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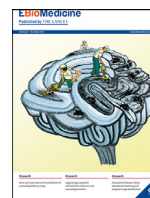
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Research paper

Early postnatal hypoferrremia in low birthweight and preterm babies: A prospective cohort study in hospital-delivered Gambian neonates

James H. Cross^a, Ousman Jarjou^a, Nuredin Ibrahim Mohammed^a, Santiago Rayment Gomez^b, Bubacarr J.B Touray^a, Andrew M. Prentice^a, Carla Cerami^{a,*}

^a Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine, Atlantic Boulevard, Fajara, P.O. Box 273, Banjul, The Gambia

^b Cardiff University, Cardiff, Wales, CF10 3AT, United Kingdom



ARTICLE INFO

Article History:

Received 8 November 2019

Revised 29 November 2019

Accepted 17 December 2019

Available online xxx

Keywords:

Nutritional immunity

Iron

Transferrin saturation

CRP

Hepcidin

Neonates

Low birthweight

Prematurity

Sub-Saharan Africa

Septicaemia

ABSTRACT

Background: Neonates, particularly those born preterm (PTB) and with low birthweight (LBW), are especially susceptible to bacterial and fungal infections that cause an estimated 225,000 deaths annually. Iron is a vital nutrient for the most common organisms causing septicaemia. Full-term babies elicit an immediate postnatal hypoferrremia assumed to have evolved as an innate defence. We tested whether PTB and LBW babies are capable of the same response.

Methods: We conducted an observational study of 152 babies who were either PTB (born ≥ 32 to < 37 weeks gestational age) and/or LBW (< 2500 g) (PTB/LBW) and 278 term, normal-weight babies (FTB/NBW). Blood was sampled from the umbilical cord vein and artery, and matched venous blood samples were taken from all neonates between 6–24 h after delivery. We measured haematological, iron and inflammatory markers.

Findings: In both PTB/LBW and FTB/NBW babies, serum iron decreased 3-fold within 12 h of delivery compared to umbilical blood (7.5 ± 4.5 vs 23.3 ± 7.1 ng/ml, $P < 0.001$, $n = 425$). Transferrin saturation showed a similar decline with a consequent increase in unsaturated iron-binding capacity. C-reactive protein levels increased over 10-fold ($P < 0.001$) and hepcidin levels doubled ($P < 0.001$). There was no difference in any of these responses between PTB/LBW and FTB/NBW babies.

Interpretation: Premature or low birthweight babies are able to mount a very rapid hypoferrremia that is indistinguishable from that in normal term babies. The data suggest that this is a hepcidin-mediated response triggered by acute inflammation at birth, and likely to have evolved as an innate immune response against bacterial and fungal septicaemia.

Trial registration: clinicaltrials.gov (NCT03353051). Registration date: November 27, 2017.

Funding: Bill & Melinda Gates Foundation (OPP1152353).

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1. Introduction

Neonatal sepsis is the third highest cause of death globally, accounting for 225,000 deaths each year [1]. It is projected that these numbers will increase as a consequence of the increasing global prevalence of antimicrobial resistance [2].

Abbreviations: AGP, Alpha-1-acid glycoprotein; CRP, C-reactive protein; ELISA, Enzyme-linked immunosorbent assay; EONS, Early-onset neonatal sepsis; FTB, Full-term birth; G6PD, Glucose-6-phosphate dehydrogenase; GCP, Good Clinical Practice; IL-6, Interleukin 6; IL-22, Interleukin 22; KGH, Kanifing General Hospital; KMC, Kanifing Municipal Council; LBW, Low birthweight; MRCC, Medical Research Council Unit The Gambia at LSHTM; NBW, Normal birthweight; PI, Principal investigator; PTB, Preterm birth; SCC, Scientific Coordinating Committee; sTfR, Soluble transferrin receptor; TIBC, Total iron-binding capacity; TLR, Toll-like receptor; TSAT, Transferrin saturation; UIBC, Unbound iron-binding capacity; WHO, World Health Organisation

* Corresponding author.

E-mail address: ccerami@mrc.gm (C. Cerami).

Physiological adaption to the postnatal environment in the first hours of life is critical for survival. At birth, babies transition from a semi-allogeneic, protected fetal setting to a microbe-rich extrauterine environment [3]. The initial mass bacterial colonisation of mucosa in the digestive, respiratory and urogenital tracts, as well as the skin, occurs during the very early neonatal period [4]. The maternal gut is the source of the majority of transmitted bacterial strains to the neonatal microbiome and common strains are also seeded from the maternal skin and the vaginal microbiome [5]. The acquisition of symbionts can positively affect gut maturation, metabolic homeostasis and immune function in early life and beyond [6], but pathogenic bacteria and fungi pose an immediate risk unless contained. Adaptive immune responses require priming in neonates, and hence innate defence mechanisms play an important role in containment.

Similarly, just before delivery or during the intrapartum period, babies can be infected by micro-organisms, which may lead to early-

Research in context

Evidence before this study

We searched PubMed and Ovid Medline for publications that detail hepcidin, serum iron and TSAT concentrations in umbilical cord blood or venous blood taken in the neonatal period that were published before July 1, 2019 with restriction to publications in the English language. We used the search terms “neonate”, “hepcidin”, “TSAT” and “serum iron”. We excluded studies on non-humans, in medically unstable neonates or studies that did not provide details of gestational age for the study population. We identified 28 studies on hepcidin, 23 on TSAT and 51 on serum iron. Collectively, 63 publications contained research on full-term (FTB) neonates, and 13 on premature (PTB) neonates. Many of the retrieved publications focused on maternal-fetal iron endowment, where umbilical cord blood was used as a proxy for early neonatal blood. Very few publications have documented iron and inflammatory variables in matched umbilical and neonatal blood samples taken in the immediate hours (0–24 h) after delivery. Analysis of the retrieved publications leads to the conclusion that neonatal hypoferrremia may exist in all neonates, however the evidence is sparse and unclear, particularly in PTB newborns.

Added value of this study

To our knowledge, this observational non-blinded study is the first to assess the independent effects of gestational age and birthweight on iron and infection parameters in healthy African newborns in the first hours of life. Our findings confirm that a very rapid hypoferrremia occurs in the early hours of postnatal life with evidence that it is mediated by an increase in hepcidin. Premature and low birthweight babies exhibited almost identical postnatal hypoferrremic responses to full-term, normal-weight newborns.

Implications of the available evidence

There is now clear evidence that neonates, including those with PTB or LBW, elicit a rapid and transient hypoferrremia probably induced, at least in part, by hepcidin. The strength and consistency of this effect indicates that it may have evolved as an innate immune response designed to protect neonates from bacterial septicemia. This suggests the possibility that a further enhancement or prolongation of hypoferrremia (for instance by mini-hepcidins), might offer an additional tool in the armoury against antibiotic-resistant infections in newborns.

Cord blood, which has high levels of serum iron and transferrin saturation [13], has frequently been used as a proxy for neonatal iron status. However, previous work by our group and others has shown that healthy, vaginally-delivered, full-term babies profoundly decrease their serum iron and TSAT levels in the first 24 h after birth; an adaptation that may have evolved as an innate defence against iron-requiring microorganisms [14]. In the current study, we hypothesised that the greater susceptibility of preterm and low birthweight babies to neonatal infections might be because they are less able to elicit this defence. We prospectively tested this in hospital-delivered Gambian neonates.

2. Participants and methods

Full details of the methods of this study can be found in the published protocol paper [15].

2.1. Study design

This observational study recruited well, hospital-delivered neonates into four different groups characterised according to birthweight and gestational age as shown in Fig. 1. The primary analysis compares all babies exhibiting preterm birth (PTB), low birthweight (LBW) or both (PTB/LBW) versus full-term (FTB), normal birthweight (NBW) (FTB/NBW) newborns. Secondary analyses examine the PTB+NBW, FTB+LBW and PTB+LBW groups separately.

2.2. Ethics, standards and informed consent

The study was approved by the Medical Research Council Unit The Gambia at London School of Hygiene and Tropical Medicine (MRCG at LSHTM) Scientific Coordinating Committee, the Joint Gambia Government/MRCG Ethics Committee (SCC1525) and the London School of Hygiene and Tropical Medicine Ethics Committee (Ref: 14316). The study was conducted according to Good Clinical Practice (GCP) standards. All participants gave written, informed consent.

2.3. Study setting

Study participants were recruited from Kanifing General Hospital (formerly Serrekunda General Hospital), in the urban Kanifing region of The Gambia, West Africa.

2.4. Recruitment, screening and enrolment

In total, we planned to identify 450 healthy newly born babies during delivery at the Kanifing General Hospital Maternity Ward. After informed consent was obtained, neonates who met the inclusion criteria were enrolled. For inclusion in the study, neonates were medically stable (no birth asphyxia nor signs of sepsis as judged by the study physician), ≥ 32 weeks gestational age and weighed ≥ 2000 g. Preterm babies (PTB) were < 37 completed weeks gestational age (assessed by New Ballard Score [16]). Term babies (FTB) had a gestational age ≥ 37 completed weeks. Low birthweight (LBW) was defined as < 2500 g in line with the usual WHO definition. Neonates, who weighed ≥ 2500 g were defined as normal birthweight (NBW). The observational period began at delivery and lasted until the end of the 7th day of life. Data collection started on the 5th July 2017 and ended on 1st February 2019.

Pregnant mothers were excluded from the study if they were below the age of 18 years, had no fetal heartbeat detected upon admission, were known to be HIV-positive, were in receipt of *Mycobacterium tuberculosis* therapy, had taken antibiotics in the last seven days, had a blood transfusion in the last month, were suffering from severe pre-eclampsia or antepartum haemorrhage, or were in another research study.

Babies were excluded at the delivery stage for the following reasons: major congenital malformations (not including polydactylism),

onset neonatal sepsis (EONS) especially if the mother is colonised with pathogenic bacteria [7]. Aspiration or ingestion of infected amniotic fluid *in utero* or infected secretions at birth are the common routes of infection.

Iron is an important commodity in the host-pathogen battle for resources [8]. It is a cofactor in numerous metabolic pathways that are critical for the human host as well as most pathogens. Therefore, systemic iron distribution is strictly regulated by the host [9]. For many human pathogens, the acquisition of iron via a variety of molecular mechanisms can enhance growth and virulence [8]. Individuals with chronically high iron states (e.g. haemochromatosis) have an enhanced risk of bacterial infection [10], as well as increased free radical redox damage [11]. Historic studies administering parental iron to Polynesian neonates infamously increased mortality rates by promoting *Escherichia coli* septicemias [12], underlining the critical role of iron in the neonatal period.

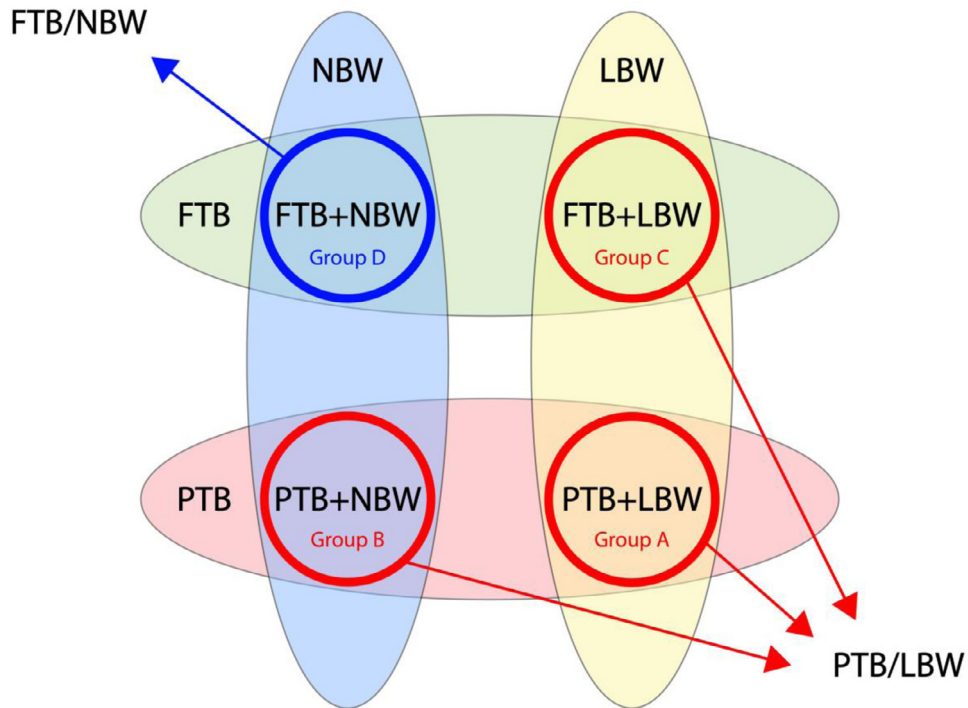


Fig. 1. Schematic diagram of all study groupings and the generation of subgroups. PTB = preterm birth, FTB = full-term birth, LBW = low birthweight and NBW = normal birthweight. FTB/NBW neonates are FTB + NBW. PTB/LBW neonates are FTB + LBW, PTB + NBW and PTB + LBW neonates.

blood transfusions given to mother or neonate, severe birth asphyxia (requiring resuscitation), neonates born via breech, vacuum or via caesarean section, or a birthweight <2000 g (in order to avoid the additional burden of a blood draw in these vulnerable neonates as guided by the Gambia Government/MRCG and LSHTM Ethics Committees).

After the delivery stage, babies were excluded following the detection of infection or illness (information gained from a venous bleed or review of systems). Neonates were also removed from the study protocol, if any medication other than intramuscular vitamin K, tetracycline eye ointment or immunisations were given. All medications given to mothers and neonates during the study period were recorded. Mothers who delivered multiple newborns were invited to enrol one of their neonates into the study.

2.5. Sample collection

Once the neonate was fully delivered, one-minute delayed cord clamping was used (following World Health Organisation (WHO) policy [17]). After the umbilical cord was removed and cleaned, a trained study nurse identified the umbilical arteries and umbilical vein. Blood was collected from each using separate blood draw equipment.

At 6–24 h post-delivery, recruited mothers and their neonates were invited to a private consultation with the study research clinician. Further demographic data were collected, along with a complete review of systems of the mother and neonate. Anthropometric data on the newborn was also collected at this point. Neuromuscular and physical maturation of each neonate was assessed using the New Ballard Score [16]. Immediately after passing the health assessment, a venous blood draw was performed on all neonates (2 ml for PTB/LBW neonates and 3.5 ml for FTB/NBW neonates).

2.6. Laboratory analyses

A full haematology panel (using a Medonic M20M GP, Boule Diagnostics, Spanga, Sweden) and glucose-6-phosphate dehydrogenase deficiency test (R&D Diagnostics Limited, Papagos, Greece) were

conducted on whole blood. Serum was separated and stored at –20 °C prior to analysis of ferritin, iron, unsaturated iron-binding capacity (UIBC), soluble transferrin receptor (sTfR), transferrin, c-reactive protein (CRP), haptoglobin and alpha-1-acid glycoprotein (AGP) using a fully automated biochemistry analyser (Cobas Integra 400 plus, Roche Diagnostic, Switzerland). Transferrin saturation (TSAT) was calculated. Serum samples were assessed for hepcidin concentration by ELISA (hepcidin-25 (human) EIA Kit, DRG, USA) with a dynamic range of 0.135–81 ng/ml.

In order to ensure a consistent assessment of haemolysis in all serum samples, batches of samples were thawed before entering the biochemistry analyser and visually scored by a single operator. A previously published specimen integrity chart for haemolysis was used as reference [18]. Samples were scored 0 (yellow 0 g/L) to 6 (dark red 8 g/L). Samples scoring ≥5 were removed from the analysis.

2.7. Sample size determination

This study targeted recruitment of 50 neonates in each of the 3 groups PTB, LBW and PTB+LBW and 300 FTB/NBW newborns. Sample size calculations for the primary outcomes of change in serum iron and TSAT between cord and the first neonatal samples were based on data from a previous study [14] and are summarised in the published protocol paper [15]. All secondary analyses are considered exploratory and were not subjected to a formal sample size calculation.

2.8. Statistical analysis

The primary analysis compared responses in the PTB and/or LBW babies combined (PTB/LBW) versus FTB/NBW. Further subgroups of the PTB/LBW group (groups A-C as summarised in Fig. 1) were also examined in the secondary analyses (see Supplementary Material). For continuous variables, baseline characteristics are presented as means (± SD) for normally distributed variables. All skewed data were transformed using the ladder command in STATA. The ladder command searches a subset of the ladder of powers for a transformation that converts the variable of

interest into a normally distributed variable. Results were confirmed graphically by the gladder command. Categorical variables are reported as proportions. The rate of missing data was small (<5%), thus we did not impute missing data. Participant characteristics and iron status indicators were compared using 2-tailed t-tests or χ^2 tests. Multiple regression was used to explore relationships between iron status indicators, participant characteristics, inflammatory and haematology markers. The relative strength of associations was assessed using standardised coefficients, which represent the effect on the outcome variable (expressed as a fraction of a standard deviation) caused by a one standard deviation difference in the predictor variable. Changes between cord and postnatal blood samples were assessed by paired t-tests. Comparisons of continuous variables between groups A-D were produced using repeat measures one-way analysis of variance. We examined the association between covariates and the three outcomes iron, TSAT and hepcidin status using linear regression models with backward elimination for variable selection. All models were adjusted for baseline (cord blood) levels. Unless otherwise stated, all hypothesis tests were two-sided at significance level of 0.05. All analyses were performed using DataDesk (Data Description Inc., Ithaca, USA) and STATA 15 (StataCorp LLC, Texas, USA).

3. Results

Fig. 2 is the CONSORT diagram summarising subject recruitment. Baseline characteristics for the 430 neonates completing the study are shown in Table 1. As per protocol, there was a large difference in birthweight between NBW (3199 ± 376 g) and LBW (2338 ± 118 g) newborns, with associated differences in length and head circumference. Gestational age was also lower in the PTB babies (35.6 ± 0.7) than FTB (39.4 ± 1.3 wk). When the LBW and PTB babies were combined as PTB/LBW, their weight and gestational ages were significantly lower than the FTB/NBW newborns. Mothers of low birthweight babies were younger (26.8 ± 6.6 y) than FTB/NBW mothers (29.7 ± 6.9 y), but there were no other differences in maternal or neonatal characteristics (Table 1 and Supplementary Table 1 & 2).

3.1. Neonatal hypoferrremia

Fig. 3 illustrates a profound hypoferrremia occurring in the first day after parturition. There were no babies that failed to show a hypoferrremic response and very few in whom the response was only moderate. Fig. 4 shows that the decline had already occurred by the beginning of our sampling window at 6 h post-delivery. Contrary to our initial hypothesis, there was no difference in the hypoferrremic response between any of the PTB, LBW or PTB/LBW groups and the controls. For all the babies combined, serum iron decreased over 3-fold from 23.3 ± 7.1 to 7.5 ± 4.5 $\mu\text{mol/L}$ ($P < 0.001$) and TSAT decreased from 51.7 ± 17.3 to $15.0 \pm 6.9\%$ ($P < 0.001$) (Table 2). The mean decrease in serum iron from cord to postnatal blood was remarkably consistent across all groups (between 15.2 and 16.5 $\mu\text{mol/L}$), equivalent to a range from 2.9 to 3.1-fold decrease (Table 3). Likewise, the spread of the decreases in TSAT between groups was very tight (between 35 and 39%), equivalent a 3.3 to 3.5-fold reduction. Correspondingly there was a substantial increase in UIBC in all groups indicating an enhanced ability to sequester any free iron (22.9 ± 10.5 to 43.5 ± 15.9 $\mu\text{mol/L}$, $P < 0.0001$ for all babies combined). Notably, ferritin, TIBC and haemoglobin levels increased from cord to the first neonatal bleed, and there was no change in transferrin; thus confirming that the decrease in serum iron was an active adaptation not related to altered haemodynamics.

Fig. 5 shows that all groups had similar hepcidin levels (20.9 ± 13.8 in PTB/LBW vs 19.4 ± 14.4 ng/ml in FTB/NBW) in their cord blood. However, the cord blood from PTB neonates had slightly higher TSAT values (54.8 ± 17.7 vs $50.2 \pm 16.9\%$, $P = 0.017$) and serum iron (24.4 ± 7.3 vs 22.7 ± 7.0 $\mu\text{mol/L}$, $P = 0.01$) in comparison to FTB neonates. In the postnatal venous blood samples all groups had similar hepcidin levels (37.4 ± 23.5 in PTB/LBW vs 38.9 ± 23.9 ng/ml in FTB/NBW). The venous blood from PTB and LBW neonates had slightly higher TSAT values ($16.1 \pm 8.4\%$ and $16.2 \pm 5.3\%$ vs $14.4 \pm 6.0\%$) in comparison to FTB neonates. There is no evidence, that these small differences in TSAT will translate into a

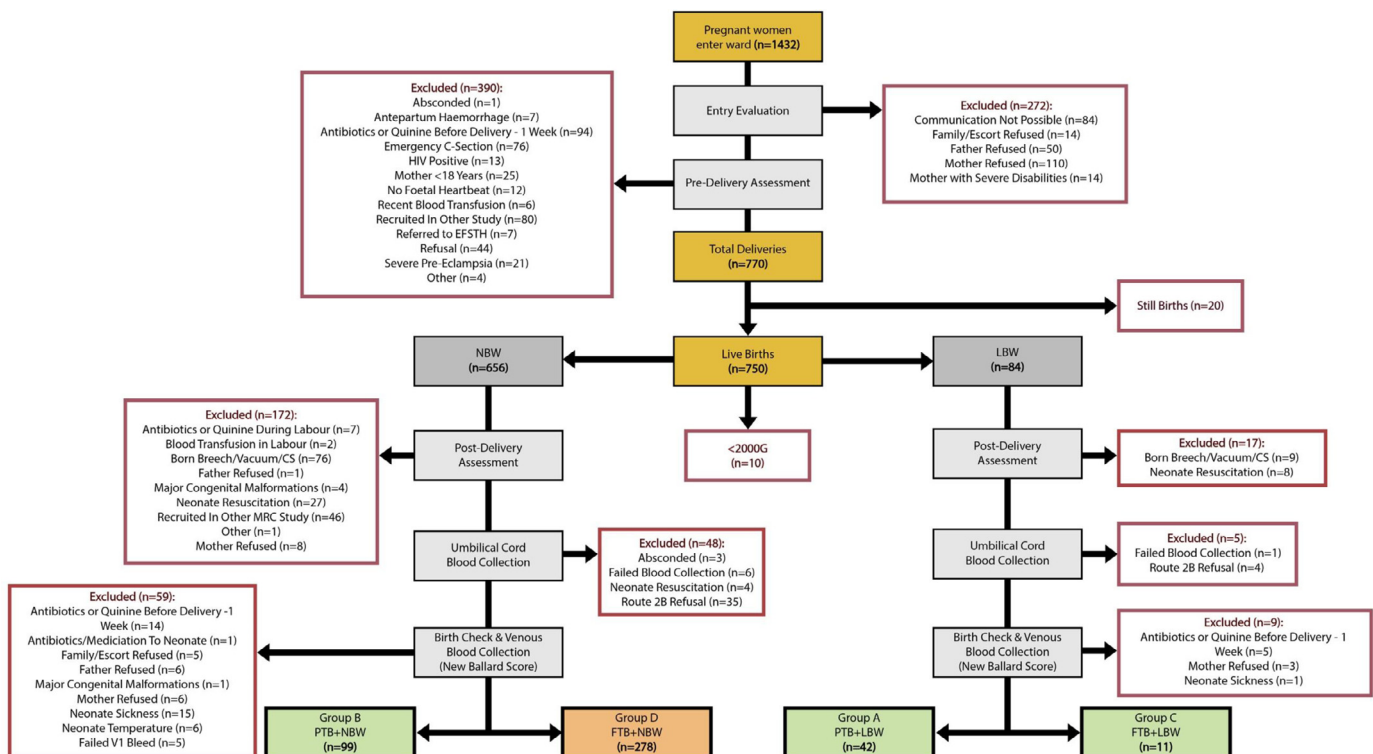


Fig. 2. CONSORT diagram for participant flow. PTB/LBW are displayed in GREEN, and FTB/NBW in ORANGE.

Table 1
Participant characteristics.

	Whole Population	FTB/NBW	PTB/LBW	<i>P</i> value	FTB	PTB	<i>P</i> value	NBW	LBW	<i>P</i> value
Number of participants (n)	430	278	152	..	289	141	..	377	53	..
Characteristic										
	Mean (± SD)									
Gestational age (Weeks)	38.1 (±2.1)	39.4 (±1.3)	35.8 (±1.1)	<0.001	39.4 (±1.3)	35.6 (±0.7)	<0.001	38.4 (±2.0)	35.9 (±1.7)	<0.001
Birthweight (g)	3092 (±453)	3299 (±368.3)	2715 (±338.5)	<0.001	3264 (±402.2)	2741 (±336.9)	<0.001	3199 (±376.3)	2338 (±117.7)	<0.001
Head circumference (cm)	34.0 (±1.2)	34.6 (±1.0)	33.0 (±0.1)	<0.001	34.5 (±1.1)	33.1 (±0.1)	<0.001	34.3 (±1.1)	32.3 (±0.8)	<0.001
Length (cm)	49.2 (±1.9)	50.0 (±1.6)	47.7 (±1.6)	<0.001	49.9 (±1.7)	47.7 (±1.6)	<0.001	49.6 (±1.7)	46.7 (±1.4)	<0.001
Maternal haemoglobin ≤7 days before delivery (g/dl)	11.6 (±1.8)	11.6 (±1.8)	11.4 (±1.8)	0.25	11.6 (±1.8)	11.5 (±1.8)	0.35	11.5 (±1.8)	11.8 (±1.7)	0.75
Age of mother (Years)	29.4 (±6.9)	29.7 (±6.9)	28.7 (±6.8)	0.16	29.6 (±7.0)	28.9 (±6.8)	0.35	29.7 (±6.9)	26.8 (±6.6)	0.003
1 min APGAR score (0–10)	9.7 (±0.7)	9.6 (±0.8)	9.7 (±0.6)	0.51	9.6 (±0.8)	9.8 (±0.6)	0.26	9.7 (±0.7)	9.6 (±0.8)	0.7
Time from admission to birth (Hours)	3.2 (±5.1)	3.2 (±5.2)	3.2 (±4.8)	0.96	3.2 (±5.2)	3.2 (±4.8)	0.94	3.2 (±5.1)	3.3 (±5.0)	0.94
Time from delivery to post-natal venous blood collection (Hours)	12.7 (±5.3)	12.7 (±5.4)	12.8 (±5.3)	0.81	12.6 (±5.3)	13.0 (±5.3)	0.47	12.8 (±5.3)	12.5 (±4.8)	0.71
	Percentage (%)									
Percentage male (%)	53.5% (224)	54.3% (151)	52.0% (79)	0.64	54.7% (158)	51.1% (72)	0.48	53.8% (203)	50.9% (27)	0.69
Percentage early term, ≥37–<38 weeks (%)	19.3% (83)	27.7% (77)	3.9% (6)	<0.001	28.7% (83)	0.0% (0)	<0.001	20.4% (77)	11.3% (6)	0.12
G6PD deficiency positive (%)	12.6% (54)	11.5% (32)	14.6% (22)	0.36	11.4% (33)	15.0% (21)	0.3	13.3% (50)	7.7% (4)	0.26
Multiple births (%)	3% (13)	2.2% (6)	4.6% (7)	0.16	2.1% (6)	4.9% (7)	0.1	2.7% (10)	5.7% (3)	0.23
Percentage of mothers on antenatal iron/folic acid (%)	82.1% (353)	81.7% (227)	82.9% (126)	0.87	81.0% (234)	84.4% (119)	0.83	83.3% (314)	73.6% (39)	0.1

Demographic, clinical and pregnancy outcome characteristics of the women and their newborns. Data are presented as arithmetic mean (± SD) and analysed by one-way analysis of variance or as a proportion (%) and analysed by Pearson χ^2 test. *P* values in bold font are considered significant based on *P* < 0.05.

clinically important difference in susceptibility to neonatal sepsis or infection. Additional comparisons can be seen in Supplementary Tables 3 & 4.

Comparisons of further iron, infection and haematological parameters in umbilical cord and venous blood can be seen in Supplementary Table 5. Analysis of the babies subdivided into groups A-D is listed in Supplementary Tables 6, 7 and 8. As anticipated, based upon the lack of difference in hypoferrremia between PTB, LBW and FTB/NBW neonates, there were no differences between the additional subgroupings of A-D.

3.2. Factors associated with the decline in serum iron and TSAT

CRP levels increased by over 10-fold between cord and postnatal blood (0.17 ± 0.6 to 2.16 ± 4.0 mg/L, *P* < 0.001) and hepcidin levels doubled (19.9 ± 14.2 to 38.4 ± 23.7 ng/ml, *P* < 0.001). Fig. 4 illustrates the timecourse of the changes.

Table 3 lists the results of regression analysis of factors associated with Day 1 serum iron. Use of standardised coefficients permits ranking in order of the effect size per standard deviation of the predictor variable. In univariate analysis CRP, hepcidin and transferrin were most strongly associated with serum iron. Note that CRP was entered as the reciprocal of the square root, so the direction of the coefficient is reversed (i.e. a high CRP was associated with a low serum iron). Haptoglobin, age of mother and birthweight were also significantly associated with serum iron. In multivariable analysis, CRP and hepcidin were the strongest correlates. The parallel analysis for TSAT showed broadly similar associations (Table 3).

3.3. Factors associated with postnatal hepcidin levels

Based on the *pre-hoc* assumption that hepcidin orchestrates the postnatal hypoferrremia, we examined the factors associated with neonatal hepcidin levels on Day 1 (Table 4). The strongest predictor was time of bleed with a negative coefficient. Examination of Fig. 4C suggests that this was because hepcidin rose very fast at, or immediately after, parturition and was already starting to decline by 6 h. Ferritin was positively associated with hepcidin, and serum iron was

negatively correlated. Surprisingly there was no evidence of a cross-sectional association between hepcidin and CRP.

4. Discussion

The rapid and profound postnatal hypoferrremia demonstrated in this study closely matches our prior findings in full-term, vaginally-delivered rural Gambian babies [14] and the results of studies elsewhere [19,20]. The 3-4-fold decrease recorded here is towards the top end of the 2–4 -fold range recorded in prior studies [14,19,20]. We have previously proposed that this may represent an evolved innate immune response designed to deprive blood-stream bacteria of iron and hence create a hostile bacteriostatic environment [14]. The current study was designed to test the hypothesis that immature or growth-restricted neonates might have a lesser ability to trigger this innate defence and that this might explain their greater susceptibility to septicemias [21]. Our hypothesis was firmly refuted. We showed that the premature and low birthweight neonates all exhibited a profound hypoferrremia during the first 24 h of life, with no detectable differences from the full-term, normal birthweight newborns. In fact, there was a remarkable similarity in the hypoferrremic response across all the study groups that underscores the efficiency of the process and supports the likelihood that it occurs by design.

The host-pathogen battle for iron has been extensively studied [22] and it has long been assumed that the hypoferrremia of the acute phase response acts as an innate defence against iron-requiring organisms. Hpcidin is a key, though not necessarily the only [23], regulator of this response [24]. Inflammatory cytokines including IL-6, IL-22 and Type-1 interferon rapidly upregulate hepcidin expression and release from the liver [25]. Hpcidin causes hypoferrremia by inhibiting the action of the transmembrane iron-exporter ferroportin in enterocytes and macrophages, thus blocking iron absorption and recycling [26]. Injection of recombinant hepcidin results in the very rapid induction of hypoferrremia in mice [27] and humans [28].

In both univariate and multivariate analysis neonatal serum iron and TSAT levels were most strongly correlated with CRP and hepcidin (Table 3) suggesting that the hypoferrremia is, at least partly, driven by an inflammatory response to the stress of the birth process and/or

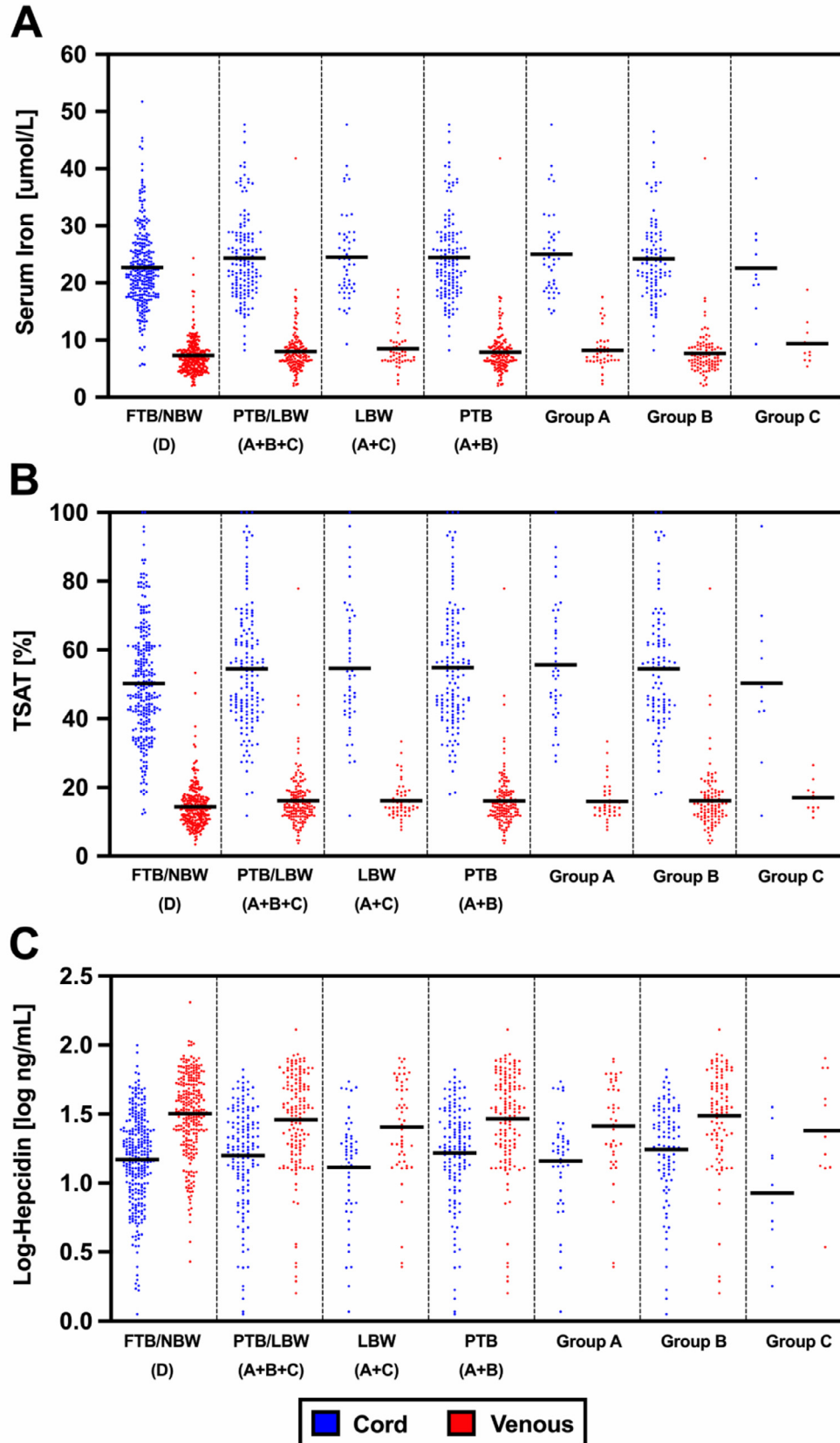


Fig. 3. Analysis of serum iron (A), TSAT (B) and hepcidin (C) in umbilical cord (BLUE) and postnatal venous blood (RED) based on all study groupings. Horizontal lines represent the arithmetic group means. FTB/NBW are FTB + NBW. PTB/LBW are FTB + LBW, PTB + NBW and PTB + LBW neonates. All group comparisons between cord and venous blood are statistically significant ($P < 0.001$). Hepcidin displayed as \log_{10} .

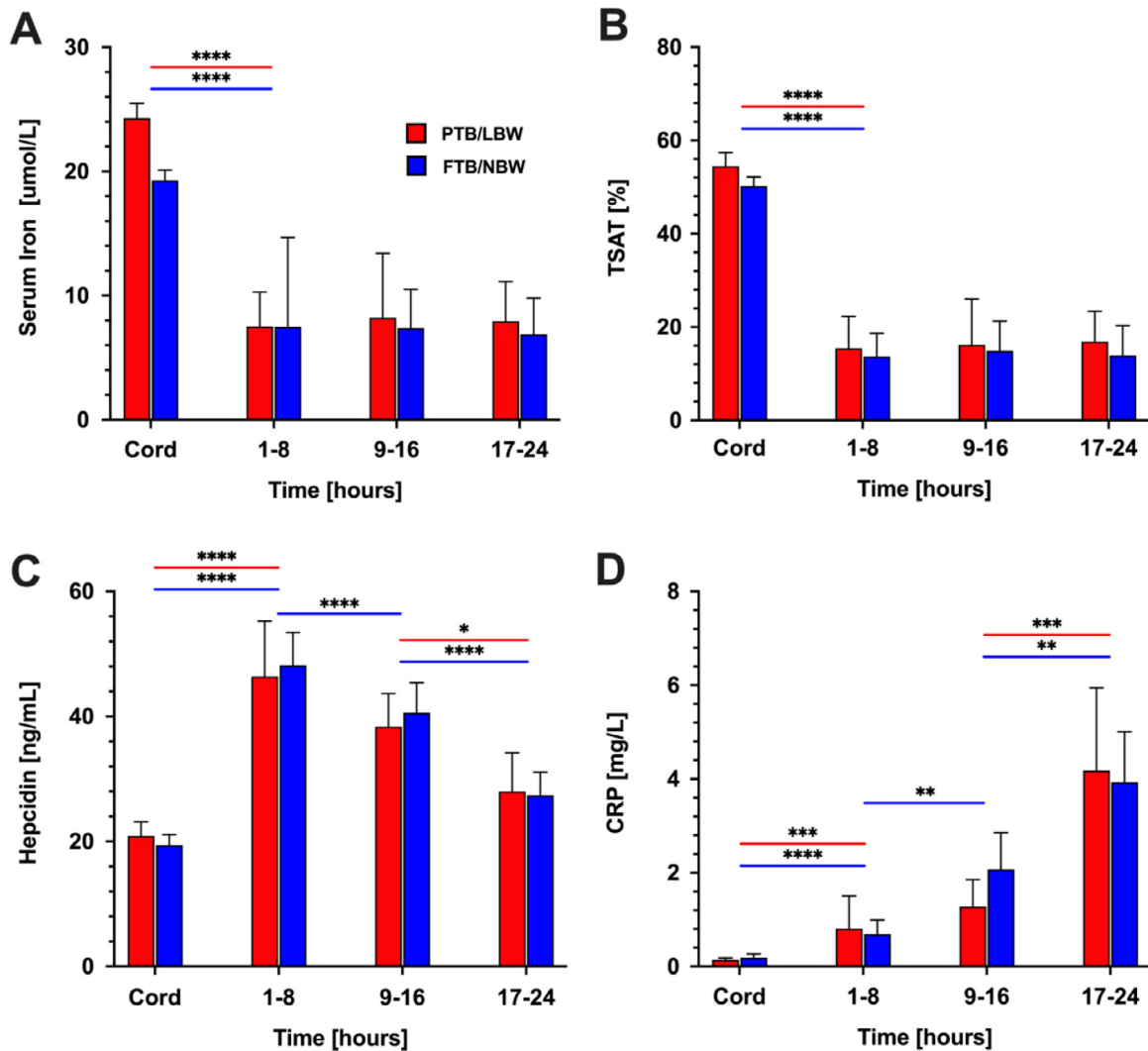


Fig. 4. Timecourse of the changes in serum iron (A), TSAT (B), hepcidin (C) and CRP (D) in the first day of life. Means \pm 95% CI. PTB/LBW are RED columns and significance lines. FTB/NBW are BLUE columns and significance lines. Columns are plotted according to mean time of bleed for the categories 0, 1–8, 9–16 and 17–24 h. **** = $P < 0.0001$, *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$. No significance line = $P > 0.05$.

the exposure of the newborn to vaginal and/or gastrointestinal microorganisms not previously encountered *in utero*. The modest proportion of variance explained in the full multivariate model (29% for serum iron and 22% for TSAT) might reflect the fact that both measures have been suppressed to close to their physiological lower limit and hence display a limited range. It is also possible that hepcidin-independent mechanisms, driven by effector molecules unmeasured in this study, are playing an additional role [23]. A full examination of the mechanism responsible for the hypoferraemia would probably require studies in an animal model (e.g. neonatal piglets).

Using *ex vivo* assays with sentinel bacteria, we have previously demonstrated that low TSAT values in serum from adults [29] and neonates [14] exerts a powerful bacteriostatic effect. In the neonatal study, growth rates of *Escherichia coli*, *Streptococcus pneumoniae*, *Streptococcus agalactiae* and *Staphylococcus aureus*, were highly significantly lower in neonatal serum than in cord serum and for each organism growth rates were significantly associated with TSAT. *S. aureus* was least responsive, possibly reflecting its ability to utilise haem iron, though it was still clearly influenced by transferrin saturation. *E. coli* was most responsive, which may explain why intramuscular iron administration to Polynesian neonates increased septicaemia rates with a major shift towards *E. coli* as the most frequently identified cause [12]. The hypoferraemia observed in the

current hospital-based study (in both PTB/LBW and FTB/NBW babies) was more profound than we previously observed in rural home deliveries [14] (TSAT declined to 15% versus 24% in rural babies) and hence the bacteriostatic effect would be expected to be even greater.

There are several strengths and limitations to our study. The large sample size allows us to confidently exclude any clinically-meaningful differences in the postnatal response of preterm and low birthweight babies compared to the full-term normal birthweight neonates. By protocol, we did not recruit mothers with complex medical histories (e.g. pre-eclampsia, antepartum haemorrhage or antenatal infection) or sick babies (e.g. with birth asphyxia or suspected sepsis). We did not recruit newborns born <32 completed weeks gestation and/or <2000 g birthweight, or those delivered via c-section, vacuum or forceps. We cannot speculate whether similar hypoferraemic responses would occur in such cases, but reciprocally we can conclude that the responses we observed are a characteristic feature of normal human birth and were not elicited by pathological circumstances. In order to allow mothers to recover from their delivery and the neonates to be stabilised and checked for inclusion, we constrained our first postnatal blood draw to after 6 h post-delivery. With many samples collected soon after this point, we have demonstrated that the reduction in circulating iron levels occurs very rapidly but we cannot state how rapidly. We did not measure pro-

Table 2
Iron Parameters (cord vs venous sample) for FTB/NBW, PTB/LBW, PTB and LBW neonates.

Sample Type	Whole Population					FTB/NBW					PTB/LBW					Preterm Birth (PTB)					Low Birthweight (LBW)				
	n	Cord	n	Venous	P value	n	Cord	n	Venous	P value	n	Cord	n	Venous	P value	n	Cord	n	Venous	P value	n	Cord	n	Venous	P value
Serum iron ($\mu\text{mol/L}$)	425	23.3 (± 7.1)	421	7.5 (± 4.5)	<0.001	275	22.7 (± 7.0)	271	7.3 (± 4.6)	<0.001	150	24.3 (± 7.3)	150	8.0 (± 4.1)	<0.001	140	24.4 (± 7.3)	139	7.8 (± 4.1)	<0.001	51	24.5 (± 7.7)	51	8.5 (± 3.4)	<0.001
UIBC ($\mu\text{mol/L}$)	423	22.9 (± 10.5)	420	43.5 (± 15.9)	<0.001	273	23.7 (± 10.4)	271	44.1 (± 18.0)	<0.001	150	21.5 (± 10.6)	149	42.2 (± 10.9)	<0.001	140	21.2 (± 9.8)	138	42.0 (± 10.9)	<0.001	51	22.1 (± 12.2)	50	43.8 (± 10.2)	<0.001
TIBC ($\mu\text{mol/L}$)	423	46.1 (± 8.0)	420	51.0 (± 17.9)	<0.001	273	46.3 (± 8.1)	271	51.4 (± 20.7)	<0.001	150	45.8 (± 7.8)	149	50.2 (± 11.3)	<0.001	140	45.6 (± 7.4)	138	49.8 (± 11.1)	<0.001	51	46.6 (± 9.3)	50	52.3 (± 11.9)	<0.001
Transferrin (g/L)	426	1.96 (± 0.3)	423	1.99 (± 0.3)	0.003	275	1.98 (± 0.3)	273	1.98 (± 0.3)	0.8335	151	1.93 (± 0.3)	150	2.00 (± 0.3)	<0.001	141	1.92 (± 0.3)	139	1.99 (± 0.3)	<0.001	52	1.94 (± 0.4)	51	2.02 (± 0.4)	0.0024
TSAT (%)	423	51.7 (± 17.3)	420	15.0 (± 6.9)	<0.001	273	50.2 (± 16.7)	271	14.4 (± 6.1)	<0.001	150	54.5 (± 18.0)	149	16.2 (± 8.2)	<0.001	140	54.8 (± 17.7)	138	16.1 (± 8.4)	<0.001	51	54.6 (± 18.7)	50	16.2 (± 5.3)	<0.001
Serum ferritin (ug/L)	426	210.5 (± 157.8)	415	381.2 (± 297.6)	<0.001	275	212.6 (± 157.7)	271	393.9 (± 312.6)	<0.001	151	206.6 (± 158.5)	149	358.6 (± 268.1)	<0.001	141	206.8 (± 154.8)	138	355.7 (± 259.6)	<0.001	52	192.1 (± 179.5)	51	366.2 (± 343.8)	<0.001
Serum AGP (g/L)	426	0.18 (± 0.1)	423	0.24 (± 0.2)	<0.001	275	0.19 (± 0.1)	273	0.25 (± 0.2)	<0.001	151	0.17 (± 0.1)	150	0.23 (± 0.2)	<0.001	141	0.16 (± 0.2)	139	0.22 (± 0.2)	<0.001	52	0.16 (± 0.1)	51	0.21 (± 0.16)	<0.001
Serum CRP (mg/L)	425	0.17 (± 0.6)	422	2.16 (± 4.0)	<0.001	275	0.19 (± 0.7)	273	2.27 (± 4.1)	<0.001	150	0.14 (± 0.2)	149	1.96 (± 3.8)	<0.001	140	0.14 (± 0.2)	138	1.99 (± 3.9)	<0.001	51	0.10 (± 0.07)	51	1.14 (± 1.8)	0.002
>5mg/L	2	0.5%	54	12.8%	<0.001	2	0.7%	37	13.6%	<0.001	0	0.0%	17	11.4%	<0.001	0	0.0%	16	11.6%	<0.001	0	0.0%	4	7.8%	0.041
>3mg/L	3	0.7%	83	19.7%	<0.001	3	1.0%	59	21.6%	<0.001	0	0.0%	24	16.1%	<0.001	0	0.0%	23	16.7%	<0.001	0	0.0%	6	11.8%	0.011
Serum hepcidin (ng/ml)	425	19.9 (± 14.2)	417	38.4 (± 23.7)	<0.001	277	19.4 (± 14.4)	270	38.9 (± 23.9)	<0.001	148	20.9 (± 13.8)	147	37.4 (± 23.5)	<0.001	138	21.5 (± 13.9)	136	37.7 (± 23.3)	<0.001	52	17.6 (± 13.0)	51	33.7 (± 22.2)	<0.001
Haemoglobin (g/dl)	414	15.3 (± 2.5)	423	19.4 (± 3.1)	<0.001	270	15.1 (± 2.3)	272	19.1 (± 2.9)	<0.001	144	15.6 (± 2.7)	151	19.9 (± 3.3)	<0.001	136	15.6 (± 2.6)	140	19.8 (± 3.3)	<0.001	48	16.3 (± 3.1)	52	20.7 (± 3.5)	<0.001

Data are presented as mean (\pm SD) and analysed by one-way analysis of variance. *P* values in bold font are considered significant based on $P < 0.05$. Skewed variables were transformed as follows: \log_{10} (serum iron), $1/\sqrt{\text{TIBC}}$, $\sqrt{\text{ferritin}}$, $1/\sqrt{\text{CRP}}$, \log_{10} (hepcidin).

Table 3

Linear regression of factors associated with postnatal serum iron and TSAT.

Predictor	Coefficient	Standard error	Standardised coefficient	P value	R2 (%)
Univariate Analysis - Serum Iron					
CRP ^a	0.135	0.023	0.258	<0.0001	15.5
Hepcidin ^b	-0.139	0.025	-0.249	<0.0001	14.2
Transferrin	0.273	0.056	0.222	<0.0001	13.7
Haptoglobin	-1.21	0.303	-0.184	<0.0001	12.2
Age of mother	-0.009	0.002	-0.152	0.001	11.1
Birthweight	-0.0001	-0.00,004	-0.149	0.001	11
Multivariate Analysis - Serum Iron					
CRP ^a	0.124	0.022	0.239	<0.0001	29.2
Hepcidin ^b	-0.11	0.025	-0.198	<0.0001	
Transferrin	0.23	0.055	0.186	<0.0001	
Age of mother	-0.008	0.002	-0.143	0.001	
Haptoglobin	-0.741	0.28	-0.114	0.008	
Birthweight	-0.00,009	0.00,003	-0.104	0.016	
Univariate Analysis - Transferrin Saturation (TSAT)					
CRP ^a	0.153	0.023	0.306	<0.0001	11.7
Haptoglobin	-1.14	0.302	-0.181	<0.0001	5.7
Hepcidin ^b	-0.085	0.025	-0.16	0.001	4.8
Birthweight	-0.0001	0.00,004	-0.153	0.001	4.7
Age of mother	-0.008	0.002	-0.151	0.002	4.7
Multivariate Analysis - Transferrin Saturation (TSAT)					
CRP ^a	0.139	0.022	0.285	<0.0001	21.8
Hepcidin ^b	-0.093	0.024	-0.253	<0.0001	
Ferritin	-0.008	0.002	0.187	<0.0001	
Age of mother	-0.68	0.285	-0.161	<0.0001	

All regressions were adjusted for 4 grades of visually assessed haemolysis. Ranked according to their standardised coefficients calculated in STATA.

^a 1/sqrt(CRP).

^b log₁₀ (hepcidin).

inflammatory cytokines (e.g. IL-6 and IL-22) which might have provided additional insights into the mechanisms eliciting hypoferraemia. Similarly, we did not attempt to address any possible hepcidin-independent mechanisms because the putative mediators remain unknown. Such studies may require animal models.

In conclusion, our results suggest that the innate postnatal iron restriction strategy in the first hours of life has evolved as an intrinsic mechanism to protect neonates from common pathogens and/or free-radical damage, and occurs regardless of gestational age or birthweight. This hypoferraemia is at least partly mediated by the hormone, hepcidin. The trigger mechanism, relation to maternal iron status, and its effect on susceptibility to systemic bacterial infections require further investigation.

Our results highlight the importance of hypoferraemia as a conserved mechanism of protection, and prompt further research into the use of iron restriction as a transient bacteriostatic mechanism to limit bacterial growth and virulence in other instances of infection. Hypoferraemia can slow the multiplication of bacterial pathogens [29], which in combination with antibiotics, could allow enough time for the adaptive immune system to fight the infection. Augmentation of innate immunity in neonates and other at-risk groups (elderly or immunocompromised) might be achieved by the use of small molecule orally-administered mini-hepcidins currently under development as hepcidin agonists [28,30].

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

We thank Kanifing Municipal Council (KMC) and Kanifing General Hospital (KGH) for their support during the study.

Funding

The research was undertaken with a research grant provided by the Bill & Melinda Gates Foundation (OPP1152353). The funding agency had no role in the design and conduct of the study, and did not have any in the collection, management, analyses or interpretation of the data nor in the preparation, review, or approval of the manuscript. The Nutrition Theme of the MRC Unit The Gambia at LSHTM are supported by core funding MC-A760-5QX00 to the MRC Unit The Gambia/MRC International Nutrition Group by the UK MRC and the UK Department for the International Development (DFID) under the MRC/DFID Concordat agreement.

Availability of Data and Materials

All data will be made available to researchers upon reasonable request to the study PI and clearance by the MRCG Scientific Coordinating and Ethics Committees.

Authors' Contributions

JHC is PhD student with MRCG at LSHTM. He contributed to the development of the protocols, study management, field data collection, development of the data analysis plan, conducting the data analysis and drafting of the manuscript.

OJ is a research clinician with MRCG at LSHTM. He contributed to the development of the protocols, study management, field data collection and patient care.

NIM is a statistician at MRCG at LSHTM. He wrote the data analysis plan in consultation with JC, CC and AMP.

SRG was a visiting B.Sc. student from Cardiff University. He contributed to the laboratory analyses and field data collection.

BJBT is a laboratory technician with MRCG at LSHTM. He contributed to the laboratory analyses and field data collection.

AMP is a Professor of International Nutrition at LSHTM and Nutrition Theme Leader at MRCG at LSHTM. He conceived the study, obtained the funding, contributed to the development of the protocols, the data analysis plan, conducting the data analysis and the drafting of the manuscript.

CC is Senior Investigator Scientist at MRCG at LSHTM and Group Leader for Iron, Infection and Anaemia. She was the PI on the trial, conceived the study, and obtained the funding. She was responsible for the overall development of the protocols, the data analysis plan, conducting the data analysis and the drafting of the manuscript.

All authors reviewed the final manuscript prior to submission.

Consent for Publication

Not applicable.

Ethics Approval and Consent to Participate

This study has been approved by The Gambia Government/MRC Joint Ethics Committee (no. SCC1525) and London School of Hygiene and Tropical Medicine Ethics Committee (ref no. 14316). The study was conducted according to Good Clinical Practice (GCP) standards. The study procedures were explained to the neonate's mother/guardians orally and in writing. A neonate was only recruited into the study after the written, informed consent was provided by the mother/guardian.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.102613.

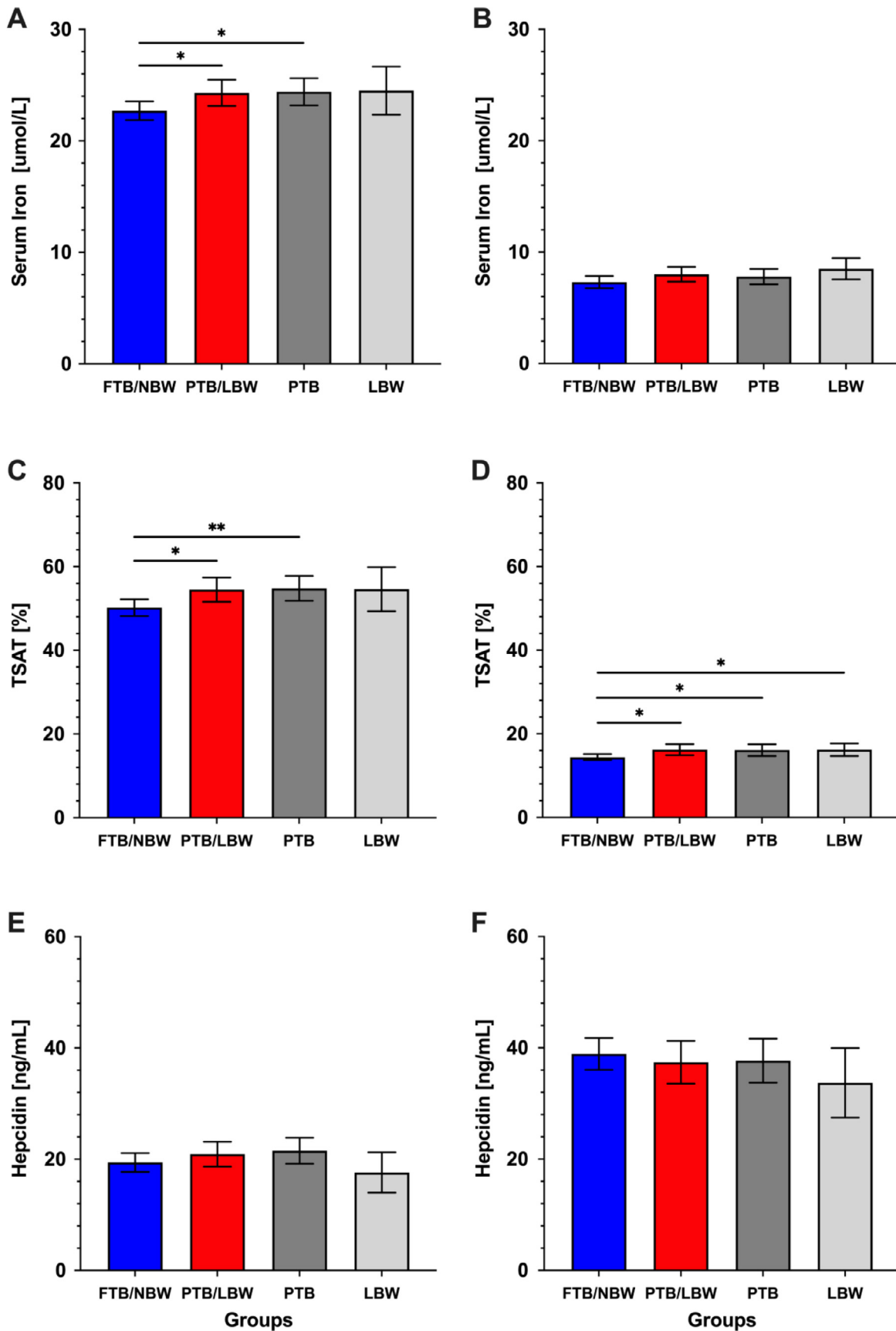


Fig. 5. Comparisons of serum iron (A = cord, B = venous), TSAT (C = cord, D = venous) and hepcidin (E = cord, F = venous) in cord and postnatal venous blood. Means \pm 95% CI. FTB/NBW are BLUE columns. PTB/LBW are RED columns. PTB are DARK GREY columns. LBW are LIGHT GREY columns. Significance lines represent the comparison of PTB/LBW, PTB or LBW groups to the FTB/NBW group. ** = $P < 0.01$, * = $P < 0.05$. No significance line = $P > 0.05$.

Table 4
Linear regression of factors associated with postnatal serum hepcidin.

Predictor	Coefficient	Standard error	Standardised coefficient	P value	R ² (%)
Univariate analysis - Serum hepcidin					
Ferritin^a	0.039	0.0005	0.354	<0.0001	14
Time of bleed	-0.0007	0.0001	-0.321	<0.0001	12
Transferrin	0.706	0.104	-0.318	<0.0001	11.5
Serum iron^b	-0.479	0.089	-0.267	<0.0001	8.2
sTfR^c	-0.36	0.092	-0.189	0.001	5
Multivariate analysis - Serum hepcidin					
Time of bleed	-0.0008	0.00009	-0.354	<0.0001	36.1
Ferritin^a	0.035	0.004	-0.319	<0.0001	
Serum iron^b	-0.413	0.078	-0.232	<0.0001	
sTfR^c	-0.286	0.086	-0.15	0.001	
Transferrin	-0.218	0.111	-0.1	0.05	

All regressions were adjusted for 4 grades of visually assessed haemolysis. Ranked according to their standardised regression coefficients calculated in STATA.

^a sqrt(ferritin).

^b log₁₀(serum iron).

^c sqrt(sTfR).

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