Universidad de Granada

FACULTAD DE MEDICINA
DEPARTAMENTO DE PEDIATRÍA

MARCADORES GENÉTICOS Y BIOQUÍMICOS EN RELACIÓN AL TRANSPORTE DE HIERRO EN EMBARAZADAS OBESAS Y DIABÉTICAS

Genetic and biochemical markers in relation to iron transport in obese and diabetics pregnant women.

TESIS DOCTORAL

Luz Mª García Valdés

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TESIS DOCTORAL

LUZ Mª GARCÍA VALDÉS

GRANADA, 2011

Memoria presentada por la licenciada

Luz Mª García Valdés

para optar al grado de Doctor en Biología

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CERTIFICAN: Que los trabajos de investigación que se exponen en la Memoria de Tesis Doctoral: **"**MARCADORES GENÉTICOS Y BIOQUÍMICOS EN RELACIÓN AL TRANSPORTE DE HIERRO EN EMBARAZADAS OBESAS Y DIABÉTICAS**",** han sido realizados en el Departamento de Pediatría de la Universidad de Granada y parcialmente en el Rowett Institute of Nutrition and Health, Aberdeen, Scotland, UK, correspondiendo fielmente a los resultados obtenidos. La presente Memoria ha sido revisada por los abajo firmantes, encontrándola conforme para ser defendida y aspirar al grado de Doctor Europeus en Biología.

Y para que conste, en cumplimiento de las disposiciones vigentes, extendemos el presente en el mes de Julio de 2011.

Prof. Dra. Dña. C. Campoy Folgoso

Prof. Dr. D. Harry McArdly **Prof. Dr. Juan A. Molina Font**

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GENERAL SUMMARY

1. Background

Optimal maternal nutrition is now widely recognised as being essential for optimal fetal growth and development and considerable interest is being shown in the way in which nutrition during pregnancy and after birth interacts to determine fetal and postnatal health. Once formed, the placenta is very efficient at transfer of nutrients to the conceptus because of the organization of the maternal and fetal vasculatures. Nonetheless, nutritional deprivation or other insults to placental function can compromise fetal development and cause adverse effects on the physiology of the offspring that can persist into adulthood.

Iron deficiency anaemia (IDA) is a common problema in pregnancy, and has been associated with adverse pregnancy outcomes. The consequences are serious for both the mother and her infant. Now we know that it is a risk factor for pre-term delivery and subsequent low birth weight.

During pregnancy, the absorbed iron is predominantly used to expand the woman's erythrocyte mass, fulfill the foetus's iron requirements and compensate for iron losses (i.e. blood losses) at delivery. Iron absorption is regulated by the size of body iron stores (Finch 1994). The increase in absorption is most pronounced after 20 weeks of gestation and peaks in late pregnancy.

During pregnancy, growth of the fetus and of the placenta, and the larger amount of circulating blood in the pregnant woman, lead to increase in the demand of nutrients, *one of them is iron.* The daily requirements for iron *for a woman in the last trimester of pregnancy* are six times greater than for a non-pregnant woman (Christensen RD 2004).

Markers of iron status are *haemoglobine, haematocrite, ferritin, transferrin, serum transferrin receptor, plasma transferrin saturation and others such serum iron, mean cell haemoglobin (MCH), mean cell volume (MCV).*

S-ferritin is considered to be a reliable blood test in the first trimester of pregnancy *for judging whether iron supplementation is necessary*. However, becomes less reliable after the 20th week due to the physiological dilution of the plasma, *which reduce more or less the concentration of S-ferritin indenpendently of changes in the iron stores* (Schwartz III, Thurnau 1995, Sandstad et al. 1996, Allen 2000). *Serum ferritin usually falls markedly between 12 and 25 wk of gestation and* the concentration reduces steadily to ~50% of normal at mid-gestation due to hemodilution and the mobilization of iron from stores to meet the increased needs of pregnancy *and expansion of the maternal blood cell mass* (Fenton, Cavill & Fisher 1977, Milman et al. 1999). Therefore, the status of iron stores in the second and third trimesters of pregnancy cannot be accurately determined by s-ferritin alone. The physiologic variations in ferritin during pregnancy may be compensated for by calculating body iron using the sTfR-to ferritin ratio (Cook, Flowers & Skikne 2003, Milman et al. 2006). Some investigators have suggested that the sTfR-to-ferritin ratio may be a better marker of body iron status than ferritin alone (Malope et al. 2001, Punnonen, Irjala & Rajamaki 1997).

During pregnancy, iron is transported *from the mother to the fetus* across the placental membrane by an active process, which is mediated via binding of maternal transferrin bound iron to transferrin receptors in placenta and subsequent transfer of iron into the fetal circulation (Fletcher, Suter 1969, Brown, Molloy & Johnson 1982). The efficiency of this transport system implies that iron deficiency in the newborn is encountered only at extreme iron deficiency in the mother, so that iron deficiency in mature newborn babies is a rare event in the developed countries. In the study by Rusia et al. (Rusia et al. 1996), serum transferrin receptor concentrations were higher in infants born to anemic mothers.

Furthermore, the ferritin-GDM association was modified by level of obesity. Obese women with high ferritin levels had a 3.5-fold increased risk of developing GDM (95% CI: 1.35, 9.27; p=0.01), whereas results were not significant among non-obese women.
Whereas placental transferrin receptor expression is increased in pregnancies complicated by diabetes mellitus, the affinity of the receptor to maternal transferrin is decreased,

probably due to hyperglycosylation of the oligosaccharides present in the binding domain (Georgieff et al. 1997). Furthermore, placental vascular disease might be present in mothers with longstanding, poorly controlled diabetes mellitus, further limiting iron transport across the placenta. Tissue iron is depleted to support the iron needs of augmented erythropoiesis under these situations. Nearly 65% of infants of diabetic mothers (IDM) have perinatal iron deficiency, as suggested by cord serum ferritin concentration <60 mg/L. In approximately 25% of these infants cord serum ferritin is <35 mg/L, suggesting significant depletion of tissue iron, including brain iron (Georgieff et al. 1990, Petry et al. 1992).

Until recently, few studies had considered body weight or body composition as factors related to iron deficiency. Many of them have shown that obesity might increase the risk of iron deficiency but, at the same time, obese subjects exhibit high serum ferritin levels.

Obesity is associated with alterations in iron metabolism. The two major characteristics are a deficit in serum iron levels and an increase in ferritin. Iron deficiency in obesity appears to be multifactorial and includes (i) A decrease in iron food intake; (ii) An impairment of intestinal iron uptake and iron release from stores because of an overexpression of hepcidin and (iii) Inadequate iron bioavailability because of inflammation. In addition, abnormal ferritin concentrations can be explained by chronic inflammation rather than by iron overload. Moreover, it appears that hypoferremia could be explained by both a true iron deficiency and a functional iron deficiency (Zafon, Lecube & Simó 2010). Pinhas-Hamiel *et al.* (Pinhas-Hamiel et al. 2003, Nead et al. 2004) have reported that low iron levels were present in 38.8% of obese children, in 12.1% of the overweight children and in only 4.4% of children of normal weigh. Another study has demonstrated that the prevalence of iron deficiency increases as body mass index (BMI) increases from normal weigh to overweight in a sample of nearly 10 thousand children and adolescents (Nead, 2004). In the adult population, one analysis from the Third National Health and Nutrition Examination Survey (NHANES III) showed that BMI was associated with significantly lower mean serum iron concentrations in women but not in men (Micozzi, Albanes & Stevens 1989). Conversely, it has also been suggested that iron

deficiency, especially perinatal iron deficiency, might lead to increased visceral adiposity (Komolova et al. 2008, McClung et al. 2008).

Serum sTfR seems to be a sensitive marker of iron deficiency in pregnancy, and by combining serum sTfR and serum ferritin measurements, the entire spectrum of iron status in pregnancy can be assessed (Carriaga et al. 1991, Åkesson et al. 1998). Three recent articles have analysed sTfR, and all of them have reported that the levels of sTfR are elevated in obese patients (Lecube et al. 2006, Freixenet et al. 2009, Yanoff et al. 2007). In addition, the chronic inflammation and increased leptin production characteristic of obesity increase hepcidin secretion from the liver (Chung et al. 2007), which, along with hepcidin produced by adipose tissue (Bekri et al. 2006), could reduce dietary iron absorption (Laftah et al. 2004).

Iron deficiency in obese individuals may result from different factors. Low iron intake, reduced iron absorption, and the sequestration of iron as a result of chronic inflammation in response to excess adiposity has been suggested among differents reasons. In regard to an iron-poor diet, low iron intake and increased iron needs have been reported among obese children and adolescents who are iron deficient (Pinhas-Hamiel et al. 2003, Nead et al. 2004, Hassapidou et al. 2006). Zimmerman et al. (Zimmermann et al. 2008) also reported that high BMI Z-scores were associated with decreased iron absorption in women independent of iron status and reduced improvement of iron status in iron-deficient children following intake of iron-fortified foods. They hypothesized that obesity may affect iron absorption through an inflammatory mediated mechanism.

The infiltration by and activation of macrophages in adipose tissue has also been linked to obesity-induced IR (Apovian et al. 2008). In accordance with this conception, obesity-associated iron abnormality has been interpreted as a feature that mimics the so-called anaemia of chronic inflammation, which is characterized by hypoferremia and high to normal serum ferritin concentration (hypoferremia and anaemia despite adequate iron stores) (Ausk, Ioannou 2008). This entity is also caused by increased inflammatory cytokines, especially IL-6, inducing increased production of the iron-regulatory hormone

hepcidin. Thus, hepcidin by means of its capacity to block iron release from macrophages, hepatocytes and enterocytes appears to be a major contributor to the hypoferremia associated with inflammation (Ganz 2006).

Hepcidin acts as an inhibitory iron regulator. Increased plasma hepcidin inhibits intestinal iron uptake and *acts* sequestering iron at the macrophage (Knutson et al. 2005), which could lead to decreased iron stores and hypoferremia. In accordance with this homeostatic model, iron loading increases hepcidin gene expression. Also, its production is suppressed by anaemia and hypoxaemia. Furthermore, hepcidin synthesis is markedly induced by infection and inflammation and because chronic disease (Park et al. 2001, Nicolas et al. 2002, Weinstein et al. 2002), and regulation mediated by cytokines, predominantly IL-6 (Nemeth, Ganz 2006).

The potential role of hepcidin in the development of iron deficiency in the obese is supported by the discovery of elevated hepcidin levels in tissue from patients with severe obesity, and the positive correlation between adipocyte hepcidin expression and BMI (Bekri et al. 2006). Besides, it has been reported that leptin up-regulates hepatic hepcidin expression, suggesting that increased leptinemia in obesity could be a contributor to aberrant iron metabolism (Chung et al. 2007). Therefore, leptin might be part of the axis that links obesity, inflammation, and hepcidin release with aberrant iron metabolism.

On the contrary, iron overload and the associated oxidative stress contribute to the pathogenesis and increase risk of type 2 diabetes and other disorders. As mentioned before, in iron overload, the accumulation interferes with the extraction, synthesis and secretion of insulin (Fernandez-Real, Lopez-Bermejo & Ricart 2002) and moderately elevated iron stores also increase the risk of type 2 diabetes (Jiang et al. 2004a).

In pregnancies complicated by maternal diabetes, the foetus is hyperglycaemic, and hiperleptinic (Cetin et al. 2000, Tapanainen et al. 2001). Newborns small for gestational age (SGA) also show a marked reduction in body fat mass at birth, which mainly reflects the decrease in lipid accumulation in adipocytes (Levy-Marchal, Jaquet 2004). Thus, a

change in the programming of the synthesis, secretion or actions of leptin may be decisive in the early origins of obesity after exposure, both above and below the needs of fetal or early neonatal life.

Recent studies seem to indicate that obesity is associated with iron deficiency although the aetiology appears to be multifactorial and includes: i) A decrease in iron food intake; ii) An impairment of intestinal iron uptake and iron release from stores because of an overexpression of hepcidin; and, iii) Inadequate iron bioavailability because of inflammation. In addition, abnormal ferritin concentrations can be explained by chronic inflammation rather than by iron overload (Yanoff et al. 2007). Recent studies are emerging suggesting an association between perinatal iron deficiency and programmed obesity in the adulthood, although the mechanism explaining this relationship is unclear.

2. General aims

- To analyse the effect of mother obesity and/or gestational diabetes during pregnancy on iron status in the mother and in the offspring and its implication in birth weight and risk of obesity.

- To study the role of obesity and/or gestational diabetes in pregnant women on the placental expression of TfR, and the mechanism involved in iron transplacental transport related to this biomarker.

- To explore the potential effect of leptin polymorphisms on the iron metabolism during pregnancy in obese and gestational diabetic mothers.

3. General Material and Methods

3.1 Subjects and study design

The subjects were participants in a longitudinal study of maternal nutrition and genetic on the foetal adiposity programming (Preobe study P06-CTS-02341), supported by Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía, Spain. This

prospective, observational study was developed during 2007 until 2010. A total of 350 pregnant women aged between 18 and 45, with singleton pregnancies, were recruited at from 12 to 20 weeks of pregnancy at the Clinical University Hospital 'San Cecilio', Ambulatorio Zaidín-Vergeles, and Hospital "Materno-Infantil" in the city of Granada, Spain. 300 pregnant women arrived to delivery following the Project protocols, drop-out 15.5% (see Figure 1).

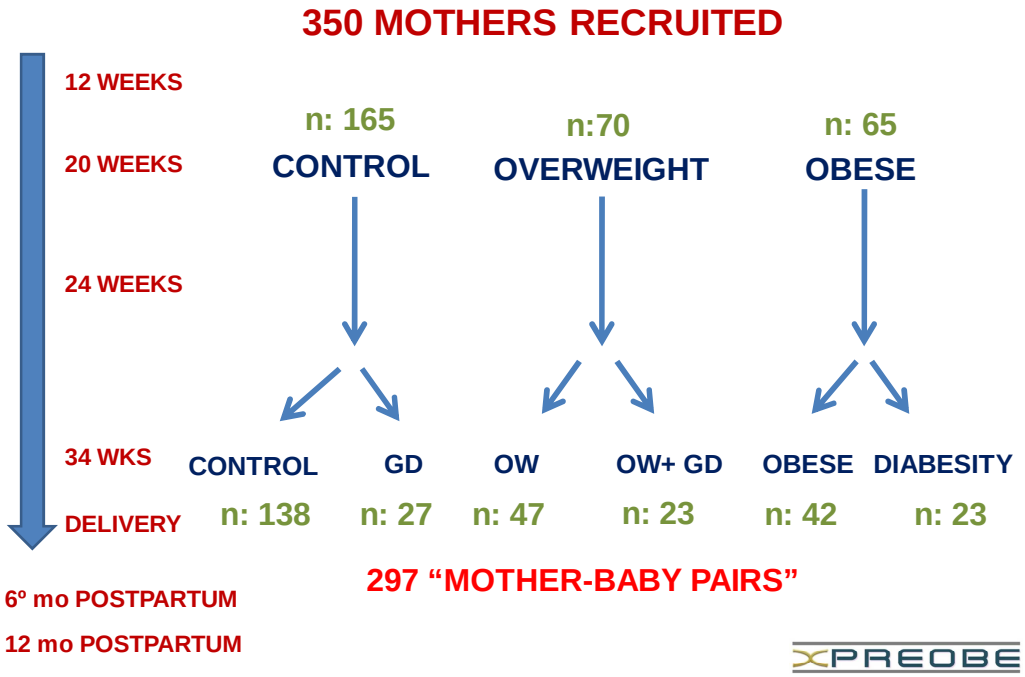
At week 20 of gestation, women were classified according to their pre-pregnancy BMI into 3 groups:

1) Control group, women with 18.5 > BMI < 25.
2) Pregnant women with overweight (BMI≥25) before the pregnancy.
3) Pregnant women with obesity (BMI≥30) before the pregnancy.

After the test of Glucose tolerance, at 34 weeks of pregnancy, the diagnosis of gestational diabetes was established, determining the following groups (see Figure 1).

1) Control group, women with 18.5 > BMI < 25.
2) Pregnant women with overweight (BMI≥25 before the pregnancy).
3) Pregnant women with obesity (BMI≥30 before the pregnancy).
4) Pregnant women with gestational diabetes (detected at week 34 & normal BMI before pregnancy).
5) Overweight (BMI≥25 before the pregnancy) pregnant women with gestational diabetes.
6) Obese (BMI≥30 before the pregnancy) pregnant women with gestational diabetes (Diabesity).

Figure 1. Study design, recruitment and distribution of the study groups.



3.1.1 Pre-pregnancy Body mass index classification

Women were classified according WHO 2009 criteria (Anonymous1995b) related to their pre-pregnancy BMI into three groups: normal-weight women (n=165) with BMI 18.5-24.9 kg/m^2; overweight women (n=70) with BMI 25-29.9 kg/m^2 and obese women (n=65) with BMI ≥30 kg/m^2 (Table 1). After the first visit at 20 weeks of pregnancy, women were examined by the obstetrician at 24 (second trimester) and 34 weeks (third trimester) and clinical parameters were recorded. Data on weight before pregnancy and before delivery were used to calculate weight gain during pregnancy. Normal weight gain ranges were from 11.5 to 16.0 kg for normal-weight women (BMI 18.5-24.9 kg/m^2); from 7.0 to 11.5 kg for overweight women (BMI 25-29.9 kg/m^2) and from 5.0-9.0 kg for obese women (BMI ≥30 kg/m^2, respectively, over pregnancy according to the Institute of Medicine (IOM) criteria (Anonymous2009). Total weight gains above these values, 16 kg for normal-weight women; 11.5 kg for overweight women and 9.0 kg for obese women, were considered excessive weight gains.

Table 1. 2009 IOM GWG Recommendations

Prepegnancy BMI category	Total weight gain (kg)	Rate of weight gain 2nd and 3rd trimester (kg/wk)
Underweight (< 18.5 kg/m^2)	12.5 - 18	0.51 (0.44 - 0.58)
Normal-weight (18.5 - 24.9 kg/m^2)	11.5 - 16	0.42 (0.35 - 0.50)
Overweight (25.0 - 29.9 kg/m^2)	7 - 11.5	0.28 (0.23 - 0.33)
Obese (≥ 30.0 kg/m^2)	5 - 9	0.22 (0.17 - 0.27)

***Calculations asume a first-trimester weight gain of 0.5 - 2.0 kg**

Maternal BMI were calculated as maternal weight in kilograms divided by height in meters squared.

Data on gestation time, birth weights of the newborns, sex, and Apgar score were also collected. Gestational age was calculated as from the last menstrual period and through ecography.

Birth weight less than 2500 g was considered low birth weight and gestational age of less than 37 weeks was considered as preterm delivery.

The characteristics of the participating women and newborns are shown in (Table 5.1, 5.2) in the results section.

3.1.2 Gestational diabetes mellitus diagnosis

Pregnant women who were diagnosed as having pre-gestational diabetes mellitus (type-1 diabetes mellitus) were excluded from the study. A total of 66 pregnant women were diagnosed gestational diabetes mellitus (GDM). Pregnant women were initially screened by measuring the plasma glucose concentration 1 h after a 50 g oral glucose challenge test (GCT) at 24-28 weeks of gestation. A diagnostic oral glucose tolerance test (OGTT) was performed on the subset of women whose plasma glucose concentrations reached or exceded the glucose threshold value (≥140 mg/dl, 7.8 mmol/L). A fasting plasma glucose level >126 mg/dl (7.0 mmol/l) or a casual plasma glucose >200 mg/dl (11.1 mmol/l)

meets the threshold for the diagnosis of diabetes, if confirmed on a subsequent day, and precludes the need for any glucose challenge. In the absence of this degree of hyperglycemia, evaluation for GDM in women with average or high-risk characteristics was developed. High risk women, including maternal age ≥ 35 years, BMI ≥ 30 Kg/m^2, relevant past obstetric and family history, impaired glucose metabolism, etc., were screening and diagnosed in the first trimester of the pregnancy. The diagnosis of GDM was made for the clinicians at the hospital based on an oral glucose tolerance test (OGTT) and the results were interpreted according to the National Diabetes Data Group (NDDG) criteria (Anonymous1979) and the Third International Workshop-Conference on Gestational Diabetes Mellitus (Metzger 1991) (Table 2). A 100g oral glucose tolerance test (OGTT) was arranged during the second trimester for all the women, except for those with risk factors that were made earlier during the first trimester.

Table 2: National Diabetes Data Group (NDDG) criteria for GD diagnosis

100g oral glucose (OGTT)	*mg/dl*	*mmol/l*
Basal	105	5.8
1h	190	10.5
2h	165	9.1
3h	145	8

Gestational diabetes was defined as two or more blood glucose values ≥ to those indicated in the table.

3.1.3 Inclusion / Exclusion Criteria

Inclusion criteria were set according to women's groups mentioned above.

Exclusion criteria are:

1- Women who wish to participate in the study should not simultaneously participate in other research studies.

2- Must be completely enclosed in one of the study of the groups without any possibility to be simultaneously incorporated on more groups of the study.

3- Mothers which are receiving any drug treatment, folate more than the 3rd first months, or DHA +/- vitamin supplements during pregnancy.

4- Mother affected by any disease other than those referred to the inclusion criteria, such hypertension or pre-eclampsia, foetal IUGR, mother infection during pregnancy, hypothyroidism / hyperthyroidism, hepatic diseases, renal disease,…

5- Mothers following an extravagant diet or vegan diet.

Table 3. Biochemical, haematological and genetic parameters analyzed in the present study

Iron Status markers	sTfR Ferritin Tranferrin TSAT index Iron Haemoglobin
Iron transport across placenta	Placental TfR
Genetic polymorphisms related to iron metabolism	Leptin Leptin receptor

3.3 Statistics

3.2.1 Power calculation

The estimation of the sample size was focused on the outcome variable, placental TfR. We considered a 0.8 % difference in TfR as a significant effect. Concerning the variation of placental TfR we assume a standard deviation of 1.5%, which is the average from several reports.

We aimed for an effect size of 0.8 % difference in TfR expression in the placental tissue. Based on the statistical model of a two factorial analysis of variance (3 different groups which are transform into 6) we achieved a statistical power of 82 % to detect 0.8 % of placental TfR difference between any of the supplements if 180 pregnancies were studied (for all estimations an error level of 0.05 is assumed). So, the power calculation shows that the sample must be at least of 30 “mother-baby” pairs /group. The sampling size was increased by 25% to avoid a reduction of the statistical significance of the results due to possible drop-outs.

3.2.2. Statistical analyses

Normality of variables was checked with the Kolmogorov-Smirnov test for samples with more than 50 subjects and Shapiro-Wilk test for smaller samples. In case of deviations from normality non parametric test were applied in the analyses.

Normal distribution of the variables was evaluated using the Kolmogorov-Smirnov test. Given their skewed distribution, serum ferritin, sTfR, sTfR/log ferritin ratio, and reticulocytes are expressed as median (range). For parametric tests, those parameters were logarithmically transformed to achieve a normal distribution. Comparisons between groups were done using Student *t* tests for continuous variables and the X^2 test for categorical variables. The relationship between continuous variables was examined by the Pearson linear correlation test in all subjects and separately in obese and non-obese subjects. A stepwise multipleregression analysis to explore the variables independently related to sTfR levels was performed. The independent variables included in the analysis

were BMI, log ferritin, serum iron, age and reticulocytes. All *p* values are based on a two-sided test of statistical significance. Significance was accepted at the level of $p < 0.05$.

Results are given in percent of total cases for qualitative variables, crude means and standard deviation for continuous variables and medians and interquartile ranges for not continuous variables.

Baseline characteristics including basal dietary fatty acid intake were compared among the four intervention groups. Differences among intervention groups for numeric variables were assessed with the analyses of variance in the normally distributed variables and Kruskal-Wallis test in the not normally distributed variables. For categorical variables Chi square tests were applied.

To evaluate the effects of overweigth, obesity and gestational diabetes on iron status and placental TfR expression, the following statistical analyses were performed. The effects of overweight and obesity as well as gestational diabetes on iron status with time were compared by using the general lineal model for repeated measures with the factor type as between subject factors and time with the three pregnancy time-points (24th and 34th week of gestation and delivery) as within subjects factor. The equality of variances was tested with the Mauchy´s test of Sphericity and for the adjustment of the degrees of freedom Sphericity assumed, lower bound and Greenhouse-Geiser corrections were applied afterwards. If significant effects were observed over time, single time-points comparisons in gestation week 30 and delivery with baseline values in the 20th week of pregnancy as well as pairwise intergroup comparisons (between the different supplementation groups) with Bonferroni corrections for multiple comparisons at the different pregnancy time-points were tested.

The effects of mother pathologies on cord blood plasma and placental TfR expression were evaluated separately with a one-way-analysis of variance or Kruskal-Wallis tests depending on the normality of variables to detect significant differences between groups. For the evaluation of pairwise intergroup differences Students-t-test or Mann-Whitney U test with corrections according to Bonferroni were applied.

For the identification of association between serum iron and ferritin and serum TfR and placental Tfr levels in maternal and fetal blood, correlation coefficients according to Spearman were calculated. The same procedure was performed for the identification of a relationship between iron relative content stored in the body and their biomarker in plasma transferrin or trasnferrin saturation index at delibery.

One way analysis of variance was used for the assessment of differences between groups in the iron, ferritin, transferrin, sTfR and placental TfR biomarkers depending on the genetic polymorphisms of Leptin analysed. In case of significance multiple comparisons with Bonferroni corrections were performed.

To evaluate the association between maternal obesity and diabetes and cord iron, transferrin, ferritin, sTFR in serum and placental TfR and the growth outcome, following statistical analysis were performed. Stepwise logistic regression analysis were performed to study the association between cord and maternal serum and placental expression of TfR. An analysis of the association between maternal and umbilical cord iron, transferrin, ferritin, sTFR in serum and placental TfR and the offspring growth were performed by means of raw and adjusted for confounders correlation coefficients which were calculated using Spearman test. Spearman and Pearson correlation coefficients were also calculated between the iron, transferrin, ferritin, sTFR in serum and placental TfR and the biomarkers of iron deficiency in the mother and offspring. Stepwise multiple lineal regression analyses were performed for the adjustment of confounders with fetal and infant growth parameters as dependent variables and each of the iron metabolism parameters studied as independent variables. All possible confounders were included in the model as covariables.

Maternal age, parity, body mass index, haematocrit at the 34th week of pregnancy and smoking habit during pregnancy, as well as length of gestation, gravidity risk factors, delivery complications and parental educational attainment and work status were taken into account in the statistical analyses as possible confounders. Infant weight, length and

head circumference at birth, Apgar score and perinatal morbidity, sex and breast feeding, as well as BMI and health status of the children at 6 months were also included.

Due to the high number of control variables only those related to the outcome variable at *P*-values <0.2 were entered as covariables in the analysis. For the identification of dependencies between clinical outcomes and confounding variables Chi Square test were used for categorical variables and the t-Student or Mann-Whitney tests for numerical variables. To identify dependencies between confounding variables and fetal and postnatal growth, Mann-Whitney test was used for categorical variables and coefficients according to Spearman were calculated for numerical variables.

In all cases the significance was assumed, if *P*-values were smaller or equal to 0.05. All computations were performed with SPSS statistical software version 15.0 (Statistical Package for Social Sciences, SPSS Inc. Chicago IL, USA).

4. Ethical Issues

The information collected was treated strictly confidential and was used only for the project.

The only invasive test in the study was maternal blood sampling, which involved a minimal risk of minor complications. In addition, no invasive test were performed in the offspring, the only samples collected from babies consisted of cord blood at delivery. Thus, there was no risk for the mothers or the babies.

During the enrolment pregnant women and their partners were informed about the study and a written informed consent was obtained prior to entry into the study. It was made clear to the subject that she could withdraw her consent at any time without any consequences for her medical treatment.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved either by the Medical Ethic Review Committee of Granada University and both Hospitals involved in the study.

Written informed consent was obtained from all subjects before their inclusion in the study after a full explanation of the study had been given by a member of the team at the first visit. Participants were assured of anonymity and confidentiality.

Chapter I: Iron status during pregnancy and effects on pregnancy outcome

3.1.1 Introduction

Intrauterine life represents a critical developmental period, during which environmental perturbations may permanently alter growth, development and metabolic regulation of the fetus in ways that predispose to disease in adulthood. Optimal maternal nutrition is now widely recognised as being essential for optimal fetal growth and development and considerable interest is being shown in the way in which nutrition during pregnancy and after birth interacts to determine fetal and postnatal health. Once formed, the placenta is very efficient at transfer of nutrients to the conceptus because of the organization of the maternal and fetal vasculatures. Nonetheless, nutritional deprivation or other insults to placental function can compromise fetal development and cause adverse effects on the physiology of the offspring that can persist into adulthood.

Iron deficiency anaemia (IDA) is a common problema in pregnancy, and has been associated with adverse pregnancy outcomes. The consequences are serious for both the mother and her infant. In the mother it is associated with an increased risk of hemorrhage, but until recently, the effect on the descendants was not so well defined. Now we know that it is a risk factor for pre-term delivery and subsequent low birth weight. There is also increasing evidence that maternal iron deficiency in pregnancy results in reduced fetal iron stores that may last well into the first year of life. This may lead to IDA in infancy which could have adverse consequences on infant development (Allen 2000).

3.1.3 Iron requirements during pregnancy

Primordial life evolved in waters rich in iron content, where iron-sulphur complexes played a central catalytic role (Martin, Russell 2003). Since then, iron has been an essential micronutrient for virtually every form of life as it plays a key role in a spectrum of biological processes. Apart from its obvious role in oxygen transport by **haemoglobin**, it is also involved in electron transport in the process of oxidative phosphorylation and function of enzymes such as hydroxylases and ribonucleotide reductase (Crichton RR

1991). However, two problems associated with the use of iron in biological system are its low solubility -at neutral pH, iron is nearly insoluble as illustrated by the very low solubility product of $Fe(OH)_3$ of $4 \cdot 10$ (Spiro 1977)- and its propensity to catalyze formation of toxic oxidants. Therefore, in physiological circumstances, iron is transported and processed by either iron binding proteins, e.g. transferrin and ferritin, or low molecular weight compounds such as citrate, amino acids and adenosine 5'-tri-phosphate (ATP).

Under physiological conditions, there is a balance between iron absorption, iron transport and iron storage in the human body. It circulates bound to plasma transferrin and accumulates within cells in the form of ferritin. More than two-thirds of body iron content is incorporated in red cells, whereas most of the remaining is found in hepatocytes and reticuloendothelial macrophages that serve as storage depots (Andrews 1999).

During pregnancy, the absorbed iron is predominantly used to expand the woman's erythrocyte mass, fulfill the foetus's iron requirements and compensate for iron losses (i.e. blood losses) at delivery. Iron absorption is regulated by the size of body iron stores (Finch 1994). Virtually all of the iron is derived from absorption and it increased markedly only after most of the storage iron had been used. Several studies on intestinal iron absorption in pregnancy have been performed (Svanberg 1975, Barrett et al. 1994, Heinrich et al. 1968), demonstrating increasing absorption with gestational age. The increase in absorption is most pronounced after 20 weeks of gestation and peaks in late pregnancy.

In normal circumstances not all the iron ingested and absorbed daily from the small intestines is needed immediately. The excess is usually stored in the bone marrow so that during periods of additional need it can be used to increase the rate of formation of haemoglobin to satisfy increased bodily needs. One such period of physical stress is pregnancy. During pregnancy, growth of the fetus and of the placenta, and the larger amount of circulating blood in the pregnant woman, lead to increase in the demand of nutrients, one of them is iron.

The daily requirements for iron for a woman in the last trimester of pregnancy are six times greater than for a non-pregnant woman (Christensen RD 2004). Comparing to a menstruating women, physiological iron requirements are three times higher in pregnancy. During the first trimester of pregnancy, iron requirements temporary decrease due to a loss of menstruation, with a daily need of approximately 0.5 mg of iron, while increase to 3.5-8.8 mg/day in the second and third trimester respectively (Schwartz III, Thurnau 1995, Allen 1997, Bothwell 2000), to meet both the requirements of the mother for the expansion of her circulating red cell mass and the demands of the developing fetus. The average requirement in the entire gestation period is ~4.4 mg/day (Bothwell 2000, Milman 2006), so that the total iron needed during the whole of pregnancy is estimated at about ~1,240 mg (Milman et al. 1999, Barrett et al. 1994, Bothwell 2000, Milman 2006, Hytten, Chamberlain 1980, Blot, Diallo & Tchernia 1999). The average requirement for a menstruating woman for the same period of time is <400 mg. The increased requirement is therefore <800 mg. This amount of iron must be acquired from the body iron store or from the diet by the end of pregnancy. Indeed, nature itself makes efforts to meet the extra need by the gradual and hightly significant increase in the absorption of iron from the gut, which is especially increased in the second half of the pregnancy (Barrett et al. 1994). However, in many women in the Western societies this biological response is not enough due likely to a low content of iron in the diet combined with poor diet habits. So, that this need cannot be met by diet alone, being derived at least partly from maternal reserves. In a well-nourished woman about half the total requirements of iron may come from iron stores. Therefore, when these reserves are already low –due to poor nutrition and/or frequent pregnancies- anemia emerge. It has been estimated that even when food intake is adequate it may take two years to replenish body iron stores after a pregnancy.

The relative importance of iron stores on the one hand and increased iron absorption on the other is best illustrated by examining iron balance during pregnancy in women from industrialized countries. Because the estimated total additional requirement during pregnancy calculated above is 800 mg, the average woman must absorb 500 mg (<2

mg/d) more iron than she required while menstruating to avoid a negative iron balance. At delivery, blood losses can reach 1290 mg of iron and in many women, it is uncertain whether diet alone can provide the additional iron needs of pregnancy (Allen 1997); the need for iron supplementation is actively debated. (Beard 1998, Hallberg 1998, Viteri 1997)

Postpartum, the mother's erythrocyte mass declines to pre-pregnancy levels, and the haemoglobin iron is recycled to body iron reserves. The net iron loss, associated with pregnancy per se, is therefore lower, approximately 630 mg (Bothwell 2000, Milman 2006).

3.1.4 Iron status during pregnancy

An adequate body iron balance is important for our well-being and quality of life (Bruner et al. 1996, Rowland et al. 1988, Beard et al. 2005). In pregnant women, a favourable iron status is a prerequisite for a good course of pregnancy, a normal development of the foetus and maturity of the newborn.

The Centers for Disease Control standards for anemia diagnosis in pregnancy reflect the "physiologic anemia" that occurs in midpregnancy. This anemia is not related to the mother's iron status (Kim et al. 1992, Anonymous1990).

3.1.1 Haematological parameters to assess iron status in pregnancy

One significant problem in identifying the relationship of adverse consequences with poor iron status is the assessment of iron status in pregnancy. Iron status and body iron can be assessed by using appropiate markers, predominantly serum ferritin, haemoglobin, serum soluble transferrin receptors (sTfR) and the sTfR/ferritin ratio.

1.2.2.1. Hemoglobin

Haemoglobin is the red pigment present in solution in the red corpuscles of the blood and its primary function is transport oxygen to all parts of the body. Iron, folic acid, others vitamins and trace elements are all required for the formation of haemoglobin, which takes place in the bone marrow.

During gestation, characteristic changes are observed in both haemoglobin and serum ferritin concentrations. The physiologic increase in plasma volume of ~50% is only partly compensated by an increase in the erythrocyte mass of ~25%. This results in haemodilution, *a hallmark of a normal pregnancy*, where the nadir haemoglobin concentration is reached at 24-32 weeks gestation. Subsequently, haemoglobin rises towards the end of the third trimester (Williams, Wheby 1992, Milman, Byg & Agger 2000). This draw an U-shaped association between the maternal hemoglobin concentrations according to the trimesters of pregnancy.

The haemoglobin concentration is still widely used as a marker for iron deficiency, mainly due to the simplicity and low cost of the analysis. However, haemoglobin as a single parameter is not valid as a biomarker to estimate iron status or body iron reserves- especially not in pregnancy where the women display various degrees of haemodilution (Koller 1982), so that haemoglobin concentrations show similar inter-individual variation i.e. women with identical erythrocyte mass can present with different haemoglobin concentrations, which may vary up to 35 g/L, making it difficult to define exactly the lower normal limit for the hemoglobin concentration in pregnant women. Moreover, there exists a broad overlap between the distribution of haemoglobin in subjects with and without iron deficiency.

1.2.2.2. Transferrin

Transferrin, an iron-binding blood plasma glycoprotein, controls the level of free iron in biological fluids (CRICHTON, CHARLOTEAUX-WAUTERS 1987). In humans, it is encoded by the *TF* gene (Yang, Lum & McGill 1984). Transferrin is a glycoprotein that binds iron very tightly but reversibly. Although iron bound to transferrin is less than 0.1% (4 mg) of the total body iron, it is the most important iron pool, with the highest rate of turnover (25 mg/24 h). Transferrin has a molecular weight of around 80 kDa and contains 2 specific high-affinity Fe(III) binding sites. The affinity of transferrin for Fe(III) is extremely high (10^{23} M^{-1} at pH 7.4) (Aisen, Leibman & Zweier 1978)but decreases progressively with decreasing pH below neutrality.

The major function of transferrin is to transport iron between sites of absorption, storage and utilization.

The liver is the main source of manufacturing transferrin, but other sources such as the brain also produce this molecule. The main role of transferrin is to deliver iron from absorption centres in the duodenum and white blood cell macrophages to all tissues. Predominantly, transferrin plays a key role where erythropoiesis and active cell division occur (Macedo, de Sousa 2008). In order for iron ion to be introduced into the cell a carrier protein is used, known as a transferrin receptor. The receptor helps maintain iron homeostasis in the cells by controlling iron concentrations (Macedo, de Sousa 2008).

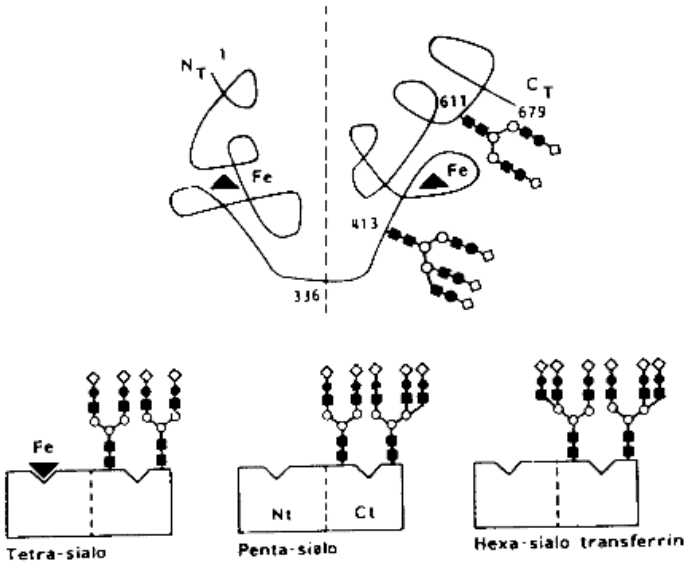


Figure 2. Transferrin (Tf) is a monomeric 80 kDa serum glycoprotein consisting of a polypeptide chain of 679 aminoacids and two N-linked complex type glycan chains that binds up to two iron atoms. The transferrin molecule comprises two homologous domains, the N-terminal and C-terminal domain, each containing one iron binding site. The carbohydrate moiety is attached to the C-terminal domain (Macgillivray et al. 1983, Dejong, Vandijk & Vaneijk 1990).

Transferrin is also associated with the innate immune system. Transferrin is found in the mucosa and binds iron, thus creating an environment low in free iron that impedes bacteria survival in a process called iron withholding. The levels of transferrin decreases in inflammation (Ritchie et al. 1999), seeming contradictory to its function.

Transferrin imbalance can have serious health effects for those with low or high serum transferrin levels. A patient with an increased serum transferrin level often suffers from iron deficiency anemia (Macedo, de Sousa 2008). A patient with decreased plasma transferrin can suffer from iron overload diseases and protein malnutrition. An absence of transferrin in the body creates a rare genetic disorder known as atransferrinemia; a condition characterized by anemia and hemosiderosis in the heart and liver that leads to many complications including heart failure. Most recently, transferrin and its receptor have been tested to diminish tumour cells by using the receptor to attract antibodies (Macedo, de Sousa 2008).

1.2.2.3. Serum transferrin receptor

Transferrin receptor-1 (TfR1) is a disulfide-linked homodimer present in the plasma membrane that binds one Tf molecule per TfR1 monomer. The transport and uptake of nonheme iron inter-organ is largely performed by the ratio transferrin (Tf)/transferrin receptor-1 (TfR1) system. When the iron–transferrin complex binds to its cellular receptor (TfR), the Tf-TfR1 complex on the cell membrane is internalized by receptor-mediated endocytosis, ultimately entering the endosomal compartment of the cytoplasm, accompanied by a proteolytic cleavage of the soluble extracelular domain of TfR into the circulation (Cotton, Thiry & Boeynaems 2000, Sherwood, Pippard & Peters 1998). Serum levels of this soluble form (sTfR) are therefore directly proportional to the tissue TfR concentration. Endosomal acidification, to a pH of <5.5, is required for release of iron from Tf. Iron is then used for celular processes, and excess iron is stored within the protein ferritin (Hentze, Muckenthaler & Andrews 2004). The size of the intracelular chelatable iron pool influences ferritin and TfR1 gene expression at the post-transcriptional level through the action of two iron-regulated RNA binding proteins, the IRPs, IRP-1 and IRP-2 (Meyron-Holtz et al. 2004, Smith et al. 2006). When cells are iron-deficient, IRP-1 and IRP-2 bind to iron-responsive elements (IREs) in the 3′- or 5′-untranslated regions of mRNA transcripts of molecules such as the TfR1 or ferritin, stabilizing them against degradation or inhibiting translation, respectively (Meyron-Holtz et al. 2004, Smith et al. 2006). This results in increased cellular iron uptake through the TfR1 and decreased intracellular iron storage within ferritin, leading to elevated levels of

intracellular iron. Iron regulatory proteins (IRPs) and iron-responsive elements (IREs), to which they bind, allow mammals to make use of the essential properties of iron while reducing its potentially toxic effects.

The transferrin receptor (TfR) is primarily expressed on the surface of erythrocytes, and can be measured in serum as soluble receptors (sTfR). The serum sTfR is assumed to be proportional to the density of TfR on erythroid cells, i.e. in the presence of iron deficiency the density of TfR and levels of sTfR increases (Baynes 1994) and the circulating sTfR level reflects cellular iron requirements (Skikne 2008). *Thus,* elevated sTfR levels are indicative of iron deficiency because erythrocytes in the bone marrow increase the presentation of membrane transferrin receptor in the presence of low levels of iron (Wish 2006). Serum sTfR renders information about celular iron deficiency, in contrast to serum ferritin which gives information about iron reserves. When iron reserves are exhausted, serum sTfR increases (Baynes 1994, Skikne 2008). Serum levels of this soluble form (sTfR) are therefore directly proportional
to the tissue TfR concentration. The circulating level of sTfR correlates inversely with body iron stores and its clinical utility as a marker of body iron status is currently being explored (Mast et al. 1998, Chang et al. 2007). Non-pregnant and pregnant women with replete iron stores have similar serum sTfR levels (Carriaga et al. 1991). In women with depleted iron stores, serum sTfR levels of >8.5 mg/L indicate iron deficiency (Carriaga et al. 1991). Serum sTfR can be a *useful indicator* to identify women with low plasma ferritin, who in addition have pronounced iron deficiency (Carriaga et al. 1991, Åkesson et al. 1998). *Therefore,* serum sTfR seems to be a sensitive marker of iron deficiency in pregnancy, and by combining serum sTfR and serum ferritin measurements, the entire spectrum of iron status in pregnancy can be assessed (Carriaga et al. 1991, Åkesson et al. 1998). The other advantages are that it can discriminate between iron deficiency and chronic anemia and that it is not influenced by infections (Hallberg 2001). In phlebotomy studies in healthy subjects, Skikne et al (Skikne, Flowers & Cook 1990) showed that the serum transferrin receptor is a sensitive index of tissue iron deficiency and is relatively unaffected by inflammation or stage of pregnancy (Carriaga et al. 1991). Its

disadvantages are its lower sensitivity for the detection of mild iron deficiency and the lack of standardization of the different methods available (Hallberg 2001).

1.2.2.4. Ferritin

In healthy subjects, serum ferritin (S-ferritin) behaves as a good biomarker for iron status being commonly used to determine the size of mobilizable iron stores in the body (Walters, Miller & Worwood 1973, Cook et al. 1974, Milman 1996, Zimmermann 2008) and seems to best discriminate between normal and iron-deficient subjects (Hallberg 2001). A serum ferritin concentration of 1 μg/L corresponds to 7-8 mg of mobilisable iron from stores (Walters, Miller & Worwood 1973, Bothwell et al. 1979). In general, ferritin levels of <30 μg/L indicate a low iron status, i.e. small or no iron reserves as verified by the absence of bone marrow haemosiderin (Milman 1996, Milman, Pedersen & Visfeldt 1983). Even if S-ferritin is influenced by the plasma dilution, a serum ferritin < 15 μg/l indicates iron deficiency in all stages of pregnancy (Blot, Diallo & Tchernia 1999) and ferritin levels of <12 μg/L are associated with IDA (Milman, Pedersen & Visfeldt 1983, Worwood 1994). S-ferritin is considered to be a reliable blood test in the first trimester of pregnancy for judging whether iron supplementation is necessary. However, becomes less reliable after the 20th week due to the physiological dilution of the plasma, which reduce more or less the concentration of S-ferritin indenpendently of changes in the iron stores (Schwartz III, Thurnau 1995, Sandstad et al. 1996, Allen 2000). This is evident from the finding that S-ferritin fell even in women who were supplied with a daily dose of 200 mg iron throughout the pregnancy (Romslo et al. 1983). Serum ferritin usually falls markedly between 12 and 25 wk of gestation and the concentration reduces steadily to ~50% of normal at mid-gestation due to hemodilution and the mobilization of iron from stores to meet the increased needs of pregnancy and expansion of the maternal blood cell mass (Fenton, Cavill & Fisher 1977, Milman et al. 1999). Therefore, the status of iron stores in the second and third trimesters of pregnancy cannot be accurately determined by s-ferritin alone. There are some other factors that affect s-ferritin concentrations. For example, s-ferritin behaves as a very sensitive acute phase reactant protein and is therefore elevated in the presence of any infectious or inflammatory process (Coenen et al. 1991, Harrison, Arosio 1996, Torti, Torti 2002,

Kalantar-Zadeh, Rodriguez & Humphreys 2004, Lim et al. 2001). This increase can be detected within hours after the onset of an inflammatory reaction (Dennison 1999). In women with inflammatory or infectious disorders, plasma ferritin can be falsely elevated, i.e. out of proportion with body iron reserves. Inflammatory status has recently been associated with obesity and the etiopathogenesis of type 2 diabetes (Nemeth, Ganz 2006, Cook 2005, Kohgo, Torimoto & Kato 2002, Visser et al. 1999, Roytblat et al. 2000, Fernandez-Real, Ricart 2003). In such conditions, plasma C reactive protein should be measured as well, in order to assess the degree of inflammation. Consumption of alcohol may also leads to increased s-ferritin (Milman, Kirchhoff 1996). Finally, other physiological factors which confound the interpretation of changes in S-ferritin are the within-subject diurnal and day-to-day variations (Dale, Burritt & Zinsmeister 2002) which in healthy, non-pregnant women amounts reach to about 15% and 20–25% respectively (Schwartz III, Thurnau 1995, Beard 1994, Ulvik 1984). In pregnancy this variation may be even more pronounced due to fluctuations of the plasma volume. This physiologic variations in ferritin may be compensated for by calculating body iron using the sTfR-to ferritin ratio (Cook, Flowers & Skikne 2003, Milman et al. 2006). Some investigators have suggested that the sTfR-to-ferritin ratio may be a better marker of body iron status than ferritin alone (Malope et al. 2001, Punnonen, Irjala & Rajamaki 1997).

1.2.2.5. Plasma transferrin saturation

During pregnancy, transferrin protein concentrations increase and the amount of iron to bind to this molecule decrease, leading to a progressive decrease in transferrin saturation and an increase in the total iron-binding capacity (Svanberg et al. 1975, Puolakka et al. 1980).

Plasma transferrin saturation is calculated from measurement of serum iron and serum transferrin. It is the ratio of serum iron to total iron binding capacity and it is expressed like a percentage that describes the occupancy of transferrin-binding sites with iron. A transferrin saturation less than 10% is considered diagnostic of iron deficiency. A saturation of <15% is considered to indicate an inadequate supply of iron to the erythrons and tissues (Worwood 1994).

1.2.2.6. Others

The other traditional indicators of iron status, e.g. serum iron, mean cell hemoglobin (MCH), mean cell volume (MCV) and transferrin saturation have the same problem as hemoglobin with wide inter-individual variations in both iron-replete and iron-deficient subjects. Thus any selected cutoff value will have either a low sensitivity or a low specificity (Hallberg 2001).

3.1.2 Anemia

Anaemia, one of the most common and widespread disorders in the world, is a global public health problem affecting both developing and developed countries with major consequences for human health as well as social and economic development. It occurs at all stages of the life cycle, but is more prevalent in pregnant women and young children.
Anaemia can be defined as 'a reduction in the oxygencarrying capacity of the blood which may be due to a reduced number of red blood cells, a low concentration of haemoglobin (Hb) or a combination of both' (Lloyd, Lewis 1996).

It is the result of a wide variety of causes that can be isolated, but more often coexist (Anonymous2004). Nutritionally related iron deficiency is the main cause of anemia throughout the world, so that iron deficiency anemia (IDA) and anaemia are often used synonymously, and the prevalence of anaemia has often been used as a proxy for IDA. In most developed countries, iron deficiency is the main cause of significant anemia during pregnancy (Yip 1997), as shown by the efficacy of iron supplementation in preventing maternal anemia (evidence of benefit). However, iron deficiency is not the sole cause of anaemia in most populations. Even in an individual, anaemia may be caused by multiple factors. These factors may be genetic, such as haemoglobinopathies; heavy blood loss as a result of menstruation, or parasite infections such as hookworms, ascaris, and schistosomiasis can lower blood haemoglobin (Hb) concentrations; acute and chronic infections, including malaria, cancer, tuberculosis, and HIV can also lower blood Hb concentrations; or nutritional, which includes iron deficiency as well as deficiencies of other vitamins and minerals, such as folate, riboflavin, vitamins A and B12, and copper

(Anonymous2004). In developing countries, especially where severe maternal anemia is more common, this nutritional factors and infections, including malaria, can often coexist with iron deficiency, contributing to anemia (Yip 1997).

Besides poor nutrition, frequent labour, multiparity, abortions, consuming excess tea or coffee after meals determined as the predictors of anemia in reproductive age women (BADHAM, ZIMMERMANN & KRAEMER 2007).

As a problem of public health, anemia was classified as none (less than 5%), mild (5 to 19.9%), moderate (20 to 39.9%) and severe (40% or more) according to the anemia prevalence at the national level by WHO. Anemia prevalence among pregnant women in Spain was reported as 17.6%; 16.3% in non-pregnant women of reproductive age and 12.9% in preschool-age children in WHO report (McLean et al. 2009).

The golden standard in the definition of iron-deficiency anaemia is an increase in haemoglobin concentration during iron therapy. This strict criterion is often not applicable in the clinical situation. Instead, an arbitrarily chosen haemoglobin concentration is used as cut-off value in the definition of anaemia.

The commonly used definition of anemia, from whatever cause, is a low hemoglobin concentration. According to WHO criteria, laboratory definition of anemia for a general population is Hb < 12 g/dL for female and < 13 g/dL for male (WHO criteria have been challenged recently 2009). As a basic screen for anaemia in pregnancy, the World Health Organization (WHO) recommended a haemoglobin (Hb) of 110 g/L (Anonymous1972, Anonymous1968) as a cut-off point throughout all pregnancy, which is now (2003) the most widely used criterion for anemia in pregnancy (Stoltzfus, Dreyfuss 1998), while the Centers for Disease Control (CDC) define anaemia as a Hb < of 110 g/L during the first and third trimesters and 105 g/L in the second trimester (Anonymous1989a).

3.1.3 Iron deficiency anemia

A negative iron balance will over time progress to iron deficient anemia characterized by microcytosis and increased amounts of protoporphyrin IX in the red blood cells (Schwartz III, Thurnau 1995).

In 2002, iron deficiency anaemia (IDA) was considered to be one of the ten most important factors contributing to the global burden of diseases and that it increases morbidity and mortality in vulnerable groups, such as preschool-aged children and pregnant women (Guilbert 2003), mainly in developing countries (Anonymous1989b). It is especially common in women of reproductive age and particularly during pregnancy. As mentioned before, the demand for iron increases about six to seven times from early pregnancy to the late pregnancy (Christensen RD 2004).

It is generally assumed that 50% of the cases of anaemia are due to iron deficiency (Anonymous2001a), but the proportion may vary among population groups and in different areas according to the local conditions. The main risk factors for IDA include a low intake of iron, poor absorption of iron from diets high in phytate or phenolic compounds, and period of life when iron requirements are especially high (i.e. growth and pregnancy).

3.1.4 Iron deficiency without anemia

Iron deficiency is the most commonly recognized nutritional deficit in either the developed or the developing world affecting an estimated two billion people (Salomon, Murray 2000). During their reproductive years women are at risk of iron deficiency due to blood loss from menstruation, in particular that 10% who suffer heavy losses (80 mL/mo). Contraceptive practice also plays a part, so that the intrauterine devices increase menstrual blood loss by 30%–50% while oral contraceptives have the opposite effect. Pregnancy is another factor. During pregnancy there is a significant increase in the amount of iron required to increase the red cell mass, expand the plasma volume and to allow for the growth of the fetal-placental unit. Finally, there is diet. Women in their

reproductive years often have a dietary iron intake that is too low to offset losses from menstruation and the increased iron requirement for reproduction (Yip 2001).

Normal hemoglobin level does not exclude ID, because individuals with normal body iron stores must lose a large amount of body iron before the hemoglobin falls below the laboratory definition of anemia.

ID can be either absolute (aID) or functional (FID). In absolute ID, iron stores are depleted; in FID, iron stores, although replete, cannot be mobilized as fast as necessary from the macrophages of the reticuloendothelial system (RES) to the bone marrow. FID occurs in anemia of inflammatory diseases because iron is trapped in the RES (Muñoz Gómez et al. 2005, Anonymous2006b). Thus, laboratory tests for investigating ID fall into two categories: measurements providing evidence of iron depletion in the body, and measurements reflecting iron-deficient RBC production (Cook 2005). The appropriate combination of these laboratory tests will help to establish a correct diagnosis of anemia and ID status (Weiss, Goodnough 2005).

The main laboratory finding to know if ID is present is low ferritin level. As mentioned before, although ferritin is an intracellular iron storage protein, small amounts of ferritin are secreted into the circulation and can be measured in the laboratory. In the presence of inflammation, a normal ferritin level (acute phase reactant) does not exclude ID, and TSAT also should be measured. As transferrin is the only iron binding protein involved in iron transport, TSAT reflects iron availability for the bone marrow. Thus, in the presence of inflammation, ID should be better defined by *n*ormal ferritin concentrations and low TSAT (FID).

The level of erythrocyte protoporphyrin, the precursor of heme, increases in patients with iron deficiency (Yip, Schwartz & Deinard 1983). An elevated erythrocyte protoporphyrin level correlates well with low serum ferritin, and can be used to screen for moderate iron deficiency without anemia (Yip, Schwartz & Deinard 1983). It should be kept in mind

that some other conditions such as infection or inflammation can also cause a significant elevation of erythrocyte protoporphyrin (Yip, Schwartz & Deinard 1983).

3.1.5 Iron deficiency and obesity

At present, obesity has been recognized as a worldwide public health problem. Excess body fat is associated with increased all-cause mortality and increased risk for several medical morbidities, including type 2 diabetes, dyslipidaemia and hypertension (Bray et al. 2008). Until recently, few studies had considered body weight or body composition as factors related to iron deficiency. Many of them have shown that obesity might increase the risk of iron deficiency but, at the same time, obese subjects exhibit high serum ferritin levels. It has been widely accepted that sTfR is a quantitative indicator of early iron deficiency and is not influenced by the acute-phase response. Thus, it can differentiate between iron deficiency anaemia and anaemia of chronic disease. Some authors have used this marker with the aim of establishing the significance of hypoferremia and hyperferritinemia. In the case of iron overload a decrease in sTfR would be expected, whereas iron deficiency must be associated with higher levels of sTfR. Three recent articles have analysed sTfR, and all of them have reported that the levels of sTfR are elevated in obese patients (Lecube et al. 2006, Freixenet et al. 2009, Yanoff et al. 2007). Studies in industrialized countries have consistently found higher rates of iron deficiency in overweight children (Pinhas-Hamiel et al. 2003, Nead et al. 2004, Wenzel, Mayer & Stults 1962, Seltzer, Mayer 1963, Scheer, Guthrie 1981, Brotanek et al. 2007) and adults (Micozzi, Albanes & Stevens 1989, Lecube et al. 2006, Yanoff et al. 2007, Whitfield et al. 2003, Rossi et al. 2001). Although the mechanism is unclear, this may be due to lower iron intakes and/or increased iron requirements in overweight individuals (Yanoff et al. 2007, Seltzer, Mayer 1963). In addition, the chronic inflammation and increased leptin production characteristic of obesity increase hepcidin secretion from the liver (Chung et al. 2007), which, along with hepcidin produced by adipose tissue (Bekri et al. 2006), could reduce dietary iron absorption (Laftah et al. 2004) (see figure 4).

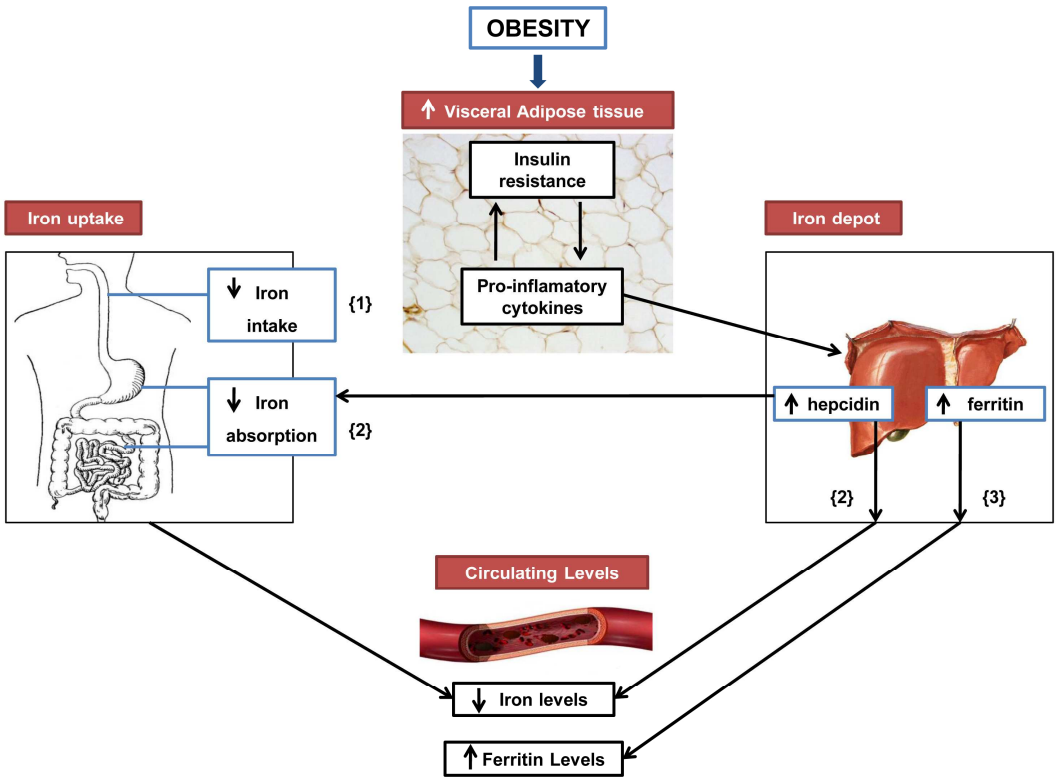


Figure 4. Mechanism that explain the relation between iron metabolism and obesity (Adapted from Zafon, Obesity Reviews 2010).1) Decrease in iron food intake. 2) Impairment in intestinal iron uptake and iron release from stores due to an overexpression of hepcidin. 3) Inadequate iron bioavailability due to inflammation.

3.1.1 Making the connection: Obesity and circulating iron levels

The best described functions of dietary iron occur through its incorporation into proteins and enzymes necessary for optimal work performance. However, iron may also function in the maintenance of body weight and composition, as a number of studies have suggested an association between iron status and obesity in paediatric and adult subjects. The inverse correlation between serum iron concentrations and adiposity was first reported in children and adolescents in the early 1960s (Wenzel, Mayer & Stults 1962, Seltzer, Mayer 1963). The first, published in 1962, demonstrated significantly lower serum iron concentrations in obese adolescents compared with controls (Wenzel, Mayer & Stults 1962). Subsequent studies have confirmed these initial results (Pinhas-Hamiel et al. 2003, Nead et al. 2004, Moayeri et al. 2006) in children and adolescents. The first of

these, a cross-sectional study published in 2003, described a greater prevalence of iron deficiency, as indicated by serum iron levels <8 mmol/L, in overweight and obese Israeli children and adolescents(Pinhas-Hamiel et al. 2003). The second study (Nead et al. 2004), using data from the third National Health and Nutrition Examination Survey (NHANES III), confirmed those findings using multivariate regression analyses to demonstrate that overweight American children were twice as likely to be iron deficient than normal-weight children. Similar associations have since been reported in adults (Lecube et al. 2006), where Lecube et al. in a study of 50 obese and 50 non-obese postmenopausal women has been also confirmed the presence of iron deficiency in obese women by measuring sTfR and reported that body mass index (BMI) was positively associated with sTfR. Interestingly, other analysis, in the adult population, from the Third National Health and Nutrition Examination Survey (NHANES III) showed that BMI was associated with significantly lower mean serum iron concentrations in women but not in men (Micozzi, Albanes & Stevens 1989). An inverse correlation was found between serum iron concentration with BMI, waist circumference, and fat mass in Hispanic women living in the United States (Chambers et al. 2006). In another recent study, Menzie et al.(Menzie et al. 2008) found significantly lower levels of serum iron and transferrin saturation (the ratio of serum iron to total iron binding capacity) in obese as compared to non-obese adult volunteers, and fat mass was shown to be a significant negative predictor of serum iron concentration. In another study, using cut-off values for serum iron and sTfR, Yanoff et al.(Yanoff et al. 2007) confirmed an increased prevalence of iron deficiency in obese as compared to non-obese adults; in that study, serum iron was significantly lower and sTfR was significantly higher in the obese individuals. Finally, Zimmermann *et al.* (Zimmermann et al. 2008) have reported that increasing obesity observed in transition countries is associated with a worsening in iron status. Hence, several studies have shown a relationship between obesity and hypoferremia, both in paediatric and adult subjects. Collectively, these reports suggest that excess adiposity may negatively affect iron status.

3.1.2 Hypothesis for possible mechanism: Diet, iron absorption and inflammation

The reason why the obese population has lower circulating iron levels remains uncertain. Iron deficiency in obese individuals may result from differents factors. Low iron intake, reduced iron absorption, and the sequestration of iron as a result of chronic inflammation in response to excess adiposity has been suggested among differents reasons. In regard to an iron-poor diet, low iron intake and increased iron needs have been reported among obese children and adolescents who are iron deficient (Pinhas-Hamiel et al. 2003, Nead et al. 2004, Hassapidou et al. 2006). However, other authors have failed to find a difference in intake of iron according to BMI. Thus, for example, Menzie et al. (Menzie et al. 2008) have reported that the obese and non-obese did not differ in total daily iron consumption, and that obesity-related hypoferremia is not associated with differences in intake of factors that affect iron absorption (such phytic acid, oxalic acid, eggs, coffee, tea, and zinc, among others), but fat mass, per se, remained a significant negative predictor of serum iron level. Zimmerman et al. (Zimmermann et al. 2008) also reported that high BMI Z-scores were associated with decreased iron absorption in women independent of iron status and reduced improvement of iron status in iron-deficient children following intake of iron-fortified foods. They hypothesized that obesity may affect iron absorption through an inflammatory mediated mechanism.

Another possible mechanism that has been suggest is that iron depletion could be as a result of the greater iron requirements of obese subjects because of their larger blood volume (Failla, Kennedy & Chen 1988).

3.1.3 Obesity and ferritin connection: Iron overload or inflammatory response?

Ferritin is an intracellular protein responsible for the sequestration, storage and release of iron, becoming the major iron storage protein involved in the regulation of iron availability. Many authors have confirmed that ferritin levels are enhanced in obese subjects (Ahmed et al. 2008, Gillum 2001, Iwasaki et al. 2005, Oshaug et al. 1995, Wrede et al. 2006). However, the significance of this finding remains controversial.

It has long been known that serum ferritin levels accurately reflects body iron stores in healthy individuals (Walters, Miller & Worwood 1973, Cook et al. 1974, Milman 1996, Zimmermann 2008). Accordingly, higher ferritin levels observed in obesity could be as a result of an increase of iron stores in a condition termed iron overload. This condition involves excess accumulation of iron in body tissues, especially in the liver. Deposited iron promotes the generation of reactive oxygen species, which can cause tissue injury and organ failure.

Certainly, there are several cofactors that affect body iron metabolism and accelerate hepatic iron overload, with alcohol, hepatic viral infections and type 2 diabetes being the most typical (Kohgo et al. 2007, Lecube et al. 2004, Lecube et al. 2006). Moirand *et al.* (Moirand et al. 1997) proposed a syndrome of iron overload in patients with increased serum ferritin and normal transferrin saturation. The vast majority (95%) of patients was overweight, hyperlipidaemic, hypertensive, or had an abnormal glucose metabolism.

Nevertheless, it should be noted that ferritin is also an acute-phase protein. Ferritin synthesis is actively regulated by the cytokines interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNFα). Plasma concentrations of IL-6 increase with obesity, unlike those of TNFα, which acts in an autocrine and paracrine fashion (Mohamed-Ali et al. 1997); in obese individuals, adipose tissue is a major determinant of plasma IL-6 concentrations, contributing as much as 30% of total body production (Mohamed-Ali et al. 1997). IL-6 increases lipolysis and fat oxidation in humans (Van Hall et al. 2003), and plasma IL-6 concentrations correlate with insulin resistance (Kern et al. 2001). Recently, IL-6 was shown directly to cause insulin resistance in the liver (Klover et al. 2003). Elevated IL-6 concentration is a predictor for development of type 2 diabetes and for myocardial infarction (Ridker et al. 2000, Pradhan et al. 2001).

In addition, several hormones such as thyroid hormone, insulin and insulin-like growth factor (IGF-1) are factors that could influence circulating ferritin levels (Zandman-Goddard, Shoenfeld 2007). Moreover, plasma ferritin is up-regulated in infections, inflammatory states, malignant diseases, and in any condition characterized by the

excessive production of toxic oxygen radicals. It is assumed that the major function of ferritin in these conditions is to reduce the bioavailability of iron, as an ancient strategy of host–defence response (Beck et al. 2002).The conception of obesity as a chronic inflammatory state has been the focus of recent attention. Adipose tissue, especially visceral adipose tissue, releases pro-inflammatory cytokines such as IL-6 and TNFα. The low-grade inflammation induced by these cytokines may contribute to the development of insulin resistance (IR), impaired glucose tolerance and type 2 diabetes (de Luca, Olefsky 2008). Moreover, the infiltration by and activation of macrophages in adipose tissue has also been linked to obesity-induced IR (Apovian et al. 2008). In accordance with this conception, obesity-associated iron abnormality has been interpreted as a feature that mimics the so-called anaemia of chronic inflammation, which is characterized by hypoferremia and high to normal serum ferritin concentration (hypoferremia and anaemia despite adequate iron stores) (Ausk, Ioannou 2008). This entity is also caused by increased inflammatory cytokines, especially IL-6, inducing increased production of the iron-regulatory hormone *hepcidin*. Thus, hepcidin by means of its capacity to block iron release from macrophages, hepatocytes and enterocytes appears to be a major contributor to the hypoferremia associated with inflammation (Ganz 2006).

3.1.4 Metabolic syndrome and ferritin

A number of reports have questioned whether iron abnormalities are related to obesity itself or whether it is determined by other associated pathologies. It should be noted that metabolic syndrome is a cluster of metabolically related cardiovascular risk factors that include, at least, obesity, IR, dyslipidaemia and hypertension (Alberti, Zimmet & Shaw 2006). Because metabolic syndrome and type 2 diabetes are conditions often observed in overweigh subjects, it could be posible that these conditions are more important factors in accounting the high ferritin levels associated with obesity than obesity itself. In fact, epidemiologic studies have found a positive association between metabolic síndrome and high serum ferritin levels (Jehn, Clark & Guallar 2004). Lecube *et al.* (Lecube et al. 2008) have found that ferritin levels were significantly higher in obese women with metabolic syndrome in comparison with obese women without metabolic syndrome.

Illouz *et al.* (Illouz et al. 2008) have shown that the waist-to-hip circumference ratio was related to serum ferritin levels in obese type 2 diabetic patients with metabolic syndrome.

3.1.5 Adipose tissue and iron metabolism: Role of Hepcidin and Leptin

Adipose tissue is an active endocrine organ and releases a number of cytokines and adipokines (Rosen, Spiegelman 2006, Lago et al. 2007), which may in turn influence iron metabolism. The relationship between adipose tissue and iron metabolism is currently a field of intense research and the role of hepcidin, a highly conserved 25 amino acid peptide hormone predominantly synthesized in the liver, has been emphasized in recent years (Nemeth, Ganz 2006). Hepcidin acts as an inhibitory iron regulator. Increased plasma hepcidin inhibits intestinal iron uptake and acts sequestering iron at the macrophage (Knutson et al. 2005), which could lead to decreased iron stores and hypoferremia. In accordance with this homeostatic model, iron loading increases hepcidin gene expression. Also, its production is suppressed by anaemia and hypoxaemia. Furthermore, hepcidin synthesis is markedly induced by infection and inflammation and because chronic disease (Park et al. 2001, Nicolas et al. 2002, Weinstein et al. 2002), and regulation mediated by cytokines, predominantly IL-6 (Nemeth, Ganz 2006). As mentioned before, obesity causes chronic inflammation (Greenberg, Obin 2006), which is associated with the expression and release of pro-inflammatory cytokines, including IL-6 and TNF-α. These pro-inflammatory cytokines may result in the release of hepcidin from the liver or adipose tissue (Bekri et al. 2006, Wrighting, Andrews 2006). Bekri *et al.* (Bekri et al. 2006) have shown that hepcidin was expressed in both visceral and subcutaneous adipose tissue and that this expression was enhanced in obese patients. The potential role of hepcidin in the development of iron deficiency in the obese is supported by the discovery of elevated hepcidin levels in tissue from patients with severe obesity, and the positive correlation between adipocyte hepcidin expression and BMI (Bekri et al. 2006). Besides, it has been reported that leptin up-regulates hepatic hepcidin expression, suggesting that increased leptinemia in obesity could be a contributor to aberrant iron metabolism (Chung et al. 2007).

On the other hand, Leptin, the first adipokine to be discovered (Zhang et al. 1994), is intriguing in this regard for 3 reasons: 1) it belongs to the family of long-chain helical cytokines (Zhang et al. 1997); 2) its circulating levels are proportional to fat mass (Considine et al. 1996); and 3) its membrane receptors exhibit structural similarity to class I cytokine receptors (Frühbeck 2006, Tartaglia et al. 1995). Interestingly, this class of receptors also includes the gp130 subunit of the IL-6 receptor family, suggesting that IL-6 and leptin may operate via a similar mode of action (Baumann et al. 1996). Therefore, leptin might be part of the axis that links obesity, inflammation, and hepcidin release with aberrant iron metabolism.

3.1.6 Obesity related to perinatal iron deficiency: Fetal Programming

Conversely, some authors has also suggested, consistent with the 'thrifty phenotype' hypothesis, that iron deficiency, especially perinatal iron deficiency, might lead to increased visceral adiposity in adulthood as result from developmental adaptations (Komolova et al. 2008, McClung et al. 2008). McClung J.P. et al (McClung et al. 2008), studied the effect of moderate iron deficiency and physical activity on body composition in a rat model and indicated that moderate iron deficiency results in the preferential accretion of body fat accompanied by corresponding reductions in lean body mass. These authors also observed that changes in body composition were affected by physical activity, suggesting that physical activity could be an effective countermeasure against some functional outcomes of moderate iron deficiency. Besides, sedentary behaviour associated with the iron-deficit state has been argued as being responsible for this accretion of adiposity (Komolova et al. 2008).

Hales and Barker coined the term 'thrifty phenotype' for individuals 'programed' to develop cardiovascular and metabolic risk factors in adulthood, such as hypertension and abdominal obesity, in response to nutritional deficiencies in fetal and early life (Hales, Barker 1992). This phenomenon was known originally as ''programming'' since the fetus was programmed to show effects long after the stressor has been removed. The concept

was refined into the ‘‘thrifty phenotype’’ which suggested that development in utero was regulated and the metabolism of the developing fetus was defined to expect a certain nutritional environment in the post-natal period (Hales, Barker 1992). If this was experienced, then there was no problem. Increased risk of disease only arose if there was an imbalance between the post-natal and prenatal nutritional intake.

In summary, recent studies seem to indicate that obesity is associated with iron deficiency although the aetiology appears to be multifactorial and includes (i) A decrease in iron food intake; (ii) An impairment of intestinal iron uptake and iron release from stores because of an overexpression of hepcidin and (iii) Inadequate iron bioavailability because of inflammation. In addition, abnormal ferritin concentrations can be explained by chronic inflammation rather than by iron overload (Yanoff et al. 2007). Recent studies are emerging suggesting an association between perinatal iron deficiency and programmed obesity in the adulthood, although the mechanism explaining this relationship is unclear.

3.1.6 Insulin, insulin resistance and iron

It is widely accepted that IR plays a pivotal role in type 2 diabetes and metabolic syndrome, as well as in some forms of obesity. A relationship between iron metabolism and glucose metabolism has been reported (Fernandez-Real, Lopez-Bermejo & Ricart 2002). Insulin causes a rapid stimulation of iron uptake by fat cells and hepatocytes (Davis, Corvera & Czech 1986). Reciprocally, iron interferes with insulin action in the liver. In addition, it has been reported that ferritin levels correlate positively with blood glucose and fasting serum insulin (Tuomainen et al. 1997), and negatively with insulin sensitivity (Dmochowski et al. 1993).

High levels of serum ferritin have been proposed as a component of IR syndrome (Fernández-Real et al. 1998). Apart from obesity, the concentration of circulating ferritin is associated with all the components of IR syndrome, such as diabetes (Ford, Cogswell 1999, Hernandez et al. 2005). In fact it has been demonstrated that ferritin levels correlate significantly with the number of IR elements (Wrede et al. 2006). Vari *et al.* (Vari et al.

2007) have reported that not only ferritin but also transferrin were significantly correlated with all parameters of metabolic syndrome in men and in both pre- and postmenopausal women. Similarly, another study has shown that in subjects having three or more criteria of metabolic syndrome, ferritin was the more important independent determinant of IR (Tsimihodimos et al. 2006). Conversely, iron depletion by phlebotomy improves IR (Valenti et al. 2007). Hence, there is evidence that hyperferritinemia observed in obesity must be interpreted in the context of the IR syndrome. However, the interacting pathways linking IR and iron are not well understood. Some authors are of the opinion that iron overload is the main cause, thus reflecting a high iron content in the liver. However, others consider that the inflammatory milieu associated with IR is the main factor responsible for hyperferritinemia. The classic work of Moirand *et al.* (Moirand et al. 1997) reported elevated liver iron concentration in liver biopsy samples of patients with elevated serum ferritin and features of metabolic syndrome. The same group of research reported the nearly constant association between higher levels of ferritin and IR in patients with unexplained hepatic iron overload demonstrated by liver biopsy (Mendler et al. 1999). Rumberger *et al.* (Rumberger et al. 2004) has shown that excess of iron increases lipolysis and subsequent raising in circulating free fatty acids could be a factor responsible for IR. Nevertheless, Brudevold *et al.* (Brudevold, Hole & Hammerstrom 2008) have recently shown that in patients with metabolic syndrome, elevated ferritin levels could not be attributed to an increase in liver iron stores and postulated other mechanisms such as inflammation. Interestingly, Olthof *et al.* (Olthof et al. 2007) have determined liver iron concentration by magnetic resonance imaging in 28 subjects. The authors have found that the correlation with serum ferritin was higher in those patients without inflammatory processes (assessed by normal serum leucocyte level, sedimentation rate and C-reactive protein) than in the group as a whole.

3.1.1 Iron metabolism and risk of gestational diabetes

As mentioned before, increasing evidence suggests that iron, a transitional metal and a strong prooxidant, influences glucose metabolism, (Fernandez-Real, Lopez-Bermejo & Ricart 2002), even in the absence of significant iron overload (review by (Rajpathak et al.

2009)). Large prospective cohort studies found that dietary iron intake, particularly heme iron derived from meat, was associated with a significant increased risk of type 2 diabetes (Rajpathak et al. 2006, De et al. 2008). Furthermore, serum ferritin levels (a biomarker of body iron stores) were positively associated with insulin resistance and diabetes risk (Tuomainen et al. 1997, Fernández-Real et al. 1998, De et al. 2008, Forouhi et al. 2007), hypertension (Piperno et al. 2002), the metabolic síndrome (Qi et al. 2007), cardiovascular risk factors, and inflammation (Williams, Poulton & Williams 2002).

In pregnant women, increased serum ferritin concentration, has also been recently reported in GDM (Lao, Chan & Tam 2001, Lao et al. 2002). Women with a history of GDM are at an elevated risk of developing type 2 diabetes (Lee et al. 2007, Yun et al. 2007, Weitzman, Harmanboehm & Maislos 1990). Lao et al. (Lao, Tam 1997) first reported that serum ferritin levels were higher among Chinese women with impaired glucose tolerance (IGT) diagnosed during 28 to 30 weeks of pregnancy compared to those with normal glucose tolerance (NGT). In another study, these investigators reported that mean ferritin levels were higher among women with GDM compared to those without GDM (Lao, Chan & Tam 2001), which was also reported in another small study among Swiss women (Bencaiova et al. 2005). In addition to these cross-sectional data, two prospective studies have also evaluated the association between serum ferritin levels and GDM (Tarim et al. 2004, Chen, Scholl & Stein 2006). In the first study among 253 Turkish women, the cumulative incidence of GDM was lower among women with ferritin levels below the median (19.7 μg/l) compared to those with levels ≥ median (4.1 vs. 13.1 per 100) (Tarim et al. 2004). In the second prospective investigation conducted among 1,456 healthy pregnant women living in New Jersey (Camden Study), the odds ratio (OR) for developing GDM was 2.35 (95% CI: 1.06, 5.22) comparing women in the highest tertile of ferritin to those in the lowest tertile (Chen, Scholl & Stein 2006). However, these results were not significant when either C-reactive protein (CRP) or prepregnant BMI was included in the model. Women in the highest tertiles of both ferritin and CRP had the highest risk compared to the rest of the women (OR: 3.30; 95% CI: 1.10, 8.41). Further, the ferritin-GDM association was modified by level of obesity. Obese women

with high ferritin levels had a 3.5-fold increased risk of developing GDM (95% CI: 1.35, 9.27; p=0.01), whereas results were not significant among non-obese women.

3.1.7 Iron status in newborn babies

The total body iron content of a newborn infant born during the third trimester is dependent on the birth weight and is approximately 75 mg/kg, being ~ 200 mg at a low birth weight of 2500 g, and 270 mg at a "normal" birth weight of 3500 g (Saddi, Shapira 1970). Approximately 60% of this iron is accreted during the third trimester of gestation (Anonymous2001b). Pregnancies resulting in large babies therefore put greater demands on the future mother's iron reserves than pregnancies resulting in small babies.

The distribution of the body iron is 75–80% in red blood cells (RBC) as hemoglobin (Hb), approximately 10% in tissues as iron-containing proteins (e.g. myoglobin and cytochromes), and the remaining 10–15% as storage iron (e.g. ferritin and hemosiderin). The storage iron content progressively increases and is reflected by cord serum ferritin concentrations >60 µg/L at full term. The iron requirements after birth are influenced by the time of onset of postnatal erythropoiesis and the rate of body growth. The iron endowment at birth and iron from external, usually dietary, sources meet this need. The period soon after birth is characterized by a 30–50% decrease in Hb secondary to cessation of erythropoiesis, lysis of senescent fetal RBC and expansion of the vascular volume. During this 'physiologic anemia' the Hb can reach 100–110 g/L between 6 and 8 weeks of age. In preterm infants, the Hb nadir can be as low as 60–80 g/L, occur 1–4 weeks earlier than full-term infants and is called 'anemia of prematurity'. An element of disordered or ineffective erythropoiesis might contribute to the earlier, more severe Hb nadir in preterm infants. The iron released during lysis of senescent RBCs (3.47 mg/g of Hb) is stored for future use and is reflected by a transient increase in serum ferritin concentration during the first month of life (Siimes, Siimes 1986). In full-term infants, this stored iron supports the iron needs of the ensuing erythropoiesis and growth until 4–6 months of age. In preterm infants, earlier iron supplementation is necessary.

Intrauterine growth restriction (IUGR), maternal smoking and poorly controlled diabetes mellitus during pregnancy are important causes of perinatal iron deficiency in developed countries. All three gestational conditions are characterized by intrauterine fetal hypoxia and augmented erythropoiesis that requires additional iron. Approximately 10% of all pregnancies are complicated by IUGR. Whereas maternal malnutrition is likely responsible in developing countries, pre-existing or pregnancy-induced maternal hypertension is responsible for IUGR in developed countries. In pregnancies associated with IUGR due to maternal hypertension, placental iron transport is decreased due to placental vascular disease and impaired uteroplacental blood flow. Approximately 50% of IUGR infants are iron deficient at birth, as suggested by cord serum ferritin concentration <60 µg/L (Chockalingam, Murphy & Ophoven 1987). The liver and brain iron concentrations are decreased in IUGR infants without a significant effect on Hb at birth. In severe cases, brain iron concentration could be decreased by 33% (Georgieff et al. 1995).

3.1.1 Influence of maternal Fe status on fetal Fe status

There are contradictory opinions with respect to the influence of maternal Fe status on fetal Fe status, and how maternal and fetal iron status are related is not entirely clear. It has been believed for a long time that maternal Fe deficiency has little or no effect on the acquisition of Fe by the fetus (MacPhail et al. 1980, Lao et al. 1991). However, a number of investigators have found a positive correlation between maternal and newborn Fe status, suggesting that the fetus is vulnerable to Fe deficiency during intrauterine life (Gaspar, Ortega & Moreiras 1993), particularly with increasing severity of maternal anemia (Singla et al. 1996). In the study by Rusia et al. (Rusia et al. 1996), serum transferrin receptor concentrations were higher in infants born to anemic mothers (Table 4).

Table 4 . Factors that influence body iron status during the perinatal period (reproduced from Rao, R., Georgieff, M.K. Iron in fetal and neonatal nutrition (2007) *Seminars in Fetal and Neonatal Medicine*, 12: 54-63).

Factors that have a negative effect
Maternal iron deficiency

Maternal diabetes mellitus
Maternal smoking
Intrauterine growth restriction
Multiple gestationa
Preterm birth
Acute and chronic fetal hemorrhage, e.g. umbilical cord accidents and fetofetal (donor twin) transfusions
Immediate clamping of the umbilical cord after birth
Exchange transfusion
Restrictive transfusion practiceb
Uncompensated phlebotomy lossesb
Recombinant erythropoietin useb
Delayed and inadequate iron supplementationb
Exclusive breast milk usebc
Ingestion of cow's milk
Factors that have a positive effect
Maternal iron supplementd
Fetofetal transfusion (recipient twin)
Delayed clamping of the umbilical cord
Liberal transfusion practiceb
Early and adequate iron supplementationb
Use of iron-fortified formulab

aIron deficiency is more likely if mother is iron deficient during pregnancy.
bThe risk of iron deficiency is greater in preterm infants than full-term infants.
cExclusive breastfeeding meets the iron needs of full-term infants during the first 4–6 months of life.
dRoutine iron supplementation of mothers with adequate iron stores is controversial.

Iron requirements are increased during pregnancy either to supply the growing fetus and placenta and for the production of increased numbers of maternal red blood cells. Extra iron requirements were considered to be met through cessation of menstrual losses, increased intestinal absorption and mobilisation of maternal iron stores. The fetus accumulates most of its iron during the last trimester of pregnancy, and the iron needs of the fetus are met at the expense of maternal iron stores. So that the newborn's iron status *may* depend on the pregnant woman's iron status. There are growing concerns that the stores of iron at birth in some infants may be insufficient to sustain optimum development during the first 6 months of life (Chaparro 2008).

During pregnancy, iron is transported from the mother to the fetus across the placental membrane by an active process, which is mediated via binding of maternal transferrin bound iron to transferrin receptors in placenta and subsequent transfer of iron into the fetal circulation (Fletcher, Suter 1969, Brown, Molloy & Johnson 1982). The efficiency

of this transport system implies that iron deficiency in the newborn is encountered only at extreme iron deficiency in the mother, so that iron deficiency in mature newborn babies is a rare event in the developed countries. At delivery, there is a correlation between the mother's and the newborn's serum ferritin (Milman, Ibsen & Christensen 1987). Children born to iron treated mothers have higher serum ferritin than children born to placebo-treated mothers (Romslo et al. 1983, Milman, Agger & Nielsen 1991, Preziosi et al. 1997). The higher ferritin levels in newborns of mother treated with iron suggest that these babies have a higher iron content and therefore a lower risk of contracting iron deficiency within the first years of life (Preziosi et al. 1997, Michaelsen, Milman & Samuelson 1995). Another important determinant of the newborn's iron status is the amount of blood transfused from the placenta before the clamping of the umbilical cord (Tyson 1992).

Maternal iron deficiency, with or without associated anemia, adversely affects fetal iron status. A maternal Hb concentration <85 g/L is associated with decreased fetal iron stores (cord serum ferritin <60 mg/L). More severe maternal anemia (Hb < 60 g/L) is associated with lower cord Hb concentration, as well as cord serum ferritin concentration <30 mg/L, a level suggestive of severe depletion of storage iron and potential brain iron deficiency (see below) (Singla et al. 1996). A maternal ferritin concentration <12 mg/L appears to be the threshold below which fetal iron accretion is affected (Jaime-Perez, Herrera-Garza & Gomez-Almaguer 2005); 14% of full-term infants born to iron-deficient mothers have a serum ferritin concentration <30 mg/L at birth. Finally, even when iron endowment appears to be adequate at birth, infants of mothers with mild to moderate iron deficiency anemia are at risk for iron deficiency throughout infancy, especially between 6 and 12 months of age (Colomer et al. 1990, Kilbride et al. 1999).

3.1.2 Influence of maternal gestational diabetes on fetal Fe status

Between 5% and 10% of pregnancies are complicated by maternal diabetes mellitus. Poorly controlled diabetes mellitus during gestation is associated with maternal and fetal hyperglycemia, fetal hyperinsulinemia, increased fetal metabolic rate and oxygen consumption. The increased fetal oxygen consumption in a relatively hypoxic intrauterine

environment stimulates erythropoiesis and expands the fetal RBC mass. The additional iron required for the augmented erythropoiesis cannot be met by increasing maternal-fetal transport. Whereas placental transferrin receptor expression is increased in pregnancies complicated by diabetes mellitus, the affinity of the receptor to maternal transferrin is decreased, probably due to hyperglycosylation of the oligosaccharides present in the binding domain (Georgieff et al. 1997). Furthermore, placental vascular disease might be present in mothers with longstanding, poorly controlled diabetes mellitus, further limiting iron transport across the placenta. Tissue iron is depleted to support the iron needs of augmented erythropoiesis under these situations. Nearly 65% of infants of diabetic mothers (IDM) have perinatal iron deficiency, as suggested by cord serum ferritin concentration <60 mg/L. In approximately 25% of these infants cord serum ferritin is <35 mg/L, suggesting significant depletion of tissue iron, including brain iron (Georgieff et al. 1990, Petry et al. 1992).

3.1.3 Maternal smoking and fetal Fe status

Maternal smoking during gestation is associated with fetal hypoxia due to carbon monoxide and decreased uteroplacental blood flow due to nicotine and catecholamine-induced vasoconstriction. The augmented erythropoiesis stimulated by fetal hypoxia results in depletion of iron stores in the offspring of these mothers (Chełchowska, Laskowska-Klita 2002, Sweet et al. 2001, Meberg et al. 1979). Cord Hb is increased and ferritin concentrations in cord blood and the placenta are decreased 40% and 20%, respectively, in infants of mothers who smoked during pregnancy (Chełchowska, Laskowska-Klita 2002). Infants born to mothers who smoked during gestation are at risk for iron deficiency at 12 and 24 months (Freeman et al. 1998).

3.1.8 Iron status in pregnancy and fetal outcomes

The traditional notion that anaemia in pregnancy, although common, was not a serious issue even for the foetus (Dallman 1989) has been revised in recent years with a number of studies suggesting a relationship between anaemia early in pregnancy and the risk of

pre-term birth, and also the reduced occurrence of LBW with iron supplementation (Scholl 2005).

Iron is one of the major trace elements required during pregnancy (Dawson, Mcganity 1987). Iron deficiency during pregnancy is still common in developed countries (Beard 1994, Milman, Clausen & Byg 1998, Robinson et al. 1998, Bergmann et al. 2002). Despite iron supplementation, 30% of pregnant women have a low serum ferritin concentration at the end of pregnancy (Kelly, Macdonald & Mcdougall 1978). Iron deficiency is associated with unwanted events like maternal anemia, premature contractions and adverse birth outcomes such as small for gestational age (SGA), preterm delivery, low birth weight (LBW) and delayed offspring neurological development, particularly if present during the first half of pregnancy (Allen 2000, Scholl 2005, Dawson, Mcganity 1987, Zhou et al. 1998, Rao, Georgieff 2007, Beard 2008, Baker et al. 2009, Goepel, Ulmer & Neth 1988). Iron-deficiency anemia can alter the proliferation of T- and B-cells, reduce the killing activity of phagocytes and neutrophils, and lower bactericidal and natural killer cell activity, thereby increasing maternal susceptibility to infections (Allen 2001). Several studies have shown that Fe deficiency during pregnancy, both in humans and in animal models, results in long-term problems for the offspring. There is evidence from animal studies that low iron intake during pregnancy and maternal iron deficiency adversely affects the offspring's blood pressure, lipid metabolism, obesity levels and other cardiovascular outcomes in the long term (Gambling et al. 2003, Lisle et al. 2003, Gambling, Dunford & McArdle 2004, Zhang et al. 2005, Andersen et al. 2006, Gambling et al. 2002, Gambling et al. 2002). Godfrey and colleagues have shown that maternal Fe status may be a risk factor for adult disease, with an increased risk of cardiovascular problems in adulthood (Godfrey et al. 1991). It induces fetal growth retardation and this effects generated in utero and early development can persist into adulthood (e.g., (Crowe et al. 1995, Godfrey et al. 1996, Godfrey, Barker 1995, Kwik-Uribe et al. 2000). It induces the increase in blood pressure (Crowe et al. 1995) diminish brain function (Walters, Miller & Worwood 1973, Kwik-Uribe et al. 2000, Rao, Jagadeesan 1996, Rao et al. 1999, Soewondo 1995) and compromise immune system development (Hallquist et al. 1992, Lockwood, Sherman 1988). *It also* can increase the

risk of infant to develop iron deficiency anaemia (IDA) during the first few years of life (Allen 2000). However, it is rare that a baby is born with IDA, unless it is premature and therefore has not had enough time to accumulate enough iron during the last trimester (Thomas 2001). So that an adecuate iron status is required from conception, throughout the pregnancy but also during lactation since iron deficiency during this period has been associated with mental retardation (Dawson, Mcganity 1987, Casanueva et al. 2003). Evidence is emerging of a strong association between foetal iron status and cognitive development, and that a shortage of iron availability in early life may have negative consequences for neural development and functioning later in life (Beard 2008, Beard 2000).

The ideal outcome of pregnancy is the delivery of a fullterm healthy infant with a birthweight of 3.1–3.6 kg. This birthweight range is associated with optimal maternal outcomes in terms of the prevention of maternal mortality and complications of pregnancy, labour and delivery, and optimal fetal outcomes in terms of preventing pre- and perinatal morbidity and mortality, and allowing for adequate fetal growth and development (Anonymous1995a). Various different types of adverse fetal environment have been found to affect birth weight with potential consequence for disease in later life, it has been thought that iron deficiency during pregnancy is one of them. Whether ID or iron deficiency anaemia (IDA) may contribute to low birth weight (LBW) have been contentious as a consequence (Rasmussen, Stoltzfus 2003). It is important also since evidence exists that LBW infants may be at risk for high blood pressure and cardiovascular disease later in life (Barker et al. 1989, Barker et al. 1990). There is ample evidence from observational studies for an association between maternal anemia (defined by hemoglobin concentration) and size at birth. This association is U-shaped, with the proportion of low birth weight infants rising with maternal hemoglobin values at the low or high end of the range (Murphy et al. 1986)(review by (Rasmussen 2001)). Lower birth weights in anemic women have been reported in several studies (e.g., (Hemminki, Rimpela 1991, Agarwal, Agarwal & Mishra 1991, Singla et al. 1997). Pregnant women's hemoglobin and newborn's birth weight are inversely correlated already from the beginning of the second trimester, before the maximum decline in hemoglobin has

occurred (Murphy et al. 1986, Sagen et al. 1984, Steer et al. 1995). It was shown in a population of unselected pregnant women, comprising women with both normal and complicated pregnancies. A low hemoglobin is associated with a low blood viscosity, which increases placental perfusion, and is assumed to result in a better nutrition of the fetus (Steer et al. 1995). From this point of view, optimum hemoglobin levels in the second trimester range between 95-115 g/L (6.0-7.1 mmol/L) (Steer et al. 1995). Welsh women, who in 13-24 weeks gestation had haemoglobin levels of <105 g/l (6.5 mmol/l), displayed a ~1.6-fold higher relative risk for preterm birth, low birth weight and prenatal mortality of their newborns than non-anaemic women (Murphy et al. 1986).

Birth weight decreases both at low hemoglobin levels of < 86 g/L (5.3 mmol/L), and at high levels of >145 g/L (9.0 mmol/L) (Steer et al. 1995). The association between a high hemoglobin in pregnant women and a low birth weight of the newborn is first of all due to poor plasma volumen expansion which results in an inadequate hemodilution in a fraction of the women, increasing blood viscosity, which may reduce placental perfusion and, thereby, the nutrition of the foetus, resulting in low birth weight (Steer et al. 1995). It predisposes to preeclampsia and eclampsia, both of which are associated with foetal growth restriction and a low birth weight of the newborn (Murphy et al. 1986, Yip 2000). In placebo-controlled studies on healthy pregnant women, there is no relationship between the women's haemoglobin and birth weight of the newborns, and there is no increased frequency of preeclampsia in the women taking iron supplements (Milman, Agger & Nielsen 1991, Milman et al. 2005). Pregnant women taking iron supplements have higher haemoglobin concentrations than women *without supplementation*. In this context, it is important to discriminate between the effect of iron supplements and the problems related to inappropriate haemodilution of pregnancy.

Due to iron deficiency anemia detected in early pregnancy is associated with a lower energy and iron intake, it results in an inadequate gestational weight gain over the whole pregnancy, and a greater than two-fold increase in the risk of preterm delivery (Scholl et al. 1992, Garn, Keating & Falkner 1981). As mentioned before, a similar U-shaped association was found between the maternal hemoglobin concentrations, i.e., anemia

according to the trimesters of pregnancy. Thus the risk of preterm delivery was approximately doubled in pregnant women with moderate-to-severe anemia during the first and second trimesters, while this relationship was reversed during the third trimester: anemia (low hemoglobin) associated with a decreased risk of preterm births (Murphy et al. 1986, Sagen et al. 1984, Klebanoff et al. 1991, Meng Lu et al. 1991, Goldenberg et al. 1996, Scanlon et al. 2000, Bondevik et al. 2001).

The biological model of iron balance, developed over many decades with the contributions of many investigators (Bothwell et al. 1979), suggest that the deleterious consequences of iron deficiency occur only after depletion of body iron stores (Dallman 1986). Elevated iron stores during pregnancy have been associated with maternal and neonatal morbidity. Women with raised ferritin levels in the third trimester of pregnancy have a greatly increased risk of preeclampsia, intrauterine growth retardation (IUGR) and preterm delivery (Scholl 2005, Rayman et al. 2002) . Some investigators reported a negative association between maternal serum ferritin and birth weight and a positive association with preterm delivery (Goldenberg et al. 1996, Tamura et al. 1996, Rondó et al. 1997). As Allen reviewed, these findings probably indicate the presence of infection, which elevates serum ferritin (Allen 2000).

In a large randomised double-blind clinical trial of iron with or without multi-micronutrient supplementation in pregnant women was shown no additional effect on birthweight from micronutrients over iron supplements alone (Ramakrishnan et al. 2003). Low iron status at the time of booking in pregnant women was found to be inversely related to placental size (Hindmarsh et al. 2000). The significance of this for the later health of the offspring is unknown but an association between increased placental size and increased blood pressure in the offspring has been noted in epidemiological studies (Barker et al. 1990).

3.1.9 Iron Suplementation and pregnancy

Large evidence from randomized control trials indicates that iron supplementation decreases the incidence of iron-deficiency anemia during pregnancy (Svanberg et al.

1975, Puolakka et al. 1980, Anonymous1996, Sjostedt et al. 1977, Taylor et al. 1982). Because iron deficiency makes a large contribution to anaemia, and iron deficiency has been related to adverse pregnancy outcomes, global efforts to reduce the anaemia burden and iron deficiency have largely been directed towards increasing intake of iron through supplementation, food fortification and diversification of the diet. Iron supplements are widely recommended and used during pregnancy worldwide (Anonymous1998, Anonymous2006a).

National medical organisations favor approaches ranging from an individual therapeutic one to proactive public health intervention; routine iron supplementation has not been recommended in Australia, New Zealand and the United Kingdom, whereas in countries like the United States and France, iron supplementation during pregnancy is standard practice (Makrides et al. 2003). In Spain, low iron dosages are used as general prophylaxis practice during second half of pregnancy, according to CDC recomendations (Anonymous1998), to achieve the requeriments through pregnancy even when iron stores are adequate. If suspects of iron deficiency or non-sufficient iron stores, prophylaxis practice should be start earlier (González de Agüero et al. 2001).

Controlled trials of iron supplementation during pregnancy have consistently demonstrated positive effects on maternal iron status at delivery. Certainly, iron supplementation during pregnancy increases maternal iron status during pregnancy including hemoglobin, serum iron, MCV, transferrin saturation, and serum ferritin, but another current concern is that iron supplements are a possible source of free radical development with the potential to cause oxidative damage to DNA, lipids and protein (Halliwell, Gutteridge 1999, Knutson et al. 1999, Lund et al. 1999). Iron overload and the associated oxidative stress contribute to the pathogenesis and increase risk of type 2 diabetes and other disorders. As mentioned before, in iron overload, the accumulation interferes with the extraction, synthesis and secretion of insulin (Fernandez-Real, Lopez-Bermejo & Ricart 2002) and moderately elevated iron stores also increase the risk of type 2 diabetes (Jiang et al. 2004a).

Studies have suggested a relationship between anaemia early in pregnancy and the risk of pre-term birth, and also the reduced occurrence of LBW with iron supplementation (Scholl 2005). However, these matters have remained controversial. Neither the Institute of Medicine Report in 1993 (Woolf 1993) or the Cochrane Review of 2001 (Mahomed 2001) were able to report either beneficial or harmful outcomes for iron supplementation (Makrides et al. 2003).

3.1.2 Objectives

- Estimate the risk of low birth weight and preterm delivery that is attributable to maternal iron deficiency.
- Determine whether overweight and obese pregnant women have an increased prevalence of iron deficiency compared with control group and the impact of this condition on the future neonatal health.
- Clarify the relationship between maternal iron status and development of adult disease in the offspring (e.g., obesity, gestational diabetes, cardiovascular disease).
- Influence of maternal iron status on neonatal iron status and the consequences for the neonatal health.
- Influence of maternal gestational diabetes on neonate iron state.
- Association of maternal iron status and risk of gestational diabetes mellitus.

3.1.3 Methodology

3.1.3 Blood sampling, hematologic assessment and biochemical parameters analysis

Maternal venous blood samples were collected at 24, 34 weeks of pregnancy and just before delivery for the hematologic and biochemical analysis. After birth, venous blood was taken from the cord immediately after a small aliquot was used for blood gas analysis to record the fetal pH at birth. The blood was collected in Vacutainer® blood collection tubes 3.0 ml, 7.5% EDTA (ref. 368857) for hematologic assessment and 8.5 ml tubes

(ref. 367953) for biochemical parameters. For biochemical assays the 8.5 ml tubes were centrifuged at 3500 r.p.m. for 10 minuts and serum was separated. Only for sTfR determination the aliquots were stored at -80ºC prior to analysis. Serum Fe was measured by enzyme-colorimetric automated methods for clinical chemistry (Modular Analytics EVO, Roche, Neuilly sur Seine Cedex, France). Ferritin, transferrin, cortisol and folate were measured by using the automatic analyser Elecsys 2010 with modular analytics E170 (Roche, Neuilly sur Seine Cedex, France). Cortisol was measured by electrochemiluminescence immunoassay (ECLIA). The transferrin saturation index (TSI) was calculated using the following formula:

$$TSI\ (\%) = (\text{serum iron } (\mu g/ml) \times 100) / (\text{serum transferrin } (mg/dl) \times 1.24)$$

3.1.4 Iron status classification

Cutoff values for Hb and hematocrit concentrations were selected on the basis of gestational age, using CDC criteria. The woman categorized as anaemic if the Hb < 110 g/L or hematocrits < 0.33 during the first and third trimesters and < 105 g/L and hematocrit < 0.32 in the second trimester (Anonymous1989a). In pregnant women that self-reported a history of current cigarette smoking (%), the Hb cutoff value was increased by +3 g/L (Anonymous1998). Pregnant women were classified as having iron deficiency anemia if the serum ferritin concentration was ≤15 µg/L and the Hb concentration was <110 g/L (1st and 3rd trimesters) or <105 g/L (2nd trimester) (Anonymous1998).

A number of other iron status markers were employed to gain further specificity and sensitivity. Serum ferritin concentrations ≤15 µg/L were used as an indicator of depleted iron stores (Perry, Yip & Zyrkowski 1995). Tissue iron deficiency was defined when sTfR concentrations exceeded 8.5 mg/L (Åkesson et al. 1998). Body iron stores were calculated from the sTfR: serum ferritin ratio (sTfR:F ratio) (Cook, Flowers & Skikne 2003). A sTfR:F ratio (both indicators expressed in units of µg/L) > 300 was also used as an indicator of depleted iron stores. This cutoff point was found to give a sensitivity of 85% and a specificity of 79% when used in pregnant women (van den Broek et al. 1998).

A measure of body iron reserves was calculated based on a formula developed using serial quantitative phlebotomy data in healthy men and non-pregnant women: (total body iron (mg/kg) = - [log (serum transferrin receptor/ serum ferritin) - 2.8229]/0.1207) (Cook, Flowers & Skikne 2003, Skikne, Flowers & Cook 1990). Although validation of this equation is not possible in pregnant women and neonates, this measure has been utilized to assess iron status in pregnant populations (Cook, Flowers & Skikne 2003, Iannotti et al. 2005). Negative values correspond to deficiency of body iron reserves. To convert the body iron concentrations from mg/kg to mmol/kg, divide values by 55.847.

Manufacturer reference were used to define the normal ranges for mean corpuscular volume (MCV), 81.0-99.0 fl; mean corpuscular hemoglobin (MCH) 27.0- 31.0 pg; mean corpuscular hemoglobin concentration (MCHC) 33.0-37.0 g/dL; and erythrocyte width distribution (RDW) 11.5-14.5%. Cut-off level used to indicate serum iron, folic acid and vitamin B12 deficiencies were 50 µg/dl, 3 ng/ml, and 148 pg/ml, respectively according to the literature (Van Den Broek, Letsky 2000, Anonymous1992) . Transferrin saturation less than 10.2% defined as low serum transferrin saturation and used as the indicator of iron deficiency anemia (Andrews 2003, Fielding 1980).

Definitions of iron status reflected the haemodilution that occurs in pregnancy (CDC) and were as follows: iron sufficiency Hb >110 g/L and SF>12 ㎎/L; iron deficiency without anaemia (ID) Hb >110 g/L and SF<12 ㎎/L; iron deficiency anaemia (IDA) Hb<110 g/L and SF<12 ㎎/L.

3.1.5 Folate classification

Serum folate concentrations < 6.80 nmol/L were used to classify any of the stages of folate depletion or deficiency (Bailey, Mahan & Dimperio 1980). To provide a more accurate measure of longer-term folate status, RBC folate status was calculated as follows: RBC folate (µg/L) = 21R × (100/*H*) where R = result, Hc = haematocrit as a percentage. A value > 453.2 nmol/L was considered normal or possibly indicative of early negative folate balance; values < 271.9 nmol/L were indicative of folate deficient erythropoiesis and anemia (Blot et al. 1981).

Chapter II: Placental iron transfer and pregnancy outcome

1. Introduction

1.1. The role of the placenta: The programming agent

Maternal obesity (prior to and during pregnancy) is present in 20-34% of pregnant women and is becoming a major health consideration for successful pregnancy outcome. Many researchers are currently investigating the effect of maternal obesity on different aspects of placental function and fetal development. Maternal obesity has been associated with both intrauterine growth restriction (IUGR) and large-for-gestational age (LGA) fetuses (Farley et al. 2009). Both conditions are characterized by altered insulin secretion and are connected to adiposity and diabetes in later life. Pre-pregnancy obesity is related to established hypertension and in some cases undiagnosed type 2 diabetes ("*Diabesity*") and it is associated with increased risk of placental dysfunction and fetal death as gestation advances.

The placenta is the first of the fetal organs to develop and has several fascinating and critical functions. As the main interface between the mother and the fetus, the placenta has three primary functions: 1) to provide an immunological barrier between the mother and fetus, 2) produce and secrete paracrine and endocrine hormones and cytokines and, 3) mediate the transfer of nutrients, oxygen, and waste products. Through these mechanisms, the initiation of maternal recognition of pregnancy, changes in local immune environment and changes in maternal cardiovascular and metabolic functions, take place (). By virtue of these roles the placenta is in a key position to play a direct role in fetal programming, i.e. by changing the patterns of developmental (hormonal) signals to the fetus or changing the pattern or amount of substrate transported to the fetus such that fetal development is altered, ultimately leading to cardiovascular or metabolic disease later in adult life. Epidemiological evidence has linked low birth weight and low placental weight to fetal programming. So, fetal growth and the long-term determination of the future offspring energy homeostasis are intimately linked to the regulation of these main functions of the placenta. It thus raised the question as to whether, maternal obesity

and/or insulin resistance, can substantially alter the key mechanisms regulating placental structural development and functionality, and therefore, expose the fetus to an inadequate growing environment.

Critical periods in placental development

The placenta is in a constant state to growth and differentiation throughout gestation showing a 40-fold increase in fetal/placental weight ratio (a measure of placental deficiency) from 6 weeks to term. This increased efficiency is achieved by the 10-fold increase in the villous volumen (vasculature) and an increase of trophoblast surface area (from 0.08 to 12.5 m^2) and a decrease in mean trophoblast thickness from 18.9 to 4.1 µm, and hence the materno-fetal diffusion distance from 55.9 to 4.8 µm (Myatt 2006). The disruption of the normal patterns of placental development will lead to a placenta with altered function. The timing of the disruption of this pattern will also be critical for placental function. Disruption during a period of angiogenesis will have different effects to disruption during a period of trophoblast growth and differentiation (Jansson et al. 2002). There is some evidence which suggests that a child of an obese or diabetic mother may suffer from exposure to a sub-optimal *in utero* environment and that these early life adversities may extend into adulthood. One primary mechanism that linked maternal nutritional status and the predisposition of metabolic disease is related to altered *placental functionalities* (Farley et al. 2009).

- ***Placental structural and functional changes in obese and diabetic pregnancies***

The biologic mechanisms underlying various risks of maternal *obesity in pregnancy* are unknown. While placental weight is related to maternal BMI, data on placental changes in obesity and diabetes in human pregnancies are still limited. Epidemiological data indicate that placental weight, albeit a crude proxy for placental structure appears to provide information on the long-term outcome for the baby (Godfrey 2002). Changes in placental growth represent an important link between perturbations in the maternal compartment (such as reduced placental blood flow, altered maternal nutrition and diabetes) and alterations in fetal growth.

Growing evidence in human and animal models of maternal obesity indicates several placental changes: *increased idiopathic villits* (Becroft, Thompson & Mitchell 2005), *macrophage infiltration and increased placental vascularity*. Maternal low grade inflammatory state with higher levels of CRP and IL-6 has been linked to placental changes in obesity. In adults this elevation of inflammatory mediators is linked to insulin resistance, suggesting that the same mechanisms may underlie the observed increases in glucose, lipids and amino acids in obese pregnancy. Maternal obesity in humans determines an increase of placental and adipose tissue macrophage infiltration, and also an increase of CD14+ expression in maternal peripheral blood mononuclear cells (PBMC) and maternal hyperleptinemia. It seems that chronic inflammation state of pre-gravid obesity is extending to in utero life with accumulation of a heterogeneous macrophage population and pro-inflammatory mediators in the placenta (Challier et al. 2008). The resulting inflammatory milieu in which the fetus develops may have critical consequences for short and long term programming of obesity (Farley et al. 2009).

Studies in rats, sheep and guinea-pigs have shown that both feed restriction and overfeeding affect development of the placental villi. A decrease of placental syncytio-trophoblast amplification factor and intact syncytiotrophoblast endoplasmic reticulum structure has been demonstrated in placental tissue from obese pregnant women; although overfeeding during the first and second trimesters results in reduced trophoblast proliferation and expression of angiogenic factors, associated with smaller cotyledons that are poorly vascularised (Reynolds et al. 2006). Moreover, cotyledon number is most affected by overnourishment during the first trimester, whereas the cotyledon size is most affected by nutritional status in the second and third trimesters (Wallace et al., 2004).

There are relatively few structural differences between placentas from mothers with diabetes and control subjects. In particular, the key measures that might be expected to influence substrate diffusion across the placenta (villous surface area, capillary surface area, villous membrane thickness) are not altered, as well as the placental morphometric diffusing capacity. Increases in capillary volume and surface area (Lobelo 2005, Wei et al. 2007), villous surface area (Whincup et al. 2008), increased total diffusive

conductance, and intervillous and trophoblast volume (Wei et al. 2007) were described and their presence or absence often ascribed to the degree of maternal diabetes control. However, a recent study has shown a reduction in the villous coefficient and elaboration index, measures of the complexity of the villous tree that would impinge upon the intervillous space, suggesting that the observed villous growth is *anisomorphic*; but there were no overall impact on villous volume or surface area, primarily due to an increased total placental volume in the T1DM pregnant women (Farley et al. 2009). So, changes in placental structure inherent in contemporary diabetic pregnancy are also minimal.

It has been demonstrated that IGF-I has a direct effect on placental development. Cord IGF-I is strongly associated with birth weight and placental weight, and IGF-I deletion or reduced receptor expression in humans are both associated with a reduction in birth weight and placental weight (Nelson et al. 2009).

- ***Regulation of placental nutrient transport***

Fetal nutrient delivery depends on the complex interaction of maternal uterine and fetal umbilical blood flow, nutrient supply, placental microstructure and transport capacity.

Placental nutrient transport has long been known to be dependent on vascular development which determines blood flow to both sides of the placenta and transport flow-limited substrates. Angiogenesis and vasculogenesis in both uteroplacental and fetal–placental circulations are important in this regard. There is abundant evidence for alterations in these parameters in pregnancies complicated by IUGR, pre-eclampsia or diabetes.

The role of the trophoblast (both amount and function), in placental transporter activity, hormone production and substrate metabolism is being recently investigated. There is evidence that changes in the activity and expression of trophoblast nutrient and ion transporters play a central role in determining fetal growth and the molecular

mechanisms regulating trophoblast transporters, which are directly related to the development of pregnancy complications and fetal programming of cardiovascular and metabolic disease (Roberts et al. 2009). Recent animal experiments have highlighted the involvement of both the vasculature and trophoblast in overall placental transport. In the over-nourished adolescent ewe, a model that results in IUGR, less proliferation of fetal trophectoderm and reduced expression of angiogenic factors in the placenta is seen. This leads to a reduction in placental mass, blood flow, fetal glucose, amino acids and oxygen concentration (Wallace et al. 2004). However, transporter activity was normal when adjusted to placental mass, so in this model placental size limits fetal growth.

It has been proposed that *'placental phenotype'* is a better representation of the intrauterine environment than birth weight. In particular, specific changes in placental nutrient transporter activity/expression characteristic of an intrauterine environment with decreased or increased delivery of nutrients(Jansson, Powell 2006), together with results on placental morphology and blood flows (Reynolds et al. 2006), constitute the *'placental phenotype'*. Placental phenotyping will provide much better information concerning the risk of developing diseases later in life than the crude proxies of intrauterine exposure that are currently used.

The concept of the placenta as a "*nutrient sensor"* has also been recently reported by (Jansson, Powell 2007). This concept introduces the idea about how the placenta coordinates nutrient transport functions with maternal nutrient availability. Thus the ability of the maternal supply line to deliver nutrients (i.e. placental blood flow, maternal nutrition, substrate and oxygen levels in maternal blood, etc.) regulates key placental nutrient transporters. With this perspective, placental transport alterations represent a mechanism to match fetal growth rate to a level which is compatible with the amount of nutrients that can be provided by the maternal supply line, making the placenta a *key player* in the regulation of fetal growth and, as a consequence, fetal programming. So, in the case of hyperglycaemia early in pregnancy (which is common even in the well-regulated patient with Type 1 Diabetes) may convey a "good nutrition" signal to the placenta, resulting in up-regulation of glucose and amino acid transporters (Jansson,

Powell 2006). There is evidence that maternal nutrition influences placental transporters and fetal growth by altering the levels of metabolic hormones, such as insulin, IGF-I and leptin, which have all been shown to regulate placental nutrient transporters (Ericsson et al. 2005b).

Maternal obesity also results in increased placental nutrient transport to the fetus (Jones, Powell & Jansson 2007). Fetal serum amino acid composition (hyperglutamatemia) and mononuclear cells (PBMC) transcriptome are different in fetuses from obese compared with non-obese pregnancies. Chronic fetal glutamate intoxication (either as a result of fetal hypoxia or maternal dietary overload) has been linked to the subsequent development of metabolic syndrome in later life. Neutral amino acid transport in the placenta inversely correlated to size at birth, and it is confirmed that placental system A (SAA) is increased in diabetes pregnancies associated with fetal macrosomia. Exogenous fetal IGF-I increases placental amino acid transfer and uptake and decreases proteolysis, facilitating organ-specific and placental growth. Therefore, although IGF-I may directly enhance placental growth via receptors expressed in trophoblast and endothelium, alternative indirect mediators like adiponectin, which have been implicated in the matching of fetal and placental weight, may contribute. The activity of the transporter for the essential amino acid leucine is increased in GDM with accelerated fetal growth, whereas an increased activity of placental SAA in both Type 1 diabetes and GDM has been found. IGF-1 stimulates SAA activity in cultured trophoblast cells, and insulin increases transport of neutral amino acids in the perfused human lobule (Nandakumaran et al. 2001) and in cultured trophoblast cells. In addition, SAA transporter activity and expression are decreased by hypoxia (Nelson et al. 2003). Leptin and insulin stimulated SAA activity uptake by 50–60% in primary villous fragments at term (Jansson et al. 2003). Furthermore, nitroxide (NO) and oxygen radicals have been shown to reduce the activity of several placental amino acid transporters (Khullar et al. 2004).

During in utero development, the fetus relies primarily on glucose as an energy substrate. In Gestational Diabetes and Type 1 Diabetes Mellitus pregnant women, there is

a steady supply of glucose even during maternal fasting because of the 30% increase in maternal hepatic glucose production in late gestation. The overall hormonal environment on the fetal side of the circulation is markedly abnormal, with median fetal insulin and leptin levels four and three times higher, respectively, than control subjects (Anonymous2008b). Maternal blood glucose, elevation of which is an obvious candidate in diabetic pregnancies, is subtly increased amongst obese women. It has been shown that even these modest increments can influence fetal growth, as evidenced by the HAPO study which showed a strong linear association between fasting maternal blood glucose in the normal range, and measures of neonatal adiposity.

Maternal insulin resistance during gestation results in increased lipolysis with increased availability of free fatty acids to be used as adipogenic substrates in the fetus. It has been shown that fetuses of obese mothers become insulin resistant in utero as estimated by umbilical cord glucose and insulin concentrations (Catalano et al. 2009). Moreover, insulin resistance in fetuses of fasted mothers (thus, in steady-state glycemic/lipemic conditions) could be estimated by HOMA-IR at birth (Catalano et al. 2009).

Accelerated fetal growth in pregnancies complicated by Type 1 diabetes, but not Gestational Diabetes (GDM), is associated with increased glucose transporter activity and protein expression in basal plasma membranes (BMs). These alterations might explain the occurrence of large babies in pregnancies complicated by Type 1 diabetes despite ‘normal’ maternal blood glucose levels (Jansson, Powell 2007). Glucose transporter activity was not affected by hormones, such as leptin, GH (growth hormone), IGF-1, insulin and cortisol, at term (Ericsson et al. 2005a). In contrast, insulin stimulated glucose uptake increases in primary villous fragments obtained at 6–8 weeks of gestation (Ericsson et al. 2005b), which may be related to the presence of the insulin-sensitive glucose transporter GLUT4 in the cytosol and microvillus plasma membranes (MVMs) of the syncytiotrophoblast in the first trimester (Ericsson et al. 2005b).

Hyperglycaemia early in pregnancy (which is common even in the well-regulated patient with Type 1 diabetes) may convey a 'good nutrition' signal to the placenta, resulting in up-regulation of glucose and amino acid transporters. Recently, Catalano et al. (2009) have shown that fatty acids are preferential lipogenic substrates for placental cells and genes for fetoplacental lipid metabolism are enhanced selectively in GDM. Moreover, Messenger RNA (*mRNA*) expression of stearoyl-CoA desaturase (*SCD*), 3-hydroxy-methylglutaryl-CoA reductase (*HMGCR*), and 3-hydroxy-methylglutaryl-CoA synthase (*HMGCS*) limiting steps for triglyceride and cholesterol synthesis may be instrumental in increasing transplacental lipid fluxes and the delivery of lipid substrates for fetal use.

Perturbations in the maternal compartment may affect the methylation status of placental genes and increase placental oxidative/nitrative stress, resulting in changes in placental function (Roberts et al. 2009). Maternal nutritional status, possibly by altering the availability of methyl donors, such as folate, has been shown to influence the methylation status of the fetal genome. DNAmethylation regulates gene expression in that hypermethylation of promoter regions is commonly associated with transcriptional repression, whereas hypomethylation often increases transcription (Gemma et al. 2009). Maternal environmental influences before or at the time of conception may alter the methylation status of trophoblast genes, which could result in a permanent change in placental structure and function.

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Other pertinent parameters include raised insulin and triglyceride concentrations, and higher concentrations of fatty acids which may contribute to increased fat accretion in the offspring.

The role of imprinted genes in the placenta

At least 60 imprinted genes have been described in humans. Paternally derived imprinted genes enhance fetal growth while maternally imprinted genes suppress fetal growth (Reik *et al.* 2003).

Changes in cellular energy levels not only promote internal pathways involved in the regulation of energy metabolism and transport, they also stimulate additional mechanisms that in turn, govern pro-inflammatory pathways and cellular functions. In fact, a close relationship exists between such pivotal functions that involved the expression and activity of some key proteins and transcription factors. Such key regulators that influence both the regulation of energy homeostasis and inflammatory process in the placenta are: the mammalian target of rapamycin (mTOR), the peroxysome-proliferator activated receptor gamma (PPAR gamma), the uncoupling protein 2 (UCP2) and the toll-like receptor 4 (TLR4). The regulation of genes involved in placental energy metabolism, pro-inflammation and DNA remodelling (UCP-2, TLR-4, DNMT-1) can determine the partitioning of energy between the mother and fetus. In

addition, they may be altered by weight gain and diabetes during pregnancy and subsequently result in long-term adverse outcomes.

Intervention strategies targeting the placenta in order to prevent or alleviate altered fetal growth and/or fetal programming are being suggested. These strategies will probably include altering placental growth and nutrient transport by maternally administered IGFs (insulin-like growth factors) and altering maternal levels of methyl donors. Another, more speculative, alternative to manipulate placental nutrient transporters is targeting the placental mTOR signalling system, which has recently been shown to regulate the placental system L amino acid transporter (Roos et al. 2005), a key transporter across the placental barrier for a number of essential amino acids. Placental mTOR inhibition may be particularly relevant in situations of fetal overnutrition and overgrowth, such as maternal diabetes, which is associated with up-regulation of placental nutrient transporters. More speculative treatment options include altering the methylation status of placental genes by, for example, maternal folate supplementation, up-regulating the activity of placental 11β-HSD-2 and decreasing placental oxidative/nitrative stress. However, in order to design more specific interventions with these placental targets, more research is needed.

Knockout of paternally expressed *Igf2* reduces placental growth while knockout of maternally expressed *p57kip2* results in placental hyperplasia. Imprinted genes control both fetal and placental growth and may therefore control both the supply (placenta) and demand (fetus) of nutrients. In addition, several imprinted genes also encode for specific transporters in trophoblast. The paternally expressed *Ata3* gene encodes a component of the system A amino acid transporter (Mizuno *et al.* 2002), while the maternally imprinted *Impt/Slc22a11* gene encodes an organic cation transporter (Dao *et al.* 1998). Imprinting is also controlled by epigenetic mechanisms including DNA methylation and histone acetylation under the control of environmental factors and nutrients (Reik *et al.* 2003). This may provide a linkage between maternal nutrition and fetal placental growth.

1.2. Effect of iron deficiency on placental gene expression: Placental Transferrin receptor

Transfer of iron from the mother to the fetus is supported by a substantial increase in maternal iron absorption during pregnancy and is regulated by the placenta (Harris 1992, Starreveld et al. 1995).

In experiments developed in rats, Gambling et al (Gambling et al. 2002) demonstrated that iron deficiency not only has direct effects on iron levels and metabolism but also alter placental function, exerting effects on other regulators of growth and development, such as *placental cytokines*, and that these changes may, in part at least, explain the deleterious consequences of maternal iron deficiency during pregnancy (Gambling et al. 2002). They found a reduced fetal growth and an increased *expression of placental cytokines*, notably TNFα, which has been associated with problems in pregnancy (Gambling et al. 2002). In subsequent experiments, they showed that iron deficiency in placental cell lines *may* change transfer of other nutrients than iron, such as amino acids, concomitantly with alteration in the expression of specific transporters.

Placental transferrin receptor (TfR) protein expression is increased in diabetic pregnancies that are complicated by low fetal iron stores, suggesting regulation of placental iron transport by fetoplacental iron status. In cell culture, iron homeostasis is regulated by coordinate stabilization of TfR mRNA and translation inactivation of ferritin mRNA by iron regulatory proteins (IRP-1 and -2) which bind to iron-responsive elements (IREs) on the respective mRNAs. Georgierff et al. determined the concentrations of IRP-1, IRP-2 and TfR mRNA in 10 placentae obtained from diabetic and non-diabetic human pregnancies with a wide range of fetoplacental iron status. The study showed that IRP-1 activity was present in human placenta and correlated closely with TfR mRNA concentration ($r=0.82$; $P=0.007$). IRP-2 activity and protein were not detected. In a second experiment, placentae were collected from 12 diabetic pregnancies, six with low fetal cord serum ferritin and placental non-heme iron concentrations, and six with normal iron status. IRP-1 activity and TfR Bmax for diferric transferrin were greater in the iron-deficient group ($P<0.05$). IRP-1 activity correlated inversely with cord serum ferritin

(r=0.75; P<0.01) and placental non-heme iron (r=0.61; P=0.05) concentration. The authors concluded that placental IRP-1 activity is directly related to TfR mRNA concentration and is more highly expressed in iron-deficient placentae. This study provides direct in vivo evidence for IRP regulation of TfR expression in the human placenta.

Kralova et al. observed consistent cytotrophoblast expression of TF in all used placenta samples, whereas the syncytiotrophoblast TF expression varied among various samples. As cytotrophoblast represents a precursor of syncytiotrophoblast and differentiates into syncytium during placenta formation, its function is important during implantation and early placentation. Therefore, the presence of TF in the extravillous cytotrophoblast suggests its possible involvement in such events.

Interestingly, strong expression of TF was also found in amniotic epithelium (Verrijt et al. 1997). However its importance here remains unclear. Although there are reports showing evidence of TF production in the placenta (Verrijt et al. 1997, Buus, Boockfor 2004), it does not necessarily mean that the increase in TF expression in the placentae after an abnormal pregnancy course is due to the increased production of TF in the cell or that TF in the cell is highly active. Another possible explanation is that the metabolism of TF in the syncytiotrophoblasts might be prevented. The explication can be connected with the presence of transferrin receptor (TfR, a transmembrane protein mediating the cellular iron uptake by binding and internalization of diferric transferrin) and its function that can influence the amount of TF in the placenta (Wada, Hass & Sussman 1979, Seligman, Schleicher & Allen 1979). Several iron transporters and regulators were characterized recently. Interestingly, these iron transporters localized in placental trophoblast cells, mainly in recycling endosomes, were found to interact. It was also suggested that the level of intracellular iron may regulate both TfR expression and TfR trafficking/transcytosis in polarized cells (Gruper et al. 2005). In cultured cytotrophoblasts, TfR levels increase in cells cultured in iron-poor medium, indicating that iron has an effect on the TfR synthesis/breakdown ratio. These cultured trophoblasts regulate iron uptake by variation of the number of surface TfRs via changes in total TfRs

and their redistribution in the membrane (Starreveld et al. 1993). Also, *in vivo* the placenta minimizes the effect of the deficiency by up-regulating the proteins involved in Fe transfer. For example, TfR levels increase inversely to maternal Fe levels (Gambling et al. 2003), which was described to occur in diabetic pregnancies that are complicated by low fetal iron stores. There the expression of TfR is increased, suggesting the regulation of placental iron transport by fetoplacental iron status (Georgieff et al. 1999).

1.3.Effects of maternal Fe status on Fe status of the placenta.

The effects of maternal Fe status on Fe status of the placenta, the organ supplying the fetus with Fe, have not been extensively studied or understood. The placenta is the point of interchange between maternal and fetal circulation, where oxygen and nutrients are transferred to the fetus and fetal waste products are removed. All these activities are essential for maintaining pregnancy and promoting fetal development. Placental Fe content at term was shown to correlate with birth weight, gestational age and placental weight, thus affecting fetal outcome. However, placenta is not usually analyzed in clinical practice.

2. Objetive

Placental tissue was collected to assess relationships between maternal iron status and placental protein expression of TfR in order to shed more light on relation between high placental TfR expression and iron defficiency in the mother and consequences for neonate´s health.

3. Methods

3.1.Stocks, Solutions, Buffers and Gel Recipes

Hepes sucrose buffer
85,6g 250mM Sucrose
4,8g 20mM Hepes
Adjust pH to 7.4 with NaOH.
Dilute to 1000ml with ultrapure water

Bradford Protein Reagent, Bio-Rad 500 0006

2mg/ml Albumin from Bovine Serum (BSA) in dH2O, Sigma A7906

Phosphate buffered saline (PBS)
Dilute 5 tablets in 1 liter of ultrapure water (1 tablet in 200ml dH2O).

Western Blotting buffers
10%APS
0.1g Ammonium persulphate
1 ml dH2O

1,5M Tris pH 8.8
45,415g 1,5M Tris
150ml Ultrapure water
Adjust pH with HCl to 8,8. Make up to 250ml with ultrapure water. Store at 4ºC.

0,5M Tris pH 6,8
15,14g 0,5M Tris
150ml Ultrapure water
Adjust pH with HCl to 6,8. Make up to 250ml with ultrapure water. Store at 4ºC.

Stock (10x) SDS Running buffer
15,15g Tris

72g Glycine
25ml SDS (20%)
Make up to 500ml with ultrapure water. Store at room temperature.

Protein sample buffer:

2 x protein sample buffer for BeWo Control (to prepare 5ml):
500µl Glycerol
625µl 0,5 Tris HCl pH 6,8
581,5µl SDS (20%)
3043,5 µl Ultrapure water
Bromophenol Blue
Leave in the roller for 1 hour to mix properly and make aliquots. Store at -20ºC.

5 x protein sample buffer for placenta samples (to prepare 10ml):
5,7ml Glycerol
1,563ml 2M Tris
1g SDS
Ultrapure water until 10ml
Bromophenol Blue
Leave in the roller for 1 hour to mix properly and make aliquots. Store at -20ºC.

Transfer buffer
29,3g Glycine
58,2g Tris
Make up 1 liter with ultrapure water and check pH (must to be around 8,3). Store at 4ºC.

Take 150 ml of 10x transfer buffer and add:
300ml Metanol
Add ultrapure water until 1,5 liter. Store at 4ºC.

Resolving gel:
15ml Ultrapure water
7,5ml 1,5M Tris pH 8,8
7,5ml 30% Acrylamide
300µl SDS 10%
100 µl APS 10%
15µl TEMED
Add APS and TEMED the last.

Stacking gel:

9ml Ultrapure water
3,78ml 0,5M Tris pH 6,8
1,98ml 30% Acrylamide
150µl SDS 10%
150µl Bromophenol Blue 1%
75µl APS 10%
15µl TEMED
Add APS and TEMED the last.

Coomassie stain
1,25g Coomassie Brilliant Blue R250
200ml Methanol
50ml Acetic acid
250ml ddH_2O

Coomassie de-stain
200ml Methanol
50ml Acetic acid
250ml ddH_2O

BeWo cell culture

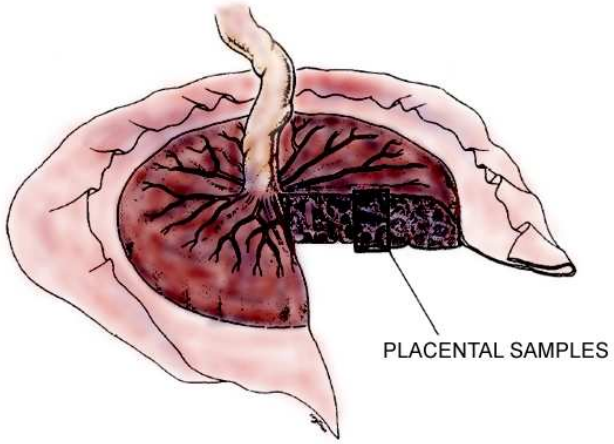
Cell Culture Medium
Penicillin/streptomycin (Lot: 1386482)
Fetal calf serum (Lot: 4/g/27OF)
Williams E Media 500 ml bottle containing glutamax, 4.2g Glucose-Pyruvate (Lot: 27866) (William's E or DMEM).
To the 500ml of Williams E Media add 50ml fetal calf serum (10% FCS), 10 ml penicillin//streptomycin (2% P/S). Mix contents by gently inverting the bottle. Store at 4ºC until used.

BeWo cell lysis buffer
50ml 50Mm Tris HCl pH 7.5
0.38g 1mM EGTA
0.37g 1mM EDTA
10ml 1% Triton x 100
2.23g 5mM Na-Pyrophosph (phosphatase inhibitor)
2.10g 50mM NaF (phosphatase inhibitor)
10ml 1mM Na-VO_3 (Protein tyrosine phosphatase inhibitor)
92.4g 0.27M Sucrose
Protease inhibitor cocktail

Unless otherwise specified all reagents were purchased from Sigma and were of the highest grade available.

3.2. Placenta samples collection

Placenta samples were collected immediately after delivery. The whole placenta was weighed, diameter was recorder and inmediatly was cut in 2 portions across the insertion of umbilical cord. A small piece 0.5×0.5×0.5 cm (200 mg) was excised from the middle of the radius of the placenta (distance between the inserction of the umbilical cord and the periphery) and washed 2 times in a NaCl 0.9% solution. Inmediatly the tissue was placed in a tube (Griener®) containing RNAlater solution (RNA stabilization reagent, Qiagen®, Cat. Nr. 76106) and completely covered in RNAlater solution. Disc samples were obtained from the identical portion of the placental plate, as regional variations in the levels of trace elements have been reported (Manci, Blackburn 1987). A visual inspection of the placenta for necrosis or any other abnormality was made by the clinicians. Samples were kept frozen at -80ºC until analysis.



3.3. BeWo cell culture and protein purification

BeWo cells culture was used like control in each gel to correct gel-to-gel variability. It has been used as a model of the placental syncytiotrophoblast in many studies.

BeWo cells are a human placental cell line that originates from a choriocarcinoma (Pattillo, Gey 1968) and demostrates many of the biochemical and morphological parameters associated with the placental syncytiotrophoblast. These cells have been used widely as an in vitro model to study placental uptake of a variety of nutrients including glucose (Vardhana, Illsley 2002), amino acids (Eaton, Sooranna 1998, Eaton, Sooranna

2000, Jones et al. 2006), and iron (Danzeisen, McArdle 1998, Van der Ende, Du Maine & Simmons 1987).

BeWo cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts., U.K.) and were cultured in 80cm^2 and 175cm^2 flasks and mantained in continuous passages with cell culture medium (William´s E Media containing Glutamax (GIBCO, Invitrogen), 10% fetal calf serum and 2% penicillin/streptomycin; Life Technologies) at 37ºC in a humidified atmosphere of 5 per cent CO_2 and 95 per cent air and were sub-cultured every 7 days and the medium changed every 2 days. The cells were made iron deficient by incubation with the iron chelator desferrioxamine (DFO). Protein Purification from BeWo cells were obtained by removing the media from the flask and adding PBS to wash the cells. After washing, PBS was removed from the flask and lysis buffer was added covering the complete surface of the flask kept on ice. Cells were removed from the wall of the flask using a long cell scraper. Lysates were transferred into 1.5ml tubes and centrifuged at 4ºC, 12000 g for 15 minuts. Supernatant was aliquoted and freezed at -80ºC and the pellet discarded.

3.4.Preparation of Placental samples to TfR determination

Proteins from placenta was obtained and protein concentration was measured by Bradford assay. TfR concentration was measured by western blot assay.

3.4.1. Protein purification from placenta tissue in RNAlater

Placental samples were taked from the freezer and placed on a glass plate on ice. A scalpel was used to cut off a small piece of frozen tissue (around 100mg). Each placental homogenates were obtained in a mix of 1ml of ultra cold hepes sucrose buffer (pH 7.4) and 40µl (tablets of inhibitor cocktail 25 fold concentrated) of protease inhibitor cocktail (Roche) to extract proteins and preserve protein functionality following cell lysis. Homogenization was done on ice in an Ultra-Turrax homogenizer at setting 1 (low

speed), 3 times 10 seconds each time, with 15 seconds breaks in between. The mixture was centrifuged () at 12000g for 15 min at 4ºC and the supernatant was aliquoted in tubes and used to measure protein concentration and frozen at -80ºC.

3.4.2. Protein concentration

Total protein content in the supernatant from placental and BeWo samples was determined by Bradford assay (Bradford 1976). The Bradford assay is a simple and accurate procedure for the estimation of soluble protein in aqueous media. The Bio-Rad assay is an acidic dye binding assay with spectrophotometric measurement at 595nm. Comparison to a standard curve provided a relative measurement of protein concentration. The samples were diluted to equalize the protein content.

3.4.3. Placental transferrin receptor determination

Western blotting (immunoblotting) was used for determination of placental TfR. This is a method used to identify individual protein zones from a sample containing a mix of proteins using specific binding of polyclonal or monoclonal antibodies. Proteins first was separated by electrophoresis, transferred onto a immobilizing membrane, detected by specific binding of antibodies and then visualised.

3.4.3.1. Polyacrylamide gel electrophoresis

Once aluminium and glass plates and spacer was cleaned with dH_2O and ethanol and assembled into the gel caster, resolving gel was prepared and applied to the SDS glass plate assembly and overlaid with a layer of butanol and allowed to set for at least 45 minuts at room temperature (rt). Once set, the butanol was discarded and the gel rinsed with dH_2O. Molten stacking gel was carefully applied to the gel cast and the combs carefully inserted. The gel was allowed to set for at least 1 hour at rt. Once set, the gels was placed into running apparatus, upper and lower chambers was filled with SDS running buffer, combs was removed under buffer, and wells was washed with buffer to remove any up polymerised material.

A volume of 20µl containing 25 µg of protein, sample buffer and ultra pure water was load in each well of the gel running apparatus. Prior to load, this mix was heated at 100ºC for 5 minuts and spun. An amount of 4µl of protein standard (See Blue Plus 2 Prestained Standard 1x, Invitrogen, Cat. No. LC5925) was heated, spun and loaded in the first well. The protein samples were run on 7,5% Acrylamide SDS-PAGE gels and separated at 20mA constant current for 1 hour 10 minuts. A BeWo cell control sample was loaded in differents positions on all of the gels and used to correct for gel-to-gel variability.

3.4.3.2. Western Blotting

Protein transfer onto a nitrocellulose membrane (Hybond ECL Nitrocellulose Membrane, Amersham Pharmacia Biotech) was by wet electroblotting for 4 hours at a constant current of 200mA (Cole-Parmer® Electrophoretic Blotting System) using a transfer buffer. After the transfer, the gel was stained with Coomassie blue to show that the protein had transferred evenly. To remove the transfer buffer remanent, the membrane was placed in a plate containing phosphate buffered saline (PBS) solution. Actin antibody was used as a loading control, for this reason at this point the membrane was cut in two half with a scalpel between 98kDa and 64kDa bands to be sure that bands of interest (TfR and β-Actin) was contained in both half. To prevent non-specific antibody binding, the membrane was then placed in a blocking solution (PBS containing 5% powdered milk) for 2 hours at rt on a shaking platform. After three 10-min washes with PBS solution to remove the blocking agent, an Anti-TFRC antibody produced in chicken (Sigma-Aldrich) (dilution 1/2000 in 5% BSA) was applied to the piece of membrane containing 98kDa band for the detection of TfR protein. The another half of the membrane containing 64kDa bands was incubate with an A5441 Monoclonal Anti-β Actin antibody produced in mouse (Sigma-Aldrich) (dilution 1/5000 in 5% non-fat milk in PBS). The membranes were incubated overnight, in a rotating platform, at 4ºC.

The membranes were placed for 2 hours, shacking at rt in a roller before three 10-min washes with PBS solution were applied. The blots were afterwards incubated for 2 hours,

on a shacking platform, at rt, with a secondary antibody anti-chicken IgG (whole molecule) peroxidase conjugate developed in rabbit A9046 (Sigma-Aldrich) (dilution 1/50000 in 5% non-fat milk in PBS) for TfR detection and a secondary antibody Rabbit anti-mouse IgG (whole molecule) peroxidase conjugate A9044 (Sigma-Aldrich) (dilution 1/5000 in 5% non-fat milk in PBS) for β Actin detection, followed again by three 10-min washes with PBS solution. Visualization of the immunologically detected proteins was achieved using the SuperSignal® West Pico Chemiluminescent Substrate detection system (Pierce Biotechnology). Equals volumes of the Stable Peroxide Solution and Luminol/Enhancer solution was mixed and applied to the membrane for 3 minuts for TfR detection and 1 minuts for β Actin detection. Processed blots were exposed to X-ray film for the optimum exposure time for BeWo control, and the films were developed using a developer solution, washed with water, covered with fixative solution, washed with water again and let dry. The film were analyzed using the ImageQuant^{Tm} TL software to asses the relative amounts of proteins by the intensity of immunoblot staining carried out by densitometry analysis. The TfR intensity signal were normalized using Actin signal to correct for sample-to-sample variability in the loading process.

Chapter 3: Leptin gene and iron metabolism

Obesity is a major global health problem and is associated with low-grade inflammation and, in a number of cases, poor iron status. Obesity is characterized by the presence of chronic low-grade inflammation and an increased risk of developing a number of chronic diseases, such as insulin resistance and type 2 diabetes (Greenberg, Obin 2006, Kahn, Hull & Utzschneider 2006).

The link between chronic diseases and anemia is well characterized. Leptin (LEP), the human homolog of the mouse obesity (ob) gene, is positioned in the chromosome 7q22-35 and is the most prominent candidate gene linked to body mass index (BMI). The leptin receptor, also identified as the diabetes gene product, is a single transmembrane protein that is established in many tissues and has several alternatively spliced isoforms. The results of linkage studies done on obese human beings using markers near the *leptin* (*LEP*)1 and *leptin receptor* (*LEPR*) gene regions are still controversial. LEP has been linked to extreme obesity in a French study (Clement et al. 1996) and a Pennsylvanian population but not in Pima Indian sibling pairs (Reed et al. 691-94). In humans, LEP and LEPR have been mapped to 7q31.3 (Green et al. 1995) and 1p31 (Winick, Stoffel & Friedman 1996), respectively.

A number of studies have noted an association between being overweight or obese and having poor iron status (Yanoff et al. 2007, Bekri et al. 2006). Adipose tissue is an active endocrine organ and releases a number of cytokines and adipokines (Lago et al. 2007), which may in turn influence iron metabolism. Leptin, the first adipokine to be discovered, is intriguing in this regard for 3 reasons: 1) it belongs to the family of long-chain helical cytokines (Zhang et al. 1997); 2) its circulating levels are proportional to fat mass (Considine et al. 1996); and 3) its membrane receptors exhibit structural similarity to class I cytokine receptors.

The expression of hepcidin, a 25 amino acid peptide hormone, in the liver is increased dramatically by inflammation and because of chronic disease (Weinstein et al. 2002).

Once released, hepcidin is thought to bind to the iron efflux protein ferroportin (Nemeth et al. 2004) and thereby act as a negative regulator of body iron homeostasis, inhibiting the release of iron recycled from senescent red blood cells by reticuloendothelial macrophages and the absorption of dietary iron by intestinal enterocytes (Laftah et al. 2004).

There is an increasing body of evidence that suggests a direct link between being overweight and having poor iron status (Yanoff et al. 2007, Bekri et al. 2006). The hypoferremia noted in obese subjects appeared to arise from a combination of 2 distinct mechanisms: 1) the development of iron deficiency (Lecube et al. 2006, Yanoff et al. 2007) and 2) the presence of chronic low-grade inflammation that resulted from the enhanced production and release of a cocktail of proinflammatory cytokines and adipokines from the adipose tissue (Lago et al. 2007). These inflammatory stimuli in turn lead to an increase in the expression of hepcidin, which once released into the circulation, impaired the recycling of iron by reticuloendothelial macrophages (Knutson et al. 2005) and the absorption of iron by duodenal enterocytes (Laftah et al. 2004, Yamaji et al. 2004), resulting in hypoferremia (Rivera et al. 2005).

The leptin can directly regulate hepatic hepcidin expression. Increased production of hepcidin in the presence of leptin was predicted to result in decreased duodenal iron absorption and impaired iron recycling from reticuloendothelial macrophages because of the inhibitory actions of hepcidin on ferroportin protein expression. Together with other stimuli, such as proinflammatory cytokines, leptin can now be added to the list of adipose-derived factors that may contribute to the hypoferremia observed in the overweight and obese population.

Obesity is a polygenic disorder, which has several candidate genes that play a role in determining the final severity. There are some SNPs of LEP gene involved in obesity physiopathology, such as A19G, A2548G in LEP gene, and Q223R in LEPR gene.

So, mutations in the leptin gene lead to defective leptin production and cause recessively inherited early onset obesity (Mammes et al. 1998). Obese individuals homozygous for the G-allele showed significantly lower leptin concentration compared to obese patients either heterozygous or homozygous for the A-allele after correction for BMI (Jiang et al. 2004b). Le Stunff et al. (Le Stunff et al. 2000) have confirmed that the recessive effect of the *LEP* G-2548A variant could potentially alter leptin expression, and female subjects with the A/A homozygote had 25% lower mean leptin levels than girls with other genotypes. Wang et al. observed (Wang et al. 2006) that the BMI of the G/G genotype was significantly higher than that of G/A and A/A genotypes in extreme obesity, and found that the *LEP* G-2548A polymorphism was associated with extreme obesity in Taiwanese aborigines.

Mutations of the promoter or the regulatory sites could affect the expression of L E P and explain the linkage of obesity with the microsatellite markers (Mammes et al. 1998). The frequencies of the LEP G/G homozygote (with Mendelian recessive and codominant models) to be higher in the extremely obese subjects (BMI >35 kg/m^2) (Wang et al. 2006). The common G allele of G-2548A is overtransmitted in the OB offspring (Jiang et al. 2004b). G-2548A was associated with a deference in BMI reduction following a low calorie diet in overweight women (Mammes et al. 1998). The G−2548A substitution either is located in a regulating site specific for LEP and a mutation creating probably correlates with regulating of the promoter regions.

It must be confirmed that genetic variations at the *L E P* locus induce changes in leptin levels or metabolism, and that these changes are associated with differences in the predisposition to obesity or in the response to a low-calorie diet. None of these variants were associated with BMI in subjects on spontaneous diet (Mammes et al. 1998).

The protein encoded by LEPR gene belongs to the gp130 family of cytokine receptors that are known to stimulate gene transcription via activation of cytosolic STAT proteins. This protein is a receptor for leptin (an adipocyte-specific hormone that regulates body weight), and is involved in the regulation of fat metabolism, as well as in a novel

hematopoietic pathway that is required for normal lymphopoiesis. Mutations in this gene have been associated with obesity and pituitary dysfunction. Alternatively spliced transcript variants encoding different isoforms have been described for this gene.

In the 223 codon in mRNA sequences the mutation C**A**G→C**G**C was detected, that corresponds to Gln→Arg change in peptide molecule. In humans, Gln223Arg polymorphisms of LEPR have been associated with higher blood pressure levels, hyperinsulinaemia, glucose intolerance, and higher BMI.

Gln223Arg polymorphism is within the region encoding the extracellular domain of the leptin receptor and may change functional characteristics of this molecule (Constantin et al. 282-86).

This mutation results in abnormal splicing of leptin-receptor transcripts and generates a mutant leptin receptor that lacks both transmembrane and intracellular domains. The mutant receptor circulates at high concentrations, binding leptin and resulting in very elevated serum leptin levels (Lahlou et al. 2000).

The association of the LEPR p.Q223R polymorphism with obesity was related to the co-dominant and dominant model, but not with the recessive model. There is hypothesis that the p.Q223R LEPR variant is associated with a BMI increase. We can propose hypothesis that variation of LEPR is participate in the union with leptin and influence on leptin serum levels. Turn, leptin levels can influence on iron metabolism.

In addition, Sharma et al. utilized microarray technology to compare hepatic gene expression changes after two types of leptin administration: one involving a direct stimulatory effect when administered peripherally (subcutaneous: SQ) and another that is indirect, involving a hypothalamic relay that suppresses food intake when leptin is administered centrally (intracerebroventricular: ICV). They found that 12 genes could be annotated to the iron ion binding group. The 9 downregulated genes were Cisd1, Haao, Cyp17a1, Dpyd, Hpd, Scd1, Cyp2c29, Ndufs8, and Sfxn1 and 3 upregulated genes of this

group were Cyba, Hpx and, Slc40a1. There is a well established link between obesity and iron metabolism. *Ob/ob* mice have higher iron absorption as compared to lean mice (Sharma et al. 2010). There is evidence in the literature that insulin resistance is associated with hepatic iron overload. A recent study found a role of Hepacidin expression in metabolic syndrome and hepatic iron overload associated with insulin resistance (Failla, Kennedy & Chen 1988). Interestingly, in this study, they also found that expression of hepcidin antimicrobial peptide 2 (Hamp2) was 3-fold and 28-fold downregulated after leptin treatment.

There is evidence in the literature that insulin resistance is associated with hepatic iron overload. A recent study found a role of Hepacidin expression in metabolic syndrome and hepatic iron overload associated with insulin resistance (Le Guenno et al. 2007). Interestingly, in Sharma found that expression of hepcidin antimicrobial peptide 2 (Hamp2) was 3-fold and 28-fold downregulated after leptin treatment.

Aims:

The main goal of this chapter is to analyse the effect of leptin polymorphisms in the different groups on iron status and in the biomarkers.

Methodology

Genotyping

Genomic DNA was prepared from whole blood by using phenol chloroform mixture and all samples were quantified using the electrophoresis in 0.8% agarose. SNPs were genotyped using the TaqMan technology (Holland et al. 1991; Livak et al. 1995) implemented on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). PCR was performed using Taq-Man Universal Master Mix (Applied Biosystems), 5 ng DNA, 900 nM of each primer, and 200 nM of each probe in a 5-ml reaction.

For the study of haplotypes of LEPR gene technology was used for genotyping Taqman probes designed by the commercial Applied Biosystems (TaqMan ® SNP Genotyping Assay, rs1137101 C_8722581_10 reference test).

Each TaqMan ® Genotyping Assay SNP is a single tube ready to be used which contains:
• Two sequence-specific primers to amplify the polymorphism of interest.
• TaqMan ® MGB probes two allele specific to detect two separate alleles of the polymorphism of interest.

Fluorescence was detected using the Real Time team ABI PRISM ® 7000 Sequence Detector.

Bioinformatic Analysis

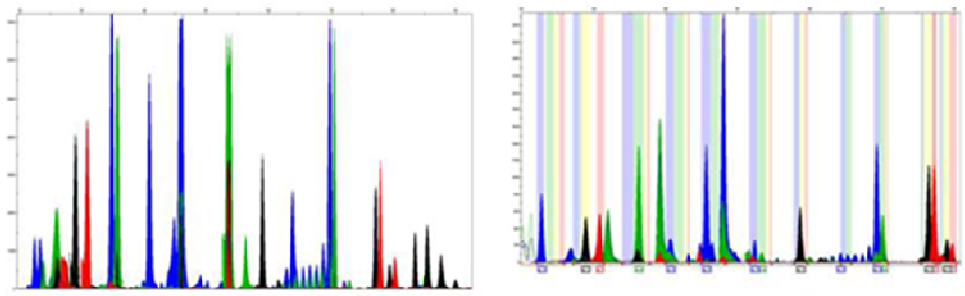
GeneMapper v1.1 software was used for automation of the genotyping of samples analyzed by capillary electrophoresis. To do this, configure the tool so that you can associate the existence of a fluorescence peak with a specific fluorochrome (bin) from among the possible alleles of a marker (panel). This way we can automate the analysis of the data analyzed in order to turn statistical tools and data analysis (SPSS, version 15.0).

For LEPR haplotype analysis the PCR data will be analyzed with ABI7000 SDS software. This software contains the algorithms necessary for testing allelic discrimination and provides the corresponding genotype in each sample for a given SNP. The software allows for the results in tables and exported to Excel compatible.

SNP	GEN	SNP (rf#)	Name HGVS	Population	Nº subjects	Alelo ancestral	A/A	A/G	G/G	A alele	G alele
LEP(+19)	LEP	rs2167270	g.5019G>A	Caucasian	124	G	0,306	0,419	0,274	0,516	0,484
LEP(-2548)	LEP	rs7799039	g.2453G>A	Caucasian	56	G	0,250	0,321	0,429	0,411	0,589
LEPR	LEPR	Rs1137101	g.177266A>G	Caucasian	198	A	0,152	0,384	0,464	0,344	0,656

SNP	Forward primer	Reverse Primer
LEP(+19)	FTGTGATCGGGCCGCTATAAG	CAGCCCAGCAGCAAATCC
LEP(-2548)	TAAGCCAAGGCAAAATTGAGG	CTTCCTGCAACATCTCAGCACT
LEPR	AACCTCTGGTTCCCCAAAAAG	AAGCCACTCTTAATACCCCCA

Sample analysis before and after the bins and panels configuration



The programe asigns one alele to the pics which comes into the asigned ranges, both in the ordinal axis (quantity of fluorescence received by the sensor in RFU), (Relative Fluorescence Units) as well as the abscis axis (size of the fragment), for one specific marker

2. Results

3.1 Descriptive analysis:

The conditions during pregnancy are listed in Table 5. This included age, smoking, hypertension, mode of delivery, duration of pregnancy, maternal body-weight gain, family history for diabetes,… among others.

A number of variables that influence both iron and body weight status, such as socioeconomic factors and physical activity were included in the analysis as residual confounders.

3.1.1 Perinatal and maternal clinical characteristics (Table 5)

In Table 5, the perinatal and clinical characteristics of the control, overweight and obese groups at 20 weeks of pregnancy are shown. There were no statistical differences in mother's age, height and gestational age between the 3 groups.

3.1.2 Maternal sociological characteristics (Table 6)

The study population was between 95% to 98% caucasican. Around 43% of the healthy mothers and the 43% of the overweight pregnant women had University studies in front of the 25.3% of the Obese group; the later also showed that none of them had Doctor studies in front of the 5.6% and the 1.5% of the control group and overweight pregnant women, respectively. The 40% of the obese women were unemployed in front of the 18% and 19% of the control and overweight ones. The 22% of the obese women had a stable employment in contrast with the 55% of the control group.

3.1.3. Neonatal clinical characteristics (Table 7)

There were no statistical differences in the anthropometric parameters of the newborn infants between the 3 groups of study, except for birth weight; the neonates born from obese mother had significantly higher birth weight than those babies born from the healthy and the overweight pregnant women.

3.1.4. Maternal daily intake of vitamins and folic acid (Table 8)

There were no statistical differences between the 6 groups established at 34 weeks of pregnancy, respect to iron and vitamin B12. However, it was demonstrated that obese women had significantly lower daily intake of folic acid and vitamin C, compared to healthy pregnant women, overweight, and all groups of gestational diabetic women.

3.1.5. Longitudinal and intergroups analysis of the haemtological and biochemical parameters during pregnancy (Table 9)

The General Model for repeated measures showed that RBC, Hb and Hto decrease significantly in the obese and diabetic women during pregnancy, and this effect is higher at the end of pregnancy. Serum Iron is significantly decreased at 34 weeks of pregnancy in obese and pregnant women suffering of diabesity. This phenomenon is reverted at delivery, when all groups show the lowest levels of pregnancy.

Serum Ferritin is also low in overweight and obese women, but increases significantly with the gestational diabetic condition, becoming significantly higher in those women affected of diabesity.

Transferrin significantly increases from 24 to 34 wks in all groups, while the TSAT index decreases. No statistical differences were demonstrated in sTfR, sTFR/Serum Ferritin ratio or in total body iron (TBI) between the 6 groups studied.

3.1.6. Relative iron status between 4 maternal groups and during pregnancy (Table 10)

The evolution of RBC, Hb and Hto in the overweight, obese and diabetic pregnant women is different than the one showed by the healthy mothers. In the later ones, these parameters decreased from 24 to 34 wks, and then increases up to the delivery. However, this behaviour is different in the overweight and obese women; not only these parameters are lower in these 3 groups compared to the control group, but also showed that there is

an intention tho increase from 24 to 34 wks, and at the end a very poor increase which determine no statistical differences between the groups studied.

Regardin serum iron the lowest level of this micronutrient was detected in the obese mothers, and specially at the 34wks of pregnancy. The depletion of serum Ferritin is also maximun at delivery in obese women respect to the other groups. Serum Ferritin increases significantly in the gestational diabetic women at delivery.

No significant differences were shown in Transferrin, TSAT index, sTFR, sTfr/sFe ratio, and TBI between the 3 periods of pregnancy studied, as well as, between the overweight, obese or diabetic pregnant women and the healthy ones.

3.1.7. Relative folate status, vitamin B12 and inflammatory state between maternal groups and during pregnancy (Table 11)

The levels of folic acid during pregnancy decreased significantly from 24 weeks of pregnancy to delivery. Most of the pregnant women developed folic acid deficiency with levels lower than 9 µg/L. Gestational Diabetes seems to be a good condition for folic acid status.
Leptin was significantly higher in overweigh, obese and diabesity pregnant women at 24 and 34 weeks of pregnancy and at delivery. Healthy pregnant women and those with gestational diabetes with a normal BMI showed leptin concentrations significantly lower.

3.1.8. Relative inflammatory state between maternal groups related to BMI and during pregnancy (Table 12)

There was no statistical differences between overweight, obese and healthy pregnant women in the immunological parameters studied.

3.1.9. Genetics polymorphism of the mothers and neonates (Table 13)

The genetic polymorphism LEP19 G→A of the leptin gene was present predominantly in the overweight and obese pregnant women in the heterocygosis form; however, the homocygosis form was present in the 1/4 of the obese women which developed gestational diabetes (25%) in front of healthy women (7%). The presence of this polymorphism in the homocygosis form in the neonates were similar to their mothers, and even higher in the case of babies born from overweight + gestational diabetic mothers.

LEP-2548 G→A genetic polymorphisms of leptin gene was present in high percentage of the women studied, preferently in healthy women, overweight and gestational diabetes (77% between heterocygosis and homocygosis). The presence of these polymorphisms in the neonate is highly related to gestational diabetes.

In relation to LEPR A→G genetic polymorphisms of leptin gene were present in a very high percentage of the groups studied.

3.1.10. Placental TfR expression depending on maternal pre-pregnancy BMI (Tables 14 and 15)

Non statistical differences were shown in the expression of placental TfR depending on maternal pre-pregnancy BMI, nor depending on gestational diabetes.

3.1.11. Correlation analysis (Tables 16, 17 and 18)

There were significant correlations between *ferritin* and *iron status indicators* at 24 and 34 weeks of pregnancy. These correlations are well established in the healthy pregnant women with iron, transferrin, TSAT indez, sTfR, VCM, HCM and RDW; however these correlations become to be less strong with overweight, obesity and finally disappear with gestational diabetes.

In the healthy pregnant women strong correlations were shown between sTfR and iron status indicators. sTfR was negatively related to serum iron, TSAT, VCM, HCM, CHCM.

No correlations were found between placental TfR expression and iron status biomarkers during pregnancy, but there were positive correlations of placental TfR expression and transferrin and negative one with TSAT index.

The placental TfR expression was not also correlated with maternal BMI and neonatal outcomes such gestational age, birth weight, placental weight/birth weight, birth longitude.

Table 5. *Perinatal and maternal clinical characteristics*

	Control	***Overweight***	***Obese***	***P value***
N	165	70	65	
Age at enrollment (years)*	31.18±4.75	32.41±4.32	30.83±5.18	0.162
Pre-pegnancy weight (kg)*	58.80±5.62	72.19±6.35	90.26±13.87	**0.000**
Height (m)*	1.63±0.06	1,63±0.06	1,63±0.06	0.353
Pre-pregnancy BMI (kg/m^2)*	22.08±1.71	27.21±1.37	34.04±4.00	**0.000**
Body fat (kg, %)				
12 wks (%)	28.24± 3.30	37.42±4.21	41.20±5.86	**0.000**
24 wks (%)	31.62±12.22	35.84±3.31	52.25±33.41	0.054
34 wks (%)	29.92±5.09	34.50±4.20	42.25±3.88	**0.000**
BMI (kg/m^2)				
12 wks	22.44±1.47	31.10±3.81	32.84±3.28	**0.000**
24 wks	25.13±2.92	29.40±2.23	35.52±3.96	**0.000**
34 wks	26.49±2.39	30.74±2.75	36.33±3.47	**0.000**
at delivery	26.57±2.73	30.76±2.85	36.05±3.42	**0.000**
Weight at delivery (kg)	71.33±8.71	82.06±10.27	94.71±12.31	**0.000**
WG over pregnancy (kg)	11.97±6.45	9.58±6.48	5.27±7.82	**0.000**
Smoking during pregnancy (%)	11.9	25.0	17.9	0.010
GD (%)	19.1	38.6	37.1	0.005
Parity				
Nuliparous (%)	50.9	33.8	33.9	0.038
Primiparous (%)	42.2	54.4	62.9	0.024
Multiparous(%)	4.3	10.3	1.6	0.001
Familiar history diabetes or obesity (%)	27.6	42.4	61.6	0.003
GA (wks)	39.33±1.55	39.13±1.92	39.55±1.45	0.613
SBP M12 (mmHg)	105.52±12.64	112.04±14.99	116.84±14.64	**0.003**
DBP M12 (mmHg)	62.50±9.61	68.25±9.61	68.74±9.13	**0.000**
SBP M24 (mmHg)	118.12±12.30	125.64±15.71	131.23±13.06	**0.000**
DBP M24 (mmHg)	66.93±8.16	70.30±9.06	73.37±9.45	**0.000**
SBP M34 (mmHg)	118.30±12.54	121.17±11.83	126.80±12.20	**0.002**
DBP M34 (mmHg)	69.79±8.85	70.49±7.89	75.14±8.25	**0.002**

**Data are presented as means±SD unless otherwise specified; BMI: Body mass index; GWG: weight gain; IOM: Institute of Medicine (Ref.); GD: gestational diabetes; IGT: Impaired glucose tolerance; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure.*

Table 6: *Mother´s sociological characteristics*

	Control	***Overweight***	***Obese***
N	165	70	65
Ethnic group			
Caucasian (%)	97.5	98.6	95.2
Gipsy (%)	0.6	0.0	1.6
African-American (%)	0.0	0.0	0.0
Asiatic (%)	0.0	0.0	0.0
American-Indian (%)	1.2	1.4	1.6
Arabic (%)	0.6	0.0	1.6
African (%)	0.0	0.0	0.0
Studies			
Primary school (%)	13.7	23.5	21.0
Secondary school (%)	18.6	14.7	17.7
Professional level (%)	19.3	17.6	35.5
University (%)	42.9	42.6	25.8
Doctor/Master (%)	5.6	1.5	0.0
Married (%)	96.3	97.1	95.2
Single/Divorced (%)	2.5	2.9	4.8
Others (%)	1.2	0.0	0.0
Unemployed (%)	18.0	19.1	40.0
Housewife (%)	8.7	10.3	15.0
Temporary employment (%)	18.0	22.1	23.3
Stable employment (%)	55.3	48.5	21.7
Residence			
Rural (%)	37.9	45.6	40.3
Urban (%)	62.1	54.4	59.7

Tabla 7. Neonatal clinical characteristics

	Control	***Overweight***	***Obese***	***P value***
N	154	62	52	
Females/males	75/79	31/31	35/17	ns
Gestational age	39.33±1.55	39.13±1.92	39.55±1.45	0.613
Preterm (<37wk gestational age) (%)	7.4	8.3	7.8	ns
Birth weight (g) (n)	3269.97±434.31	3262.79±520.69	3462.45±500.99	**0.022**
Height (cm)	50.41±1.86	49.26±6.53	50.92±2.82	0.219
Low birth weight (< 2500g) (%)	4.5	6.6	0.0	
Head circumference (cm)	34.61±1.26	34.58±1.22	34.34±1.52	0.582
Placental weight (g)	486.69±116.91	495.53±118.87	527.31±119.35	0.280
Placental long diameter (cm)	19.57± 2.41	19.64± 2.42	19.75±2.34	0.973
Placental small diameter (cm)	17.86±1.92	18.15±3.46	18.10±1.66	0.943
Ratio Placental/Fetal weight	0.148±0.03	0.153±0.036	0.153±0.036	0.371
Apgar 1 minute	9	9	9	0.802
Apgar 5 minutes	10	10	10	0.129

Table 8: Daily intake of micronutrients during pregnancy

	Control	Overweight	Obese	GD	Overweight + GD	Diabesity	P value
Iron*	12.48±2.38	10.93±3.30	10.89±4.69	13.31±2.87	12.93±2.81	11.77±3.56	0.205
Folic Acid#	310.64±77.17^{ab}	257.33±77.59^{b}	251.78±131.84^{b}	359.60±100.01^{ab}	401.32±126.69^{a}	280.55±117.44^{ab}	**0.003**
Vitamin B12#	5.76±2.91	4.70±2.27	5.01±2.70	8.59±9.62	4.59±2.92	5.01±2.57	0.182
Vitamin C*	167.42±78.27	134.74±67.10	117.00±90.89	195.48±67.89	216.54±94.76	132.16±46.42	**0.034**

**mg/day; # µg/day*

Table 9: Longitudinal and intergroups analysis of the haematological and biochemical data during pregnancy

		Control		*Overweight*		*Obese*		*GD*	*Overweight + GD*	*Diabesity*	*P value*
RBC (10^6µl)	24 wks	4.01±0.58	B	4.10±0.64	A	3.98±0.36		3.79±0.32	3.73±0.29	3.85±0.26	0.646
	34 wks	3.92±0.39[a]	B	3.88±0.51[a]	B	3.92±0.28[a]		3.84±0.29[a]	3.92±0.48[a]	4.09±0.33[a]	0.632
	40 wks	4.34±0.71[a]	A	3.99±0.49[ab]	AB	3.91±0.48[ab]		3.83±0.46[b]	4.14±0.48[ab]	3.88±0.45[ab]	0.005
	Newborn	4.81±0.52		4.80±0.45		4.67±0.57		4.59±0.83	4.66±0.23	4.77±0.56	0.814
Hb (g/dL)	24 wks	12.43±1.62	A	12.52±1.95	B	12.14±1.03	B	11.28±0.77	11.15±0.68	11.78±0.82	0.247
	34 wks	11.80±1.22[a]	B	11.52±1.73[a]	A	11.40±0.86[a]	A	11.64±1.27[a]	11.53±1.27[a]	11.85±0.78[a]	0.401
	40 wks	12.64±2.02[a]	A	11.39±1.41[bc]	A	11.22±1.63[bc]	A	11.44±1.59[abc]	12.10±1.22[abc]	11.09±1.39[abc]	0.002
	Newborn	16.87±1.68		16.87±1.45		16.08±1.90		16.43±3.18	16.20±1.10	16.87±1.74	0.705
Hc (%)	24 wks	35.49±4.76	B	36.23±5.72	B	34.92±3.07		32.98±2.10	32.47±2.69	34.32±1.89	0.437
	34 wks	34.19±3.58[a]	B	33.55±4.74[a]	A	33.35±2.25[a]		33.73±3.09[a]	33.28±4.14[a]	34.71±2.08[a]	0.430
	40 wks	37.22±6.17[a]	A	33.52±4.24[b]	A	32.51±4.84[b]		33.76±4.46[ab]	35.89±2.73[ab]	33.11±4.24[ab]	0.002
	Newborn	51.76±5.95		50.88v5.06		49.09±5.61		49.49±9.40	50.46±4.18	51.00±5.72	0.799
MCV (fl)	24 wks	88.74±4.26	B	88.30±5.20	B	87.89±4.15	B	87.06±3.14	87.12±6.21	89.17±4.30	0.892
	34 wks	87.22±5.32[a]	A	86.55±5.62[a]	A	85.11±3.71[a]	A	87.74±4.65[a]	85.18±6.43[a]	85.23±5.52[a]	0.226
	40 wks	85.99±6.94[a]	A	83.95±5.11[a]	A	83.03±4.88[a]	A	88.28±5.15[a]	87.15±4.90[a]	85.32±4.51[a]	0.143
	Newborn	107.68±5.56		106.06±4.48		103.56±7.79		107.64±3.50	108.21±6.15	106.98±2.10	0.335
MCH (pg)	24 wks	31.14±1.91	B	30.55±2.09	B	30.55±1.81	B	29.80±1.73	29.87±1.92	30.50±0.62	0.239
	34 wks	30.13±2.09[a]	A	29.75±2.32[a]	A	28.99±1.90[a]	A	30.25±2.30[a]	29.61±2.84[a]	29.08±1.54[a]	0.105
	40 wks	29.24±2.53[a]	C	28.59±2.22[a]	A	28.66±1.61[a]	A	29.89±2.14[a]	29.30±1.44[a]	28.60±1.84[a]	0.491
	Newborn	35.11±1.51		35.19±1.62		34.52±1.93		35.73±1.40	34.74±1.88	35.48±1.55	0.580
MCHC (g/dL)	24 wks	35.07±1.12	B	34.61±1.38		34.77±1.53		34.22±1.60	34.30±0.79	34.25±1.44	0.162
	34 wks	34.54±1.26[a]	A	34.37±0.91[a]		34.18±1.31[a]		34.46±1.36[a]	34.72±1.34[a]	34.13±1.14[a]	0.564
	40 wks	34.03±1.34[a]	C	34.07±1.52[a]		36.48±7.81[a]		33.89±1.65[a]	33.66±1.39[a]	33.52±0.87[a]	0.483
	Newborn	32.64±1.30		33.23±0.89		32.76±0.78		33.21±1.17	32.14±1.27	33.16±1.11	0.223
RDW (%)	24 wks	13.96±0.91	B	14.04±0.65	B	13.91±0.62	B	14.02±0.57	14.07±0.69	13.73±1.31	0.964
	34 wks	14.26±1.19[a]	C	14.14±0.93[a]	B	14.14±0.86[a]	B	14.38±1.30[a]	14.77±2.40[a]	14.48±1.23[a]	0.576
	40 wks	15.02±1.70	A	14.99±0.94	A	15.18±1.56	A	15.21±2.20	14.85±1.89	15.33±1.05	0.979
	Newborn	16.95±0.83		16.91±0.76		16.79±0.85		16.94±1.18	17.08±1.07	17.67±1.61	0.514
Serum Iron (µg/dL)	24 wks	85.06±33.61		71.08±23.21		69.74±25.70		90.60±36.75	86.00±14.85	98.28±50.80	0.072
	34 wks	86.25±54.51[a]		78.36±42.68[a]		61.77±21.40[a]		99.47±74.27[a]	101.31±62.09[a]	67.80±31.52[a]	0.046
	40 wks	84.81±52.00[a]		74.03±43.37[a]		71.42±32.42[a]		73.80±39.00[a]	85.14±47.03[a]	81.45±43.32[a]	0.812
	Newborn	175.33±48.19		160.00±4.24		133.33±54.93		168.33±14.57	164.57±44.28	156.00±69.07	0.911

Serum Ferritin (µg/L)	24 wks	22.416±15.79	A	31.59±7.62	A	26.00±18.37		24.50±19.65	38.52±26.13	28.87±15.17	0.654
	34 wks	17.37±12.28^{abc}	B	14.00±6.56^{bc}	B	14.97±9.63^{c}		23.21±11.27^{abc}	25.69±14.84^{a}	15.29±8.49^{abc}	0.002
	40 wks	25.28±14.11^{ac}	A	19.63±7.35^{abc}	AB	16.30±6.22^{ab}		32.25±14.61^{ac}	32.10±17.01^{abc}	45.08±65.57^{abc}	0.000
	Newborn	302.97±46.13		211.65±27.65		160.57±37.29		219.37±120.19	233.00±144.96	58.33±50.44	0.030
Transferrin (mg/dL)	24 wks	373.43±49.72	B	361.66±73.75	B	365.87±63.65	B	384.60±53.03	384.75±38.97	351.86±29.70	0.735
	34 wks	415.36±57.42^{a}	A	420.39±50.82^{a}	A	426.32±67.80^{a}	A	434.37±42.34^{a}	416.62±69.03^{a}	400.73±47.38^{a}	0.232
	40 wks	399.31±68.68^{a}	B	399.29±56.92^{a}	A	400.31±63.36^{a}	AB	374.20±72.87^{a}	359.96±134.13^{a}	342.05±110.33^{a}	0.119
	Newborn	195.67±13.79		224.00±14.14		194.33±19.29		203.00±10.39	207.71±25.27	212.33±40.00	0.744
TSAT index (%)	24 wks	18.96±8.54		24.98±56.18		15.73±5.99		19.46±8.03	18.22±3.81	22.14±10.40	0.756
	34 wks	17.36±11.17^{a}		15.29±8.45^{a}		12.22±5.43^{a}		18.46±12.93^{a}	20.50±13.90^{a}	14.08±7.77^{a}	0.069
	40 wks	17.88±12.60^{a}		15.01±8.73^{a}		14.56±6.73^{a}		16.51±9.41^{a}	18.26±9.55^{a}	18.58±7.51^{a}	0.734
	Newborn	72.03±17.56		57.70±2.12		56.50±26.35		67.07±7.45	65.40±22.33	60.77±27.44	0.942
sTfR (nmol/L)	24 wks	17.27±5.47		16.67±6.11		20.24±6.19		-	-	-	0.320
	34 wks	21.51±6.53		22.37±7.02		21.82±6.98		28.24±18.50	21.67±11.44	21.98±8.23	0.684
	40 wks	20.79±9.18		21.67±5.41		23.92±8.33		24.67±17.44	19.44±10.81	15.53±6.05	0.731
	Newborn	33.15±9.55		31.66±5.69		30.06±8.87		28.02±10.09	40.37±12.89	35.00±16.96	0.634
sTfR/serum Ferritin Ratio	24 wks	1.22±0.95		1.19±1.27		1.19±1.46		-	0.98±0.75	0.89±0.75	0.963
	34 wks	1.96±1.59		2.31±2.32		2.06±1.56		2.41±3.21	1.46±1.73	2.14±1.42	0.939
	40 wks	1.31±1.41		1.65±1.12		1.48±0.75		1.12±1.23	1.00±0.89	1.03±0.87	0.918
TBI (mg/Kg)	24 wks	24.02±6.24		25.02±7.03		25.18±7.14		-	23.57±7.0	24.38±7.0	0.959
	34 wks	20.34±6.63		18.69±5.71		19.89±7.00		21.04±9.21	24.17±8.52	19.35±7.50	0.698
	40 wks	24.57±7.54		20.81±5.30		21.01±4.04		25.97±7.99	26.53±8.81	26.14±9.65	0.398

Relative iron status between 6 maternal groups and during pregnancy.
a, b, c: Differences between intervention groups. Values with different letters are statistically different (P<0.05). A, B, C: Differences between pregnancy time points. Values with different letters are statistically different (P<0.05).
RBC: Red Blood Cells; Hb Haemoglobin; Hc: Haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: Red Blood Cells (RBC) distribution width = (STDEV/VCM) × 100; TSAT index: transferring saturation index; sTfR: serum transferrin receptor; TBI: total body iron = [log (serum transferring receptor/serum ferritin) – 2,8229] /0,1207).

Table 10: Relative iron status between 4 maternal groups and during pregnancy

		Control		*Overweight*		*Obese*		*GD**	*P value*
RBC (10^6µl)	24 wks	4.01±0.58	B	4.10±0.64	A	3.98±0.36		3.80±0.27	0.355
	34 wks	3.92±0.40	B	3.88±0.51	B	3.92±0.28		3.94±0.38	0.928
	40 wks	4.34±0.71[a]	A	3.99±0.49[ab]	AB	3.91±0.48[ab]		3.94±0.47[b]	0.002
Hb (g/dL)	24 wks	12.43±1.62	A	12.52±1.95	A	12.14±1.03	A	11.45±0.777	0.102
	34 wks	11.80±1.22	B	11.52±1.73	B	11.40±0.86	B	11.66±1.14	0.375
	40 wks	12.64±2.02[a]	A	11.39±1.41[b]	B	11.22±1.63[b]	AB	11.53±1.45[b]	0.001
Hc (%)	24 wks	35.49±4.76	B	36.23±5.72	A	34.92±3.07		33.38±2.18	0.220
	34 wks	34.19±3.58	B	33.55±4.74	B	33.35±2.25		33.86±3.23	0.622
	40 wks	37.22±6.17[a]	A	33.52±4.24[b]	AB	32.51±4.84[b]		34.19±4.01[b]	0.001
MCV (fl)	24 wks	88.74±4.26	A	88.30±5.19	A	87.89±4.15	A	87.95±4.33	0.754
	34 wks	87.22±5.32	B	86.55±5.62	B	85.11±3.71	B	86.19±5.55	0.238
	40 wks	85.99±6.94	B	83.95±5.11	B	83.03±4.88	B	87.08±4.91	0.075
MCH (pg)	24 wks	31.14±1.91	A	30.55±2.09	A	30.55±1.81	A	30.10±1.38	0.096
	34 wks	30.13±2.09	B	29.74±2.32	B	28.99±1.90	B	29.71±2.32	0.082
	40 wks	29.24±2.53	C	28.59±2.22	B	28.66±1.61	B	29.34±1.89	0.468
MCHC (gr/dl)	24 wks	35.07±1.12[a]	A	34.61±1.30[a]		34.17±1.53[a]		34.25±1.27[a]	0.047
	34 wks	34.54±1.26	B	34.37±0.91		34.18±1.31		34.45±1.29	0.530
	40 wks	34.03±1.34	C	34.07±1.52		34.61±1.06		33.71±1.35	0.192
RDW (%)	24 wks	13.96±0.91	B	14.04±0.65	B	13.91±0.62	B	13.92±0.91	0.926
	34 wks	14.26±1.19	C	14.14±0.93	C	14.14±0.86	B	14.54±1.69	0.405
	40 wks	15.02±1.70	A	14.99±0.94	A	15.18±1.56	A	15.14±1.80	0.965
Serum iron (µg/dl)	24 wks	85.06±33.61[a]		71.08±23.21[b]		69.74±25.70[b]		92.81±38.27[a]	0.008
	34 wks	86.25±54.51		78.36±42.68		61.77±21.40		90.56±60.97	0.070
	40 wks	84.81±52.00		74.08±43.38		71.42±32.42		79.34±41.73	0.597
Serum Ferritin (µg/L)	24 wks	22.16±15.79	A	31.59±67.62	A	26.00±18.37		29.92±19.02	0.409
	34 wks	17.37±12.28[ab]	A	14.00±6.56[b]	B	14.97±9.63[ab]		21.63±12.38[a]	0.010
	40 wks	25.28±14.11[a]	AB	19.63±7.35[ab]	AB	16.30±6.22[b]		36.37±38.81[a]	0.003
Transferrin (mg/dL)	24 wks	373.43±49.72	A	361.66±73.75		365.87±63.65	B	370.31±41.10	0.694
	34 wks	415.37±57.42	B	420.39±50.82		426.32±67.79	AB	418.60±54.35	0.808
	40 wks	399.31±68.68	A	399.29±56.92		400.31±63.36	AB	359.92±102.34	0.055
TSAT index (%)	24 wks	18.96±8.54		24.98±56.18		15.73±5.99		20.32±8.14	0.066
	34 wks	17.36±11.17		15.29±8.45		12.22±5.43		17.80±12.02	0.066
	40 wks	17.89±12.60		15.01±8.73		14.56±6.73		17.66±8.68	0.475

sTfR (nmol/L)	24 wks	17.13±5.48	B	16.28±6.12	B	20.53±6.45		17.90±3.58	0.286
	34 wks	21.51±6.53	A	22.38±7.02	A	21.82±6.98		24.19±13.39	0.715
	40 wks	20.79±9.18	A	21.67±5.41	A	23.92±8.3		21.10±13.53	0.793
	Newborn	33.15±9.55		31.66±5.69		30.06±8.87		33.82±12.82	0.890
sTfR/sFe Ratio	24 wks	1.21±0.94	B	1.19±1.27	B	1.20±1.46	A	0.93±0.06	0.989
	34 wks	1.93±1.58	A	2.31±2.32	A	2.06±1.56	A	2.03±2.23	0.921
	40 wks	1.29±1.39	B	1.65±1.12	AB	1.48±0.70	A	1.07±1.00	0.619
TBI (mg/Kg)	24 wks	24.03±6.16	A	25.02±7.03	A	25.18±7.14		23.97±0.57	0.930
	34 wks	20.50±6.62	B	18.69±5.71	B	19.89±7.00		21.49±8.25	0.666
	40 wks	24.68±7.47	A	20.81±5.30	B	21.01±4.04		26.17±7.85	0.094

a, b, c: Differences between intervention groups. Values with different letters are statistically different ($P<0.05$). A, B, C: Differences between pregnancy time points. Values with different letters are statistically different ($P<0.05$).
GD*: GD normal weight + GD overweight+ GD obese. RBC: Red Blood Cells; Hb Haemoglobin; Hc: Haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MPV: mean platelet volume; RDW: Red Blood Cells (RBC) distribution width = (STDEV/VCM) × 100; TSAT index: transferring saturation index; sTfR: serum transferrin receptor; sTfR/sFe Ratio: serum transferrin receptor/serum ferritin; TBI: total body iron = [log (serum transferring receptor/serum ferritin) – 2,8229] /0,1207).

Table 11: Relative folate status, vitamin B12 and inflammatory state between maternal groups and during pregnancy

		Normal	***Overweight***	***Obese***	***GD***	***Overweight + GD***	***Diabesity***	***P value***
Folic Acid	24 wks	15.19±4.26	14.05±5.00	12.56±4.72	-	-	-	**0.010**
	34 wks	13.98±4.62	12.51±5.55	10.22±4.91	16.39±3.84	14.36±4.68	14.33±4.56	**0.000**
	40 wks	13.54±4.82	9.84±5.05	8.66±4.83	15.28±4.79	10.74±5.55	13.93±3.88	**0.000**
Vitamin B12	24 wks	322.91±121.84	272.77±132.41	248.58±56.59	-	-	-	0.370
	34 wks	276.70±103.25	165.50±99.0	259.77±63.78	268.24±62.73	363.05±169.50	257.84±101.18	0.545
	40 wks	310.92±109.94	193.75±73.61	163.32±57.81	268.96±67.77	261.06±118.21	206.02±25.35	0.111
C-RP	34 wks	0.47±0.50	0.56±0.26	1.07±0.88	0.33±0.15	1.65±3.40	1.22±1.47	0.438
	40 wks	0.92±0.94	1.34±1.17	1.85±2.34	1.37±1.24	1.79±1.27	2.37±4.36	0.684
IL-6	24 wks	58.41±92.93	117.90±174.34	17.25±18.88	-	-	-	0.395
	34 wks	186.10±448.46	119.33±165.73	22.16±17.49	11.11±8.91	3.56±1.02	30.54±14.99	0.918
	40 wks	240.15±335.34	148.77±106.70	32.96±34.77	135.02±110.0	75.86±81.92	-	0.603
Leptin	24 wks	12.01±7.68	30.31±26.46	29.77±6.48	-	-	-	**0.032**
	34 wks	14.08±15.41	32.95±35.41	42.80±23.58	8.49±3.77	8.03±3.11	28.39±14.99	0.065
	40 wks	13.60±13.76	34.27±38.21	18.59±10.23	11.30±10.04	15.15±1.97	35.44±14.23	0.181
	Newborn	14.61±16.09	33.50±30.86	48.98±71.91	71.21±30.89	33.53±56.84	15.05±9.99	**0.024**

a, b, c: Differences between intervention groups. Values with different letters are statistically different (P<0.05). A, B, C: Differences between pregnancy time points. Values with different letters are statistically different (P<0.05). IL-6: interleukin 6; C-RP: C-Reactive protein

Table 12: Evolution of Inflamatory biomarkers in pregnant women depending on BMI

	weeks	*Normal*		*Overweight*		*Obese*	*P value*
Resistin	24	46.14±63.26		27.72±22.66		41.17±33.90	0.972
	34	32.21±46.64		65.32±94.22		16.52±16.93	0.925
	40	163.87±285.91		237.97±218.27		35.47±27.94	0.650
Adiponectin	24	32060.27±28929.58		31736.82±30903.47	B	23386.28±12245.61	0.249
	34	26197.23±21869.33		15092.61±18878.64	B	31236.93±20334.09	0.280
	40	40506.81±29567.46		76344.99±16956.81	A	49976.83±32701.24	0.791
IL-1	24	0.63±1.17		0.28± 0.32		0.12±0.00	0.594
	34	1.18±4.00		0.12± 0.00		0.12±0.00	0.821
	40	0.48±1.23		0.38± 0.65		0.75±1.41	0.849
Il-6	24	85.41±92.93	AB	117.90±174.34		17.25±18.87	0.395
	34	174.37±442.21	B	102.79± 157.49		23.35±16.28	0.803
	40	235.94±328.95	A	130.54±101.15		32.96±34.77	0.276
IL-10	24	149.22±418.05		248.73±617.14		26.15±34.38	0.822
	34	78.17±130.73		109.56±121.55		66.32±56.47	0.775
	40	324.12±505.66		255.49±302.57		30.35±36.04	0.397

a, b, c: Differences between intervention groups. Values with different letters are statistically different ($P<0.05$). A, B, C: Differences between pregnancy time points. Values with different letters are statistically different ($P<0.05$).

Table 13: Prevalence of Leptin genetic polymorphisms of the mothers and their offspring in the healthy, overweight, obese and gestational diabetic condition groups.

		Normal (%)	*Overweight (%)*	*Obese (%)*	*GD (%)*	*Overweight+GD (%)*	*Diabesity (%)*
LEP19	**GA**	**46.5**	**56.7**	**52.9**	**46.2**	**25.0**	**37.5**
Mother	**AA**	**7.0**	**6.7**	**17.6**	**7.7**	**12.5**	**25.0**
	GG	**46.5**	**36.7**	**29.4**	**46.2**	**62.5**	**37.5**
LEP19	**GA**	**46.5**	**46.7**	**52.9**	**53.8**	**25.0**	**28.6**
Neonate	**AA**	**9.3**	**10.0**	**11.8**	**0.0**	**25.0**	**14.3**
	GG	**44.2**	**43.3**	**35.3**	**46.2**	**50.0**	**57.1**
LEP2548	**GA**	**46.5**	**50.0**	**41.2**	**61.5**	**25.0**	**37.5**
Mother	**AA**	**25.6**	**16.7**	**23.5**	**15.4**	**37.5**	**25.0**
	GG	**27.9**	**33.3**	**35.3**	**23.1**	**37.5**	**37.5**
LEP2548	**GA**	**48.8**	**66.7**	**52.9**	**61.5**	**12.5**	**28.6**
Neonate	**AA**	**16.3**	**3.3**	**23.5**	**7.7**	**25.0**	**42.9**
	GG	**34.9**	**30.0**	**23.5**	**30.8**	**62.5**	**28.9**
LEPR	**GA**	**53.5**	**43.3**	**47.1**	**69.2**	**25.0**	**25.0**
Mother	**AA**	**11.6**	**23.3**	**5.9**	**7.7**	**25.0**	**25.0**
	GG	**34.9**	**33.3**	**47.1**	**23.1**	**50.0**	**50.0**
LEPR	**GA**	**46.5**	**46.7**	**29.4**	**61.5**	**12.5**	**57.1**
Neonate	**AA**	**18.6**	**23.3**	**23.5**	**7.7**	**25.0**	**14.3**
	GG	**34.9**	**30.0**	**47.1**	**30.8**	**62.5**	**28.6**

Tabla 14: Placental TfR expression depending on materna pre-preganancy BMI

	Control	*Overweight*	*Obese*	*P value*
N	85	36	32	
TfR/Actin	0.40±0.72	0.50±1.07	0.53±0.71	0.842
TfR/Placental weight	0.0007±0.0022	0.0008±0.0031	0.0007±0.005	0.636

Tabla 15: Placental TfR expression depending on maternal pre-pregnancy BMI and gestational diabetes

	Normal	*Overweight*	*Obese*	*GD*	*Overweight GD*	*Obese GD*	*P value*
N	74	25	21	12	11	11	
TfR/Actin	0.41±0.75	0.39±1.18	0.58±0.81	0.37±0.43	0.75±0.77	0.43±0.47	0.778
TfR-Actin/Plac W	0.0009±0.0018	0.0008±0.0026	0.0016±0.0027	0.0007±0.0008	0.0013±0.0007	0.0011±0.0004	0.791

Plac W: Placental weight

Correlation analysis

Table 16 (i): Correlation between ferritin and iron status indicators

		Hb	Hc	Iron	Tf	TSAT index	VCM	sTfR	HCM	CHCM	RDW	RBC
Control												
24 weeks	*P-value*	0.809	0.554	**0.000**	**0.000**	**0.000**	0.133	**0.036**	**0.047**	0.157	**0.001**	0.307
	r	-0.024	-0.059	0.322	-0.486	0.432	0.148	-0.325	0.195	0.140	0.308	-0.101
34 weeks	*P-value*	0.106	0.271	**0.001**	**0.000**	**0.000**	**0.013**	**0.009**	**0.002**	0.089	0.100	0.715
	r	0.158	0.108	0.293	-0.355	0.344	0.239	-0.406	0.293	0.166	0.161	-0.036
At Delivery	*P-value*	0.697	0.812	0.876	**0.001**	0.499	0.177	**0.000**	**0.021**	**0.013**	0.197	0.388
	r	0.045	-0.028	0.018	-0.362	0.077	0.157	-0.584	0.264	0.284	0.150	-0.100
Overweight												
24 weeks	*P-value*	0.842	0.681	0.985	**0.000**	**0.000**	0.548		0.325	0.438	0.388	0.537
	r	-0.034	-0.071	0.003	-0.855	0.986	0.104		0.169	0.133	-0.148	-0.106
34 weeks	*P-value*	**0.041**	**0.047**	**0.016**	0.065	**0.007**	**0.012**	0.235	**0.016**	0.390	0.667	0.391
	r	0.342	0.334	0.398	-0.311	0.442	0.414	-0.304	0.400	0.148	0.074	0.147
At Delivery	*P-value*	0.960	0.776	0.769	0.147	0.818	0.229	0.188	0.195	0.475	0.719	0.333
	r	-0.011	-0.063	-0.063	-0.305	-0.050	0.261	-0.374	0.280	0.157	-0.079	-0.211
Obese												
24 weeks	*P-value*	0.671	0.600	0.312	**0.017**	0.871	**0.063**	0.173	0.206	0.798	0.066	0.684
	r	0.082	0.102	-0.188	-0.426	-0.030	0.350	-0.498	0.242	-0.050	-0.346	-0.079
34 weeks	*P-value*	**0.010**	0.072	**0.007**	**0.015**	**0.001**	**0.030**	0.050	**0.024**	0.084	0.829	0.686
	r	0.463	0.333	0.473	-0.434	0.581	0.397	-0.552	0.412	0.321	-0.041	0.077
At Delivery	*P-value*	0.889	0.810	0.342	0.924	0.241	0.229	0.363	0.375	0.256	0.167	0.761
	r	0.040	0.068	0.231	-0.023	0.283	0.330	-0.323	0.247	-0.313	0.376	-0.086

Table 16 (ii): Correlation between ferritin and iron status indicators

		Hb	Hc	Iron	Tf	TSAT index	VCM	sTfR	HCM	CHCM	RDW	RBC
Gest Diabetes												
24 weeks	*P-value*	0.819	0.600	0.748	**0.022**	0.376	0.837	-	0.315	0.279	0.602	0.616
	r	0.142	-0.319	0.199	-0.930	0.513	0.129	-	0.571	0.606	-0.318	-0.307
34 weeks	*P-value*	**0.013**	**0.049**	0.318	0.122	0.214	0.238	0.066	0.066	**0.039**	0.203	0.158
	r	0.556	0.458	0.242	-0.367	0.229	0.284	0.430	0.430	0.477	-0.306	0.337
At Delivery	*P-value*	0.812	0.875	0.297	0.228	0.631	0.628	0.136	0.530	0.841	0.383	0.851
	r	0.070	0.046	-0.289	-0.331	-0.135	0.142	-0.622	0.183	0.059	0.253	-0.055
Overweight+GD												
24 weeks	*P-value*	0.636	0.704	0.716	0.208	0.390	0.330	-	0.240	0.969	0.934	0.671
	r	-0.364	-0.296	0.284	-0.792	0.610	-0.670	-	-0.760	0.031	-0.066	0.329
34 weeks	*P-value*	0.613	0.752	**0.029**	**0.003**	**0.003**	0.338	0.063	0.378	0.595	**0.018**	0.700
	r	-0.137	-0.086	0.544	-0.692	0.686	-0.257	-0.787	-0.237	-0.144	0.583	0.104
At Delivery	*P-value*	0.554	0.380	0.436	0.107	0.875	0.911	**0.024**	0.806	0.853	0.536	0.529
	r	0.229	0.334	-0.278	-0.540	-0.057	-0.044	-0.976	-0.096	-0.073	0.239	0.243
Neonate	*P-value*	0.347	0.433	**0.006**	0.286	**0.008**	0.789	.	0.816	0.849	0.114	**0.003**
	r	-0.653	-0.567	0.896	-0.471	0.886	-0.211	.	-0.184	0.151	-0.886	-0.997
Diabesity												
24 weeks	*P-value*	0.517	0.913	0.317	0.177	0.337	0.178	-	0.553	0.218	0.692	0.405
	r	0.334	-0.058	-0.445	-0.574	-0.429	-0.632	-	-0.308	0.589	-0.209	0.422
34 weeks	*P-value*	0.805	0.812	0.091	**0.017**	**0.045**	**0.007**	0.090	**0.010**	0.398	0.127	0.075
	r	-0.073	0.070	0.451	-0.605	0.524	0.684	-0.744	0.661	-0.245	-0.428	-0.491
At Delivery	*P-value*	0.475	0.545	0.084	**0.000**	0.644	0.075	0.362	0.115	0.713	0.184	0.906
	r	-0.256	-0.218	-0.519	-0.952	-0.149	-0.587	-0.843	-0.530	-0.134	0.457	0.043

Table 17(i): Correlation between sTfR and iron status indicators

		Hb	***Hc***	***Iron***	***Tf***	***TSAT index***	***VCM***	***HCM***	***CHCM***	***RDW***	***RBC***
Control											
24 weeks	*P-value*	0.188	0.189	**0.003**	0.065	**0.001**	0.180	0.318	0.986	0.567	0.504
	r	-0.210	-0.210	-0.449	0.287	-0.476	-0.214	-0.160	0.003	-0.092	-0.107
34 weeks	*P-value*	**0.000**	**0.001**	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	0.100	0.617	0.139
	r	-0.548	-0.491	-0.562	0.521	-0.567	-0.554	-0.587	-0.260	0.080	-0.235
At Delivery	*P-value*	0.685	0.948	**0.001**	**0.000**	**0.000**	**0.000**	**0.000**	**0.009**	0.344	0.119
	r	-0.070	0.011	-0.530	0.602	-0.614	-0.663	-0.695	-0.431	0.162	0.264
Overweight											
24 weeks	*P-value*	0.665	0.526	0.184	**0.005**	**0.042**	0.174	0.635	0.279	**0.033**	0.752
	r	-0.110	-0.160	-0.319	0.614	-0.471	-0.335	-0.120	0.270	0.503	-0.080
34 weeks	*P-value*	0.761	0.788	0.120	0.625	0.091	0.940	0.870	0.867	0.480	0.795
	r	0.080	0.070	-0.392	0.128	-0.423	-0.020	-0.043	-0.044	0.184	0.068
At Delivery	*P-value*	0.712	0.815	0.734	0.301	0.906	0.929	0.513	0.213	0.370	0.708
	r	-0.104	0.066	0.100	0.298	0.035	-0.025	-0.183	0.342	0.249	0.106
Obese											
24 weeks	*P-value*	**0.017**	**0.024**	0.475	**0.032**	0.249	0.999	0.597	0.379	0.143	**0.042**
	r	-0.730	-0.699	-0.274	0.711	-0.429	0.000	-0.191	-0.313	0.498	-0.649
34 weeks	*P-value*	**0.020**	**0.032**	**0.012**	0.146	**0.004**	**0.028**	0.066	0.596	**0.039**	0.331
	r	-0.634	-0.594	-0.673	0.427	-0.737	-0.606	-0.524	-0.162	0.576	-0.293
At Delivery	*P-value*	**0.006**	**0.003**	0.970	0.380	0.733	0.066	**0.034**	0.578	**0.042**	**0.013**
	r	-0.898	-0.922	-0.014	0.312	-0.124	-0.724	-0.793	-0.257	0.772	-0.859

Table 17 (ii): Correlation between sTfR and iron status indicators

		Hb	Hc	Iron	Tf	TSAT index	VCM	HCM	CHCM	RDW	RBC
Gest Diabetes											
34 weeks	*P-value*	0.056	0.074	0.448	0.893	0.419	**0.009**	**0.009**	0.095	0.068	0.645
	r	-0.743	-0.710	-0.345	-0.063	-0.366	-0.877	-0.881	-0.676	0.720	0.214
At Delivery	*P-value*	0.899	0.766	0.165	0.100	0.089	0.901	0.417	0.517	0.071	0.734
	r	0.068	0.157	-0.588	0.669	-0.685	0.066	-0.412	0.335	0.774	0.179
Overweight + GD											
34 weeks	*P-value*	0.265	0.091	0.540	0.064	0.293	0.154	0.030	0.026	0.886	0.015
	r	0.543	0.742	-0.317	0.785	-0.518	-0.659	-0.854	-0.866	-0.076	0.897
At Delivery	*P-value*	0.801	0.963	0.993	**0.006**	0.752	0.063	0.378	0.711	0.176	0.295
	r	0.199	-0.037	0.007	0.994	-0.248	-0.937	-0.622	0.289	0.824	0.705
Diabesity											
34 weeks	*P-value*	0.471	0.690	0.224	0.088	0.132	**0.044**	**0.030**	0.362	**0.046**	0.175
	r	-0.369	-0.210	-0.583	0.747	-0.686	-0.823	-0.854	-0.457	0.819	0.636
At Delivery	*P-value*	0.149	0.135	0.174	0.211	0.157	0.647	0.480	**0.019**	0.752	0.160
	r	0.973	0.978	0.963	0.946	0.970	-0.526	-0.729	-1.000	0.380	0.968

Table 18 (i): Correlations between placental expression of TfR and iron status indicators.

		Hb	***Hc***	***Serum iron***	***sFe***	***Transferrin***	***TSAT index***	***sTfR***	***MCV***	***MCH***	***MCHC***	***RDW***
24 weeks	*P-value*	0.568	0.562	0.303	0.486	0.259	0.781	0.396	0.317	0.371	0.907	0.842
	r	-0.053	-0.053	0.095	-0.064	0.104	-0.026	0.106	-0.092	-0.082	-0.011	0.018
	N	120	120	120	120	120	120	66	120	120	120	120
34 weeks	*P-value*	0.484	0.261	0.521	0.109	0.772	0.250	0.487	0.427	0.159	0.171	0.821
	r	0.059	0.095	0.054	0.136	0.025	0.097	0.078	-0.067	-0.119	-0.116	-0.019
	N	142	142	142	141	142	142	81	142	142	142	142
At Delivery	*P-value*	0.237	0.729	0.628	0.739	0.946	0.772	0.446	0.811	0.051	0.105	0.575
	r	-0.106	-0.031	0.043	-0.029	0.006	0.025	0.090	-0.022	-0.175	-0.146	-0.051
	N	125	125	132	131	132	132	74	125	125	125	125
Neonate	*P-value*	0.929	0.829	0.112	0.121	**0.030**	**0.037**	0.092	0.563	0.205	0.326	0.177
	r	-0.010	0.025	-0.462	-0.452	0.599	-0.581	0.257	-0.066	-0.145	-0.113	-0.154
	N	78	78	13	13	13	13	44	78	78	78	78

Hb Haemoglobin; Hc: Haematocrit; sFe: serum Ferritin; TSAT index; sTfR: serum transferrin receptor; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: Red Blood Cells (RBC) distribution width.

Table 19: Correlation between placental expression of TfR and maternal BMI and birth outcomes.

		Maternal BMI	Gestational age	Birth weight	Placental/birth weight	Birth Longitude
	P-value	0.393	0.574	0.292	0.200	0.988
	r	0.069	-0.046	-0.086	-0.106	-0.001
	N	154	151	153	148	120

1. Discussion

Iron deficiency is the most common nutrient deficiency in the world globally impacting 1.62 billion people with highest rates in pregnant women and children (Anonymous2008a). Reduced neonatal iron status at birth has been associated with impaired mental and psychomotor function, altered temperament, impaired neonatal auditory recognition memory and impaired auditory brainstem response (Siddappa et al. 2004, Tamura et al. 2002, Wachs et al. 2005, Amin et al. 2010). During the third trimester of pregnancy the majority of fetal iron stores (75 mg Fe/kg body weight) are acquired (Widdowson, Spray 1951), therefore understanding mechanisms of placental iron transport during the third trimester of pregnancy is important to facilitating adequate iron endowment at birth.

The placenta is a key regulatory organ that is essential for fetal nutrient transport. Humans have a hemochorial placenta; maternal blood is in direct contact with the fetal chorionic villi. In the mature hemochorial placenta there are only two layers separating maternal and fetal blood, the syncytiotrophoblast (STB), and fetal endothelial cells (Fuchs, Ellinger 2004). The STB is able to selectively regulate transport of oxygen and essential nutrients to the fetus while also allowing for the excretion of fetal waste products (ex. carbon dioxide) to be picked up and cleared by maternal circulation. Iron must be actively transported across the STB against a concentration gradient in order to meet the fetus's large iron demands. Thus the efficiency of placental iron transfer may set the stage for postnatal iron status and the subsequent risk of developing iron deficiency in infancy. At delivery the neonate typically has accrued a large amount of storage iron with normative serum ferritin concentrations averaging 134 mg/L at a time when most pregnant women have exhausted their ferritin reserves (Siddappa et al. 2007).

Previous research has indicated that potential compensatory mechanisms such as increased maternal iron absorption and increased placental iron transport may act to mitigate the risk of iron deficiency in the fetus (O'Brien et al. 1999, O'Brien et al. 2003). The mechanisms and regulatory signals for increased placental iron transport remain

unclear. Regulation of key placental iron transporters may allow for greater iron uptake and transfer to the fetus. Some researchers have suggested that this regulation occurs primarily at uptake rather than efflux stages of placental iron transport (Gambling et al. 2003). Transferrin receptor (TfR) is expressed primarily on the apical side of the STB membrane where it is responsible for binding maternal diferric transferrin (Petry et al. 1994). Maternal iron is then delivered to the placenta by receptor mediated endocytosis as reviewed by Srai et al. (Srai, Bomford & McArdle 2002). This involves clathrin-mediated invagination of the maternal diferric transferrineTfR complex, acidification and iron release (potentially through DMT-1) and the recycling of apotransferrin back into maternal circulation. TfR density in the placenta has been shown to correspond with increased placental iron uptake and iron availability and is believed to be a major determinant of placental iron transfer (Gambling et al. 2001),(Bierings et al. 1992). Previous animal and cell culture data have demonstrated that maternal iron deficiency leads to increased transferrin receptor mRNA and protein expression (Gambling et al. 2001). Similar upregulation of placental TfR expression has been shown in pregnancies complicated with diabetes (Petry et al. 1994). However, the role of maternal and neonatal iron status on regulating placental TfR expression is inconclusive in normal healthy populations (Bradley et al. 2004, Langini et al. 2006, Li, Yan & Bai 2008).

In the pregnant women participants in the PREOBE study, anaemia or iron deficiency were not observed, suggesting that iron supply to the mother and fetus were adequate. Our study has several limitations. We did not measured hepcidin, all of which may have provided important data on potential mechanisms. The association between iron status and obesity is one that should be explored further, as obesity and iron deficiency are diseases that continue to evolve worldwide, and both have significant public health implications. The present study demonstrate that the obese and diabetic pregnant women have some problems related to iron intake and vitamin C, which were lower than in healthy or diabetic women.

As relatively new analytes, sTfR and sTfR-Index are little used at present in routine iron screens although sTfR concentrations have correlated with marrow iron stores in a

heterogenous group of patients (Means Jr. et al. 1999), and sTfR has been noted in preliminary studies as a potentially specific and sensitive marker of ID in pregnancy (Åkesson et al. 1998, Åkesson et al. 2002) that is not influenced by inflammation. In the healthy pregnant women strong correlations were shown between sTfR and iron status indicators. sTfR was negatively related to serum iron, TSAT, VCM, HCM and CHCM. The placental TfR expression was not correlated with maternal BMI and neonatal outcomes such gestational age, birth weight, placental weight/birth weight, birth longitude.

MCV is considered a robust index of maternal iron status in women without hemoglobinopathies (Godfrey, et al, 1991; Tam & Lao, 1999). In our study, MCV level in the study group (85.34±10.8 fl) was significantly higher than in the control group (77.69±6.46 fl). There were significant correlations between *ferritin* and *iron status indicators* at 24 and 34 weeks of pregnancy. These correlations are well stablished in the healthy pregnant women with iron, transferrin, TSAT indez, sTfR, VCM, HCM and RDW; however these correlations become to be less strong with overweight, obesity and finally disappear with gestational diabetes.

The high amount of variability in iron status indicators need the adjustment of research designs to incorporate multiple measurements or the incorporation of confidence limits around measurements when individuals are assigned to iron-sufficient or -deficient categories. Without this confidence in the accurate diagnosis of iron status, it is difficult to address the real causal relationship of iron status with functional outcomes.

There has been shown a relationship between maternal transferrin and placental TfR expression; the level of placental TfR expressed are regulated by transferrin level in mother's serum. In the present study, no correlations were found between placental TfR expression and iron status biomarkers during pregnancy, but there were positive correlations of placental TfR expression and transferrin and negative one with TSAT index in umbilical cord.

It has been demonstrated that higher ferritine levels is associated to a low expression of placental TfR. In the present study diabetic mothers showed higher ferritine levels and lower placental TfR. This mechanisms should affect the iron availability to the fetus. However, no statistical differences were demonstrated in the iron status in the neonate between the studied groups, suggesting other factors involved in the lower neurological development demonstrated in this children.

Placental TfR was not correlated to maternal BMI and neonatal outcomes such such gestational age, birth weight, placental weight/birth weight, birth longitude.

If only the women who developed iron deficiency anemia before or early in pregnancy were at increased risk of delivering preterm this might mean that a mechanism that involves iron could be integral to the outcome of pregnancy. Allen (Allen 2001) suggested 3 potential mechanisms whereby maternal IDA might give rise to preterm delivery: hypoxia, oxidative stress, and infection. Chronic hypoxia from anemia could initiate a stress response, followed by the release of CRH by the placenta, the increased production of cortisol by the fetus, and an early delivery. Increased oxidative stress in iron deficient women that was not offset by endogenous or dietary antioxidants could damage the maternal-fetal unit and result in preterm delivery. With reduced immune function and increased risk of infection among iron deficient women, there would be an increased production of cytokines, secretion of CRH, and production of prostaglandin, increasing risk of a preterm birth.

There is an increasing body of evidence that suggests a direct link between being overweight and having poor iron status (Pinhas-Hamiel et al. 2003, Nead et al. 2004, Lecube et al. 2006, Yanoff et al. 2007, Bekri et al. 2006, Wenzel, Mayer & Stults 1962, Seltzer, Mayer 1963). The hypoferremia noted in obese subjects appeared to arise from a combination of 2 distinct mechanisms: 1) the development of iron deficiency (Lecube et al. 2006, Yanoff et al. 2007) and 2) the presence of chronic low-grade inflammation that resulted from the enhanced production and release of a cocktail of proinflammatory cytokines and adipokines from the adipose tissue (Rosen, Spiegelman 2006, Lago et al. 2007). These inflammatory stimuli in turn lead to an increase in the expression of

hepcidin, which once released into the circulation, impaired the recycling of iron by reticuloendothelial macrophages (Knutson et al. 2005) and the absorption of iron by duodenal enterocytes (Laftah et al. 2004, Yamaji et al. 2004), resulting in hypoferremia (Weinstein et al. 2002, Rivera et al. 2005). In the present study, there were no statistical differences between overweight, obese and healthy pregnant women in the inmunological parameters studied.

Inflammatory cytokines have been shown to induce ferritin synthesis in experimental models (Rao, Georgieff 2007), and sTfR is assumed to reliably reflect the degree of tissue iron supply. The lack of association, therefore, between ferritin and sTfR in our results supports the presence of inflammation.

The relationships between obesity, serum iron, measures of iron intake, iron stores and inflammation are a very exciting topic nowadays. We hypothesized that both inflammation-induced sequestration of iron and true iron deficiency were involved in the hypoferremia of obesity. The data from the PREOBE study shows that there is not statistical differences in CRP, IL6 between the study groups. Leptin was significantly higher in overweight, obese and diabesity pregnant women at 24 and 34 weeks of pregnancy and at delivery. Healthy pregnant women and those with gestational diabetes with a normal BMI showed leptin concentrations significantly lower. Regarding serum iron the lowest level of this micronutrient was detected in the obese mothers, and specially at the 34wks of pregnancy. The depletion of serum Ferritin is also maximun at delivery in obese women respect to the other groups. Serum Ferritin increases significantly in the gestational diabetic women at delivery.

The evolution of RBC, Hb and Hto in the overweight, obese and diabetic pregnant women is different than the one showed by the healthy mothers. In the later ones, these parameters decreased from 24 to 34 wks, and then increases up to the delivery. However, this behavior is different in the overweight and obese women; not only these parameters are lower in these 3 groups compared to the control group, but also showed that there is

an intention to increase from 24 to 34 wks, and at the end a very poor increase which determine no statistical differences between the groups studied. No significant differences were shown in Transferrin, TSAT index, sTFR, sTfr/sFe ratio, and TBI between the 3 periods of pregnancy studied, as well as, between the overweight, obese or diabetic pregnant women and the healthy ones. Maternal Hb and serum ferritin showed a highly significant positive correlation ($r=0.92$; $p<0.001$) indicating that iron deficiency was the most dominant factor in the causation of anemia amongst them.

The General Model for repeated measures showed that RBC, Hb and Hto decrease significantly in the obese and diabetic women during pregnancy, and this effect is higher at the end of pregnancy. Serum Iron is significantly decreased at 34 weeks of pregnancy in obese and pregnant women suffering of diabesity. This phenomenon is reverted at delivery, when all groups show the lowest levels of pregnancy. Serum Ferritin is also low in overweight and obese women, but increases significantly with the gestational diabetic condition, becoming significantly higher in those women affected of diabesity. There was this hypothesis that high iron stores in GDM women could be due to nutritional improvement in pregnant women (Lao & Ho, 2004), in addition, excess iron can affect insulin synthesis and secretion, and enhance oxidation of lipids which in turn decreases glucose utilization in muscles and increase gluconeogenesis in liver, thus leading to liver mediated insulin resistance. Accordingly, further studies are needed to show the role of increased maternal iron status from prophylactic iron supplementation and nutritional improvement in the development of GDM. The prevalence of iron deficiency resulted higher in obese and diabesity mothers compared to the control group during the 3rd trimestre of pregnancy.

Transferrin significantly increases from 24 to 34 wks in all groups, while the TSAT index decreases. No statistical differences were demonstrated in sTfR, sTFR/Serum Ferritin ratio or in total body iron (TBI) between the 6 groups studied.

Iron supplementation alone or in combination with folic acid has been associated with the well being of the mother and fetus. It leads to a significant reduction in anemia incidence during pregnancy and, thus, plays a vital role in reducing maternal morbidity and mortality. In the present study, the levels of folic acid during pregnancy decreased significantly from 24 weeks of pregnancy to delivery. Most of the pregnant women involved in this study developed folic acid deficiency with levels lower than 9 µg/L. On the contrary Diabetic mothers are included in a regular protocol for diet and insuline control. In fact, in the present study, diabetic mothers showed better nutritional status of folic acid, probably due to the dietetic control established.

Gestational diabetes mellitus (GDM) increases the risk of macrosomia and perinatal morbidity and mortality for the fetus, while presaging a long-term risk of development of type 2 diabetes for the mother (Anonymous2000, Clark et al. 1997). The mechanisms involved in the development of GDM are not completely understood. It is increasingly being recognized that there is a systemic inflammation in GDM, as indicated by higher levels of serum C-reactive protein (CRP) and/or interleukin-6 (Wolf et al. 2003, Qiu et al. 2004). Inflammation is usually associated with obesity because adipocytes from adipose tissue can secrete proinflammatory cytokines (Kriketos et al. 2004).

In addition, obese women with high ferritin levels had a 3.5-fold increased risk of developing GDM (95% CI 1.35–9.27, $P < 0.01$), whereas nonobese women did not. These data thus suggest that the impact of high serum ferritin on the risk of GDM is, at least in part, mediated by obesity (Chen, 2006). There is an extensive body of data suggesting that higher iron stores are associated with risk of type 2 diabetes in nonpregnant subjects (Jiang et al. 2004a, Tuomainen et al. 1997, Ford, Cogswell 1999, Wilson et al. 2003, Salonen et al. 1998). In pregnant women, Lao et al. found that higher Hb (>13 g/dl) was an independent risk for GDM (Lao et al. 2002) and that women with iron deficiency anemia had a reduced risk of GDM (Lao, Ho 2004). Higher Hb (>130 g/l) during early pregnancy was not associated with increased risk for GDM did not support the hypothesis that higher serum ferritin reflects excess iron stores in patients with GDM.

The data obtained in the present study do not confirm this hypotesis completely, but suggest a relationship between ferritin and iron status and the risk to developm GDM, but this risk was also mediated by mother BMI.

More studies are needed to clarify all the important mechanisms where iron is involved related to human growth and development.

2. Conclusions

1. Obesity in pregnancy is related to lower study levels, unemployement and less oportunities for a estable job.

2. The babies born from obese mothers showed higher birth weight.

3. Obesity during pregnancy is linked to a significant lower daily intake of folic acid and vitamin C, compared to healthy pregnant women or overweight ones. This factor could have importance in terms of programming effects in the fetus and neonate.

4. A significant decrease of serum iron and ferritin have been demonstrated in obese and diabesity pregnant women in the last trimestre of pregnancy, leading the fetus in a high risk for a correct growth and development.

5. Transferrin significantly increases from 24 to 34 weeks of pregnancy in all groups studied, while TSAT index decreases.

6. sTfR, sTfR/serum ferritin ratio or total body iron (TBI) did not show any difference between the groups studied, so these should not be considered as biomarkers of iron deficiency.

7. The present study has demonstrated a different evolution of RBC, Hb and Hto during pregnancy in overweight, obese and diabetic pregnant women.

8. Obese women showed the lowest level of serum iron during pregnancy, specially at 34 weeks. The potential consequences for the fetal and offspring growth and development require more studies. However, these results suggest the need of control carefully this micronutrient in overweight and obese women during pregnancy.

9. Folic acid deficiency were shown in the 35% of the pregnant women involved in the study; most of them were obese.

10. Gestational Diabetes is a risk factor to maintain high leptin levels during pregnancy which will interact on the fetus growth and development, and in the programming of the offspring adipose tissue mechanism to produce leptin.

11. The LEP19 G→A polymorphisms of the leptin gene are present in the overweight and obese pregnant women.

12. The LEP-2548 G→A genetic polymorphisms of leptin gene is present in healthy, overweight and gestational diabetes (77%). Its presence in the neonate is highly related to gestational diabetes.

References

Weight Gain During Pregnancy IOM. Reexamining the guidelines. 2009, National Academies Press, Washington, DC.

"
Worldwide prevalence of anaemia 1993-2005." 2008a, in , eds. B. de Benoist, E. McLea, I. Egli & M. Cogswell, pp. 1-40.

"Hyperglycemia and Adverse Pregnancy Outcomes", 2008b, *New England Journal of Medicine,* vol. 358, no. 19, pp. 1991-2002.

"Iron and folate supplementation. Standards for maternal and neonatal care. Integrated Management
of Pregnancy and Childbirth (IMPAC).", 2006a, vol. 1.8.

"Prevalence and causes of iron deficiency anaemia" 2006b, in *Disorders of iron homeostasis, erythrocytes, erythropoiesis.* , eds. C. Beaumont, P. Beris, Y. Beuzard & C. Brugnara, European School of Haematology, Paris, pp. 409-419.

"Focusing on anaemia : towards an integrated approach for effective anaemia control : joint statement by the World Health Organization and the United Nations Children's Fund", 2004, .

Iron deficiency anaemia: assessment, prevention, and control. A guide for programme managers, 2001a, World Health Organization (WHO/NHD/01.3), Geneva.

"Panel on Micronutrients Subcommittee on Upper Reference Levels of Nutrients and of Interpretation and Uses of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes Food and Nutrition Board, Institute of Medicine. Iron." 2001b, in *Dietary reference intakes for vitamin a, vitamink, arsenic,boron, chromium, copper, iodine, iron,manganese, molybdenum, nickel, silicon, vanadium and zinc.* National Academy Press, Washington, DC., pp. 290-393.

"Gestational diabetes mellitus", 2000, vol. 23 (Supp1 1), pp. S77–S79.

"Recommendations to prevent and control iron deficiency in the United States. Centers for Disease Control and Prevention.", 1998, *MMWR.Recommendations and reports : Morbidity and mortality weekly report.Recommendations and reports / Centers for Disease Control,* vol. 47, no. RR-3, pp. 1-29.

"Screening for iron deficiency anemia -- including iron prophylaxis" 1996, in *Guide to clinical preventive services,* 2nd ed edn, U.S. Preventive Services Task Force. International Medical Publishing, Alexandria, VA., pp. 231-46.

"Maternal anthropometry and pregnancy outcomes. A WHO Collaborative Study.", 1995a, *Bulletin of the World Health Organization*, vol. 73 Suppl, pp. 1-98.

"Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee.", 1995b, *World Health Organization technical report series*, vol. 854, pp. 1-452.

"
The prevalence of anaemia in women.", 1992, .

Institute of Medicine Report. Nutrition during pregnancy
1990, , National Academy Press, Washington DC.

"CDC criteria for anemia in children and childbearing-aged women.", 1989a, *MMWR.Morbidity and mortality weekly report*, vol. 38, no. 22, pp. 400-404.

Requirements of Vitamin A, Iron, Folate and Vitamin B12. Report of a Joint FAO/WHO Expert Group. 1989b, Food and Agricultural Organization, World Health Organization, Geneva.

"Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance", 1979, *Diabetes*, vol. 28, no. 12, pp. 1039-1057.

"Nutritional anaemias. Report of a WHO group of experts.", 1972, *World Health Organization - Technical Report Series*, vol. 503, pp. 1-29.

"Nutritional anaemias. Report of a WHO scientific group.", 1968, *World Health Organization - Technical Report Series*, vol. 405, pp. 5-37.

Agarwal, K.N., Agarwal, D.K. & Mishra, K.P. 1991, "Impact of anaemia prophylaxis in pregnancy on maternal haemoglobin, serum ferritin and birth weight", *Indian Journal of Medical Research - Section B Biomedical Research Other Than Infectious Diseases*, vol. 94, no. AUG., pp. 277-280.

Ahmed, F., Coyne, T., Dobson, A. & McClintock, C. 2008, "Iron status among Australian adults: findings of a population based study in Queensland, Australia", *Asia Pacific Journal of Clinical Nutrition*, vol. 17, no. 1, pp. 40-47.

Aisen, P., Leibman, A. & Zweier, J. 1978, "Stoichiometric and site characteristics of the binding of iron to human transferrin", *Journal of Biological Chemistry*, vol. 253, no. 6, pp. 1930-1937.

Åkesson, A., Bjellerup, P., Berglund, M., Bremme, K. & Vahter, M. 2002, "Soluble transferrin receptor: Longitudinal assessment from pregnancy to postlactation", *Obstetrics and gynecology*, vol. 99, no. 2, pp. 260-266.

Åkesson, A., Bjellerup, P., Berglund, M., Bremme, K. & Vahter, M. 1998, "Serum transferrin receptor: A specific marker of iron deficiency in pregnancy", *American Journal of Clinical Nutrition*, vol. 68, no. 6, pp. 1241-1246.

Alberti, K.G.M.M., Zimmet, P. & Shaw, J. 2006, "Metabolic syndrome?a new world-wide definition. A Consensus Statement from the International Diabetes Federation", *Diabetic Medicine*, vol. 23, no. 5, pp. 469-480.

Allen, L.H. 2001, "Biological mechanisms that might underlie iron's effects on fetal growth and preterm birth", *Journal of Nutrition*, vol. 131, no. 2, pp. 581S-589S.

Allen, L.H. 2000, "Anemia and iron deficiency: Effects on pregnancy outcome", *American Journal of Clinical Nutrition*, vol. 71, no. 5 SUPPL., pp. 1280S-1284S.

Allen, L.H. 1997, "Pregnancy and iron deficiency: Unresolved issues", *Nutrition reviews*, vol. 55, no. 4, pp. 91-101.

Amin, S.B., Orlando, M., Eddins, A., MacDonald, M., Monczynski, C. & Wang, H. 2010, "In Utero Iron Status and Auditory Neural Maturation in Premature Infants as Evaluated by Auditory Brainstem Response", *Journal of Pediatrics*, vol. 156, no. 3, pp. 377-381.

Andersen, H.S., Gambling, L., Holtrop, G. & McArdle, H.J. 2006, "Maternal iron deficiency identifies critical windows for growth and cardiovascular development in the rat postimplantation embryo", *Journal of Nutrition*, vol. 136, no. 5, pp. 1171-1177.

Andrews, N.C. 2003, "Iron studies: Normal and abnormal" in

Clinical hematology and oncology: presentation, diagnosis, and treatment., eds. B. Furie, P.A. Cassileth, M.B. Atkins & R.J. Mayer, 1 edition edn, Churchill Livingstone, Elsevier, United States.

Andrews, N.C. 1999, "Medical progress: Disorders of iron metabolism", *New England Journal of Medicine*, vol. 341, no. 26, pp. 1986-1995.

Apovian, C.M., Bigornia, S., Mott, M., Meyers, M.R., Ulloor, J., Gagua, M., McDonnell, M., Hess, D., Joseph, L. & Gokce, N. 2008, "Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects", *Arteriosclerosis Thrombosis and Vascular Biology*, vol. 28, no. 9, pp. 1654-1659.

Ausk, K.J. & Ioannou, G.N. 2008, "Is obesity associated with anemia of chronic disease? A population-based study", *Obesity*, vol. 16, no. 10, pp. 2356-2361.

BADHAM, J., ZIMMERMANN, M.B. & KRAEMER, K. 2007, *The guidebook nutritional anaemia,* Sight and Life.

Bailey, L.B., Mahan, C.S. & Dimperio, D. 1980, "Folacin and Iron Status in Low-Income Pregnant Adolescents and Mature Women", *American Journal of Clinical Nutrition,* vol. 33, no. 9, pp. 1997-2001.

Baker, P.N., Wheeler, S.J., Sanders, T.A., Thomas, J.E., Hutchinson, C.J., Clarke, K., Berry, J.L., Jones, R.L., Seed, P.T. & Poston, L. 2009, "A prospective study of micronutrient status in adolescent pregnancy", *American Journal of Clinical Nutrition,* vol. 89, no. 4, pp. 1114-1124.

Barker, D.J.P., Bull, A.R., Osmond, C. & Simmonds, S.J. 1990, "Fetal and placental size and risk of hypertension in adult life", *British medical journal,* vol. 301, no. 6746, pp. 259-262.

Barker, D.J.P., Osmond, C., Golding, J., Kuh, D. & Wadsworth, M.E.J. 1989, "Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease", *British medical journal,* vol. 298, no. 6673, pp. 564-567.

Barrett, J.F.R., Whittaker, P.G., Williams, J.G. & Lind, T. 1994, "Absorption of non-haem iron from food during normal pregnancy", *British medical journal,* vol. 309, no. 6947, pp. 79-82.

Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.-. & Tartaglia, L.A. 1996, "The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, no. 16, pp. 8374-8378.

Baynes, R.D. 1994, "Iron deficiency" in *Iron metabolism in health and disease*, eds. J.H. Brock, J.W. Halliday, M.J. Pippard & L.W. Powell, Saunders, London, pp. 204-207.

Beard, J.L. 2008, "Why iron deficiency is important in infant development", *Journal of Nutrition,* vol. 138, no. 12, pp. 2534-2536.

Beard, J.L. 2000, "Effectiveness and strategies of iron supplementation during pregnancy", *American Journal of Clinical Nutrition,* vol. 71, no. 5, pp. 1288S-1294S.

Beard, J.L. 1998, "Weekly iron intervention: The case for intermittent iron supplementation", *American Journal of Clinical Nutrition,* vol. 68, no. 2, pp. 209-212.

Beard, J.L. 1994, "Iron deficiency: Assessment during pregnancy and its importance in pregnant adolescents", *American Journal of Clinical Nutrition,* vol. 59, no. 2 SUPPL., pp. 502S-510S.

Beard, J.L., Hendricks, M.K., Perez, E.M., Murray-Kolb, L.E., Berg, A., Vernon-Feagans, L., Irlam, J., Isaacs, W., Sive, A. & Tomlinson, M. 2005, "Maternal iron deficiency anemia affects postpartum emotions and cognition", *Journal of Nutrition,* vol. 135, no. 2, pp. 267-272.

Beck, G., Ellis, T.W., Habicht, G.S., Schluter, S.F. & Marchalonis, J.J. 2002, "Evolution of the acute phase response: iron release by echinoderm (Asterias forbesi) coelomocytes, and cloning of an echinoderm ferritin molecule", *Developmental and comparative immunology,* vol. 26, no. 1, pp. 11-26.

Becroft, D.M., Thompson, J.M. & Mitchell, E.A. 2005, "Placental villitis of unknown origin: Epidemiologic associations", *American Journal of Obstetrics and Gynecology,* vol. 192, no. 1, pp. 264-271.

Bekri, S., Gual, P., Anty, R., Luciani, N., Dahman, M., Ramesh, B., Iannelli, A., Staccini-Myx, A., Casanova, D., Ben Amor, I., Saint-Paul, M., Huet, P., Sadoul, J., Gugenheim, J., Srai, S.K.S., Tran, A. & Le Marchand-Brustel, Y. 2006, "Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH", *Gastroenterology,* vol. 131, no. 3, pp. 788-796.

Bencaiova, G., Krafft, A., Burkhardt, T. & Zimmermann, R. 2005, "Hemoglobinopathies, body iron stores and gestational diabetes mellitus", *Haematologica,* vol. 90, no. 8, pp. 1138-1139.

Bergmann, R.L., Gravens-Müller, L., Hertwig, K., Hinkel, J., Andres, B., Bergmann, K.E. & Dudenhausen, J.W. 2002, "Iron deficiency is prevalent in a sample of pregnant women at delivery in Germany", *European Journal of Obstetrics Gynecology and Reproductive Biology,* vol. 102, no. 2, pp. 155-160.

Bierings, M., van Eijk, H.G., Baert, M.R.M. & van Dijk, H.P. 1992, "Regulation of transferrin receptor expression in term human cytotrophoblasts.", *Trophoblast Res,* vol. 6, pp. 237-42.

Blot, I., Diallo, D. & Tchernia, G. 1999, "Iron deficiency in pregnancy: Effects on the newborn", *Current opinion in hematology,* vol. 6, no. 2, pp. 65-70.

Blot, I., Papiernik, E., Kaltwasser, J.P., Werner, E. & Tchernia, G. 1981, "Influence of Routine Administration of Folic-Acid and Iron during Pregnancy", *Gynecologic and obstetric investigation,* vol. 12, no. 6, pp. 294-304.

Bondevik, G.T., Lie, R.T., Ulstein, M. & Kvale, G. 2001, "Maternal hematological status and risk of low birth weight and preterm delivery in Nepal", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 80, no. 5, pp. 402-408.

Bothwell, T.H., Charlton, R.W., Cook, J.D. & Finch, C.A. 1979, *Iron metabolism in man.* Blackwell Scientific Publications, Oxford.

Bothwell, T.H. 2000, "Iron requirements in pregnancy and strategies to meet them", *American Journal of Clinical Nutrition,* vol. 72, no. 1 SUPPL., pp. 257S-264S.

Bradford, M.M. 1976, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", vol. 7;72, pp. 248-54.

Bradley, J., Leibold, E.A., Harris, Z.L., Wobken, J.D., Clarke, S., Zumbrennen, K.B., Eisenstein, R.S. & Georgieff, M.K. 2004, "Influence of gestational age and fetal iron status on IRP activity and iron transporter protein expression in third-trimester human placenta", *American Journal of Physiology - Regulatory Integrative and Comparative Physiology,* vol. 287, no. 4 56-4, pp. R894-R901.

Bray, G.A., Laine, C., Goldmann, D. & Wilson, J.F. 2008, "Obesity", vol. ITC4, pp. 1-16.

Brotanek, J.M., Gosz, J., Weitzman, M. & Flores, G. 2007, "Iron deficiency in early childhood in the United States: Risk factors and racial/ethnic disparities", *Pediatrics,* vol. 120, no. 3, pp. 568-575.

Brown, P.J., Molloy, C.M. & Johnson, P.M. 1982, "Transferrin receptor affinity and iron transport in the human placenta", *Placenta,* vol. 3, no. 1, pp. 21-27.

Brudevold, R., Hole, T. & Hammerstrom, J. 2008, "Hyperferritinemia Is Associated with Insulin Resistance and Fatty Liver in Patients without Iron Overload", *PLoS One,* vol. 3, no. 10, pp. Article No.: e3547.

Bruner, A.B., Joffe, A., Duggan, A.K., Casella, J.F. & Brandt, J. 1996, "Randomised study of cognitive effects of iron supplementation in non-anaemic iron-deficient adolescent girls", *Lancet,* vol. 348, no. 9033, pp. 992-996.

Buus, R.M. & Boockfor, F.R. 2004, "Transferrin expression by placental trophoblastic cells", *Placenta,* vol. 25, no. 1, pp. 45-52.

Carriaga, M.T., Skikne, B.S., Finley, B., Cutler, B. & Cook, J.D. 1991, "Serum transferrin receptor for the detection of iron deficiency in pregnancy", *American Journal of Clinical Nutrition,* vol. 54, no. 6, pp. 1077-1081.

Casanueva, E., Pfeffer, F., Drijanski, A., Fernandez-Gaxiola, A.C., Gutierrez-Valenzuela, V. & Rothenberg, S.J. 2003, "Iron and folate status before pregnancy and anemia during pregnancy", *Annals of Nutrition and Metabolism,* vol. 47, no. 2, pp. 60-63.

Catalano, P.M., Presley, L., Minium, J. & Mouzon, S.H.-. 2009, "Fetuses of obese mothers develop insulin resistance in utero", *Diabetes care,* vol. 32, no. 6, pp. 1076-1080.

Cetin, I., Morpurgo, P.S., Radaelli, T., Taricco, E., Cortelazzi, D., Bellotti, M., Pardi, G. & Beck-Peccoz, P. 2000, "Fetal plasma leptin concentrations: Relationship with different intrauterine growth patterns from 19 weeks to term", *Pediatric research*, vol. 48, no. 5, pp. 646-651.

Challier, J.C., Basu, S., Bintein, T., Minium, J., Hotmire, K., Catalano, P.M. & Hauguel-de Mouzon, S. 2008, "Obesity in Pregnancy Stimulates Macrophage Accumulation and Inflammation in the Placenta", *Placenta*, vol. 29, no. 3, pp. 274-281.

Chambers, E.C., Heshka, S., Gallagher, D., Wang, J., Pi-Sunyer, F.X. & Pierson, J.,Richard N. 2006, "Serum Iron and Body Fat Distribution in a Multiethnic Cohort of Adults Living in New York City", *Journal of the American Dietetic Association*, vol. 106, no. 5, pp. 680-684.

Chang, J., Bird, R., Clague, A. & Carter, A. 2007, "Clinical utility of serum soluble transferrin receptor levels and comparison with bone marrow iron stores as an index for iron-deficient erythropoiesis in a heterogeneous group of patients", *Pathology*, vol. 39, no. 3, pp. 349-353.

Chaparro, C.M. 2008, "Setting the Stage for Child Health and Development: Prevention of Iron Deficiency in Early Infancy", *Journal of Nutrition*, vol. 138, no. 12, pp. 2529-2533.

Chełchowska, M. & Laskowska-Klita, T. 2002, "Effect of maternal smoking on some markers of iron status in umbilical cord blood.", *Roczniki Akademii Medycznej w Bialymstoku (1995)*, vol. 47, pp. 235-240.

Chen, X., Scholl, T.O. & Stein, T.P. 2006, "Association of elevated serum ferritin levels and the risk of gestational diabetes mellitus in pregnant women: The Camden study", *Diabetes care*, vol. 29, no. 5, pp. 1077-1082.

Chockalingam, U.M., Murphy, E. & Ophoven, J.C. 1987, "Cord transferrin and ferritin values in newborn infants at risk for prenatal uteroplacental insufficiency and chronic hypoxia", *Journal of Pediatrics*, vol. 111, no. 2, pp. 283-286.

Christensen RD, O.R. 2004, "Anaemias unique to pregnancy and the perinatal period" in *Wintrobe's clinical hematology*, eds. J.P. Greer, J. Foerster, N.J. Lukens, G.M. Rodgers, F. Paraskevas & B. Glader, 11th edition edn, Lippincott Williams and Wilkins, USA, pp. 1467-1486.

Chung, B., Matak, P., McKie, A.T. & Sharp, P. 2007, "Leptin increases the expression of the iron regulatory hormone hepcidin in HuH7 human hepatoma cells", *Journal of Nutrition*, vol. 137, no. 11, pp. 2366-2370.

Clark, C.M., Qiu, C., Amerman, B., Porter, B., Fineberg, N., Aldasouqi, S. & Golichowski, A. 1997, "Gestational diabetes: should it be added to the syndrome of insulin resistance?", vol. 20, pp. 867– 871.

Clement, K., Garner, C., Hager, J., Philippi, A., LeDuc, C., Carey, A., Harris, T.J.R., Jury, C., Cardon, L.R., Basdevant, A., Demenais, F., GuyGrand, B., North, M. & Froguel, P. 1996, "Indication for linkage of the human OB gene region with extreme obesity", *Diabetes*, vol. 45, no. 5, pp. 687-690.

Coenen, J.L.L.M., Van Dieijen-Visser, M.P., Van Pelt, J., Van Deursen, C.T.B.M., Fickers, M.M.F., Van Wersch, J.W.J. & Brombacher, P.J. 1991, "Measurements of serum ferritin used to predict concentrations of iron in bone marrow in anemia of chronic disease", *Clinical chemistry*, vol. 37, no. 4, pp. 560-563.

Colomer, J., Colomer, C., Gutierrez, D., Jubert, A., Nolasco, A., Donat, J., Fernandez-Delgado, R., Donat, F. & Alvarez-Dardet, C. 1990, "Anaemia during pregnancy as a risk factor for infant iron deficiency: Report from the Valencia infant Anaemia Cohort (VIAC) study", *Paediatric and perinatal epidemiology*, vol. 4, no. 2, pp. 196-204.

Considine, R.V., Sinha, M.K., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Nyce, M.R., Ohannesian, J.P., Marco, C.C., Mckee, L.J., Bauer, T.L. & Caro, J.F. 1996, "Serum immunoreactive-leptin concentrations in normal-weight and obese humans", *New England Journal of Medicine*, vol. 334, no. 5, pp. 292-295.

Cook, J.D. 2005, "Diagnosis and management of iron-deficiency anaemia", *Best Practice and Research: Clinical Haematology*, vol. 18, no. 2 SPEC. ISS., pp. 319-332.

Cook, J.D., Flowers, C.H. & Skikne, B.S. 2003, "The quantitative assessment of body iron", *Blood*, vol. 101, no. 9, pp. 3359-3364.

Cook, J.D., Lipschit.Da, Miles, L.E.M. & Finch, C.A. 1974, "Serum Ferritin as a Measure of Iron Stores in Normal Subjects", *American Journal of Clinical Nutrition*, vol. 27, no. 7, pp. 681-687.

Cotton, F., Thiry, P. & Boeynaems, J.M. 2000, "Measurement of soluble transferrin receptor by immunoturbidimetry and immunonephelometry", *Clinical biochemistry*, vol. 33, no. 4, pp. 263-267.

Crichton RR 1991, "The importance of iron in biological systems" in *Inorganic biochemistry of iron metabolism*, ed. Crichton RR, Chichester: Ellis Horwood Lim, , pp. 29-58.

CRICHTON, R.R. & CHARLOTEAUX-WAUTERS, M. 1987, "Iron transport and storage", *European Journal of Biochemistry*, vol. 164, no. 3, pp. 485-506.

Crowe, C., Dandelar, P., Fox, M., Dhingra, K., Bennet, L. & Hanson, M.A. 1995, "The effects of anaemia on heart, placenta and body weight, and blood pressure in fetal and neonatal rats", *Journal of Physiology,* vol. 488, no. 2, pp. 515-519.

Dale, J.C., Burritt, M.F. & Zinsmeister, A.R. 2002, "Diurnal variation of serum iron, iron-binding capacity, transferrin saturation, and ferritin levels", *American Journal of Clinical Pathology,* vol. 117, no. 5, pp. 802-808.

Dallman, P.R. 1989, "Iron-Deficiency - does it Matter", *Journal of internal medicine,* vol. 226, no. 5, pp. 367-372.

Dallman, P.R. 1986, "Biochemical Basis for the Manifestations of Iron-Deficiency", *Annual Review of Nutrition,* vol. 6, pp. 13-40.

Danzeisen, R. & McArdle, H.J. 1998, "Copper and iron interactions in a placental cell line (BeWo)", *Biochemical Society transactions,* vol. 26, no. 2.

Davis, R.J., Corvera, S. & Czech, M.P. 1986, "Insulin stimulates cellular iron uptake and causes the redistribution of intracellular transferrin receptors to the plasma membrane", *Journal of Biological Chemistry,* vol. 261, no. 19, pp. 8708-8711.

Dawson, E.B. & Mcganity, W.J. 1987, "Protection of Maternal Iron Stores in Pregnancy", *Journal of Reproductive Medicine,* vol. 32, no. 6, pp. 478-487.

de Luca, C. & Olefsky, J.M. 2008, "Inflammation and insulin resistance", *FEBS letters,* vol. 582, no. 1, pp. 97-105.

De, C.L., Li, H., Sui, J.L., Zhao, Z., Li, X. & Zhong, M.L. 2008, "Body iron stores and dietary iron intake in relation to diabetes in adults in north China", *Diabetes care,* vol. 31, no. 2, pp. 285-286.

Dejong, G., Vandijk, J.P. & Vaneijk, H.G. 1990, "The Biology of Transferrin", *Clinica Chimica Acta,* vol. 190, no. 1-2, pp. 1-46.

Dennison, H.A. 1999, "Limitations of ferritin as a marker of anemia in end stage renal disease.", *ANNA journal / American Nephrology Nurses" Association,* vol. 26, no. 4, pp. 409-414; quiz 419.

Dmochowski, K., Finegood, D.T., Francombe, W., Tyler, B. & Zinman, B. 1993, "Factors Determining Glucose-Tolerance in Patients with Thalassemia Major", *Journal of Clinical Endocrinology & Metabolism,* vol. 77, no. 2, pp. 478-483.

Eaton, B.M. & Sooranna, S.R. 2000, "Transport of large neutral amino acids into BeWo cells", *Placenta,* vol. 21, no. 5-6, pp. 558-564.

Eaton, B.M. & Sooranna, S.R. 1998, "In vitro modulation of L-arginine transport in trophoblast cells by glucose", *European journal of clinical investigation*, vol. 28, no. 12, pp. 1006-1010.

Ericsson, A., Hamark, B., Jansson, N., Johansson, B.R., Powell, T.L. & Jansson, T. 2005a, "Hormonal regulation of glucose and system A amino acid transport in first trimester placental villous fragments", *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, vol. 288, no. 3 57-3, pp. R656-R662.

Ericsson, A., Hamark, B., Powell, T.L. & Jansson, T. 2005b, "Glucose transporter isoform 4 is expressed in the syncytiotrophoblast of first trimester human placenta", *Human Reproduction*, vol. 20, no. 2, pp. 521-530.

Failla, M.L., Kennedy, M.L. & Chen, M.L. 1988, "Iron-Metabolism in Genetically-Obese (Ob/ob) Mice", *Journal of Nutrition*, vol. 118, no. 1, pp. 46-51.

Farley, D., Tejero, M.E., Comuzzie, A.G., Higgins, P.B., Cox, L., Werner, S.L., Jenkins, S.L., Li, C., Choi, J., Dick Jr., E.J., Hubbard, G.B., Frost, P., Dudley, D.J., Ballesteros, B., Wu, G., Nathanielsz, P.W. & Schlabritz-Loutsevitch, N.E. 2009, "Feto-placental Adaptations to Maternal Obesity in the Baboon", *Placenta*, vol. 30, no. 9, pp. 752-760.

Fenton, V., Cavill, I. & Fisher, J. 1977, "Iron stores in pregnancy", *British journal of haematology*, vol. 37, no. 1, pp. 145-149.

Fernandez-Real, J.M., Lopez-Bermejo, A. & Ricart, W. 2002, "Cross-talk between iron metabolism and diabetes", *Diabetes*, vol. 51, no. 8, pp. 2348-2354.

Fernandez-Real, J.M. & Ricart, W. 2003, "Insulin resistance and chronic cardiovascular inflammatory syndrome", *Endocrine reviews*, vol. 24, no. 3, pp. 278-301.

Fernández-Real, J.-., Ricart-Engel, W., Arroyo, E., Balançá, R., Casamitjana-Abella, R., Cabrero, D., Fernández-Castañer, M. & Soler, J. 1998, "Serum ferritin as a component of the insulin resistance syndrome", *Diabetes care*, vol. 21, no. 1, pp. 62-68.

Fielding, J. 1980, *Serum Iron and Iron Binding Capacity*, Churchill Livingstone, New-york.

Finch, C. 1994, "Regulators of iron balance in humans", *Blood*, vol. 84, no. 6, pp. 1697-1702.

Fletcher, J. & Suter, P.E.N. 1969, "Transport of Iron by Human Placenta", *Clinical science*, vol. 36, no. 2, pp. 209-&.

Ford, E.S. & Cogswell, M.E. 1999, "Diabetes and serum ferritin concentration among US adults", *Diabetes care,* vol. 22, no. 12, pp. 1978-1983.

Forouhi, N.G., Harding, A.H., Allison, M., Sandhu, M.S., Welch, A., Luben, R., Bingham, S., Khaw, K.T. & Wareham, N.J. 2007, "Elevated serum ferritin levels predict new-onset type 2 diabetes: Results from the EPIC-Norfolk prospective study", *Diabetologia,* vol. 50, no. 5, pp. 949-956.

Freeman, V.E., Mulder, J., Van't Hof, M.A., Hoey, H.M.V. & Gibney, M.J. 1998, "A longitudinal study of iron status in children at 12, 24 and 36 months", *Public health nutrition,* vol. 1, no. 2, pp. 93-100.

Freixenet, N., Remacha, A., Berlanga, E., Caixas, A., Gimenez-Palop, O., Blanco-Vaca, F., Bach, V., Baiget, M., Sanchez, Y., Felez, J. & Miguel Gonzalez-Clemente, J. 2009, "Serum soluble transferrin receptor concentrations are increased in central obesity. Results from a screening programme for hereditary hemochromatosis in men with hyperferritinemia", *Clinica Chimica Acta,* vol. 400, no. 1-2, pp. 111-116.

Frühbeck, G. 2006, "Intracellular signalling pathways activated by leptin", *Biochemical Journal,* vol. 393, no. 1, pp. 7-20.

Fuchs, R. & Ellinger, I. 2004, "Endocytic and transcytotic processes in villous syncytiotrophoblast: Role in nutrient transport to the human fetus", *Traffic,* vol. 5, no. 10, pp. 725-738.

Gambling, L., Charania, Z., Hannah, L., Antipatis, C., Lea, R.G. & McArdle, H.J. 2002, "Effect of iron deficiency on placental cytokine expression and fetal growth in the pregnant rat", *Biology of reproduction,* vol. 66, no. 2, pp. 516-523.

Gambling, L., Danzeisen, R., Fosset, C., Andersen, H.S., Dunford, S., Srai, S.K.S. & McArdle, H.J. 2003, "Iron and copper interactions in development and the effect on pregnancy outcome", *Journal of Nutrition,* vol. 133, no. 5, pp. 1554S-1556S.

Gambling, L., Danzeisen, R., Gair, S., Lea, R.G., Charania, Z., Solanky, N., Joory, K.D., Srai, S.K.S. & McArdle, H.J. 2001, "Effect of iron deficiency on placental transfer of iron and expression of iron transport proteins in vivo and in vitro", *Biochemical Journal,* vol. 356, pp. 883-889.

Gambling, L., Dunford, S. & McArdle, H.J. 2004, "Iron deficiency in the pregnant rat has differential effects on maternal and fetal copper levels", *Journal of Nutritional Biochemistry,* vol. 15, no. 6, pp. 366-372.

Gambling, L., Dunford, S., Wallace, D.I., Zuur, G., Solanky, N., Srai, S.K.S. & McArdle, H.J. 2003, "Iron deficiency during pregnancy affects postnatal blood pressure in the rat", *Journal of Physiology,* vol. 552, no. 2, pp. 603-610.

Ganz, T. 2006, "Molecular pathogenesis of anemia of chronic disease", *Pediatric Blood & Cancer,* vol. 46, no. 5, pp. 554-557.

Garn, S.M., Keating, M.T. & Falkner, F. 1981, "Hematological Status and Pregnancy Outcomes", *American Journal of Clinical Nutrition,* vol. 34, no. 1, pp. 115-117.

Gaspar, M.J., Ortega, R.M. & Moreiras, O. 1993, "Relationship between iron status in pregnant women and their newborn babies. Investigation in a Spanish population", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 72, no. 7, pp. 534-537.

Gemma, C., Sookoian, S., Alvarĩas, J., García, S.I., Quintana, L., Kanevsky, D., González, C.D. & Pirola, C.J. 2009, "Maternal pregestational BMI is associated with methylation of the PPARGC1A promoter in newborns", *Obesity,* vol. 17, no. 5, pp. 1032-1039.

Georgieff, M.K., Berry, S.A., Wobken, J.D. & Leibold, E.A. 1999, "Increased placental iron regulatory protein-1 expression in diabetic pregnancies complicated by fetal iron deficiency", *Placenta,* vol. 20, no. 1, pp. 87-93.

Georgieff, M.K., Landon, M.B., Mills, M.M., Hedlund, B.E., Faassen, A.E., Schmidt, R.L., Ophoven, J.J. & Widness, J.A. 1990, "Abnormal iron distribution in infants of diabetic mothers: Spectrum and maternal antecedents", *Journal of Pediatrics,* vol. 117, no. 3, pp. 455-461.

Georgieff, M.K., Mills, M.M., Gordon, K. & Wobken, J.D. 1995, "Reduced neonatal liver iron concentrations after uteroplacental insufficiency", *Journal of Pediatrics,* vol. 127, no. 2, pp. 308-311.

Georgieff, M.K., Petry, C.D., Mills, M.M., McKay, H. & Wobken, J.D. 1997, "Increased N-glycosylation and reduced transferrin-binding capacity of transferrin receptor isolated from placentae of diabetic women", *Placenta,* vol. 18, no. 7, pp. 563-568.

Gillum, R.F. 2001, "Association of serum ferritin and indices of body fat distribution and obesity in Mexican American men - the Third National Health and Nutrition Examination Survey", *International journal of obesity,* vol. 25, no. 5, pp. 639-645.

Godfrey, K., Robinson, S., Barker, D.J.P., Osmond, C. & Cox, V. 1996, "Maternal nutrition in early and late pregnancy in relation to placental and fetal growth", *British medical journal,* vol. 312, no. 7028, pp. 410-414.

Godfrey, K.M. 2002, "The role of the placenta in fetal programming - A review", *Placenta,* vol. 23, pp. S20-S27.

Godfrey, K.M. & Barker, D.J.P. 1995, "Maternal nutrition in relation to fetal and placental growth", *European Journal of Obstetrics Gynecology and Reproductive Biology,* vol. 61, no. 1, pp. 15-22.

Godfrey, K.M., Redman, C.W.G., Barker, D.J.P. & Osmond, C. 1991, "The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight", *British journal of obstetrics and gynaecology*, vol. 98, no. 9, pp. 886-891.

Goepel, E., Ulmer, H.U. & Neth, R.D. 1988, "Premature Labor Contractions and the Value of Serum Ferritin during Pregnancy", *Gynecologic and obstetric investigation*, vol. 26, no. 4, pp. 265-273.

Goldenberg, R.L., Tamura, T., DuBard, M., Johnston, K.E., Copper, R.L. & Neggers, Y. 1996, "Plasma ferritin and pregnancy outcome", *American Journal of Obstetrics and Gynecology*, vol. 175, no. 5, pp. 1356-1359.

González de Agüero, R., Sobreviela, M., Torrijo, C. & Fabre, E. 2001, "Alimentación y nutrición materna durante el embarazo" in *Manual de Asistencia al Embarazo Normal*, ed. E. Fabre González, 2ª ed edn, Editorial Ino Reproducciones, , pp. pp 265-313.

Green, E.D., Maffei, M., Braden, V.V., Proenca, R., Desilva, U., Zhang, Y.Y., Chua, S.C., Leibel, R.L., Weissenbach, J. & Friedman, J.M. 1995, "The Human Obese (Ob) Gene - Rna Expression Pattern and Mapping on the Physical, Cytogenetic, and Genetic Maps of Chromosome-7", *Genome research*, vol. 5, no. 1, pp. 5-12.

Greenberg, A.S. & Obin, M.S. 2006, "Obesity and the role of adipose tissue in inflammation and metabolism", *American Journal of Clinical Nutrition*, vol. 83, no. 2, pp. 461S-465S.

Gruper, Y., Bar, J., Bacharach, E. & Ehrlich, R. 2005, " Transferrin receptor colocalizes and interacts with the emochromatosis factor (HFE) and the divalent metal transporter-1 (DMT1) in trophoblast cells.", *J Cell Physiol*, vol. 204, pp. 901-912.

Guilbert, J.-. 2003, "The world health report 2002 - Reducing risks, promoting healthy life [2]", *Education for Health*, vol. 16, no. 2, pp. 230.

Hales, C.N. & Barker, D.J.P. 1992, "Type-2 (On-Insulin-Dependent) Diabetes-Mellitus - the Thrifty Phenotype Hypothesis", *Diabetologia*, vol. 35, no. 7, pp. 595-601.

Hallberg, L. 1998, "Daily iron supplementation: why it is necessary.", *Hallberg L. Daily iron supplementation: why it is necessary. Am J Clin Nutr 1998 ; 68 : 213-7.*, vol. 68, pp. 213-217.

Hallberg, L. 2001, *Perspectives on nutritional iron deficiency*.

Halliwell, B. & Gutteridge, J.M. 1999, *Free radicals in medicine and biology*, 2nd ed edn, Clarendon Press, Oxford.

Hallquist, N.A., McNeil, L.K., Lockwood, J.F. & Sherman, A.R. 1992, "Maternal-iron-deficiency effects on peritoneal macrophage and peritoneal natural-killer-cell cytotoxicity in rat pups", *American Journal of Clinical Nutrition,* vol. 55, no. 3, pp. 741-746.

Harris, E.D. 1992, "New insights into placental iron transport", *Nutrition reviews,* vol. 50, no. 11, pp. 329-331.

Harrison, P.M. & Arosio, P. 1996, "The ferritins: Molecular properties, iron storage function and cellular regulation", *Biochimica et Biophysica Acta - Bioenergetics,* vol. 1275, no. 3, pp. 161-203.

Hassapidou, M., Fotiadou, E., Maglara, E. & Papadopoulou, S.K. 2006, "Energy intake, diet composition, energy expenditure, and body fatness of adolescents in Northern Greece", *Obesity,* vol. 14, no. 5, pp. 855-862.

Heinrich, H.C., Bartels, H., Heinisch, B., Hausmann, K., Kuse, R. & Humke, W. 1968, "Intestinale 59Fe-Resorption und pra¨latenter Eisenmangel wa¨hrend der Gravidita¨t des Menschen.", vol. 46, pp. 199-202.

Hemminki, E. & Rimpela, U. 1991, "Iron supplementation, maternal packed cell volume, and fetal growth", *Archives of Disease in Childhood,* vol. 66, no. 4 SUPPL., pp. 422-425.

Hentze, M.W., Muckenthaler, M.U. & Andrews, N.C. 2004, "Balancing acts: Molecular control of mammalian iron metabolism", *Cell,* vol. 117, no. 3, pp. 285-297.

Hernandez, C., Lecube, A., Carrera, A. & Simo, R. 2005, "Soluble transferrin receptors and ferritin in Type 2 diabetic patients", *Diabetic Medicine,* vol. 22, no. 1, pp. 97-101.

Hindmarsh, P.C., Geary, M.P.P., Rodeck, C.H., Jackson, M.R. & Kingdom, J.C.P. 2000, "Effect of early maternal iron stores placental weight and structure", *Lancet,* vol. 356, no. 9231, pp. 719-723.

Hytten, F. & Chamberlain, G. 1980, *Clinical physiology in obstetrics.* 1st ed edn, Blackwell Scientific Publication, Oxford.

Iannotti, L.L., O'Brien, K.O., Chang, S.C., Mancini, J., Schulman-Nathanson, M., Liu, S.Y., Harris, Z.L. & Witter, F.R. 2005, "Iron deficiency anemia and depleted body iron reserves are prevalent among pregnant African-American adolescents", *Journal of Nutrition,* vol. 135, no. 11, pp. 2572-2577.

Illouz, F., Roulier, V., Rod, A., Gallois, Y., Pelle, C.-., Aube, C., Rohmer, V., Ritz, P. & Ducluzeau, P.H. 2008, "Distribution of adipose tissue: Quantification and

relationship with hepatic steatosis and vascular profiles of type 2 diabetic patients with metabolic syndrome", *Diabetes & metabolism,* vol. 34, no. 1, pp. 68-74.

Iwasaki, T., Nakajima, A., Yoneda, M., Yamada, Y., Mukasa, K., Fujita, K., Fujisawa, N., Wada, K. & Terauchi, Y. 2005, "Serum ferritin is associated with visceral fat area and subcutaneous fat area", *Diabetes care,* vol. 28, no. 10, pp. 2486-2491.

Jaime-Perez, J.C., Herrera-Garza, J.L. & Gomez-Almaguer, D. 2005, "Sub-Optimal Fetal Iron Acquisition under a Maternal Environment", *Archives of Medical Research,* vol. 36, no. 5, pp. 598-602.

Jansson, N., Greenwood, S.L., Johansson, B.R., Powell, T.L. & Jansson, T. 2003, "Leptin stimulates the activity of the system A amino acid transporter in human placental villous fragments", *Journal of Clinical Endocrinology and Metabolism,* vol. 88, no. 3, pp. 1205-1211.

Jansson, T., Ekstrand, Y., Björn, C., Wennergren, M. & Powell, T.L. 2002, "Alterations in the activity of placental amino acid transporters in pregnancies complicated by diabetes", *Diabetes,* vol. 51, no. 7, pp. 2214-2219.

Jansson, T. & Powell, T.L. 2007, "Role of the placenta in fetal programming: Underlying mechanisms and potential interventional approaches", *Clinical science,* vol. 113, no. 1-2, pp. 1-13.

Jansson, T. & Powell, T.L. 2006, "Human Placental Transport in Altered Fetal Growth: Does the Placenta Function as a Nutrient Sensor? - A Review", *Placenta,* vol. 27, no. SUPPL., pp. 91-97.

Jehn, M., Clark, J.M. & Guallar, E. 2004, "Serum ferritin and risk of the metabolic syndrome in U.S adults", *Diabetes care,* vol. 27, no. 10, pp. 2422-2428.

Jiang, R., Manson, J.E., Meigs, J.B., Ma, J., Rifai, N. & Hu, F.B. 2004a, "Body Iron Stores in Relation to Risk of Type 2 Diabetes in Apparently Healthy Women", *Journal of the American Medical Association,* vol. 291, no. 6, pp. 711-717.

Jiang, Y., Wilk, J.B., Borecki, I., Williamson, S., DeStefano, A.L., Xu, G., Liu, J., Ellison, R.C., Province, M. & Myers, R.H. 2004b, "Common variants in the 5 ' region of the leptin gene are associated with body mass index in men from the National Heart, Lung, and Blood Institute Family Heart Study", *American Journal of Human Genetics,* vol. 75, no. 2, pp. 220-230.

Jones, H.N., Ashworth, C.J., Page, K.R. & McArdle, H.J. 2006, "Expression and adaptive regulation of amino acid transport system A in a placental cell line under amino acid restriction", *Reproduction,* vol. 131, no. 5, pp. 951-960.

Jones, H.N., Powell, T.L. & Jansson, T. 2007, "Regulation of Placental Nutrient Transport - A Review", *Placenta,* vol. 28, no. 8-9, pp. 763-774.

Kahn, S.E., Hull, R.L. & Utzschneider, K.M. 2006, "Mechanisms linking obesity to insulin resistance and type 2 diabetes", *Nature,* vol. 444, no. 7121, pp. 840-846.

Kalantar-Zadeh, K., Rodriguez, R.A. & Humphreys, M.H. 2004, "Association between serum ferritin and measures of inflammation, nutrition and iron in haemodialysis patients", *Nephrology Dialysis Transplantation,* vol. 19, no. 1, pp. 141-149.

Kelly, A.M., Macdonald, D.J. & Mcdougall, A.N. 1978, "Observations on Maternal and Fetal Ferritin Concentrations at Term", *British journal of obstetrics and gynaecology,* vol. 85, no. 5, pp. 338-343.

Kern, P.A., Ranganathan, S., Li, C., Wood, L. & Ranganathan, G. 2001, "Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance", *American Journal of Physiology - Endocrinology and Metabolism,* vol. 280, no. 5 43-5, pp. E745-E751.

Khullar, S., Greenwood, S.L., McCord, N., Glazier, J.D. & Ayuk, P.T.-. 2004, "Nitric oxide and superoxide impair human placental amino acid uptake and increase Na+ permeability: Implications for fetal growth", *Free Radical Biology and Medicine,* vol. 36, no. 3, pp. 271-277.

Kilbride, J., Baker, T.G., Parapia, L.A., Khoury, S.A., Shuqaidef, S.W. & Jerwood, D. 1999, "Anaemia during pregnancy as a risk factor for iron-deficiency anaemia in infancy: A case-control study in Jordan", *International journal of epidemiology,* vol. 28, no. 3, pp. 461-468.

Kim, I., Hungerford, D.W., Yip, R., Kuester, S.A., Zyrkowski, C. & Trowbridge, F.L. 1992, "Pregnancy nutrition surveillance system--United States, 1979-1990.", *MMWR.CDC surveillance summaries : Morbidity and mortality weekly report.CDC surveillance summaries / Centers for Disease Control,* vol. 41, no. 7, pp. 25-41.

Klebanoff, M., Shiono, P., Selby, J., Trachtenberg, A. & Graubard, B. 1991, "Anemia and spontaneous preterm birth", *International Journal of Gynecology & Obstetrics,* vol. 36, no. 4, pp. 345-345.

Klover, P.J., Zimmers, T.A., Koniaris, L.G. & Mooney, R.A. 2003, "Chronic Exposure to Interleukin-6 Causes Hepatic Insulin Resistance in Mice", *Diabetes,* vol. 52, no. 11, pp. 2784-2789.

Knutson, M.D., Oukka, M., Koss, L.M., Aydemir, F. & Wessling-Resnick, M. 2005, "Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1 overexpression and down-regulated by hepcidin", *Proceedings of the*

National Academy of Sciences of the United States of America, vol. 102, no. 5, pp. 1324-1328.

Knutson, M.D., Walter, P.B., Mendoza, C., Ames, B.N. & Viteri, F.E. 1999, "Effects of daily and weekly oral iron supplements on iron status and lipid peroxidation in women", *Faseb Journal*, vol. 13, no. 5, pp. A698-A698.

Kohgo, Y., Torimoto, Y. & Kato, J. 2002, "Transferrin receptor in tissue and serum: Updated clinical significance of soluble receptor", *International journal of hematology*, vol. 76, no. 3, pp. 213-218.

Kohgo, Y., Ikuta, K., Ohtake, T., Torimoto, Y. & Kato, J. 2007, "Iron overload and cofactors with special reference to alcohol, hepatitis C virus infection and steatosis/insulin resistance", *World Journal of Gastroenterology*, vol. 13, no. 35, pp. 4699-4706.

Koller, O. 1982, "The clinical significance of hemodilution during pregnancy", *Obstetrical and Gynecological Survey*, vol. 37, no. 11, pp. 649-652.

Komolova, M., Bourque, S.L., Nakatsu, K. & Adams, M.A. 2008, "Sedentariness and increased visceral adiposity in adult perinatally iron-deficient rats", *International journal of obesity*, vol. 32, no. 9, pp. 1441-1444.

Kriketos, A.D., Greenfield, J.R., Peake, P.W., Furler, S.M., Denyer, G.S., Charlesworth, J.A. & Campbell, L.V. 2004, "Inflammation, insulin resistance, and adiposity - A study of first-degree relatives of type 2 diabetic subjects", *Diabetes care*, vol. 27, no. 8, pp. 2033-2040.

Kwik-Uribe, C.L., Gietzen, D., German, J.B., Golub, M.S. & Keen, C.L. 2000, "Chronic marginal iron intakes during early development in mice result in persistent changes in dopamine metabolism and myelin composition", *Journal of Nutrition*, vol. 130, no. 11, pp. 2821-2830.

Laftah, A.H., Ramesh, B., Simpson, R.J., Solanky, N., Bahram, S., Schümann, K., Debnam, E.S. & Srai, S.K.S. 2004, "Effect of hepcidin on intestinal iron absorption in mice", *Blood*, vol. 103, no. 10, pp. 3940-3944.

Lago, F., Dieguez, C., Gómez-Reino, J. & Gualillo, O. 2007, "The emerging role of adipokines as mediators of inflammation and immune responses", *Cytokine and Growth Factor Reviews*, vol. 18, no. 3-4, pp. 313-325.

Lahlou, N., Clement, K., Carel, J.C., Vaisse, C., Lotton, C., Le Bihan, Y., Basdevant, A., Lebouc, Y., Froguel, P., Roger, M. & Guy-Grand, B. 2000, "Soluble leptin receptor in serum of subjects with complete resistance to leptin - Relation to fat mass", *Diabetes*, vol. 49, no. 8, pp. 1347-1352.

Langini, S.H., De Portela, M.L., Lázzari, A., Ortega Soler, C.R. & Lönnerdal, B. 2006, "Do indicators of maternal iron status reflect placental iron status at delivery?", *Journal of Trace Elements in Medicine and Biology,* vol. 19, no. 4, pp. 243-249.

Lao, T.T., Chan, L.Y., Tam, K.-. & Ho, L.-. 2002, "Maternal hemoglobin and risk of gestational diabetes mellitus in Chinese women", *Obstetrics and gynecology,* vol. 99, no. 5, pp. 807-812.

Lao, T.T., Chan, P.L. & Tam, K.F. 2001, "Gestational diabetes mellitus in the last trimester - A feature of maternal iron excess?", *Diabetic Medicine,* vol. 18, no. 3, pp. 218-223.

Lao, T.T. & Ho, L.F. 2004, "Impact of iron deficiency anemia on prevalence of gestational diabetes mellitus", *Diabetes care,* vol. 27, no. 3, pp. 650-656.

Lao, T.T., Loong, E.P.L., Chin, R.K.H., Lam, C.W.K. & Lam, Y.M. 1991, "Relationship between newborn and maternal iron status and haematological indices", *Biology of the neonate,* vol. 60, no. 5, pp. 303-307.

Lao, T.T. & Tam, K.-. 1997, "Maternal serum ferritin and gestational impaired glucose tolerance", *Diabetes care,* vol. 20, no. 9, pp. 1368-1369.

Le Guenno, G., Chansaume, E., Ruivard, M., Morio, B. & Mazur, A. 2007, "Study of iron metabolism disturbances in an animal model of insulin resistance", *Diabetes research and clinical practice,* vol. 77, no. 3, pp. 363-370.

Le Stunff, C., Le Bihan, C., Schork, N.J. & Bougneres, P. 2000, "A common promoter variant of the leptin gene is associated with changes in the relationship between serum leptin and fat mass in obese girls", *Diabetes,* vol. 49, no. 12, pp. 2196-2200.

Lecube, A., Hernandez, C., Genesca, J., Esteban, J.I., Jardi, R., Garcia, L. & Simo, R. 2004, "Diabetes is the main factor accounting for the high ferritin levels detected in chronic hepatitis C virus infection", *Diabetes care,* vol. 27, no. 11, pp. 2669-2675.

Lecube, A., Hernandez, C., Genesca, J. & Simo, R. 2006, "Glucose abnormalities in patients with hepatitis C virus infection - Epidemiology and pathogenesis", *Diabetes care,* vol. 29, no. 5, pp. 1140-1149.

Lecube, A., Hernández, C., Pelegrí, D. & Simó, R. 2008, "Factors accounting for high ferritin levels in obesity", *International journal of obesity,* vol. 32, no. 11, pp. 1665-1669.

Lecube, A., Carrera, A., Losada, E., Hernandez, C., Simo, R. & Mesa, J. 2006, "Iron deficiency in obese postmenopausal women", *Obesity,* vol. 14, no. 10, pp. 1724-1730.

Lee, A.J., Hiscock, R.J., Wein, P., Walker, S.P. & Permezel, M. 2007, "Gestational diabetes mellitus: Clinical predictors and long-term risk of developing type 2 Diabetes - A retrospective cohort study using survival analysis", *Diabetes care,* vol. 30, no. 4, pp. 878-883.

Levy-Marchal, C. & Jaquet, D. 2004, "Long-term metabolic consequences of being born small for gestational age", *Pediatric Diabetes,* vol. 5, no. 3, pp. 147-153.

Li, Y., Yan, H. & Bai, B. 2008, "Change in iron transporter expression in human term placenta with different maternal iron status", *European Journal of Obstetrics Gynecology and Reproductive Biology,* vol. 140, no. 1, pp. 48-54.

Lim, M.K., Lee, C.K., Ju, Y.S., Cho, Y.S., Lee, M.-., Yoo, B. & Moon, H.-. 2001, "Serum ferritin as a serologic marker of activity in systemic lupus erythematosus", *Rheumatology international,* vol. 20, no. 3, pp. 89-93.

Lisle, S.J.M., Lewis, R.M., Petry, C.J., Ozanne, S.E., Hales, C.N. & Forhead, A.J. 2003, "Effect of maternal iron restriction during pregnancy on renal morphology in the adult rat offspring", *British Journal of Nutrition,* vol. 90, no. 1, pp. 33-39.

Lloyd, C. & Lewis, V. 1996, "Diseases associated with pregnancy" in *Myles Textbook for Midwives,* eds. V.R. Bennett & I.K. Brown, 12th edn edn, Churchill Livingstone, Edinburgh, pp. 333-64.

Lobelo, F. 2005, "Fetal programming and risk of metabolic syndrome: Prevention efforts for high-risk populations [13]", *Pediatrics,* vol. 116, no. 2, pp. 519.

Lockwood, J.F. & Sherman, A.R. 1988, "Spleen natural killer cells from iron-deficient rat pups manifest an altered ability to be stimulated by interferon", *Journal of Nutrition,* vol. 118, no. 12, pp. 1558-1563.

Lund, E.K., Wharf, S.G., Fairweather-Tait, S.J. & Johnson, I.T. 1999, "Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers", *American Journal of Clinical Nutrition,* vol. 69, no. 2, pp. 250-255.

Macedo, M.F. & de Sousa, M. 2008, "Transferrin and the transferrin receptor: Of magic bullets and other concerns", *Inflammation and Allergy - Drug Targets,* vol. 7, no. 1, pp. 41-52.

Macgillivray, R.T.A., Mendez, E., Shewale, J.G., Sinha, S.K., Linebackzins, J. & Brew, K. 1983, "The Primary Structure of Human-Serum Transferrin - the Structures of 7 Cyanogen-Bromide Fragments and the Assembly of the Complete Structure", *Journal of Biological Chemistry,* vol. 258, no. 6, pp. 3543-3553.

MacPhail, A.P., Charlton, R.W., Bothwell, T.H. & Torrance, J.D. 1980, "The relationship between maternal and infant iron status", *Scandinavian journal of haematology,* vol. 25, no. 2, pp. 141-150.

Mahomed, K. 2001, "Iron supplementation in pregnancy (Cochrane Review)." in *The Cochrane Library. Update Software,* Issue 4 edn, The Cochrane Library., Oxford, UK.

Makrides, M., Crowther, C.A., Gibson, R.A., Gibson, R.S. & Skeaff, C.M. 2003, "Efficacy and tolerability of low-dose iron supplements during pregnancy: a randomized controlled trial", *American Journal of Clinical Nutrition,* vol. 78, no. 1, pp. 145-153.

Malope, B.I., MacPhail, A.P., Alberts, M. & Hiss, D.C. 2001, "The ratio of serum transferrin receptor and serum ferritin in the diagnosis of iron status", *British journal of haematology,* vol. 115, no. 1, pp. 84-89.

Mammes, O., Betoulle, D., Aubert, R., Giraud, V., Tuzet, S., Petiet, A., Colas-Linhart, N. & Fumeron, F. 1998, "Novel polymorphisms in the 5 ' region of the LEP gene - Association with leptin levels and response to low-calorie diet in human obesity", *Diabetes,* vol. 47, no. 3, pp. 487-489.

Manci, E.A. & Blackburn, W.R. 1987, "Regional variations in the levels of zinc, iron, copper, and calcium in the term human placenta", *Placenta,* vol. 8, no. 5, pp. 497-502.

Martin, W. & Russell, M.J. 2003, "On the origins of cells: a hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells", *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* vol. 358, no. 1429, pp. 59-83.

Mast, A.E., Blinder, M.A., Gronowski, A.M., Chumley, C. & Scott, M.G. 1998, "Clinical utility of the soluble transferrin receptor and comparison with serum ferritin in several populations", *Clinical chemistry,* vol. 44, no. 1, pp. 45-51.

McClung, J.P., Andersen, N.E., Tarr, T.N., Stahl, C.H. & Young, A.J. 2008, "Physical activity prevents augmented body fat accretion in moderately iron-deficient rats", *Journal of Nutrition,* vol. 138, no. 7, pp. 1293-1297.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D. & De Benoist, B. 2009, "Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005", *Public health nutrition,* vol. 12, no. 4, pp. 444-454.

Means Jr., R.T., Allen, J., Sears, D.A. & Schuster, S.J. 1999, "Serum soluble transferrin receptor and the prediction of marrow aspirate iron results in a heterogeneous group of patients", *Clinical and laboratory haematology,* vol. 21, no. 3, pp. 161-167.

Meberg, A., Haga, P., Sande, H. & Foss, O.P. 1979, "Smoking during pregnancy: hematological observations in the newborn", *Acta Paediatrica Scandinavica,* vol. 68, no. 5, pp. 731-734.

Mendler, M.-., Turlin, B., Moirand, R., Jouanolle, A.-., Sapey, T., Guyader, D., Le Gall, J.-., Brissot, P., David, V. & Deugnier, Y. 1999, "Insulin resistance-associated hepatic iron overload", *Gastroenterology,* vol. 117, no. 5, pp. 1155-1163.

Meng Lu, Z., Goldenberg, R.L., Cliver, S., Cutter, G. & Brankson, M. 1991, "The relationship between maternal hematocrit and pregnancy outcome", vol. 77, pp. 190-4.

Menzie, C.M., Yanoff, L.B., Denkinger, B.I., McHugh, T., Sebring, N.G., Calis, K.A. & Yanovski, J.A. 2008, "Obesity-related hypoferremia is not explained by differences in reported intake of heme and nonheme, iron or intake of dietary factors that can affect iron absorption", *Journal of the American Dietetic Association,* vol. 108, no. 1, pp. 145-148.

Metzger, B.E. 1991, "Summary and recommendations of the Third International Workshop-Conference on Gestational Diabetes Mellitus", *Diabetes,* vol. 40, no. SUPPL. 2, pp. 197-201.

Meyron-Holtz, E.G., Ghosh, M.C., Iwai, K., LaVaute, T., Brazzolotto, X., Berger, U.V., Land, W., Ollivierre-Wilson, H., Grinberg, A., Love, P. & Rouault, T.A. 2004, "Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis", *EMBO Journal,* vol. 23, no. 2, pp. 386-395.

Michaelsen, K.F., Milman, N. & Samuelson, G. 1995, "A longitudinal study of iron status in healthy infants: effects of early iron status, growth velocity and dietary factors", *Michaelsen KF, Milman N, Samuelson G. A longitudinal study of iron status in healthy infants: effects of early iron status, growth velocity and dietary factors. Acta Paediatr Scand 1995; 84: 1035–44.,* vol. 84, pp. 1035-44.

Micozzi, M.S., Albanes, D. & Stevens, R.G. 1989, "Relation of Body Size and Composition to Clinical Biochemical and Hematologic Indexes in United-States Men and Women", *American Journal of Clinical Nutrition,* vol. 50, no. 6, pp. 1276-1281.

Milman, N. 2006, "Iron and pregnancy - A delicate balance", *Annals of Hematology,* vol. 85, no. 9, pp. 559-565.

Milman, N. 1996, "Serum ferritin in Danes: Studies of iron status from infancy to old age, during blood donation and pregnancy", *International journal of hematology,* vol. 63, no. 2, pp. 103-135.

Milman, N., Agger, A.O. & Nielsen, O.J. 1991, "Iron Supplementation during Pregnancy - Effect on Iron Status Markers, Serum Erythropoietin and Human Placental-

Lactogen - a Placebo Controlled-Study in 207 Danish Women", *Danish medical bulletin,* vol. 38, no. 6, pp. 471-476.

Milman, N., Bergholt, T., Byg, K.-., Eriksen, L. & Graudal, N. 1999, "Iron status and iron balance during pregnancy. A critical reappraisal of iron supplementation", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 78, no. 9, pp. 749-757.

Milman, N., Bergholt, T., Eriksen, L., Byg, K.-., Graudal, N., Pedersen, P. & Hertz, J. 2005, "Iron prophylaxis during pregnancy - How much iron is needed? A randomized dose-response study of 20-80 mg ferrous iron daily in pregnant women", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 84, no. 3, pp. 238-247.

Milman, N., Byg, K.-. & Agger, A.O. 2000, "Hemoglobin and erythrocyte indices during normal pregnancy and postpartum in 206 women with and without iron supplementation", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 79, no. 2, pp. 89-98.

Milman, N., Byg, K.-., Bergholt, T., Eriksen, L. & Hvas, A.-. 2006, "Body iron and individual iron prophylaxis in pregnancy - Should the iron dose be adjusted according to serum ferritin?", *Annals of Hematology,* vol. 85, no. 9, pp. 567-573.

Milman, N., Clausen, J. & Byg, K.-. 1998, "Iron status in 268 Danish women aged 18-30 years: Influence of menstruation, contraceptive method, and iron supplementation", *Annals of Hematology,* vol. 77, no. 1-2, pp. 13-19.

Milman, N., Ibsen, K.K. & Christensen, J.M. 1987, "Serum Ferritin and Iron Status in Mothers and Newborn-Infants", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 66, no. 3, pp. 205-211.

Milman, N. & Kirchhoff, M. 1996, "Relationship between serum ferritin, alcohol intake, and social status in 2235 Danish men and women", *Annals of Hematology,* vol. 72, no. 3, pp. 145-151.

Milman, N., Pedersen, N.S. & Visfeldt, J. 1983, "Serum ferritin in healthy Danes: Relation to marrow haemosiderin iron stores", *Danish medical bulletin,* vol. 30, no. 2, pp. 115-120.

Moayeri, H., Bidad, K., Zadhoush, S., Gholami, N. & Anari, S. 2006, "Increasing prevalence of iron deficiency in overweight and obese children and adolescents (Tehran Adolescent Obesity Study)", *European journal of pediatrics,* vol. 165, no. 11, pp. 813-814.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S. & Coppack, S.W. 1997, "Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-α, in vivo", *Journal of Clinical Endocrinology and Metabolism,* vol. 82, no. 12, pp. 4196-4200.

Moirand, R., Mortaji, A.M., Loreal, O., Paillard, F., Brissot, P. & Deugnier, Y. 1997, "A new syndrome of liver iron overload with normal transferrin saturation", *Lancet,* vol. 349, no. 9045, pp. 95-97.

Muñoz Gómez, M., Campos Garríguez, A., García Erce, J.A. & Ramírez Ramírez, G. 2005, "Pathophysiology of iron metabolism: Diagnostic and therapeutic implications", *Nefrologia,* vol. 25, no. 1, pp. 9-19.

Murphy, J.F., Newcombe, R.G., O'Riordan, J., Coles, E.C. & Pearson, J.F. 1986, "RELATION OF HAEMOGLOBIN LEVELS IN FIRST AND SECOND TRIMESTERS TO OUTCOME OF PREGNANCY", *The Lancet,* vol. 327, no. 8488, pp. 992-995.

Myatt, L. 2006, "Placental adaptive responses and fetal programming", *Journal of Physiology,* vol. 572, no. 1, pp. 25-30.

Nandakumaran, M., Makhseed, M., Al-Rayyes, S., Akanji, A.O. & Sugathan, T.N. 2001, "Effect of insulin on transport kinetics of alpha-aminoisobutyric acid in the perfused human placental lobule in vitro", *Pediatrics International,* vol. 43, no. 6, pp. 581-586.

Nead, K.G., Halterman, J.S., Kaczorowski, J.M., Auinger, P. & Weitzman, M. 2004, "Overweight children and adolescents: A risk group for iron deficiency", *Pediatrics,* vol. 114, no. 1, pp. 104-108.

Nelson, D.M., Smith, S.D., Furesz, T.C., Sadovsky, Y., Ganapathy, V., Parvin, C.A. & Smith, C.H. 2003, "Hypoxia reduces expression and function of system A amino acid transporters in cultured term human trophoblasts", *American Journal of Physiology - Cell Physiology,* vol. 284, no. 2 53-2, pp. C310-C315.

Nelson, S.M., Coan, P.M., Burton, G.J. & Lindsay, R.S. 2009, "Placental structure in type 1 diabetes: Relation to fetal insulin, leptin, and IGF-I", *Diabetes,* vol. 58, no. 11, pp. 2634-2641.

Nemeth, E., Tuttle, M.S., Powelson, J., Vaughn, M.B., Donovan, A., Ward, D.M., Ganz, T. & Kaplan, J. 2004, "Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization", *Science,* vol. 306, no. 5704, pp. 2090-2093.

Nemeth, E. & Ganz, T. 2006, "Regulation of iron metabolism by hepcidin", *Annual Review of Nutrition,* vol. 26, pp. 323-342.

Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X., Devaux, I., Beaumont, C., Kahn, A. & Vaulont, S. 2002, "The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation", *Journal of Clinical Investigation,* vol. 110, no. 7, pp. 1037-1044.

O'Brien, K.O., Zavaleta, N., Abrams, S.A. & Caulfield, L.E. 2003, "Maternal iron status influences iron transfer to the fetus during the third trimester of pregnancy", *American Journal of Clinical Nutrition,* vol. 77, no. 4, pp. 924-930.

O'Brien, K.O., Zavaleta, N., Caulfield, L.E., Yang, D.X. & Abrams, S.A. 1999, "Influence of prenatal iron and zinc supplements on supplemental iron absorption, red blood cell iron incorporation, and iron status in pregnant Peruvian women", *American Journal of Clinical Nutrition,* vol. 69, no. 3, pp. 509-515.

Olthof, A.W., Sijens, P.E., Kreeftenberg, H.G., Kappert, P., Irwan, R., van der Jagt, E.J. & Oudkerk, M. 2007, "Correlation between serum ferritin levels and liver iron concentration determined by MR imaging: impact of hematologic disease and inflammation", *Magnetic resonance imaging,* vol. 25, no. 2, pp. 228-231.

Oshaug, A., Bugge, K.H., Bjonnes, C.H., Borchiohnsen, B. & Neslein, I.L. 1995, "Associations between Serum Ferritin and Cardiovascular Risk-Factors in Healthy-Young Men - a Cross-Sectional Study", *European journal of clinical nutrition,* vol. 49, no. 6, pp. 430-438.

Park, C.H., Valore, E.V., Waring, A.J. & Ganz, T. 2001, "Hepcidin, a Urinary Antimicrobial Peptide Synthesized in the Liver", *Journal of Biological Chemistry,* vol. 276, no. 11, pp. 7806-7810.

Pattillo, R.A. & Gey, G.O. 1968, "The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro.", *Cancer research,* vol. 28, no. 7, pp. 1231-1236.

Perry, G.S., Yip, R. & Zyrkowski, C. 1995, "Nutritional risk factors among low-income pregnant US women: The centers for disease control and prevention (CDC) pregnancy nutrition surveillance system, 1979 through 1993", *Seminars in perinatology,* vol. 19, no. 3, pp. 211-221.

Petry, C.D., Eaton, M.A., Wobken, J.D., Mills, M.M., Johnson, D.E. & Georgieff, M.K. 1992, "Iron deficiency of liver, heart, and brain in newborn infants of diabetic mothers", *Journal of Pediatrics,* vol. 121, no. 1, pp. 109-114.

Petry, C.D., Wobken, J.D., Mckay, H., Eaton, M.A., Seybold, V.S., Johnson, D.E. & Georgieff, M.K. 1994, "Placental Transferrin Receptor in Diabetic Pregnancies with Increased Fetal Iron Demand", *American Journal of Physiology-Endocrinology and Metabolism,* vol. 267, no. 4, pp. E507-E514.

Pinhas-Hamiel, O., Newfield, R.S., Koren, I., Agmon, A., Lilos, P. & Phillip, M. 2003, "Greater prevalence of iron deficiency in overweight and obese children and adolescents", *International journal of obesity,* vol. 27, no. 3, pp. 416-418.

Piperno, A., Trombini, P., Gelosa, M., Mauri, V., Pecci, V., Vergani, A., Salvioni, A., Mariani, R. & Mancia, G. 2002, "Increased serum ferritin is common in men with essential hypertension", *Journal of hypertension,* vol. 20, no. 8, pp. 1513-1518.

Pradhan, A.D., Manson, J.E., Rifai, N., Buring, J.E. & Ridker, P.M. 2001, "C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus", *Journal of the American Medical Association,* vol. 286, no. 3, pp. 327-334.

Preziosi, P., Prual, A., Galan, P., Daouda, H., Boureima, H. & Hercberg, S. 1997, "Effect of iron supplementation on the iron status of pregnant women: consequences for newborns", *American Journal of Clinical Nutrition,* vol. 66, no. 5, pp. 1178-1182.

Punnonen, K., Irjala, K. & Rajamaki, A. 1997, "Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency", *Blood,* vol. 89, no. 3, pp. 1052-1057.

Puolakka, J., Jänne, O., Pakarinen, A., Järvinen, P.A. & Vihko, R. 1980, "Serum ferritin as a measure of iron stores during and after normal pregnancy with and without iron supplements.", *Acta Obstetricia et Gynecologica Scandinavica, Supplement,* vol. 95, pp. 43-51.

Qi, L., Van Dam, R.M., Rexrode, K. & Hu, F.B. 2007, "Heme iron from diet as a risk factor for coronary heart disease in women with type 2 diabetes", *Diabetes care,* vol. 30, no. 1, pp. 101-106.

Qiu, C.F., Sorensen, T.K., Luthy, D.A. & Williams, M.A. 2004, "A prospective study of maternal serum C-reactive protein (CRP) concentrations and risk of gestational diabetes mellitus", *Paediatric and perinatal epidemiology,* vol. 18, no. 5, pp. 377-384.

Rajpathak, S., Ma, J., Manson, J., Willett, W.C. & Hu, F.B. 2006, "Iron intake and the risk of type 2 diabetes in women: A prospective cohort study", *Diabetes care,* vol. 29, no. 6, pp. 1370-1376.

Rajpathak, S.N., Crandall, J.P., Wylie-Rosett, J., Kabat, G.C., Rohan, T.E. & Hu, F.B. 2009, "The role of iron in type 2 diabetes in humans", *Biochimica et Biophysica Acta - General Subjects,* vol. 1790, no. 7, pp. 671-681.

Ramakrishnan, U., González-Cossío, T., Neufeld, L.M., Rivera, J. & Martorell, R. 2003, "Multiple micronutrient supplementation during pregnancy does not lead to greater infant birth size than does iron-only supplementation: A randomized controlled trial in a semirural community in Mexico", *American Journal of Clinical Nutrition,* vol. 77, no. 3, pp. 720-725.

Rao, J. & Jagadeesan, V. 1996, "Lipid peroxidation and activities of antioxidant enzymes in iron deficiency and effect of carcinogen feeding", *Free Radical Biology and Medicine*, vol. 21, no. 1, pp. 103-108.

Rao, R., De Ungria, M., Sullivan, D., Wu, P., Wobken, J.D., Nelson, C.A. & Georgieff, M.K. 1999, "Perinatal brain iron deficiency increases the vulnerability of rat hippocampus to hypoxic ischemic insult", *Journal of Nutrition*, vol. 129, no. 1, pp. 199-206.

Rao, R. & Georgieff, M.K. 2007, "Iron in fetal and neonatal nutrition", *Seminars in Fetal and Neonatal Medicine*, vol. 12, no. 1, pp. 54-63.

Rasmussen, K.M. 2001, "Is there a causal relationship between iron deficiency or iron-deficiency anemia and weight at birth, length of gestation and perinatal mortality?", *Journal of Nutrition*, vol. 131, no. 2 SUPPL. 2, pp. 590S-603S.

Rasmussen, K.M. & Stoltzfus, R.J. 2003, "New evidence that iron supplementation during pregnancy improves birth weight: new scientific questions.", *The American Journal of Clinical Nutrition*, vol. 78, no. 4, pp. 673-674.

Rayman, M.P., Barlis, J., Evans, R.W., Redman, C.W.G. & King, L.J. 2002, "Abnormal iron parameters in the pregnancy syndrome preeclampsia", *American Journal of Obstetrics and Gynecology*, vol. 187, no. 2, pp. 412-418.

Reynolds, L.P., Caton, J.S., Redmer, D.A., Grazul-Bilska, A.T., Vonnahme, K.A., Borowicz, P.P., Luther, J.S., Wallace, J.M., Wu, G. & Spencer, T.E. 2006, "Evidence for altered placental blood flow and vascularity in compromised pregnancies", *Journal of Physiology*, vol. 572, no. 1, pp. 51-58.

Ridker, P.M., Rifai, N., Stampfer, M.J. & Hennekens, C.H. 2000, "Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men", *Circulation*, vol. 101, no. 15, pp. 1767-1772.

Ritchie, R.F., Palomaki, G.E., Neveux, L.M., Navolotskaia, O., Ledue, T.B. & Craig, W.Y. 1999, "Reference distributions for the negative acute-phase serum proteins, albumin, transferrin and transthyretin: A practical, simple and clinically relevant approach in a large cohort", *Journal of clinical laboratory analysis*, vol. 13, no. 6, pp. 273-279.

Rivera, S., Nemeth, E., Gabayan, V., Lopez, M.A., Farshidi, D. & Ganz, T. 2005, "Synthetic hepcidin causes rapid dose-dependent hypoferremia and is concentrated in ferroportin-containing organs", *Blood*, vol. 106, no. 6, pp. 2196-2199.

Roberts, V.H.J., Smith, J., McLea, S.A., Heizer, A.B., Richardson, J.L. & Myatt, L. 2009, "Effect of Increasing Maternal Body Mass Index on Oxidative and Nitrative Stress in The Human Placenta", *Placenta*, vol. 30, no. 2, pp. 169-175.

Robinson, S., Godfrey, K., Denne, J. & Cox, V. 1998, "The determinants of iron status in early pregnancy", *British Journal of Nutrition*, vol. 79, no. 3, pp. 249-255.

Romslo, I., Haram, K., Sagen, N. & Augensen, K. 1983, "Iron requirement in normal pregnancy as assessed by serum ferritin, serum transferrin saturation and erythrocyte protoporphyrin determinations", *British journal of obstetrics and gynaecology*, vol. 90, no. 2, pp. 101-107.

Rondó, P.H.C., Abbott, R., Rodrigues, L.C. & Tomkins, A.M. 1997, "The influence of maternal nutritional factors on intrauterine growth retardation in Brazil", *Paediatric and perinatal epidemiology*, vol. 11, no. 2, pp. 152-166.

Roos, S., Palmberg, I., Saljo, K., Powell, T.L. & Jansson, T. 2005, "Expression of placental mammalian target of rapamycin (mTOR) is altered in relation to fetal growth and mTOR regulates leucine transport.", *Placenta*, vol. 26, no. 8-9, pp. A9-A9.

Rosen, E.D. & Spiegelman, B.M. 2006, "Adipocytes as regulators of energy balance and glucose homeostasis", *Nature*, vol. 444, no. 7121, pp. 847-853.

Rossi, E., Bulsara, M.K., Olynyk, J.K., Cullen, D.J., Summerville, L. & Powell, L.W. 2001, "Effect of hemochromatosis genotype and lifestyle factors on iron and red cell indices in a community population", *Clinical chemistry*, vol. 47, no. 2, pp. 202-208.

Rowland, T.W., Deisroth, M.B., Green, G.M. & Kelleher, J.F. 1988, "The effect of iron therapy on the exercise capacity of nonanemic iron-deficient adolescent runners", *American Journal of Diseases of Children*, vol. 142, no. 2, pp. 165-169.

Roytblat, L., Rachinsky, M., Fisher, A., Greemberg, L., Shapira, Y., Doudevani, A. & Gelman, S. 2000, "Raised interleukin-6 levels in obese patients", *Obesity research*, vol. 8, no. 9, pp. 673-675.

Rumberger, J.M., Peters Jr., T., Burrington, C. & Green, A. 2004, "Transferrin and iron contribute to the lipolytic effect of serum in isolated adipocytes", *Diabetes*, vol. 53, no. 10, pp. 2535-2541.

Rusia, U., Flowers, C., Madan, N., Agarwal, N., Sood, S. & Sikka, M. 1996, "Serum transferrin receptor levels in the evaluation of iron deficiency in the neonate", *Acta Paediatrica Japonica (Overseas Edition)*, vol. 38, no. 5, pp. 455-459.

Saddi, R. & Shapira, G. 1970, "Iron requirements during growth" in *Iron deficiency*, eds. L. Hallberg, H.G. Harwerth & A. Vanotti, Academic Press, London, pp. 183–98.

Sagen, N., Nilsen, S.T., Kim, H.C., Bergsjo, P. & Koller, O. 1984, "Maternal Hemoglobin Concentration is Closely Related to Birth-Weight in Normal

Pregnancies", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 63, no. 3, pp. 245-248.

Salomon, J.A. & Murray, C.J.L. 2000, "The epidemiological transition revisited: new compositional models for mortality by age, sex and cause.", .

Salonen, J.T., Tuomainen, T.P., Nyyssonen, K., Lakka, H.M. & Punnonen, K. 1998, "Relation between iron stores and non-insulin dependent diabetes in men: case-control study", *British medical journal,* vol. 317, no. 7160, pp. 727-727.

Sandstad, B., Borch-Iohnsen, B., Milman, N., Graudal, N., Galloe, A. & Agger, A.O. 1996, "6. Serum ferritin and selective iron prophylaxius in pregnancy? [6]", *Journal of internal medicine,* vol. 240, no. 1, pp. 47-50.

Scanlon, K.S., Yip, R., Schieve, L.A. & Cogswell, M.E. 2000, "High and low hemoglobin levels during pregnancy: differential risks for preterm birth and small for gestational age", *Obstetrics & Gynecology,* vol. 96, no. 5, Part 1, pp. 741-748.

Scheer, J.C. & Guthrie, H.A. 1981, "Hemoglobin criteria with respect to obesity", *American Journal of Clinical Nutrition,* vol. 34, no. 12, pp. 2748-2751.

Scholl, T.O. 2005, "Iron status during pregnancy: Setting the stage for mother and infant", *American Journal of Clinical Nutrition,* vol. 81, no. 5, pp. 1218S-1222S.

Scholl, T.O., Hediger, M.L., Fischer, R.L. & Shearer, J.W. 1992, "Anemia Vs Iron-Deficiency - Increased Risk of Preterm Delivery in a Prospective-Study", *American Journal of Clinical Nutrition,* vol. 55, no. 5, pp. 985-988.

Schwartz III, W.J. & Thurnau, G.R. 1995, "Iron deficiency anemia in pregnancy", *Clinical obstetrics and gynecology,* vol. 38, no. 3, pp. 443-454.

Seligman, P.A., Schleicher, R.B. & Allen, R.H. 1979, "Isolation and Characterization of the Transferrin Receptor from Human-Placenta", *Journal of Biological Chemistry,* vol. 254, no. 20, pp. 9943-9946.

Seltzer, C.C. & Mayer, J. 1963, "Serum Iron and Iron-Binding Capacity in Adolescents .2. Comparison of Obese and Nonobese Subjects", *American Journal of Clinical Nutrition,* vol. 13, no. 6, pp. 354-&.

Sharma, A., Bartell, S.M., Baile, C.A., Chen, B., Podolsky, R.H., McIndoe, R.A. & She, J. 2010, "Hepatic Gene Expression Profiling Reveals Key Pathways Involved in Leptin-Mediated Weight Loss in ob/ob Mice", *Plos One,* vol. 5, no. 8, pp. e12147.

Sherwood, R.A., Pippard, M.J. & Peters, T.J. 1998, "Iron homeostasis and the assessment of iron status", *Annals of Clinical Biochemistry,* vol. 35, pp. 693-708.

Siddappa, A.M., Georgieff, M.K., Wewerka, S., Worwa, C., Nelson, C.A. & DeRegnier, R.A. 2004, "Iron deficiency alters auditory recognition memory in newborn infants of diabetic mothers", *Pediatric research,* vol. 55, no. 6, pp. 1034-1041.

Siddappa, A.M., Rao, R., Long, J.D., Widness, J.A. & Georgieff, M.K. 2007, "The assessment of newborn iron stores at birth: A review of the literature and standards for ferritin concentrations", *Neonatology,* vol. 92, no. 2, pp. 73-82.

Siimes, A.S.I. & Siimes, M.A. 1986, "Changes in the concentration of ferritin in the serum during fetal life in singleton and twins", *Early human development,* vol. 13, no. 1, pp. 47-52.

Singla, P.N., Tyagi, M., Kumar, A., Dash, D. & Shankar, R. 1997, "Fetal growth in maternal anaemia", *Journal of tropical pediatrics,* vol. 43, no. 2, pp. 89-92.

Singla, P.N., Tyagi, M., Shankar, R., Dash, D. & Kumar, A. 1996, "Fetal iron status in maternal anemia", *Acta Paediatrica, International Journal of Paediatrics,* vol. 85, no. 11, pp. 1327-1330.

Sjostedt, J.E., Manner, P., Nummi, S. & Ekenved, G. 1977, "Oral iron prophylaxis during pregnancy. A comparative study on different dosage regimens", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 56, no. sup 60.

Skikne, B.S. 2008, "Serum transferrin receptor", *American Journal of Hematology,* vol. 83, no. 11, pp. 872-875.

Skikne, B.S., Flowers, C.H. & Cook, J.D. 1990, "Serum transferrin receptor: A quantitative measure of tissue iron deficiency", *Blood,* vol. 75, no. 9, pp. 1870-1876.

Smith, S.R., Ghosh, M.C., Ollivierre-Wilson, H., Hang Tong, W. & Rouault, T.A. 2006, "Complete loss of iron regulatory proteins 1 and 2 prevents viability of murine zygotes beyond the blastocyst stage of embryonic development", *Blood Cells, Molecules, and Diseases,* vol. 36, no. 2, pp. 283-287.

Soewondo, S. 1995, "The effect of iron deficiency and mental stimulation on Indonesian children's cognitive performance and development", *Kobe Journal of Medical Sciences,* vol. 41, no. 1-2, pp. 1-17.

Spiro, T.G. 1977, "Chemistry and biochemistry of iron" in *Proteins of iron metabolism*, eds. E.B.". "Brown, P.". "Aisen, J.". "Fielding & H.H.". "Crichton, Grune and Stratton, New York, pp. 23-32.

Srai, S.K.S., Bomford, A. & McArdle, H.J. 2002, "Iron transport across cell membranes: molecular understanding of duodenal and placental iron uptake", *Best Practice & Research Clinical Haematology,* vol. 15, no. 2, pp. 243-259.

Starreveld, J.S., Kroos, M.J., Van Suijlen, J.D.E., Verrijt, C.E.H., Van Eijk, H.G. & Van Dijk, J.P. 1995, "Ferritin in cultured human cytotrophoblasts: Synthesis and subunit distribution", *Placenta,* vol. 16, no. 4, pp. 383-395.

Starreveld, J.S., Van Dijk, H.P., Kroos, M.J. & Van Eijk, H.G. 1993, "Regulation of transferrin receptor expression and distribution in vitro cultured human cytotrophoblasts", *Clinica Chimica Acta,* vol. 220, no. 1, pp. 47-60.

Steer, P., Alam, M.A., Wadsworth, J. & Welch, A. 1995, "Relation between Maternal Hemoglobin Concentration and Birth-Weight in Different Ethnic-Groups", *British medical journal,* vol. 310, no. 6978, pp. 489-491.

Stoltzfus, R.J. & Dreyfuss, M.L. 1998, *Guidelines for the use of iron supplements to prevent and treat iron deficiency anemia,* International Nutritional Anemia Consultative Group (INACG), International Life Sciences Institute, Ilsi Press, Washington.

Svanberg, B. 1975, "Absorption of iron in pregnancy", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 54, no. sup 48.

Svanberg, B., Arvidsson, B., Norrby, A., Rybo, G. & Sölvell, L. 1975, "Absorption of supplemental iron during pregnancy - a longitudinal study with repeated bone-marrow studies and absorption measurements.", *Acta Obstetricia et Gynecologica Scandinavica, Supplement,* , no. 48, pp. 87-108.

Sweet, D.G., Savage, G., Tubman, T.R.J., Lappin, T.R.J. & Halliday, H.L. 2001, "Study of maternal influences on fetal iron status at term using cord blood transferrin receptors", *Archives of Disease in Childhood: Fetal and Neonatal Edition,* vol. 84, no. 1, pp. F40-F43.

Tamura, T., Goldenberg, R.L., Hou, J.R., Johnston, K.E., Cliver, S.P., Ramey, S.L. & Nelson, K.G. 2002, "Cord serum ferritin concentrations and mental and psychomotor development of children at five years of age", *Journal of Pediatrics,* vol. 140, no. 2, pp. 165-170.

Tamura, T., Goldenberg, R.L., Johnston, K.E., Cliver, S.P. & Hickey, C.A. 1996, "Serum ferritin: A predictor of early spontaneous preterm delivery", *Obstetrics and gynecology,* vol. 87, no. 3, pp. 360-365.

Tapanainen, P., Leinonen, E., Ruokonen, A. & Knip, M. 2001, "Leptin concentrations are elevated in newborn infants of diabetic mothers", *Hormone research,* vol. 55, no. 4, pp. 185-190.

Tarim, E., Kilicdag, E., Bagis, T. & Ergin, T. 2004, "High maternal hemoglobin and ferritin values as risk factors for gestational diabetes", *International Journal of Gynecology and Obstetrics,* vol. 84, no. 3, pp. 259-261.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Woolf, E.A., Monroe, C.A. & Tepper, R.I. 1995, "Identification and expression cloning of a leptin receptor, OB-R", *Cell*, vol. 83, no. 7, pp. 1263-1271.

Taylor, D.J., Mallen, C., McDougall, N. & Lind, T. 1982, "Effect of iron supplementation on serum ferritin levels during and after pregnancy", *British journal of obstetrics and gynaecology*, vol. 89, no. 12, pp. 1011-1017.

Thomas, B. 2001, *Manual of Dietetic Practice*, 3rd edn edn, Blackwell Publishing, Oxford.

Torti, F.M. & Torti, S.V. 2002, "Regulation of ferritin genes and protein", *Blood*, vol. 99, no. 10, pp. 3505-3516.

Tsimihodimos, V., Gazi, I., Kalaitzidis, R., Elisaf, M. & Siamopoulos, K.C. 2006, "Increased serum ferritin concentrations and liver enzyme activities in patients with metabolic syndrome", *Metabolic Syndrome and Related Disorders*, vol. 4, no. 3, pp. 196-203.

Tuomainen, T.P., Nyyssonen, K., Tervahauta, A., Korpela, H., Lakka, T., Kaplan, G.A. & Salonen, J.T. 1997, "Body iron stores are associated with serum insulin and blood glucose concentrations - Population study of 1,013 eastern Finnish men", *Diabetes care*, vol. 20, no. 3, pp. 426-428.

Tyson, J.E. 1992, "Immediate care of the newborn infant" in *Effective care of the newborn*, eds. J.C. Sinclair & M.B. Bracken, Oxford University Press, Oxford, pp. 21-39.

Ulvik, R.J. 1984, "Stability of serum ferritin in healthy subjects", *Haematologia*, vol. 17, no. 4, pp. 433-438.

Valenti, L., Fracanzani, A.L., Dongiovanni, P., Bugianesi, E., Marchesini, G., Manzini, P., Vanni, E. & Fargion, S. 2007, "Iron depletion by phlebotomy improves insulin resistance in patients with nonalcoholic fatty liver disease and hyperferritinemia: Evidence from a case-control study", *American Journal of Gastroenterology*, vol. 102, no. 6, pp. 1251-1258.

Van Den Broek, N.R. & Letsky, E.A. 2000, "Etiology of anemia in pregnancy in south Malawi", vol. 72, pp. 247-56.

van den Broek, N.R., Letsky, E.A., White, S.A. & Shenkin, A. 1998, "Iron status in pregnant women: which measurements are valid?", vol. 103(3), pp. 817-24.

Van der Ende, A., Du Maine, A. & Simmons, C.F. 1987, "Iron metabolism in BeWo chorion carcinoma cells. Transferrin-mediated uptake and release of iron", *Journal of Biological Chemistry,* vol. 262, no. 18, pp. 8910-8916.

Van Hall, G., Steensberg, A., Sacchetti, M., Fischer, C., Keller, C., Schjerling, P., Hiscock, N., Møller, K., Saltin, B., Febbraio, M.A. & Pedersen, B.K. 2003, "Interleukin-6 stimulates lipolysis and fat oxidation in humans", *Journal of Clinical Endocrinology and Metabolism,* vol. 88, no. 7, pp. 3005-3010.

Vardhana, P.A. & Illsley, N.P. 2002, "Transepithelial glucose transport and metabolism in BeWo choriocarcinoma cells", *Placenta,* vol. 23, no. 8-9, pp. 653-660.

Vari, I.S., Balkau, B., Kettaneh, A., Andre, P., Tichet, J., Fumeron, F., Caces, E., Marre, M., Grandchamp, B., Ducimetiere, P. & DESIR Study Grp 2007, "Ferritin and transferrin are associated with metabolic syndrome abnormalities and their change over time in a general population - Data from an Epidemiological Study on the Insulin Resistance Syndrome (DESIR)", *Diabetes care,* vol. 30, no. 7, pp. 1795-1801.

Verrijt, C.E.H., Kroos, M.J., Verhoeven, A.J.M., vanEijk, H.G. & vanDijk, J.P. 1997, "Transferrin in cultured human term cytotrophoblast cells: Synthesis and heterogeneity", *Molecular and cellular biochemistry,* vol. 173, no. 1-2, pp. 177-181.

Visser, M., Bouter, L.M., McQuillan, G.M., Wener, M.H. & Harris, T.B. 1999, "Elevated C-reactive protein levels in overweight and obese adults", *Jama-Journal of the American Medical Association,* vol. 282, no. 22, pp. 2131-2135.

Viteri, F.E. 1997, "Iron supplementation for the control of iron deficiency in populations at risk", *Nutrition reviews,* vol. 55, no. 6, pp. 195-209.

Wachs, T.D., Pollitt, E., Cueto, S. & Jacoby, E. 2005, "Relation of neonatal iron status to individual variability in neonatal temperament", *Developmental psychobiology,* vol. 46, no. 2, pp. 141-153.

Wada, H.G., Hass, P.E. & Sussman, H.H. 1979, "Transferrin Receptor in Human Placental Brush-Border Membranes - Studies on the Binding of Transferrin to Placental Membrane-Vesicles and the Identification of a Placental Brush-Border Glycoprotein with High-Affinity for Transferrin", *Journal of Biological Chemistry,* vol. 254, no. 24, pp. 2629-2635.

Walters, G.O., Miller, F.M. & Worwood, M. 1973, "Serum ferritin concentration and iron stores in normal subjects", *Journal of clinical pathology,* vol. 26, no. 10, pp. 770-772.

Wang, T.N., Huang, M.C., Chang, W.T., Ko, A.M.S., Tsai, E.M., Liu, C.S., Lee, C.H. & Ko, Y.C. 2006, "G-2548A polymorphism of the leptin gene is correlated with extreme obesity in Taiwanese aborigines", *Obesity,* vol. 14, no. 2, pp. 183-187.

Wei, J.-., Li, H.-., Sung, F.-., Lin, C.-., Chiang, C.-., Li, C.-. & Chuang, L.-. 2007, "Birth weight correlates differently with cardiovascular risk factors in youth", *Obesity*, vol. 15, no. 6, pp. 1609-1616.

Weinstein, D.A., Roy, C.N., Fleming, M.D., Loda, M.F., Wolfsdorf, J.I. & Andrews, N.C. 2002, "Inappropriate expression of hepcidin is associated with iron refractory anemia: Implications for the anemia of chronic disease", *Blood*, vol. 100, no. 10, pp. 3776-3781.

Weiss, G. & Goodnough, L.T. 2005, "Anemia of chronic disease", *New England Journal of Medicine*, vol. 352, no. 10, pp. 1011-1023+1059.

Weitzman, S., Harmanboehm, I. & Maislos, M. 1990, "Gestational Diabetes and Risk of Developing Iddm", *Diabetes care*, vol. 13, no. 2, pp. 186-186.

Wenzel, B.J., Mayer, J. & Stults, H.B. 1962, "Hypoferraemia in Obese Adolescents", *Lancet*, vol. 2, no. 7251, pp. 327-&.

Whincup, P.H., Kaye, S.J., Owen, C.G., Huxley, R., Cook, D.G., Anazawa, S., Barrett-Connor, E., Bhargava, S.K., Birgisdottir, B.E., Carlsson, S., De Rooij, S.R., Dyck, R.F., Eriksson, J.G., Falkner, B., Fall, C., Forsén, T., Grill, V., Gudnason, V., Hulman, S., Hyppönen, E., Jeffreys, M., Lawlor, D.A., Leon, D.A., Minami, J., Mishra, G., Osmond, C., Power, C., Rich-Edwards, J.W., Roseboom, T.J., Sachdev, H.S., Syddall, H., Thorsdottir, I., Vanhala, M., Wadsworth, M. & Yarbrough, D.E. 2008, "Birth weight and risk of type 2 diabetes a systematic review", *JAMA - Journal of the American Medical Association*, vol. 300, no. 24, pp. 2886-2897.

Whitfield, J.B., Treloar, S., Zhu, G., Powell, L.W. & Martin, N.G. 2003, "Relative importance of female-specific and non-female-specific effects on variation in iron stores between women", *British journal of haematology*, vol. 120, no. 5, pp. 860-866.

Widdowson, E.M. & Spray, C.M. 1951, "
Chemical development in utero.", *Arch Dis Child*, vol. 26; (127), pp. 205-14.

Williams, M.D. & Wheby, M.S. 1992, "Anemia in pregnancy", *Medical Clinics of North America*, vol. 76, no. 3, pp. 631-647.

Williams, M.J.A., Poulton, R. & Williams, S. 2002, "Relationship of serum ferritin with cardiovascular risk factors and inflammation in young men and women", *Atherosclerosis*, vol. 165, no. 1, pp. 179-184.

Wilson, J.G., Lindquist, J.H., Grambow, S.C., Crook, E.D. & Maher, J.F. 2003, "Potential role of increased iron stores in diabetes", *American Journal of the Medical Sciences*, vol. 325, no. 6, pp. 332-339.

Winick, J.D., Stoffel, M. & Friedman, J.M. 1996, "Identification of microsatellite markers linked to the human leptin receptor gene on chromosome 1", *Genomics*, vol. 36, no. 1, pp. 221-222.

Wish, J.B. 2006, "Assessing iron status: Beyond serum ferritin and transferrin saturation", *Clinical Journal of the American Society of Nephrology*, vol. 1, pp. S4-S8.

Wolf, M., Sandler, L., Hsu, K., Vossen-Smirnakis, K., Ecker, J.L. & Thadhani, R. 2003, "First-trimester C-roactive protein and subsequent gestational diabetes", *Diabetes care*, vol. 26, no. 3, pp. 819-824.

Woolf, S.H. 1993, "Routine Iron Supplementation during Pregnancy - Review Article", *Jama-Journal of the American Medical Association*, vol. 270, no. 23, pp. 2848-2854.

Worwood, M. 1994, "Laboratory determination of iron status" in *Iron metabolism in health and disease*, eds. J.H. Brock, J.W. Halliday, M.J. Pippard & L.W. Powell, Saunders, London, pp. 449–476.

Wrede, C.E., Buettner, R., Bollheimer, L.C., Scholmerich, J., Palitzsch, K.D. & Hellerbrand, C. 2006, "Association between serum ferritin and the insulin resistance syndrome in a representative population", *European Journal of Endocrinology*, vol. 154, no. 2, pp. 333-340.

Wrighting, D.M. & Andrews, N.C. 2006, "Interleukin induces hepcidin expression through STAT3", *Blood*, vol. 108, no. 9, pp. 3204-3209.

Yamaji, S., Sharp, P., Ramesh, B. & Srai, S.K. 2004, "Inhibition of iron transport across human intestinal epithelial cells by hepcidin", *Blood*, vol. 104, no. 7, pp. 2178-2180.

Yang, F., Lum, J.B. & McGill, J.R. 1984, "Human transferrin: cDNA characterization and chromosomal localization", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 9 I, pp. 2752-2756.

Yanoff, L.B., Menzie, C.M., Denkinger, B., Sebring, N.G., McHugh, T., Remaley, A.T. & Yanovski, J.A. 2007, "Inflammation and iron deficiency in the hypoferremia of obesity", *International journal of obesity*, vol. 31, no. 9, pp. 1412-1419.

Yip, R. 2001, "Iron" in *Present knowledge in nutrition*, eds. B. Bowman & R.M. Russell, 8th edn, ILSI Press, Washington DC, pp. 311-318.

Yip, R. 2000, "Significance of an abnormally low or high hemoglobin concentration during pregnancy: Special consideration of iron nutrition", *American Journal of Clinical Nutrition*, vol. 72, no. 1 SUPPL., pp. 272S-279S.

Yip, R. 1997, "The challenge of improving iron nutrition: Limitations and potentials of major intervention approaches", *European journal of clinical nutrition,* vol. 51, no. SUPPL. 4, pp. S16-S24.

Yip, R., Schwartz, S. & Deinard, A.S. 1983, "Screening for iron deficiency with the erythrocyte protoporphyrin test", *Pediatrics,* vol. 72, no. 2, pp. 214-219.

Yun, S., Kabeer, N.H., Zhu, B.P. & Brownson, R.C. 2007, "Modifiable risk factors for developing diabetes among women with previous gestational diabetes.", *Preventing chronic disease,* vol. 4, no. 1.

Zafon, C., Lecube, A. & Simó, R. 2010, "Iron in obesity. An ancient micronutrient for a modern disease", *Obesity Reviews,* vol. 11, no. 4, pp. 322-328.

Zandman-Goddard, G. & Shoenfeld, Y. 2007, "Ferritin in autoimmune diseases", *Autoimmunity Reviews,* vol. 6, no. 7, pp. 457-463.

Zhang, F., Basinski, M.B., Beals, J.M., Briggs, S.L., Churgay, L.M., Clawson, D.K., DiMarchi, R.D., Furman, T.C., Hale, J.E., Hsiung, H.M., Schoner, B.E., Smith, D.P., Zhang, X.Y., Wery, J.-. & Schevitz, R.W. 1997, "Crystal structure of the obese protein leptin-E100", *Nature,* vol. 387, no. 6629, pp. 206-209.

Zhang, J., Lewis, R.M., Wang, C., Hales, N. & Byrne, C.D. 2005, "Maternal dietary iron restriction modulates hepatic lipid metabolism in the fetuses", *American Journal of Physiology - Regulatory Integrative and Comparative Physiology,* vol. 288, no. 1 57-1, pp. R104-R111.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J.M. 1994, "Positional cloning of the mouse obese gene and its human homologue", *Nature,* vol. 372, no. 6505, pp. 425-432.

Zhou, L.-., Yang, W.-., Hua, J.-., Deng, C.-., Tao, X. & Stoltzfus, R.J. 1998, "Relation of hemoglobin measured at different times in pregnancy to preterm birth and low birth weight in Shanghai, China", *American Journal of Epidemiology,* vol. 148, no. 10, pp. 998-1006.

Zimmermann, M.B., Zeder, C., Muthayya, S., Winichagoon, P., Chaouki, N., Aeberli, I. & Hurrell, R.F. 2008, "Adiposity in women and children from transition countries predicts decreased iron absorption, iron deficiency and a reduced response to iron fortification", *International journal of obesity,* vol. 32, no. 7, pp. 1098-1104.

Zimmermann, M.B. 2008, "Methods to assess iron and iodine status", *British Journal of Nutrition,* vol. 99, pp. S2-S9.