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Behavioural consequences at 5 years of neonatal iron deficiency in a low-risk maternal-infant cohort

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Short Running Title Behavioural consequences of neonatal iron deficiency

Abbreviations BASELINE: Babies after SCOPE: Evaluating the Longitudinal Impact using Neurological and Nutritional Endpoints; BSID-III: Bayley Scales of Infant and Toddler Development, 3rd Edition; CBCL: Child Behaviour Checklist; hsCRP: high sensitivity C-reactive protein; KBIT-2: Kaufman Brief Intelligence Test, 2nd Edition; SCOPE: Screening for Pregnancy Endpoints; SGA: small-for-gestational age.

Trial Registration This manuscript utilises data from two large prospective cohort studies, which were observational in design with no intervention aspect. Both studies were registered with clinical registries; BASELINE Study NCT01498965 (www.clinicaltrials.gov) and SCOPE Study ACTRN12607000551493 (http://www.anzctr.org.au).

Data described in the manuscript will be made available upon request pending application and approval from the Corresponding Author and the BASELINE Study PI team.

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ABSTRACT

Background Iron is critical to the developing brain, however fetal iron accretion is compromised by several maternal and pregnancy-related factors. Little consideration has been given to the long-term neurological consequences of neonatal iron deficiency, especially in generally healthy, low-risk populations.

Objective We aimed to investigate the association between neonatal iron deficiency and neurological development at 2 and 5 years of age.

Design We measured umbilical cord serum ferritin concentrations in the prospective maternal-infant Cork BASELINE Birth Cohort. Lifestyle and clinical data were collected from 15 weeks' of gestation to 5 years of age. Standardised neurological assessments were performed at 2 (Bayley Scales of Infant Development/Child Behaviour Checklist [CBCL]) and 5 (Kaufman Brief Intelligence Test/CBCL) years.

Results Among 697 maternal-infant pairs, median [IQR] cord ferritin concentrations were 200.9 [139.0,265.8] µg/L; 8% had neonatal iron deficiency (ferritin <76µg/L). Using fully adjusted models, there was no association between neonatal iron deficiency and cognitive or behavioural outcomes at 2 or 5 years. We conducted an *a priori* sensitivity analysis in 306 high-risk children, selected using known risk factors for neonatal iron deficiency (smoking/obesity/C-section delivery/small-for-gestational age birth).. In this high-risk subgroup, children with iron deficiency at birth (12%) had similar cognitive outcomes, but the behavioural assessments showed higher internalising (9.0 [5.3,12.0] vs. 5.0 [3.0,10.0], *P*=0.006; adj. estimate [95% CI]: 2.8 [0.5,5.1], *P*=0.015) and total (24.5 [15.3,40.8] vs. 16.0 [10.0,30.0], *P*=0.009; adj. estimate [95% CI]: 6.6 [0.1,13.1], *P*=0.047) problem behaviour scores at 5 years compared to those born iron sufficient.

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Conclusions We have demonstrated lasting behavioural consequences of neonatal iron deficiency in high-risk children from our generally healthy, low-risk maternal-infant cohort. While larger investigations are warranted, this study provides strong association data to suggest that interventions and strategies targeting the fetal and neonatal period should be prioritised for the prevention of iron deficiency and its neurological consequences.

Keywords Iron deficiency; behaviour; internalising problems; externalising problems, newborn infants; neonatal; maternal health; maternal obesity; Caesarean section; birth cohort.

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INTRODUCTION

While much of brain development in early life is preprogramed, there are a number of important biological and environmental factors that influence brain development (1). The influence of nutrition is controllable, or at least, modifiable (2). Iron is especially critical to the developing brain, as it is central to the neuronal processes of myelination, energy metabolism and neurotransmitter production (3). Iron deficiency during periods of rapid brain development is therefore associated with long-lasting, often irreversible adverse consequences for cognition, motor development and behaviour (4, 5). Worryingly, these translate into long-term negative effects on mental health, academic performance, career and earning potential, representing a real challenge for society.

The late fetal and early neonatal period is characterised by significant vulnerability to iron deficiency. We know that infants born premature often have reduced iron stores at birth (6), however, the old adage that healthy, term infants are born with enough iron reserves for 6 months is not always true. While maternal iron deficiency is an acknowledged contributor to neonatal iron deficiency (7, 8), a multitude of other common maternal and pregnancy-related factors reduce fetal iron accretion in utero. Pregnancy complications including gestational diabetes mellitus, hypertension and fetal growth restriction, in addition to maternal health and lifestyle factors such as smoking and obesity all compromise neonatal iron stores (9-11). Early-life events, including delivery by Caesarean section and immediate umbilical cord clamping, further add to this cumulative risk of neonatal iron deficiency (12, 13), predisposing infants to iron deficiency later in infancy and childhood (14).

Despite this, little consideration has been given to the long-term neurological consequences of neonatal iron deficiency. Investigations to date have been limited but evidence of poorer language ability, motor function and auditory system development in children born iron

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deficient has been reported (15-20). Although, many of these investigations have been constrained by inadequate sample sizes with short-term follow-up in very heterogenous, high-risk cohorts.

Our primary objective was to investigate associations between neonatal iron deficiency at birth, defined as a cord ferritin concentration <76 µg/L (15) and neurological development up to 5 years of age in term-born participants of a prospective, maternal-infant cohort in Ireland. On the basis of our extensive research in this low-risk cohort, including the largest European study of iron nutrition among young children (21); an association of poor cognitive outcomes among children with low iron status who are not classed as iron deficient (22); and identification of 4 key risk factors for neonatal iron deficiency, including maternal smoking, maternal obesity, delivery by Caesarean section and small-for-gestational age (SGA) birth (9), our second objective was to further investigate these associations in the subgroup of children identified as high risk for neonatal iron deficiency using these 4 risk factors as selection criteria.

SUBJECTS AND METHODS

Cohort design

This study is an analysis of participants that were recruited to the Cork BASELINE (Babies after SCOPE: Evaluating the Longitudinal Impact using Neurological and Nutritional Endpoints) Birth Cohort Study (www.clinicaltrials.gov NCT01498965), a follow-on to the SCOPE (Screening for Pregnancy Endpoints) Ireland study (http://www.anzctr.org.au ACTRN12607000551493), between March 2008 and January 2011. Low-risk, nulliparous women with a singleton pregnancy were recruited to SCOPE (*n* 1768) before 15 weeks' gestation from Cork University Maternity Hospital, as part of an international study aimed at investigating early indicators of pregnancy complications (23).

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The Cork BASELINE Birth Cohort was established with three main objectives: to investigate the effect of intrauterine growth restriction and early nutrition on neurological development; to ascertain the incidence and determinants of food allergy and eczema in early childhood; and to examine the effect of early infant feeding and nutritional status on growth and development in childhood.

Research midwives collected information on maternal socioeconomic status, education, relationship status, lifestyle, dietary factors and a complete medical history, in addition to anthropometric and clinical measurements throughout pregnancy. At 20 weeks' gestation, women in the SCOPE Ireland study provided written informed consent to the BASELINE Study for their infants (*n* 1537), who were then followed prospectively from birth through to 5 years of age. Detailed information on early life environment, diet, health, growth and development was gathered by interviewer-led questionnaires and clinical assessments at day 2, and 2, 6 and 12 months and at 2 and 5 years (24). Follow-up assessments at 5 years were completed in December 2016. Both studies were conducted in accordance with the Declaration of Helsinki and ethical approval was granted by the Clinical Research Ethics Committee of the Cork teaching hospitals (SCOPE: ECM5(10) 05/02/2008, BASELINE: ECM5(9) 01/07/2008).

Neurological assessments

Participants completed detailed neurological assessments; the Bayley Scales of Infant and Toddler Development 3rd Edition (BSID-III) at 2 years, the Kaufman Brief Intelligence Test 2nd Edition (KBIT-2) at 5 years and the Child Behaviour Checklist (CBCL) at 2 and 5 years. **Figure 1** provides a brief overview of cohort design and assessments included in the current study.

BSID-III

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The BSID-III is an individually administered assessment of developmental functioning (25), that was administered by a research psychologist trained in its administration and scoring. The assessment consists of 5 scales: cognition, receptive communication, expressive communication, fine motor and gross motor, with receptive and expressive communication combined to form a language composite score and fine and gross motor combined to form a motor composite score. Published normative scores for the composite scales are a mean of 100 with a standard deviation of 15; developmental delay was defined as a score less than 85. The BSID-III was administered to a pre-selected subgroup (*n* 460) of the birth cohort to examine the effect of fetal growth on developmental outcomes (26).

KBIT-2

The KBIT-2 is a brief, individualised test to measure verbal and non-verbal intelligence in children from 4 years, identifying individuals who may be at risk of academic problems or developmental delay (27). The KBIT-2, administered by a research nurse trained in test administration and interpretation, consists of 3 subtests, 2 of which are verbal (Verbal Knowledge and Riddles) and 1 non-verbal (Matrices). The subtests involve individually administered verbal and non-verbal tasks that do not require reading or spelling but consists of verbal questions, illustrations and visual stimuli. The verbal subtests assess verbal concept formation, word meaning and reasoning, while the non-verbal subtest assesses fluid reasoning, visual processing and problem solving. The verbal and non-verbal scales are tallied, standardised for age and transformed into a composite IQ score. The standard score for each component has a mean of 100 and a standard deviation of 15; scores less than 85 represent developmental delay.

CBCL

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Emotional and behavioural problems were assessed by the CBCL (28). The CBCL is a 99-item validated screener completed by parents/caregivers, indicating the frequency of behaviours in their child over the past 2 months on a 3-point scale (not true, sometimes true or very/often true), with increasing scores indicating increasing problems. The Internal Problem Score is made up of scores from 4 individual syndrome scales: emotionally reactive, anxious/depressed, somatic complaints (physical complaints e.g. nausea/headaches) and withdrawn. The External Problem Score is made up of scores from 2 individual syndrome scales: attention problems and aggressive behaviour. Summing the Internal Problem Score and the External Problem Score with 2 further individual scale scores: sleep problems and other problems, provides a Total Problem Score. For this analysis, scores ≥93rd percentile were denoted as abnormal.

Biological samples and analytical methods

A venous blood sample was collected from the umbilical cord at birth (*n* 727) and at 2 (*n* 706) and 5 years (*n* 485) of age in consenting cohort participants, as described in our previous explorations in this cohort (9, 21). Biochemical analyses were undertaken in the Cork Centre for Vitamin D and Nutrition Research Laboratory in University College Cork. Serum ferritin concentrations were measured by immunoturbidimetric assay using the RX Monaco Clinical Chemistry Analyser (Randox Laboratories Ltd., Co. Antrim, UK), employing a method standardised against the World Health Organisation's International Standard for ferritin (NIBSC 94/572). High sensitivity C-reactive protein (hsCRP) was measured concurrently as a marker of inflammation; infants with a hsCRP >5 mg/L were subsequently excluded from the analysis. Given the highly stretched distribution of cord ferritin concentrations and the need for international comparison, neonatal iron deficiency was defined as a cord ferritin concentration <76 µg/L in this study, as per the extensively used definition suggested by Tamura and colleagues (15).

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Data analysis

All term-born participants with valid umbilical cord ferritin measurements and at least 1 neurological assessment at 2 and 5 years were eligible for inclusion in this study. Data were analysed using IBM SPSS® for Windows™ version 24 (IBM Corp., Armonk, NY, USA) and Statistical Analysis System (SAS) version 9.4 (SAS Institute Inc., Cary, NC, USA). Distributions of all variables were tested using Kolmogorov-Smirnov tests and descriptive statistics (means and standard deviations, medians and interquartile ranges or percentages and frequencies, where appropriate) were determined for all variables. Comparisons between categorical variables, including participant characteristics, were made using Chi square tests, while Mann-Whitney U tests were employed for continuous variables, including the neurological outcome scores at 2 and 5 years.

Multiple linear regression was used to explore the association between neonatal iron deficiency (defined as a cord ferritin concentration <76 µg/L) and neurological outcomes at 2 and 5 years. Each analysis was performed in participants with a complete dataset of umbilical cord ferritin concentrations and the relevant neurological outcome of interest; imputation was not used for any missing outcome or covariate data. Separate linear regression models were built for each predictor-outcome association, with initial associations between neonatal iron deficiency (and other potential confounders) with the neurological outcomes assessed by univariate linear regression in which the significance level was set at alpha = 0.25.

Multivariate models were then built, with adjustment for covariates based on statistical significance from the univariate analysis (alpha = 0.1). At this stage, any non-significant covariates from the univariate analysis were only kept in the final model if deemed clinically relevant and potentially on the clinical pathway of this association. In the exploration of

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associations in the total sample, the *a priori* decision was taken to include infant sex, maternal education and mode of delivery in the final models. Given the multifaceted nature of neurological development in childhood, final models were adjusted for a number of maternal and infant characteristics, particularly measures of socioeconomic status (e.g. household income, nationality), maternal education and maternal health and lifestyle (e.g. age at delivery, smoking status, alcohol consumption, BMI, mode of delivery). Further adjustment for concurrent iron status (ferritin at 2 or 5 years, depending on timing of outcome assessment) was considered in Model 2, although these models were limited to smaller sample sizes, as concurrent iron status was not a prerequisite for inclusion in this study.

Linearity and constant error variance were then evaluated through scatter plots and the White test, for both the outcome and each of the predictors in the model. The distribution of residuals was normal for all multivariate models, as assessed through histograms, normal probability plots and the Shapiro-Wilk test. Associations were expressed as adjusted estimates with 95% confidence intervals (95% CI) and statistical significance was set at $P < 0.05$ in final models.

For the *a priori* sensitivity analysis in high-risk children, the previously identified risk factors of maternal obesity, maternal smoking, delivery by Caesarean section and SGA birth (9) were used as selection criteria to identify high-risk children. Associations between neonatal iron deficiency and neurological development at 2 and 5 years were explored within this subgroup employing the same linear regression methodology as outlined above. Further analyses were carried out within each individual risk factor subgroup but these analyses were insecure due to the low numbers of participants in each individual subgroup.

RESULTS

Subject characteristics

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Cord ferritin concentrations were measured in 727 infants, but 26 preterm infants (<37 weeks' gestation) and 4 with an elevated hsCRP were excluded. Ferritin concentrations at 2 and 5 years were available in 352 and 246 of these participants, respectively. The principal characteristics of the 697 maternal-infant pairs included in this study are presented in **Table 1**. The median age of mothers was 31 years, with the majority Caucasian and highly educated. A quarter of deliveries were by Caesarean section but delayed umbilical cord clamping was not routinely employed during vaginal or Caesarean deliveries during the study period. Almost three-quarters of infants were predominantly breastfed at hospital discharge, decreasing significantly by 2 months of age, with a very low prevalence of iron supplement use during infancy and childhood (<2%).

Median [IQR] cord ferritin concentrations were 200.9 [139.0, 265.8] µg/L (mean ± SD 198.6 ± 79.3 µg/L), but a wide range, from 6.9 to 385.4 µg/L was observed. The distribution of ferritin concentrations at birth, 2 and 5 years in study participants is presented in **Supplemental Table 1**. Neonatal iron deficiency, indicated by cord ferritin <76 µg/L, was evident in 8% (*n* 52) of newborns. Of the 52 participants born with ferritin concentrations <76 µg/L, deficiency was reversed in all but 1 child at 2 years and in all at 5 years, based on data from our earlier investigations in the cohort (21). Of those that were born iron sufficient, 4 and 3% had developed iron deficiency at 2 and 5 years, respectively.

Developmental profile

All participants had complete data for at least 1 neurological assessment at 2 (BSID-III: *n* 229, CBCL: *n* 597) and 5 years (KBIT-2: *n* 537, CBCL: *n* 579). At 2 years, the median [IQR] BSID-III cognitive composite score was 95.0 [90.0, 100.0], lower than language (109.0 [100.0, 115.0]) and motor (103.0 [97.0, 110.0]) scores. Developmental delay was indicated in 6, 7 and 2% of participants in the cognitive, language and motor subscales,

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respectively. The median [IQR] CBCL total problem score was 19.0 [10.0, 29.0]; <5% of participants had abnormal behaviour at 2 years.

Behavioural scores at 5 years were similar, with a total problem score of 17.0 [9.0, 30.0]; although 12% had abnormal internalising behaviour problems. The median [IQR] KBIT-2 IQ composite score at 5 years was 104.0 [99.0, 110.0], with scores of 109.0 [101.0, 114.0] and 100.0 [94.0, 104.0] for the verbal and non-verbal scales, respectively. The prevalence of developmental delay was <3%.

To note, while we previously reported an adverse effect of low ferritin and mean corpuscular volume on cognition at 2 years in this cohort (22), no cross-sectional associations between iron status indices and CBCL behavioural outcomes at 2 or 5 years were observed (data not shown).

Association between neonatal iron deficiency and neurological outcomes: total sample

Neonatal iron deficiency (ferritin <76 µg/L) was not associated with cognitive or behavioural outcomes at 2 years (**Table 2**). At 5 years, while no differences in KBIT-2 scores were observed, children born iron deficient had higher CBCL externalising problem scores compared to those born iron sufficient. This association was attenuated following adjustment for potential confounders, including indicators of maternal health, education and socioeconomic status, in the final multivariate models (model 1 adj. estimate [95% CI]: 1.3 [-0.9, 3.5], **Table 3**).

Association between neonatal iron deficiency and neurological outcomes: sensitivity analysis in high-risk children

Of the 306 children identified as high risk for neonatal iron deficiency, using the predefined selection criteria of maternal obesity, maternal smoking, delivery by Caesarean section and SGA, 12% (*n* 38) had serum ferritin <76 µg/L.

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Fourteen of the 52 infants that were born iron deficient in the total sample were not identified as high risk in our sensitivity analysis. Iron deficiency in these infants was most likely due to the presence of additional risk factors outside of those used as selection criteria. Two infants were born to mothers with gestational hypertension, 1 following gestational diabetes mellitus, 4 were born to overweight mothers and 10 to mothers that were ex-smokers.

There were no differences in BSID-III or KBIT-2 scores, but high-risk children born iron deficient had higher CBCL scores at 5 years compared to high-risk children who were iron sufficient at birth (**Table 4**). Following adjustment, the associations with internalising (adj. estimate [95% CI]: 2.8 [0.5, 5.1]) and total problem scores (adj. estimate [95% CI]: 6.6 [0.1, 13.1]) remained significant (**Table 5**). Further adjustment for concurrent iron status in model 2 resulted in the same estimates but a widening of the confidence intervals. Caution is advised around these models, given the reduced sample size and extensive adjustments.

Individual risk factor analysis

Further analyses were performed in the individual subgroups of children delivered by Caesarean section (*n* 172) and born to mothers with obesity (*n* 85). While these analyses were hampered by inadequate sample sizes (or not feasible as in the case of children born to smoking mothers (*n* 73) or SGA (*n* 54)), we have presented the findings to provide additional information.

The risks of Caesarean section delivery or being born to a mother with obesity themselves were not associated with any neurological outcomes at 2 or 5 years. Additionally, neonatal iron deficiency was not associated with cognitive or intelligence outcomes at 2 or 5 years in either subgroup.

Children delivered by Caesarean section who were born iron deficient had consistently poorer behavioural outcomes at 2 and 5 years when compared to children delivered by Caesarean

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section who were born iron sufficient (**Supplemental Table 3**). This association was robust to adjustment for internal problems at 2 years (adj. estimate [95% CI]: 3.0 [0.8, 5.2]), and internal (adj. estimate [95% CI]: 4.0 [1.4, 6.6]), external (adj. estimate [95% CI]: 3.2 [0.5, 5.9]) and total (adj. estimate [95% CI]: 8.8 [1.8, 15.9]) problem scores at 5 years (**Supplemental Table 5**). Children born iron deficient to obese mothers had higher behavioural scores at 5 years compared to those born iron sufficient to mothers with obesity (**Supplemental Table 4**). Associations with internal (adj. estimate [95% CI]: 6.2 [0.1, 12.4]), external (adj. estimate [95% CI]: 8.4 [2.1, 14.6]) and total (adj. estimate [95% CI]: 21.5 [4.5, 38.5]) problem scores were robust to confounding (**Supplemental Table 5**).

DISCUSSION

In this largest investigation to date of neurological outcomes and neonatal iron status, we have described lasting behavioural consequences of being born with iron deficiency. In our low-risk maternal-infant cohort, children born iron deficient that were identified as high-risk for neonatal iron deficiency had poorer behavioural outcomes at 5 years of age.

Investigations into the long-term neurological consequences of neonatal iron deficiency have been limited and confined mainly to short-term neurophysiological outcomes in heterogeneous populations (15, 18, 29, 30). Our study represents the first and largest report of long-lasting internalising and externalising behavioural consequences of neonatal iron deficiency. These findings are consistent with previous reports in older infants. In Lozoff's Costa Rican cohort, children with chronic, severe iron deficiency between 12-23 months of age had an increased risk of behavioural problems throughout childhood and adolescence (31, 32). Those that were iron deficient in infancy were also more likely to be single, experience negative emotions, and feel detached or dissociated at 25 years of age (4).

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In contrast to previous studies, our observation of a consistent association between neonatal iron deficiency and behaviour is in a relatively homogenous cohort of term-born children in a high-resource setting, whose mothers were highly-educated and predominately Caucasian. This is important, particularly given the multifaceted nature of neurological development during early life, with a number of widely acknowledged environmental and genetic influences (1). To further account for these potential influences, all our final models were adjusted for measures of socioeconomic status, maternal education and health and lifestyle. Maternal characteristics appeared to have the most significant influence in our analysis, further highlighting the wide number of factors that can influence neurological development in early childhood. Moreover, these children in a population at low risk of malnutrition were generally healthy, growing normally and well-nourished, demonstrated by the almost complete correction of iron deficiency by 2 years of age. This correction can be attributed in part to the consumption of a diet rich in iron-fortified products (21) and the likely redistribution of tissue iron in the case of depleted iron stores induced by high-risk situations such as maternal smoking.

In contrast to earlier reports from Tamura (15) and Geng (33), we found no evidence of an association between neonatal iron deficiency and cognitive or intelligence outcomes. The less severe degree of iron deficiency observed in our generally healthy cohort from a high-resource setting may explain our lack of association between neonatal iron status and cognition. Although, a large prospective cohort in Benin also reported no association between neonatal iron status at birth and cognitive outcomes at 1 year (34). This, coupled with the overall normal cognitive profile observed in our cohort, suggests that the observed behavioural consequences in our study are not attributed to cognitive disturbances.

The direction and magnitude of the effect of iron deficiency on brain development is dependent on the timing of the deficiency and the iron requirement of brain regions at that

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time (35, 36). Compelling evidence from in vitro and animal models indicates a direct effect of iron on the synthesis of the monoamine neurotransmitters, namely dopamine, serotonin and norepinephrine (37, 38). These neurotransmitters promote, amplify, inhibit or attenuate neuronal signalling and are thus central to the formation of the neuronal networks underlying behaviour, higher order cognitive and emotional processes in infants (39). Iron deficiency during critical periods of monoamine metabolism, most crucially in late gestation, can therefore result in altered neurotransmitter metabolism and function (40, 41), providing a highly plausible mechanistic pathway for our observed behavioural consequences.

The behavioural consequences of neonatal deficiency were apparent in the children identified as high risk in this study, using our previously identified risk factors as selection criteria. Maternal obesity during pregnancy has long been associated with adverse health outcomes for mother and infant (42), but recent evidence suggests it is also an independent risk factor for iron deficiency (9, 10, 43). The chronic pro-inflammatory state of obesity is thought to result in an overexpression of the iron-regulatory hormone, hepcidin, inhibiting iron absorption, resulting in poorer iron status of the mother and foetus (10). The profound impact of maternal obesity and delivery by Caesarean section in the present study, two previously unacknowledged risk factors for neonatal iron deficiency, is particularly concerning, given the rising rates of both worldwide (44, 45). Interventions targeting these risks are likely the best way to prevent iron deficiency in newborn infants, particularly focused on improving overall maternal health and lifestyle prior to pregnancy. Furthermore, the routine employment of delayed cord clamping during all deliveries, including those by Caesarean section, has been shown to improve neonatal iron status after birth (13).

We have demonstrated long-lasting behavioural consequences of neonatal iron deficiency in high-risk children, beyond the time of the deficiency itself and despite correction of the deficiency. We noted behavioural consequences at 5 years, when a child develops a more

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stable pattern of behaviour and interaction with their environment. Altered behaviour in early childhood has been shown to inhibit a child's interactions with their environment, caregivers and teachers (2, 46). This inhibition appears to further undermine the development of children's self-regulatory skills including the ability to modulate affect, attention and behaviour (47). Recent evidence from a prospective longitudinal study spanning 3 decades also reported lasting effects of inhibited behaviour and temperament in early life on adult functioning, particularly in social and mental health (48). Early social-emotional development is therefore widely considered an important determinant of future educational attainment, mental health, job and earning potential and overall quality of life (36, 49). The behavioural consequences of iron deficiency represent a real cost and burden to our society, therefore, the earlier the developing brain is protected from suboptimal iron status, the better it is for long-term health and prosperity.

As the prevention of neonatal iron deficiency is not always feasible, particularly when deficiency is secondary to conditions such as prematurity or pregnancy complications, screening strategies to facilitate the early detection of iron deficiency in infants are pertinent to enable prompt treatment, thus ensuring normal neurological development in at-risk individuals (50). However, screening for iron deficiency in newborn infants has not received attention in research or clinical practice. Current screening strategies, such as those employed in North America, mainly target older children, or measure haemoglobin, which is not an indicator of iron deficiency, and are inadequate to protect infants from iron deficiency and its associated neurological effects (36, 51). The development of better screening strategies, encompassing screening tools and relevant point-of-care tests, to facilitate the early detection of iron deficiency in newborn infants, especially those at high risk, should be considered a priority.

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While this study is observational, it is strengthened by the longitudinal, prospective design of our cohort, with its multidisciplinary research team and use of validated, standardised neurological assessments. The CBCL is a parent-reported assessment of behaviour, potentially subject to bias, but it is one of the most widely used, validated assessments of behaviour in the research setting. In contrast to previous investigations in the field, our study population is a homogenous cohort of generally healthy children in a low-risk setting, with preterm infants excluded, given their complex nutritional and developmental profile. Maternal educational attainment and household income were used as proxy measures for parental intelligence in this study, an important determinant of child development. Our approach of carrying out sensitivity analyses in high-risk children is novel, although some explorations were constrained by inadequate sample sizes. The study was powered to detect a medium, yet clinically significant effect size with >80% power in the total sample.

In conclusion, we have demonstrated lasting behavioural consequences of neonatal iron deficiency in high-risk children from our generally healthy, low-risk maternal-infant cohort. This study provides strong association data to suggest that this hypothesis should be tested further, with larger investigations warranted, perhaps by an individual participant data meta-analysis. This study also serves as a timely reminder of the importance of the fetal and neonatal period to life-long health and well-being. Interventions targeting the fetal and neonatal period should therefore be prioritised, as they represent the best opportunity for the prevention of neonatal iron deficiency and its long-lasting neurological consequences.

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388 The authors' responsibilities were as follows - EKM and MEK: designed the research; EKM:
389 conducted the research and analysed the data; DMM, JO'BH, LCK, ADI and MEK: principal
390 investigators of the BASELINE and SCOPE cohorts, responsible for project inception and
391 oversight; EKM and MEK: wrote the manuscript and had primary responsibility for the final
392 content and all authors: read and approved the final manuscript. The authors have no
393 conflicts of interest relevant to this article to disclose.

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Table 1 Principal characteristics of the study population and as a function of neonatal iron status[1]

	Total Population *n* = 697	**Ferritin <76µg/L** *n* = 52	**Ferritin ≥76µg/L** *n* = 645
Maternal Characteristics[2]			
Age at delivery (yrs)	31.0 [29.0, 33.0]	31.0 [29.3, 34.0]	31.0 [29.0, 33.0]
Caucasian	99 (689)	100 (52)	99 (637)
University/third level educated	89 (618)	79 (41)	90 (577)[3]
Household income <€21,000 pa	6 (40)	8 (4)	6 (36)
Relationship status, single	4 (29)	4 (2)	4 (27)
Body Mass Index			
Overweight (≥25kg/m^2)	29 (201)	33 (17)	29 (184)
Obese (≥30kg/m^2)	12 (85)	21 (11)	12 (74)
Smoking Status			
Non-smoker	51 (353)	37 (19)	52 (334)[3]
Ex-smoker	38 (267)	44 (23)	38 (244)
Smoker	11 (73)	19 (10)	10 (63)
Mode of Delivery			
Vaginal	75 (505)	62 (32)	76 (473)
Elective Caesarean Section	7 (49)	16 (8)	7 (41)
Emergency Caesarean Section	18 (123)	22 (11)	17 (109)
Infant Characteristics			
Gender, male	52 (364)	54 (28)	52 (336)
Birth weight (kg)	3.5 [3.3, 3.8]	3.5 [3.2, 3.7]	3.5 [3.3, 3.8]
Gestational age (wks)	40.3 [39.4, 41.0]	39.7 [39.1, 40.9]	40.3 [39.4, 41.1][3]
Small-for-gestational age	8 (54)	15 (8)	7 (46)
Feeding			
Breastfed at hospital discharge	74 (517)	77 (40)	74 (477)
Breastfed at 2 months	33 (229)	23 (12)	34 (217)
Age complementary feeding (wks)	20.0 [17.0, 22.0]	19.0 [17.3, 22.0]	20.0 [17.0, 22.0]

[1] Data presented as medians [interquartile range] or percentages (*n*).

[2] Maternal data collected at 15 weeks' gestation unless otherwise stated.

[3] $P<0.05$ for comparison with cord ferritin <76 µg/L group.

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Table 2 Neurological outcomes at 2 and 5 years in children born with cord ferritin concentrations < and ≥76 µg/L[1]

	n	<76 µg/L	*n*	≥76 µg/L	P value[2]
Cord ferritin (µg/L)		**51.7 [31.7, 63.9]**		**213.4 [153.4, 269.5]**	
Two Year Assessment					
CBCL internal problems	47	4.0 [2.0, 8.0]	550	4.0 [1.0, 7.0]	0.307
CBCL external problems	47	9.0 [5.0, 14.0]	550	8.0 [4.0, 12.0]	0.172
CBCL total problems	47	22.0 [13.0, 32.0]	550	19.0 [10.0, 29.0]	0.173
BSID cognition	16	95.0 [91.3, 100.0]	213	95.0 [90.0, 100.0]	0.925
BSID language	14	103.0 [94.0, 110.0]	177	109.0 [100.0, 115.0]	0.322
BSID motor	16	103.0 [94.0, 100.0]	183	103.0 [97.0, 110.0]	0.381
Five Year Assessment					
CBCL internal problems	45	7.0 [2.5, 12.0]	534	5.0 [3.0, 9.3]	0.089
CBCL external problems	45	7.0 [4.0, 12.5]	534	5.0 [2.0, 10.0]	0.018
CBCL total problems	45	21.0 [14.0, 35.0]	534	17.0 [9.0, 29.0]	0.054
KBIT verbal	37	101.0 [99.0, 105.0]	499	109.0 [101.0, 114.0]	0.254
KBIT non-verbal	37	97.0 [94.0, 100.0]	500	100.0 [94.0, 104.0]	0.320
KBIT IQ composite	37	101.0 [99.0, 105.0]	497	104.0 [99.0, 110.0]	0.070

[1] Values are medians [interquartile range].

[2] Difference between groups explored by Mann-Whitney U test.

BSID: Bayley Scales of Infant and Toddler Development; CBCL: Child Behaviour Checklist; KBIT: Kaufman Brief Intelligence Test.

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Table 3 Associations between neonatal iron deficiency, defined as cord ferritin <76 µg/L, and neurological outcomes at 2 and 5 years

	Unadjusted Estimate (95% CI)	***n***	**Model 1 Adjusted Estimate (95% CI)**	***n***	**Model 2 Adjusted Estimate[11] (95% CI)**	***n***
Two Year Assessment						
CBCL internal problems	0.7 (-0.6, 2.0)	596	0.3 (-1.0, 1.7)[1]	564	-0.2 (-2.0, 1.7)	282
CBCL external problems	0.8 (-1.1, 2.6)	596	0.4 (-1.5, 2.4)[1]	564	-0.6 (-3.5, 2.2)	282
CBCL total problems	2.3 (-1.9, 6.6)	596	0.9 (-3.5, 5.4)[2]	563	-2.7 (-9.0, 3.7)	282
BSID cognition	0.5 (-4.1, 5,2)	228	2.1 (-2.9, 7.1)[3]	208	4.2 (-1.8, 10.1)	104
BSID language	-4.7 (-12.3, 3.0)	190	-2.0 (-9.5, 5.5)[4]	177	2.4 (-7.5, 12.3)	86
BSID motor	-3.0 (-8.5, 2.6)	198	-3.9 (-9.4, 1.6)[5]	191	-5.1 (-11.7, 1.4)	101
Five Year Assessment						
CBCL internal problems	1.9 (-0.03, 3.8)	578	1.2 (-0.9, 3.2)[6]	529	0.1 (-3.1, 3.1)	217
CBCL external problems	2.1 (-0.02, 4.2)	578	1.3 (-0.9, 3.5)[7]	529	0.2 (-3.3, 3.6)	217
CBCL total problems	4.8 (-0.7, 10.4)	578	2.3 (-3.5, 8.2)[7]	529	-0.9 (-9.8, 8.1)	217
KBIT verbal	-1.6 (-4.9, 1.7)	535	-0.4 (-3.7, 3.0)[8]	508	-3.4 (-8.0, 1.1)	222
KBIT non-verbal	-1.1 (-4.0, 1.7)	536	-0.9 (-4.5, 2.8)[9]	344	-1.7 (-6.8, 3.4)	155
KBIT IQ composite	-2.1 (-4.8, 0.7)	533	-1.6 (-4.8, 1.6)[10]	344	-3.0 (-7.2, 1.2)	158

[1] Model adjusted for infant sex, age at complementary feeding, maternal age, country of birth, education, smoking and alcohol status in early pregnancy and mode of delivery.

[2] Model adjusted for infant sex, age at complementary feeding, maternal age, country of birth, education, smoking and alcohol status and BMI in early pregnancy and mode of delivery.

[3] Model adjusted for infant sex, early feeding and age at complementary feeding, maternal education, smoking status in early pregnancy, mode of delivery and household income.

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[4] Model adjusted for infant sex, gestational age, early feeding and age at complementary feeding, maternal education, preeclampsia diagnosis and mode of delivery.

[5] Model adjusted for infant sex, maternal education and mode of delivery.

[6] Model adjusted for infant sex, age at complementary feeding, maternal age, education, smoking and alcohol status and BMI in early pregnancy, mode of delivery and household income.

[7] Model adjusted for infant sex, age at complementary feeding, maternal age, marital status, education, smoking and alcohol status and BMI in early pregnancy, mode of delivery and household income.

[8] Model adjusted for infant sex and birth weight, maternal education, country of birth, BMI in early pregnancy, mode of delivery and household income.

[9] Model adjusted for infant sex, early feeding and age at complementary feeding, maternal education, marital status and mode of delivery.

[10] Model adjusted for infant sex, birth weight and early feeding, maternal education, marital status, country of birth, BMI in early pregnancy, mode of delivery and household income.

[11] Model 2 = model 1 plus adjustment for concurrent (at 2 or 5 years, depending on timing of outcome assessment) serum ferritin concentrations. Ferritin concentrations at 2 and 5 years only available for a subgroup of the sample.

BSID: Bayley Scales of Infant and Toddler Development; CBCL: Child Behaviour Checklist; KBIT: Kaufman Brief Intelligence Test.

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Table 4 Neurological outcomes at 2 and 5 years in subgroup of high-risk children born with cord ferritin concentrations < and ≥76 µg/L[1]

	n	<76 µg/L	*n*	≥76 µg/L	P value[2]
Cord ferritin (µg/L)		**47.3 [29.3, 59.5]**		**190.9 [143.8, 262.2]**	
Two Year Assessment					
CBCL internal problems	33	5.0 [2.0, 10.5]	231	4.0 [2.0, 7.0]	0.117
CBCL external problems	33	9.0 [4.5, 14.5]	231	8.0 [4.0, 13.0]	0.603
CBCL total problems	33	24.0 [14.0, 32.5]	231	19.0 [11.0, 32.0]	0.287
BSID cognition	12	95.0 [91.3, 100.0]	106	95.0 [90.0, 100.0]	0.760
BSID language	10	103.0 [94.0, 110.0]	85	109.0 [100.0, 115.0]	0.555
BSID motor	11	103.0 [94.0, 110.0]	89	103.0 [97.0, 110.0]	0.416
Five Year Assessment					
CBCL internal problems	32	9.0 [5.3, 12.0]	225	5.0 [3.0, 10.0]	0.006
CBCL external problems	32	7.5 [4.0, 14.8]	225	5.0 [2.0, 10.0]	0.023
CBCL total problems	32	24.5 [15.3, 40.8]	225	16.0 [10.0, 30.0]	0.009
KBIT verbal	26	106.5 [101.0, 113.3]	215	107.0 [100.0, 114.0]	0.889
KBIT non-verbal	26	97.0 [94.0, 100.3]	215	98.0 [94.0, 104.0]	0.467
KBIT IQ composite	26	100.5 [98.5, 105.3]	215	103.0 [99.0, 109.0]	0.339

[1] Values are medians [interquartile range].

[2] Difference between groups explored by Mann-Whitney U test.

BSID: Bayley Scales of Infant and Toddler Development; CBCL: Child Behaviour Checklist; KBIT: Kaufman Brief Intelligence Test.

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Table 5 Associations between neonatal iron deficiency, defined as cord ferritin <76 µg/L, and behavioural outcomes at 2 and 5 years in high-risk children

	Unadjusted Estimate (95% CI)	***n***	**Model 1 Adjusted Estimate (95% CI)**	***n***	**Model 2 Adjusted Estimate[6] (95% CI)**	***n***
Two Year Assessment						
CBCL internal problems	1.3 (-0.3, 2.9)	263	1.0 (-0.5, 2.6)[1]	262	1.0 (-1.2, 3.2)	139
CBCL external problems	0.1 (-2.4, 2.6)	263	-0.2 (-2.7, 2.4)[2]	254	-0.3 (-3.8, 3.2)	135
CBCL total problems	2.1 (-3.4, 7.6)	263	1.2 (-4.3, 6.7)[3]	262	0.9 (-6.8, 8.6)	139
Five Year Assessment						
CBCL internal problems	3.2 (1.0, 5.5)	256	2.8 (0.5, 5.1)[4]	246	2.8 (-0.8, 6.4)	103
CBCL external problems	2.9 (0.4, 5.4)	256	2.4 (-0.1, 4.9)[5]	246	2.4 (-1.5, 6.4)	103
CBCL total problems	8.0 (1.5, 14.6)	256	6.6 (0.1, 13.1)[5]	246	6.6 (-3.6, 16.8)	103

[1] Model adjusted for infant sex, maternal age, education, country of birth, smoking and alcohol status in early pregnancy and mode of delivery.

[2] Model adjusted for infant sex and early feeding, maternal age, education, country of birth, smoking status in early pregnancy and mode of delivery.

[3] Model adjusted for infant sex, maternal age, education, country of birth, smoking status in early pregnancy and mode of delivery.

[4] Model adjusted for infant sex and age at complementary feeding, maternal age, education, smoking and alcohol status in early pregnancy and mode of delivery.

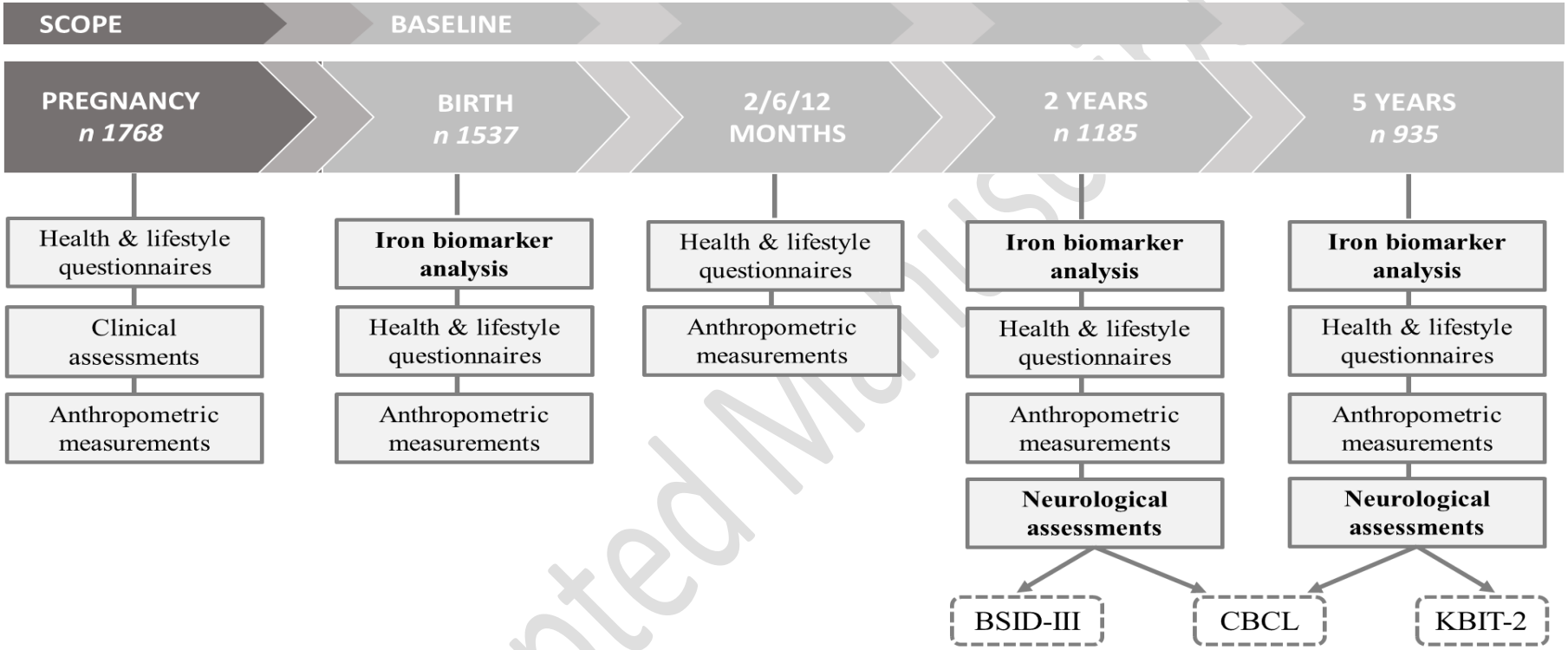
[5] Model adjusted for infant sex and age at complementary feeding, maternal age, education, smoking in early pregnancy and mode of delivery.

[6] Model 2 = model 1 plus adjustment for concurrent (at 2 or 5 years, depending on timing of outcome assessment) serum ferritin concentrations. Ferritin concentrations at 2 and 5 years only available for a subgroup of the sample.

CBCL: Child Behaviour Checklist.

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Figure 1 Overview of cohort design, including timeline of assessments conducted in the current study



BSID-III: Bayley Scales of Infant and Toddler Development 3rd Edition; CBCL: Child Behaviour Checklist; KBIT-2: Kaufman Brief Intelligence Test 2nd Edition

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