lifestyle factors. This enabled researchers to obtain a holistic view of the factors that may influence, first, the maternal iron status and then, the effect of adjusting prenatal iron supplementation, maternal iron status at the end of gestation, and child neurodevelopment. Additionally, the potential ongoing inflammatory processes were monitored by considering C-reactive protein concentrations as a confounding factor in the analyses. This helped to account for the possible effects of inflammation on both maternal iron status and child neurodevelopment. Moreover, the determination of *HFE* gene mutations allowed for the evaluation of the effect of genetic variability on iron metabolism and the potential impact of personalized iron supplementation. The measurement of serum ferritin and haemoglobin concentrations in both the first and third trimesters of gestation allowed for the ruling out of the possible effect of natural fluctuations in maternal iron status during pregnancy and the estimation of the true effect of prenatal iron supplementation on the child's neurodevelopment. Finally, the comprehensive and detailed assessment of children's neuropsychological functions, using internationally recognised and reliable tests, was another notable strength of the research.

Although the studies in this thesis provide valuable insights into the effect of adjusting prenatal iron supplementation on maternal iron status and child neurodevelopment, as well as the determinants of maternal iron status, it is important to interpret the findings with caution due to some limitations that may affect their validity.

As for the evaluation of the determinants of maternal iron status, the observational approach used both in the MoBa cohort and the ECLIPSES study may limit the external validity of the results as women's characteristics vary across populations. The studies only included non-anaemic women mostly Caucasians, which may limit the generalizability of the findings to other ethnic groups. Additionally, the interpregnancy interval was not available in the ECLIPSES study, limiting the interpretation of parity as a predictor of iron status. Dietary assessment using questionnaires is susceptible to misreporting bias, although potential bias was deemed to be low in these studies. Finally, recent blood donations, which reduce iron stores, were not considered. Regarding the ECLIPSES study as a randomized controlled trial, substantial drop-out could have weakened the statistical accuracy and negatively affected the intervention results. This is otherwise common in population-based intervention studies with long follow-ups and possible selection bias was addressed by comparing the characteristics of participants included and not included in the analyses, with no differences found between them. Additionally, maternal iron status at delivery was not available for inclusion in the analyses as women gave birth in hospitals that did not participate in the study. As for the assessment of child neurodevelopment, although the BSID-III is an internationally used and recognized tool for assessing the child's cognitive function, the neurodevelopmental assessment shows low stability in early childhood, which could have led to estimates of nullity. Finally, residual confounding due to unmeasured or unknown risk factors that may occur even after adjustment for known potential confounders must be considered.

Conclusions

This section summarises the main findings derived from a thorough analysis of the research objectives. It also aims to provide a clear and concise summary of the research contributions to the field, along with their implications and possible avenues for future research. The conclusions drawn from this thesis are the culmination of rigorous research, offering valuable insights and paving the way for future developments in the field.

In relation to maternal iron status:

- In women without anaemia in early pregnancy, administering prenatal iron supplementation adjusted to their specific iron needs has been shown to enhance maternal iron status, effectively reducing the risk of both iron deficiency and iron excess towards the end of gestation.

- In terms of maternal haematological status, the best results were obtained after prenatal iron supplementation as follows: daily 80 mg when women started pregnancy with normal haemoglobin concentrations but iron deficiency, daily 40 mg when they started pregnancy with normal haemoglobin concentrations and good iron reserves or when they started pregnancy with normal-high haemoglobin concentrations and iron deficiency, and daily 20 mg when they started pregnancy with normal-high haemoglobin concentrations and replete iron stores.

- Genetic mutations in the *HFE* gene play a role in the effect of prenatal iron supplementation, increasing the risk of haemoconcentration.

- Factors such as body mass index in early pregnancy, parity, smoking, and diet have been found to be associated with iron deficiency without anaemia during the early stages of pregnancy. Therefore, it is important for pregnancy planning policies to prioritize the management of iron status in underweight, multiparous, or vegetarian women, as they are at a higher risk of developing iron deficiency.

- Factors identified as predictors of maternal iron status in early and mid-pregnancy in our population and in the Norwegian population were similar. This has powerful clinical implications, as it provides a set of characteristics that are so strongly linked to iron status that they go beyond possible country-specific cultural and lifestyle differences. Thus, this facilitates decision-making when targeting public health policies towards specific population groups.

In relation to child neurodevelopment:

- In women without anaemia in early pregnancy, administering prenatal iron supplementation adjusted to their actual iron needs has been shown to enhance cognitive functioning and full IQ in their children at 4 years of age. However, no improvements in cognitive, language and motor development were observed when children were evaluated at 40 days of age.

- In women without anaemia in early pregnancy, administering prenatal iron supplementation adjusted to their actual iron needs has been shown to reduce behavioural and psychological problems in their children at 4 years of age.

- In terms of neuropsychological and behavioural development of children at 4 years of age, the best results were obtained after prenatal iron supplementation as follows: daily 80 mg when women started pregnancy with normal haemoglobin concentrations but iron deficiency, daily 40 mg when they started pregnancy with normal haemoglobin concentrations and good iron reserves, and daily 20 mg when they started pregnancy with normal-high haemoglobin concentrations and moderately high iron stores.

As final conclusions, highlighting the clinical implications of the findings of this thesis:

- Properly addressing iron needs during pregnancy through prenatal iron supplementation adjusted to individual requirements reduces the risk of iron imbalances in late pregnancy and positively impacts the long-term neurodevelopment of children.

- The experimental findings of this thesis support the benefits of high-dose prenatal iron supplementation for the neuropsychological and behavioural development of children born to women with low initial iron stores, even without anaemia. Conversely, for women with sufficient iron stores, low iron doses are more beneficial.

- When prescribing prenatal iron supplementation, it is essential to consider factors such as baseline iron stores, sociodemographic characteristics, genetic conditions, and lifestyle of pregnant women.

- Relying only on haemoglobin levels to determine the appropriate prenatal iron dosage is insufficient. Early pregnancy assessment of serum ferritin, which reflects maternal iron reserves, should be universally conducted as a routine diagnostic test for pregnant women and women of childbearing age intending to conceive, enabling healthcare professionals to comprehensively evaluate iron status and accurately prescribe prenatal iron supplementation.

- Genetic screening for mutations in the *HFE* gene can provide valuable insights into prenatal supplement use decisions. However, due to its high cost, it is recommended primarily for women with haemoglobin levels at the higher end of the normal range.

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Annexes

ANNEX 1. OTHER SCIENTIFIC CONTRIBUTIONS

To provide a comprehensive overview of the research conducted during this doctoral thesis, this annex is dedicated to including additional scientific contributions to the area that have been published in addition to the main body of the thesis.

- Lucía Iglesias-Vázquez, Anne-Claire Binter, Josefa Canals, Carmen Hernández-Martínez, Núria Voltas, Albert Ambròs, Laura Pérez-Crespo, Mònica Guxens, Victoria Arija. Association between prenatal exposure to air pollution and child's cognitive, language, and motor function in ECLIPSES study. Environmental Research, 2022. DOI: 10.1016/j.envres.2022.113501

- Victoria Arija, Cristina Jardí, Cristina Bedmar, Andrés Díaz, Lucía Iglesias, Josefa Canals. Supplementation of Infant Formula and Neurodevelopmental Outcomes: a systematic review. Current Nutrition Reports, 2022. DOI: 10.1007/s13668-022-00410-7

- Lucía Iglesias-Vázquez, Núria Serrat, Cristina Bedmar, Meritxell Pallejà-Millán, Victoria Arija. Prenatal folic acid supplementation and folate status in early pregnancy: ECLIPSES study. British Journal of Nutrition, 2021. DOI: 10.1017/S0007114521004840

- Lucía Iglesias-Vázquez, Victoria Arija, Núria Aranda, et al. Factors associated with serum ferritin levels and iron excess: results from the EPIC-EurGast study. European Journal of Nutrition, 2021. DOI: 10.1007/s00394-021-02625-w

- Andrés Díaz-López*, Lucía Iglesias-Vázquez*, Meritxell Pallejà-Millán, Cristina Rey Reñones, Gemma Flores Mateo, Victoria Arija. Association between iron status and incident type 2 diabetes: a population-based cohort study. Nutrients, 2020. DOI: 10.3390/nu12113249

- Lucía Iglesias Vázquez, Josefa Canals, Núria Voltas, Cristina Jardí, Carmen Hernández, Cristina Bedmar, Joaquín Escribano, Núria Aranda, Rosa Jiménez, Josep María Barroso, Blanca Ribot, Victoria Arija. Does the fortified milk with high iron dose improve the neurodevelopment of healthy infants? Randomized controlled trial. BMC Pediatrics, 2019. DOI: 10.1186/s12887-019-1679-0

- Lucía Iglesias Vázquez, Edith Valera, Marcela Villalobos, Mónica Tous, Victoria Arija. Prevalence of Anemia in Children from Latin America and the Caribbean and Effectiveness of Nutritional Interventions: Systematic Review and Meta–Analysis. Nutrients, 2019. DOI: 10.3390/nu11010183

- Mónica Tous Marcela, Villalobos, Lucía Iglesias Vázquez, Silvia Fernández-Barrés, Victoria Arija. Vitamin D status during pregnancy and offspring outcomes: a systematic review and meta-analysis of observational studies. European Journal of Clinical Nutrition, 2019. DOI: 10.1038/s41430-018-0373-x

- Lucía Iglesias Vázquez, Josefa Canals, Victoria Arija. Review and meta-analysis found that prenatal folic acid was associated with a 58% reduction in autism but had no effect on mental and motor development. Acta Paediatrica, 2019. DOI:10.1111/apa.14657

ANNEX 2. INTERNATIONAL RESEARCH COLLABORATIONS

This annex aims to document the enriching experiences of international research stays undertaken during this doctoral thesis, emphasizing the valuable opportunities for collaboration, knowledge exchange, and cross-cultural learning that have contributed to the overall research journey.

- **Université du Québec à Rimouski (UQAR)**
  Department of Biology, Chemistry and Geography. Québec, Canada
  Responsible: Dr Richard Saint-Louis
  Duration: 27 March to 9 July 2023

- **Poznań University of Life Sciences**
  Department of Human Nutrition and Dietetics. Poznań, Poland
  Responsible: Dr Joanna Suliburska
  Duration: 30 January to 12 February 2023

- **Cochrane Centre Chile**
  Unit of Public Health, University of Valparaíso. Valparaíso, Chile
  Responsible: Dr Eva Madrid
  Duration: 1 December to 31 December 2022

- **Norwegian Institute of Public Health (NIPH)**
  Unit of Environmental Toxicology and Epidemiology. Oslo, Norway
  Responsible: Dr Cathrine Thompsen
  Duration: 29 January to 15 April 2018