Haemoconcentration risk at the end of pregnancy: effects on neonatal behaviour

Nuria Aranda[1,4],†, Carmen Hernández-Martínez[2,4],†, Victoria Arija[1,3,4], Blanca Ribot[1,4] and Josefa Canals[2,4],*

[1]Nutrition and Public Health Unit, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Reus, Spain: [2]Department of Psychology, Faculty of Educational Sciences and Psychology, Universitat Rovira i Virgili, Ctra. Valls s/n, 43007 Tarragona, Spain: [3]Unitat de Suport a la Recerca Tarragona–Reus, Institut Universitari d'Investigació en Atenció Primària Jordi Gol, Tarragona, Spain: [4]Research Group in Nutrition and Mental Health (NUTRISAM), Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili, Reus, Spain

Abstract

Objective: To determine the associations between haemoconcentration at the end of pregnancy (third trimester and delivery) and neonatal behaviour in healthy pregnant women supplemented with moderate doses of Fe.
Design: A prospective longitudinal study in which obstetric and clinical history, maternal toxic habits, maternal anxiety and Hb levels were recorded at the third trimester and delivery. Neonatal behaviour was assessed at 48–72 h of age using the Neonatal Behavioral Assessment Scale.
Setting: Unit of Obstetrics and Gynaecology of the Sant Joan University Hospital in Reus, Tarragona (Spain).
Subjects: A total of 210 healthy and well-nourished pregnant women and their full-term, normal-weight newborns.
Results: The results showed that, after adjusting for confounders, in the third trimester the risk of haemoconcentration (6·2 % of pregnant women) was related to decreased neonatal state regulation ($B = -1·273$, $P = 0·006$) and alertness ($B = -1·848$, $P = 0·006$) scores. In addition, the risk of haemoconcentration at delivery (12·0 % of pregnant women) was also related to decreased neonatal state regulation ($B = -0·796$, $P = 0·021$) and poor robustness and endurance ($B = -0·921$, $P = 0·005$) scores.
Conclusions: Our results show that the risk of haemoconcentration at the end of pregnancy is related to the neonate's neurodevelopment (and self-regulation capabilities), suggesting that Fe supplementation patterns and maternal Fe status during pregnancy are important factors for neurodevelopment which may be carefully controlled.

In recent years, various studies have investigated prenatal Fe status and its effects on maternal and fetal health. It is well established that Fe deficiency (ID) is highly prevalent during pregnancy (18–40 %) in both developing and developed countries[1] and is related to adverse obstetric outcomes such as low birth weight, preterm delivery[2–6] and behavioural, emotional and cognitive outcomes throughout infant development[7–10]. This is because, during fetal development, Fe is essential for Hb synthesis and is an important factor in neuronal synapse formation, myelination and the synthesis of certain neurotransmitters in the central nervous system (CNS)[11].

To prevent ID during pregnancy, several countries have launched generalized and routine Fe supplementation programmes. Most of these are generally beneficial to maternal–fetal health but, in some cases, they may contribute to increased Fe and Hb levels and thus lead to haemoconcentration[12]. Because this condition is less prevalent (13·3 %)[13,14], its effects are less studied.

High ferritin levels have been related to oxidative stress[2,15–18] and to reduced absorption of other important minerals[19], and haemoconcentration has been related to increased blood viscosity and blood pressure[20], which impedes the placental–fetal blood flow and interferes with the fetal supply of oxygen and nutrients[21,22]. All of these conditions may interfere during the course of pregnancy with the general development of the fetus and the development of its CNS. In fact, high Fe levels and haemoconcentration have been related to premature birth and low birth weight[13,14,20,21,23–25] but there are few

---

studies that have related these conditions with neurodevelopmental outcomes. The CNS is the most complex and vulnerable organ in the human body and its development is also correspondingly an extremely complex and vulnerable period[26]. This process is largely dependent on the appropriate release of oxygen, protein, energy and micronutrients, and an inadequate supply of these may contribute to alterations in neurodevelopment[27]. Tamura *et al.*[28] studied the relationship between cord ferritin concentrations and cognitive development and found that infants with cord ferritin levels in the highest quartile showed lower IQ (intelligence quotient) scores at 5 years old. Those authors attributed these results to possible maternal infections or neuronal damage caused by oxidative stress resulting from Fe overload. In relation to the amount of Fe supplementation, the study by Hanieh *et al.*[29] compared a daily Fe supplementation pattern with a twice weekly Fe supplementation pattern and found that children of mothers with a daily Fe supplementation pattern scored lower in a cognitive test at 6 months old; in fact, 40 % of women with this pattern of supplementation were found to have ferritin levels greater than 41 µg/l and normal Hb concentrations, results that were attributed to oxidative stress processes related to high Fe levels. There are certain aspects of these studies that deserve special attention. First, their main aims were to examine the effects on different levels of ID (serum cord ferritin, Hb concentrations), which meant that they paid little attention to results related to elevated ferritin or high Hb concentrations. Second, cognitive development was assessed at 4 months and 4–6 years old, ages at which other multiple factors can also affect infant cognitive development, including maternal education and psychopathology, obstetric conditions, mother–infant attachment, infant breast-feeding, educational programmes, genetic factors, nutritional status, etc.[30–35]. Third, the infant's own Fe status also affects her/his cognitive development[36] and this variable was not taken into account. Consequently, to study the long-term effects of pregnancy Hb concentrations, several variables must be taken into account. However, neonatal behavioural characteristics shortly after birth are not influenced by these longer-term postnatal variables, although prenatal conditions must be also taken into account. Several studies have shown that prenatal maternal toxic habits, socio-economic status and anxiety, among other factors, are related to neonatal behaviour[30,31,33,37], suggesting that relationships must be adjusted for them. To our knowledge, to date there are no data regarding the potentially harmful effect of high Hb levels on neurobehavioural development in newborns.

Another important issue is the moment during gestation at which these harmful conditions occur. As Georgieff suggested[38], the effect of any nutrient deficiency or overabundance on brain development will be governed by the principle of timing, dose and duration. In this regard, Hernández-Martínez *et al.*[10] found that the effects of ID on CNS development varied depending on the trimester of pregnancy in which the ID occurred and depending on the level of ID. Although late pregnancy seems to be a vulnerable period for nutritional insults[8,39,40], there are no studies that investigate whether the effect of elevated levels of Fe on neurodevelopment is different depending on the point during pregnancy when this condition occurs.

Given all this information, it is clear that further studies are needed on the possible effects of high Hb concentrations in pregnant women on newborn neurodevelopment. Consequently, the main aim of the present study was to assess the effects of haemoconcentration at the end of pregnancy on neonatal behaviour after controlling for confounding factors. We hypothesized that high Hb concentrations at the end of pregnancy will be related to poor neonatal development.

Methods

Sample

Participants were pregnant women followed from the first trimester of gestation to childbirth. These women were volunteers and the Unit of Obstetrics and Gynaecology of the Sant Joan University Hospital in Reus (Spain) carried the recruitment out over 4 years (from 2004 to 2008).

The inclusion criteria for the study were being a healthy pregnant Caucasian woman older than 18 years with a singleton pregnancy at gestational week ≤11. Exclusion criteria applied across the duration of the study were: having a chronic illness or a possible inflammation (serum ferritin (SF) >62 µg/l and transferrin saturation <16 %)[41,42]; having a multiple pregnancy; not meeting the prescribed Fe supplementation during pregnancy; not complying with the scheduled clinical visits; not having blood drawn at each clinical visit; and giving birth at another hospital.

A total of 299 well-nourished Spanish pregnant women were recruited, of which 27·8 % were excluded for fetal loss, having incomplete biochemical parameters or having gone to another hospital to give birth. In addition, of the 216 women, six had a possible inflammation that could alter biochemical parameters and were excluded. Hence, the final sample was composed of 210 mothers aged from 18 to 43 years and their neonates (47·6 % boys and 52·4 % girls). There were no significant differences in psychosocial and sociodemographic characteristics between the excluded and the included women. The statistical power of the results in terms of sample size, standard deviation from the Neonatal Behavioral Assessment Scale (NBAS) scores and the number of women with and without haemoconcentration risk at the end of pregnancy and partum is 87 %.

Procedure and study design

The present longitudinal study was conducted according to the guidelines laid down in the Declaration of Helsinki

and all procedures involving pregnant women were approved by the Ethics Committee of the Sant Joan University Hospital in Reus. Women admitted to the study were recruited during their first prenatal care visit (at gestational week ≤11) by gynaecologists from the Sant Joan University Hospital in Reus and written informed consent was obtained from all of them.

Study data were recorded during their pregnancy consultations. At week 10, consultation, clinical and obstetric histories were recorded, and blood samples were collected for laboratory analysis. At week 15, the obstetrician pre-scribed 40 mg Fe/d in accordance with the study protocol and the guidelines of the Spanish Ministry of Health and Consumption[43]. This dose was adapted in the following clinical visits according to maternal Hb and SF levels. At 24 and 34 weeks, the clinical and obstetric histories were revised, blood samples were collected, and a member of research team (in a separate room to encourage true responses) carried out a semi-structured interview on adherence to the Fe supplementation. At delivery, blood samples were collected and obstetric and neonatal variables were recorded (gestational age, neonatal birth weight and gender). In the immediate postpartum period (between 48 and 72 h after delivery) retrospective maternal anxiety and neonatal behaviour were assessed by two trained examiners (κ accordance 0·9) in a room at the hospital with optimal conditions. The examination of the newborn was completed in 20–25 min and was carried out midway between feedings. Not all newborns could be scored on the habituation items because they did not achieve the optimal state of con-sciousness (state 1, 2 or 3).

Measures

Hb concentrations in the blood were measured immedi-ately at the clinical visits using a Coulter GENS analyser (Coulter, Hialeah, FL, USA). Serum and plasma were stored at −80°C in our biobank and thawed immediately prior to batch analyses to reduce inter-batch variation in analysis. SF was determined by turbidimetric immuno-assay as described by Gomez et al.[44]. According to the WHO recommendations[45,46], Hb values were codified into three categories: (a) anaemia (Hb < 105 g/l in the second trimester and <110 g/l in the third trimester and at delivery, and depleted Fe stores as SF < 12 µg/l); (ii) nor-mal (Hb between 105 and 130 g/l in the second trimester and between 110 and 130 g/l in the third trimester); and (iii) risk of haemoconcentration (Hb > 130 g/l)[24].

Neonatal behaviour was assessed at 48–72 h of age using the Spanish translation of the NBAS[47]. The NBAS assesses the behaviour of the newborn within the dynamic context of the infant–caregiver relationship. The NBAS contains twenty-eight behavioural items scored for optimal performance on a 9-point scale (except for one item, 'smiles', for which the scoring criterion is to record the number of times a smile is observed during the exam); seven supplementary items, also scored on a 9-point

scale; and eighteen reflex items that are designed to identify gross neurological abnormalities and which are scored on a 4-point scale. Behavioural items are classified into six clusters: (i) habituation, which evaluates the neonate's ability to respond to and inhibit discrete stimuli while asleep (four items); (ii) social-interactive, which evaluates the neonate's ability to attend to visual and auditory stimuli and the quality of overall alertness (seven items); (iii) the motor system, which evaluates neonate motor performance, quality of movement and muscular tone (five items); (iv) state organization, which evaluates infant arousal irritability and lability of states (four items); (v) state regulation, which evaluates the neonate's ability to regulate her/his state when faced with increasing levels of stimulation (four items); and (vi) the autonomic system, which records signs of stress related to homeostatic adjustments in the CNS (four items, 'smiles' included). The following supplementary items assess several aspects of overall infant behaviour during assessment: quality of alertness (assesses the infant attentional state), cost of attention (assesses the effort that the infant makes to keep an optimal state of alertness), examiner facilitation (assesses the effort that the examiner makes to help the infant to keep a good state of alertness), general irritability (assesses the infant reactions of irritability), robustness and endurance (assesses the general physical resistance), state regulation (assesses the infant general self-regulation capabilities) and examiner's emotional response (asses-ses the emotional response that the examiner feels during the examination).

Maternal anxiety was assessed using the Spanish ver-sion of the State Trait Anxiety Inventory (STAI)[48]. This self-report questionnaire contains forty items that assess state anxiety, which is the level of transient and situational anxiety; and trait anxiety, the level of dispositional and stable trait anxiety. For the present study, we used trait anxiety as a confounding variable.

The socio-economic level of the family was assessed using the Hollingshead Index[49]. This index estimates the individual's social status by grouping occupations into nine categories (from unskilled to highly skilled work) and by grouping the level of education into seven categories (from non-completed primary education to completed higher education). The status score is estimated by multiplying the occupation-scale value by 5 and the education-scale value by 3 and then combining the two scores.

Statistical analysis

All statistical analyses were carried out using the statistical software package IBM SPSS Statistics Version 22.0.

All variables were checked for normality of distribution. All variables were normally distributed, and are presented as percentages or as means and standard deviations.

The Bonferroni correction was applied to control for the increase in type I error due to multiple comparisons; the significance level was 0·025.

ANOVA was performed to test the differences in neonatal behaviour between the different groups of Hb levels and a Bonferroni *post hoc* analysis was applied.

To study if the risk of haemoconcentration is a significant predictor of neonatal behaviour, unadjusted and adjusted multiple linear regression models were performed for each NBAS cluster and supplementary item, and for the third trimester and delivery. Prior to these analyses, collinearity between variables was checked and the collinear variables (maternal SF level, umbilical cord blood ferritin and gestational age at birth) were deleted from the analysis. The Hb level variable was transformed into two dummy variables (dummy 1: anaemia *v.* normal Hb levels; dummy 2: haemoconcentration risk *v.* normal Hb levels) for entry in the multiple linear regression. In the unadjusted multiple linear regression models, the entry method was used to introduce the Hb level variable, and in the adjusted models, the entry method was used to enter the Hb level variable and the stepwise method to enter the confounding variables. The candidate variables for entry in the models were: infant birth weight at delivery (grams); socio-economic status of the family (total score); prenatal tobacco smoke exposure (no, yes); infant gender (female, male); and prenatal anxiety maternal trait (total score).

Results

Descriptive data of the sample
The sample characteristics are shown in Table 1. In general, the socio-economic status of the families was medium–high (92 %) and the level of trait anxiety was moderate (mean 15·4 (SD 7·9)) in comparison to Spanish data. In relation to neonatal variables, the mean gestational age was 39·3 (SD 1·4) weeks and the mean birth weight was 3231·2 (SD 403·7) g.

All the mothers included in the analysis systematically received Fe supplements from about week 15 and the mean pharmacological Fe supplementation was 43·9 (SD 26·6) mg/d. The prevalence of haemoconcentration risk in the third trimester was 6·2 % and at delivery was 12·0 %. The prevalence of anaemia and low SF levels increased in the second and third trimesters. At delivery, 53·4 and 21·5 % of women had low Fe stores and anaemia, respectively.

Neonatal behaviour differences between Hb level groups at different stages of pregnancy
Table 2 shows differences in NBAS clusters between groups of Hb levels in the third trimester of pregnancy and at delivery. The first and second trimesters were not included in the analyses due to the low number of women in the haemoconcentration risk group.

Children of mothers with higher levels of Hb (>130 g/l) in the third trimester scored significantly lower in the state

Table 1 Characteristics of the sample of healthy and well-nourished pregnant women and their full-term, normal-weight newborns (*n* 210), Reus, Spain, 2004–2008

	Mean or %	SD or *n*
Mothers		
Age (years; mean and SD)	31·7	4·3
Socio-economic status of the family (% and *n*)		
Low	8·0	17
Medium	47·5	100
High	44·5	93
Smoker† (% and *n*)	20·0	42
Fe dose of supplementation (mg/d; mean and SD)	43·9	26·6
Trait anxiety score (mean and SD)	15·4	7·9
Hb level (g/l; mean and SD)		
At week 10	125·7	8·0
At week 24	113·1	8·6
At week 34	114·2	9·6
At delivery	116·9	10·9
Anaemia (% and *n*)		
At week 10 (Hb < 110 g/l)	2·9	6
At week 24 (Hb < 105 g/l)	14·9	31
At week 34 (Hb < 110 g/l)	28·2	59
At delivery (Hb < 110 g/l)	21·5	45
Hb > 130 g/l (% and *n*)		
At week 10	28·4	59
At week 24	1·4	3
At week 34	6·2	13
At delivery	12·0	25
Newborns		
Gender of newborn is male (% and *n*)	47·6	100
Gestational age (weeks; mean and SD)	39·3	1·4
Birth weight (g; mean and SD)	3231·2	403·7
Umbilical-cord ferritin (μg/l; mean and SD)	125·2	1·8

†Active or second-hand smoker.

regulation cluster (mean 4·6 (SD 2·0)) than children of women with normal levels (mean 5·8 (SD 1·5); *P* = 0·020). No significant differences were found between these conditions at birth in the bivariate analysis.

Prediction of neonatal behaviour by levels of Hb
Unadjusted and adjusted multiple linear regression models were performed to assess the effect of haemoconcentration risk during pregnancy on neonatal behaviour (Table 3). In the third trimester, the presence of risk of haemoconcentration was related to a decreased score in the state regulation cluster (*B* = − 1·273, *P* = 0·006) and to a lower alert quality score (*B* = − 1·848, *P* = 0·006). At delivery, the risk of haemoconcentration was also related to a decreased score in the state regulation cluster (*B* = − 0·796, *P* = 0·021) and a poor robustness and endurance supplementary item score (*B* = − 0·921, *P* = 0·005). No other variables in the analysis were significant.

Discussion

We carried out a longitudinal study of a population of well-nourished pregnant women to analyse the effect of

Table 2 Descriptive data of neonatal behaviour depending on Hb levels of mothers at the end of pregnancy in the sample of healthy and well-nourished pregnant women and their full-term, normal-weight newborns (*n* 210), Reus, Spain, 2004–2008

	Third trimester						Delivery					
	Anaemia (Hb < 110 g/l) (*n* 59)		Normal Hb (Hb = 110–130 g/l) (*n* 138)		Haemoconcentration risk (Hb > 130 g/l) (*n* 13)		Anaemia (Hb < 110 g/l) (*n* 45)		Normal Hb (Hb = 110–130 g/l) (*n* 140)		Haemoconcentration risk (Hb > 130 g/l) (*n* 25)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NBAS clusters												
Habituation	7·8	1·2	7·5	1·5	7·6	1·1	7·4	1·7	7·7	1·2	7·3	1·6
Orientation	5·6	1·6	5·8	1·6	5·2	1·1	5·8	1·6	5·8	1·6	5·4	2·2
Motor	5·4	0·8	5·3	1·0	5·1	0·8	5·3	0·9	5·3	0·9	5·3	1·0
State organization	3·6	1·1	3·8	0·9	3·9	0·8	3·9	0·9	3·7	1·0	3·9	0·6
State regulation*	5·8	1·4	5·8	1·5	4·6	2·0	5·9	1·4	5·8	1·5	5·1	1·7
Autonomic stability	7·2	0·9	7·1	1·1	6·9	1·4	7·3	0·8	7·1	1·1	7·1	1·2
NBAS supplementary items												
Alertness	5·2	2·3	5·4	2·2	3·9	2·9	5·4	2·3	5·3	2·3	4·6	2·6
Effort to keep attention	5·8	2·1	5·7	1·8	4·5	1·8	6·0	1·8	5·7	1·9	4·9	2·0
Examiner facilitation	5·4	2·2	5·5	2·1	4·5	2·7	5·3	2·1	5·5	2·1	5·0	2·5
General irritability	5·6	2·5	5·7	2·2	4·3	2·7	5·8	2·3	5·7	2·3	4·6	2·3
Robustness endurance	5·8	2·4	5·6	2·3	4·3	2·6	5·6	2·6	5·7	2·2	4·8	2·5
State regulation	6·1	2·5	6·2	2·1	5·1	2·8	6·2	2·3	6·2	2·2	5·8	2·4
Examiner's emotional response	6·1	2·3	6·1	2·1	5·1	2·4	6·1	2·1	6·0	2·2	5·7	2·3

NBAS, Neonatal Behavioral Assessment Scale.
*Significant *P* values from Bonferroni *post hoc* analysis: normal Hb group *v.* haemoconcentration risk group (*P* = 0·020).

haemoconcentration at the end of pregnancy on neonatal behaviour. Our results show that, as we hypothesized, increased maternal Hb concentration at the end of the pregnancy is related to poor state regulation behaviour, alertness, robustness and endurance in the neonate.

Previous studies have described relationships between haemoconcentration during pregnancy and the risk of low birth weight, premature delivery and fetal loss[13,21,22,25] and our results show for the first time relationships between pregnancy haemoconcentration and neonatal behaviour as a sign of fetal neurodevelopment.

The healthy pregnant women who participated in the study were Caucasian, with a medium–high socio-economic level, no known previous obstetric pathology, and typical general and obstetric characteristics in terms of gestational age and previous deliveries. In addition, the women took a moderate Fe supplementation with a mean of 43·9 mg/d in accordance with the study protocol and the guidelines of the Spanish Ministry of Health and Consumption[43]. Fe supplementation is recommended by international organizations in moderate doses (<60 mg/d)[50] although these moderates doses could be excessive in women who start pregnancy with good Fe stores and with alterations in the haemochromatosis gene (*HFE*) that increase the intestinal absorption of Fe[13,51]. Mutations in the *HFE* gene are common in our population, where 47 % of women carry one of the three major mutations[52].

To define groups of Hb levels and depletion of Fe stores we used the limits established by international organizations[45]. SF is considered the best biochemical parameter for monitoring a deficient Fe status in pregnancy in the absence of infection or inflammation because it correctly identifies the women without Fe stores[53]. However, a known limitation of SF is that it also increases with acute or chronic inflammation, malignancy or liver disease. As transferrin saturation does not increase in the presence of inflammation, we used both parameters to detect inconsistent values (high SF and low transferrin saturation) that may hide a possible inflammation with ID, as suggested by some authors[42], and we excluded six women in this situation from the analysis. To define haemoconcentration, we considered high levels of Hb without taking into account the total plasma volume. This is a limitation because the total plasma volume helps to differentiate the haemodilution status of normal pregnancy from haemoconcentration. However, measuring blood viscosity is laborious and expensive, and therefore, in everyday clinical practice and in many studies, Hb concentration is reported as a measure of haemoconcentration because its relationship with blood viscosity is linear when haematocrit is <0·5, which is usually the case in pregnancy[54]. Considering all this, we were not able to state that the women with Hb > 130 g/l had haemoconcentration; therefore, we defined them as being at risk of haemoconcentration[24]. Our results show that as pregnancy progresses, the percentage of women with depleted Fe stores and anaemia increases. Furthermore, 6·2 % of pregnant women are at risk of haemoconcentration in the third trimester and 12·0 % at delivery. These data are similar to those published by the WHO in its global database on anaemia[1] and also to the results from other European studies[21,25,55], confirming that ID is elevated

Table 3 Prediction of neonatal behaviour for high levels of Hb at the end of pregnancy in the sample of healthy and well-nourished pregnant women and their full-term, normal-weight newborns (*n* 210), Reus, Spain, 2004–2008

	Independent variables	*B*	*P* value	Model parameters
Third trimester				
State regulation cluster	Unadjusted model			$R^2_{c \times 100} = 3{\cdot}6$; $F_{2,203} = 3{\cdot}8$; $P = 0{\cdot}024$
	Hb levels			
	Normal *v.* haemoconcentration risk	−1·186	0·007	
	Normal *v.* anaemia	−0·023	0·924	
	Adjusted model			$R^2_{c \times 100} = 5{\cdot}0$; $F_{2,189} = 3{\cdot}8$; $P = 0{\cdot}018$
	Hb levels			
	Normal *v.* haemoconcentration risk	−1·273	0·006	
	Normal *v.* anaemia	−0·061	0·809	
Alert quality	Unadjusted model			$R^2_{c \times 100} = 2{\cdot}7$; $F_{2,203} = 2{\cdot}6$; $P = 0{\cdot}075$
	Hb levels			
	Normal *v.* haemoconcentration risk	−1·475	0·020	
	Normal *v.* anaemia	−0·317	0·406	
	Adjusted model			$R^2_{c \times 100} = 5{\cdot}3$; $F_{3,189} = 3{\cdot}7$; $P = 0{\cdot}020$
	Hb levels			
	Normal *v.* haemoconcentration risk	−1·848	0·006	
	Normal *v.* anaemia	−0·376	0·320	
	Weight of the newborn (g)	0·001	0·027	
Delivery				
State regulation cluster	Unadjusted model			$R^2_{c \times 100} = 2{\cdot}9$; $F_{2,203} = 3{\cdot}0$; $P = 0{\cdot}052$
	Hb levels			
	Normal *v.* haemoconcentration risk	−0·748	0·023	
	Normal *v.* anaemia	−0·048	0·683	
	Adjusted model			$R^2_{c \times 100} = 3{\cdot}4$; $F_{2,189} = 2{\cdot}7$; $P = 0{\cdot}019$
	Hb levels			
	Normal *v.* haemoconcentration risk	−0·796	0·021	
	Normal *v.* anaemia	−0·079	0·804	
Robustness and endurance	Unadjusted model			$R^2_{c \times 100} = 3{\cdot}5$; $F_{2,203} = 2{\cdot}8$; $P = 0{\cdot}062$
	Hb levels			
	Normal *v.* haemoconcentration risk	−1·153	0·023	
	Normal *v.* anaemia	−0·667	0·172	
	Adjusted model			$R^2_{c \times 100} = 9{\cdot}2$; $F_{3,189} = 5{\cdot}2$; $P = 0{\cdot}002$
	Hb levels			
	Normal *v.* haemoconcentration risk	−0·921	0·005	
	Normal *v.* anaemia	−0·492	0·304	
	Weight of the newborn (g)	0·001	0·002	

Candidate variables in the adjusted models: infant birth weight at delivery (grams); socio-economic status of the family; prenatal exposure to tobacco smoke (no, yes); infant gender (female, male); and maternal prenatal trait anxiety.

even in industrialized countries and that a high percentage of women are at risk of haemoconcentration.

Neonatal behaviour is a phenotypical manifestation of the CNS development and is biologically determined by an interaction between genetics and environmental intra-uterine variables[56]. In addition to prenatal nutritional status, other environmental variables can affect prenatal neurodevelopment such as maternal prenatal infections, prenatal toxic habits, prenatal emotional states, etc. and the NBAS is a good and sensitive instrument for detecting differences between exposed groups[9,30,31,33,37,57–60]. These and other variables affect prenatal neurodevelopment by interacting and even modifying gene expression[26]. Therefore, our analysis has taken these aspects into account to disentangle the compound variables that can affect neurodevelopment. Our results show that, after controlling for confounding variables, the risk of haemoconcentration in the third trimester and at delivery is related to poor state regulation cluster scores and infant alert quality and robustness and endurance; that is to say, in general, these infants show more problems with

self-regulation, being more excitable and showing more irritability during the examination, and the examiner perceives them to be weaker and to have more attention problems. These results suggest that the third trimester of pregnancy is a critical period for neurodevelopment, when the CNS is more vulnerable to environmental insults possibly due to the speed of several neurological changes, including synapse formation and myelination, and the rapid increase in brain volume (up to 260 % in the third trimester and more than double in the first year of life)[8,39,40].

The poor alertness and self-regulation behaviours shown by infants prenatally exposed to high Hb concentration levels may indicate that they are more prone to developing the cognitive problems observed by several authors[28,29,61]. In this regard, previous studies have shown that lower scores in the NBAS clusters that assess infant irritability were good predictors of verbal and total IQ at 6 years old[62] and infant psychopathological symptoms[63]. The neurobehavioural characteristics shown by neonates of mothers with high Hb concentrations may be explained in several ways. One explanation

is that high Hb concentrations are related to high blood viscosity which can directly reduce the perfusion and oxygenation of the placental and fetal tissues by reducing the blood flow and the efficiency of the nutrient exchange processes, thus decreasing the oxygen and nutrients available to the fetus[20–22]. Furthermore, the reduction in nutrient transfer can reduce the availability of the substrate substances needed by the placental hormones that stimulate fetal growth[64] and can increase plasma cortisol in response to hypoxia[65]. Other authors suggest that an alternative explanation for the inverse association between Hb levels and fetal growth (and probably fetal CNS development) may be provided by the direct role of Hb in nitric oxide regulation and endothelial function. Hb binds and inactivates nitric oxide (by endothelium-derived smooth-muscle relaxation factor), which leads to vaso-constriction and consequently hypertension and placental ischaemia[66]. A recent study shows a direct link between reduced cerebral oxygenation and impaired brain growth in fetuses with congenital heart disease and raises the possibility that *in utero* brain development could be improved with maternal oxygen therapy[67]. On the other hand, among the causes of haemoconcentration are excessive Fe supplementation and pregnancy is a state where there is an increased propensity to produce free radicals, particularly in the presence of Fe[12], which suggests that both Fe deficiency and excess could produce excessive mitochondrial damage and oxidative stress[68] that could also affect the development of the fetus and its CNS[67]. Several authors have observed the negative influence of high ferritin levels during pregnancy on maternal and infant health[2,16–18,24,69,70] and cognitive development[28], both of which could be due to increased oxidative stress caused by excess Fe. In this regard, oligodendrocyte injury in the developing brain[71] has been related to prenatal oxidative stress and an excessive generation of free radicals in the intra-uterine environment, which increases the risk of neurodevelopment disorders such as schizophrenia in adulthood[57,67]. Fe is essential for Hb synthesis and is important to the CNS in that it contributes to neuronal synapse formation, myelination and the synthesis of certain neurotransmitters[11]. However, as in other studies[25,72], we did not observe a negative effect from anaemia. It is likely that the physiological haemodilution that normally occurs in pregnancy can mask these results and that the Hb cut-off of <110 g/l for anaemia in pregnancy endorsed by the WHO is set too high for the present study population[69].

Our study has some strengths and limitations. While the prospective longitudinal data are methodologically strong, our final sample is modest, and we cannot rule out the possibility of residual confounding. We have included several confounders in the analysis, but it is possible that other confounders affect the relationships studied.

Taking all this information into account, our data allow us to conclude that high levels of Hb at the end of pregnancy are related to neonatal problems in self-regulation (excitability and irritability), alertness and with a poor robustness in terms of the neonatal behavioural development indicators measured. We suggest and support the practice of tailoring the pattern of Fe supplementation during pregnancy to the individual character-istics of each pregnant woman in order to improve fetal development.

References

1. World Health Organization (2008) *Worldwide Prevalence of Anaemia 1993–2005. WHO Global Database on Anaemia.* Geneva: WHO.
2. Aranda N, Ribot B, Garcia E *et al.* (2011) Pre-pregnancy iron reserves, iron supplementation during pregnancy, and birth weight. *Early Hum Dev* **87**, 791–797.
3. Rasmussen K (2001) Is there a causal relationship between iron deficiency or iron-deficiency anemia and weight at birth, length of gestation and perinatal mortality? *J Nutr* **131**, 2S-2, 590S–601S.
4. Ribot B, Aranda N, Viteri F *et al.* (2012) Depleted iron stores without anaemia early in pregnancy carries increased risk of lower birthweight even when supplemented daily with moderate iron. *Hum Reprod* **27**, 1260–1266.
5. Allen LH (1993) Iron deficiency anemia increases risk of preterm delivery. *Nutr Rev* **51**, 49–52.
6. Haider BA, Olofin I, Wang M *et al.* (2013) Anaemia, prenatal iron use, and risk of adverse pregnancy outcomes: systematic review and meta-analysis. *BMJ* **346**, f3443.
7. Oyemade UJ, Cole OJ, Johnson AA *et al.* (1994) Prenatal predictors of performance on the Brazelton neonatal behavioural assessment scale. *J Nutr* **124**, 6 Suppl, 1000S–1005S.

8. Thompson RA & Nelson CA (2001) Developmental science and the media: early brain development. *Am Psychol* **56**, 5–15.

9. Cucó G, Fernandez-Ballart J, Arija V *et al.* (2005) Effect of B$_1$-, B$_6$- and iron intake during pregnancy on neonatal behavior. *Int J Vitam Nutr Res* **75**, 320–326.

10. Hernández-Martínez C, Canals J, Aranda N *et al.* (2011) Effects of iron deficiency on neonatal behavior at different stages of pregnancy. *Early Hum Dev* **87**, 165–169.

11. Georgieff MK & Rao R (2001) The role of nutrition in cognitive development. In *Handbook in Developmental Cognitive Neuroscience*, pp. 491–504 [CA Nelson and M Luciana, editors]. Cambridge, MA: MIT Press.

12. Casanueva E & Viteri FE (2003) Iron and oxidative stress in pregnancy. *J Nutr* **133**, 5 Suppl. 2, 1700S–1708S.

13. Aranda N, Ribot B, Viteri F *et al.* (2013) Predictors of haemoconcentration at delivery: association with low birth weight. *Eur J Nutr* **52**, 1631–1639.

14. Arija V, Ribot B & Aranda N (2013) Prevalence of iron deficiency states and risk of haemoconcentration during pregnancy according to initial iron stores and iron supplementation. *Public Health Nutr* **16**, 1371–1378.

15. Aisen P, Cohen G & Kang JO (1990) Iron toxicosis. *Int Rev Exp Pathol* **31**, 1–46.

16. Casanueva E, Viteri F, Mares-Galindo M *et al.* (2006) Weekly iron as a safe alternative to daily supplementation for non-anemic pregnant women. *Arch Med Res* **37**, 674–682.

17. Papadopoulou E, Stratakis N, Roumeliotaki T *et al.* (2013) The effect of high doses of folic acid and iron supplementation in early-to-mid pregnancy on prematurity and fetal growth retardation: the mother–child cohort study in Crete, Greece (Rhea study). *Eur J Nutr* **52**, 326–336.

18. Khambalia AZ, Aimone A, Nagubandi P *et al.* (2016) High maternal iron status, dietary iron intake and iron supplement use in pregnancy and risk of gestational diabetes mellitus: a prospective study and systematic review. *Diabet Med* **33**, 1211–1221.

19. Sandstrom B (2001) Micronutrient interactions: effects on absorption and bioavailability. *Br J Nutr* **85**, Suppl. 2, S181–S185.

20. Ziaei S, Norrozi M, Faghihzadeh S *et al.* (2007) A randomised placebo-controlled trial to determine the effect of iron supplementation on pregnancy outcome in pregnant women with haemoglobin ≥13.2 g/dl. *BJOG* **114**, 684–688.

21. Gaillard R, Eilers PH, Yassine S *et al.* (2014) Risk factors and consequences of maternal anaemia and elevated haemoglobin levels during pregnancy: a population-based prospective cohort study. *Paediatr Perinat Epidemiol* **28**, 213–226.

22. Cordina M, Bhatti S, Fernandez M *et al.* (2015) Association between maternal haemoglobin at 27–29 weeks gestation and intrauterine growth restriction. *Pregnancy Hypertens* **5**, 339–345.

23. Steer P, Alam MA, Wadsworth J *et al.* (1995) Relation between maternal haemoglobin concentration and birth weight in different ethnic groups. *BMJ* **310**, 489–491.

24. Peña-Rosas J & Viteri F (2009) Effects and safety of preventive oral iron or iron + folic acid supplementation for women during pregnancy. *Cochrane Database Syst Rev* issue 4, CD004736.

25. Cung TG, Paus AS, Aghbar A *et al.* (2014) Stillbirths at a hospital in Nablus, 2010: a cohort study. *Glob Health Action* **7**, 25222.

26. Ellison PT (2010) Fetal programming and fetal psychology. *Infant Child Dev* **19**, 6–20.

27. Massaro AN, Rothbaum R & Aly H (2006) Fetal brain development: the role of maternal nutrition, exposures and behaviors. *Eur J Paediatr Neurol* **4**, 1–9.

28. Tamura T, Goldenberg RL, Hou J *et al.* (2002) Cord serum ferritin concentrations and mental and psychomotor development of children at five years of age. *J Pediatr* **140**, 165–170.

29. Hanieh S, Ha TT, Simpson JA *et al.* (2013) The effect of intermittent antenatal iron supplementation on maternal and infant outcomes in rural Viet Nam: a cluster randomised trial. *PLoS Med* **10**, e1001470.

30. Hernández-Martínez C, Arija V, Balaguer A *et al.* (2008) Do the emotional states of pregnant women affect neonatal behavior? *Early Hum Dev* **84**, 745–750.

31. Hernández-Martínez C, Arija V, Escribano J *et al.* (2010) Does maternal anxiety affect neonatal behaviour differently in boys and girls? *Early Hum Dev* **86**, 209–211.

32. Conroy S, Pariante CM, Marks MN *et al.* (2012) Maternal psychopathology and infant development at 18 months: the impact of maternal personality disorder and depression. *J Am Acad Child Adolesc Psychiatry* **51**, 51–61.

33. Koutra K, Chatzi L, Roumeliotaki T *et al.* (2012) Sociodemographic determinants of infant neurodevelopment at 18 months of age: Mother–Child Cohort (Rhea Study) in Crete, Greece. *Infant Behav Dev* **35**, 48–59.

34. Ding Y, Xu X, Wang Z *et al.* (2014) The relation of infant attachment to attachment and cognitive and behavioural outcomes in early childhood. *Early Hum Dev* **90**, 459–464.

35. Victora CG, Horta BL, de Mola CL *et al.* (2015) Association between breastfeeding and intelligence, educational attainment, and income at 30 years of age: a prospective birth cohort study from Brazil. *Lancet Glob Health* **3**, e199–e205.

36. Angulo-Barroso RM, Schapiro L, Liang W *et al.* (2011) Motor development in 9-month-old infants in relation to cultural differences and iron status. *Dev Psychobiol* **53**, 196–210.

37. Hernández-Martínez C, Arija V, Escribano J *et al.* (2012) A longitudinal study on the effects of maternal smoking and secondhand smoke exposure during pregnancy on neonatal neurobehavior. *Early Hum Dev* **88**, 403–408.

38. Georgieff MK (2007) Nutrition and the developing brain: nutrient priorities and measurement. *Am J Clin Nutr* **85**, issue 2, 614S–620S.

39. Dobbing JN & Sands J (1973) Quantitative growth and development of human brain. *Arch Dis Child* **48**, 757–767.

40. Rao R & Georgieff MK (2000) Early nutrition and brain development. In *The Effects of Early Adversity on Neurobehavioral Development. Symposium on Child Psychology*, pp. 1–30 [CA Nelson, editor]. Hillsdale, NJ: Erlbaum Associates.

41. Chen X, Scholl TO & Stein TP (2006) Association of elevated serum ferritin levels and the risk of gestational diabetes mellitus in pregnant women: the Camden study. *Diabetes Care* **29**, 1077–1082.

42. Rambod M, Kovesdy CP & Kalantar-Zadeh K (2008) Combined high serum ferritin and low iron saturation in hemodialysis patients: the role of inflammation. *Clin J Am Soc Nephrol* **3**, 1691–1701.

43. Ministerio de Sanidad y Consumo (2006) *Guía para la Prevención de Defectos Congénitos*. Madrid: Ministerio de Sanidad y Consumo; available at http://www.msssi.gob.es/profesionales/prestacionesSanitarias/publicaciones/DefectosCongenitos.htm

44. Gomez F, Simo JM, Camps J *et al.* (2000) Evaluation of a particle-enhanced turbidimetric immunoassay for the measurement of ferritin: application to patients participating in an autologous blood transfusion program. *Clin Biochem* **33**, 191–196.

45. World Health Organization (2011) *Haemoglobin Concentrations for the Diagnosis of Anaemia and Assessment of Severity. Vitamin and Mineral Nutrition Information System*. Geneva: WHO.

46. World Health Organization, United Nations University & UNICEF (2001) *Iron Deficiency Anaemia: Assessment, Prevention, and Control. A Guide for Programme Managers. WHO/NHD/01.3*. Geneva: WHO.

47. Brazelton TB & Nugent JK (1997) *Escala para la Evaluación del Comportamiento Neonatal*. Barcelona: Paidós.

48. Spielberger CD, Gorsuch RL & Lushene RE (1994) *STAI Cuestionario de Ansiedad Estado Rasgo* [adaptación española: Nicolás Seisdedos Cubero]. Madrid: TEA Ediciones.

49. Hollingshead AB (2011) Four factor index of social status. *Yale J Sociol* **8**, 21–52.

50. World Health Organization (2006) *Iron and Folate Supplementation. Standards for Maternal and Neonatal Care. Integrated Management of Pregnancy and Childbirth (IMPAC)*. Geneva: WHO.

51. Aranda N, Viteri FE, Montserrat C et al. (2010) Effects of C282Y, H63D, and S65C HFE gene mutations, diet, and lifestyle factors on iron status in a general Mediterranean population from Tarragona, Spain. *Ann Hematol* **89**, 767–773.

52. Aranda N, Viteri FE, Fernández-Ballart J et al. (2007) Frequency of the hemochromatosis gene (HFE) 282C → Y, 63H → D, and 65S → C mutations in a general Mediterranean population from Tarragona, Spain. *Ann Hematol* **86**, 17–21.

53. Walsh T, O'Broin SD, Cooley S et al. (2011) Laboratory assessment of iron status in pregnancy. *Clin Chem Lab Med* **49**, 1225–1230.

54. Yip R (2000) Significance of an abnormally low or high hemoglobin concentration during pregnancy: special consideration of iron nutrition. *Am J Clin Nutr* **72**, 1 Suppl., 272S–279S.

55. Scholl TO (2005) Iron status during pregnancy: setting the stage for mother and infant. *Am J Clin Nutr* **81**, issue 5, 1218S–1222S.

56. Brazelton TB & Nugent JK (2011) *The Neonatal Behavioral Assessment Scale*, 4th ed. Cambridge: Mac Keith Press.

57. Debnath M, Venkatasubramanian G & Berk M (2015) Fetal programming of schizophrenia: select mechanisms. *Neurosci Biobehav Rev* **49**, 90–104.

58. Kingston D, McDonald S, Austin M et al. (2015) Association between prenatal and postnatal psychological distress and toddler cognitive development: a systematic review. *PLoS One* **10**, e126929.

59. Glover V (2014) Maternal depression, anxiety and stress during pregnancy and child outcome; what needs to be done. *Best Pract Res Clin Obstet Gynaecol* **28**, 25–35.

60. O'Donnell KJ, Glover V, Barker ED et al. (2014) The persisting effect of maternal mood in pregnancy on childhood psychopathology. *Dev Psychopathol* **26**, 393–403.

61. Yang L, Ren AG, Liu JM et al. (2010) Influence of hemoglobin level during early gestation on the development of cognition of pre-school children. *Zhonghua Liu Xing Bing Xue Za Zhi* **31**, 1353–1358.

62. Canals J, Hernández-Martínez C, Esparó G et al. (2011) Neonatal Behavioral Assessment Scale as a predictor of cognitive development and IQ in full-term infants: a 6-year longitudinal study. *Acta Paediatr* **100**, 1331–1337.

63. Canals J, Esparó G & Fernández-Ballart JD (2006) Neonatal behaviour characteristics and psychological problems at 6 years. *Acta Paediatr* **95**, 1412–1417.

64. Barker DJ (1998) *In utero* programming of chronic disease. *Clin Sci (Lond)* **95**, 115–128.

65. Iwamoto HS, Murray MA & Chernausek SD (1992) Effects of acute hypoxemia on insulin-like growth factors and their binding proteins in fetal sheep. *Am J Physiol* **263**, 1151–1156.

66. Kim-Shapiro DB, Schechter AN & Gladwin MT (2006) Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler Thromb Vasc Biol* **26**, 697–705.

67. Sun L, Macgowan CK, Sled JG et al. (2015) Reduced fetal cerebral oxygen consumption is associated with smaller brain size in fetuses with congenital heart disease. *Circulation* **131**, 1313–1323.

68. Walter PB, Knutson MD, Paler-Martinez A et al. (2002) Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. *Proc Natl Acad Sci U S A* **99**, 2264–2269.

69. Viteri FE, Casanueva E, Tolentino MC et al. (2012) Antenatal iron supplements consumed daily produce oxidative stress in contrast to weekly supplementation in Mexican non-anemic women. *Reprod Toxicol* **34**, 125–132.

70. Peña-Rosas JP, De-Regil LM, Gomez Malave H et al. (2015) Intermittent oral iron supplementation during pregnancy. *Cochrane Database Syst Rev* issue 10, CD009997.

71. Shen Y, Yu HM, Yuan TM et al. (2007) Intrauterine infection induced oligodendrocyte injury and inducible nitric oxide synthase expression in the developing rat brain. *J Perinat Med* **35**, 203–209.

72. Ribot B, Aranda N, Giralt M et al. (2013) Effect of different doses of iron supplementation during pregnancy on maternal and infant health. *Ann Hematol* **92**, 221–229.