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Iron and Infection: Neonatal Iron Transition

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Of the

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Faculty of Epidemiology & Population Health

LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE

Funded By: Bill and Melinda Gates Foundation

Research Group Affiliations: MRC International Nutrition Group & MRC Unit The Gambia at LSHTM
STATEMENT OF OWN WORK

I, James H. Cross, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

James H. Cross

13th December 2019
ACKNOWLEDGEMENTS

I am incredibly grateful for the four years I have spent being able to conduct research at MRC Unit The Gambia at LSHTM.

My supervisor, Carla Cerami, has taught me a vast number of lessons about the world of work over the last six years. The care, compassion, and energy you have given me over half a decade have inspired me to build a career in global health research, with an emphasis on giving back to society and focusing on the people that need it most. Your scientific and technical advice has led me to try and ask the correct questions and work in a manner that gets things done. These are attitudes, which I hope to continue in my future career. I am inspired by your courage and spirit toward pushing a ‘can do’ attitude. Your constant availability at any day or time, your sense of humour and friendship have been invaluable when times became difficult. Thank you for all you have given me, and I hope you are proud of what we have both achieved working together.

To our theme leader and my associate supervisor, Andrew Prentice, you have overseen my work for my entire time at MRCG. Your caring comments and advice on aspects that relate to work, my career, research and life have helped me every step of the way. Thank you for accepting me into this wonderful family-like research group and supporting me in my transition from an undergraduate teenager to an individual that hopes to continue a career in global health research. Your leadership and mentorship to me and others has been inspiring.

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Touray, Sheriffo Jarjou, Nuradin Ibrahim Mohammed and Bakary Sonko. Thank you for the endless hours of high-quality work you have completed for this study.

Malcolm Velasco and Santiago Rayment Gomez, thank you for coming to The Gambia and becoming part of our team. Your hard work and everyday support were vital to the success of this study, my thesis and my sanity!

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I want to conclude with a special mention of appreciation to my grandmother, Ann. Nan, your strength, passion and determination, along with your honest love of people, life and common sense have been guiding lights for me during this task. Our whole family would not be here without you, you’re a role model for me, and I hope that you can rest and be content with what I have currently achieved, and my plans for the future.

**Note:** Thanks to Dr Noémi Roy and Dr Gladys Latunde-Dada for their guidance and advice during the examination process, along with making my viva as enjoyable and thought-provoking as possible.
ABSTRACT

Neonates, particularly those born preterm (PTB) and low birthweight (LBW), are especially susceptible to bacterial infections that cause an estimated 225,000 deaths annually. Iron is a vital substrate for the most common organisms causing septicaemia. Full-term babies elicit an immediate post-natal hypoferremia assumed to have evolved as an innate defence. This thesis aimed to test whether preterm and low birthweight newborns are capable of a similar response.

A longitudinal observational study was conducted in 430 hospital-delivered Gambian babies. Demographic, anthropometric and haematological data were collected from 152 babies who were either PTB (between ≥32-<37 weeks gestational age) and/or LBW (<2500g) (PTB/LBW) and 278 full-term, normal-weight babies (FTB/NBW). Blood was sampled from the umbilical cord and matched venous blood samples from all neonates between 6-24 hours after delivery. An additional matched venous blood sample was taken from all full-term, normal birth weight newborns between 24-192 hours of life. In both FTB/NBW and PTB/LBW neonates, serum iron decreased 3-fold compared to umbilical blood concentrations within 12h of delivery (23·3±0·35 vs 7·5±0·22 ng/ml, P<0.001, n=425). Hepcidin levels doubled (27·0±0·96 vs 52·9±1·63 ng/ml, P<0·001, n=425). In FTB/NBW neonates, a steady increase in serum iron and TSAT follows (to 16.5±3.9µmol/L and 36.7±9.2% respectively by 136-192hrs post-delivery), even in the presence of relatively high serum hepcidin levels (45.2±19.1ng/ml) suggestive of hepcidin resistance possibly caused by iron saturation of macrophages.

Our findings confirm that a very rapid hypoferremia occurs in the early hours of post-natal life with evidence that it is mediated by an increase in hepcidin. The strength and consistency of this effect in all neonates indicates that it may have evolved as an innate immune response designed to protect newborns from bacterial septicaemia.
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<tr>
<td>AGA</td>
<td>Appropriate for Gestational Age</td>
</tr>
<tr>
<td>AGP</td>
<td>Alpha 1-Acid Glycoprotein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>APP</td>
<td>Antimicrobial Protein and Peptides</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette–Guérin</td>
</tr>
<tr>
<td>BMGF</td>
<td>Bill &amp; Melinda Gates Foundation</td>
</tr>
<tr>
<td>BMP6</td>
<td>Bone Morphogenetic Protein 6</td>
</tr>
<tr>
<td>CDA</td>
<td>Cord Arterial Blood</td>
</tr>
<tr>
<td>CDV</td>
<td>Cord Venous Blood</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CRE</td>
<td>Carbapenem-Resistant Enterobacteriaceae</td>
</tr>
<tr>
<td>CRF</td>
<td>Case Report Form</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CT Scan</td>
<td>Computerized Tomography Scan</td>
</tr>
<tr>
<td>CyTOF</td>
<td>Cytometry by Time Of Flight</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DFID</td>
<td>Department for International Development</td>
</tr>
<tr>
<td>DHS</td>
<td>Demographic and Health Survey</td>
</tr>
<tr>
<td>DMT-1</td>
<td>Divalent Metal Transporter 1</td>
</tr>
<tr>
<td>ECOWAS</td>
<td>Economic Community of West African States</td>
</tr>
<tr>
<td>eCRF</td>
<td>Electronic Case Report Form</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EFSTH</td>
<td>Edward Francis Small Teaching Hospital</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>--------------------------------</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assays</td>
</tr>
<tr>
<td>EONS</td>
<td>Early-Onset Neonatal Sepsis</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended Spectrum Beta-Lactamases</td>
</tr>
<tr>
<td>FTB</td>
<td>Full-Term Birth</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-Colony Stimulating Factor</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GBS</td>
<td>Group B Streptococci</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practice</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<tr>
<td>HICs</td>
<td>High Income Countries</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>ID</td>
<td>Identification</td>
</tr>
<tr>
<td>IDE</td>
<td>Integrated Development Environment</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor-1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-22</td>
<td>Interleukin 22</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IMCI</td>
<td>Integrated Management of Childhood Illness</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KGH</td>
<td>Kanifing General Hospital</td>
</tr>
<tr>
<td>KMC</td>
<td>Kangaroo Mother Care</td>
</tr>
<tr>
<td>LBW</td>
<td>Low Birth Weight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SBA</td>
<td>Skilled Birth Attendant</td>
</tr>
<tr>
<td>SCC</td>
<td>Scientific Coordinating Committee</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for Gestational Age</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard Operating Procedure</td>
</tr>
<tr>
<td>SSPs</td>
<td>Study Specific Protocols</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>sTfR</td>
<td>Soluble Transferrin Receptor</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TFR1</td>
<td>Transferrin Receptor 1</td>
</tr>
<tr>
<td>TIBC</td>
<td>Total Iron-Binding Capacity</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TSAT</td>
<td>Transferrin Saturation</td>
</tr>
<tr>
<td>UIBC</td>
<td>Unbound Iron-Binding Capacity</td>
</tr>
<tr>
<td>V1</td>
<td>Venous Bleed 1</td>
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<tr>
<td>V2</td>
<td>Venous Bleed 2</td>
</tr>
<tr>
<td>V3</td>
<td>Venous Bleed 3</td>
</tr>
<tr>
<td>V4</td>
<td>Venous Bleed 4</td>
</tr>
<tr>
<td>VLBW</td>
<td>Very Low Birthweight</td>
</tr>
<tr>
<td>VPTB</td>
<td>Very Preterm Birth</td>
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<tr>
<td>WBC</td>
<td>White Blood Cell</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>YICSS</td>
<td>Young Infants Clinical Signs Studies</td>
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</table>
GLOSSARY OF KEY TERMS

Hypoferremia: A deficiency of iron in the blood.

Nutritional immunity: To prevent infection from pathogenic organisms by restricting access to essential metals.

Neonatal sepsis: A type of infection in neonates that specifically refers to the presence of bacteria in the blood stream (due to meningitis, pneumonia, pyelonephritis or gastroenteritis) in the setting of fever. Early-onset (appearing 0-3 days of life) and late-onset (appearing >4 days of life) are further levels of categorisation.

Bacteriostatic: Substance or process that reversibly inhibits growth or reproduction of bacteria. Different from bactericidal (capable of killing bacteria outright).

Hepcidin: A 25-amino acid peptide (HAMP gene) exclusively synthesized by the liver that is the main regulator of systemic iron metabolism. Its primary role is to inhibit iron efflux into the blood by binding to the transmembrane protein, ferroportin.
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin saturation:</td>
<td>The percentage of transferrin molecules that are bound to two iron ions in the ferric form (Fe$^{3+}$). This is a ratio of serum iron concentration and the total iron-binding capacity (TIBC) expressed as a percentage.</td>
</tr>
<tr>
<td>TIBC (total iron-binding capacity):</td>
<td>An indirect measure of the amount of total serum transferrin (apotransferrin, monotransferrin, diferric transferrin) concentration in the circulation.</td>
</tr>
<tr>
<td>Post-natal:</td>
<td>The period immediately after the birth of a child.</td>
</tr>
<tr>
<td>Preterm birth:</td>
<td>Babies born alive before 37 weeks of pregnancy are completed. Sub-categories included extremely preterm (&lt;28 weeks), very preterm (28-32 weeks) and moderate to late preterm (32-37 weeks).</td>
</tr>
<tr>
<td>Low birthweight:</td>
<td>Babies born with a birth weight of &lt;2500g.</td>
</tr>
</tbody>
</table>
Chapter 1 – Background to Thesis

Summary of Chapter

In this chapter, I briefly introduce the background information and rationale to this thesis. This involves discussing the previous work completed by the HYPO-G Study at MRCG Unit The Gambia by lead investigator Dr Sarah Prentice, which laid the foundations for this further research. I then define the aims, objectives and structure of my research degree. Additionally, I describe the role I played in all aspects of the study, with further information on the research degree timeline. Funding and the subsequent publications produced are also detailed. This chapter then reviews the high-level background literature to this thesis, including research into neonatal mortality linked to infection, the transition to extrauterine life and the role iron plays in infectious disease.
1.1 Introduction and Rationale

1.1.1 Why study neonatal iron metabolism?

Neonatal sepsis is the third highest cause of death globally, accounting for 225,000 deaths each year.\textsuperscript{1} Neonatal infections cause an estimated 23% of all neonatal deaths, with neonatal sepsis alone accounting for 15%. The increasing global threat of antimicrobial resistance will no doubt exacerbate these figures in the future.\textsuperscript{2}

Humans undergo the most complex physiological adaption in their life during the transition from a semi-allogeneic, protected foetal setting to a microbe-rich extrauterine environment.\textsuperscript{3,4} The initial mass bacterial colonisation of mucosa in the digestive, respiratory, urogenital tracts, as well as the skin\textsuperscript{5,6} occurs during the very early neonatal period and can positively affect gut maturation,\textsuperscript{7} metabolic homeostasis and immune function\textsuperscript{8–10} in early life and beyond. Early-onset neonatal sepsis (EONS) occurs in <72 hours of life, with most causative pathogens being transmitted vertically from mother to infant before or during delivery.\textsuperscript{11}

Iron is a cofactor in numerous metabolic pathways that are critical for the human host as well as most pathogens, making it an important mineral in the host-pathogen battle for resources.\textsuperscript{12} Therefore, systemic iron distribution is usually strictly regulated.\textsuperscript{13} The assimilation of iron from its human host via a plethora of molecular mechanisms (e.g. iron transporters and siderophores\textsuperscript{14}), results in increased growth\textsuperscript{15} and virulence\textsuperscript{16} of many human pathogens. For example, individuals with chronically high iron states (e.g. hemochromatosis), not only have increased free radical redox damage,\textsuperscript{17} but an enhanced risk of infection, especially from iron-dependent species of bacteria.\textsuperscript{18,19}
The hormone hepcidin is the primary regulator of iron homeostasis.²⁰ Host inflammatory mediators, IL-6,²¹ IL-22²² and IFN-α,²³ have been observed to increase the transcription of hepcidin through several Toll-like receptor (TLR) ligands²⁴ and STAT3 signalling.²⁵,²⁶ Hepcidin binds to the transmembrane protein ferroportin in macrophages, hepatocytes and enterocytes, resulting in its internalization and degradation.²⁷–²⁹ Consequently, enteric absorption from dietary iron is reduced, and iron is sequestered in macrophages, which causes a reduction in the extracellular iron concentration. Research carried out in animal models has shown that when the innate immune system is activated, it elicits a hypoferremic response which then limits the risk and severity of bacterial infections.³⁰–³²

Previous research by our group has suggested that the hepcidin concentration in full-term neonates increases over the first hours of life, which is linked to a significant reduction in serum iron and transferrin saturation over a similar time period.³³ Nevertheless, an accurate and reliable representation of iron homeostasis in all neonates (in particular preterm and/or low birthweight) is still lacking. The process of reviewing this mechanism between multiple studies was previously made harder by the lack of inter-assay standardisation of hepcidin ELISA assays until the recent research conducted by Van der Vorm et al.³⁴

1.1.2 What has previous work by our group provided towards the field?

Before the start of this research degree, previous research conducted at MRC Unit The Gambia showed the presence of hepcidin-mediated neonatal hypoferremia in vaginally-delivered healthy full-term neonates in rural Kiang Keneba.³³ This proof-of-concept observational study observed the following key learning points:

- Normal healthy term neonates display a rapid suppression of serum iron and TSAT within the first 6-12 hours post-partum.
• This response is thought to last for 2-3 days, followed by a slow increase up to 92 hours.

• There is a strong negative correlation between hepcidin and serum iron during this period, suggesting that hepcidin regulates this response through the redistribution of iron into circulating macrophages.

• These findings were correlated to levels of IL-6, suggesting that inflammatory stimulation of hepcidin is in part, the driving mechanism of systemic hypoferremia.

• Growth rates of the ex vivo micro cultured neonatal sepsis causing bacteria Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae and Streptococcus agalactiae were significantly lower in neonate venous serum (6-24 hr time period) as compared to cord serum.

• Each organism’s growth rates was significantly associated with TSAT level in the serum.

To conclude, the evidence to date suggests that neonatal hypoferremia takes place immediately post-partum in healthy term neonates, but there is limited research into the effects of gestational age and birthweight on this method of protection. Our research group pilot data suggests this change in iron distribution could provide a bacteriostatic method of protection to neonates in the first hours of life, when immune defences are immature. The rationale to conduct my research was to confirm this earlier discovery of acute neonatal hypoferremia in full-term neonates in another population, and to test whether preterm (PTB) and/or low birthweight (LBW) infants are equally effective in sequestering iron away from the bloodstream. If this was not the case, we aimed to describe the proportion of such babies that may show ineffective or blunted hypoferremia.
1.2 PhD Aims and Objectives

Overall Aim: To characterise the effects that gestational age and birthweight have on iron distribution immediately after birth and during the first week of life in healthy Gambian neonates.

Specific Objectives:

- Conduct a comprehensive review of hepcidin, serum iron and TSAT concentration in neonates in umbilical cord and venous blood (up to 1 month of age).

- Characterise iron metabolism in full-term, preterm and low birthweight neonates at birth and during the first 24 hours of life. Do premature and/or low birthweight neonates have a defect in their ability to sequester iron at 6-24 hours after birth in comparison to full-term newborns with normal birthweight?

- Describe how concentrations of hepcidin, serum iron and TSAT change in full-term neonates (only) at birth and during the first seven days of life.

Figure 1.1 PhD study logo (NeoInnate Study).
The PhD conceptional framework is displayed in Figure 1.2. This PhD is split into preparation (A) and observation (B) phases. B is itself divided into two sections, with Part B1 focusing on a comparison of iron homeostasis in first hours of life between full-term, normal birthweight (FTB+NBW) neonates and preterm (PTB) and/or low birthweight (LBW) neonates. Part B2 focuses on iron and infection parameters in FTB+NBW neonates after the first week of life only.

![PhD conceptional framework](image)

**Figure 1.2** PhD conceptional framework.

### 1.3 PhD Outline

This thesis is formatted in a research paper style in accordance with the London School of Hygiene & Tropical Medicine write-up regulations. The manuscripts and published articles are included without any adaption for this thesis. As a result, repetition is common between chapters in regard to materials and methods. This has been reduced with the production of a protocol paper (Chapter 4). If supplementary material is part of the manuscripts or publications, it has been added at the end of the chapter. Each chapter begins with a summary page outlining the content of each chapter.
This thesis contains seven chapters, one of which is a published protocol paper (Chapter 4), one is a published original research publication (Chapter 5) and two submitted manuscripts (Chapter 3 and 6). An outline of all the chapters is as follows:

**Chapter 1:** An introduction to the thesis content and structure, with detailed information on the PhD aims, objectives, rationale, candidate involvement, publications and timeframe.

**Chapter 2:** An introduction to the study setting at Kanifing General Hospital and the surrounding communities.

**Chapter 3:** A prepared (submitted) manuscript containing a literature review of the concentrations of hepcidin, serum iron and TSAT in newborn blood in the first weeks of life.

**Chapter 4:** A published research article containing the methodology of all study methods involved in the production of the two original articles (Chapters 5 & 6). It should be noted that it was not possible to complete secondary objective (III) in this publication (e.g. ex vivo bacterial growth in neonatal serum) due to the lack of adequate blood sample volumes from the initial blood draws.

**Chapter 5:** A published research article comparing the concentrations of hepcidin, serum iron and TSAT in full-term, normal birthweight newborns against preterm and/or low birthweight neonates in the first hours of life.

**Chapter 6:** A prepared (submitted) manuscript describing concentrations of hepcidin, serum iron and TSAT in full term, normal birthweight newborns during the first week of life only.
Chapter 7: A final discussion chapter to highlight the overall study findings, limitations of this thesis and reflection on what future research should be conducted in the field of neonatal iron homeostasis and beyond.

1.4 Candidate’s Involvement

The original idea of assessing iron and inflammation makers in newborns at birth and shortly afterwards in order to assess if hypoferremia was present came from Dr Sarah Prentice, Dr Carla Cerami (Higher Scientific Officer for MRC Unit The Gambia) and Professor Andrew Prentice (Nutrition Theme Leader for MRC Unit The Gambia). Dr Carla Cerami obtained the funding from the Bill and Melinda Gates Foundation. I assisted in completing the narrative and budget components of the grant (OPP1152353). I worked closely with Dr Carla Cerami (primary supervisor) to develop and implement this observational study and received technical support from Professor Andrew Prentice (secondary supervisor).

As seen in Chapter 3, I was responsible for searching the literature, analysing data and formulating the first draft of the narrative review. Dr Carla Cerami and Professor Andrew Prentice (co-authors) supervised me and provided technical support and feedback during the production of the review.

The methodology of the NeoInnate Study (Chapter 4) was prepared predominantly from pilot research conducted by Dr Sarah Prentice and Professor Andrew Prentice. Dr Carla Cerami and I adapted previous study procedures and protocols to fit with a change in the study setting. Dr Ousman Jarjou and the nursing team provided clinical insight into the development of study-specific protocols (SPPs). Mr Bakary Sonko oversaw the development of data handling and management, ensuring all aspects followed MRCG data management
policies. Dr Nuradin Ibahim Mohammed provided statistical oversight to ensure the study was adequately powered for our formal analysis.

My role during the NeoInnate Study was a PhD student, along with co-investigator and project manager. I assisted in the writing of the funding application and regular reports to the Bill and Melinda Gates Foundation (BMGF). I assisted in the production of a study budget, monitored spending and procurement/logistics daily. I developed all clinical, data and laboratory study-specific protocols (SSPs). I orchestrated the renovation of a new study-specific laboratory at Kanifing General Hospital (with the help of construction workers, labours and senior hospital staff). I assisted in the production of a successful ethics application to MRCG at LSHTM Scientific Coordinating Committee, MRCG at LSHTM/Gambian Government Joint Ethics Committee and London School of Hygiene and Tropical Medicine Ethics Committee, alongside assisting with the formal registration of the study at ClinicalTrials.gov. I actively supported the communication between Kanifing General Hospital and the NeoInnate study team, holding regular meetings with senior hospital administration. I worked with a team of MRCG at LSHTM staff to arrange a study-specific open day at Kanifing General Hospital, working with hospital administration, local media, international and local non-governmental organisations (NGOs), religious leaders, national and local government and the local population of Kanifing. Furthermore, I aided the assembly of a study team consisting of a research clinician, eight clinical nurses, ten laboratory technicians and two international students by shortlisting candidates, helping conduct interviews and working closely with the Human Resources Department (MRCG at LSHTM). I coordinated the fulfilment of daily project administration and laboratory management (staff appraisal, organising subject community visits, producing staff rotas, and running laboratory controls). I conducted regular shift work at the NeoInnate laboratory, completing primary laboratory analysis and supporting the study nurse team. I developed and verified all electronic data records on the study-specific REDCap (Research Electronic Data Capture) database, executing data curation and management leading to formal
statistical analysis in collaboration with study team members (Dr Carla Cerami and Prof Andrew Prentice) and the study statistician (Dr Nuradin Ibrahim Mohammed). Additionally, I conducted all secondary biochemical processes, sample handling and analysis (ELISA and biochemistry analysis). I provided regular training days to Kanifing General Hospital and NeoInnate Study staff on study procedures, theory and protocols. This work led me to the scientific writing of the manuscripts seen in Chapters 5 and 6.

While here, I summarised my contribution to the contents of this thesis, it is essential to note that this study was designed, conducted and analysed in a collaborative approach. I have provided the names and roles of everyone involved in these areas in Annex 1.11.

1.5 PhD Publications

Published Papers:


- **Cross JH, Jarjou O, Mohammed NI, et al.** Early postnatal hypoferremia in low birthweight and preterm babies. *EBioMedicine* 2020;52;102613. (Chapter 5)

Re-Submitted Manuscript:


**Poster Presentations:**


**Conference Talks:**


• London School of Hygiene and Tropical Medicine – M. Phil. / Ph.D. Upgrading Seminar. 30th September 2017. Iron and Infection: Neonatal Iron Transition (NeoInnate Study).


There was one other paper that I was involved in as part of my laboratory work at MRC Unit The Gambia at LSHTM during my PhD registration. This was in the same field (iron homeostasis) as my thesis, but was unrelated to my PhD research:

1.6 PhD Timeframe

The PhD registration period ran from 28th September 2015 until 31st December 2019. A Gantt Chart of all PhD and study activities can be observed in Annex 1.12.

1.7 Funding

The research was undertaken with a research grant provided by the Bill & Melinda Gates Foundation (OPP1152353), under principal investigator Dr Carla Cerami (primary supervisor). 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 manuscripts. This research grant was used to pay for all my costs, including my travel and PhD stipend. 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.

1.8 Ethics

This study has been approved by The Gambia Government/MRC Joint Ethics Committee (no. SCC1525) (Annex 1.13-1.14) and Ethics Committee of London School of Hygiene and Tropical Medicine (LSHTM) (ref no. 14316) (Annex 1.15). This study was registered with clinicaltrials.gov (NCT03353051) on 27th November 2017.
1.9 Background

1.9.1 Ending Preventable Neonatal Mortality

Since the formation of the World Health Organisation (WHO) Millennium Development Goal 4, significant progress in under-5 child survival has been made over the last two decades.\textsuperscript{35} Neonatal deaths have decreased from 5 million (1990) to 2.5 million (2019).\textsuperscript{35} Despite this, nearly two million of these newborns die in their first week of life,\textsuperscript{36} with 24-45\% of all neonatal deaths occurring in the first 24 hours of life.\textsuperscript{37} With the substantial reduction in under-5 mortality rates occurring mostly in the older cohort, a more significant proportion of under-5 deaths now occur in the neonatal period. This has been estimated as an increase from 40\% in 1990 to 47\% globally in 2019.\textsuperscript{35}

With the creation of the Sustainable Development Goal 3.2 in 2015, health professionals, academics and policymakers are aiming to end preventable deaths of all children under the age of five by 2030.\textsuperscript{38} Part of this is a target for all countries to reduce their neonatal mortality rate to <12 per 1000 live births. Emphasis has also been placed on the ongoing major global health challenge of high rates of stillbirth. It is estimated that a similar number of babies are dying each year before delivery (e.g. 2-3 million).\textsuperscript{39}
Preterm birth, intrapartum-related complications and infections (including sepsis and pneumonia) are the most common causes of neonatal death. Many of these deaths are due to gaps in the continuum of pre- and post-natal care. This includes pre-pregnancy, antenatal, intrapartum, delivery, postpartum, and postnatal periods for both the mother and her newborn.

1.9.2 Neonatal Mortality in Low- and Middle-Income Countries (LMICs)

Analysis of global neonatal mortality rates has highlighted substantial disparities at a country and regional level. Almost 99% of all neonatal deaths occur in low- and middle-income countries. In particular, the geographical region of Sub-Saharan Africa is struggling to stem the tide, with the highest neonatal mortality rate globally of 28 per 1000 births (2018). This suggests that African newborns are nearly nine times more likely to die in the first month of life than a child born in a high-income country. An extreme example of how much work remains to be done over the next decade is that some regional neonatal death rates in LMICs reach close to 46 deaths per 1000 births.

Simple interventions related to antenatal care, education, nutrition, and maternal health can all help reduce the prevalence of preterm and low birthweight newborns. One of the most effective is the low-cost mass drug distribution of oral iron and folic acid supplementation to pregnant women during pregnancy. This has been shown to reduce the risk of maternal iron deficiency and anaemia at term in the mother, leading to a reduced risk of delivering a preterm and/or low birthweight neonate.
Antenatal care is critical for reducing adverse outcomes in mother and baby. The WHO currently recommend that mothers receive a minimum of eight antenatal care contact visits during their pregnancy. However, only 65% of pregnant mothers in the world achieve four visits before delivery. This is significantly worse in LMICs. Moreover, this analysis does not take into account the skill level of the healthcare provider or the quality of the antenatal care received. The lack of pregnancy-related healthcare education and the high burden of adverse pregnancy and newborn outcomes in LMICs, results in mothers not seeking medical care when necessary. This can apply to both the onset of labour and newborn illness. The lack of regular attendance to antenatal care contact visits can also result in issues relating to micronutrient supplementation, hypertension, immunisations, sexual health screening, and acquiring insecticide-treated mosquito nets. Barriers to these interventions may include physical, financial and cultural aspects.

WHO guidelines support the delivery of a baby by a skilled birth attendant (SBA) at a health facility. Unlike previously, high proportions of deliveries in HICs and LMICs now take place in healthcare facilities. The reason for this recommendation was to offer the opportunity to deliver in a safe and clean environment, while providing skilled, good quality essential newborn and maternal care. This policy was designed to allow for the identification of high-risk neonates and promotes their management quickly and effectively. However in LMICs, this has often resulted in more pregnant mothers delivering in unhygienic hospitals with inadequate health infrastructure or technology. The upsurge in healthcare facility deliveries has also increased the risk of newborns and mothers acquiring hospital-associated infections, which commonly possess antimicrobial-resistance mechanisms. This is made worse by the fact that many maternity and neonatal wards lack a continuous water supply, basic resuscitation equipment, resources for hand hygiene and waste disposal. Similarly, the ratio of skilled healthcare professionals to the number of beds is often far lower than recommended by the WHO. This situation is deteriorating in public hospitals in LMICs faster than anywhere else in the world due to staff attrition to different
countries or to newly established private healthcare facilities. This results in services being overstretched, leading to a reduced quality of care. Mothers as a result may then feel less inclined to remain in the delivery facility for the recommended 24 hours after birth. Ironically, this is the period in which most maternal and neonatal complications present. Studies also suggest that many newborns born in LMICs struggle to receive thermal protection immediately after delivery (e.g. kangaroo mother care (KMC)), hygienic umbilical cord care or early and exclusive breastfeeding. All of these interventions are associated with a reduced risk of infection-related deaths in the newborn.

However, high-quality data are required to better understand the fundamental issues affecting newborns and their ability to survive and thrive. Analysis of global mortality rates and their causes has shown that both are influenced by inadequate coverage and poor quality of data. This can result in the misdirection of funds, resulting in inappropriate or weak interventions. Birth registration is a fundamental human right and is vital to economic, social and health planning, yet birth registration is at a prevalence of just 46% in sub-Saharan Africa. The lack of registered births, deaths, and accurate birthweight and delivery records may result in a misunderstanding of the true scale of the issue relating to neonatal mortality. Now there is greater advocacy to collect accurate, timely and disaggregated newborn data from LMICs, which can be used to support evidence-based decision-making, programming and planning.

1.9.3 Neonatal Mortality in The Gambia

The Gambia has achieved the Millennium Development Goals of improving the under-5 mortality rate, immunisation coverage, the proportion of the population using an improved drinking water source, primary schools enrolment and reduction in malaria disease burden by the 2015 deadline. Much less progress has been made in the reduction of neonatal
mortality rates. National neonatal death levels, as a proportion of under-5 deaths, have increased from 31% in 1990 to 45% in 2017. Like many other LMICs, preterm birth, intrapartum complications and sepsis are responsible for nearly three quarters of all newborn deaths (Figure 1.4). On this record, it is unlikely The Gambia can meet its Sustainable Development Goal 3.2 for neonatal mortality by 2030 unless vast and effective interventions are put into practice.

Figure 1.4 Distribution of newborn deaths by cause in The Gambia. Source: Estimates generated by the WHO and Maternal and Child Epidemiology Estimation Group (MCEE) 2018 (http://data.unicef.org).68

Issues that face neonatal healthcare in The Gambia are similar to those in other LMICs. High levels of poverty, along with a shortage of adequately trained staff and a significant urban bias (66%) in the distribution of the workforce are amongst the issues.69 In general, Gambian healthcare system suffers from a limited amount of medical supplies and a lack of durable infrastructure and technology.70 The proportions of birth registration, skilled birth attendants present at delivery, postnatal care for newborns and exclusive breastfeeding proportions show that reasonable improvements can be easily made (Table 1.1).
### Table 1.1 Health-related statistics for The Gambia

<table>
<thead>
<tr>
<th>Maternal and Neonatal Health-Related Statistics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Births Per Year (000):</td>
<td>81 (2016)</td>
</tr>
<tr>
<td>Total Under-5 Population (000):</td>
<td>360 (2016)</td>
</tr>
<tr>
<td>Birth Registration (%):</td>
<td>72% (2013)</td>
</tr>
<tr>
<td>Total Maternal Deaths Per Year:</td>
<td>590 (2015)</td>
</tr>
<tr>
<td>Neonatal Mortality Rate (per 1000 live births)</td>
<td>22 (2013)</td>
</tr>
<tr>
<td>Total Stillbirth Rate (per 1000 total births):</td>
<td>24 (2015)</td>
</tr>
<tr>
<td>Neonatal Deaths Per Year (as % of all &lt;5 deaths):</td>
<td>45% (2015)</td>
</tr>
<tr>
<td>Total Under-5 Deaths Per Year (000):</td>
<td>5 (2016)</td>
</tr>
<tr>
<td>Skilled Birth Attendant (%):</td>
<td>57% (2013)</td>
</tr>
<tr>
<td>Postnatal Care for Neonates (%):</td>
<td>6% (2013)</td>
</tr>
<tr>
<td>Exclusive Breastfeeding (%):</td>
<td>47% (2013)</td>
</tr>
<tr>
<td>Antenatal Care, 4+ Visits (%):</td>
<td>78% (2013)</td>
</tr>
<tr>
<td>Low Birthweight Prevalence (%):</td>
<td>10% (2010)</td>
</tr>
<tr>
<td>Iron/Folic Acid Supplementation During Pregnancy (%):</td>
<td>45% (2013)</td>
</tr>
<tr>
<td>Physician Density (per 1000 population)</td>
<td>0.11 (2015)</td>
</tr>
<tr>
<td>Nurse and Midwife Density (per 1000 population)</td>
<td>1.62 (2015)</td>
</tr>
</tbody>
</table>

**Table 1.1 Health-related statistics for The Gambia. Source:** Countdown to 2030, Maternal, Newborn and Child Survival, Healthy Newborn Network.67

### 1.9.4 Maternal and Newborn Care at Kanifing General Hospital

Annually, Kanifing General Hospital (KGH) provides antenatal care to 500–700 pregnant mothers (unpublished hospital data, 2017), with many presenting very late in their pregnancy. This is thought to be due to pregnant mothers failing to see the advantages gained by early uptake of antenatal care. It is viewed as a curative rather than preventative measure.71 Local healthcare centres report that few mothers meet the new requirement for eight antenatal visits, with close to half receiving the old requirement of four antenatal visits.72 This significantly differs from the data produced by a Gambian demographic health survey (DHS).70
Antenatal care at Kanifing General Hospital is provided by a group of dedicated, skilled and experienced nurses and midwives. However, as in other hospitals in LMICs, barriers to their care remain. Blood pressure monitoring, urine sampling, blood sampling, tetanus protection, iron supplementation, fetal monitoring and health education are seven routine components of antenatal care that are available at Kanifing General Hospital. Nonetheless, it is rare for all to be available at one antenatal visit. Critical for detecting pre-eclampsia, the lack of back up or charged (i.e. lack of electrical supply) electrical blood pressure monitors results in blood pressure measures being missed. Similarly, due to the lack of full blood count laboratory supplies and broken haemoglobinometers, many weeks’ worth of haemoglobin measurements can be lost. This increases the risk of maternal anaemia not being diagnosed, and subsequently could lead to increases rates of stillbirths, low birth weight and preterm babies.\(^7\) During the initiation of this study, it also became apparent that only one Pinard horn for fetal monitoring was available throughout the whole hospital.

The total number of births at Kanifing General Hospital equals close to 3500–4500 per year (based on hospital data from 2014-2015, unpublished). This is 5-6-fold higher than the number receiving antenatal care at the hospital. The percentage of these that are live, low birth weight neonates (<2.5kg) is approximately 10% (based on hospital data from 2014-2015, unpublished). Further details relating to delivery care at Kanifing General Hospital can be seen in Section 2.1.3.

1.9.5 Contribution of Neonatal Infections

Neonatal sepsis is the third highest cause of death globally, accounting for 225,000 deaths each year.\(^1\) This results in neonatal infections (e.g. bacterial sepsis, meningitis, pneumonia, and tetanus) causing an estimated 23% of all neonatal deaths. Neonatal sepsis alone accounts for 15% of these deaths,\(^36\) with the majority occurring in Sub-Saharan Africa.\(^7\)
Intrauterine and neonatal infections do not just cause death, but also can result in substantial long-term multisystem morbidity, affecting not just the individual but the local community as well as national productivity.\textsuperscript{75, 76}

Early-onset neonatal sepsis (EONS) is defined as bacteraemia or bacterial meningitis occurring within \textless 72 hours of life, with most causative pathogens being transmitted vertically from mother to infant before or during delivery.\textsuperscript{77} Maternal chorioamnionitis, prematurity, fetal distress (i.e. fetal tachycardia or passage of meconium), prolonged rupture of membranes and maternal colonisation with group B streptococcus (GBS) are all deemed influential risk factors.\textsuperscript{77} Quite different to this is late-onset neonatal sepsis, which occurs after this point (>72 hours), with transmission often occurring horizontally from the surrounding delivery environment.\textsuperscript{78} As a result, many of the causative microorganisms are nosocomially derived.

In a recent highly informative review conducted by Okomo \textit{et al.},\textsuperscript{78} it is suggested that aetiology data of neonatal infections in sub-Saharan Africa is of poor quality with publications rarely using the STROBE-NI reporting guidelines. Studies that solely observe the epidemiology of neonatal infections in Gambian newborns are lacking. This is despite a high burden of infectious disease in this population and region. In 1999, Mulholland \textit{et al.} suggested that the most important causes of serious, very young infant infections were \textit{Streptococcus pneumoniae}, \textit{Staphylococcus aureus}, and \textit{Salmonella} spp.\textsuperscript{80} New data analysis by Okomo \textit{et al.} now suggests that \textit{Staphylococcus aureus}, \textit{Klebsiella} spp. and \textit{Escherichia coli} are the most common causes of bacteraemia and sepsis in African newborns.\textsuperscript{79} This change in aetiology over the last twenty years is thought to be due advances in delivery and newborn care along with the introduction of several new vaccines (e.g. Pneumococcal conjugate vaccines and Haemophilus influenza type b).\textsuperscript{81} Though not given directly to the newborn, Okomo \textit{et al.} speculates that both vaccines have reduced the transmission of pathogenic isolates in the community. Subsequently, herd immunity could be
affecting the colonisation rates and densities of these and other species of pathogenic and commensal bacteria in the newborn.

Part of the reason why *S. aureus* is found to be a predominant cause of neonatal sepsis in developing countries is believed to be due to its ability to colonise the skin of newborns, mothers and caregivers.\(^{82,83}\) However, care must be taken with interpreting its apparent high prevalence, as inadequate site sterilisation when blood cultures are taken may also lead to increased rates of false detection.\(^{84}\) Confirmed *S. aureus* sepsis is strongly associated with higher mortality rates as compared to other bacterial pathogens. This is partly due to metastatic complications in disease progression\(^ {85}\) and its strong association with sepsis in low birthweight newborns.\(^{86}\)

Enterobacteriaceae such *E. coli* and *Klebsiella* spp. are widespread in the maternal genital tract, and hence neonates acquire these isolates commonly via their gastrointestinal or respiratory tracts during delivery.\(^{87}\) Okomo *et al.* observed *Klebsiella* spp. to be the second leading cause of neonatal infections in their study.\(^ {81}\) A potential reason for this may be its strong association to nosocomial settings,\(^ {88}\) the capacity to produce biofilms\(^ {89}\) and its ability to display multidrug-resistant phenotypes by producing extended-spectrum β-lactamases (ESBLs) or carbapenemases.\(^ {90}\) With regards to *E. coli*, strains that commonly cause neonatal sepsis often express plasmids, which are essential in processes such as iron acquisition and virulence.\(^ {91}\) Interestingly, *E. coli* and *Klebsiella* strains are associated with risk factors such as prematurity and very low birthweight.\(^ {90}\)

### 1.9.6 Diagnosis and Treatment of Neonatal Sepsis

Diagnosis of neonatal sepsis remains difficult, partly as there is no accepted definition. Identification with high accuracy and specificity is a challenge not only in developing
countries but even in well-equipped tertiary healthcare facilities in HIC settings. Blood cultures are the gold standard for the diagnosis of neonatal sepsis. Because of economic, logistical and infrastructure-related restrictions, developing countries face multiple challenges when implementing blood culture-based diagnostic testing. Microbiological identification of a pathogen isolated from blood cultures has high specificity, but sensitivity is low. This is supported by the Aetiology of Neonatal Infections in South Asia (ANISA) study, conducted in Bangladesh, India, and Pakistan. This was designed to investigate the incidence of possible severe bacterial infection (pSBI) episodes in the first two months of life and estimate the proportions of bacterial and viral causes. This large study implemented the use of the latest metagenomic approaches, and still only detected 28% of all causative organisms of pSBI. Reasons for low sensitivity of microbiological identification include the collection of small neonatal blood volumes, the presence of low or intermittent isolate concentrations and maternal intrapartum antibiotic exposure. In order to combat this issue, the WHO launched the Integrated Management of Childhood Illness (IMCI) algorithm as part of the WHO Young Infants Clinical Signs Studies (YICSS). This has led to the production of a seven clinical signs diagnosis to use when laboratory services are not available. This clinical algorithm is associated with 85% sensitivity and 75% specificity for severe bacterial infection in the first week of life. Other investigations that are thought to assist in the diagnosis if appropriate include full blood count, chest radiograph, CRP measurement, CSF or skin swabs.

The WHO Integrated Management of Childhood Illness (IMCI) policy suggests that prophylactic intramuscular (IM) or intravenous (IV) ampicillin and gentamicin should be given to neonates with documented risk factors for neonatal sepsis. WHO recommends that this should continue for two days following which the neonate should be reassessed. In LMIC settings, however, the use of parenteral therapies in newborns may be limited by the availability of inpatient neonatal care, transportation to healthcare facilities, economic and
social factors. New interventions to prevent severe neonatal infections are required and are of great interest to the maternal and newborn research community.

1.9.7 The Threat of Antimicrobial-Resistant Infections

It has been estimated that the number of people who will die as a result of antibiotic-resistant infections will rise from 700,000 to 10 million each year by 2050. Antibiotic resistance is a major global health threat, with nearly half of the pathogens that cause severe neonatal bacterial infections worldwide reported to be resistant to the first-line (ampicillin or penicillin, and gentamicin) and second-line (third-generation cephalosporins) WHO-recommended treatments. Laxminarayan et al. produced estimates suggest 30% of all neonatal deaths can be attributable to multidrug-resistant pathogens. A disproportionate number of these deaths occur in developing countries, and the rates are increasing. In particular, a small group of studies conducted in Sub-Saharan Africa show a high prevalence of resistance to recommended empirical therapies to neonatal sepsis.

In developing countries, gram-negative bacteria have frequently been reported as the cause of neonatal sepsis. This is a worrying trend, with many gram-negative bacteria intrinsically resistant to many antibiotics, along with the ability to transfer genetic material of new resistance mechanisms between species. Klebsiella spp. and E. coli can both can carry extended-spectrum beta-lactamase (ESBL) producing plasmids, enabling them to become resistant to a wide variety of penicillin and cephalosporin antibiotics. In this scenario, carbapenems are the last remaining treatment option, however, the availability of second-line antibiotics in developing countries is known to be poor. K. pneumoniae is also the leading cause of infections caused by carbapenem-resistant bacteria worldwide. The acquisition of an enzyme called New Delhi metallo-beta-lactamase (NDM-1) is a significant reason for this. Bacteria with this genetic element can cause disease with increased
morbidity and mortality, especially in preterm newborns. Previous authors have commented that strains harbouring these antimicrobial-resistant components are a massive threat to neonatal units, particularly in LMIC hospitals as neonates are subjected to poor infection control and high antimicrobial exposure.

With regards to *S. aureus* antimicrobial resistance, methicillin-resistant *Staphylococcus aureus* (MRSA) infections have now spread worldwide, with some of the fastest rates of the increase occurring in community settings. The WHO now reports that in some locations of Africa, 80% of *S. aureus* infection are caused by MRSA. Nevertheless, optimism remains, with MRSA still remaining sensitive to glycopeptides such as vancomycin and teicoplanin, and other antibiotics like linezolid, tigecycline, and daptomycin. However, many of these drug options are expensive, regularly unavailable in LMICs and have potent side-effects on newborns.

### 1.9.8 Interventions to Combat Neonatal Infections

*Staphylococcus aureus, Klebsiella spp.,* and *Escherichia coli* are all pathogens associated with poor hygiene in hospital environments, suggesting that new interventions are required in sub-Saharan hospitals to increase infection control from surfaces, hands and water. One such intervention is the use of a bundle of hygiene-based interventions for a hospital maternity ward. A study that implemented such a bundle provided enhanced infection prevention and control training, clean delivery kits (e.g. a blade, cord thread, antiseptics), text message reminders, alcohol hand rub, timed environmental cleaning and the introduction of bathing babies >1.5 kg with 2% chlorhexidine gluconate. This study subsequently observed reductions in sepsis and death rates in hospitalised neonates in a high-risk setting in a developing country. These kinds of interventions offer simplicity and
reasonable levels of sustainability, with the production of standardised protocols helping to
improve aseptic practices in the delivery rooms and neonatal units.

A further intervention toward the prevention of neonatal sepsis in LMICs is the use of oral
azithromycin given to the mother during labour. Azithromycin is a macrolide antibiotic that
has a broad antimicrobial activity. Azithromycin has also been found to reduce the proportion
of low birthweight births by a quarter, when given intrapartum to the mother for the last few
months of pregnancy. Equally, it has been able to reduce overall under-5 mortality when
given in the form of a mass drug administration trial. More recent studies have shown that
azithromycin during labour can significantly reduce bacterial carriage (e.g. mainly S. aureus,
GBS and S. pneumoniae) both in the mother and her newborn. This suggests that it halts
the vertical transmission of neonatal sepsis-causing organisms from mother to child and
hence may lower their risk of neonatal sepsis, pneumonia and meningitis. It is now believed
that this simple one dose intervention could dramatically improve neonatal mortality in
LMICs. However, questions remain about the effect MDA of antibiotics will have on the
levels of antimicrobial resistance in the mother, newborn and the broader community.

Symbiotics are a combination of probiotics and prebiotics; the latter is added to promote
growth and sustain the colonisation of the probiotic bacterial strain. Studies looking at the
effects of symbiotics, prebiotics and probiotics have grown more popular over recent years.
This is primarily due to the naive immune system of the neonate and the undeveloped
gastrointestinal microbiome making it easier to establish probiotic strains. One of the main
successes of this area of study is the use of probiotics to reduce the incidence and severity
of necrotising enterocolitis in premature infants. Now, research suggests that
symbiotics can be used to reduce neonatal sepsis mortality rates in LMICs. One of the
most prominent studies using symbiotics, is the one conducted by Panigrahi et al. This
was a randomised, double-blind, placebo-controlled oral symbiotic trial of Lactobacillus
plantarum plus fructo-oligosaccharide in India. This study identified that a single symbiotic
preparation was protective against neonatal sepsis and death in the first week of life. This is
an exciting finding, that may relate to the findings of our study, as *Lactobacillus plantarum* is an organism that does not require iron for growth, and instead uses manganese in many of its metalloenzymes. The establishment of *Lactobacillus plantarum* colonisation at birth could be out competing pathogenic species (i.e. that require iron) for space and niches, due to the hypoferremic conditions. Panigrahi *et al.* believe that these findings suggest that this cost-effective method could be used in developing countries to reduce the rates of neonatal sepsis.

**1.9.9 Transition to Extrauterine Life**

The higher rates of mortality relating to severe bacterial infections in newborns are suggested to be partially due to the immense physiological and immunological strain on the neonate in the first few days of life. The transition from fetal to extrauterine life leads to all organ systems being involved in major instantaneous adaptions to respiratory exchange, cardiovascular flow, endocrine function, haematological maturation, substrate metabolism and thermogenesis.

Though the sterile womb paradigm versus *in utero* colonisation hypothesis continues to divide, childbirth is still characterised by a neonate descending from a semi-allogeneic, protected environment to one that is far more abundant in a diverse array of microbes. This is the initial focal point of mass bacterial colonisation of the skin and gastrointestinal tract of the neonate, resulting in changes to nutritional and immunological functions in early life and beyond.

Regarding iron homeostasis, as seen in our study, increases in newborn haematocrit and haemoglobin occur within the first hours post-delivery. This is likely due to post-delivery dehydration, as well as vasomotor instability and venous pooling. Similarly, serum ferritin
levels increase due to the physiological haemolysis of fetal red blood cells, which contain ferritin in high concentrations. A frequent side-effect of this red cell turnover is hyperbilirubinemia (i.e. physiological or idiopathic jaundice), which is a common clinical issue in most (60-80%) neonates in the first weeks of life.

**1.9.10 Immune Responses of the Neonate**

Neonates are also partly hindered by possessing a functionally immature and developing immune system. The broad-range killing capacity of the innate immune system consists of granulocytes, antigen-presenting cells, NK cells and γδ-T cells. Neutrophils are the predominant cell type of the innate immune system, with their role being to engulf and degrade pathogens during infection. Nevertheless, neonatal neutrophils at birth can be measured as low as $1.5 \times 10^9$ cells/L blood, compared to $4.4 \times 10^9$ cells/L in adults. Neonatal neutrophils also possess qualitative deficiencies in respect to low levels of expression of TLR4 (critical for IL-6 expression), cell surface L-selectin, Mac-1 (critical for transmigration) and neutrophil extracellular traps (NETs). These deficiencies are even more pronounced in preterm neonates. Similarly, DC cells, γδ-T cells and monocytes are also all in low concentrations in the neonate and exhibit low expression levels of MHC-II, CD80, CD86, CD40 and ICAM-115. This is thought to inhibit their ability to activate antigen-specific B and T cell populations.

The immune system of premature neonates is further underdeveloped when compared to full-term newborns. Antimicrobial proteins and peptides (APPs) are released to destroy pathogens by iron-binding, enzymatic destruction, zinc deprivation and membrane pore formation. Such proteins and peptides are secreted into the vernix caseosa found coating the skin, respiratory and gastrointestinal tracts of newborn babies. However, the
Production of APPs and vernix caseosa is positively correlated with gestational age,\textsuperscript{140} potentially leading to early preterms being at higher risk of infection. Preterms also start life with a lower endowment of antigen-specific IgG than full-term neonates, resulting in reduced opsonisation and phagocytosis. This is due to IgG being transferred across the placenta from the maternal circulation in large amounts after 32 weeks of gestation.\textsuperscript{140} In full-term neonates, the complement pathways (classical, alternative and lectin) are generally similar to adult levels, this aids the destruction of bacterial cells. However, these pathways are also all immature in preterm neonates.\textsuperscript{141}

**Figure 1.5** An overview of innate immune deficiencies of preterm and low birthweight babies. Adapted from Melville et al.\textsuperscript{140}

Immunological deficiencies in neonates (exaggerated in low birthweight and preterm babies) no doubt reflect in the typical clinical presentation of neonatal sepsis. This presentation is
often rapid in progression, with many nonspecific clinical signs. This is further confirmed by unusual haematological laboratory results coming from newborn blood samples. Our study experienced such results, with even healthy newborns found to have increased white blood cell counts from cord and venous samples; though we suspect that this might be an artefact of nucleated red blood cells being identified as granulocytes.

1.9.11 Iron in Infection and Immunity

Iron is a cofactor in numerous metabolic pathways that are critical for the human host. Transition metals (e.g. Fe, Cu and Mg) are commonly built-in to metalloenzymes, storage proteins and transcription factors. This results in children and pregnant woman requiring adequate iron stores for growth and development. The lack of such stores leads to iron deficiency and anaemia, which subsequently negatively affects the immune response to infection. However, the catalytic action of these metals also potentiates their toxicity when in high concentrations (i.e. equalling tissue damage and inflammation), so the levels of these metals must be well regulated. The mechanisms that regulate the toxicity of free transition metals in the body, additionally function as a countermeasure against local pathogens (Figure 1.6). This is seen clinically with individuals suffering from high iron states (e.g. haemochromatosis), not only resulting in free radical redox damage but also an enhanced risk of infection from iron-dependent species of bacteria. This host-pathogen battle for resources is due to iron also being a vital commodity for growth, replication and virulence of most pathogenic microorganisms. Subsequently, pathogens have evolved counteractions to the host’s iron-restriction mechanisms and assimilate iron from their human host.
Neonatal sepsis-causing bacterial pathogens can produce a plethora of molecular mechanisms such as siderophores and iron-specific channels (Table 1.2). These iron acquisition genes are frequently concentrated on high pathogenicity islands.\textsuperscript{147}

**Figure 1.6** The host-pathogen battle of iron.
Table 1.2 Iron sources of neonatal sepsis causing bacteria. Source: Adapted from Andrews et al.\textsuperscript{196}

In 2000-2001, three independent research groups discovered the hormone hepcidin.\textsuperscript{197–199} Hepcidin is now understood to be the master regulator of human iron homeostasis, and consequently iron-restriction.\textsuperscript{200} Host inflammatory mediators, IL-6\textsuperscript{21}, IL-22\textsuperscript{22} and Type 1 interferon\textsuperscript{23} increase transcription of hepcidin in the liver through several toll-like receptor (TLR) ligands\textsuperscript{24} and STAT3 signalling.\textsuperscript{24,25} This results in the internalization of the transmembrane protein ferroportin in macrophages and enterocytes. This leads to the breakdown of ferroportin in the lysosome. Consequently, enteric absorption of dietary iron is reduced, and sequestration of iron in macrophages causes a decrease in systemic iron concentration in the circulation.\textsuperscript{201} In neonates, due to the relatively small amounts of iron absorbed enterically, iron restriction mainly takes place in circulating macrophages. Hepcidin synthesis is also known to be controlled by high iron stores, hypoxia, and erythropoiesis (Figure 1.7).\textsuperscript{200}

Figure 1.7 Hepcidin-induced iron homeostasis. Source: Adapted from Ganz et al.\textsuperscript{200}
The human host also produces many host chaperone molecules such as transferrin, lactoferrin, haptoglobin, hemopexin (Figure 1.8) to limit the acquisition of iron and its moieties (haem and haemoglobin), thus making iron harder to sequester from the human host.  

**Figure 1.8** The sequestration of iron and its moieties. Source: Adapted from Parrow et al.  

### 1.9.12 Dysregulation of Iron Homeostasis

For the reasons stated above, iron homeostasis needs to be maintained at all times. Iron supplementation is widely regarded as one of the most critical public health interventions in low and middle incoming countries, reducing the morbidity and mortality linked to iron deficiency and anaemia. However, reports have suggested that iron supplementation in areas with high burdens of infectious disease can increase the risk of severe bacteria morbidity and mortality. We suggest that a study conducted by our team (and led by the candidate) partly explains the mechanism. In the previous FeVir study, we collected the blood of 48 adult male Gambian subjects immediately before and four hours after taking 400 mg ferrous sulphate orally. We then performed full blood counts and analysis of serum biochemical parameters, including serum iron, transferrin, ferritin, and transferrin saturation (TSAT). Additionally, we inoculated these paired serum samples with different sepsis causing organisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella enterica* serovar Typhimurium and *Escherichia coli* in an *ex vivo* bacterial growth assay. This study concluded that *ex vivo* bacterial growth over twelve hours (36h for Y.
*Enterococcus* enterocolitica) was significantly increased by supplemental iron and strongly correlated with TSAT and serum iron concentration (Annex 1.16).

A clinical study by Barry et al. showed similar results in a different context. Barry et al. discovered that Polynesian neonates were twenty-fold more likely to have neonatal sepsis compared to European neonates. This research uncovered that this was due to intramuscular iron dextran injections given to the neonate if they were thought to be anaemic. Barry et al. also described that there was not only a difference in the incidence of sepsis and mortality between those given iron dextran injections but additionally, a difference in the type of causative organism. Further data showed that iron dextran had impaired the nutritional immunity of the newborns and changed the causative organism from *S. aureus* to *E. coli*. This finding associates closely with the differences in growth seen between *S. aureus* and *E. coli* isolates in response to TSAT level seen in our previous FeVir study.

Both the *ex vivo* and *in vivo* studies suggest that lower iron concentrations in the blood are vital to limit the growth of bacteria and severity of sepsis caused by common neonatal sepsis-causing pathogens.

### 1.9.13 Pre-Analytical Effects on Hepcidin Measurement

As hepcidin is the master regulator of iron homeostasis, its measurement can be crucial to the diagnosis of several iron-related disorders. However, its accurate measurement is complicated. Hepcidin is at the centre of the iron, infection and inflammation axis. Significant increases in hepcidin concentration occur in response to iron administration and/or inflammatory stimuli. Subsequently, this can cause several clinical conditions and treatments to confound systemic hepcidin measurements. This is observed for chronic
kidney disease (increase),\textsuperscript{207} erythrocyte transfusions (increase),\textsuperscript{208} alcohol abuse (decrease)\textsuperscript{209} and chronic hepatitis C viral infection (decrease)\textsuperscript{210} all having an effect. These observations result in the need for a detailed medical history to be collected when researching the molecular mechanisms related to iron homeostasis in the human host.

Serum hepcidin concentrations also vary significantly between healthy subjects, as evidenced by the published reference ranges.\textsuperscript{211,212} Itkonen \textit{et al.} noted that reference values for adult females (18–50 yrs) ranged between 0.4–9.2 nmol/L, 0.7–16.8 nmol/L for females >50 yrs and for adult males (≥ 18 yrs) between 1.1–15.6 nmol/L.\textsuperscript{212} These results suggest lower hepcidin levels are observed in pre-menopausal women, as well as a significant difference between sexes (i.e. higher concentrations in men). In-subject variation is also detected, with fasting and diurnal rhythm both found to play a role.\textsuperscript{213,214} This is seen with serum hepcidin concentrations being found to be lowest in the early morning, increase over the day, followed by a return to more moderate levels by evening.

Further complications of measuring hepcidin concentration in human urine, serum and plasma come from laboratory-based issues. Of the two main methods of measuring hepcidin, mass spectrometry-based methods (MS) are expensive but are accurate, more reproducible and measure only the native complete form of hepcidin, hepcidin-25.\textsuperscript{215} Enzyme-linked immunosorbent assay-based methods (ELISA) are more cost-effective; however, these methods additionally measure the other isoforms of hepcidin, hepcidin-20 and hepcidin-22.\textsuperscript{215} The effect of these different test attributes on the clinical diagnosis of iron-related disorders is still unclear. The measurement of hepcidin by ELISA-based methods is further hindered by hepcidin being a relatively small size (i.e. 25 amino acids) with few antigenic epitopes.\textsuperscript{216} Antibody generation required for the production of the ELISA-based method is hampered by the high degree of genetic conservation between animal species, making it a challenge to produce viable antibodies from exposure to the protein in
the host animal.\textsuperscript{217} Production of synthetic alternatives is also challenging due to hepcidin's complex hairpin-like conformation with four disulphide bonds.\textsuperscript{218}

An essential aspect of the laboratory handling process of samples requiring serum hepcidin measurement is the monitoring of freeze-thaw cycles. Serum hepcidin is reported to only remain stable for two to four freeze-thaw cycles, dependent on the analysis method.\textsuperscript{219,220} Additionally, Itkonen \textit{et al.} has noted that hepcidin concentration starts to decrease after 24-48 hours storage at room temperature, six days at 4°C, 42 days at −20°C, and over two years at −80°C.\textsuperscript{212} This is not bettered by hepcidin's ability to aggregate and adhere to plastic laboratory test tubes and pipette tips.\textsuperscript{221}

Previously, the analysis of hepcidin concentration has been hindered by the lack of a commutable calibrator, a reference material, and a reference method.\textsuperscript{221} Subsequently, analysis of serum hepcidin levels from various publications share correlation; however, absolute values regularly vary considerably between the ELISA test kits used (\textit{Chapter 3}). This is observed with low values being associated with the use of DRG ELISA kits, and high values linked with the use of Intrinsic Lifesciences and Bachem ELISA kits. To combat this, Van der Vorm \textit{et al.} produced a commutable candidate reference material that was a native, lyophilized plasma with cryoprotectant.\textsuperscript{34} This material was refined and is now freely available for purchase.\textsuperscript{222} However, this product was not available at the time of laboratory analysis during this study.

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Chapter 2 – PhD Study Setting

Summary of Chapter

This chapter provides a brief geographical and economic overview of the Kanifing region and The Gambia as a whole. This is then followed by a description of the work environment at the study hospital, Kanifing General Hospital. Lastly, I describe the environment in which community visits take place around the Kanifing and Kombo regions, in the homes of study participants. All ethical, political and logistical issues faced in each of the study settings are detailed.
2.1 PhD Study Setting

2.1.1 The Republic of The Gambia

The Republic of The Gambia is the smallest mainland African nation, formed around the River Gambia. As part of West Africa, it is surrounded by Senegal and has a population of 2.3 million people (2018). Islam is practiced by 95% of the country’s population, with twelve main ethnic groups, the largest of which are Mandinka and Fula. The Gambia has a market-based economy, focused around tourism and traditional subsistence agriculture.

Figure 2.1 Map of The Gambia. Arrow shows study site location at Kanifing General Hospital. Source: © OpenStreetMap contributors.

A countrywide political problem faced by the study was the removal of the previous President of The Gambia, Yahya Abdul-Aziz Jemus Junkung Jammeh. The Gambia experienced a contested election in December 2016, which provoked a constitutional crisis. The situation was resolved by military intervention from the neighbouring countries from the Economic Community of West African States (ECOWAS). The new Gambian president was inaugurated towards the end of January 2017, but even after the inauguration, the Gambian government and society were in a state of flux and political uncertainty. This affected the
timing of three aspects of the study, including ethical approvals, community sensitisation, and study initiation.

2.1.2 Kanifing Municipality

The Kanifing region is made up predominately of the large town, Serrekunda. Kanifing region is a peri-urban area with a population of around 370,000 and is 10 km to the southwest of the capital, Banjul. Serrekunda was initially made up of nine villages that have merged into a sprawling urban area.

2.1.3 Study Sites: Kanifing General Hospital, The Gambia

![Figure 2.2 Map of Kanifing, The Gambia. Source: © OpenStreetMap contributors.](image)
Kanifing General Hospital, located in the Kanifing district, is one of six general hospitals in The Gambia. It is a public, government-run facility that first opened in 2010. Named initially Serrekunda General Hospital (SGH) it contains approximately 130 beds, with services and units such as an accident and emergency unit, out-patient unit, CT scanning department, maternity, antenatal and paediatric units. Recent investment has led to the formation of a small neonatal care unit. Complex clinical cases are commonly referred to The Edward Francis Small Teaching Hospital (EFSTH) in Banjul, as it is the only tertiary government referral hospital. Seven paediatricians staff the maternity and paediatric wards, along with sixteen senior nurses/midwives. During the study period, usually, there were four to six trained nurses working in the maternity ward during the day shift, with an equal number of nurse attendants. Staffing levels would commonly reduce during the night shift. Previously, the hospital has experienced periodic shortages of medicines, supplies, staff, electricity and water.
Figure 2.3 Kanifing General Hospital Maternity Ward. NeoInnate study team recruiting (top left), cord bleeding (top right), electronic data collecting (bottom left) at Kanifing General Hospital Maternity Ward (bottom right). Note: all imaged individuals have provided photographic consent.

Annually, Kanifing General Hospital (KGH) provides antenatal care to 500–700 pregnant mothers (unpublished - hospital data). Mothers receiving antenatal care at other local primary health-care facilities increase the total number of births at the hospital to 3500–4500 per year (unpublished - hospital data). The percentage of these that are live, low birthweight neonates (<2.5kg) is approximately 10% (hospital data 2016-2018, unpublished). During the study period, rates of stillbirth were at times poorly documented by hospital maternity ward staff, as seen in other institutions in low- and middle-income countries (LMICs).5

The maternity ward was split into two large wards, with one being used for pre- and post-delivery mothers and their neonates. There was also an adjacent eight-bed open-plan delivery ward. The delivery ward used a single partially broken neonatal resuscitator, which was commonly occupied by multiple neonates (1-3) at peak admission periods. The delivery ward also possessed limited respiratory support in the form of a single electrical oxygen concentrator. Thanks to the help from MRCG at LSHTM, both issues were overcome quickly in order to ensure service quality greatly improved. Other barriers to care included the lack of implementing Kangaroo mother care (KMC) in the hospital at the beginning of the study. As a result of a local implementation study, we were able to train several doctors and senior nurses on the care protocols. Infection control was also deemed to be poor quality in the maternity ward during the initiation of the study. As a result, we ensured new cleaning equipment was available on a monthly basis, in addition to investment into new waste systems, weighing scale, delivery equipment, storage devices and worktables.
A study-specific issue we experienced at Kanifing General Hospital was that mothers who delivered at the hospital were not always receiving antenatal care at the same health facility. At the beginning of the study, this resulted in many newborns being missed to recruitment. Anecdotal evidence suggested that mothers who were approached at antenatal visits at Kanifing General Hospital were often choosing to deliver at home or a primary level healthcare facility. Subsequently, our study team worked with local midwives and nurses to ensure mothers were informed of the benefits and risks associated with delivery at Kanifing General Hospital and being part of the NeoInnate Study. Furthermore, we also produced an additional recruitment route to ensure mothers receiving antenatal care outside the hospital could equally have their newborn recruited to the NeoInnate Study. This was a two-stage process, in which on arrival to the maternity ward if the mother was found to be capable, she would consent to the collection and storage of cord blood in the maternity ward. After a few hours, and when the mother was deemed to be in the right frame of mind after the delivery process, she was asked as to whether the cord blood sample and her neonate could be full recruited into the study. If at this point, the mother refused to give consent, the cord blood sample and her data were removed from the fridge and study server, respectively. This aspect of the study protocol required high-quality consent training focused on detailed discussions concerning different scenarios with the study staff.
2.1.4 Study Sites: Community Visits (West Coast Region)

Figure 2.4 Community study visits to the homes of recruited newborns. *Note: all imaged individuals have provided photographic consent.*

Neonates were visited at their home address between 24-192 hours post-delivery. Neonates originated from a wide area of villages across the Kombo region, the closest of which was from fifty metres away from the hospital, with the furthest coming from Faraba Banta (41.2 km away). Gambian homes were generally made of concrete with open windows and tin rooftops. Occupancies were shared between large family groups, as is common in the region. At each visit, a physical examination of the neonate was completed, followed by the collection of a venous blood sample, if the neonate was born full-term, normal birthweight. For further details of community visit protocols, see Chapter 4.6
Figure 2.5 Community study visits involved a complete review of systems and the collection of neonatal vital signs data. Note: all imaged individuals have provided photographic consent.

2.1.5 Study Sites: Laboratory Work (Kanifing General Hospital and MRCG Keneba Laboratory)

During the study site setup process, we became aware that laboratory facilities on-site at the hospital would be required. Kanifing General Hospital provided our study team with a disused storeroom, which we converted into a study-specific laboratory. This fully functioning laboratory contained temperature control, UPS, internet, refrigeration, locked storage and sample processing equipment (i.e. centrifuge, full blood count machine, sample rollers, pipettes). Twelve Kanifing General Hospital and three MRCG laboratory technicians
provided round-the-clock laboratory service. This design ensured the implementation of the study led to direct capacity building in the hospital. Full haematological analyses, serum separation and sample storage were performed in this laboratory. On completing the sample collection phase of the NeoInnate Study, samples were transported to MRCG Keneba field station (Kiang region) on dry ice. Secondary laboratory analysis including glucose-6-phosphate dehydrogenase deficiency testing (G6PD), biochemical analysis (serum ferritin, serum iron, unbound iron-binding capacity (UIBC), soluble transferrin receptor (sTfR), transferrin, C-reactive protein (CRP), haptoglobin, and alpha-1-acid glycoprotein (AGP)) and hepcidin ELISA testing were performed on a batch basis by two study staff members.

**Figure 2.6** Laboratory bench-top work during PhD study. Primary sample processing was completed in a purpose-built renovated laboratory at Kanifing General Hospital for NeoInnate study (*right*). MRC Keneba Microbiology Laboratory (*bottom left*) was the location for secondary sample processing (hepcidin ELISA - *top left*) and analysis.
2.2 References


Chapter 3 - Hepcidin, serum iron and transferrin saturation in full term and premature infants during the first month of life: A review of existing evidence in humans (Review)

Summary of Chapter

BACKGROUND: Iron sequestration is a fundamental aspect of innate immunity. Evidence suggests that neonates actively regulate their iron distribution at birth and in early postnatal life, in order to sequester iron away from microbial pathogens.

OBJECTIVE: To review existing evidence of serum iron, transferrin saturation and hepcidin levels in full-term and premature infants during the first month of life.

METHODS: We reviewed literature retrieved from PubMed and Ovid Medline containing data on umbilical cord and venous blood concentrations of hepcidin, serum iron and transferrin saturation in human neonates from 0-1 month of age. After the standardisation of the hepcidin values based on inter-assay cross-calibration studies, weighted mean averages were produced for cord and venous blood using available data. Similar weighted means were produced for serum iron and TSAT.

RESULTS: Data from 59 studies were used to create reference ranges for full-term neonates over the first month of life. In full-term neonates, venous blood hepcidin increases 2-3-fold over the first month of life (to reach 61.1 ng/mL; CI: 20.1-102.0 ng/mL) compared to umbilical cord blood (29.7 ng/mL; CI: 21.1-38.3 ng/mL). Cord blood has high levels of serum iron (28.5 μmol/L; CI: 26.0-31.1 μmol/L) and TSAT (51.7%; CI: 46.5-56.9%). Following a short-lived immediate postnatal hypoferremia, iron and TSAT rebounded to approximately
half the levels in the cord by the end of the first month. There was insufficient data to formulate reference ranges for preterm babies.

**CONCLUSION:** Evidence shows that full-term neonates experience an early postnatal hypoferremia as an innate immune defence, probably mediated by hepcidin. It is unclear whether preterm babies are able to elicit the same defence.
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Primary Supervisor Dr Carla Cerami

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James H. Cross is a PhD student with MRCG at LSHTM. He contributed to the literature search, the data analysis and the drafting of the manuscript.

SECTION E

Student Signature

Date 08/09/2019

Supervisor Signature

Date 08/09/2019
Title

Hepcidin, serum iron and transferrin saturation in full term and premature infants during the first month of life: A review of existing evidence in humans

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Abbreviations:

AGA       Appropriate for gestational age
CI        Confidence Interval
CRP       C-reactive protein
DFID      Department for International Development
DMT-1     Divalent metal transporter 1
EIA       Enzyme immunoassay
ELISA     Enzyme-linked immunosorbent assays
EPO       Erythropoietin
FTB       Full-term birth
IL-6  Interleukin 6
IQR   Interquartile range
MRC   Medical Research Council
MS    Mass spectrometry
NHNES National Health and Nutrition Examination Survey
PTB   Preterm birth
SD    Standard Deviation
SGA   Small for gestational age
sTfR  Soluble transferrin receptor
TFR1  Transferrin receptor 1
TLR4  Toll-like receptor 4
TSAT  Transferrin saturation
WHO   World Health Organisation

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**Competing Interests:** The authors declare that they have no competing interests.
3.1 ABSTRACT

BACKGROUND: Iron sequestration is a fundamental aspect of innate immunity. Evidence suggests that neonates actively regulate their iron distribution at birth and in early postnatal life, in order to sequester iron away from microbial pathogens.

OBJECTIVE: To review existing evidence of serum iron, transferrin saturation and hepcidin levels in full-term and premature infants during the first month of life.

METHODS: We reviewed literature retrieved from PubMed and Ovid Medline containing data on umbilical cord and venous blood concentrations of hepcidin, serum iron and transferrin saturation in human neonates from 0-1 month of age. After the standardisation of the hepcidin values based on inter-assay cross-calibration studies, weighted mean averages were produced for cord and venous blood using available data. Similar weighted means were produced for serum iron and TSAT.

RESULTS: Data from 59 studies were used to create reference ranges for full-term neonates over the first month of life. In full-term neonates, venous blood hepcidin increases 2-3-fold over the first month of life (to reach 61.1 ng/mL; CI: 20.1-102.0 ng/mL) compared to umbilical cord blood (29.7 ng/mL; CI: 21.1-38.3 ng/mL). Cord blood has high levels of serum iron (28.5 μmol/L; CI: 26.0-31.1 μmol/L) and TSAT (51.7%; CI: 46.5-56.9%). Following a short-lived immediate postnatal hypoferremia, iron and TSAT rebounded to approximately half the levels in the cord by the end of the first month. There was insufficient data to formulate reference ranges for preterm babies.

CONCLUSION: Evidence shows that full-term neonates experience an early postnatal hypoferremia as an innate immune defence, probably mediated by hepcidin. It is unclear whether preterm babies are able to elicit the same defence.

WORD COUNT ABSTRACT: 260/300

KEYWORDS: Nutritional immunity, host-pathogen interaction, hepcidin, neonates, hypoferremia, transferrin, serum iron
3.2 INTRODUCTION

Iron homeostasis during pregnancy

Three important mediators of hepcidin synthesis: iron status, inflammation, and erythropoiesis, are all altered during pregnancy.\(^1\)\(^{-4}\) Iron demand on the mother increases significantly to support expanded maternal erythropoiesis and iron requirements of the growing fetus.\(^5\)\(^{-9}\) During pregnancy, the placenta transfers \(~270\)mg of iron from the mother to the fetus via the placenta.\(^10\),\(^11\) Syncytiotrophoblasts in the placental villi take up transferrin-bound iron from the maternal circulation by endocytosis via transferrin receptor 1 (TFR1) (Figure 1).\(^12\)\(^{-14}\) As reviewed in Fisher et al.,\(^15\) iron is released from TFR1 and transferred from clathrin-coated vesicles into the syncytiotrophoblast cytoplasm by DMT-1,\(^16\) Zrt/Irt-like protein ZIP8,\(^17\) and ZIP14,\(^18\) collectively. Ferroportin transports iron out of placental syncytiotrophoblasts, and then ceruloplasmin, hephaestin, and zyklopen may all play a role\(^19\)\(^{-21}\) in helping the iron pass through the endothelium to reach the fetal circulation and bind to fetal transferrin.\(^13\)

Maternal control of fetal and early neonatal iron metabolism

Increases in maternal and fetal iron absorption occur in the second and third trimester,\(^22\),\(^23\) when maternal hepcidin decreases to trigger increased duodenal iron absorption,\(^24\) splenic macrophage iron recycling, and the release of maternal hepatic iron stores.\(^25\)\(^{-27}\) The resulting increased circulating maternal iron is then freely available for transfer to the fetus. Factors that are thought to contribute to maternal hepcidin suppression in the second and third trimester include maternal iron deficiency, erythropoiesis in the mother or fetus,\(^27\) oestrogen,\(^28\) and progesterone receptor membrane component-1.\(^29\) Conflicting evidence now exists as to whether pregnancy-induced plasma dilution may also play a role.\(^15\),\(^30\)
Fetal control of fetal and early neonatal iron metabolism

Eighty percent of all the iron transferred from the mother to the fetus occurs in the last trimester.\textsuperscript{31} An illustration of the fetal demand for iron (amounting to 1.6-2.0 mg/kg per day\textsuperscript{32}) is that umbilical cord blood contains a higher serum iron concentration than in the maternal circulation and at delivery babies have higher total body iron per kilo than that measured in their mothers or in healthy adults.\textsuperscript{33–44} This pattern is seen even in anaemic mothers and their babies.\textsuperscript{32,43,45,46} The relative roles of maternal and fetal hepcidin levels in controlling placental iron transport are unclear and may change during the course of gestation.\textsuperscript{25,26,30,42,45–55} As iron becomes more available in the last months of pregnancy, the fetus synthesizes hepcidin probably to control the rate of placental iron transfer and thereby to protect itself from iron-overload.\textsuperscript{15,30,56} Evidence showing the importance of fetal hepcidin includes: 1) umbilical cord hepcidin concentrations at birth are higher than maternal levels before and during delivery\textsuperscript{26,44,57–60} and 2) in pregnancies with multiple gestations, differences in cord hepcidin between siblings explained a greater fraction of variability in cord hemoglobin, serum ferritin, sTfR, and EPO than maternal hepcidin levels.\textsuperscript{49}

Placental control of fetal and early neonatal iron metabolism

The placenta may also independently regulate iron transfer to the fetus in some scenarios.\textsuperscript{61} A reduction of ferroportin expression on the apical fetal-facing membrane of placental syncytiotrophoblasts during maternal iron deficiency, in addition to increased expression of TFR1 on the maternal-facing side supports this hypothesis.\textsuperscript{30} Sangkhae \textit{et al.} propose that during maternal iron deficiency, iron is held in the placenta to ensure that its metabolic homeostasis is maintained. Placental protein synthesis and critical transfer mechanisms can then continue, ensuring the more detrimental condition of placental dysfunction does not occur. These findings were observed in murine and \textit{in vivo} human trophoblast models, but not in respect to the human pregnancies analysed.\textsuperscript{30}
Impact of labour and delivery on hepcidin

Childbirth is an intensely stressful event. Inflammatory pathways (including IL-6) are induced at the onset of human labor, even in the absence of intrauterine infection.\textsuperscript{62-69} Initiating stimuli for IL-6 production and release could involve the endocrine events of labor,\textsuperscript{68-70} mechanical distension of the membranes and cervix (smooth muscle),\textsuperscript{62,70-73} placental hypoxia and/or hypo-perfusion,\textsuperscript{70,74} fetal hypoxia-acidemia,\textsuperscript{75} pain\textsuperscript{76} or exposure to infective agents.\textsuperscript{67,69,70,77} The production of IL-6 leads to an increase in hepcidin levels along with a massive influx of immune cells (predominantly neutrophils) into the cervix, decidua, myometrium, chorioamnionic membranes and amniotic fluid.\textsuperscript{68,78} This further exacerbates the rise in IL-6 and other cytokines.\textsuperscript{76,79} The increase in post-delivery maternal hepcidin concentrations is larger with caesarean section deliveries (5.5-fold increase) as compared to standard vaginal deliveries (3-fold increase).\textsuperscript{80} This is most likely due to the surgical procedure and the subsequent inflammation. Similar increases in serum hepcidin are seen postoperatively during other abdominal surgeries.\textsuperscript{81} The effect of this maternal rise in hepcidin before, during and immediately after childbirth on the late fetal/early neonatal iron status is unknown, though like IL-6,\textsuperscript{82} hepcidin is not thought to cross the placenta.\textsuperscript{83}

Effects of infection on neonatal serum hepcidin levels

Intra-amniotic infections can cause an increase in fetal hepcidin.\textsuperscript{84} Multiple studies have documented an association between chorioamnionitis, perinatal acidosis and neonatal sepsis with high umbilical cord hepcidin concentrations.\textsuperscript{84-89} For example, an extremely high cord concentration (437.6 ng/mL) was found in a neonate with confirmed \textit{Enterococcus faecalis} early-onset sepsis.\textsuperscript{87} Similarly, very-low birth weight, premature neonates with late-onset culture-confirmed sepsis, exhibit elevated levels of hepcidin.\textsuperscript{90} Nevertheless, despite the well-documented regulatory pathways of infection and inflammation on iron regulation, it is important to note that multiple publications have shown a lack of correlation between hepcidin, IL-6 and CRP in sick neonates.\textsuperscript{87,91} This is likely due to differences in the
biochemical kinetics of these molecules. IL-6 concentrations spike very early in the course of perinatal infection, whereas the rise of CRP is delayed.

**Standardising hepcidin measurements**

Multiple assays, including mass spectrometry (MS) and immunochemistry ELISA methods, are available to quantify hepcidin in various body fluids (urine, serum and plasma).\(^9^2\) However, in the studies included in this review, none of these methods are calibrated using the same standards and, as a result, there are significant differences in hepcidin values between studies.\(^9^3,9^4\)

In 2016, Van der Vorm *et al.* harmonized many of the available hepcidin ELISA assays using native, lyophilized plasma with cyrolyoprotectant as a commutable candidate reference material.\(^9^3\) Linear equations were formulated to standardize the hepcidin assays.\(^9^3\) These equations can now be used to conduct post-hoc standardization of non-calibrated test results, aiding the retrospective comparison of data from previous publications. We have used these equations in this review to generate standardized hepcidin values *(Supplementary Table 1)*. The production of standardized reference material, which was refined in 2019, is available for purchase allowing hepcidin measurements to be standardized in all laboratories.\(^9^4\)

**3.3 METHODS**

In March 2019, we reviewed the literature searching two databases: PubMed and Ovid Medline with no restrictions on language or the year of publication. The original search was for human studies only. Corresponding authors of extracted publications were not contacted. One individual carried out the inclusion/exclusion process of the retrieved studies, and there was no assessment of bias or the quality of studies as seen in a systematic review process.
Table 1 displays the search strategy used. Figure 2 shows the flow diagram of the literature search. The search generated publications containing data on cord and venous concentrations of hepcidin, serum iron and transferrin saturation in the neonatal period. Studies that analyzed healthy neonates were included. Mean, median or range of the gestational age of the study population was a requirement for inclusion. Neonates >37 weeks at delivery were regarded as full-term neonates (FTB). Studies or study groups with a gestational <37 weeks were classed as premature (PTB) neonates. Retrieved publications had to report a mean time of bleed 0-720 hours post-delivery to be analysed. Mean (SD or 95% CI), or median (range, IQR, or 95% CI) data were extracted from the included publications. Studies reporting mean (95% CI) were included in the calculation of weighted means (95% CI) and the associated Figures 2-5. Reference ranges for adults and children were presented for comparison.\textsuperscript{95,96} Many retrieved publications did not stratify results by birthweight; as a result, this variable was not recorded in Tables 2-4. Publications were not stratified by sample type (serum or plasma) due to the overall lack of studies. If multiple publications on the same study population were retrieved, only one was included in the analysis. This was the case with Ervasti et al.\textsuperscript{97,98} and Sweet et al.,\textsuperscript{99} as previously mentioned in Lorenz et al.\textsuperscript{100}

The standardization of hepcidin values using different ELISA assays was performed using the slopes and intercepts from Van der Vorm et al.\textsuperscript{93} This was performed for studies that used ELISA test kits from DRG (hepcidin-25 (human) EIA Kit, DRG, USA), Bachem (hepcidin-25 EIA Kit, Bachem, USA) and Intrinsic Lifesciences (Intrinsic Hepcidin ELISA Kit, Intrinsic Lifesciences, USA). It was not possible to standardize hepcidin values acquired using the ELISA from Hangzhou Eastbiopharm (Hangzhou Eastbiopharm Co. Ltd. Hangzhou, Zhejiang, China) and mass spectroscopy (MCProt Biotechnology, Kanazawa, Japan), used in Basu et al.\textsuperscript{52} and Ichinomiya et al.,\textsuperscript{85} respectively. Prohepcidin was not included in the analysis as it is a poor proxy for biochemically active hepcidin-25.\textsuperscript{101-106}
The software Stata IC version 15 (StataCorp LP, College Station, Texas, USA) and R (R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, 2020, [https://www.R-project.org](https://www.R-project.org)) were used to analyse data. To calculate the confidence interval (CI) around the weighted mean, the weighted variance was calculated using the `wtd.var` function from the R package Hmisc. The standard error derived from this weighted variance was then used to calculate the t-statistic (i.e. weighted mean divided by weighted standard error), from which the 95% CI was derived. GraphPad Prism version 8 (GraphPad Software, San Diego, California, USA) software was used to produce the graphical representation of the results.

### 3.4 RESULTS

The initial search of two electronic databases for three different iron markers yielded 13,931 publications. After the exclusion of duplicated studies and selection criteria filtering, 20 publications were included in the analysis for hepcidin, 23 publications for TSAT and 51 publications for serum iron. Many of these studies were found to contain information on multiple parameters of interest. Overall, we identified 59 publications containing data on hepcidin, serum iron or TSAT in FTB neonates. Sixteen publications were found to contain data on PTB neonates.

In publications detailing the effects of cord clamping interventions, all retrieved cord blood values were from groups that underwent 60 seconds of delayed cord clamping. This is consistent with current WHO policy.¹⁰⁷ Cord blood weighted mean values are generated in Tables 2-7, and are represented by a dashed line in Figures 3-5 and α (95% CI) in Figures 6-8.
**Hepcidin**

Standardized weighted mean umbilical cord blood hepcidin levels were higher in FTB neonates (29.7 ng/mL; CI: 21.1-38.3 ng/mL) vs PTB neonates (8.4 ng/mL; CI: 2.0-14.7 ng/mL) (**Figure 3A and 3B and Tables 2 and 3**). Full-term cord blood hepcidin levels were 2-fold higher than in adult male (13.1 ng/mL; CI: 1.4-43.2 ng/mL) and female (10.6 ng/mL; CI: 1.4-43 ng/mL) references ranges (**Table 2**). FTB standardized venous hepcidin levels increased (61.1 ng/mL; CI: 20.1-102.0 ng/mL) over the first four days of life (**Figure 6A**). This trend is unclear for PTB neonates due to the lack of studies (**Table 3 and Figure 6B**). No studies were retrieved that assessed post-delivery venous blood samples >77 hours in FTB or >168 hours in PTB.

**TSAT**

The weighted mean TSAT in cord blood was higher in FTB neonates (51.7%; CI: 46.5-56.9%) compared to PTB neonates (36.5%; CI: 0.8-72.1%) (**Tables 4 and 5 and Figure 4**). Cord blood TSAT in FTB neonates was double the reference levels found in adults (23.5%; CI: 12-38.8%) and children aged 1-5 years (19.4%; CI: 8.2-32.9%) (**Table 4**). The weighted mean average of TSAT decreased 2-fold from cord blood to venous blood in FTB neonates (down to 25.2%; CI: 20.1-30.3%) (**Figure 7A**). This hypoferric response in FTB neonates was followed by a steady increase from 21.8% (CI: 18.8-24.7%) to 44.2% (CI: 32.1-57.8%). No trend was identifiable in PTB neonates due to the lack of data (**Table 5 and Figure 7B**).

**Serum Iron**

Unlike TSAT values, serum iron levels in cord blood were higher in PTB (46.8 μmol/L; CI: 29.7-63.8 μmol/L) neonates compared to FTB neonates (28.5 μmol/L; CI: 26.0-31.1 μmol/L) (**Figure 5**). Like TSAT, a similar 2-fold decrease in the weighted mean average of venous blood compared to cord blood is seen in FTB (13.8 μmol/L; CI: 10.8-16.9 μmol/L) (**Table 6**), and PTB neonates (16.2 μmol/L; CI: 15.3-17.0 μmol/L) (**Table 7**). **Figure 8** suggests that
after the initial reduction (in the first 48 hours of life), levels of serum iron remain consistent over the first month of life in PTB (B) and FTB neonates (A). Serum iron was lowest between 0-48 hours post-delivery (Table 6 and Table 7).

3.5 DISCUSSION

Standardization of hepcidin

To our knowledge, this is the first review to retrospectively compare serum hepcidin concentrations between studies, using post-hoc standardized values to produce calculated weighted mean averages in umbilical cord and venous blood. Pre-standardized hepcidin values in cord and peripheral blood share good correlation between methods. However, absolute values vary considerably between the ELISA test kits used (Table 2-3). A trend of low values is associated with the use of DRG ELISA kits, and high values linked with the use of Intrinsic Lifesciences and Bachem ELISA kits.

Hypoferremia in FTB neonates

The weighted mean average for cord blood hepcidin was calculated using data from 11 studies. Almost all included studies reported a mean value between 11-41ng/mL, apart from Kulik-Rechberger et al. This study reported a much higher cord blood hepcidin value (67.9ng/mL; CI: 59.3-76.5ng/mL) as seen in Figure 3A. In addition, this study also recorded higher hepcidin levels in venous samples collected at 72 hours (92.9ng/mL; CI: 83.3-102.3ng/mL),

When all the data are reviewed together (Figure 6A), hepcidin increases from within the first 2-11 hours of life and then continues to increase up to 82 hours post-delivery. At all times the hepcidin levels are much higher than those recorded in adults. This excess hepcidin
production may provide a quick, comprehensive and relatively long-lasting (0-3 days) hypoferremic response to aid protection during this vulnerable period\textsuperscript{108}. After the first few days, TSAT gradually increases as do serum iron levels, eventually reaching a plateau at approximately 1 month of age.

**Iron metabolism biomarker data gaps in first month of life in full-term babies**

Gaps in the time course of the concentration of hepcidin, TSAT and serum iron in the first month of life in full-term neonates still exist. This hinders our understanding of neonatal iron metabolism, particularly because hepcidin, TSAT and serum iron are transient and dynamic iron parameters. At the point in which hypoferremia is believed to be maximal, publications detailing the concentration in early (<12 hours) venous samples are lacking in both groups (FTB n=2, PTB n=1). Further research at this time point is required to fully elicit the strength and consistency of this response, as well as understanding the process in greater detail.

**Lack of data on preterm neonates during the first 24 hours**

After analysis of the current literature, the extent of the role that hypoferremia plays in neonates with a gestational age less than 37 weeks is still unclear. This is primarily due to the limited number of publications documenting hepcidin (n=5), TSAT (n=6) and serum iron (n=13) in the first month of life in preterm neonates. The variability between the studies is vast and further complicated by the complex, intensive and inconsistent care of premature neonates worldwide.

Data analysis of the retrieved publications suggests that preterm neonates have lower cord hepcidin than in full-term neonates, infants and healthy adults. Weighted cord mean values are 3-fold higher in full-term (29.7ng/mL; CI: 21.1-38.3ng/mL) neonates compared to preterm (8.4ng/mL; CI: 2.0-14.7ng/mL) neonates. We speculate that this could be due to very early preterm neonates (<30 weeks’ gestation) possessing circulatory monocytes with decreased surface expression of TLR4, lower mRNA expression of TLR4 and reduced cytokine
An effect on the production of IL-6 at delivery, might then lead to a reduced ability to stimulate hepcidin expression as suggested in full-term babies.

Our analysis proposes that peripheral venous hepcidin values in preterm neonates increase to 44ng/mL at 168 hours. However, decreases in TSAT between the cord and venous samples are not observed (36.5% to 45.6%). We propose that this is due to a lack of data on TSAT levels in preterm neonates over the first hours of life, potentially due to the complex ethical questions around bleeding preterm neonates so early in postnatal life. This results in the collection of skewed data, focusing only on later time points in the first month of life.

**Limitations**

The aim of this review was to evaluate our current knowledge on neonatal iron homeostasis in preterm and full-term neonates. As a result of the dearth of publications detailing the parameters of interest during this period, our review has several limitations. Firstly, many studies do not stratify their study groups by gestational age (preterm: <37 weeks, full term: >37 weeks). Subsequently, we have had to assign each study group or population by the mean gestational age. This will result in a reduction of any natural variation potentially caused by gestational age between the reviewed populations. This is also the case with respect to birth weight.

Similarly, the studies on preterm neonates are made up of multiple small sample size subgroups with different gestational ages. Due to the lack of preterm studies, we have had to combine these study groups to formulate weighted means and figures. This in itself, could distort the impact of gestational age on our results, since data from the very early preterm newborns is combined with that from the late preterm neonates.
The retrieval of gestational age was a crucial aspect of the search strategy; however, few studies document the method used. There are large differences in the accuracy of different techniques.\textsuperscript{110}

Post-hoc standardization of different hepcidin ELISA kits has, to our knowledge, never been completed before with retrospective data. However, care should be given to the accuracy of the standardized values, as standardization was only possible for DRG, Bachem and Intrinsic Lifesciences ELISA test kits. Studies that used alternative methods\textsuperscript{111} were not included in summary statistics.

An essential criterion of inclusion in this publication was that all neonatal data came from healthy newborns. However, documentation of labor practices and postnatal care, along with postnatal medication lack detail in the publications retrieved. Vaginal delivery is commonly referred to as the method of delivery; however, the use of inflammation-inducing forceps or vacuum delivery is not widely reported.

**Conclusion**

Currently available data suggests that hepcidin, serum iron and TSAT levels for adults and infants are much lower than those found in cord blood and venous blood from neonates during the first month of life. We have strengthened the evidence that full-term neonates possess the ability to produce a hepcidin-mediated hypoferremic response post-delivery. Whether this mechanism is found in PTB neonates is still unclear. This is predominately due to the lack of studies on healthy preterm neonates during the first hours of life. If premature or low birthweight neonates are unable to mount a hypoferremic response, this could enhance their risk of early neonatal infections. Conversely, if the hypoferremic response is seen in both preterm and full-term neonates, it will further support the hypothesis that regulation of iron distribution plays a fundamental role as an innate mechanism of protection against infection.
In summary, serum hepcidin is likely triggered by the inflammatory effect of labor and delivery. We suggest that this intrinsic mechanism of protection protects newborns with immature immune systems to transition from a semi-allogeneic, protected fetal setting to a microbe-rich extrauterine environment.\textsuperscript{112,113} Hepcidin-induced hypoferremia then potentially provides a broad action innate bacteriostatic action to invading micro-organisms, when physiological adaption to postnatal life is so critical for survival.

**Acknowledgements:**

We thank Dr Andrew Armitage for his advice and support in formulating standardised hepcidin values.

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Authors’ Contributions:
J.H.C., A.M.P. and C.C. designed the research; J.H.C. conducted the search strategy and analysed data; J.H.C., A.M.P. and C.C. wrote the paper. All authors reviewed the final manuscript prior to submission.

Consent for Publication:
Not applicable.
TABLE LEGENDS:

Table 1: Literature Search Strategy. Searches conducted via PubMed and Ovid Medline.

Table 2: Hepcidin (ng/mL) in full-term newborns over the neonatal period. Values from Basu et al\textsuperscript{111} were not standardized because the study used the Hangzhou Eastbiopharm ELISA, which was not part of the Van der Vorm et al. analysis.\textsuperscript{93} Extracted standard deviations were converted to 95% confidence intervals. Median (IQR or 95% CI) were not included in weighted means. Reference ranges were obtained from Hepcidin Analysis (Radboudumc).\textsuperscript{96}

Table 3: Hepcidin (ng/mL) in preterm newborns over the neonatal period. Ichinomiya et al\textsuperscript{85} was not standardized because the study used a mass spectrometry based method that was not part of the Van der Vorm et al. analysis.\textsuperscript{93} Extracted standard deviations were converted to 95% confidence intervals. Median (IQR or 95% CI) were not included in weighted means. Reference ranges were obtained from Hepcidin Analysis (Radboudumc).\textsuperscript{96}

Table 4: Transferrin saturation (%) in full-term newborns over the neonatal period. Extracted standard deviations were converted to 95% confidence intervals. Median (IQR or 95% CI) were not included in weighted means. Reference ranges are taken from the National Health and Nutrition Examination Survey, 1999–2000.\textsuperscript{114}

Table 5: Transferrin saturation (%) in preterm newborns over the neonatal period. Extracted standard deviations were converted to 95% confidence intervals. Median (IQR or 95% CI) were not included in weighted means. AGA group\textsuperscript{a} and SGA group\textsuperscript{b} can be identified in Figure 3B using the superscripted letters. Reference ranges were obtained from the National Health and Nutrition Examination Survey, 1999–2000.\textsuperscript{114}
Table 6: Serum iron (μmol/L) in full-term newborns over the neonatal period. Extracted standard deviations were converted to 95% confidence intervals. Median (IQR or 95% CI) were not included in weighted means. Reference ranges are generated from the National Health and Nutrition Examination Survey, 1999–2000.\textsuperscript{114}

Table 7: Serum iron (μmol/L) in preterm newborns over the neonatal period. Extracted standard deviations were converted to 95% confidence intervals. Median (IQR or 95% CI) were not included in weighted means. AGA group\textsuperscript{c}, SGA group\textsuperscript{d}, 30-36 wks\textsuperscript{g} and 24-29 wks\textsuperscript{h} can be identified in Figure 4B using the superscripted letters. Ru et al, 2018\textsuperscript{115} is referenced as \textsuperscript{e}. Ru et al, 2018\textsuperscript{116} is referenced as \textsuperscript{f}. Reference ranges are generated from the National Health and Nutrition Examination Survey, 1999–2000.\textsuperscript{114}

TABLES:

Table 1: Literature Search Strategy

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Table 2: Hepcidin concentration (ng/mL) in full-term newborns over the neonatal period.

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<td>ELISA (Bachem)</td>
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<td>Cord (Serum)</td>
<td>17.85 (4.75-69.2)</td>
<td>24.1 (3.1-44.2)</td>
</tr>
<tr>
<td>Cao et al.</td>
<td>2014</td>
<td>USA</td>
<td>57</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Serum)</td>
<td>131.8 (109-155)</td>
<td>41.7 (34.5-48.9)</td>
</tr>
<tr>
<td>Cao et al.</td>
<td>2016</td>
<td>USA</td>
<td>98</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Serum)</td>
<td>121.5 (105-138)</td>
<td>38.3 (33.2-43.6)</td>
</tr>
<tr>
<td>Delaney et al.</td>
<td>2019</td>
<td>USA</td>
<td>108</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Serum)</td>
<td>92.13 (91.9-92.3)</td>
<td>29.2 (29.1-29.2)</td>
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<tr>
<td>Dosch et al.</td>
<td>2016</td>
<td>USA</td>
<td>47</td>
<td>ELISA (DRG)</td>
<td>Cord (Plasma)</td>
<td>13.4 (11.7-15.1)</td>
<td>17.8 (15.4-20.2)</td>
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<tr>
<td>Garcia-Valdes et al.</td>
<td>2015</td>
<td>Spain</td>
<td>52</td>
<td>ELISA (DRG)</td>
<td>Cord (Serum)</td>
<td>18.01 (15.1-20.9)</td>
<td>24.3 (20.2-28.4)</td>
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<tr>
<td>Hoppe et al.</td>
<td>2018</td>
<td>Sweden</td>
<td>15</td>
<td>ELISA (Bachem)</td>
<td>Cord (Serum)</td>
<td>30.5 (21.7-38.8)</td>
<td>19.5 (13.9-24.8)</td>
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<tr>
<td>Kulik-Rechberger et al.</td>
<td>2016</td>
<td>Poland</td>
<td>44</td>
<td>ELISA (DRG)</td>
<td>Cord (Serum)</td>
<td>48.98 (42.9-55.1)</td>
<td>67.9 (59.3-76.5)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>44</td>
<td>ELISA (DRG)</td>
<td>Venous - 72 hours (Serum)</td>
<td>66.79 (60-73.5)</td>
<td>92.9 (83.3-102.3)</td>
</tr>
<tr>
<td>Lee et al.</td>
<td>2016</td>
<td>USA</td>
<td>104</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Serum)</td>
<td>87.4 (74.4-103)</td>
<td>27.7 (23.6-32.6)</td>
</tr>
<tr>
<td>Lorenz et al.</td>
<td>2014</td>
<td>Germany</td>
<td>100</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Plasma)</td>
<td>103.9 (61.4-149.2)</td>
<td>32.9 (19.6-47.1)</td>
</tr>
<tr>
<td>Prentice et al.</td>
<td>2019</td>
<td>The Gambia</td>
<td>81</td>
<td>ELISA (Bachem)</td>
<td>Cord (Serum)</td>
<td>43.8 (36.8-52.3)</td>
<td>27.9 (23.5-33.4)</td>
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<td>53</td>
<td>ELISA (Bachem)</td>
<td>Venous - 6 hours (2-11) (Serum)</td>
<td>79.4 (68.1-92.4)</td>
<td>50.7 (43.5-58.9)</td>
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<tr>
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<td></td>
<td></td>
<td>21</td>
<td>ELISA (Bachem)</td>
<td>Venous - 29 hours (26-34) (Serum)</td>
<td>45.9 (36.5-57.8)</td>
<td>29.3 (23.3-36.9)</td>
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<tr>
<td></td>
<td></td>
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<td>33</td>
<td>ELISA (Bachem)</td>
<td>Venous - 77 hours (74-82) (Serum)</td>
<td>87.1 (73.8-102.7)</td>
<td>55.6 (47.1-65.5)</td>
</tr>
<tr>
<td>Rehu et al.</td>
<td>2010</td>
<td>Finland</td>
<td>116</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Serum)</td>
<td>71.6 (60.8-84.4)</td>
<td>22.8 (19.4-26.77)</td>
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<tr>
<td>Ru et al.</td>
<td>2018</td>
<td>USA</td>
<td>50</td>
<td>ELISA (Bachem)</td>
<td>Cord (Serum)</td>
<td>17 (12-24.2)</td>
<td>10.9 (7.7-15.5)</td>
</tr>
<tr>
<td>Slomka et al.</td>
<td>2013</td>
<td>Poland</td>
<td>54</td>
<td>ELISA (DRG)</td>
<td>Cord (Serum)</td>
<td>18.50 (2.75-35.13)</td>
<td>25 (2.8-48.4)</td>
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<tr>
<td>Young et al.</td>
<td>2011</td>
<td>USA</td>
<td>19</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Serum)</td>
<td>61.0 (26.4-95.6)</td>
<td>19.4 (8.6-30.3)</td>
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</tbody>
</table>

**Weighted Mean (Cord)**

<table>
<thead>
<tr>
<th>Hepcidin (ng/mL) Mean (95% CI)</th>
<th>Standardized Hepcidin (ng/mL) Mean (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>73.2 (48.1-98.3)</td>
<td>29.7 (21.1-38.3)</td>
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</table>

N/A = Not available
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<thead>
<tr>
<th>Weighted Mean (Venous)</th>
<th>72.7 (48.3-97.2)</th>
<th>N/A</th>
<th>61.1 (20.1-102.0)</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (Median (95% CI))</td>
<td>Men: 13.1 (1.4-43.2) ng/mL</td>
<td>Women: 10.6 (1.4-43) ng/mL (hepcidinanalysis.com, 2019)</td>
<td></td>
<td></td>
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<tr>
<td>Infants (Median (95% CI))</td>
<td>11.9 (3.3-37.7) ng/mL (hepcidinanalysis.com, 2019)</td>
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</table>
Table 3: Hepcidin concentration (ng/mL) in preterm newborns over the neonatal period.

<table>
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<tr>
<th>Reference</th>
<th>Year</th>
<th>Location</th>
<th>n</th>
<th>Test Type</th>
<th>Type of Sample</th>
<th>Study Group</th>
<th>Hepcidin (ng/mL)</th>
<th>Standardized Hepcidin (ng/mL)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean (95% CI)</td>
<td>Median (IQR or 95% CI)</td>
</tr>
<tr>
<td>Delaney et al.</td>
<td>2019</td>
<td>USA</td>
<td>126</td>
<td>ELISA (Bachem)</td>
<td>Cord (Serum)</td>
<td></td>
<td>13.78 (13.6-14)</td>
<td>8.8 (8.7-9)</td>
</tr>
<tr>
<td>Ichinomiya et al.</td>
<td>2017</td>
<td>Japan</td>
<td>92</td>
<td>Mass Spec (MCProt)</td>
<td>Cord (Serum)</td>
<td></td>
<td>7.3 (2.85-16.38)</td>
<td>N/A</td>
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<tr>
<td>Lorenz et al.</td>
<td>2014</td>
<td>Germany</td>
<td>40</td>
<td>ELISA (Intrinsic)</td>
<td>Cord (Plasma)</td>
<td>24-29 wks</td>
<td>26.9 (13.5-63.1)</td>
<td>8.7 (4.5-20.1)</td>
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<td>45.9 (24.7-74.5)</td>
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<td></td>
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<td>26.9 (13.5-63.1)</td>
<td>8.7 (4.5-20.1)</td>
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<td></td>
<td>45.9 (24.7-74.5)</td>
<td>N/A</td>
</tr>
<tr>
<td>Ru et al.</td>
<td>2018</td>
<td>USA</td>
<td>92</td>
<td>ELISA (Bachem)</td>
<td>Cord (Serum)</td>
<td></td>
<td>12.1 (9.2-15.7)</td>
<td>7.8 (5.9-10.1)</td>
</tr>
<tr>
<td>Uijterschout et al.</td>
<td>2015</td>
<td>Netherlands</td>
<td>85</td>
<td>ELISA (Bachem)</td>
<td>Venous - 168 hours (Serum)</td>
<td>69.6 (14.6-180.1)</td>
<td>44.4 (9.4-114.8)</td>
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<td>69.6 (14.6-180.1)</td>
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<td></td>
<td></td>
<td>69.6 (14.6-180.1)</td>
<td>N/A</td>
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<tr>
<td>Adults (Median (95% CI))</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>13.1 (1.4-43.2)</td>
<td>Women: 10.6 (1.4-43) ng/mL</td>
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<tr>
<td>Infants (Median (95% CI))</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.9 (3.3-37.7)</td>
<td>(hepcidinanalysis.com, 2019)</td>
</tr>
</tbody>
</table>
Table 4: Transferrin saturation (%) in full-term newborns over the neonatal period.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Location</th>
<th>n</th>
<th>Type of Sample</th>
<th>TSAT (%) Mean (95% CI)</th>
<th>Median (IQR or 95% CI or Range)</th>
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<tbody>
<tr>
<td>Al-Tawil et al.</td>
<td>2012</td>
<td>Egypt</td>
<td>90</td>
<td>Venous - 24 hours</td>
<td>25 (24.6-25.4)</td>
<td></td>
</tr>
<tr>
<td>Ali et al.</td>
<td>2016</td>
<td>USA</td>
<td>64</td>
<td>Cord</td>
<td>59.2 (53.9-64.5)</td>
<td></td>
</tr>
<tr>
<td>Anderson et al.</td>
<td>2011</td>
<td>Sweden</td>
<td>162</td>
<td>Venous - 48 hours</td>
<td>23 (21.9-24.1)</td>
<td></td>
</tr>
<tr>
<td>Balogh et al.</td>
<td>2007</td>
<td>Hungary</td>
<td>20</td>
<td>Cord</td>
<td>23 (21.9-24.1)</td>
<td>60.5 (14-90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Venous - 39 hours</td>
<td>60.5 (14-90)</td>
<td></td>
</tr>
<tr>
<td>Basu et al.</td>
<td>2015</td>
<td>India</td>
<td>15</td>
<td>Cord</td>
<td>61.8 (54.7-68.9)</td>
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<tr>
<td>El-Farrash et al.</td>
<td>2012</td>
<td>Egypt</td>
<td>30</td>
<td>Cord</td>
<td>49.5 (42.5-56.5)</td>
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<tr>
<td>Ervasti et al.</td>
<td>2007</td>
<td>Finland</td>
<td>199</td>
<td>Cord</td>
<td>55 (52.4-57.6)</td>
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<tr>
<td>Haga et al.</td>
<td>1980</td>
<td>Norway</td>
<td>21</td>
<td>Cord</td>
<td>55 (33.8-76.2)</td>
<td></td>
</tr>
<tr>
<td>Kalem et al.</td>
<td>2019</td>
<td>Turkey</td>
<td>380</td>
<td>Cord</td>
<td>55.87 (54.8-56.9)</td>
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<tr>
<td>Kelly et al.</td>
<td>1978</td>
<td>Scotland</td>
<td>115</td>
<td>Cord</td>
<td>58.8 (55.6-62)</td>
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<tr>
<td>Kitajima et al.</td>
<td>2010</td>
<td>Japan</td>
<td>8</td>
<td>Cord</td>
<td>15.1 (8.3-27.5)</td>
<td>44.2 (32.1-57.8)</td>
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<td></td>
<td>Venous - 720 hours</td>
<td>15.1 (8.3-27.5)</td>
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<tr>
<td>Kleven et al.</td>
<td>2007</td>
<td>USA</td>
<td>26</td>
<td>Cord</td>
<td>42 (32.4-51.6)</td>
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<tr>
<td>Mashakho et al.</td>
<td>1991</td>
<td>DRC</td>
<td>166</td>
<td>Cord</td>
<td>32.3 (30.1-34.5)</td>
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<tr>
<td>Milman et al.</td>
<td>1987</td>
<td>Denmark</td>
<td>74</td>
<td>Cord</td>
<td>48 (32-71)</td>
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<td>Venous - 120 hours</td>
<td>33 (21-48)</td>
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<tr>
<td>Prentice et al.</td>
<td>2019</td>
<td>The Gambia</td>
<td>81</td>
<td>Cord</td>
<td>47.6 (43.7-51.5)</td>
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<td>Venous - 6 hours (2-11)</td>
<td>24.4 (21.2-27.6)</td>
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<td></td>
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<td>Venous - 29 hours (26-34)</td>
<td>21.8 (18.8-24.7)</td>
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<td>Venous - 77 hours (74-82)</td>
<td>30.9 (26.9-34.8)</td>
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<tr>
<td>Puolakka et al.</td>
<td>1980</td>
<td>Finland</td>
<td>47</td>
<td>Cord</td>
<td>53 (49-57)</td>
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<tr>
<td>Rehu et al.</td>
<td>2010</td>
<td>Finland</td>
<td>116</td>
<td>Cord</td>
<td>50.6 (44.5-57.5)</td>
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<tr>
<td>Rois et al.</td>
<td>1975</td>
<td>USA</td>
<td>26</td>
<td>Cord</td>
<td>61.2 (55.9-66.5)</td>
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<td>Study</td>
<td>Year</td>
<td>Country</td>
<td>Sample Size</td>
<td>Type</td>
<td>Value</td>
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<tr>
<td>Slomka et al.</td>
<td>2013</td>
<td>Poland</td>
<td>49</td>
<td>Cord</td>
<td>58.1 (51.7-73.6)</td>
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<tr>
<td>Yamada et al.</td>
<td>2014</td>
<td>Brazil</td>
<td>21</td>
<td>Cord</td>
<td>47.7 (40.2-55.2)</td>
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<td></td>
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<td>21</td>
<td>Venous - 720 hours</td>
<td>39.8 (34.7-44.9)</td>
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<tr>
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<td></td>
<td>21</td>
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</tbody>
</table>

- **Weighted Mean (Cord)**: 51.7 (46.5-56.9) N/A
- **Weighted Mean (Venous)**: 25.2 (20.1-30.3) N/A

- **Adults (Median (95% CI))**: 23.5 (12-38.8) % (10-90th percentiles) - NHNES
- **Infants (Median (95% CI))**: 19.4 (8.2-32.9) % (10-90th percentiles) - NHNES
Table 5: Transferrin saturation (%) in preterm newborns over the neonatal period.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Location</th>
<th>n</th>
<th>Type of Sample</th>
<th>Study Group</th>
<th>TSAT (%)</th>
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</thead>
<tbody>
<tr>
<td>Celik et al.</td>
<td>2015</td>
<td>Turkey</td>
<td>42</td>
<td>Venous - 648 hours (288-1872)</td>
<td></td>
<td>46.5 (41.2-51.8)</td>
</tr>
<tr>
<td>Haga et al.</td>
<td>1980</td>
<td>Norway</td>
<td>23</td>
<td>Cord</td>
<td>AGA Groupa</td>
<td>48 (39.8-56.2)</td>
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<tr>
<td></td>
<td></td>
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<td>6</td>
<td>Cord</td>
<td>SGA Groupb</td>
<td>41 (23.4-58.6)</td>
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<tr>
<td>Ichinomiya et al.</td>
<td>2017</td>
<td>Japan</td>
<td>92</td>
<td>Cord</td>
<td></td>
<td>87.2 (68.3-100)</td>
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<tr>
<td>Kitajima et al.</td>
<td>2010</td>
<td>Japan</td>
<td>13</td>
<td>Cord</td>
<td></td>
<td>64.3 (15.8-88.9)</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>13</td>
<td>Venous - 720 hours</td>
<td></td>
<td>33.2 (17.1-79.5)</td>
</tr>
<tr>
<td>Lackmann et al.</td>
<td>1998</td>
<td>Germany</td>
<td>15</td>
<td>Venous (&lt;1 hour)</td>
<td>&lt;32 wks</td>
<td>39 (5-83)</td>
</tr>
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<td></td>
<td></td>
<td>22</td>
<td>Venous (&lt;1 hour)</td>
<td>33-34 wks</td>
<td>36 (7-87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26</td>
<td>Venous (&lt;1 hour)</td>
<td>35-36 wks</td>
<td>31 (13-60)</td>
</tr>
<tr>
<td>Yamada et al.</td>
<td>2014</td>
<td>Brazil</td>
<td>25</td>
<td>Cord</td>
<td></td>
<td>24.8 (18.5-31.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25</td>
<td>Venous - 720 hours</td>
<td></td>
<td>44.1 (37.3-50.9)</td>
</tr>
</tbody>
</table>

Weighted Mean (Cord) 36.5 (0.8-72.1) N/A

Weighted Mean (Venous) 45.6 (30.4-60.9) N/A

Adults (Median (95% CI)) 23.5 (12-38.8) % (10-90th percentiles) - NHNES

Infants (Median (95% CI)) 19.4 (8.2-32.9) % (10-90th percentiles) - NHNES
Table 6: Serum iron concentration (μmol/L) in full-term newborns over the neonatal period.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Location</th>
<th>n</th>
<th>Type of Sample</th>
<th>Serum Iron (μmol/L) Mean (95% CI or Range)</th>
<th>Median (IQR or 95% CI or Range)</th>
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</thead>
<tbody>
<tr>
<td>Ahlsten et al.</td>
<td>1989</td>
<td>Sweden</td>
<td>20</td>
<td>Cord</td>
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<td>Ali et al.</td>
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<td>64</td>
<td>Cord</td>
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<td>Amarnath et al.</td>
<td>1989</td>
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<td>Anderson et al.</td>
<td>2011</td>
<td>Sweden</td>
<td>162</td>
<td>Venous - 48 hours</td>
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<td>The Gambia</td>
<td>193</td>
<td>Cord</td>
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<td>114</td>
<td>Cord</td>
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<td>Jordan</td>
<td>92</td>
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<td>Greece</td>
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<td>Busarira et al.</td>
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<td>Chong et al.</td>
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<td>Delaney et al.</td>
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<td>108</td>
<td>Cord</td>
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<td>El-Farrash et al.</td>
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<td>1980</td>
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<td>Lao et al.</td>
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<td>Lee et al.</td>
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<td>Mezdoud et al.</td>
<td>2017</td>
<td>Algeria</td>
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<td>2018</td>
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<td>Sweet et al.</td>
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<td>Yamada et al.</td>
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Table 7: Serum iron concentration (μmol/L) in preterm newborns over the neonatal period.

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<th>Study Group</th>
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<td>Celik et al.</td>
<td>2015</td>
<td>Turkey</td>
<td>42</td>
<td>Venous - 648 hours (288-1872)</td>
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<td>Delaney et al.</td>
<td>2019</td>
<td>USA</td>
<td>126</td>
<td>Cord</td>
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<td>7</td>
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<td>Ichinomiya et al.</td>
<td>2017</td>
<td>Japan</td>
<td>92</td>
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<td>Lackmann et al.</td>
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<td>Ru et al.</td>
<td>2018</td>
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<td>91</td>
<td>Cord</td>
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FIGURE LEGENDS

Figure 1: Placental iron transfer between mother and fetus. Fe$^{2+}$ = ferrous iron, Fe$^{3+}$ = ferric iron, Tf = transferrin, Apo-Tf = unsaturated transferrin, Fetal Tf = fetal-derived transferrin, NTBI = non-transferrin bound iron. Syncytiotrophoblasts in the placental villi take up transferrin-bound iron from the maternal circulation by endocytosis via transferrin receptor 1 (TFR1). Iron is released from TFR1 in acidified endosomes and transferred into the syncytiotrophoblast cytoplasm by DMT-1, Zrt/Irt-like protein ZIP8, and ZIP14, collectively. Ferroportin transports iron out of placental syncytiotrophoblasts, and then ceruloplasmin, hephaestin, and zyklopen oxidise Fe$^{2+}$ to Fe$^{3+}$ helping it pass through the endothelium to reach the fetal circulation. It is still unclear as to whether newly transported iron enters the fetal circulation as NTBI or bound to fetal transferrin. Fetal-derived hepcidin is believed to regulate ferroportin expression on the fetal basal-side of placental syncytiotrophoblasts. Maternal-derived hepcidin is believed to play a role in regulating TFR1 expression on the maternal-side of the placental syncytiotrophoblasts. This figure is adapted from Sangkhae and Nemeth et al, 2018.

Figure 2: Flow diagram of the literature search and selection criteria. Retrieving publications on hepcidin, TSAT or serum iron in neonates over the first month of life.

Figure 3: Hepcidin (ng/mL) in cord blood: (A) full term neonates, (B) preterm neonates. Standardized means from each publication are plotted with error bars showing 95% confidence intervals. Dashed line shows the weighted mean of all publications found in the figure. Median values were not included in this figure.
Figure 4: Transferrin saturation (%) in cord blood: (A) full term neonates, (B) preterm neonates. Means from each publication are plotted with error bars showing 95% confidence intervals. Dashed line shows the weighted mean of all publications found in the figure. a shows Haga et al, AGA group. b shows Haga et al SGA group. Median values were not included in this figure.

Figure 5: Serum iron (μmol/L) in cord blood: (A) full term neonates, (B) preterm neonates. Means from each publication are plotted with error bars showing 95% confidence intervals. Dashed line shows the weighted mean of all publications found in the figure. c shows AGA neonates in Haga et al. d shows SGA neonates in Haga et al. Ru et al, 2018 is referenced as e. Ru et al, 2018 is referenced as f. g shows 30-36 wks neonates in Sweet et al. h shows 24-29 wks neonates in Sweet et al. Median values were not included in this figure.

Figure 6: Hepcidin (ng/mL) over the neonatal period: (A) full term neonates, α shows the weighted mean (95%CI) for all studies seen in Figure 2A. β, γ and ε shows Prentice et al. δ shows Kulik-Rechberger et al. (B) preterm neonates, α shows the weighted mean (95%CI) for all studies seen in Figure 2B. β shows Uijterschout et al.

Figure 7: Transferrin saturation (%) over the neonatal period: (A) full term neonates, α shows the weighted mean (95%CI) for all studies seen in Figure 3A. β shows Prentice et al. γ shows Al-Tawil et al. δ shows Prentice et al. ε shows Balogh et al. ϕ shows Anderson et al. η shows Milman et al. i shows Kitajima et al. ϕ shows Yamada et al. (B) preterm neonates, α shows the
weighted mean (95%CI) for all studies seen in Figure 3B. β shows Lackmann et al.127 χ shows Celik et al.128 δ shows Yamada et al.126 ε shows Kitajima et al.125 All values are mean (95%CI), unless marked with ° median (range) and • median (95%CI). Lackmann et al, 1998 (β) data from the three study groups (<32 wks, 33-34 wks and 35-36 wks) was averaged as all groups are classed as PTB neonates and are bled at the same time of life.127

Figure 8: Serum iron (μmol/L) over the neonatal period: (A) full term neonates, α shows the weighted mean (95%CI) for all studies seen in Figure 2A. β shows Prentice et al.108 χ shows Patidar et al.129 δ shows Prentice et al.108 ε shows Balogh et al.101 φ shows Szabo et al.130 γ shows Anderson et al.124 η shows Prentice et al.108 τ shows Milman et al.40 ϕ shows Tsuzuki et al.131 κ shows Tiker et al.132 λ shows Yapakci et al.133 μ shows Ozkiraz et al.134 ν shows Yamada et al.126 (B) preterm neonates, α shows the weighted mean (95%CI) for all studies seen in Figure 2B. β shows Lackmann et al.127 χ shows Tiker et al.132 δ shows Tiker et al.132 ε shows Tsuzuki et al.131 φ shows Schiza et al.135 γ shows Yapakci et al.133 η shows Celik et al.128 τ shows Yamada et al.126 All values are mean (95%CI), unless marked with * mean (range), ° median (range) and • median (95%CI). Lackmann et al, 1998 (β) data from the three study groups (<32 wks, 33-34 wks and 35-36 wks) was averaged as all groups are classed as PTB neonates and are bled at the same time of life.127
Figure 1: Placental iron transfer between mother and fetus
Figure 2: Flow diagram of the literature search.

- **Hepcidin**
  - Ovid MedLine (n=97)
  - PubMed (n=177)
  - Records Identified Filtered by Species (Humans) (n=274)
  - Duplicates Removed (n=79)
  - Publications in Analysis (n=20)

- **TSAT**
  - Ovid MedLine (n=119)
  - PubMed (n=333)
  - Records Identified Filtered by Species (Humans) (n=452)
  - Duplicates Removed (n=117)
  - Publications in Analysis (n=23)

- **Serum Iron**
  - Ovid MedLine (n=2809)
  - PubMed (n=10396)
  - Records Identified Filtered by Species (Humans) (n=13205)
  - Duplicates Removed (n=2485)
  - Publications in Analysis (n=51)

**Reasons for Exclusion (n=11047):**

**Full-Term Publications (n=59) | Preterm Publications (n=16)**
Figure 3: Standardized hepcidin concentration (ng/mL) in cord blood.
Figure 4: Transferrin saturation (%) in cord blood.
Figure 5: Serum iron concentration (μmol/L) in cord blood
Figure 6: Hepcidin concentration (ng/mL) over the neonatal period

Figure 6A - Hepcidin in FTB neonates

Figure 6B - Hepcidin in PTB neonates
Figure 7: Transferrin saturation (%) over the neonatal period

Figure 7A - TSAT in FTB neonates

Figure 7B - TSAT in PTB neonates
Figure 8: Serum iron concentration (μmol/L) over the neonatal period

Figure 8A - Serum iron in FTB neonates

Figure 8B - Serum iron in PTB neonates
Title

Hepcidin, serum iron and transferrin saturation in full term and premature infants during the first month of life: A review of existing evidence in humans.

Authors

James H. Cross, BSc¹, Andrew M. Prentice, PhD¹, Carla Cerami, MD¹*.

Affiliations

¹Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine, Atlantic Boulevard, Fajara, P.O. Box 273, Banjul, The Gambia.

3.7 SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Supplementary Table 1 - Post-hoc hepcidin standardisation

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<td>0.711 (0.674-0.748)</td>
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Supplementary Table 1: Previously documented regression relationships used to conduct post-hoc hepcidin standardisation. These regression relationships were extract from Table 2 of van der Vorm et al.¹

SUPPLEMENTARY REFERENCES

Chapter 4 - Neonatal iron distribution and infection susceptibility in full term, preterm and low birthweight babies in urban Gambia: study protocol for an observational study (Methodology)

Summary of Chapter

BACKGROUND: Neonatal infection is the third largest cause of death in children under-five worldwide. Nutritional immunity is the process by which the host innate immune system limits nutrient availability to invading organisms. Iron is an essential micronutrient for both microbial pathogens and their mammalian hosts. Changes in iron availability and distribution have significant effects on pathogen virulence and on the immune response to infection. Our previously published data shows that, during the first 24 hours of life, full-term neonates have reduced overall serum iron. Transferrin saturation decreases rapidly from 45% in cord blood to ~20% by six hours post-delivery.

METHODS: To study neonatal nutritional immunity and its role in neonatal susceptibility to infection, we will conduct an observational study on 300 full-term normal birthweight (FTB+NBW), 50 preterm normal birthweight (PTB+NBW), 50 preterm low birthweight (PTB+LBW) and 50 full-term low birthweight (FTB+LBW), vaginally-delivered neonates born at Kanifing General Hospital, The Gambia. We will characterize and quantify iron-related nutritional immunity during the early neonatal period and use ex vivo sentinel bacterial growth assays to assess how differences in serum iron affect bacterial growth. Blood samples will be collected from the umbilical cord (arterial and venous) and at serial time points from the neonates over the first week of life.

DISCUSSION: Currently, little is known about nutritional immunity in neonates. In this study, we will increase understanding of how nutritional immunity may protect neonates from
infection during the first critical days of life by limiting the pathogenicity and virulence of neonatal sepsis causing organisms by reducing the availability of iron. Additionally, we will investigate the hypothesis that this protective mechanism may not be activated in preterm and low birthweight neonates, potentially putting these babies at an enhanced risk of neonatal infection.

*NOTE: The secondary study objective (III) of assessing the bacterial growth of common neonatal pathogens in neonatal sera was not achieved due to the lack of sufficient blood sample volume.*
RESEARCH PAPER COVER SHEET - METHODOLOGY PAPER

Please note that a cover sheet must be completed for each research paper included within a thesis.

**SECTION A – Student Details**

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<th>Surname/Family Name</th>
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<td>Mr.</td>
<td>James Henry</td>
<td>Cross</td>
<td>Iron and Infection: Neonatal Iron Transition</td>
<td>Dr Carla Cerami</td>
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If the Research Paper has previously been published please complete Section B, if not please move to Section C.

**SECTION B – Paper already published**

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<th>Gates Open Research</th>
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</thead>
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| When was the work published?  | 21st May 2019 (First Published)  
                              | 15th October 2019 (Latest Published) |

If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

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<tr>
<td>Was the work subject to academic peer review?</td>
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*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.*
Keywords
Nutritional Immunity, Host-Pathogen Interaction, Hepcidin, Neonates, Hypoperfomeria, Transferrin, The Gambia, Sub-Saharan Africa

Corresponding author: Carla Cerami (ccerami@mrc.gm)

Author roles: Cross JH: Data Curation, Formal Analysis, Investigation, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; Jarjou O: Investigation, Project Administration, Supervision, Writing – Review & Editing; Mohammed NI: Formal Analysis, Writing – Review & Editing; Prentice AM: Conceptualization, Formal Analysis, Writing – Review & Editing; Cerami C: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing.

Competing interests: No competing interests were disclosed.

Grant information: This study was supported by the Bill & Melinda Gates Foundation [OPP1152353].
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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First published: 21 May 2019, 3:1469 (https://doi.org/10.12686/gatopenres.12963.1)

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?

Please list the paper’s authors in the intended authorship order:

Stage of publication

Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

This paper was proposed at the start of the study by myself and my supervisor to reference all materials and methods for the subsequent original publications. I coordinated the development of study protocols and data analysis plan. Additionally, I produced the first draft of this paper for review by co-authors. Dr Carla Cerami, Dr Ousman Jarjou and Professor Andrew Prentice all helped re-structure the first draft and provided suggestions towards making edits. Dr Nuredin Ibrahim Mohammed advised in the formal statistical analysis plan.

SECTION E

Student Signature

Date 08/08/2019
Supervisor Signature

Date 08/08/2019
STUDY PROTOCOL

Neonatal iron distribution and infection susceptibility in full term, preterm and low birthweight babies in urban Gambia: study protocol for an observational study. [version 1; peer review: 2 approved]

James H. Cross, Ousman Jarjour, Nuredin Ibrahim Mohammed, Andrew M. Prentice, Carla Cerami

MRC Unit The Gambia at the London School of Hygiene & Tropical Medicine, Fajara, The Gambia

v1 First published: 21 May 2019, 3:1469 (https://doi.org/10.12688/gatesopenres.12963.1)

Abstract

Background: Neonatal infection is the third largest cause of death in children under five worldwide. Nutritional immunity is the process by which the host innate immune system limits nutrient availability to invading organisms. Iron is an essential micronutrient for both microbial pathogens and their mammalian hosts. Changes in iron availability and distribution have significant effects on pathogen virulence and on the immune response to infection. Our previously published data shows that, during the first 24 hours of life, full-term neonates have reduced overall serum iron. Transferrin saturation decreases rapidly from 45% in cord blood to ~20% by six hours post-delivery.

Methods: To study neonatal nutritional immunity and its role in neonatal susceptibility to infection, we will conduct an observational study on 300 full-term normal birth weight (FTB+NBW), 50 preterm normal birth weight (PTB+NBW), 50 preterm low birth weight (PTB+LBW) and 50 full-term low birth weight (FTB+LBW), vaginally-delivered neonates born at Kanifing General Hospital, The Gambia. We will characterize and quantify iron-related nutritional immunity during the early neonatal period and use ex vivo sentinel bacterial growth assays to assess how differences in serum iron affect bacterial growth. Blood samples will be collected from the umbilical cord (arterial and venous) and at serial time points from the neonates over the first week of life.

Discussion: Currently, little is known about nutritional immunity in neonates. In this study, we will increase understanding of how nutritional immunity may protect neonates from infection during the first critical days of life by limiting the pathogenicity and virulence of neonatal sepsis causing organisms by reducing the availability of iron. Additionally, we will investigate the hypothesis that this protective mechanism may not be activated in preterm and low birth weight neonates, potentially putting these babies at an enhanced risk of neonatal infection.

Trial registration: clinicaltrials.gov (NCT03353051) 27/11/2017

Open Peer Review

Reviewer Status ✔ ✔

Invited Reviewers

1 
2

version 2
published 15 Oct 2019

version 1
published 21 May 2019

1 Deborah Stefanova, ETH Zurich, Zurich, Switzerland

2 Veena Sangkhare, University of California, Los Angeles (UCLA), Los Angeles, USA

Any reports and responses or comments on the article can be found at the end of the article.
Keywords
Nutritional Immunity, Host-Pathogen Interaction, Hepcidin, Neonates, Hypoferremia, Transferrin, The Gambia, Sub-Saharan Africa

Corresponding author: Carla Cerami (ccerami@mrc.mr)

Author roles: Cross JH: Data Curation, Formal Analysis, Investigation, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; Jarjour O: Investigation, Project Administration, Supervision, Writing – Review & Editing; Mohammed NI: Formal Analysis, Writing – Review & Editing; Prentice AM: Conceptualization, Formal Analysis, Writing – Review & Editing; Cerami C: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This study was supported by the Bill & Melinda Gates Foundation [OPP1152353].

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How to cite this article: Cross JH, Jarjour O, Mohammed NI et al. Neonatal iron distribution and infection susceptibility in full term, preterm and low birthweight babies in urban Gambia: study protocol for an observational study. [version 1; peer review: 2 approved]

First published: 21 May 2019, 3:1469 (https://doi.org/10.12688/gatesopenres.12963.1)
Abbreviations
PTB = Preterm birth
FTB = Fullterm birth
LBW = Low birthweight
NBW = Normal birthweight
WHO = World Health Organisation
TSAT = Transferrin saturation
MDG = Millennium development goal
GBS = Group B Streptococcus
EONS = Early onset neonatal sepsis
YICSS = Young Infants Clinical Signs Studies
KGH = Kanifing General Hospital
MRCG = Medical Research Council Unit The Gambia at LSHTM
KMC = Kanifing Municipal Council
UIBC = Unbound iron-binding capacity
IL6 = Interleukin 6
IL22 = Interleukin 22
sTfR = Soluble transferrin receptor
CRP = C-reactive protein
AGP = Alpha 1-acid glycoprotein
HIV = Human immunodeficiency virus
TB = Tuberculosis
EDTA = Ethylenediaminetetraacetic acid
V1 = Venous bleed 1
V2 = Venous bleed 2
IM = Intramuscular
ELISA = Enzyme-linked immunosorbent assay
ID = Identification
CRF = Case report form
eCRF = Electronic case report form
ANOVA = Analysis of variance
IDE = Integrated development environment
CyTOF = Cytometry by time of flight
STAT3 = Signal transducer and activator of transcription 3
TLR = Toll-like receptor
SOP = Standard operating procedure
OD = Optical density
WBC = White blood cell
LPS = Lipopolysaccharide

Introduction
Neonatal infections – challenges in low-income settings
Neonatal infection is the third largest cause of death in children under-five worldwide and is an ongoing major global public health challenge (Sustainable Development Goal 3.2). Between 1990 and 2016 maternal and under-five child mortality has decreased by half. However, the proportion of neonatal deaths among under-five deaths increased from 37% (1990) to 44% (2013). Today, approximately 2.8 million children die annually during the neonatal period – the first 28 days of life. Of these, 73% die within the first six days of life. An increasing proportion of child deaths are in sub-Saharan Africa, with 60–80% of newborn deaths occurring in low birthweight (LBW) neonates (<2500g at birth). 95% of all LBW neonates are born in low-income countries. However, the situation is likely to be worse than documented, as neonatal deaths in developing countries are commonly under reported and the records commonly contain errors. It is estimated that about one third of deaths in the first month of life, are caused by infections including bacterial sepsis, meningitis, pneumonia, neonatal tetanus, and diarrhoea.

Evidence is lacking on the aetiology of neonatal infections in developing countries, especially from community settings. However, the limited data suggests that Klebsiella species, Escherichia coli, and Staphylococcus aureus are common causes of early onset sepsis (EONS). The available antibiotic susceptibility data suggests that pathogens associated with neonatal sepsis in developing countries are often resistant to WHO-recommended empiric antibiotics. Antibiotic resistance has emerged with potency over the last few decades due to a multitude of complex reasons. Antibiotic overuse, inappropriate prescribing, inadequate diagnostics, extensive agricultural use, availability of new few antibiotics, and the ease of transportation of resistant bacteria are among the factors contributing to the rise. Equally, bacteria have the ability to rapidly mutate (with or without drug selection pressure) and horizontally transfer genetic material between species (i.e. non-human pathogens) of bacteria. Neonates are particularly at risk from antibiotic resistant organisms because they generally succumb before alternative antibiotic regimes can be tried.

Diagnosis of neonatal sepsis with high specificity remains challenging in developing countries. A widely used tool developed by the World Health Organization Young Infants Clinical Signs Studies (YICSS), which includes seven clinical signs to aid diagnosis, has only an 85% sensitivity and 75% specificity for severe bacterial infection during the first week of life. Microbiological identification of a pathogen isolated from blood cultures is one of the most important factors in the host-pathogen battle for resources. Bacteria and other pathogens have evolved a wide variety of mechanisms to acquire iron from the nutrient rich host (e.g. siderophores and iron specific channels) to aid growth and virulence, with a number of iron acquisition genes concentrated on high pathogenicity islands.

Nutritional immunity
Iron is critical for the human host and most pathogens. Iron is one of the most important factors in the host-pathogen battle for resources. Bacteria and other pathogens have evolved a wide variety of mechanisms to acquire iron from the nutrient rich host (e.g. siderophores and iron specific channels) to aid growth and virulence, with a number of iron acquisition genes concentrated on high pathogenicity islands.
Nutritional immunity describes the normal physiological innate processes used by the host to combat infection by limiting nutrient availability. Key among these processes is the ability to rapidly decrease the circulating concentration of iron (and other transition metals) in response to an infection. The hypoferrremia of inflammation is mediated by the hormone, hepcidin. Research completed in 2000–2001 by three independent research groups led to the discovery of the hepcidin hormone, and the important function it plays in many aspects of iron metabolism. Hepcidin is now understood to be the master regulator of iron homeostasis. Unlike any other micronutrient, iron is regulated by a hormone that responds to both infection and nutritional status. The host inflammatory mediators, IL6, IL22 and Type I interferon, have been found to increase transcription of hepcidin through several Toll-like receptor (TLR) ligands and STAT3 signalling resulting in decreased systemic iron concentrations in the circulation. This multifaceted mechanism limits nutrient availability to extracellular invading microorganisms. The system is well documented in mouse models, but less so in human studies. However, it is clear that humans with excessive levels of serum iron (e.g. due to hemochromatosis) are predisposed to infection with iron-dependent species of bacteria.

Neonatal hypoferrremia

Although iron metabolism in adults and older children is well studied, the kinetics of iron handling in the early neonatal period, a time of intense physiological change, are poorly understood. Childbirth results in a neonate moving from a semi-allogeneic, protected and nearly sterile environment to one that is abundant in a diverse array of microbes. The delivery process is the initial focal point for the mass bacterial colonisation of the skin and gastrointestinal tract of the neonate. Post-natal iron metabolism in neonates is controlled by an array of different signals, such as hypoxia, erythropoietic drive, maternal and foetal iron stores, iron homeostasis, and the important function it plays in many aspects of iron metabolism.

The study described here will shed light on the effects of preterm and low birthweight neonates born by elective caesarean section in full term, healthy vaginally delivered newborns, compared to neonates born by normal vaginal delivery. The study is the initial phase of a larger study to understand the effects of low birthweight and preterm delivery on iron metabolism and handling in the neonatal period. The secondary objective of this study is to characterise iron metabolism, handling and recycling in full term neonates at birth and during the first 7 days of life.

Protocol

Study site

Study participants will be recruited from Kanifing General Hospital (KGH), formerly Serrekunda General Hospital, in the Kanifing region of The Gambia, West Africa. Serrekunda is a large town, forming a peri-urban area with a population of around 340,000, and is 13km to the southwest of the capital, Banjul. Serrekunda was originally made up of nine villages that have merged into a sprawling urban area. Annually, Kanifing General Hospital (KGH) provides antenatal care to 500–700 pregnant mothers. Mothers receiving antenatal care at other local health-care facilities increase the total number of births at the hospital to 3500–4500 per year. The percentage of these that are live, low birthweight neonates (<2.5kg) is approximately 10%. Specimen samples will be subjected to primary processing on-site at KGH, followed by transport to Medical Research Council Unit The Gambia at LSHTM (MRCG) for storage and analysis.

Participants

In total, 450 healthy newborns will be included during delivery at the Kanifing General Hospital Maternity Ward (Figure 1) starting in July 2017. Pregnant mothers must be over the age of eighteen years. After informed consent is obtained, neonates who meet the inclusion criteria will be enrolled into the study. For inclusion in the study, neonates must be healthy, medically stable, greater than 32 weeks gestational age and weigh more than 2000g. To be considered preterm (PTB) the neonates will be < 37 weeks gestational age (assessed by New Ballard Score) and ≥ 32 weeks gestational age. All neonates with a gestational age ≥ 37 weeks will be considered full term (FTB). To be considered low birthweight (LBW) the...
Figure 1. Main study flow chart of all study procedures and exclusion criteria. Group A will contain neonates characterised by preterm birth and low birthweight (PTB+LBW); Group B will contain neonates characterised by preterm birth and normal birthweight (PTB+NBW); Group C will contain neonates characterised by full term birth and low birthweight (FTB+LBW); Groups D1, D2 and D3 will all contain babies characterised by full term birth and normal birthweight (FTB+NBW). In this study, preterm is defined <37 weeks gestation and low birthweight is <2500g. Exclusion criteria (α): Father refused, mother refused, family/escort refused, communication not possible or mother with severe disabilities. Exclusion criteria (β): Antibiotics or antimalarials given before delivery (<24 hours), referred to tertiary level health facility, abscended, known HIV-positive, severe pre-eclampsia, receiving TB treatment, antepartum haemorrhage, recent blood transfusion (within the last month), no foetal heartbeat, mother <18 years, refusal, recruited to another study and emergency caesarean section. Exclusion criteria (γ): Recruited to another study on-site, refusal, blood transfusion given in labour, antibiotics or antimalarials given during labour, neonate requires resuscitation (1 min APGAR), neonatal weight <2000g, neonate born breech, neonate born via vacuum delivery, neonate born caesarean section, foetal stillbirth, macerated stillbirth and major congenital malformations. Exclusion criteria (δ): Failed cord blood collection (serum tubes), failed cord blood collection (EDTA), cord blood processed >3 hours, neonate requires resuscitation (10 min APGAR), absconded and route 2B refusal. Exclusion criteria (ε): Mother birth check refusal, father birth check refusal, family escort birth check refusal, mother <18 years, recruited to another study on-site, antibiotics or antimalarials given to mother before delivery (<24 hours), neonate has had surgery, neonates sibling twin was recruited, neonate given antibiotics (other than tetracycline eye ointment), neonate given iron supplementation, neonatal sickness (tone, activity, feeding, heart rate, respiratory rate, abnormal anterior fontanelle), neonatal temperature (<36.5°C or >37.5°C), major congenital malformations (neonate), New Ballard Score (<32 weeks), failed V1 (serum), failed V1 (EDTA), failed V1 (both EDTA and serum), mother V1 bleed refusal, father V1 bleed refusal, and family/escort V1 bleed refusal. Exclusion criteria (ζ): neonatal sickness (tone, activity, feeding, heart rate, respiratory rate, abnormal anterior fontanelle), neonatal temperature (<36.5°C or >37.5°C), neonate has had surgery, neonate given antibiotics (other than tetracycline eye ointment), neonate given iron supplementation, failed V2 bleed, Mother community/V2 bleed refusal, father community/V2 bleed refusal, and family community/V2 bleed refusal.
neonates will weigh < 2500g. All neonates weighing ≥ 2500 g will be considered normal birthweight (NBW).

The study groups are:

Group A (PTB+LBW): Neonates who are both preterm and low birthweight.

Group B (PTB+NBW): Neonates who are preterm and normal birthweight.

Group C (FTB+LBW): Neonates who are full term but low birthweight.

Group D (FTB+NBW): Neonates who are full term and normal birthweight.

In addition to the main study, 300 FTB neonates of the 450 neonates will also be included into a sub-study, which aims to describe serum iron markers in full term babies (Group D, FTB+NBW only) over the first week of life.

Study design

This is a proof-of-concept, observational cohort study (Groups A, B, C and D) with an embedded short prospective cohort study (Group D divided into D1, D2 and D3).

Entry evaluation

Consent and enrolment. There are two routes into the study enrolment (Figure 2). Pregnant mothers who are receiving antenatal care on-site at KGH, will be approached at an antenatal visit and voluntarily sensitised to the study requirements and protocol (Route 1). Pregnant women, who are sensitised will not be required at that point to give written or verbal consent. This group will be provided with study information sheets and encouraged to discuss study participation with their family. When the pregnant woman returns to KGH Maternity Ward to deliver (some mothers will choose to deliver at other healthcare facilities), she will be asked to read the full study information sheet (or have it read to her by a study nurse if she is not literate) and provide formal written consent to the study involvement for their neonate (see Extended data).

Route 2 will provide an alternative route of enrolment for pregnant mothers, that would like their neonate to be part of the study but have been receiving antenatal care at another facility before delivering at KGH maternity ward. In route 2, healthy, pregnant women will enter the KGH ward to deliver and will be approached to provide written formal consent to umbilical cord blood collection and storage only. No testing or laboratory processes will be conducted on their sample, until full study consent is gained post-delivery. The cord blood sample will be stored at 4°C (within the maternity ward), until the mother’s pain and discomfort subside (2–6 hours post-delivery). At this point, the mother and/or father will be invited to provide written formal consent on full study enrolment. If, at this point, mother and/or father refuse full study consent post-delivery, the previously collected personal information and umbilical cord sample will be safely discarded.

Pre-delivery screening

In both Route 1 and Route 2 enrolment, mothers must provide written consent before assessment of personal information (antenatal card) and questioning can begin. After consenting, mothers will be asked for their demographic information and their personal contact details. Pregnant mothers will be excluded from the study if they are below the age of 18 years, have no foetal heartbeat detected upon admission, known to be HIV-positive, in receipt of Mycobacterium tuberculosis therapy, taken antibiotics in the last 24 hours, had a blood transfusion in the last month, suffering from severe pre-eclampsia or antepartum haemorrhage, or in another research study. Mothers can refuse to be part of the study at any stage of the study protocol. Pregnant women that are referred at this point to a tertiary level healthcare facility, will be excluded from the study.

Delivery procedures, post-delivery screening and umbilical cord blood collection

Delivery procedures and screening. Study nurses will assist clinical KGH maternity ward staff in the delivery process and collect data via electronic case report form (eCRF) on their designated study tablets. Neonates will be excluded at the delivery stage of the study for the following reasons: major congenital malformations (not including polydactylism), blood transfusions given to mother or neonate, severe birth asphyxia (requiring resuscitation), neonates born via breech, vacuum or via caesarean section, or a birthweight <2000g. After the delivery stage of the study protocol, neonates can be excluded from the study following the detection of infection or illness (information gained from full blood count analysis or review of systems). Neonates will also be removed from the study protocol, if medication is given (not including intramuscular vitamin K, tetracycline eye ointment or any immunisations). All medication that is given to mothers and neonates will be recorded. Mothers will be able to refuse study participation at any stage. Mothers that deliver multiple newborns will only be invited to consent and enrol one of their neonates into the study.

Umbilical cord blood collection. Once the neonate is fully delivered, one-minute delayed cord clamping will be used (following World Health Organisation (WHO) policy5). During the one-minute delay, the one-minute APGAR score will be conducted. If the neonate requires resuscitation, the neonate will be excluded from the study. After the umbilical cord has been removed and cleaned, a trained study nurse will identify the umbilical arteries and the umbilical vein. Blood will be collected from both. The tubes will be placed in the cool box for 1-3 hours before transfer to the study laboratory for primary processing. If the mother is enrolled by route 2, the mother will be asked to provide written consent to full study recruitment before the sample is sent for primary laboratory processing.

Hospital assessment and 1st venous blood draw

Hospital health assessment (study recruitment and group allocation). At 6–24 hours post-delivery, recruited mothers and their neonates will be invited to a private consultation with the study research clinician. Further demographic data will
Figure 2. NeoInnate Study enrolment route and blood draw design. Group A contains neonates characterised by preterm birth and low birthweight (PTB+LBW); Group B contains neonates characterised by preterm birth and normal birthweight (PTB+NBW); Group C contains neonates characterised by full term birth and low birthweight (FTB+LBW); Groups D1, D2 and D3 all contain babies characterised by full term birth and normal birthweight (FTB+NBW).
be collected, along with a photograph of the antenatal card to gather gestational age data (fundal height, last menstrual period and ultrasound), mother’s last haemoglobin level before delivery (dated), known sickle cell status, neonate immunisations, and medication given to the mother (pre, during and post-delivery) and the neonate. A complete review of systems of the mother and neonate plus anthropometric data on the newborn will then be collected. Neuromuscular and physical maturation of each neonate will be assessed using the New Ballard Score.10

Neonates will be excluded if they score less than 32 weeks of gestation. From this assessment, the neonate will be assigned to a specific study group. If the neonate is allocated to the Group D (FTB+NBW) group, the neonate will be allocated to a randomised bleed group (≥24 hours - <80 hours (Group D1); ≥80 hours - <136 hours (Group D2); and ≥136 - <192 hours (Group D3)). Failure to meet the inclusion criteria at this stage of the study protocol, will result in exclusion from the study.

1st venous blood draw (all neonates). A blood sample will be collected from all neonates that have passed the inclusion criteria in the hospital health assessment. Immediately after the health assessment, a venous blood draw will be performed (6–24 hours post-delivery). PTB and/or LBW neonates will donate 2ml of venous blood. FTB+NBW neonates will donate 3.5ml of venous blood. All samples will reach the laboratory within three hours post collection for primary processing.

Community health assessment and 2nd venous blood draw

Community health assessment. Study nurses will visit all mothers or enrolled neonates at their homes at least once. At that visit, a physical examination of the neonate will be completed. The following information will also be collected: neonatal immunisation history, a complete review of systems of the mother and baby, and any medication given to the mother or neonate since delivery. Mothers will also be provided with health education and study contact details (should the neonate become unwell).

2nd venous blood draw Group D (FTB+NBW) only. At this point, if the mother and neonate are deemed to have passed the screening process and the neonate is in Group D, then the neonate will have its second and last venous blood draw (3.5ml). All samples will reach the laboratory within three hours post collection for primary processing.

Laboratory evaluations

Blood samples. Whole blood samples will be assessed for: full haematology panel (using a Medonic M20M GP), glucose-6-phosphate dehydrogenase deficiency and sickle trait. All serum samples collected will be assessed by ELISA for the following: IL6, IL22, free haem, hepcidin, hemopexin, lipocalin-2, lactoferrin, and foetal haemoglobin. Additionally, serum ferritin, serum iron, UIBC, soluble transferrin receptor (sTfR), transferrin, C-reactive protein (CRP), haptoglobin, and alpha-1-acid glycoprotein (AGP) will be assessed using a fully automated biochemistry analyser (Cobas Integra 400 plus) Additionally, umbilical WBC will be processed and analysed for exploratory secondary analysis 4.

Bacterial growth assays. Ex vivo growth of bacteria (including clinical and laboratory isolates of Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Enterobacter spp., Enterococcus spp., and Salmonella Typhimurium) in participant serum as in Cross et al. (2015)11 will be performed.

Study outcomes

The primary outcome variables will be TSAT (transferrin saturation) and serum iron.

The secondary outcome variables will be hepcidin; hemopexin; haptoglobin; IL22; free serum haem and haemoglobin; foetal haemoglobin; lactoferrin; lipocalin-2; IL6; C-reactive protein; alpha-1-acid glycoprotein; transferrin concentration; soluble transferrin receptor; unbound iron-binding capacity; ferritin; haemoglobin; WBCs types and numbers in cord blood samples and ex vivo bacterial growth.

Data entry, handling, storage and security

All protocol-required field data will be captured electronically on an electronic eCRF or a paper case report form (CRF) that will be completed for each included participant. After giving written consent the pregnant women will be given a study identification number, which will be used in all future datasets for subject anonymity. Field data will be collected verbally and from antenatal cards by study nurses. Collected data will be entered in real time using eCRFs developed on top of a REDCap (Research Electronic Data Capture) database and published on Samsung Galaxy Tab 3 SM-T111 handheld devices. Collected data will be transported to the database via a direct secure connection over the 4G mobile network. Laboratory related data will be extracted directly from laboratory equipment and uploaded to the database. Any data collected on the paper format will be double entered by a trained data entry clerk. The local co-investigator will review all forms and identify any errors prior to data entry or to marking data as complete. The study data will also be validated through automated and manual validation methods implemented in the study database application system. The study database will be custom-developed. All paper CRF will be stored in a locked file archive. Electronic data will be stored on the local dedicated server maintained at MRCG. The study will be conducted in compliance with Good Clinical Practice. Study personal security measures will include controlled access limited to authorised users only, physical security, remove identifiable information (anonymization), avoidance of third-party cloud storage and password protection.

Sample size and power

This study will target recruitment of 150 “exposed” neonates which will include a target of 50 neonates in each Group A, B and C. 300 neonates will be recruited for Group D (“unexposed”). The study will have constraints from time, budget, loss to follow up, haemolysis during sample collection, insufficient blood volume and the distribution of new births in each group at the Kanifing General Hospital.

Based on this, we have run simulations (Stata/IC 15.1) to calculate the power to detect a range of differences comparing groups for example Groups D and A with respect to the primary
Outcomes TSAT and serum iron. We did not calculate power for the secondary outcomes, which are considered exploratory. The simulation was run using a linear regression model assuming a lognormal distribution for the response variables TSAT and serum iron levels 6–24 hours after birth. Data from a previous study (Prentice S, personal communication) was used to obtain mean and SD estimates for TSAT and serum iron both at baseline and 6–24 hours after birth. The predictor variables were the Groups (A–D) with Group D as the reference. The model was adjusted for the baseline (cord blood levels). We also examined the power assuming a normal distribution for TSAT (i.e. without log transformation). The significance level considered was 0.05 and the simulation was run for 100000 iterations. This process was repeated for the following four different sample size scenarios which we refer to as N1, N2, N3 and N4 respectively:

- N1 Group A = Group B = Group C = 50 neonates
- N2 Group A = Group B = 25 neonates and Group C = 50 neonates
- N3 Group A = Group B = 10 neonates and Group C = 50 neonates
- N4 Group A = Group B = 50 neonates and Group C = 10 neonates

For all the above four cases, D = 300.

The simulation results for the baseline adjusted model with log transformation show that for sample size scenario N1, the minimum mean differences that can be detected with 80% would increase (sizes of 0.35 and 0.39 respectively. The power drops substantially (power were about 4% and 2.5 µmol/L for TSAT and serum iron respectively (Figure 3A and 3B). These correspond to effect sizes of 0.35 and 0.39 respectively. The power drops substantially if smaller numbers were to be recruited as in scenarios N2 (A=B=25) and N3 (A=B=10). Under N2 and N3, the minimum mean differences that can be detected with 80% would increase to about 5.8% and 9.1% for TSAT (Figure 3A) and 3.3 µmol/L and 5 µmol/L for serum iron (Figure 3B). The results for scenario N4 can be considered as subset of N1-N3 by rearranging Groups A, B and C.

Statistical analysis

The primary research objective is to examine if preterm and/or low birthweight neonates ("Exposed") have a reduced ability to sequester iron at 6-24 hours after birth in comparison to full term neonates with normal birthweight ("Unexposed")?

We hypothesize that FTB+NBW (Group D) neonates on average will have lower values of TSAT and serum iron compared to "Exposed" (PTB or LBW babies) (Figure 4). Initially, we will analyse all "Exposed" (Groups A+B+C) vs "Unexposed" (Group D). Each neonate will be further classified by his or her gestational age (premature vs. full term) and birthweight (low vs. normal) in a 2x2 table (Table 1). Linear regression models will be used in order to evaluate the difference in mean between each Group A-C and D; that is where D will be the reference group. TSAT and serum iron levels will be log transformed before fitting the models (if necessary). Both the unadjusted and adjusted (for the cord blood level) mean differences together with the 95% CI will be calculated.

In the second stage of analysis, we will assess the effect of potential confounding variables using the regression models. Covariates to be considered include the specific time of measurement, demographic and health variables. The time effect may not be linear, and this will be investigated in the further regression models. To reduce the effects of multiple testing, data analysis will be driven by a predefined primary study hypothesis. Any exploratory analyses conducted (in the absence of predefined study hypotheses) will be considered hypothesis-generating, rather than confirmatory. In order to reduce the levels of missing and inaccurately entered data into the database, all clinical, demographic and laboratory data will be entered in real time via electronic data capture, with automated and manual validation methods implemented. The study design does not provide for the recruitment of equal numbers of subjects in each month of the year (or during the dry vs wet seasons). The Gambia has a higher birth rate during the months of September-December in comparison to other months.

In order to remove this potential source of bias, we will adjust for month of birth and/or season in the regression analysis. If the missing data rates is more than 5%, we will consider imputation. The follow-up duration is relatively short. Thus, we expect little bias from loss to follow-up. If loss to follow-up rate is considerably different between groups, we will perform sensitivity analyses to examine the robustness of results. We will also consider sensitivity analysis fitting a multivariate regression model where the main outcomes of interest (including TSAT, serum iron and hepcidin) will be jointly regressed to the same set of predictors.

The analysis for the secondary objectives are described below:

I. Characterise how iron metabolism, handling and recycling differs between full term, preterm and low birthweight neonates at birth and during the first 24 hours of life.

A similar strategy will be used as for the primary objective. Regression modelling will be used to evaluate the difference in means between each Group A-C and D; D will be the reference ("Unexposed") group. The effects of potential confounding variables will also be assessed using further regression modelling.

II. Describe iron metabolism, handling and recycling in full term neonates at birth and during the first 7 days of life.

Analysis of the longitudinal data will involve generalised estimating equations incorporating time of measurement. We will include spline terms at each time point to evaluate the change in the outcomes (all primary and secondary outcome parameters) over time during the transition period from cord to 24–79; 80–135; 136–192 hours after birth. Note that this will only include data collected from Group D neonates and will not be a comparison between Groups A–C and D neonates.

III. Determine if sera from preterm and low birthweight neonates supports a greater level of ex-vivo growth of
Figure 3. Estimated power to detect a given difference between Groups A vs D based on simulation using a linear regression model adjusted for baseline for three sample size scenarios. N1 (Group A=Group B=Group C=50 neonates); N2 (Group A=Group B=25 neonates and Group C=50 neonates); N3 (Group A=Group B =10 neonates and Group C=50 neonates); N4 (Group A=Group B=50 neonates and Group C =10 neonates).

Figure 4. An example of hypothetical scenario for TSAT values between the groups to be compared. In this example: (i) Time 0 refers to average cord blood levels (ii) Time 6-24 refers to the mean level in the 6-24 hour period after birth. (iii) T1, T2, represent TSAT in 1 and 2 above and AT=T2-T1 for full term, normal birthweight (Group D) (iv.) T1', T2' and AT'=T2'-T1' same as above but for the premature, low birthweight (Group A). Hypothesis: H₀: T2=T2' vs. H₁: T2≠T2'.

Table 1. Four combinations in total for exposure: a: Pre, Low; b: Pre, Normal; c: Full, Low and d: Full, Normal.

<table>
<thead>
<tr>
<th>Birthweight (BW)</th>
<th>Term</th>
<th>Pre</th>
<th>Low</th>
<th>Normal</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Full</td>
<td>C</td>
<td>B</td>
<td>D</td>
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Changes in iron availability modulate the growth. Growth assays will be fitted to a standard form of the logistic equation:

\[ N_t = \frac{K}{1 + \left( \frac{K-N_0}{N_0} \right)e^{-rt}} \]

Here, the population size at the beginning of the growth curve is given by \( N_0 \). The carrying capacity is given by \( K \). The intrinsic growth rate of the population is \( r \). We will generate the best fitting values of \( K \), \( r \) and \( N_0 \) for the growth curve data. Additionally, for each bacterium, we compare the time at which the population density reaches \( \frac{1}{2} K \) (inflection point), the fastest possible generation time (doubling time) and the area under the logistic curve obtained by taking the integral of the logistic equation. This will be used to assess growth curves from different sample types (Cord vs V1) and between the four study groups.

IV. Characterize frequencies and functionality of neutrophils, monocytes, dendritic cells, NK cells, B cells, T cells (D8 and CD4) in cord blood from full term, premature and low birthweight neonates.

Microorganisms that are common causes of neonatal sepsis in Africa and The Gambia (Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Enterobacter spp., Enterococcus spp., and S. Typhimurium) in comparison to sera from FTB+NBW neonates.

The bacterial growth will be analysed in a similar method as described in Cross et al. (2015) in order to determine if
Exploratory analysis will be conducted using linear regressions modelling.

Statistical analyses will be performed using STATA (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC); R (R Foundation for Statistical Computing, Vienna, Austria,) and Data Desk (Data Description Inc Ithaca NY). All files used will have an accompanying data dictionary. Annotated STATA do-files or R files will be used to describe any data transformations and statistical tests used.

Dissemination of findings
The study results will be published in relevant peer-reviewed journals and key findings will be presented at international scientific meetings. Data sharing will be in agreement with the sponsor policy on research data sharing and with the Bill & Melinda Gates Foundation Global Access requirements.

Study status
The study is in the data collection phase.

Discussion
Humans and bacteria are involved in an on-going tug of war over iron. Each side has evolved complicated and varied iron-acquisition mechanisms in an effort to turn the tide of war in their own favour50. Nutritional immunity describes the processes by which the human host tries to starve invading organisms of nutrients, especially iron.

This study aims to determine if premature and low birthweight babies have a defect in their ability to sequester iron during the first 24 hours of life. The study design will produce a detailed and extensive picture of iron metabolism in neonates. To our knowledge, no other study has tried to analyse such a large and diverse collection of iron and infection variables in neonates born in Sub Saharan Africa. The study will enrol subjects who are all at an increased risk of neonatal infection, and subsequent sepsis and death.

A potential limitation of this study is the inadequacy of using the New Ballard Score as the only method of gestational aging. Original and New Ballard Score are reported to overestimated gestational age compared to ultrasound and in particular, misclassify preterm infants as term newborns59. Additionally, newborn clinical assessments as a whole, tend to underestimate gestational age in growth-restricted neonates50. The gold standard of gestational aging is an ultrasound in the first trimester33. However, this procedure is rarely correctly completed in this study population. If it is documented on the mother’s antenatal records, care will be taken to record it. Limits of the study also include that HIV status, TB status and iron supplementation given are all gained from the antenatal records of the mother. Furthermore, antenatal records will not contain all information on medication given in every mothers’ pregnancy. As a result, care will be made to extensively question participants mother’s during verbal one-to-one consultation with our study research clinician.

In conclusion, our overarching study goal is to evaluate the likelihood that novel products designed to induce hypoferremia (potentially via mini-hepcidins50) may be useful in the future for the prevention of neonatal sepsis in high risk babies. This could be produced by a transient redistribution of iron away from the circulation, thus applying a bacteriostatic brake on any bacteria that have crossed into the baby’s systemic circulation and hence boosting host survival in vulnerable newborns. We hope this may ultimately help reduce the use of antibiotics in maternal and neonatal wards worldwide.

Ethical approval
This study has been approved by The Gambia Government/MRC Joint Ethics Committee (no. SCC1525) and Ethics Committee of London School of Hygiene and Tropical Medicine (ref no. 14316). The study procedures will be explained to the neonate’s mother/guardians orally or in writing. A neonate is only recruited into the study after the consent form has been signed/thumb printed by the mother/guardian.

This study was registered with clinicaltrials.gov (NCT03353051) on 27 November 2017.

Data availability
Underlying data
No data are associated with this article.

Extended data

This project contains the following extended data:
- SCC1525v2__NeoInnate_Consent form Route 1_v3 Approved8Nov17.docx (Route 1 consent and information sheet)


This project contains the following extended data:
- SCC1525v2__NeoInnate_Consent form Route 2_Part 1__ (UCB Collection) - Labour Ward_v1.1-Approved 8Nov17.docx (Route 2 consent and information sheet part 1)

Figshare: Cross et al. Gates Open Research SCC1525v2__NeoInnate_Consent form Route 2_Part 2_(Post-Delivery) - ANC
This project contains the following extended data:

- SCC1525v2_NeoInnate_Consent form Route 2 Part 2 (Post-Delivery) - ANC Outside SGH v1-Approved
- 8Nov17 (Route 2 consent and information sheet part 2)

Data are available under the terms of the Creative Commons Attribution 4.0 International License (CC-BY 4.0).

References

1. WHO: WHO | Every Newborn: an action plan to end preventable deaths. WHO (World Health Organization), 2014. Reference Source

Grant information

This study was supported by the Bill & Melinda Gates Foundation [OPP1152553].

The funding agency had no role in the design and conduct of the study, and will not have any in the collection, management, analyses or interpretation of the data nor in the preparation, review, or approval of the manuscript.

Acknowledgements

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


52. WHO | Delayed umbilical cord clamping for improved maternal and infant health and nutrition outcomes. WHO. 2018. Reference Source


Chapter 5 – Early postnatal hypoferremia in low birthweight and preterm babies: A prospective cohort study in hospital-delivered Gambian neonates (Main Paper)

Summary of Chapter

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 globally. Iron is a vital nutrient for the most common organisms causing septicaemia. Full-term babies elicit an immediate postnatal hypoferremia 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 (<2500g) (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 hrs after delivery. We measured haematological, iron and proinflammatory biomarkers.

FINDINGS: In both PTB/LBW and FTB/NBW babies, serum iron decreased 3-fold within 12hrs of delivery compared to umbilical blood (7·5±4·5 vs 23·3±7·1ng/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 hypoferremia that is indistinguishable from that in normal term babies. The data suggest that this is a hepcidin-mediated response triggered by acute non-infective inflammation at birth, and likely to have evolved as an innate immune response against bacterial and fungal septicaemia.
RESEARCH PAPER COVER SHEET - MAIN PAPER

Please note that a cover sheet must be completed for each research paper included within a thesis.

SECTION A – Student Details

Student ID Number LSH158152
First Name(s) James Henry
Surname/Family Name Cross
Thesis Title Iron and Infection: Neonatal Iron Transition
Primary Supervisor Dr Carla Cerami

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?
When was the work published?

If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

Have you retained the copyright for the work?*  
Choose an item.  
Was the work subject to academic peer review?  
Choose an item.

*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published? EbioMedicine

Please list the paper’s authors in the intended authorship order: James H. Cross, Ousman Jarjou, Nuredin Ibrahim Mohammed, Santiago Rayment Gomez, Bubacarr J.B Touray, Andrew M. Prentice, Carla Cerami.

Stage of publication In press

SECTION D – Multi-authored work
For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

James H. Cross 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.

SECTION E

Student Signature

Date 08/11/2019

Supervisor Signature

Date 08/11/2019
Title

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

Authors

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Running Title:

Neonatal hypoferremia in premature and low birthweight babies
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 20 studies on hepcidin, 23 on TSAT and 51 on serum iron. Collectively, 59 publications contained research on full-term (FTB) neonates, and 16 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 (0-24h) after delivery. Analysis of the retrieved publications leads to the conclusion that neonatal hypoferremia 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 Gambian newborns in the first hours of life. Our findings confirm that a very rapid hypoferremia 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 hypoferremic responses to full-term, normal-weight newborns.
Implications of all the available evidence:

There is now clear evidence that neonates, including those with PTB or LBW, elicit a rapid and transient hypoferremia 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 septicaemia. This suggests the possibility that a further enhancement or prolongation of hypoferremia (for instance by mini-hepcidins), might offer an additional tool in the armoury against antibiotic-resistant infections in newborns.
5.1 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 globally. Iron is a vital nutrient for the most common organisms causing septicaemia. Full-term babies elicit an immediate postnatal hypoferremia assumed to have evolved as an innate defence. We tested whether PTB and LBW babies are capable of the same response.

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INTERPRETATION: Premature or low birthweight babies are able to mount a very rapid hypoferremia that is indistinguishable from that in normal term babies. The data suggest that this is a hepcidin-mediated response triggered by acute non-infective 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).
KEYWORDS: Nutritional immunity, iron, transferrin saturation, CRP, hepcidin, neonates, low birthweight, prematurity, sub-Saharan Africa, septicaemia.
5.2 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.\(^2\)

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 extraterine 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-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. Individuals with chronically high iron states (e.g. haemochromatosis) have an enhanced risk of bacterial infection, as well as increased free radical redox damage. Historic studies administering parenteral iron to Polynesian neonates infamously increased mortality rates by promoting Escherichia coli septicemias, underlining the critical role that iron plays in the neonatal period.

Cord blood, which has high levels of serum iron and transferrin saturation, 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 24h after birth- an adaptation that may have evolved as an innate defence against iron-requiring microorganisms. 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.

5.3 PARTICIPANTS AND METHODS

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

Study Design
This observational study recruited well, hospital-delivered neonates into four different groups characterised according to birthweight and gestational age as shown in Figure 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) newborns (FTB/NBW). Secondary analyses examine the PTB+NBW, FTB+LBW and PTB+LBW groups separately.
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.

Study setting

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

Recruitment, screening and enrolment

In total, we planned to identify 450 healthy newly born babies during delivery at the Kanifing General Hospital (KGH) 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 attending study physician), ≥32 weeks gestational age and weighed ≥2000g. Preterm babies (PTB) were <37 completed weeks gestational age (assessed by New Ballard Score\textsuperscript{16}). Term babies (FTB) had a gestational age ≥37 completed weeks. Low birthweight (LBW) was defined as <2500g in line with the usual WHO definition. Neonates who weighed ≥2500g were defined as normal birthweight (NBW). The observational period began at delivery and lasted until the end of the 7\textsuperscript{th} day of life. Data collection started on the 5\textsuperscript{th} July 2017 and ended on 1\textsuperscript{st} 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), blood transfusions given to mother or neonate, severe birth asphyxia (requiring resuscitation), neonates born via breech, vacuum or via caesarean section, or a birthweight <2000g (in order to avoid the additional burden of a blood draw in these vulnerable neonates as guided by the Joint Gambia Government/MRCG Ethics Committee).

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.

**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 6-24h 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).

**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. Hepcidin reference material refined by Diepeveen et al\(^{18}\) was not used due to the lack of availability at the time of conducting laboratory analyses.

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.\(^{19}\) Samples were scored 0 (yellow 0 g/L) to 6 (dark red 8 g/L). Samples scoring ≥5 were removed from the analysis. All serum samples experienced one freeze-thaw cycle before biochemical analyses was conducted.

**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.

**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 Figure 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 χ² 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).
5.4 RESULTS

**Figure 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±376g) and LBW (2338±118g) 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±1·3wk). 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±6·6y) than FTB/NBW mothers (29·7±6·9y), but there were no other differences in maternal or neonatal characteristics (Table 1 and Supplementary Table 1 & 2).

**Neonatal hypoferremia**

**Figure 3** illustrates a profound hypoferremia occurring in the first day after parturition. There were no babies that failed to show a hypoferremic response and very few in whom the response was only moderate. **Figure 4** shows that the decline had already occurred by the beginning of our sampling window at 6h post-delivery. Contrary to our initial hypothesis, there was no difference in the hypoferremic 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±4·5μmol/L (P<0·001) and TSAT decreased from 51·7±17·3 to 15·0±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 16·5μmol/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 3·4 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±15·9μmol/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.

Figure 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, P=0·3) in their cord blood. However, the cord blood from PTB neonates had slightly higher TSAT values (54·8±17·7 vs 50·2±16·9%, P=0·017) and serum iron (24·4±7·3 vs 22·7±7·0 μmol/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±23·9ng/ml in FTB/NBW, P=0·5). The venous blood from PTB and LBW neonates had slightly higher TSAT values (16·1±8·4% and 16·2±5·3% vs 14·4±6·0%, both P=0·01) in comparison to FTB neonates. There is no evidence that these small differences in TSAT will translate into a 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 hypoferremia between PTB, LBW and FTB/NBW neonates, there were no differences between the additional subgroupings of A-D.

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±4·0mg/L, P<0·001) and hepcidin levels doubled (19·9±14·2 to 38·4±23·7ng/ml, P<0·001). Figure 4 illustrates the timecourse of the changes.
Table 3 lists the results of the 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).

Factors associated with postnatal hepcidin levels

Based on the *pre-hoc* assumption that hepcidin orchestrates the postnatal hypoferremia, 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 Figure 4C suggests that this was because hepcidin rose very fast at, or immediately after, parturition and was already starting to decline by 6h. 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.

5.5 DISCUSSION

The rapid and profound postnatal hypoferremia demonstrated in this study closely matches our prior findings in full-term, vaginally-delivered rural Gambian babies\textsuperscript{14} and the results of studies elsewhere.\textsuperscript{20,21} The 3-4-fold decrease recorded here is towards the top end of the 2-4-fold range recorded in prior studies.\textsuperscript{14,20,21} 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.\textsuperscript{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.\textsuperscript{22} Our hypothesis was firmly refuted. We showed that the premature and low birthweight neonates all exhibited a profound hypoferremia during the first 24h of life, with no detectable differences from the full-term, normal birthweight newborns. In fact, there was a remarkable similarity in the hypoferremic response across all the study groups that underscores the efficiency of the process and supports the likelihood that it occurs by the process of evolution.

The host-pathogen battle for iron has been extensively studied\textsuperscript{23} and it has long been assumed that the hypoferremia of the acute phase response acts as an innate defence against iron-requiring organisms. Hepcidin is a key, though not necessarily the only,\textsuperscript{24} regulator of this response.\textsuperscript{25} Inflammatory cytokines including IL-6, IL-22 and Type-1 interferon rapidly upregulate hepcidin expression and release from the liver.\textsuperscript{26} Hepcidin causes hypoferremia by inhibiting the action of the transmembrane iron-exporter ferroportin in enterocytes and macrophages, thus blocking iron absorption and recycling.\textsuperscript{27} Injection of recombinant hepcidin results in the very rapid induction of hypoferremia in mice\textsuperscript{28} and humans.\textsuperscript{29}

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 hypoferremia is, at least partly, driven by an inflammatory response to the stress of the birth process and/or the exposure of the newborn to vaginal and/or gastrointestinal micro-organisms not previously encountered \textit{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 too 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.\textsuperscript{24} A full examination of
the mechanism responsible for the hypoferremia would probably require studies in an animal model. Neonatal piglets may be a suitable model due to their low iron endowment at birth, low iron milk, fast growth rate and similarities to human anatomy and physiology. These factors result in young porcine models often becoming iron deficient and anaemic without the need for low-iron diets (i.e. as seen in murine models).

Using *ex vivo* assays with sentinel bacteria, we have previously demonstrated that low TSAT values in serum from adults and neonates 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. The hypoferremia 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 (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 <2000g birthweight, or those delivered via c-section, vacuum or forceps. We cannot speculate whether similar hypoferremic 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 6h 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-inflammatory cytokines (e.g. IL-6 and IL-22) which might have provided additional insights into the mechanisms eliciting hypoferremia. 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 hypoferremia 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 hypoferremia 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. Hypoferremia can slow the multiplication of bacterial pathogens, 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.
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
MRCG = 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
V1 = Venous bleed 1
WHO = World Health Organisation
Acknowledgements:
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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.

Competing Interests:
The authors declare that they have no competing interests.

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.
5.6 REFERENCES


Figure and Table Legends:

Figure 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.

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

Figure 3: Analysis of serum iron (A), TSAT (B) and hepcidin (C) in umbilical cord (BLUE) and post-natal 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}.

Figure 4: Timecourse of the changes in serum iron (A), TSAT (B), hepcidin (C) and CRP (D) in the first day of life. Means ± 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 hours. **** = P<0·0001, *** = P<0·001, ** = P<0·01, * = P<0·05. No significance line = P>0·05.

Figure 5: Comparisons of serum iron (A = cord, B = venous), TSAT (C = cord, D = venous) and hepcidin (E = cord, F = venous) in cord and post-natal venous blood. Means ± 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 1: Participant Characteristics. 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 χ² test. P values in bold font are considered significant based on P<0·05.

Table 2: Iron Parameters (cord vs venous sample) for FTB/NBW, PTB/LBW, PTB and LBW neonates. Data are presented as mean (± 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: log₁₀ (serum iron), 1/sqrt (TIBC), sqrt (ferritin), 1/sqrt (CRP), log₁₀ (hepcidin).

Table 3: Linear regression of factors associated with postnatal serum iron and TSAT. All regressions were adjusted for 4 grades of visually assessed haemolysis. Ranked according to their standardized coefficients calculated in STATA. a 1/sqrt(CRP), b log₁₀ (hepcidin).

Table 4: Linear regression of factors associated with postnatal serum hepcidin. All regressions were adjusted for 4 grades of visually assessed haemolysis. Ranked according to their standardized regression coefficients calculated in STATA. a sqrt(ferritin), b log₁₀ (serum iron), c sqrt(sTfR).
Figure 1
Figure 3

A. Serum Iron [μmol/L]

B. TSAT [%]

C. Log-Hepcidin [log ng/mL]
Figure 4

A

Serum iron [μmol/L]

PTB/LBW
FTB/NBW

Cord 1-8 9-16 17-24

Time [hours]

B

TSAT [%]

Cord 1-8 9-16 17-24

Time [hours]

C

Hepcidin [ng/mL]

Cord 1-8 9-16 17-24

Time [hours]

D

CRP [mg/L]

Cord 1-8 9-16 17-24

Time [hours]
Figure 5

A  B

Serum Iron [μmol/L]

FTB/NBW  PTB/LBW  PTB  LBW

FTB/NBW  PTB/LBW  PTB  LBW

C  D

TSAT [%]

FTB/NBW  PTB/LBW  PTB  LBW

FTB/NBW  PTB/LBW  PTB  LBW

E  F

Hepcidin [ng/mL]

FTB/NBW  PTB/LBW  PTB  LBW

FTB/NBW  PTB/LBW  PTB  LBW
Table 1 – Participant characteristics

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<tr>
<td>Age of mother (Years)</td>
<td>29·4 (±6·9)</td>
<td>29·7 (±6·9)</td>
<td>28·7 (±6·8)</td>
<td>0·16</td>
<td>29·6 (±7·0)</td>
<td>28·9 (±6·8)</td>
<td>0·35</td>
<td>29·7 (±6·9)</td>
<td>26·8 (±6·6)</td>
<td>0·003</td>
</tr>
<tr>
<td>1 min APGAR score (0-10)</td>
<td>9·7 (±0·7)</td>
<td>9·6 (±0·8)</td>
<td>9·7 (±0·6)</td>
<td>0·51</td>
<td>9·6 (±0·8)</td>
<td>9·8 (±0·6)</td>
<td>0·26</td>
<td>9·7 (±0·7)</td>
<td>9·6 (±0·8)</td>
<td>0·7</td>
</tr>
<tr>
<td>Time from admission to birth (Hours)</td>
<td>3·2 (±5·1)</td>
<td>3·2 (±5·2)</td>
<td>3·2 (±4·8)</td>
<td>0·96</td>
<td>3·2 (±5·2)</td>
<td>3·2 (±4·8)</td>
<td>0·94</td>
<td>3·2 (±5·1)</td>
<td>3·3 (±5·0)</td>
<td>0·94</td>
</tr>
<tr>
<td>Time from delivery to postnatal blood collection (Hours)</td>
<td>12·7 (±5·3)</td>
<td>12·7 (±5·4)</td>
<td>12·8 (±5·3)</td>
<td>0·81</td>
<td>12·6 (±5·3)</td>
<td>13·0 (±5·3)</td>
<td>0·47</td>
<td>12·8 (±5·3)</td>
<td>12·5 (±4·8)</td>
<td>0·71</td>
</tr>
<tr>
<td>Percentage male (%)</td>
<td>53·5% (224)</td>
<td>54·3% (151)</td>
<td>52·0% (79)</td>
<td>0·64</td>
<td>54·7% (158)</td>
<td>51·1% (72)</td>
<td>0·48</td>
<td>53·8% (203)</td>
<td>50·9% (27)</td>
<td>0·69</td>
</tr>
<tr>
<td>Percentage early term, ≥37-&lt;38 weeks (%)</td>
<td>19·3% (83)</td>
<td>27·7% (77)</td>
<td>3·9% (6)</td>
<td>&lt;0·001</td>
<td>28·7% (83)</td>
<td>0·0% (0)</td>
<td>&lt;0·001</td>
<td>20·4% (77)</td>
<td>11·3% (6)</td>
<td>0·12</td>
</tr>
<tr>
<td>G6PD deficiency positive (%)</td>
<td>12·6% (54)</td>
<td>11·6% (32)</td>
<td>14·6% (22)</td>
<td>0·36</td>
<td>11·4% (33)</td>
<td>15·0% (21)</td>
<td>0·3</td>
<td>13·3% (50)</td>
<td>7·7% (4)</td>
<td>0·26</td>
</tr>
<tr>
<td>Multiple births (%)</td>
<td>3% (13)</td>
<td>2·2% (6)</td>
<td>4·6% (7)</td>
<td>0·16</td>
<td>2·1% (6)</td>
<td>4·9% (7)</td>
<td>0·1</td>
<td>2·7% (10)</td>
<td>5·7% (3)</td>
<td>0·23</td>
</tr>
<tr>
<td>Percentage of mothers on antenatal iron/folic acid (%)</td>
<td>82·1% (353)</td>
<td>81·7% (227)</td>
<td>82·9% (126)</td>
<td>0·87</td>
<td>81·0% (234)</td>
<td>84·4% (119)</td>
<td>0·83</td>
<td>83·3% (314)</td>
<td>73·6% (39)</td>
<td>0·1</td>
</tr>
</tbody>
</table>
Table 2 - Changes in iron status, inflammation and hepcidin between cord and postnatal blood

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Whole Population</th>
<th>Preterm Birth (PTB)</th>
<th>Low Birthweight (LBW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cord</td>
<td>Venous</td>
<td>P value</td>
</tr>
<tr>
<td>Serum iron</td>
<td>423</td>
<td>23.3 (±7.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>(μmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UIBC</td>
<td>429</td>
<td>23.9 (±10.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(μmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIBC</td>
<td>429</td>
<td>61.0 (±8.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(μmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>424</td>
<td>1.99 (±0.3)</td>
<td>0.0001</td>
</tr>
<tr>
<td>(g/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>424</td>
<td>51.7 (±17.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>426</td>
<td>210.5 (±157.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(μg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum AGP</td>
<td>426</td>
<td>0.18 (±0.1)</td>
<td>-0.00003</td>
</tr>
<tr>
<td>(g/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum CRP</td>
<td>425</td>
<td>0.17 (±0.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>444</td>
<td>11.6 (±3.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(g/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Table values are mean ± standard deviation.
Table 3 - Factors associated with neonatal serum iron and TSAT at 6-24h post-partum

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>Standardised coefficient</th>
<th>P value</th>
<th>R2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate Analysis - Serum Iron</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP a</td>
<td>0.135</td>
<td>0.023</td>
<td>0.258</td>
<td>&lt;0.0001</td>
<td>15.5</td>
</tr>
<tr>
<td>Hepcidin b</td>
<td>-0.139</td>
<td>0.025</td>
<td>-0.249</td>
<td>&lt;0.0001</td>
<td>14.2</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.273</td>
<td>0.056</td>
<td>0.222</td>
<td>&lt;0.0001</td>
<td>13.7</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>-1.21</td>
<td>0.303</td>
<td>-0.184</td>
<td>&lt;0.0001</td>
<td>12.2</td>
</tr>
<tr>
<td>Age of mother</td>
<td>-0.009</td>
<td>0.002</td>
<td>-0.152</td>
<td>0.001</td>
<td>11.1</td>
</tr>
<tr>
<td>Birthweight</td>
<td>-0.0001</td>
<td>-0.0004</td>
<td>-0.149</td>
<td>0.001</td>
<td>11.1</td>
</tr>
<tr>
<td><strong>Multivariate Analysis - Serum Iron</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP a</td>
<td>0.124</td>
<td>0.022</td>
<td>0.239</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Hepcidin b</td>
<td>-0.11</td>
<td>0.025</td>
<td>-0.198</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.23</td>
<td>0.055</td>
<td>0.186</td>
<td>&lt;0.0001</td>
<td>29.2</td>
</tr>
<tr>
<td>Age of mother</td>
<td>-0.008</td>
<td>0.002</td>
<td>-0.143</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>-0.741</td>
<td>0.28</td>
<td>-0.114</td>
<td>0.008</td>
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</tr>
<tr>
<td>Birthweight</td>
<td>-0.0009</td>
<td>-0.0003</td>
<td>-0.104</td>
<td>0.016</td>
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</tr>
<tr>
<td><strong>Univariate Analysis - Transferrin Saturation (TSAT)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP a</td>
<td>0.153</td>
<td>0.023</td>
<td>0.306</td>
<td>&lt;0.0001</td>
<td>11.7</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>-1.14</td>
<td>0.302</td>
<td>-0.181</td>
<td>&lt;0.0001</td>
<td>5.7</td>
</tr>
<tr>
<td>Hepcidin b</td>
<td>-0.085</td>
<td>0.025</td>
<td>-0.16</td>
<td>0.001</td>
<td>4.8</td>
</tr>
<tr>
<td>Birthweight</td>
<td>-0.0001</td>
<td>0.0004</td>
<td>-0.153</td>
<td>0.001</td>
<td>4.7</td>
</tr>
<tr>
<td>Age of mother</td>
<td>-0.008</td>
<td>0.002</td>
<td>-0.151</td>
<td>0.002</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>Multivariate Analysis - Transferrin Saturation (TSAT)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP a</td>
<td>0.139</td>
<td>0.022</td>
<td>0.285</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Hepcidin b</td>
<td>-0.093</td>
<td>0.024</td>
<td>-0.253</td>
<td>&lt;0.0001</td>
<td>21.8</td>
</tr>
<tr>
<td>Ferritin</td>
<td>-0.008</td>
<td>0.002</td>
<td>0.187</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Age of mother</td>
<td>-0.68</td>
<td>0.285</td>
<td>-0.161</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
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</table>
Table 4 - Factors associated with neonatal serum hepcidin at 6-24h post-partum

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>Standardised coefficient</th>
<th>P value</th>
<th>R² (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate Analysis - Serum Hepcidin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin a</td>
<td>0·039</td>
<td>0·0005</td>
<td>0·354</td>
<td>&lt;0·0001</td>
<td>14</td>
</tr>
<tr>
<td>Time of bleed</td>
<td>-0·0007</td>
<td>0·0001</td>
<td>-0·321</td>
<td>&lt;0·0001</td>
<td>12</td>
</tr>
<tr>
<td>Transferrin</td>
<td>-0·706</td>
<td>0·104</td>
<td>-0·318</td>
<td>&lt;0·0001</td>
<td>11·5</td>
</tr>
<tr>
<td>Serum iron b</td>
<td>-0·479</td>
<td>0·089</td>
<td>-0·267</td>
<td>&lt;0·0001</td>
<td>8·2</td>
</tr>
<tr>
<td>sTfR c</td>
<td>-0·36</td>
<td>0·092</td>
<td>-0·189</td>
<td>0·001</td>
<td>5</td>
</tr>
<tr>
<td>Multivariate Analysis - Serum Hepcidin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time of bleed</td>
<td>-0·0008</td>
<td>0·00009</td>
<td>-0·354</td>
<td>&lt;0·0001</td>
<td></td>
</tr>
<tr>
<td>Ferritin a</td>
<td>0·035</td>
<td>0·004</td>
<td>0·319</td>
<td>&lt;0·0001</td>
<td>36·1</td>
</tr>
<tr>
<td>Serum iron b</td>
<td>-0·413</td>
<td>0·078</td>
<td>-0·232</td>
<td>&lt;0·0001</td>
<td></td>
</tr>
<tr>
<td>sTfR c</td>
<td>-0·286</td>
<td>0·086</td>
<td>-0·15</td>
<td>0·001</td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>-0·218</td>
<td>0·111</td>
<td>-0·11</td>
<td>0·05</td>
<td></td>
</tr>
</tbody>
</table>
Title
Early postnatal hypoferremia in low birthweight and preterm babies

Authors
James H. Cross, BSc¹, Ousman Jarjou, MBChB¹, Nuredin Ibrahim Mohammed, PhD¹, Santiago Rayment Gomez², Bubacarr J.B Touray, BSc¹, Andrew M. Prentice, PhD¹, Carla Cerami, MD¹.

Affiliations
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²Cardiff University, Cardiff, Wales, CF10 3AT, United Kingdom.

5.7 SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS
The full study protocol has been previously published.¹
### SUPPLEMENTARY RESULTS

**Supplementary Table 1 – Participant Characteristics (Groups A-D)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Whole Population</th>
<th>Group A (PTB+LBW)</th>
<th>Group B (PTB+NBW)</th>
<th>Group C (FTB+LBW)</th>
<th>Group D (FTB+NBW)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Participants (n)</td>
<td>430</td>
<td>42</td>
<td>99</td>
<td>11</td>
<td>278</td>
<td></td>
</tr>
<tr>
<td>Gestational age (Weeks)</td>
<td>38.1 (±2.1)</td>
<td>35.1 (±0.9)</td>
<td>35.7 (±0.5)</td>
<td>38.6 (±1.3)</td>
<td>39.4 (±1.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3092 (±453.9)</td>
<td>2326 (±122.6)</td>
<td>2916 (±225.9)</td>
<td>2380 (±89.2)</td>
<td>3299 (±368.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>34.0 (±1.2)</td>
<td>32.2 (±0.7)</td>
<td>33.4 (±0.8)</td>
<td>32.7 (±1.0)</td>
<td>34.6 (±1.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>49.2 (±1.9)</td>
<td>46.6 (±1.4)</td>
<td>48.2 (±1.4)</td>
<td>47.0 (±1.5)</td>
<td>50.0 (±1.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maternal hemoglobin ≤7 days before delivery (g/dl)</td>
<td>11.6 (±1.8)</td>
<td>12.2 (±1.5)</td>
<td>11.3 (±1.8)</td>
<td>10.8 (±2.0)</td>
<td>11.6 (±1.8)</td>
<td>0.29</td>
</tr>
<tr>
<td>Age of mother (Years)</td>
<td>29.4 (±6.9)</td>
<td>26.9 (±6.6)</td>
<td>29.8 (±6.7)</td>
<td>26.4 (±6.9)</td>
<td>29.7 (±6.9)</td>
<td>0.03</td>
</tr>
<tr>
<td>1 min APGAR score (0-10)</td>
<td>9.65 (±0.7)</td>
<td>9.6 (±0.7)</td>
<td>9.8 (±0.48)</td>
<td>9.2 (±1.3)</td>
<td>9.6 (±0.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>Time from admission to birth (Hours)</td>
<td>3.23 (±5.1)</td>
<td>3.2 (±4.9)</td>
<td>3.2 (±4.8)</td>
<td>3.7 (±5.5)</td>
<td>3.2 (±5.2)</td>
<td>0.99</td>
</tr>
<tr>
<td>Time from delivery to V1 blood collection (Hours)</td>
<td>12.7 (±5.3)</td>
<td>13.0 (±4.9)</td>
<td>13.0 (±5.5)</td>
<td>10.5 (±3.8)</td>
<td>12.7 (±5.4)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Percentage (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage male (%)</td>
<td>53.5% (224)</td>
<td>47.6% (20)</td>
<td>52.5% (52)</td>
<td>63.6% (7)</td>
<td>54.3% (151)</td>
<td>0.76</td>
</tr>
<tr>
<td>Percentage early term, ≥37–≤38 weeks (%)</td>
<td>19.3% (83)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>54.5% (6)</td>
<td>27.7% (77)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G6PD deficiency positive (%)</td>
<td>12.6% (54)</td>
<td>7.1% (3)</td>
<td>18.2% (18)</td>
<td>9.1% (1)</td>
<td>11.5% (32)</td>
<td>0.23</td>
</tr>
<tr>
<td>Multiple births (%)</td>
<td>3% (13)</td>
<td>7.1% (3)</td>
<td>4% (4)</td>
<td>0% (0)</td>
<td>2.2% (6)</td>
<td>0.28</td>
</tr>
<tr>
<td>Percentage of mother on antenatal iron/folic acid (%)</td>
<td>82.1% (353)</td>
<td>76.2% (32)</td>
<td>87.9% (87)</td>
<td>63.6% (7)</td>
<td>81.7% (227)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

**Supplementary Table 1**: Baseline Characteristics. Demographic, clinical and pregnancy outcome characteristics of the women and their newborns for groups A-D. Data are presented as mean (± SD) and analysed by ANOVA or as n (%) and analysed by χ2 test. P values in bold font are considered significant based on P<0.05.
## Supplementary Table 2: Maternal Ethnicity

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<thead>
<tr>
<th>Ethnic Group</th>
<th>Whole Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FTB/NBW</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Aku</td>
<td>0.9% (4)</td>
</tr>
<tr>
<td>Balanta</td>
<td>0.9% (4)</td>
</tr>
<tr>
<td>Fula</td>
<td>18.4% (79)</td>
</tr>
<tr>
<td>Jola</td>
<td>13.3% (57)</td>
</tr>
<tr>
<td>Mandinka</td>
<td>38.4% (165)</td>
</tr>
<tr>
<td>Manjago</td>
<td>0.7% (3)</td>
</tr>
<tr>
<td>Sarahule</td>
<td>5.1% (22)</td>
</tr>
<tr>
<td>Serere</td>
<td>6.3% (27)</td>
</tr>
<tr>
<td>Wollof</td>
<td>11.9% (51)</td>
</tr>
<tr>
<td>Other</td>
<td>4.2% (15)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Health Outcome</th>
<th>FTB/NBW</th>
<th>PTB/LBW</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Full Term Birth (FTB)</td>
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<tr>
<td>Preterm Birth (PTB)</td>
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<td>NBW (FTB+LBW)</td>
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<td>LBW (FTB+LBW)</td>
<td>11.5% (16)</td>
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<td>P value</td>
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<th>Group A (FTB+LBW)</th>
<th>Group B (FTB+NBW)</th>
<th>Group C (FTB+LBW)</th>
<th>Group D (FTB+NBW)</th>
<th>P value</th>
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<td>0.4% (4)</td>
<td>2% (2)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>1.4% (4)</td>
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Supplementary Table 2: Ethnicity of mother. Data are presented as n (%) and analysed by χ2 test. P values in bold font are considered significant based on P < 0.05. No significant differences between groups was observed.
### Supplementary Table 3 – Comparison of iron status, inflammation and haematological parameters in cord samples

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Whole Population</th>
<th>PTB/LBW</th>
<th>FTB/NBW</th>
<th>Full-Term Birth (FTB)</th>
<th>Preterm Birth (PTB)</th>
<th>Normal Birthweight (NBW)</th>
<th>Low Birthweight (LBW)</th>
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<td>n</td>
<td>Cord</td>
<td>P value</td>
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<td>Cord</td>
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<td>Serum iron (μmol/L)</td>
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<td>23.3 (±7.1)</td>
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<td>24.3 (±7.3)</td>
<td>0.02</td>
<td>285</td>
<td>22.7 (±7.0)</td>
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<td>22.7 (±7.0)</td>
<td>140</td>
<td>24.4 (±7.3)</td>
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<td>26.9</td>
<td>23.1 (±7.0)</td>
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<tr>
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<td>19.0 (±10.6)</td>
<td>273</td>
<td>23.7 (±10.4)</td>
<td>0.04</td>
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<td>23.8 (±10.8)</td>
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<td>21.2 (±9.8)</td>
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<td>23.0 (±10.3)</td>
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<td>1.98 (±0.4)</td>
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<td>1.92 (±0.3)</td>
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<td>206.8</td>
<td>213.0 (±179.5)</td>
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<td>&gt;5mg/L</td>
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<td>&gt;3mg/L</td>
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<td>N/A</td>
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<td>Serum hepcidin (ng/ml)</td>
<td>19.9</td>
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<td>21.5 (±13.9)</td>
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<td>21.6 (±13.0)</td>
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<td>15.6 (±2.7)</td>
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<td>15.1 (±2.4)</td>
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<td>15.1 (±2.4)</td>
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<td>15.6 (±2.6)</td>
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<td>15.1</td>
<td>15.1 (±2.4)</td>
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<td>43.8 (±8.1)</td>
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<td>43.7 (±8.7)</td>
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<td>42.3 (±8.7)</td>
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<td>Soluble transferrin receptor (mg/L)</td>
<td>6.0 (±2.0)</td>
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<td>0.03 (±0.09)</td>
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<td>Undetectable haptoglobin (%)</td>
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<td>42.7%</td>
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<td>43.8%</td>
<td>0.76</td>
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<td>Mean corpuscular volume (fl)</td>
<td>98.6</td>
<td>98.5 (±8.1)</td>
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<td>97.8 (±8.3)</td>
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<td>97.7 (±8.4)</td>
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<td>Mean corpuscular haemoglobin (g)</td>
<td>35.8 (±2.4)</td>
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<td>35.0 (±2.4)</td>
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<td>35.9 (±1.0)</td>
<td>0.5</td>
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<td>35.8 (±1.0)</td>
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<td>35.8 (±1.0)</td>
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<td>35.8 (±1.0)</td>
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<tr>
<td>White blood cell count (×10⁹/L)</td>
<td>13.0 (±5.3)</td>
<td>12.3 (±4.6)</td>
<td>269</td>
<td>13.3 (±7.0)</td>
<td>0.07</td>
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<td>13.3 (±7.5)</td>
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<td>Lymphocyte count (×10⁹/L)</td>
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<td>4.67 (±2.2)</td>
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<td>4.77 (±3.5)</td>
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<td>4.82 (±3.5)</td>
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<td>Lymphocyte percentage (%)</td>
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<td>35.2 (±7.9)</td>
<td>269</td>
<td>35.2 (±7.9)</td>
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<td>MDC cell count (×10⁹/L)</td>
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<td>1.05 (±0.5)</td>
<td>269</td>
<td>1.05 (±0.6)</td>
<td>0.15</td>
<td>277</td>
<td>1.05 (±0.6)</td>
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<td>MDC cell percentage (%)</td>
<td>8.27</td>
<td>8.52 (±3.0)</td>
<td>269</td>
<td>8.15 (±2.4)</td>
<td>0.17</td>
<td>277</td>
<td>8.17 (±2.5)</td>
</tr>
</tbody>
</table>

P values indicate significance levels.
| Parameter                                   | Unit/L | Value 1 | SD 1 | Value 2 | SD 2 | Value 3 | SD 3 | Value 4 | SD 4 | Value 5 | SD 5 | Value 6 | SD 6 | Value 7 | SD 7 | Value 8 | SD 8 | Value 9 | SD 9 | Value 10 | SD 10 | Value 11 | SD 11 | Value 12 | SD 12 |
|--------------------------------------------|--------|---------|------|---------|------|---------|------|---------|------|---------|------|---------|------|---------|------|---------|------|---------|------|---------|------|---------|------|
| Granulocyte count (unit/L)                |        | 7.30    | 0.68 | 8.68    | 0.68 | 7.48    | 0.67 | 7.47    | 0.67 | 6.66    | 0.68 | 7.41    | 0.67 | 7.41    | 0.67 | 6.66    | 0.68 | 7.41    | 0.67 | 7.41    | 0.67 | 6.66    | 0.68 |
| Granulocyte percentage (%)                |        | 55.5    | 5.3  | 53.3    | 5.3  | 56.6    | 5.6  | 56.4    | 5.6  | 53.7    | 5.6  | 56.4    | 5.6  | 56.4    | 5.6  | 53.7    | 5.6  | 56.4    | 5.6  | 56.4    | 5.6  |
| Red blood cell count (unit/L)             |        | 4.12    | 0.36 | 4.04    | 0.36 | 4.20    | 0.40 | 4.03    | 0.40 | 4.22    | 0.40 | 4.02    | 0.40 | 4.02    | 0.40 | 4.22    | 0.40 | 4.02    | 0.40 | 4.02    | 0.40 |
| Red blood cell distribution width (%)     |        | 15.2    | 1.6  | 16.4    | 1.6  | 15.2    | 1.6  | 15.2    | 1.6  | 15.2    | 1.6  | 15.2    | 1.6  | 15.2    | 1.6  | 15.2    | 1.6  | 15.2    | 1.6  | 15.2    | 1.6  |
| Red blood cell distribution width - absolute (fl) |  | 81.0    | 0.0  | 81.5    | 0.0  | 81.3    | 0.0  | 81.4    | 0.0  | 81.6    | 0.0  | 81.3    | 0.0  | 81.3    | 0.0  | 81.5    | 0.0  | 81.3    | 0.0  | 81.3    | 0.0  |
| Mean platelet volume (fl)                 |        | 8.34    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 | 8.37    | 0.35 |
| Platelet distribution width (%)           |        | 43.3    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  | 43.5    | 2.9  |
| Platelet distribution width - absolute (fl) |  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  | 0.2    | 0.0  |
| Platelet count (unit/L)                   |        | 241.4   | 46.9 | 236.7   | 46.9 | 243.8   | 46.9 | 243.2   | 46.9 | 237.5   | 46.9 | 243.3   | 46.9 | 243.3   | 46.9 | 237.5   | 46.9 | 243.3   | 46.9 |
| Haemolysis score (0-6)                    |        | 39.0    | 5.4  | 35.8    | 5.4  | 35.8    | 5.4  | 36.5    | 5.4  | 36.5    | 5.4  | 36.5    | 5.4  | 36.5    | 5.4  | 36.5    | 5.4  | 36.5    | 5.4  | 36.5    | 5.4  |

**Supplementary Table 3:** Further Iron, Infection and Haematological Parameters (umbilical cord samples) for FTB/NBW, PTB/LBW, FTB, PTB, NBW and LBW neonates. Data are presented as mean (± SD) and analysed by ANOVA. *P* values in bold font are considered significant based on *P*<0.05. *a* = Platelet Crit (%) is a measure of total platelet mass.
### Supplementary Table 4 – Comparison of iron status, inflammation and haematological parameters in venous samples

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<thead>
<tr>
<th>Sample Type</th>
<th>Whole Population</th>
<th>PTB/LBW</th>
<th>FTB/NBW</th>
<th>Full-Term Birth (FTB)</th>
<th>Preterm Birth (PTB)</th>
<th>Normal Birthweight (NBW)</th>
<th>Low Birthweight (LBW)</th>
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<td>n</td>
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<td>8·0 (±4·1)</td>
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<td>7·3 (±4·6)</td>
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<td>35·6 (±10·1)</td>
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<td>Lymphocyte count (×10^3/L)</td>
<td>425</td>
<td>8·1 (±4·5)</td>
<td>151</td>
<td>9·4 (±5·0)</td>
<td>271</td>
<td>9·8 (±5·6)</td>
<td>0·04</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0·9</td>
</tr>
<tr>
<td>MID cell count (×10^3/L)</td>
<td>425</td>
<td>1·48 (±1·0)</td>
<td>151</td>
<td>1·37 (±1·0)</td>
<td>271</td>
<td>1·55 (±1·1)</td>
<td>0·08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0·2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>0·1</td>
</tr>
<tr>
<td>MID cell percentage (%)</td>
<td>425</td>
<td>9·67 (±5·4)</td>
<td>151</td>
<td>9·43 (±5·0)</td>
<td>271</td>
<td>9·81 (±5·6)</td>
<td>0·49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0·2</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0·9</td>
</tr>
</tbody>
</table>

**Note:** The table shows a comparison of iron status, inflammation, and haematological parameters in venous samples across different groups. The data includes values such as percentage, count, and concentration levels, along with statistical significance (P value) for comparison.
Supplementary Table 4: Further Iron, Infection and Haematological Parameters (venous samples) for FTB/NBW, PTB/LBW, FTB, PTB, NBW and LBW neonates. Data are presented as mean (± SD) and analysed by ANOVA. *P* values in bold font are considered significant based on *P* < 0.05.
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Whole Population</th>
<th>FTB/NBW</th>
<th>FTB/LBW</th>
<th>Preterm Birth (PTB)</th>
<th>Low Birthweight (LBW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>414</td>
<td>42.7 (±0.6)</td>
<td>54.7 (±3.0)</td>
<td>&lt;0.001</td>
<td>270 42.7 (±0.6)</td>
</tr>
<tr>
<td>Platelet distribution width (%)</td>
<td>552</td>
<td>6.0 (±2.0)</td>
<td>7.0 (±2.2)</td>
<td>&lt;0.001</td>
<td>273 5.9 (±2.0)</td>
</tr>
<tr>
<td>Haptoglobin (%)</td>
<td>226</td>
<td>0.02 (±0.07)</td>
<td>0.02 (±0.06)</td>
<td>0.02</td>
<td>272 0.03 (±0.07)</td>
</tr>
<tr>
<td>Undetectable haptoglobin (%)</td>
<td>46</td>
<td>42.7%</td>
<td>34.0%</td>
<td>0.001</td>
<td>192 43.0%</td>
</tr>
<tr>
<td>Mean corpuscular volume (fL)</td>
<td>614</td>
<td>98 (±0.8)</td>
<td>98 (±0.8)</td>
<td>0.05</td>
<td>270 97.8 (±6.3)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin (pg)</td>
<td>414</td>
<td>35.1 (±2.4)</td>
<td>35.9 (±2.4)</td>
<td>&lt;0.001</td>
<td>270 35.0 (±2.4)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin concentration (pg/dL)</td>
<td>414</td>
<td>35.8 (±1.0)</td>
<td>35.6 (±1.0)</td>
<td>&lt;0.001</td>
<td>270 35.6 (±1.0)</td>
</tr>
<tr>
<td>White blood cell count (unit/L)</td>
<td>412</td>
<td>13.0 (±5.3)</td>
<td>12.5 (±5.5)</td>
<td>&lt;0.001</td>
<td>269 13.5 (±5.6)</td>
</tr>
<tr>
<td>Lymphocyte count (unit/L)</td>
<td>412</td>
<td>7.21 (±3.3)</td>
<td>7.21 (±3.2)</td>
<td>&lt;0.001</td>
<td>269 7.4 (±3.7)</td>
</tr>
<tr>
<td>MID cell count (unit/L)</td>
<td>412</td>
<td>1.02 (±0.5)</td>
<td>1.48 (±1.0)</td>
<td>&lt;0.001</td>
<td>269 1.05 (±0.6)</td>
</tr>
<tr>
<td>Granulocyte count (unit/L)</td>
<td>412</td>
<td>1.85 (±0.5)</td>
<td>1.95 (±0.4)</td>
<td>&lt;0.001</td>
<td>269 2.13 (±0.7)</td>
</tr>
<tr>
<td>Red blood cell count (Unit/L)</td>
<td>412</td>
<td>5.6 (±1.0)</td>
<td>5.7 (±1.0)</td>
<td>&lt;0.001</td>
<td>269 5.6 (±1.0)</td>
</tr>
<tr>
<td>Red blood cell distribution width (fL)</td>
<td>412</td>
<td>152 (±1.8)</td>
<td>154 (±1.6)</td>
<td>&lt;0.001</td>
<td>270 154 (±1.6)</td>
</tr>
<tr>
<td>Red blood cell distribution width - absolute (fL)</td>
<td>412</td>
<td>81 (±0.6)</td>
<td>82 (±0.5)</td>
<td>&lt;0.001</td>
<td>270 80 (±0.9)</td>
</tr>
<tr>
<td>Platelet count (unit/L)</td>
<td>412</td>
<td>241.4 (±8.9)</td>
<td>260.9 (±4.6)</td>
<td>&lt;0.003</td>
<td>269 243.8 (±8.9)</td>
</tr>
<tr>
<td>Mean platelet volume (fL)</td>
<td>409</td>
<td>9.3 (±0.1)</td>
<td>8.6 (±0.2)</td>
<td>0.001</td>
<td>269 8.7 (±0.7)</td>
</tr>
<tr>
<td>Platelet distribution width (%)</td>
<td>409</td>
<td>4.3 (±1.2)</td>
<td>4.3 (±1.3)</td>
<td>0.007</td>
<td>269 4.5 (±3.1)</td>
</tr>
<tr>
<td>Platelet distribution width - absolute (fL)</td>
<td>409</td>
<td>11.8 (±1.1)</td>
<td>12.0 (±1.3)</td>
<td>0.009</td>
<td>267 11.8 (±1.3)</td>
</tr>
<tr>
<td>Platelet crit (%)</td>
<td>409</td>
<td>2.0 (±0.6)</td>
<td>2.2 (±0.7)</td>
<td>0.001</td>
<td>269 2.01 (±0.7)</td>
</tr>
<tr>
<td>Platelet large cell ratio (%)</td>
<td>409</td>
<td>17.8 (±5.3)</td>
<td>18.5 (±5.6)</td>
<td>0.001</td>
<td>267 18.5 (±5.4)</td>
</tr>
<tr>
<td>Platelet large cell count (unit/L)</td>
<td>409</td>
<td>41.4 (±1.4)</td>
<td>46.5 (±1.8)</td>
<td>&lt;0.001</td>
<td>267 42.1 (±1.4)</td>
</tr>
<tr>
<td>Haemolysis score (0-6)</td>
<td>0</td>
<td>166</td>
<td>39.0%</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>------------------------</td>
<td>----</td>
<td>-----</td>
<td>--------</td>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>144</td>
<td></td>
<td>34.0%</td>
<td>43</td>
<td>10.2%</td>
</tr>
<tr>
<td>2</td>
<td>78</td>
<td></td>
<td>18.4%</td>
<td>213</td>
<td>50.8%</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td></td>
<td>8.3%</td>
<td>150</td>
<td>35.6%</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td></td>
<td>0.0%</td>
<td>15</td>
<td>3.5%</td>
</tr>
</tbody>
</table>

**Supplementary Table 5:** Further Iron, Infection and Haematological Parameters (Umbilical cord and venous sample) for FTB/NBW, PTB/LBW, PTB and LBW neonates. Data are presented as mean (± SD) and analysed by ANOVA. *P* values in bold font are considered significant based on *P*<0.05.
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Whole Population</th>
<th>Group A (PTB+LBW)</th>
<th>Group B (PTB+NBW)</th>
<th>Group C (FTB+LBW)</th>
<th>Group D (FTB+NBW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum iron (μmol/L)</td>
<td>425</td>
<td>7.5 (4.5-11.6)</td>
<td>40</td>
<td>8.2 (3.3)</td>
<td>22.6 (7.9)</td>
</tr>
<tr>
<td>Haptoglobin (g/L)</td>
<td>182</td>
<td>35.6 (2.9-38)</td>
<td>9</td>
<td>35.9 (3.9)</td>
<td>35.7 (3.9)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Serum hepcidin (ng/mL)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>TIBC (μmol/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>TIBC (mg/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Soluble transferrin receptor (mg/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Transferrin (mg/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Serum ferritin (μg/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Hemoglobin saturation (%)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Mean corpuscular volume (fL)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin (pg)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin concentration (g/dL)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>White blood cell count (x10^9/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Lymphocyte count (x10^9/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Lymphocyte percentage (%)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>RBC count (x10^12/L)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>RBC volume (fL)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>RBC haemoglobin (pg)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>RBC haemoglobin concentration (g/dL)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>RBC haemoglobin saturation (%)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin (pg)</td>
<td>101</td>
<td>86 (46-96)</td>
<td>35</td>
<td>86 (46-96)</td>
<td>86 (46-96)</td>
</tr>
</tbody>
</table>
**Supplementary Table 6:** Iron, Infection and Haematological Parameters (umbilical cord and venous samples) for groups A, B, C and D neonates. Data are presented as mean (± SD) and analysed by ANOVA. *P* values in bold font are considered significant based on *P*<0.05.
### Supplementary Table 7 – Comparison of iron status, inflammation and haematological parameters in cord samples (Group A-D)

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Whole Population</th>
<th>Group A (PTB+LBW)</th>
<th>Group B (PTB+NBW)</th>
<th>Group C (FTB+LBW)</th>
<th>Group D (FTB+NBW)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Cord</td>
<td>n</td>
<td>Cord</td>
<td>n</td>
<td>Cord</td>
</tr>
<tr>
<td>Serum iron (μmol/L)</td>
<td>425</td>
<td>23·3 (±7·1)</td>
<td>99</td>
<td>24·2 (±7·2)</td>
<td>10</td>
<td>22·6 (±7·9)</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>423</td>
<td>51·7 (±17·3)</td>
<td>99</td>
<td>54·5 (±17·7)</td>
<td>10</td>
<td>50·4 (±23·2)</td>
</tr>
<tr>
<td>Serum hepcidin (ng/ml)</td>
<td>425</td>
<td>19·9 (±14·2)</td>
<td>96</td>
<td>22·7 (±14·1)</td>
<td>10</td>
<td>12·7 (±11·6)</td>
</tr>
<tr>
<td>UIBC (μmol/L)</td>
<td>423</td>
<td>22·9 (±10·6)</td>
<td>99</td>
<td>21·2 (±9·8)</td>
<td>10</td>
<td>26·4 (±18·8)</td>
</tr>
<tr>
<td>TIBC (μmol/L)</td>
<td>423</td>
<td>46·1 (±8·0)</td>
<td>99</td>
<td>45·4 (±7·0)</td>
<td>10</td>
<td>49·0 (±12·6)</td>
</tr>
<tr>
<td>Serum ferritin (ug/L)</td>
<td>426</td>
<td>210·5 (±157·8)</td>
<td>99</td>
<td>214·1 (±146·7)</td>
<td>10</td>
<td>203·8 (±214·4)</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>414</td>
<td>15·3 (±2·5)</td>
<td>96</td>
<td>15·3 (±2·4)</td>
<td>8</td>
<td>15·8 (±4·5)</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>414</td>
<td>42·7 (±7·4)</td>
<td>96</td>
<td>42·7 (±7·3)</td>
<td>8</td>
<td>44·7 (±13·3)</td>
</tr>
<tr>
<td>Transferrin (g/L)</td>
<td>426</td>
<td>1·96 (±0·3)</td>
<td>99</td>
<td>1·92 (±0·3)</td>
<td>10</td>
<td>2·08 (±0·6)</td>
</tr>
<tr>
<td>Soluble transferrin receptor (mg/L)</td>
<td>424</td>
<td>6·0 (±2·0)</td>
<td>99</td>
<td>5·74 (±1·6)</td>
<td>10</td>
<td>7·00 (±3·7)</td>
</tr>
<tr>
<td>Haptoglobin (g/L)</td>
<td>426</td>
<td>0·02 (±0·07)</td>
<td>99</td>
<td>0·03 (±0·0)</td>
<td>10</td>
<td>0·02 (±0·03)</td>
</tr>
<tr>
<td>Undetectable haptoglobin (%)</td>
<td>182</td>
<td>42·7%</td>
<td>20</td>
<td>47·6%</td>
<td>4</td>
<td>40%</td>
</tr>
<tr>
<td>Serum AGP (g/L)</td>
<td>426</td>
<td>0·18 (±0·1)</td>
<td>99</td>
<td>0·17 (±0·2)</td>
<td>10</td>
<td>0·22 (±0·1)</td>
</tr>
<tr>
<td>Serum CRP (mg/L)</td>
<td>425</td>
<td>0·17 (±0·6)</td>
<td>99</td>
<td>0·16 (±0·3)</td>
<td>10</td>
<td>0·14 (±0·1)</td>
</tr>
<tr>
<td>&gt;5mg/L</td>
<td>2</td>
<td>0·5%</td>
<td>0</td>
<td>0·0%</td>
<td>0</td>
<td>0·0%</td>
</tr>
<tr>
<td>&gt;3mg/L</td>
<td>3</td>
<td>0·7%</td>
<td>0</td>
<td>0·0%</td>
<td>0</td>
<td>0·0%</td>
</tr>
<tr>
<td>Mean corpuscular volume (fl)</td>
<td>414</td>
<td>98·0 (±6·2)</td>
<td>96</td>
<td>98·2 (±5·4)</td>
<td>8</td>
<td>95·3 (±11·4)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin (pg)</td>
<td>414</td>
<td>35·1 (±2·4)</td>
<td>96</td>
<td>35·3 (±2·2)</td>
<td>8</td>
<td>33·9 (±4·3)</td>
</tr>
</tbody>
</table>
| Mean corpuscular haemoglobin concentration (g/dl) | 414 | 35·8 (±1·0) | 96 | 35·9 (±1·0) | 8 | 35·5 (±1·1) | 270 | 35·8 (±0·9) | 0·17 |}
| White blood cell count (unit/L)   | 412              | 13·0 (±5·3)       | 95                | 12·7 (±4·4)       | 8                 | 14·7 (±6·6)  | 269    | 13·3 (±5·7)  | 0·04   |
| Lymphocyte count (unit/L)         | 412              | 4·73 (±3·1)       | 95                | 4·6 (±2·0)        | 8                 | 6·6 (±3·6)  | 269    | 4·77 (±3·5)  | 0·32   |
| Lymphocyte percentage (%)         | 412              | 36·2 (±8·3)       | 95                | 36·2 (±7·9)       | 8                 | 43·4 (±6·4)  | 269    | 35·2 (±7·6)  | <0·001 |
| MID cell count (unit/L)           | 412              | 1·02 (±0·6)       | 95                | 0·97 (±0·4)       | 8                 | 1·23 (±0·5)  | 269    | 1·05 (±0·6)  | 0·49   |
| MID cell percentage (%)           | 412              | 8·27 (±2·6)       | 95                | 8·04 (±2·0)       | 8                 | 8·99 (±3·5)  | 269    | 8·15 (±2·4)  | 0·009  |
### Supplementary Table 7: Iron, infection and haematological parameters (umbilical cord samples) for groups A, B, C, and D neonates. Data are presented as mean (± SD) and analysed by ANOVA. $P$ values in bold font are considered significant based on $P<0.05$.  

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Unit/L</th>
<th>Mean (± SD)</th>
<th>Groups</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte count (unit/L)</td>
<td>412</td>
<td>7.21 (±2.7)</td>
<td>40</td>
<td>5.43 (±2.6)</td>
<td>95</td>
</tr>
<tr>
<td>Granulocyte percentage (%)</td>
<td>412</td>
<td>55.5 (±9.1)</td>
<td>40</td>
<td>48.7 (±10.2)</td>
<td>95</td>
</tr>
<tr>
<td>Red blood cell count (unit/L)</td>
<td>414</td>
<td>4.36 (±0.7)</td>
<td>40</td>
<td>4.6 (±0.8)</td>
<td>96</td>
</tr>
<tr>
<td>Red blood cell distribution width (%)</td>
<td>414</td>
<td>15.2 (±1.1)</td>
<td>40</td>
<td>15.1 (±0.8)</td>
<td>96</td>
</tr>
<tr>
<td>Red blood cell distribution width - absolute (fl)</td>
<td>414</td>
<td>81.0 (±8.6)</td>
<td>40</td>
<td>82.4 (±7.8)</td>
<td>96</td>
</tr>
<tr>
<td>Platelet count (unit/L)</td>
<td>414</td>
<td>241.4 (±86.9)</td>
<td>40</td>
<td>226.3 (±76.4)</td>
<td>96</td>
</tr>
<tr>
<td>Mean platelet volume (fl)</td>
<td>409</td>
<td>8.34 (±0.8)</td>
<td>39</td>
<td>8.16 (±0.8)</td>
<td>95</td>
</tr>
<tr>
<td>Platelet distribution width (%)</td>
<td>409</td>
<td>43.3 (±2.8)</td>
<td>39</td>
<td>43.0 (±2.9)</td>
<td>95</td>
</tr>
<tr>
<td>Platelet distribution width - absolute (fl)</td>
<td>409</td>
<td>11.8 (±1.2)</td>
<td>39</td>
<td>11.5 (±1.1)</td>
<td>95</td>
</tr>
<tr>
<td>Platelet crit (%)</td>
<td>409</td>
<td>0.2 (±0.06)</td>
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<td>0.183 (±0.05)</td>
<td>95</td>
</tr>
<tr>
<td>Plate large cell ratio (%)</td>
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<td>17.8 (±5.3)</td>
<td>39</td>
<td>16.6 (±5.0)</td>
<td>95</td>
</tr>
<tr>
<td>Plate large cell count (unit/L)</td>
<td>409</td>
<td>41.4 (±14.2)</td>
<td>39</td>
<td>36.5 (±12.2)</td>
<td>95</td>
</tr>
<tr>
<td>Haemolysis score (0-6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>166</td>
<td>39.0%</td>
<td>13</td>
<td>31.00%</td>
<td>37</td>
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<tr>
<td>1</td>
<td>144</td>
<td>34.0%</td>
<td>10</td>
<td>23.8%</td>
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</tr>
<tr>
<td>2</td>
<td>78</td>
<td>18.4%</td>
<td>13</td>
<td>31.00%</td>
<td>13</td>
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<td>3</td>
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<td>8.3%</td>
<td>6</td>
<td>14.3%</td>
<td>8</td>
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<tr>
<td>4</td>
<td>4</td>
<td>0.0%</td>
<td>0</td>
<td>0.00%</td>
<td>0</td>
</tr>
</tbody>
</table>

*Supplementary Table 7:* Iron, infection and haematological parameters (umbilical cord samples) for groups A, B, C, and D neonates. Data are presented as mean (± SD) and analysed by ANOVA. $P$ values in bold font are considered significant based on $P<0.05$.  

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## Supplementary Table 8 – Comparison of iron status, inflammation and haematological parameters in venous samples (Groups A-D)

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Whole Population</th>
<th>Group A (PTB+LBW)</th>
<th>Group B (PTB+NBW)</th>
<th>Group C (FTB+LBW)</th>
<th>Group D (FTB+NBW)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Type</td>
<td>n</td>
<td>Venous</td>
<td>n</td>
<td>Venous</td>
<td>n</td>
<td>Venous</td>
</tr>
<tr>
<td>Serum iron (μmol/L)</td>
<td></td>
<td>421</td>
<td>40</td>
<td>82 (±3.5)</td>
<td>99</td>
<td>7.7 (±4.4)</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td></td>
<td>420</td>
<td>39</td>
<td>15.9 (±5.5)</td>
<td>99</td>
<td>16.2 (±9.3)</td>
</tr>
<tr>
<td>Serum hepcidin (ng/ml)</td>
<td></td>
<td>417</td>
<td>40</td>
<td>33.6 (±21.1)</td>
<td>96</td>
<td>39.4 (±24.0)</td>
</tr>
<tr>
<td>UIBC (μmol/L)</td>
<td></td>
<td>420</td>
<td>39</td>
<td>43.4 (±10.1)</td>
<td>99</td>
<td>41.5 (±11.2)</td>
</tr>
<tr>
<td>TIBC (μmol/L)</td>
<td></td>
<td>420</td>
<td>39</td>
<td>51.6 (±11.6)</td>
<td>99</td>
<td>49.1 (±10.9)</td>
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<tr>
<td>Serum ferritin (μg/L)</td>
<td></td>
<td>416</td>
<td>40</td>
<td>358.5 (±340.0)</td>
<td>98</td>
<td>354.6 (±220.7)</td>
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<tr>
<td>Haemoglobin (g/dl)</td>
<td></td>
<td>423</td>
<td>41</td>
<td>20.8 (±3.3)</td>
<td>99</td>
<td>19.4 (±3.1)</td>
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<tr>
<td>Haematocrit (%)</td>
<td></td>
<td>423</td>
<td>41</td>
<td>59.4 (±10.1)</td>
<td>99</td>
<td>54.5 (±9.4)</td>
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<tr>
<td>Transferrin receptor (mg/L)</td>
<td></td>
<td>421</td>
<td>40</td>
<td>7.47 (±2.3)</td>
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<td>6.67 (±1.8)</td>
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<tr>
<td>Haptoglobin (g/L)</td>
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<td>423</td>
<td>40</td>
<td>0.01 (±0.01)</td>
<td>99</td>
<td>0.02 (±0.05)</td>
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<tr>
<td>Undetectable haptoglobin (%)</td>
<td></td>
<td>144</td>
<td>10</td>
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<td>28.3%</td>
</tr>
<tr>
<td>Serum AGP (g/L)</td>
<td></td>
<td>423</td>
<td>40</td>
<td>0.24 (±0.2)</td>
<td>99</td>
<td>0.24 (±0.2)</td>
</tr>
<tr>
<td>Serum CRP (mg/L)</td>
<td></td>
<td>422</td>
<td>40</td>
<td>1.01 (±0.2)</td>
<td>98</td>
<td>2.39 (±4.5)</td>
</tr>
<tr>
<td>&gt;5mg/L %</td>
<td></td>
<td>54</td>
<td>3</td>
<td>7.5%</td>
<td>13</td>
<td>13.3%</td>
</tr>
<tr>
<td>&gt;3mg/L %</td>
<td></td>
<td>83</td>
<td>5</td>
<td>12.5%</td>
<td>18</td>
<td>18.4%</td>
</tr>
<tr>
<td>Mean corpuscular volume (fl)</td>
<td></td>
<td>423</td>
<td>41</td>
<td>100.1 (±8.1)</td>
<td>99</td>
<td>98.3 (±5.4)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin (pg)</td>
<td></td>
<td>423</td>
<td>41</td>
<td>35.2 (±2.5)</td>
<td>99</td>
<td>35.1 (±2.1)</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin conc (g/dl)</td>
<td></td>
<td>423</td>
<td>41</td>
<td>35.2 (±0.9)</td>
<td>99</td>
<td>35.7 (±0.9)</td>
</tr>
<tr>
<td>White blood cell count (unit/L)</td>
<td></td>
<td>422</td>
<td>41</td>
<td>13.1 (±4.2)</td>
<td>99</td>
<td>15.8 (±4.8)</td>
</tr>
<tr>
<td>Lymphocyte count (unit/L)</td>
<td></td>
<td>422</td>
<td>41</td>
<td>3.7 (±1.2)</td>
<td>99</td>
<td>4.0 (±1.5)</td>
</tr>
<tr>
<td>Lymphocyte percentage (%)</td>
<td></td>
<td>422</td>
<td>41</td>
<td>30.5 (±9.6)</td>
<td>99</td>
<td>26.8 (±8.5)</td>
</tr>
<tr>
<td>MID cell count (unit/L)</td>
<td></td>
<td>422</td>
<td>41</td>
<td>1.25 (±0.9)</td>
<td>99</td>
<td>1.42 (±1.0)</td>
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<tr>
<td>MID cell percentage (%)</td>
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<td>41</td>
<td>9.79 (±5.2)</td>
<td>99</td>
<td>9.27 (±4.9)</td>
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</tbody>
</table>
Supplementary Table 8: Iron, infection and haematological parameters (venous samples) for groups A, B, C, and D neonates. Data are presented as mean (± SD) and analysed by ANOVA. P values in bold font are considered significant based on $P < 0.05$. 

| Granulocyte count (unit/L) | 422  | 10·3 (±4·1) | 41  | 8·12 (±3·4) | 99  | 10·32 (±3·9) | 11  | 9·02 (±2·8) | 271 | 10·67 (±4·2) | 0·0017 |
| Granulocyte percentage (%) | 422  | 63·4 (±8·6) | 41  | 59·8 (±8·5) | 99  | 64·0 (±7·8) | 11  | 63·3 (±6·6) | 271 | 63·7 (±8·8) | 0·04 |
| Red blood cell count (unit/L) | 423  | 5·58 (±1·0) | 41  | 5·9 (±1·0) | 99  | 5·5 (±0·9) | 11  | 6·11 (±1·2) | 272 | 5·53 (±0·9) | 0·018 |
| Red blood cell distribution width (%) | 423  | 15·4 (±1·3) | 41  | 15·2 (±0·8) | 99  | 15·4 (±1·0) | 11  | 15·6 (±1·1) | 272 | 15·4 (±1·4) | 0·69 |
| Red blood cell distribution width - absolute (fl) | 423  | 82·2 (±8·5) | 41  | 83·8 (±8·4) | 99  | 82·5 (±7·9) | 11  | 79·2 (±11·5) | 272 | 82·0 (±8·6) | 0·39 |
| Platelet count (unit/L) | 423  | 260·4 (±94·6) | 41  | 256·9 (±86·1) | 99  | 269·4 (±101·0) | 11  | 234·3 (±84·3) | 272 | 258·7 (±94·0) | 0·35 |
| Mean platelet volume (fl) | 419  | 8·47 (±0·8) | 41  | 8·35 (±0·8) | 96  | 8·38 (±0·7) | 11  | 8·46 (±0·8) | 271 | 8·52 (±0·8) | 0·3 |
| Platelet distribution width (%) | 419  | 43·7 (±3·2) | 41  | 43·2 (±3·1) | 96  | 43·4 (±2·8) | 11  | 44·2 (±2·7) | 271 | 43·9 (±3·3) | 0·26 |
| Platelet distribution width - absolute (fl) | 419  | 12·0 (±1·3) | 41  | 11·7 (±1·3) | 96  | 11·8 (±1·1) | 11  | 12·1 (±1·3) | 271 | 12·1 (±1·4) | 0·19 |
| Platelet crit (%) | 419  | 0·22 (±0·07) | 41  | 0·212 (±0·06) | 96  | 0·226 (±0·07) | 11  | 0·190 (±0·07) | 271 | 0·215 (±0·07) | 0·35 |
| Plate large cell ratio (%) | 419  | 18·8 (±5·5) | 41  | 17·9 (±5·3) | 96  | 18·1 (±4·7) | 11  | 19·3 (±5·4) | 271 | 19·2 (±5·8) | 0·21 |
| Plate large cell count (unit/L) | 419  | 46·5 (±15·8) | 41  | 44·1 (±11·3) | 96  | 47·2 (±14·1) | 11  | 41·8 (±13·0) | 271 | 46·9 (±17·0) | 0·52 |
| Haemolysis score (0-6) | 0  | 0·0% | 3  | 7·5% | 6  | 6·1% | 0  | 0·00% | 33  | 12·2% | 0·2 |
|   | 1  | 43  | 10·2% | 18  | 45·00% | 57  | 57·6% | 1  | 9·1% | 135 | 49·8% | 0·018 |
|   | 2  | 213 | 50·6% | 18  | 45·00% | 33  | 33·3% | 3  | 27·3% | 92  | 34·00% | 0·51 |
|   | 3  | 150 | 35·6% | 1  | 4·5% | 3  | 3·00% | 7  | 63·6% | 11  | 4·1% | <0·001 |
|   | 4  | 15  | 3·5% | 0  | 0·00% | 0  | 0·00% | 0  | 0·00% | 0  | 0·00% | -- |
SUPPLEMENTARY REFERENCES

Chapter 6 – Iron homeostasis in full term, normal birthweight Gambian neonates over the first week of life (FTB/NBW Paper)

Summary of Chapter

BACKGROUND: Human neonates elicit a profound hypoferremia to protect against bacterial and fungal sepsis on their first day of life.

OBJECTIVE: We examined the transience of this effect by measuring iron and its chaperone proteins, inflammatory and hematological parameters over the first post-partum week.

DESIGN: We prospectively studied term (>37 completed gestational weeks), normal weight (>2500g) newborns at Kanifing General Hospital, The Gambia. Blood was sampled from the umbilical cord vein (CDV) and artery (CDA). Neonatal venous blood was sampled at 6-24h (V1) in all babies, who were then randomized to a second blood draw at 25-80h (V2), 81-136h (V3) or 137-192h (V4). Hepcidin, serum iron, transferrin saturation, transferrin, haptoglobin, CRP, AGP, sTfR, ferritin, TIBC, UIBC and full blood count were assayed.

RESULTS: Two hundred and seventy-eight neonates (278, 54.3% males, gestational age 39.4±1.3wk, birth weight 3299±368g) were enrolled. We confirmed the profound early postnatal decrease in serum iron (CDV=22.7±7.0 µmol/L to V1=7.3±4.3 µmol/L, P<0.0001) and TSAT (50.2±16.7% to 14.4±6.1%, P<0.0001). Both variables increased steadily to reach 16.5±3.9 µmol/L and 36.7±9.2% at V4 (P for trend <0.0001 for each). Hepcidin increased rapidly after birth (CDV=19.4±14.4 ng/ml to V1=38.9±23.9 ng/ml, P<0.0001) then dipped before rising again by V4. Inflammatory markers increased from V1 onwards. Network
analysis revealed a disconnect between the correlations observed in CDV and V2-V4 and those in V1. Surprisingly, serum iron and TSAT were only weakly influenced by hepcidin.

**CONCLUSIONS:** Rapid anti-infective post-natal hypoferremia in human neonates is transient. The later rise in serum iron despite very high hepcidin indicates hepcidin resistance possibly caused by macrophage saturation with iron released from degradation of fetal erythrocytes. Pharmacological prolongation of hypoferremia might offer an ancillary tool in the armoury against antimicrobial resistance, however, it would need to overcome or circumvent the hepcidin resistance we now report.
RESEARCH PAPER COVER SHEET - FTB/NBW PAPER

Please note that a cover sheet must be completed for each research paper included within a thesis.

SECTION A – Student Details

Student ID Number  LSH158152          Title       Mr.
First Name(s)       James Henry
Surname/Family Name  Cross
Thesis Title        Iron and Infection: Neonatal Iron Transition
Primary Supervisor  Dr Carla Cerami

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?
When was the work published?
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

Have you retained the copyright for the work?*  Choose an item.  Was the work subject to academic peer review?  Choose an item.

*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published? American Journal of Clinical Nutrition

Please list the paper’s authors in the intended authorship order: James H. Cross, Ousman Jarjou, Nuredin Ibrahim Mohammed, Santiago Rayment Gomez, Bubacarr J.B Touray, Andrew M. Prentice, Carla Cerami.

Stage of publication Undergoing revision
SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

James H. Cross 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.

SECTION E

Student Signature
Date 08/11/2019

Supervisor Signature
Date 08/11/2019
Title
Iron homeostasis in full term, normal birthweight Gambian neonates over the first week of life.

Authors
James H. Cross¹, Ousman Jarjou¹, Nuredin Ibrahim Mohammed¹, Santiago Rayment Gomez², Bubacarr J.B Touray¹, Andrew M. Prentice¹, Carla Cerami³*.

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+220 787 5756

Running Title:
Neonatal hypoferremia in full term, normal birthweight neonates
6.1 ABSTRACT

BACKGROUND: Human neonates elicit a profound hypoferremia to protect against bacterial and fungal sepsis on their first day of life.

OBJECTIVE: We examined the transience of this effect by measuring iron and its chaperone proteins, inflammatory and hematological parameters over the first postpartum week.

DESIGN: We prospectively studied term (>37 completed gestational weeks), normal weight (>2500g) newborns at Kanifing General Hospital, The Gambia. Blood was sampled from the umbilical cord vein (CDV) and artery (CDA). Neonatal venous blood was sampled at 6-24h (V1) in all babies, who were then randomized to a second blood draw at 25-80h (V2), 81-136h (V3) or 137-192h (V4). Hepcidin, serum iron, transferrin saturation, transferrin, haptoglobin, CRP, AGP, sTfR, ferritin, TIBC, UIBC and full blood count were assayed.

RESULTS: Two hundred and seventy-eight neonates (278, 54.3% males, gestational age 39.4±1.3wk, birth weight 3299±368g) were enrolled. We confirmed the profound early postnatal decrease in serum iron (CDV=22.7±7.0 µmol/L to V1=7.3±4.3 µmol/L, P<0.0001) and TSAT (50.2±16.7% to 14.4±6.1%, P<0.0001). Both variables increased steadily to reach 16.5±3.9 µmol/L and 36.7±9.2% at V4 (P for trend <0.0001 for each). Hepcidin increased rapidly after birth (CDV=19.4±14.4 ng/ml to V1=38.9±23.9 ng/ml, P<0.0001) then dipped before rising again by V4. Inflammatory markers increased from V1 onwards. Network analysis revealed a disconnect between the correlations observed in CDV and V2-V4 and those in V1. Surprisingly, serum iron and TSAT were only weakly influenced by hepcidin.

CONCLUSIONS: Rapid anti-infective postnatal hypoferremia in human neonates is transient. The later rise in serum iron despite very high hepcidin indicates hepcidin resistance possibly caused by macrophage saturation with iron released from degradation of fetal erythrocytes. Pharmacological prolongation of hypoferremia might offer an ancillary tool
in the armoury against antimicrobial resistance, however, it would need to overcome or circumvent the hepcidin resistance we now report.

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

**FUNDING:** Bill & Melinda Gates Foundation (OPP1152353).

**WORD COUNT:** 296/300

**Keywords:** Nutritional immunity, host-pathogen interaction, hepcidin, neonates, hypoferremia, transferrin, The Gambia, sub-Saharan Africa

### 6.2 INTRODUCTION

During pregnancy, the mother increases iron absorption and turnover of erythrocytes to provide for the growing fetus.\(^1\) As maternal hepcidin decreases during the third trimester, placental iron transfer rises.\(^2\) This leads to higher cord blood TSAT and serum iron levels compared to those of the mother at delivery,\(^3\)-\(^5\) even in anemic mothers.\(^6\) To protect the fetus against possible iron overload during the last trimester, fetal-derived hepcidin regulates iron transfer via degradation of ferroportin on placental syncytiotrophoblasts.\(^7\) As a result, umbilical cord hepcidin concentrations of term neonates are higher than those of the mother, before and during delivery.\(^8\)-\(^10\)

Using murine and *in vivo* human trophoblast models, evidence also suggests that the placenta may independently regulate iron transfer to the fetus in specific circumstances.\(^11\) A reduction of ferroportin expression on the apical fetal-facing membrane of placental syncytiotrophoblasts and increased expression of transferrin receptor 1 (TFR1) on the maternal-facing side have been observed in the setting of maternal iron deficiency. Placental metabolic homeostasis is subsequently maintained, protecting the fetus from the more detrimental condition of placental dysfunction.\(^11\)
In laboring mothers at term, cord levels of IL-6 increase 4-fold even in the absence of evident infection. Since the placenta is impermeable to IL-6, these high cord blood levels suggest that labour could be associated with a fetal-neonatal inflammatory response, potentially triggered by labour-related mechanisms or exposure to infectious agents. Immediately after delivery, newborns face the most complex multi-organ physiological adaption that they will experience in their entire lives. Increased IL-6 levels in the newborn are thought to assist with organ system transition at birth (e.g. cytokine-induced synthesis of lung surfactant proteins) and the activation of the immune system in the newborn. IL-6 is also known to activate the JAK-STAT pathway, leading to the induction of hepcidin synthesis. In previous studies we and others have demonstrated a rapid and profound hypoferremia occurring within the first few hours after delivery. This is assumed to have evolved as a defence against early-onset neonatal sepsis (EONS) and remains robust in premature and low-birthweight babies. Several studies have shown that post-natal peripheral hepcidin and prohepcidin (precursor) levels are higher than those in cord blood. We previously observed an initial increase in hepcidin within the first 12 hours of life in healthy newborns, positively correlated to raised IL-6 levels, and we confirmed this in low birth weight and premature newborns. Our data suggested that the IL-6-hepcidin-ferroportin axis plays a partial, but probably not exclusive, role in orchestrating the hypoferremia.

Here, we examine the duration of the hypoferremia and the likely regulatory influences in full-term, normal birthweight babies by analysing serial blood samples over the first week of life.
6.3 SUBJECTS AND METHODS

Full details of the NeoInnate Study (clinicaltrials.gov, NCT03353051) can be found in the published protocol paper.23

Study Design

The NeoInnate Study tested whether preterm and/or low birthweight babies were capable of inducing acute hypoferremia previously noted in full-term, normal birthweight babies. Results of the primary outcomes have been presented elsewhere.18 Here we describe the pre-planned secondary analysis of longitudinal changes in iron, hematological and inflammatory parameters over the first week of life within the term, normal weight babies from the FTB/NBW group. All babies were sampled from the cord artery (CDA) and vein (CDV) and had an early postnatal draw (V1) at 6-24h. For the longitudinal analysis over the first week of life and to avoid more than two blood draws per baby, the babies were then randomly allocated to a second blood draw at 25-80h (V2), 81-136h (V3) or 137-192h (V4) (Figure 1). Randomisation of second blood draw group allocation was completed using a random number calculator (GraphPad QuickCalcs, GraphPad Software INC, CA 92037, USA). This allocation was adapted according to study working hours and access to the newborn. Data collection started on the 5th July 2017 and ended on 1st February 2019.

Ethics, standards and informed consent

The trial 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/MRC Ethics Committee (SCC1525) and the London School of Hygiene and Tropical Medicine Ethics Committee (Ref:14316) and conducted according to Good Clinical Practice (GCP) standards. All participants gave written, informed consent.
**Study setting**

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

**Recruitment, screening and enrolment**

We planned to enrol 300 neonates into this longitudinal arm of the NeoInnate Study. For inclusion in this aim of the study, neonates were healthy, medically stable (i.e. not requiring resuscitation and with no signs of sepsis) with a gestational age ≥37 completed weeks (assessed by New Ballard Score\textsuperscript{24}) and weighed ≥2500g.

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 TB 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), blood transfusions given to mother or neonate, severe birth asphyxia (requiring resuscitation), neonates born via breech, vacuum or via caesarean section.

After the delivery stage, babies were excluded following the detection of infection or illness (i.e. 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 was 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.
Sample collection

Once the neonate was fully delivered, one-minute delayed cord clamping was used (following World Health Organisation (WHO) policy\textsuperscript{25}). After the umbilical cord was removed and cleaned, a trained study nurse identified the umbilical arteries (CDA) and umbilical vein (CDV). Blood was collected from each using separate blood draw equipment.

At 6-24 hours post-delivery, recruited mothers and their neonates were invited to a private consultation with the study research clinician. Demographic data were collected, along with a complete review of systems of the mother and neonate, and newborn anthropometry. Neuromuscular and physical maturation of each neonate was assessed using the New Ballard Score\textsuperscript{24}. Immediately after passing the health assessment, a 3.5ml venous blood draw was performed on all neonates (V1).

During the community visit at the home of the neonate, a review of systems in the mother and child were conducted by a research nurse. This was followed by collecting data on medication, behaviour and immunisations of the neonate after leaving the hospital. A further sample of 3.5ml venous blood was then collected (V2-4) if the neonate was regarded as healthy.

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 fresh 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. Samples were scored 0 (yellow 0 g/L of hemoglobin) to 6 (dark red 8 g/L of hemoglobin). Samples scoring ≥5 were removed from the analysis.

**Sample size determination**

Sample size calculations for the primary outcomes of the NeoInnate Study were based on data from a previous study and are summarised in the protocol paper. The secondary outcomes presented here were not subjected to a formal sample size analysis.

**Statistical analysis**

Statistical analysis and preparation of figures were conducted using STATA v15.1 (Stat-Corp LP, College Station, TX, USA), DataDesk version 7.0.2 (Data Description Inc) and GraphPad Prism (GraphPad Software INC, CA 92037, USA). For continuous variables, baseline characteristics are presented as means (± SD) for normally distributed variables. All skewed data (hepcidin, CRP, AGP, sTfR and ferritin) 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 (%). Iron and inflammation markers were compared using ordinary least squares analysis of variance (OLS ANOVA), using Scheffé’s post-hoc tests to control for multiple testing (V1 vs V2, V3 or V4). All hypothesis tests were two-sided at a significance level of 0.05. The rate of missing data was small (<5%), thus we did not impute missing data. Weighted Pearson network analysis was conducted using the Shiny Network
Application (https://jolandakos.shinyapps.io/NetworkApp/). The network was formatted using the Fruchterman-Reingold Algorithm only showing moderate or strong (>0.3) associations between nodes. V2, V3 and V4 nodes were combined to aid visualisation.

6.4 RESULTS

A CONSORT diagram summarising subject recruitment is shown in Figure 2. There were 278 neonates with paired cord blood and V1 samples. Of these, 224 provided a second venous blood sample (V2, V3 or V4).

Neonatal characteristics
Baseline characteristics are shown in Table 1. Newborns were healthy vaginally delivered babies, with a mean gestational age of 39.4 ± 1.3wk and mean birthweight of 3299 ± 368g. Many of the mothers (81.7%) received iron and folic acid during pregnancy as per WHO guidelines. Mean anthropometric measurements of all neonates fell within the 25th and 75th centiles of the WHO growth charts for gestational age. The mean times of bleed were: V1 = 12.7 ± 0.32h; V2 = 57.6 ± 15.6h; V3 = 105.8 ± 17.0h; and V4 = 156.7 ± 21.0h.

Changes in iron and chaperone proteins in the first week of life
Iron metabolism parameters over the first week of life can be seen in Table 2 and Figure 3. Following the acute hypoferremia on Day 1 (V1 = 7.3 ± 4.6μmol/L vs CDV = 22.7 ± 7.0μmol/L, P<0.0001) there was a steady increase in serum iron to 16.5 ± 3.9μmol/L at V4 (P for trend <0.0001) (Fig 3A). There was a slight decrease in transferrin levels (Fig 3B) but TIBC levels remained relatively constant (Fig 3C) and hence TSAT levels mirrored those for serum iron (Fig 3D). Following the acute postnatal hypoferremia (V1 = 14.4 ± 6.0% vs CDV = 50.2 ± 16.7%, P<0.0001) TSAT increased steadily to 36.7 ± 9.2% at V4 (P for trend <0.0001). UIBC consequently decreased from V1 to V4 (from 44.1 ± 18.0μmol/L to 29.5 ±
9.6μmol/L, P for trend <0.0001) (**Fig 3E**). Ferritin levels were very high at V1 (393 ± 313μg/L) and showed a non-significant decline to 355 ± 182μg/L at V4 (**Fig 3F**).

**Changes in hepcidin and inflammatory markers in the first week of life**

Hepcidin levels in cord blood were high (19.4 ± 14.4ng/ml) and doubled immediately after birth to reach 38.9 ± 23.9ng/ml at V1 (P<0.0001). There was then a slight non-significant dip followed by a steady rise to 45.2 ± 19.1ng/ml at V4 (P for trend <0.0001) (**Fig 4A**). CRP levels were low in cord blood (0.2 ± 0.7 mg/L) and increased 10-fold immediately after birth (to 2.3 ± 4.1mg/L at V1, P<0.0001) followed by a further steep rise to 5.6 ± 9.3mg/L at V2 (P<0.0001 vs V1) and then a decline to 0.9 ± 1.5 by V4 (P<0.0001 for trend) (**Fig 4B**). AGP showed a small increase after birth and then a further slow rise to V4 (P<0.0001) (**Fig 4C**). The heme-binding inflammatory-response protein haptoglobin increased between CDV and V1 (from 0.02 ± 0.1g/L to 0.1 ± 0.2g/L, P<0.0001) and then declined slightly to 0.08 ± 0.1g/L at V4 (P for trend <0.0001) (**Fig 4D**).

**Changes in hematological indices in the first week of life**

Cord hemoglobin (15.1 ± 2.3g/dl) and hematocrit (42.2 ± 7.0%) were high and there was a further increase by V1 (hemoglobin to 19.5 ± 2.9g/dl, hematocrit to 55.0 ± 9.7%, P<0.0001 for both increases). This was followed by a steady decline back towards cord-like levels by V4 (hemoglobin to 16.9 ± 2.9g/dl, hematocrit to 46.9 ± 9.0%, P<0.0001 for trend in both cases) (**Fig 5A,5B**). Soluble transferrin receptor levels dropped markedly reflecting the fact that there was a net breakdown of haemoglobin during the first week of life and an abundance of circulating iron to supply the needs of any on-going erythropoiesis (**Fig 5C**).

**Pearson correlation network analysis of iron and inflammation markers**

Weighted Pearson correlation network analysis between the iron and inflammation markers in all samples (CDV, V1 and V2-4) are shown in **Figure 6**. The unconnected nodes for birthweight and gestational age reflect their lack of influence on any of the markers. As
expected, there were consistent associations between serum iron, TSAT, UIBC and TIBC at all time points, but the V1 nodes were notably separated from CDV and V2-V4 underscoring the unusual nature of the immediate post-natal hypoferremia. Hemoglobin and hematocrit followed a similar pattern, with cord values only correlating to V2-V4 values. The inflammatory markers CRP, AGP and haptoglobin were generally associated as would be expected (see lower part of the network) but ferritin, also an inflammatory marker, was notably separate. The most surprising feature was that hepcidin featured on the periphery of the network and was not associated with the inflammatory markers or serum iron.

**Comparisons of iron and inflammation markers in arterial (CDA) and venous cord blood (CDV)**

*Figure 7* illustrates the comparisons between iron and inflammation markers in venous and arterial cord blood. For most markers there were no differences. TSAT was slightly higher in venous blood (50.5 ± 1.0%) than arterial cord blood (46.9 ± 1.0%, \( P<0.0001 \)). Conversely, arterial blood had higher ferritin (276.9 ± 14.7 vs 215.7 ±10.1µg/L, \( P<0.0001 \)), UIBC (27.1 ± 0.7 vs 23.4 ± 0.7µmol/L, \( P<0.0001 \)) and TIBC (49.8 ± 0.57 vs 46.1 ± 0.51µmol/L, \( P<0.0001 \)). There were no differences in any red blood cell indices between venous and arterial cord blood, but white cell counts (with the exception of granulocyte count) were significantly higher in arterial blood (*Table 3*). Conversely platelet counts were significantly higher in venous blood.

### 6.5 DISCUSSION

This study demonstrates that, following the previously reported\textsuperscript{17,18} acute postnatal hypoferremia, serum iron and TSAT steadily revert towards cord-like levels over the first week of life. We interpret this as further evidence that the early hypoferremia has evolved to
help newborns navigate the hazardous journey as they progress from an almost sterile intra-uterine existence to the heavy bacterial exposures of extra-uterine life.

Our prior analyses\textsuperscript{17,18} using data from independent studies, revealed that the early hypoferremia was, at least in part, driven by an inflammatory response to the birth process, eliciting a rapid IL-6-mediated rise in hepcidin. Hepcidin blocks the release of iron from enterocytes and macrophages\textsuperscript{1} and thereby reduces serum iron through the dual actions of preventing iron absorption and recirculation. In neonates who receive insignificant amounts of dietary iron on Day 1, the latter mechanism dominates and the hypoferremia represents a temporary redistribution of iron away from the extracellular plasma where it would enhance the growth of any ingressing bacteria or fungi.\textsuperscript{17} It is likely that additional hepcidin-independent mechanisms play an additional role in the hypoferremia.\textsuperscript{29}

A surprising element of the current data is that iron and TSAT levels start to revert to normal despite hepcidin levels that continue to rise over the first week reaching values that are 3-4-fold higher than observed in healthy adults.\textsuperscript{30} Furthermore, there was no correlation between hepcidin and serum iron or TSAT in the V2-4 samples. This unexpected disconnect between the relatively high levels of serum iron and TSAT coupled with high hepcidin concentrations suggests that early neonatal iron metabolism is desensitised to the action of hepcidin and thus the sequestration of intracellular iron is not maintained. This could be because the intracellular iron pools are saturated in the early post-partum period. We propose that macrophage cellular iron pools are increased in the first hours of life, initially due to the physiological hemolysis of fetal erythrocytes,\textsuperscript{31} followed by the uptake of transferrin-iron complexes.\textsuperscript{32} Erythrophagocytosis and the recycling of iron released from the catabolism of heme also add to intracellular iron levels.\textsuperscript{33} This is further exacerbated by the effects of inflammation-induced hepcidin excess at 6-24 hours post-delivery, leading to hepcidin-induced co-degradation\textsuperscript{34} and/or hepcidin occlusion\textsuperscript{35} of the transmembrane iron transporter, ferroportin. We propose the initial hepcidin levels reduce expression of ferroportin on
macrophage cell membranes, thus eliciting the immediate post-natal hypoferremia, but that complete removal of all ferroportin molecules from the cell membrane is not achieved. This is supported by in vivo hepcidin challenge experiments showing a halving of ferroportin expression within 4 hours. However, the complete removal of ferroportin was not achieved. Previous authors have suggested that excess levels of hepcidin more likely result in the blocking of the central cavity of ferroportin, rather than its internalization. We propose that as hepcidin levels are consistently high during the first week of life, intermittent binding and releasing of hepcidin to the central cavity of individual ferroportin molecules may allow for slow rates of iron efflux into the circulation. We hypothesize that this results in a gradual increase in serum iron and TSAT, even in the high hepcidin environment, as seen in Figures 3 and 4. Previous research suggests that a potential cause of occlusion over internalization and the differences between tissues in sensitivity to hepcidin could be due to the differing endocytic machinery present in different cell types, or differing ferroportin glycosylation. Further research in respect to neonatal macrophages is required.

Other studies have shown that following the neonatal period, circulating hepcidin levels decline to those similar to or lower than those observed in cord blood. Increased expression of growth factors (i.e. IGF-1, HGF, EGF, PDGF-BB) is thought to be the cause of downregulation of hepcidin transcription. Our research shows that this trend does not begin until after the first week of life.

Differences in hepcidin concentration between males and females are observed in adults and children. Previous research has noted significantly lower hepcidin concentrations in male neonates compared to females in the first week of life. Though initially thought to be due to iron status and CRP concentration differences, it has recently been suggested that higher testosterone levels may be the cause. Though we did not measure testosterone levels in our samples, no significant difference in cord, V1 (6-24 hrs) or V2-4 (24-192 hours) hepcidin concentration was observed between sexes.
CRP levels peaked between 24-80 hours post-delivery, with again a surprising absence of correlation with hepcidin concentrations. This is despite the well-documented regulatory pathways of infection and inflammation on iron regulation.\textsuperscript{46} Previous studies have suggested that the lack of correlation between hepcidin, IL-6 and CRP is due to differences in the kinetics of these proinflammatory biomarkers during the infection process. IL-6 concentrations spike very early in the course of infection or inflammation, hepcidin then follows and the rise of CRP is delayed and with a further delay to AGP release.\textsuperscript{47}

The great majority of mothers in this study reported that they received iron and folic acid in pregnancy as per Gambian government guidelines but 52\% remained anaemic in the last week before delivery. Despite this, ferritin levels in cord blood were high (CDV: 213 ± 158µg/L) and levels almost doubled immediately after delivery to 394 ± 313µg/L at V1. It has previously been suggested that this is due to the physiological hemolysis of fetal red blood cells, which contain ferritin in high concentrations.\textsuperscript{22} Similarly, we found elevated levels of haptoglobin, peaking at 0.1 ± 0.2g/L at 24-80 hours of life. We suggest this is another layer of nutritional immunity, as haptoglobin binds to hemoglobin, further restricting iron availability to invading microorganisms.\textsuperscript{48}

We also recorded increases in newborn hemoglobin and hematocrit taken within the first hours of life (6-24 hours) compared to cord blood. This is likely to be in part due to postnatal dehydration, as well as vasomotor instability and venous pooling.\textsuperscript{49} The decreases in hemoglobin and hematocrit over the first week of life are likely a response to the higher ambient oxygen concentration \textit{ex-utero}.\textsuperscript{49}

We undertook the comparative analysis of iron markers and inflammation in arterial and venous cord blood to ensure that non-standardized sampling of ‘cord blood’ in prior studies did not affect the comparisons between cord and postnatal samples. Serum iron was identical between sampling sites, and although there were significant differences for TIBC,
and hence reciprocally for TSAT, the differences were only in the order of 6-7%. Transferrin levels were similar, suggesting that differences in non-transferrin iron-binding compounds account for the small difference in TIBC and TSAT.

Cord arteriovenous samples show significant differences in all platelet parameters. This may be the result of the placenta being an active site of platelet production as previously suggested by Woods et al.50

There are several strengths and limitations to our study. The large sample size and the relative homogeneity of responses across most analytes provides confidence in the trends observed. A limitation is that maternal iron markers in mid-gestation, parturition and after delivery were not measured. This would have provided information as to what effect maternal iron and inflammation status had on the neonatal iron marker fluctuations we studied. Measurement of pro-inflammatory cytokines (e.g. IL-6 and IL-22) and growth factors (i.e. IGF-1, HGF, EGF and PDGF-BB) may have provided additional insights into the regulation of postnatal iron metabolism. Variables governing the hepcidin-independent regulation of iron redistribution are not yet known so could not be measured in our study. The effects of diurnal rhythm, iron supplementation and infection were not assessed and could be a direction for future research.

In conclusion, our results suggest that early postnatal hypoferremia is a fast-acting, yet short-lived adaptation likely to have evolved to protect the newborn from infection at the time they are most vulnerable. This is followed by a period of hepcidin desensitisation, or leaky intracellular iron sequestration, as iron efflux into the serum continues even in the presence of high serum hepcidin concentrations. The reduced need for iron for erythropoiesis during the first week of life could also increase the concentration of serum iron. This interpretation is supported by the observed decrease in sTfR levels over the first week indicating that erythroid tissues were not demanding iron. We have previously shown18 that, in principle, the
duration of postnatal hypoferremia might be extendable through the administration of mini-hepcidins as an ancillary tool against antimicrobial-resistant infections. The new data presented here suggests that any such intervention would need to overcome or circumvent the hepcidin resistance we report in the first week of life.

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Abbreviations:

AGP = Alpha 1-acid glycoprotein
CDA = Cord arterial blood
CDV = Cord venous blood
CRP = C-reactive protein
EDTA = Ethylenediaminetetraacetic acid
EGF = Epidermal growth factor
ELISA = Enzyme-linked immunosorbent assay
G6PD = Glucose-6-phosphate dehydrogenase
GCP = Good Clinical Practice
HGF = Hepatocyte growth factor
IGF-1 = Insulin-like growth factor-1
IL-6 = Interleukin 6
IL-22 = Interleukin 22
KGH = Kanifing General Hospital
KMC = Kanifing Municipal Council
MRCG = Medical Research Council Unit The Gambia at LSHTM
PDGF-BB = Platelet-derived growth factor - BB
ROS = Reactive oxygen species
SCC = Scientific Coordinating Committee
sTfR = Soluble transferrin receptor
TfR1 = Transferrin receptor
TIBC = Total iron-binding capacity
TSAT = Transferrin saturation
UIBC = Unbound iron-binding capacity
WHO = World Health Organisation
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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 a 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 JHC, 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 Anemia. 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.

Competing Interests:

The authors declare that they have no competing interests.

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.
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**Figure Legends:**

**Figure 1: Study recruitment and blood draw design.** Mothers were approached on entering the KGH maternity ward, The Gambia. This was followed by the consenting process, recruitment and delivery data collection. At delivery, venous (CDV) and arterial (CDA) cord blood was collected after one-minute delayed cord clamping. The neonate was weighed after cord blood collection. At 6-24 hours post-delivery, the research study clinician conducted a health check of the mother and newborn. New Ballard Score was used to establish gestational age. If the neonate was deemed healthy, the V1 blood draw was completed. Follow-up in the community was conducted 24-216 hours post-delivery. This involved a health check of mother and newborn by the study nurse. If deemed healthy, the newborn was bled again (V2: ≥24 hours - <80 hours, V3: ≥80 hours - <136 hours, V4: ≥136 - <192 hours).

**Figure 2: CONSORT diagram for participant flow.** Two hundred and seventy-eight full-term, normal birthweight neonates were recruited to the study. Red outlined boxes represent all neonates excluded.

**Figure 3: Changes in iron and chaperone proteins in the first week of life**
Blood was drawn from the umbilical cord vein (CDV) at birth and from the dorsum of the hand at serial time points for each individual (>6-≤24hrs and >24-≤216hrs). Dots represent individual measurements. The blue line is a loess fit curve with 95% confidence intervals shaded in red. A = serum iron, B = transferrin, C = TIBC, D = TSAT, E = UIBC, F = ferritin.

**Figure 4: Changes in hepcidin and inflammatory markers in the first week of life**
Blood was drawn from the umbilical cord vein at birth and from the dorsum of the hand at serial time points for each individual (>6-≤24hrs and >24-≤216hrs). Dots represent individual
measurements. The blue line is a loess fit curve with 95% confidence intervals shaded in red. A = hepcidin, B = CRP, C = AGP, D = haptoglobin.

Figure 5: Changes in hematological indices in the first week of life
Blood was drawn from the umbilical cord vein at birth and from the dorsum of the hand at serial time points for each individual (>6-≤24hrs and >24-≤216hrs). Dots represent individual measurements. The blue line is a loess fit curve with 95% confidence intervals shaded in red. A = hemoglobin, B = hematocrit, C = sTfR.

Figure 6: Weighted Pearson correlation network analysis of iron status and inflammation variables between cord and post-natal blood samples. Produced using the Shiny Network Application (https://jolandakos.shinyapps.io/NetworkApp/). This analysis is formatted into the “spring” layout that uses the Fruchterman-Reingold Algorithm placing the more strongly correlated nodes closer together. Node colours define sample type (YELLOW = CDV, BLUE = V1, PINK = V2-4). The direction and size of the Pearson correlation between two nodes is represented using the colour (RED = Negative, GREEN = Positive) and thickness of an edge. Cord = umbilical cord blood. V1 = venous blood from dorsum of the hand at >6-≤24hrs. V2-4 = venous blood from dorsum of the hand at >24-≤216hrs. Fer = ferritin, Hep = hepcidin, Hct = hematocrit, Hgb = hemoglobin, GA = gestational age, BW = birthweight, CRP = C-reactive protein, Hapt = haptoglobin, AGP = alpha 1-acid glycoprotein, sTfR = soluble transferrin receptor, UIBC = unbound iron-binding capacity, TIBC = total iron-binding capacity, Trans = transferrin, fe = serum iron, TSAT = transferrin saturation.

Figure 7: Comparison of iron status, inflammation and hematological parameters in cord arterial (CDA = BLUE) and venous (CDV = RED) blood. Box plots represent the arithmetic mean with whiskers representing minimum and maximum values. * = P<0.05. No significance line = P>0.05.
Tables

Table 1: Demographic, clinical and pregnancy outcome characteristics of the women and their newborns.

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<th>V3</th>
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<td>Time from Delivery to V2 Blood Collection (Hours)</td>
<td>-</td>
<td>57.2 (±15.6)</td>
<td>57.6 (±15.6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Time from Delivery to V3 Blood Collection (Hours)</td>
<td>-</td>
<td>105.8 (±17.0)</td>
<td>-</td>
<td>105.8 (±17.0)</td>
<td>-</td>
</tr>
<tr>
<td>Time from Delivery to V4 Blood Collection (Hours)</td>
<td>-</td>
<td>156.7 (±21.0)</td>
<td>-</td>
<td>-</td>
<td>156.7 (±21.0)</td>
</tr>
<tr>
<td>Percentage Male (%)</td>
<td>55.5% (30)</td>
<td>54.3% (151)</td>
<td>46.4% (26)</td>
<td>58.0% (58)</td>
<td>54.4% (37)</td>
</tr>
<tr>
<td>G6PD Deficiency Positive (%)</td>
<td>14.8% (8)</td>
<td>11.5% (32)</td>
<td>8.9% (5)</td>
<td>10.0% (10)</td>
<td>13.2% (9)</td>
</tr>
<tr>
<td>Multiple Births (%)</td>
<td>0.0% (0)</td>
<td>2.2% (6)</td>
<td>1.8% (1)</td>
<td>4.0% (4)</td>
<td>1.5% (1)</td>
</tr>
<tr>
<td>Percentage of Mother on Antenatal Iron/Folic Acid (%)</td>
<td>81.5% (44)</td>
<td>81.7% (227)</td>
<td>83.9% (47)</td>
<td>80.0% (80)</td>
<td>82.3% (56)</td>
</tr>
</tbody>
</table>

Data are presented as arithmetic mean (± SD) or as a proportion (%). Exclusions represent newborns successfully bled at cord and V1 timepoints but who were lost to follow up or sick before V2-4 blood draw was completed.
Table 2: Comparison of iron status, inflammation and hematological parameters in cord and post-natal samples.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>n</th>
<th>CDV</th>
<th>n</th>
<th>V1</th>
<th>n</th>
<th>V2</th>
<th>n</th>
<th>V3</th>
<th>n</th>
<th>V4</th>
<th>n</th>
<th>V1 vs V2 (P value)</th>
<th>V1 vs V3 (P value)</th>
<th>V1 vs V4 (P value)</th>
<th>Trend (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Iron (µmol/L)</td>
<td>275</td>
<td>22.7±7.0</td>
<td>271</td>
<td>7.3±4.6</td>
<td>54</td>
<td>10.1±3.2</td>
<td>83</td>
<td>14.7±4.7</td>
<td>62</td>
<td>16.5±3.9</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>273</td>
<td>50.2±16.7</td>
<td>271</td>
<td>14.4±6.1</td>
<td>54</td>
<td>20.2±8.1</td>
<td>91</td>
<td>32.2±13.0</td>
<td>61</td>
<td>36.7±9.2</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>Serum Hepcidin (ng/ml)</td>
<td>277</td>
<td>19.4±14.4</td>
<td>270</td>
<td>38.9±23.9</td>
<td>55</td>
<td>32.7±18.4</td>
<td>82</td>
<td>45.9±17.7</td>
<td>62</td>
<td>45.2±19.1</td>
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<td>ns</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>UIBC (µmol/L)</td>
<td>273</td>
<td>23.7±10.4</td>
<td>271</td>
<td>44.1±18.0</td>
<td>54</td>
<td>41.5±10.3</td>
<td>91</td>
<td>32.4±10.2</td>
<td>61</td>
<td>29.5±9.6</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>TIBC (µmol/L)</td>
<td>273</td>
<td>46.3±8.1</td>
<td>271</td>
<td>51.4±20.7</td>
<td>54</td>
<td>51.6±16.8</td>
<td>91</td>
<td>46.9±9.6</td>
<td>61</td>
<td>46.0±10.5</td>
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<td>Serum Ferritin (ug/L)</td>
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<td>212.6±157.7</td>
<td>269</td>
<td>393.9±312.6</td>
<td>54</td>
<td>343.7±206.0</td>
<td>83</td>
<td>311.8±131.3</td>
<td>62</td>
<td>354.6±181.8</td>
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</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>270</td>
<td>15.1±2.3</td>
<td>272</td>
<td>19.1±2.9</td>
<td>49</td>
<td>19.5±2.9</td>
<td>85</td>
<td>18.1±2.9</td>
<td>64</td>
<td>16.9±2.9</td>
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<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>270</td>
<td>42.2±7.0</td>
<td>272</td>
<td>53.9±8.9</td>
<td>49</td>
<td>55.0±9.7</td>
<td>85</td>
<td>50.5±8.8</td>
<td>64</td>
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<tr>
<td>Transferrin (g/L)</td>
<td>275</td>
<td>2.0±0.3</td>
<td>273</td>
<td>2.0±0.3</td>
<td>56</td>
<td>1.9±0.3</td>
<td>93</td>
<td>1.7±0.2</td>
<td>62</td>
<td>1.7±0.2</td>
<td>ns</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Soluble Transferrin Receptor (mg/L)</td>
<td>273</td>
<td>6.0±2.0</td>
<td>271</td>
<td>6.9±2.2</td>
<td>54</td>
<td>6.6±2.0</td>
<td>92</td>
<td>6.1±2.2</td>
<td>62</td>
<td>5.1±1.3</td>
<td>ns</td>
<td>0.003</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin (g/L)</td>
<td>275</td>
<td>0.02±0.1</td>
<td>273</td>
<td>0.03±0.7</td>
<td>56</td>
<td>0.1±0.2</td>
<td>93</td>
<td>0.08±0.2</td>
<td>62</td>
<td>0.08±0.1</td>
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<td>0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Serum AGP (g/L)</td>
<td>275</td>
<td>0.2±0.1</td>
<td>273</td>
<td>0.3±0.2</td>
<td>56</td>
<td>0.4±0.2</td>
<td>93</td>
<td>0.4±0.1</td>
<td>62</td>
<td>0.4±0.1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Serum CRP (mg/L)</td>
<td>275</td>
<td>0.2±0.7</td>
<td>273</td>
<td>2.3±4.1</td>
<td>55</td>
<td>5.6±9.3</td>
<td>83</td>
<td>2.0±2.2</td>
<td>62</td>
<td>0.9±1.5</td>
<td>&lt;0.0001</td>
<td>ns</td>
<td>0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean Corpuscular Volume (fl)</td>
<td>270</td>
<td>97.8±6.3</td>
<td>272</td>
<td>97.7±6.0</td>
<td>49</td>
<td>97.6±5.1</td>
<td>85</td>
<td>96.0±5.9</td>
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<tr>
<td>Mean Corpuscular Hemoglobin (pg)</td>
<td>270</td>
<td>35.1±2.4</td>
<td>272</td>
<td>34.8±2.4</td>
<td>49</td>
<td>34.8±2.1</td>
<td>85</td>
<td>34.5±2.2</td>
<td>64</td>
<td>34.1±2.2</td>
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<td>ns</td>
<td>ns</td>
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<tr>
<td>Mean Corpuscular Hemoglobin Concentration (g/dl)</td>
<td>270</td>
<td>35.8±1.0</td>
<td>272</td>
<td>35.6±1.1</td>
<td>49</td>
<td>35.6±1.5</td>
<td>85</td>
<td>35.9±0.9</td>
<td>64</td>
<td>36.2±1.1</td>
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<td>0.0002</td>
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<tr>
<td>White Blood Cell Count (unit/L)</td>
<td>269</td>
<td>13.3±5.7</td>
<td>271</td>
<td>16.4±5.8</td>
<td>48</td>
<td>10.5±4.4</td>
<td>93</td>
<td>8.8±3.0</td>
<td>64</td>
<td>9.2±2.5</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>Lymphocyte Count (unit/L)</td>
<td>269</td>
<td>4.8±3.5</td>
<td>271</td>
<td>4.2±2.9</td>
<td>48</td>
<td>3.1±1.6</td>
<td>93</td>
<td>3.3±1.2</td>
<td>64</td>
<td>3.5±1.0</td>
<td>0.01</td>
<td>0.01</td>
<td>ns</td>
<td>0.0005</td>
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</tr>
<tr>
<td>Lymphocyte Percentage (%)</td>
<td>269</td>
<td>35.2±7.9</td>
<td>271</td>
<td>26.5±8.8</td>
<td>48</td>
<td>31.1±9.3</td>
<td>93</td>
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<tr>
<td>MID Cell Count (unit/L)</td>
<td>269</td>
<td>1.04±0.8</td>
<td>271</td>
<td>1.54±1.0</td>
<td>48</td>
<td>1.1±0.8</td>
<td>93</td>
<td>1.2±0.8</td>
<td>64</td>
<td>1.7±0.8</td>
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<td>ns</td>
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<tr>
<td>MID Cell Percentage (%)</td>
<td>269</td>
<td>8.1±2.4</td>
<td>271</td>
<td>9.8±5.6</td>
<td>48</td>
<td>11.5±7.0</td>
<td>93</td>
<td>14.8±7.8</td>
<td>64</td>
<td>18.9±8.1</td>
<td>ns</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>Granulocyte Count (unit/L)</td>
<td>269</td>
<td>7.5±2.7</td>
<td>271</td>
<td>10.7±4.2</td>
<td>48</td>
<td>6.3±4.0</td>
<td>93</td>
<td>4.2±1.9</td>
<td>64</td>
<td>4.1±1.8</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>Granulocyte Percentage (%)</td>
<td>269</td>
<td>56.6±8.7</td>
<td>271</td>
<td>63.7±8.8</td>
<td>48</td>
<td>57.4±9.3</td>
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<td>46.4±9.2</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Red Blood Cell Count (unit/L)</td>
<td>270</td>
<td>4.3±0.7</td>
<td>272</td>
<td>5.5±0.9</td>
<td>49</td>
<td>5.6±1.0</td>
<td>95</td>
<td>5.3±0.9</td>
<td>64</td>
<td>5.0±0.9</td>
<td>ns</td>
<td>ns</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
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<tr>
<td>Red Blood Cell Distribution Width (%)</td>
<td>270</td>
<td>15.2±1.2</td>
<td>272</td>
<td>15.4±1.4</td>
<td>49</td>
<td>15.6±2.8</td>
<td>85</td>
<td>15.1±0.9</td>
<td>64</td>
<td>15.1±2.2</td>
<td>ns</td>
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<tr>
<td>Parameter</td>
<td>V1</td>
<td>V2</td>
<td>V3</td>
<td>V4</td>
<td>V5</td>
<td>V6</td>
<td>P-value</td>
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</tr>
<tr>
<td>Red Blood Cell Distribution Width - Absolute (fl)</td>
<td>270: 80.7 (±8.9)</td>
<td>272: 82.0 (±8.6)</td>
<td>49: 82.5 (±15.7)</td>
<td>66: 77.9 (±8.0)</td>
<td>64: 75.3 (±7.0)</td>
<td>ns</td>
<td>0.004</td>
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<td></td>
</tr>
<tr>
<td>Platelet Count (unit/L)</td>
<td>270: 243.8 (±92.8)</td>
<td>272: 258.7 (±94.0)</td>
<td>49: 261.2 (±90.8)</td>
<td>66: 234.8 (±97.0)</td>
<td>64: 267.7 (±94.0)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean Platelet Volume (fl)</td>
<td>267: 8.4 (±0.8)</td>
<td>271: 8.5 (±0.8)</td>
<td>48: 8.5 (±0.9)</td>
<td>93: 8.7 (±0.8)</td>
<td>64: 9.3 (±0.7)</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution Width (%)</td>
<td>267: 43.5 (±3.0)</td>
<td>271: 43.9 (±3.3)</td>
<td>48: 44.5 (±3.5)</td>
<td>93: 45.4 (±3.6)</td>
<td>64: 46.4 (±2.7)</td>
<td>ns</td>
<td>0.05</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>Platelet Distribution Width - Absolute (fl)</td>
<td>267: 11.8 (±1.3)</td>
<td>271: 12.1 (±1.4)</td>
<td>48: 12.1 (±1.6)</td>
<td>93: 12.5 (±1.5)</td>
<td>64: 13.3 (±1.2)</td>
<td>ns</td>
<td>0.003</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>Platelet Crit (%)</td>
<td>267: 0.201 (±0.07)</td>
<td>271: 0.215 (±0.07)</td>
<td>48: 0.2 (±0.1)</td>
<td>93: 0.2 (±0.07)</td>
<td>64: 0.2 (±0.08)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.009</td>
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<td></td>
</tr>
<tr>
<td>Plate Large Cell Ratio (%)</td>
<td>267: 18.0 (±5.4)</td>
<td>271: 19.2 (±5.8)</td>
<td>48: 19.4 (±6.3)</td>
<td>93: 21.1 (±5.9)</td>
<td>64: 24.3 (±5.0)</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Plate Large Cell Count (unit/L)</td>
<td>267: 42.1 (±14.6)</td>
<td>271: 46.9 (±17.0)</td>
<td>48: 48.4 (±17.4)</td>
<td>93: 47.6 (±17.8)</td>
<td>64: 63.1 (±21.8)</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

Data are presented as mean (± SD) and analysed by ordinary least squares analysis of variance (OLS ANOVA). Number of available results differs by each parameter, due to limitations in blood sample volume for some participants. Scheffé’s post-hoc tests were conducted between V1 vs V2, V3 and V3 groups. P values in bold font are considered significant based on P<0.05. Hepcidin (log), ferritin (log), sTfR (log), AGP (Sqrt) and CRP (log) were all transformed to form a normal distribution before ordinary least squares analysis of variance (OLS ANOVA) and reverse transformation for the listings above.
Table 3: Comparison of iron status, inflammation and hematological parameters in cord venous and arterial blood.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>n</th>
<th>Cord (Arterial)</th>
<th>n</th>
<th>Cord (Venous)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Iron (μmol/L)</td>
<td>258</td>
<td>22.7 (±6.9)</td>
<td>275</td>
<td>22.7 (±7.0)</td>
<td>0.8</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>255</td>
<td>47.1 (±15.8)</td>
<td>273</td>
<td>50.2 (±16.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum Hepcidin (ng/ml)</td>
<td>249</td>
<td>19.7 (±14.4)</td>
<td>277</td>
<td>19.4 (±14.4)</td>
<td>0.34</td>
</tr>
<tr>
<td>UIBC (μmol/L)</td>
<td>255</td>
<td>27.1 (±11.3)</td>
<td>273</td>
<td>23.7 (±10.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TIBC (μmol/L)</td>
<td>257</td>
<td>49.8 (±9.1)</td>
<td>273</td>
<td>46.3 (±8.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum Ferritin (ug/L)</td>
<td>241</td>
<td>277.0 (±234.2)</td>
<td>275</td>
<td>212.6 (±157.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>242</td>
<td>15.2 (±2.6)</td>
<td>270</td>
<td>15.1 (±2.3)</td>
<td>0.53</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>260</td>
<td>43.0 (±7.5)</td>
<td>270</td>
<td>42.2 (±7.0)</td>
<td>0.16</td>
</tr>
<tr>
<td>Transferrin (g/L)</td>
<td>258</td>
<td>2.0 (±0.3)</td>
<td>275</td>
<td>2.0 (±0.3)</td>
<td>0.48</td>
</tr>
<tr>
<td>Soluble Transferrin Receptor (mg/L) (log)</td>
<td>260</td>
<td>6.0 (±1.9)</td>
<td>273</td>
<td>6.0 (±2.0)</td>
<td>0.86</td>
</tr>
<tr>
<td>Haptoglobin (g/L)</td>
<td>260</td>
<td>0.02 (±0.05)</td>
<td>275</td>
<td>0.02 (±0.06)</td>
<td>0.64</td>
</tr>
<tr>
<td>Serum AGP (g/L)</td>
<td>260</td>
<td>0.2 (±0.1)</td>
<td>275</td>
<td>0.2 (±0.1)</td>
<td>0.97</td>
</tr>
<tr>
<td>Serum CRP (mg/L)</td>
<td>242</td>
<td>0.2 (±0.03)</td>
<td>275</td>
<td>0.2 (±0.07)</td>
<td>0.27</td>
</tr>
<tr>
<td>Mean Corpuscular Volume (fl)</td>
<td>241</td>
<td>98.2 (±6.2)</td>
<td>270</td>
<td>97.8 (±6.3)</td>
<td>0.07</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin (pg)</td>
<td>241</td>
<td>35.1 (±2.5)</td>
<td>270</td>
<td>35.1 (±2.4)</td>
<td>0.9</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin Concentration (g/dl)</td>
<td>241</td>
<td>35.8 (±1.1)</td>
<td>270</td>
<td>35.8 (±1.0)</td>
<td>0.09</td>
</tr>
<tr>
<td>White Blood Cell Count (unit/L)</td>
<td>241</td>
<td>14.2 (±6.1)</td>
<td>269</td>
<td>13.3 (±5.7)</td>
<td>0.004</td>
</tr>
<tr>
<td>Lymphocyte Count (unit/L)</td>
<td>241</td>
<td>5.5 (±3.7)</td>
<td>269</td>
<td>4.8 (±3.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lymphocyte Percentage (%)</td>
<td>241</td>
<td>38.0 (±9.1)</td>
<td>269</td>
<td>35.2 (±7.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MID Cell Count (unit/L)</td>
<td>241</td>
<td>1.2 (±0.8)</td>
<td>269</td>
<td>1.0 (±0.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MID Cell Percentage (%)</td>
<td>241</td>
<td>8.8 (±3.2)</td>
<td>269</td>
<td>8.1 (±2.4)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Granulocyte Count (unit/L)</td>
<td>241</td>
<td>7.5 (±3.0)</td>
<td>269</td>
<td>7.5 (±2.7)</td>
<td>0.53</td>
</tr>
<tr>
<td>Granulocyte Percentage (%)</td>
<td>241</td>
<td>53.2 (±9.4)</td>
<td>269</td>
<td>56.6 (±8.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Red Blood Cell Count (unit/L)</td>
<td>242</td>
<td>4.4 (±0.8)</td>
<td>270</td>
<td>4.3 (±0.7)</td>
<td>0.44</td>
</tr>
<tr>
<td>Red Blood Cell Distribution Width (%)</td>
<td>242</td>
<td>15.3 (±1.0)</td>
<td>270</td>
<td>15.2 (±1.2)</td>
<td>0.11</td>
</tr>
<tr>
<td>Red Blood Cell Distribution Width - Absolute (fl)</td>
<td>242</td>
<td>81.5 (±9.2)</td>
<td>270</td>
<td>80.7 (±8.9)</td>
<td>0.008</td>
</tr>
<tr>
<td>Platelet Count (unit/L)</td>
<td>242</td>
<td>193.0 (±86.7)</td>
<td>270</td>
<td>243.8 (±92.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean Platelet Volume (fl)</td>
<td>237</td>
<td>8.6 (±0.8)</td>
<td>267</td>
<td>8.4 (±0.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet Distribution Width (%)</td>
<td>237</td>
<td>44.6 (±3.4)</td>
<td>267</td>
<td>43.5 (±3.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet Distribution Width - Absolute (fl)</td>
<td>237</td>
<td>12.3 (±1.4)</td>
<td>267</td>
<td>11.8 (±1.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet Crit (%)</td>
<td>237</td>
<td>0.2 (±0.07)</td>
<td>267</td>
<td>0.2 (±0.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plate Large Cell Ratio (%)</td>
<td>237</td>
<td>20.4 (±5.9)</td>
<td>267</td>
<td>18.0 (±5.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plate Large Cell Count (unit/L)</td>
<td>237</td>
<td>42.1 (±14.6)</td>
<td>267</td>
<td>42.1 (±14.6)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are presented as mean (± SD) and analysed by one-way analysis of variance. P values in bold font are considered significant based on P<0.05. Hepcidin (log), ferritin (log), sTfR
(log), AGP (Sqrt) and CRP (log) are all transformed to form a normal distribution before one-way analysis of variance (ANOVA) and reverse transformation for the listings above.
Figure 1: Study recruitment and blood draw design.
Figure 2: CONSORT diagram for participant flow.
Figure 3: Changes in iron and chaperone proteins in the first week of life.
Figure 4: Changes in hepcidin and inflammatory markers in the first week of life.
Figure 5: Changes in hematological indices in the first week of life.
Figure 6: Weighted Pearson correlation network analysis of iron status and inflammation variables between cord and postnatal blood samples.
Figure 7: Comparison of iron status, inflammation and hematological parameters in cord arterial (CDA = BLUE) and venous (CDV = RED) blood.
Chapter 7 - Discussion

Summary of Chapter

This chapter focuses on describing a flowing narrative of the overall content of this thesis. It begins with a description of the essential aspects of study design, the critical learning gained from previous pilot studies and the literature review process. A detailed summary of what was discovered during the NeoInnate Study and how it addressed critical research gaps then follows.

The subsequent section then notes several of the main issues and learning points faced during the research study process. At each point in this section, we outline the problem, the science behind it and how our research team overcame it.

Finally, this discussion recommends four potential future research areas. This firstly focuses on the role the placenta plays in the maternal-fetal iron transfer process. This is followed by a review of possible triggers of the neonatal hypoferremic response (e.g. hepcidin-dependent and hepcidin-independent mechanisms), and what research is required in order to understand these mechanisms further. Thirdly, this section describes the current research into harnessing hypoferremia to combat other infections and how this area is moving forward. This includes the current development of therapies that use iron chaperone proteins (e.g. haptoglobin, lactoferrin and lipocalin-2), bacterial iron-uptake mechanisms, and hepcidin agonists to treat bacterial infections. Lastly, this chapter outlines how future research should focus on the role of macrophages in the neonatal hypoferremic response and the kinetics of hepcidin-resistance post-hypoferremia.
7.1 A Review of the NeolInnate Study

7.1.1 NeolInnate Study: Where Did It Begin? (Chapter 1)

In light of the evidence that suggests hepcidin-induced hypoferremia is protective against severe bacterial infections,¹ the findings of our team's pilot study were an exciting, unexpected finding. This PhD study was formulated after the previous research by Prentice et al. (HYPO-G study).² This study was a non-blinded randomised controlled trial conducted on 120 full-term, normal birthweight Gambian neonates. Infants were randomised to receive Bacillus Calmette–Guérin (BCG) vaccination on the day of birth or after study completion at four days of age. Blood samples for investigation of iron parameters, hepcidin, IL-6 and red blood cell parameters were collected at birth (i.e. umbilical cord) and time-points up to 96 hours of age. This study found that regardless of whether neonates received BCG vaccination or not, that during the first 24 hours of life full-term neonates actively reduce the serum iron concentration and transferrin saturation of the blood. This was found to occur as early as six hours postpartum and lasted for two to three days. This response was in strong correlation with hepcidin, suggesting that hepcidin regulates this response as seen in previous mechanistic studies.³ This finding was also correlated to levels of IL-6, suggesting that inflammatory stimulation was the trigger. Using a micro-adaptation of the ex vivo bacterial growth assay previously described in our FeVir study,⁴ we showed that the lack of iron similarly reduced growth rates of E. coli, S. aureus, S. pneumoniae and S. agalactiae (common neonatal sepsis causing pathogens) inoculated into venous samples collected at 6-24 hours of life, in comparison to cord samples. The growth rates were significantly associated with TSAT level in the serum.²

Supported by similar but smaller reductions in serum iron concentration and TSAT observed in the first week of life in other studies,⁵-⁸ this observation suggested that the neonatal
hypoferremia may have evolved as a mechanism designed to protect neonates from infection during the first critical days of life. This is a result of limiting the pathogenicity and virulence of invading bacterial organisms. However, more diverse populations with a range of birthweights and gestational ages were required to confirm this. This is especially the case in the light of evidence that suggests preterm neonates suffer deficiencies in Toll-like receptors (TLRs), a vital component of the inflammation-induced hypoferremic response. We further hypothesised that this might lead to a dampening of this response, or a complete failure to reduce systemic iron concentrations. We believed that this might, in part, explain why being premature and/or low birthweight may be risk factors for neonatal sepsis.

7.1.2 A Review of Iron Homeostasis Over the First Month of Life (Chapter 3)

This research studentship started with a systematic search of literature relating to hepcidin, serum iron and transferrin saturation levels in full-term and preterm newborns during the first month of life (Chapter 3). Retrieved data suggested that hepcidin, serum iron and TSAT levels for adults and infants are much lower than those observed in cord blood and venous blood during the first month of life. Accumulated data further strengthened our previously defined hypothesis that full-term neonates possess the ability to produce a hepcidin-mediated hypoferremic response post-delivery. Nonetheless, data concerning preterm neonates was lacking and subsequently it was unclear as to whether they produce a similar response. This dearth of studies is presumably due to their relatively unstable medical condition, and the complex ethical issues around bleeding premature neonates. This review also highlighted a lack of data from blood samples collected in the first hours of life (6-24 hours) and over the first week, even in term babies.

Previously, Van der Vorm et al. formulated a series of linear equations to assist in the standardisation of each commonly used hepcidin measurement assay. In our review, we
used these equations to standardise results from previous publications. This allowed the
calculation of weighted mean averages in cord and venous blood for full-term and preterm
neonates. We believe the main strength of this review was that it is the first review to
compare retrospective serum hepcidin concentrations between studies, using standardised
values. This is the first review of hepcidin, serum iron and TSAT in cord blood and venous
samples over the first weeks of life, that was stratified by gestational age. The learning
points from this review for the following observational study were the importance of
collecting of very early postnatal venous blood (6-24 hours), the use of the DRG hepcidin
ELISA kit, the importance of detailed labour and delivery data, and standardisation of cord
blood collection protocols (Chapter 4).

Weaknesses of the review included the allocation of each study group or population in each
publication to a mean gestational age. This was due to limited access to raw study data. It is
suspected that this has reduced any natural variation potentially caused by gestational age
between the reviewed populations. Equally, due to studies rarely stating the average birth
weight of each population, analysis stratified by birthweight was not possible. Furthermore,
the retrieval of gestational age was a crucial aspect of the search strategy; however, few
studies documented the method used to calculate it. Due to the substantial differences in the
accuracy of different techniques, the true mean gestational age of each population may
vary by differing amounts.

7.1.3 Early Postnatal Hypoferremia in Low Birthweight and Preterm Babies
(Chapter 5)

Initiation of the NeolInnate Study then began, with the formation of a protocol and data
analysis plan. The primary objective of the NeolInnate study was to ascertain if there was a
defect in the ability of preterm and low birthweight newborns to elicit a hypoferremic
response immediately after delivery (Chapter 5). This observational study ended with the recruitment of 152 babies who were either preterm (PTB) (born ≥32 to <37 weeks gestational age) and/or low birth weight (LBW) (<2500g) (PTB/LBW) and 278 term (FTB), normal-weight babies (NBW) (FTB/NBW). Blood was sampled from the umbilical cord vein and artery, as well as matched venous blood samples taken from all neonates between 6-24 hours after delivery. In both PTB/LBW and FTB/NBW babies, we observed serum iron decreased 3-fold within 12 hrs of delivery compared to umbilical blood, and transferrin saturation showed a similar decline with a consequent increase in unsaturated iron-binding capacity. C-reactive protein levels increased over 10-fold and hepcidin levels doubled over the same period. We can now conclude that there was no difference in any of these responses between PTB/LBW and FTB/NBW babies. This result suggests that the innate postnatal iron restriction strategy in the first hours of life has evolved as an intrinsic mechanism to protect all neonates from common pathogens and/or free-radical damage, regardless of gestational age or birthweight. This finding now prompts further research into the use of iron restriction as a transient bacteriostatic mechanism to limit bacterial growth and virulence in other instances of infection.

One of the limitations of this study includes that 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). This was deliberate; however, the question remains as to whether neonates with suspected sepsis, have sepsis due (in part) to them lacking this innate immune response. This question will remain challenging to assess as it will be difficult to access the exact point of the initiation of the infection and to what degree any change in inflammation or iron restriction is due to the presence of the infection itself. Additionally, we did not recruit newborns born <32 completed weeks gestation and/or <2000g birth weight, or those delivered via caesarean section, vacuum or forceps. As a result, we cannot speculate whether similar hypoferremic responses occur in these newborns. However, reciprocally, we can reason that the responses we observed in the
NeoInnate Study are a characteristic feature of normal human birth and not due to pathological circumstances.

7.1.4 Iron Homeostasis in Full Term, Normal Birthweight Gambian Neonates Over The First Week of Life (Chapter 6)

The final objective of the NeoInnate Study was to describe the longitudinal changes in iron metabolism and haematological parameters after the initial hypoferremic response at 6-24 hours post-delivery. This was achieved by the collection of a second venous sample from all full-term, normal birth weight newborns later in the first week of life. This resulted in 224 of the 278 full-term, normal birthweight neonates (FTB/NBW) providing a cord blood sample, and two peripheral venous blood samples at 6-24h and 24-192h of age. This publication (Chapter 6) highlights that levels of hepcidin quickly return to previous mean cord-like levels at 24-48 hrs, and then subsequently increase to very high levels at 80-136hrs post-delivery. Serum iron and TSAT levels also increased during this period. This observation suggests that the early postnatal hypoferremia is a fast-acting, yet short-lived, adaptation that is followed by a period of hepcidin desensitisation or leaky intracellular iron sequestration. We suggest that this is the explanation for increased iron efflux into the serum, even in the presence of high serum hepcidin concentrations. This increase in circulating serum iron could be heightened by the reduced need for iron for erythropoiesis over the same period. A marker of this is the decreasing sTfR level over the first week of life. Weaknesses in research directed toward this objective include the lack of data relating to the quality of breastfeeding during the first week of life. This would have allowed us to control the analysis of V2-4 samples for the effect of enteric iron absorption from breast milk or formula (though both are likely to contribute small amounts of iron). Similarly, the measurement of maternal iron markers before (i.e. midgestation and parturition) and after delivery could have provided greater detail into the role of iron endowment between the mother and fetus and also the
part that labour-induced inflammation plays in the recorded hypoferremic response. Though maternal iron markers have been analysed in relation to cord iron markers,\textsuperscript{14,15} we believe no analysis has been completed concerning the change in serum iron and TSAT we have recorded. This analysis could provide further evidence as to what is triggering this phenomenon. The measurement of pro-inflammatory cytokines (e.g. IL-6 and IL-22) and growth factors (e.g. IGF-1, HGF, EGF and PDGF-BB) in all samples would have provided greater insight into the regulation of neonatal iron homeostasis.

7.2 Neolnnate Study: Study Design, Issues Faced and Learning Points for the Future

The Neolnnate study was a challenging study to design, due to its time-sensitivity, complicated exclusion criteria and complex ethical issues.

The review of the previous literature conducted at the start of the Neolnnate Study provided insight into the importance of collecting accurate delivery and labour data. We were particularly concerned that the inflammation believed to be responsible for the hypoferremic response in the neonate, may be confounded by pro- or anti-inflammatory stimuli received during labour and delivery. This included the effect of differing medication prescribed (i.e. gentamycin during labour),\textsuperscript{16} and delivery methods.\textsuperscript{17} As a consequence of this review, the subsequent study collected a detailed assessment of the labour process for each delivery. This study design also ensured that all recruited neonates had a cord blood sample collected using a standardised protocol, as previous studies have shown differing delays between cord-cutting can significantly affect early neonatal iron metabolism.\textsuperscript{18} Importantly, it also added much to the standard of care offered to study participants.
A fundamental aspect of the study design was the allocation of all neonates to four independent groups (e.g. FTB+NBW, FTB+LBW, PTB+NBW, PTB+LBW). This has allowed for the analysis of the independent effects of both birthweight and prematurity on neonatal hypoferremia. However, we did struggle to recruit full-term, low birthweight neonates during the study recruitment period. As birthweight is a predictor of prematurity, I believe this could be due to the research clinician, who conducted the New Ballard Score (NBS) of each neonate, not being blinded to the birthweight before NBS completion. Thus, there could be a degree of misclassification. The study research clinician was however, well trained in NBS and was deliberately unaware of specific group recruitment rates.

An important ethical issue facing the design of this study was the earliest time point in which a neonate could be bled post-delivery. Our team and hospital staff concluded that six hours would be the ideal time. This aspect of study design allowed the mother to recover physically and psychologically before providing full consent to the recruitment of the neonate and collection of the V1 sample. It also allowed clinical staff to assess if the mother or newborn were experiencing any medical complications. This led to the assessment of gestational age and anthropometrics at 6-24 hours post-delivery being a less stressful experience for the neonate, after being warmed and exposed to early breastfeeding.

Another of the most ethically complex study design aspects was the size of the blood volume collected from all recruited neonates, especially those born preterm and low birthweight. A sample volume of 3.5 ml of blood was required from each venous bleed of a full-term, normal birth weight neonate. This was calculated from the data produced by Howie et al., which suggest to the Gambia Government/MRC Joint Ethics Committee to allow a single blood draw of 2.4% total blood volume. With regards to maximum cumulative draw volume allowed, Howie et al., 2011 referenced multiple institutions that allowed >10ml of whole blood from a 3 kg birth weight neonate to be taken for research purposes throughout one to two months. These volumes were deemed to provided ‘minimal risk’ to the neonate.
Regarding preterm neonates, venous bleed volumes used in prior studies varied according to their weight, with an average of ~2ml per venous bleed. In our study, each blood draw tube was marked with a specific volume allowed from each neonate. As another layer of protection, all neonates received a complete review of systems by a research clinician or senior study nurse before any bleed was conducted. This ensured that the subject's health was of the highest priority. Furthermore, the study was designed in order to reduce the number of bleeds for each neonate. We were aware that repetitive bleeding of healthy neonates, would be unpopular with their families and expose each neonate to repetitive episodes of stress. As a result, we allocated each full-term, normal birthweight individual into a specific study group, in order to repetitively measure iron and inflammation markers at specific ranges of time over the first week of life using a maximum of two bleeds per baby.

Bleeding neonates is notoriously difficult. At the start of the study, the study team faced the complex challenge of completing 750 neonatal peripheral bleeds. On bleeding the first study participant, we became aware that the long tubing section of the vacutainer set-up was resulting in the loss of the pressure required to complete a successful blood draw. An extended study meeting successfully overcame this hurdle, where we were able to safely adapt the blood draw equipment, while still maintaining its sterility. However, in some cases, the blood volume collected was not high enough to conduct the ex vivo bacterial growth assay on some serum samples. This was primarily due to the difficulty of bleeding neonates, resulting in clotting taking place before the required sample volumes could be reached. Efforts were made to miniaturise the assay; however, the reduced assay-well volume caused a breakdown in the relationship previously documented between bacterial growth and TSAT. Sadly, as a result, one of the study objectives set out in the protocol (e.g. ex vivo bacterial growth assay) could not be achieved.

Before the study initiation, our study team was informed that bleeding neonates in the community would be an impossible challenge, primarily due to the interference from
family on the bleeding procedure. Experienced studies teams had suggested that the study design should ask the mother and their newborn to return to the maternity ward during this period. However, we were aware of experiences in other communities in The Gambia, where many neonates would have been lost to follow up if this design was adopted. With no other option available to acquire samples from discharged neonates between 24-192 hour post-delivery, we recruited an immensely experienced neonatal nurse with good local connections to conduct all community bleeds. We also worked as a study team to deliver safe and effective study protocols using the knowledge of local practices and cultural structures to drive this protocol. This resulted in 224/278 community bleeds being completed.

The NeoInnate Study suffered toward the end of the study from a lack of funds. This was primarily due to the issues relating to delays in the study initiation and the recruitment rate. The lack of funds resulted in our study being unable to examine how hypoferremia relates to maternal iron and inflammation makers (before, during and after labour). Similarly, this also led to the inability to measure IL-6, IL-22, haptoglobin (by ELISA assay) and hemopexin in all blood samples. This analysis would have informed us as to the role that inflammation and other chaperone molecules play in the reported hypoferremic response. The use of the Cobas 400+ biochemical analyser to detect haptoglobin concentrations in cord blood in this study, has also resulted in us not being to detect mother-neonate pairs that have experienced intrauterine infections as previously stated in Buhimschi et al.,20 If we had been able to afford haptoglobin ELISA test kits, this would not have been the case.
7.3 Recommendations For Future Research

7.3.1 Role of the Placenta in Maternal-Fetal Iron Transfer

As detailed in Chapter 3, sufficient iron stores are critical for a healthy pregnancy. In the placental villi, syncytiotrophoblasts uptake transferrin-bound iron from the maternal circulation via transferrin receptor 1 (TFR1) (Chapter 3 - Figure 1). TFR1 releases iron into an acidified clathrin-coated vesicle, where it is then transported into the cytoplasm of the syncytiotrophoblast by DMT-1, Zrt/Irt-like protein ZIP8 and ZIP14, collectively. Ferroportin, ceruloplasmin, hephaestin, and zyklopen all assist with the transportation of iron into the fetal circulation, where it binds to fetal transferrin. However, the regulation of maternal-placental-fetal iron transport mechanisms remains unclear. A recent publication by Sangkhae et al. provides additional details on the effects of iron-deficient, iron-replete and iron-overload conditions on the regulation of this process in mice, humans and human trophoblasts.

As seen in previous publications, Sangkhae et al. detected maternal hepcidin suppression during the pregnancy to ensure increased dietary iron intake and a raised systemic serum iron concentration. The suppression of hepcidin at this time is believed to be regulated by several factors including erythropoiesis in the mother or fetus, oestrogen, and progesterone receptor membrane component-1. Conflicting evidence now exists as to whether pregnancy-induced plasma dilution may also play a role.

In instances of iron-overload, both maternal and fetal hepcidin is produced at higher quantities in order to restrict further dietary uptake and protect the fetus from oxidative damage via placental iron transfer. However, questions remain as to whether fetal iron endowment is regulated by fetal or placental hepcidin in iron-replete or iron-deficient conditions.
conditions. Previous publications have remarked that in a state of maternal iron deficiency, the fetus could be regarded as the "perfect parasite" as human fetal iron endowment is maintained regardless of maternal iron status.\textsuperscript{21} However, Sangkhae et al. recently recorded conflicting evidence in murine models.\textsuperscript{29} Instead, iron transfer to the murine fetus under iron-deficient conditions was restricted. Decreased expression of ferroportin on the fetal side of the placenta is understood to be the cause. Interestingly, also observed was increased TFR1 expression on the maternal-facing membrane of the placental syncytiotrophoblasts. This suggests that during maternal iron deficiency, iron is held in the placenta to ensure that placental metabolic homeostasis is maintained. Sufficient iron concentration of the placenta is believed to ensure oxidative phosphorylation can continue, ensuring adequate levels of ATP are still produced.\textsuperscript{29} This allows the continuation of placental protein synthesis and other critical transfer functions, protecting against placental dysfunction.

Nonetheless, these findings were only observed in the murine and \textit{in vivo} human trophoblast models.\textsuperscript{29} When similar analyses were conducted on human pregnancies, expression of TFR1 protein increased, yet ferroportin expression did not change. Sangkhae et al. have proposed that this may be the case due to the scarcity of severely iron deficient human mothers in their study setting.\textsuperscript{29} Further research is now required to fully understand these interspecies differences, and what role placental iron regulation has on the hypoferremic response discussed here.

\textbf{7.3.2 Potential Triggers of Early Postnatal Hypoferremia}

\textbf{7.3.2.1 IL-6 and the JAK/STAT3 Pathway}

The initiation of hepcidin synthesis via the actions of IL-6/JAK/STAT3 pathway has been extensively characterised.\textsuperscript{37} IL-6 begins by binding to its receptor activating the formation of
a hexameric plasma-membrane signalling assembly. This is formed of IL-6, IL-6 receptor, and a gp130 subunit.\textsuperscript{38} Activation of JAK2 then follows, enabling the phosphorylation of its tyrosine residues and those of subsequent downstream STAT molecules. The phosphorylated STAT molecules then dimerise, translocate to the nucleus, and target the transcription promoters of the \textit{HAMP} gene.\textsuperscript{39} Transcription of the \textit{HAMP} gene results in the synthesis of new hepcidin proteins, which are later released into the circulation. Hepcidin then binds a single ferroportin protein on the plasma membrane of enterocytes, macrophages, and hepatocytes. This causes the endocytosis and co-degradation of both bound proteins in the lysosome.\textsuperscript{40} Hence, enteric absorption of dietary iron is reduced, and sequestration of iron in macrophages causes a reduction in serum iron concentration.\textsuperscript{3}

Our study has now confirmed that hepcidin-mediated neonatal hypoferremia exists in all healthy neonates (\textit{Chapter 5}). Potential triggers for this mechanism remain unclear, as IL-6 was not analysed. However, previous research has found that inflammation (signalled by IL-6 and other cytokines) is involved in the onset and progression of human labour at term in the mother,\textsuperscript{41–48} in the absence of intrauterine infection. Similarly, inflammatory markers are also detected in the cord blood of the neonate.\textsuperscript{49} Previous authors have suggested that the instigating stimuli for this could involve the endocrine events of labour,\textsuperscript{47,48,50} mechanical distension of the membranes and cervix (smooth muscle),\textsuperscript{41,50–53} placental hypoxia and/or hypoperfusion,\textsuperscript{50,54} fetal hypoxia-acidemia,\textsuperscript{55} maternal pain\textsuperscript{56} or exposure to infective agents.\textsuperscript{46,48,50,57} We suggest that due to the impermeability of the placenta to IL-6,\textsuperscript{49,58} independent stimuli of the increased inflammatory response are taking place in the mother and newborn. This theory is supported by evidence that there are significantly lower levels of IL-6 in the newborn than the mother.\textsuperscript{59} We propose that this increase in IL-6 leads to the previously reported large influx of immune cells (predominantly neutrophils) into the cervix, decidua, myometrium, chorioamnionic membranes and amniotic fluid.\textsuperscript{47,60} We speculate that a similar process may occur in the neonate, with the activation of circulating neutrophils around the body at delivery. Labour is associated with neutrophilia in the early neonatal
Both neonatal neutrophil activation and antigen expression correlate with the length of labour. Decreased proportions of T cells expressing CD2, CD3 and CD4, as well as increased expression of CD16 and CD56 on natural killer cells from vaginally-delivered newborns have been reported. This is in contrast to those born by elective caesarean section. Interestingly, another publication has documented significantly higher cord hepcidin levels in neonates born via standard vaginal delivery and second stage caesarean section than those born via elective caesarean section. This evidence suggests that stimuli on the neonate that cause the proposed inflammation-induced hypoferremia may occur during the process of labour, rather than delivery. This is reinforced by a study by Weinschenk et al., that did not observe a difference in the increased neonatal neutrophil activation between modes of delivery after the initiation of labour. We propose that the stimuli during labour could be mechanical trauma caused by contractions in the latent and active phases of labour, or the rupture of membranes leading to the exposure of the neonate to the uterine microbiome. Both proposed stimuli would increase in severity as the length of labour progressed. In this study, we have analysed the time between arrival to the maternity ward and time of delivery, in relation to hypoferremic response. However, as many factors are responsible for the time of arrival at the hospital before delivery, especially in LMICs with the lack of transport infrastructure and financial constraints, it is no surprise that no significant effect was observed. Future studies measuring the length of labour accurately in relation to hypoferremia could clarify this. As to whether this effect is a deliberate action to generate the hypoferremic response is unknown. Previous authors have suggested this pro-inflammatory response may also assist in organ system transition at birth (e.g. the lungs and cytokine-induced synthesis of surfactant proteins) and/or the activation of the immune system in the newborn.

Since hepcidin and IL-6 are not thought to cross the placenta, we have proposed that this labour-induced inflammatory response leads to the increase in hepcidin expression in the newborn and hence to the systemic sequestration of iron during and immediately after
labour/delivery has taken place. Further studies are required to confirm this, with a specific focus on the differences in iron and inflammation markers in the newborn over the first 48 hours of life between vaginally, elective caesarean and emergency caesarean section deliveries. However, this hypothetical study would be challenging due to the complex confounding clinical reasons for elective and emergency caesarean section delivery.

7.3.2.2 Additional Pro-Inflammatory Regulators of Hepcidin Expression

Additional research is also required to understand the role of other pro-inflammatory regulators of hepcidin expression have on early postnatal iron homeostasis.

IL-22 is a member of the IL-10–related family of cytokines, predominately expressed by lymphocytes. The binding of IL-22 to its receptor leads to activation of Jak1 and Tyk2, causing the phosphorylation of tyrosine molecules on STAT1, STAT3, STAT5. The subsequent transcription of the HAMP gene results in the synthesis of hepcidin and the reduction of circulating serum iron levels. The distinct familial differences between IL-22 and IL-6, lead the IL-22 pathway to work independently of IL-6-mediated hepcidin expression.

IL-22 also mediates the production of other antimicrobial proteins and acute-phase reactant proteins in the liver. These include the chaperone protein responsible for restricting bacterial uptake of human haemoglobin in the blood, haptoglobin. This finding, besides the weak activation of IL-22 from exposure to bacterial LPS, makes additional research into this cytokine pathway necessary. This research would provide further insight as to whether contact with microorganisms during the delivery process is the exclusive trigger of neonatal hypoferremia.

Similar to IL-22, IL-1 is a pro-inflammatory molecule that can work independently of the IL-6/JAK/STAT3 signalling pathway to induce hepcidin expression. The synthesis of hepcidin
in hepatocytes in both wild-type and IL-6 pathway knockout mice supports this observation. Nonetheless, the action of IL-1 is suggested to more commonly be additive to the mechanism of IL-6. STAT3 and NF-κB pathway crossover is believed to be the cause. This is supported by murine models showing increased hepcidin synthesis in response to LPS inoculation, compared to those that received IL-6 treatment. IL-1 binds to its receptor, IL-1R, causing the phosphorylation of the cellular protein, IKK. Phosphorylation and degradation of pathway inhibitor, IκB then follows, resulting in the activation of NF-κB. NF-κB promotes the expression of C/EBPδ, which then binds to P-Smad and promotes the transcription of the HAMP gene. IL-1Ra is a structurally similar molecule to IL-1; however, it binds to IL-1 receptor in a competitive nature and does not induce any intracellular response. At birth, levels of IL-1Ra increase for the first days of life. This suggests that IL-1-mediated inflammation and subsequent hepcidin synthesis may be unfavourable to early neonatal life, which is in conflict with the conclusions of our study (Chapters 5 and 6). An analysis of IL-1 and its relationship with the mechanisms of neonatal hypoferremia is required.

Pro-inflammatory regulators of hepcidin expression also include activin b, leptin, oncostatin M and leukaemia inhibitory factor (LIF). However, the strength of their independent hypoferremia-inducing qualities in the setting of bacterial infection in the human host remain unknown.

7.3.2.3 Hepcidin-Independent Mechanisms of Hypoferremia

Ferroportin (FPN1) is the only cell-surface transmembrane protein known to export ferrous iron out of mammalian cells. Subsequently, FPN1 expression levels, play a key role in the control of cellular and systemic iron concentrations. Duodenal enterocytes, placental syncytiotrophoblasts, hepatocytes and reticuloendothelial macrophages are the predominant
cell types that express FPN1. As seen in Figure 1.7, erythropoiesis, hypoxia, iron status, and inflammation are all reported to alter the expression levels of FPN1. This is primarily controlled via the action of its master regulator, hepcidin. Inhibition of ferroportin begins by hepcidin binding, inducing endocytosis and co-degradation of both molecules. This results in a reduction in iron export and an increase in pooled intracellular iron.

Nonetheless, FPN1 can also be regulated via hepcidin-independent mechanisms leading to a reduction in systemic serum iron concentration. Whether hepcidin-independent mechanisms contribute to the production of early neonatal hypoferremia (via the direct suppressive effects of inflammation on FPN1) remains undetermined.

One such potential hepcidin-independent mechanism is the activation of TLR2/6, leading to the direct downregulation of FPN1 mRNA synthesis. This results in intracellular iron sequestration and systemic hypoferremia. Previous authors speculate that TLR2 and TLR6 do not activate hepcidin upregulation, offering redundancy to the mechanism of hypoferremia, enabling a faster, broader and more effective immune response. These same studies have shown that bacterial lipopeptides (e.g. Mycoplasma-derived FSL1 and bacterial LPS) that target TLR2 and TLR6 promptly reduce ferroportin mRNA production for over three hours. This is supported by TLR2-knockout models exhibiting no effect on ferroportin expression in response to FSL1 inoculation. Further analyses are now required to understand if whole pathogens can trigger a similar response. This information would strengthen TLR2/6 as essential mediators of iron redistribution and may offer an alternative, more direct drug target compared to hepcidin-mediated pathways.

Tumour necrosis factor-α (TNF-α) is a cytokine produced by many cell types, including the primary producers: macrophages, Langerhans cells, and Kupffer cells. Following synthesis and release, TNF-α binds to either tumour necrosis factor receptor 1 or 2 (TNFR1 and TNFR2) resulting in the initiation of mitogen-activated protein kinase (MAP kinase), caspase,
and NF-κB pathways (amongst others).\textsuperscript{92} In 1989, Alvarez-Hernández \textit{et al.} proposed that TNF-α causes systemic hypoferremia within the first six hours of inoculation, while total iron-binding capacity remains constant.\textsuperscript{93} Laftah \textit{et al.} uncovered a similar reduction in serum iron recorded in the first 3 hours after intraperitoneal inoculation of TNF-α.\textsuperscript{94} These findings are supported by evidence that TNF-α mediated hypoferremia is initiated within the first eight hours after caecal ligation and puncture in wild-type murine models.\textsuperscript{95} No such response was detected in TNF-α deficient model. As no change in serum hepcidin concentration was linked to any of these responses,\textsuperscript{93–95} studies suggest hepcidin-independent mechanisms regulate this method of protection, resulting in the downregulation in FPN1 transcription. TNF-α mediated hypoferremia may regulate a proportion of the early neonatal hypoferremia we have uncovered; however, additional research is needed to understand its role.

\subsection*{7.3.3 Harnessing Iron to Fight Infections}

As previous research by our group has shown, limited iron concentrations reduce bacterial growth rates of sepsis-causing pathogens in human serum (\textit{Annex 1.16}).\textsuperscript{4} The NeoInnate Study discussed here has also shown that iron-sequestration is a potentially fundamental aspect of the early life innate immune response. In light of the innate and adaptive immunological differences between preterm, full-term and low birthweight newborns,\textsuperscript{96} the observation that neonatal hypoferremia is maintained in the population, regardless of gestational age or weight, suggests that it offers a significant benefit to the newborn. As a result, it is possible to consider that the limitation of iron sources to invading organisms in a multitude of other infection types could offer a partial (in combination with antibiotics) or complete (with the use of the host's immune system) treatment option. We hypothesise that limiting the growth and replication rates of invading organisms by providing a transient bacteriostatic mechanism of protection, would allow the host's neutrophils to engulf and destroy bacterial cells. We also propose that reduced replication rates of the bacterial
pathogen may allow for a reduction in the recommended dose, frequency and/or duration of current antibiotic treatments. We wonder whether combining iron-sequestration plus antibiotics may allow for toxic drug regimens and classes to be made available to individuals with a decreased maximum tolerable dose. This is particularly the case with neonates, as they possess unique physiological processes compared to adults leading to differences in drug absorption, distribution, metabolism, and elimination.97

An added benefit of this proposed treatment method would be that it is defined as combination therapy. Evidence suggests that antibiotic combination therapies already offer an increased benefit against blood infections, including those caused by carbapenem-resistant K. pneumonia (i.e. a common neonatal pathogen).98 We speculate that similar observations may be seen by inducing transient hypoferremia, without causing long term adverse effects to iron homeostasis (Chapter 6). This proposed treatment strategy may allow for the use of older disused drugs to be redeployed, as observed with other drug combination therapies.99 Furthermore, the use of antibiotic combination therapy has been found to reduce the spread of antimicrobial resistance.100 This observation, along with most microbial pathogens requiring iron for growth and virulence, and bacterial iron acquisition mechanisms being genetically coded by pathogenic housekeeping genes, suggests that hypoferremia-antibiotic therapies may offer a reduction in the creation of new antimicrobial resistance mechanisms. Evidence of the effect that iron chelation therapy could offer in combination with an antibiotic can already be seen in the study conducted by Coraca-Huber et al.101 This study showed that this form of treatment significantly reduced the formation of staphylococcal biofilms during infections. It is worth noting that hepcidin has also been previously described to have direct bactericidal effects on bacterial pathogens.102

The sequestration of iron and its moieties by native chaperone molecules in order to produce a protective response against invading organisms could also offer a new and novel treatment option. A study that supports this is that of Remy et al.,103 the main finding of
which is the positive effect haptoglobin treatment has on reducing canine pneumonia and sepsis in animal models. Haptoglobin works by binding to cell-free haemoglobin in the blood and other body fluids, reducing its availability as an iron source to many bacterial pathogens.\textsuperscript{104} Cell-free haemoglobin is regularly elevated during sepsis, with the level of increase correlating with a higher rate of mortality.\textsuperscript{105} Remy \textit{et al.} have shown that in a canine \textit{S. aureus} pneumonia model with septic shock, that human haptoglobin concentrate infusions lead to the binding with canine cell-free haemoglobin. This results in its clearance and lowering of the overall level of iron within the circulation. The resulting effect is an increase in survival in the canine model. The theory behind this is an increase in the internalisation of haptoglobin-haemoglobin complexes, thus limiting access to iron for extracellular organisms. This suggests that haptoglobin therapy can enhance innate host immunity, supporting the notion it offers a novel approach to treat systemic bacterial infections. Further clinical trials in humans are required to confirm this. This use of haptoglobin is particularly relevant to neonates due to enhanced erythrocyte turnover in early postnatal life leading to the release of haemoglobin, along with the suggestion that haptoglobin synthesis is increased in response to intrauterine infection.\textsuperscript{20} This supports the hypothesis it may be an effective method of innate protection.

Another treatment option in the host-pathogen battle for iron is the use of lipocalin-2. Lipocalin-2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is released by several cell types (e.g. hepatocytes, pneumocytes, renal epithelial cells, and vascular smooth muscle cells) as a result of inflammation, ischemia and infection.\textsuperscript{106} Recent research has shown that lipocalin-2 is produced in response to the infection of macrophages by intracellular bacterial pathogens, such as \textit{Brucella abortus}.\textsuperscript{107} Lipocalin-2 it thought to prevent iron uptake by \textit{B. abortus} by two mechanisms: 1) by stripping iron from iron-laden siderophores and 2) intracellular levels of lipocalin-2 in macrophages inhibit further iron uptake by macrophages during infection. This results in the starvation of the pathogen of an iron source, which is critical for growth, virulence and replication. This suggests that
synthetically increasing the concentration of lipocalin-2 in the host's extracellular environment could also protect individuals against extracellular pathogens which commonly cause sepsis.\textsuperscript{108}

Lactoferrin is a glycoprotein belonging to the innate immune system, found in saliva, blood, tears and human milk. Lactoferrin's central role is to bind to iron, with additional immunostimulatory, antimicrobial and anti-inflammatory roles.\textsuperscript{109} Due to the structural similarities between bovine and human lactoferrin, the biological function is similar.\textsuperscript{110} A Cochrane review of six studies detailing its use in preterm neonates has shown that oral supplementation of lactoferrin with or without probiotics reduced the risk of late-onset necrotizing enterocolitis, bacterial and fungal sepsis.\textsuperscript{111} Equally, the use of recombinant lactoferrin (i.e. Talactoferrin) offers some promise as it does not require pasteurisation, increasing its biological activity.\textsuperscript{112} Interestingly, concentrations of lactoferrin are high in colostrum,\textsuperscript{113} again suggesting that iron-restriction is an essential aspect of neonatal innate immune defence in the gut.\textsuperscript{114} How this might be linked to circulating levels of neonatal iron remains unclear.

Lastly, research is also progressing toward harnessing the use of the bacterium's iron uptake machinery in order to combat the infections it causes. This comes in the form of the manipulation of the action of bacterial siderophores and the bacterial influx transport channels. Small-molecule siderophores are produced and released by nearly all bacterial species.\textsuperscript{115} As seen in Figure 1.6 and Table 1.2, the redundancy of these iron-uptake systems allows bacteria to bind and consume different forms of iron. This function of multiple ligand targets by a plethora of siderophore types is predominately due to their differing chemical structures of the iron-chelating chemical groups (i.e. hydroxamate, α-hydroxylcarboxylate and catechol).\textsuperscript{115} The ability to synthesise these molecules is often transferred between bacterial isolates by mobile genetic elements.\textsuperscript{116} Recently, developments have been made to exploit these iron-siderophore uptake systems in common bacterial pathogens.
by using siderophores conjugated to antibiotics. This new form of antibiotics has been focused on gram-negative bacteria due to the threat they pose with regards to antimicrobial resistance and their vastly characterised bacterial iron uptake mechanisms. One drug discovery candidate that is showing early promise is cefiderocol. The new expedited development program of cefiderocol has led to a recent international, double-blind, randomised phase III clinical trial of the efficacy and safety of cefiderocol in patients with nosocomial pneumonia. These results highlight that cefiderocol met the non-inferiority comparison to high dose meropenem in all-cause mortality at 14 days after initiation of treatment. Cefiderocol, a siderophore-cephalosporin drug, which uses the bacterium's active transport machinery in a "Trojan horse" style to gain entry past the cell wall. As previously stated, cefiderocol is now of particular use against carbapenem-resistant gram-negative pathogens, including those expressing New Delhi metallo-β-lactamase-mediated carbapenem resistance, as seen in cases of neonatal sepsis (Section 1.9.7). Additionally, in vitro activity against isolates from the global surveillance studies, SIDERO-WT and SIDERO-CR have shown that cefiderocol is highly effective against current strains of P. aeruginosa, A. baumannii, B. cepacia, B. pseudomallei and S. maltophilia. Several of these have recently been noted in cases of invasive bacterial infection in neonates in sub-Saharan Africa.

7.3.4 Hepcidin Agonists as Hypoferremic Therapies

Here, we speculate that the use of hepcidin agonists or inducers may be the hypoferremic therapy that is required to produce this iron-restricted environment in the host. There are several therapeutic inducers of hepcidin expression; these include BMP6, TMRSS6-silencing oligonucleotides and a number of small molecules. One of the inducers that has progressed in its development is IONIS-TMRSS6-LRX (Ionis Pharmaceuticals, Inc.). This molecule is in phase 2 clinical trials and induces hepcidin expression by silencing
Tmprss6 mRNA by using lipid nanoparticles linked to small interfering RNAs (siRNAs) (ClinicalTrials.gov Identifier: NCT04059406).

A hepcidin agonist of note is minihepcidin PR73, which works by inhibiting the function of the transmembrane protein, ferroportin. PR73 has been shown to have possible protective effects in animal models against systemic bacterial infection. Researchers have uncovered that animal models deficient in hepcidin, when given PR73, are protected against siderophilic bacteria such as *Vibrio vulnificus* and *Yersinia enterocolitica*. Additionally, PR73 is observed to be protective against non-siderophilic bacteria such as *Klebsiella pneumoniae* or *Escherichia coli*. This is due to the PR73 mechanism of action leading to a systemic hypoferremic state in the model, repressing bacterial growth, and subsequently reducing the likelihood of mortality. These findings are especially remarkable as a similar deficiency of hepcidin, seen in hereditary hemochromatosis, leads to increased mortality when infected with siderophilic bacteria. This suggests that these observations may be relatable to humans. Currently, all of the research relating to the use of PR73 is in the preclinical phase.

Two hepcidin agonists that have reached clinical trials are LJPC-401 (La Jolla Pharmaceutical Company) and PTG-300 (Protagonist Therapeutics Inc.). PTG-300 is now in phase II and has previously been shown to reduce serum iron concentration in cynomolgus monkeys. During phase I trials, the drug was shown to be tolerated by study participants and reduced their serum iron concentration in a dose-dependent manner. Hypoferremic responses were maintained for almost 144 hours. The LJPC-401 phase 1 trial reports similar findings with reduced serum iron concentration of the blood in a dose-dependent manner. Researchers found that the maximal point of hypoferremia occurred at eight-hours post-injection.
Though the clinical trials referenced suggest that induced hepcidin levels produce a fast-acting and short-lived response, it is still unclear what effect hormonal augmentation would have in the sick neonate in the immediate period after treatment, or the long-term effects (i.e. erythropoiesis, microbiome and iron deficiency). Further research is required in humans to assess how we might produce a balanced therapy, focused on 1) maintaining iron-sequestration for a required time allowing for iron starvation in invading organisms, and 2) to not produce significant adverse side effects to human iron homeostasis. Realistically it will be many years before trials in neonates would be seriously contemplated.

7.3.5 Hepcidin-Resistance

Assessing our data on iron and inflammation markers in full-term, normal birthweight neonates over the first week of life (Chapter 6) leads us to question the validity of the proposed methods of treatment for severe bacterial infections in newborns seen in Sections 7.3.3 and 7.3.4. The hepcidin-induced hypoferremia observed in the NeoInnate Study was a robust and fast-acting response to early postpartum life. Nevertheless, it was not maintained for longer than 72 hours of life in most individuals. This is even the case despite hepcidin levels remaining higher than adult levels in the majority of the full term, normal birthweight study participants over the first week of life. From our data, we suggest that hepcidin-resistance may potentially halt the continuation of this protective mechanism after 48 hours post-delivery. This is observed in Chapter 6 (Figures 3 and 4) were hepcidin levels are maintained at high levels at 48 hours onwards, with serum iron and TSAT levels increasing over the same period. We hypothesise that this is due to the effect of high dose excess hepcidin, causing occlusion rather than degradation of ferroportin on the membrane of neonatal macrophages. Previous research has shown that excess hepcidin can preferentially cause occlusion, leading to the intermittent binding and releasing of hepcidin to the central cavity of individual ferroportin molecules. We propose that this may allow for
the steady and slow increase of serum iron concentration over the following days. This finding is noteworthy, as dietary iron absorption during this period is very low. Therefore, any increase in serum iron is unlikely to be caused due to enterocyte iron efflux.

A previous study in suckling 15-day old mouse models has found a potentially related discovery in the context of early life hepcidin resistance. Frazer et al. aimed to elicit if, as previous studies had suggested, iron absorption in suckling mammals was resilient to stimuli that ordinarily decreased absorption in adults. Interestingly, this study uncovered that enterocyte ferroportin was hyporesponsive to the effect of circulating serum hepcidin during the suckling period. Immunofluorescence assays suggested that this finding was not due to changes in ferroportin localisation, making it inaccessible to the actions of hepcidin, as previously reported by Theurl et al. concerning hepcidin resistance in the retinal pigment epithelium. Similarly, this could be the possible cause of hepcidin-resistance in our study, due to impermeable physical barriers between molecules of ferroportin and hepcidin in a subset of cell types responsible for iron release at the latter part of the first week of life. On balance, this is unlikely to be the case as we believe circulating and splenic macrophages are the target cells for iron sequestration during neonatal hypoferremia.

Furthermore, Frazer et al. found that decreases in serum iron levels occurred after hepcidin stimulation, suggesting that hepcidin activity was present in some cell types. However, this was not the case concerning enterocytes. After further laboratory analysis, ferroportin in these murine enterocytes was shown to be smaller than standard adult ferroportin molecules. The authors suggested that this may be due to early life murine ferroportin being spliced or glycosylated differently. This evidence suggests that in murine models, age-specific modification of ferroportin is found in the transmembrane of enterocytes. Further research is required to assess if that is the case with respect to human neonatal macrophage-expressed ferroportin molecules.
Similar to our reports, Frazer et al. note that hepcidin-resistance could be the result of high hepcidin excess favouring the occlusion of ferroportin rather than its degradation. As seen in Aschemeyer et al., hepcidin is thought to bind to the central metal-binding cavity, leading to the hinderance of the conformational changes required for iron efflux. Additionally, we submit that with the hepcidin levels in excess, intermittent binding and releasing of hepcidin to the central cavity of individual ferroportin molecules may allow for slow rates of iron efflux into the circulation (Chapter 6). This leads us to consider that there could be scope for research into the optimum dose of minihepcidin required to swing the balance back into the favour of degradation rather than occlusion, should our hypothesis be found to be correct. Equally, it would also be interesting to access the endocytic machinery of neonatal macrophages to uncover if deficiencies in degradation mechanisms may be the cause of these proposed reasons for hepcidin-resistance.

Why hepcidin-resistance occurs in young mice and humans remains unclear. However, it could be that both the hyporesponsiveness of murine enterocytes in 15-day mice pups and the hepcidin-resistance uncovered in our study in human neonates is a semi-protective mechanism against the iron deficiency in early life, which is particularly common. It may be possible that human neonates are trying to strike a balance between inducing protective hypoferremia at the point it is most required to protect against infection (i.e. 24 hours of life) and ensuring serum iron is still available to the tissues responsible for erythropoiesis and growth.

Further research is required to determine whether neonatal ferroportin is differentially spliced or glycosylated and if so, what cell types this occurs in. It is also crucial that we establish whether, like adult ferroportin, the expression of the hypothetical variant form of ferroportin suggested here, is less or more affected by inflammation and by what mechanism.
Until these questions are answered, it would not be worthwhile supporting the use of hepcidin agonist or inducers to combat neonatal systemic infections.

7.4 Conclusions

The research of this thesis has contributed three key components to our present understanding of neonatal iron homeostasis. Firstly, it has evaluated our current knowledge of iron and inflammation markers (hepcidin, serum iron and TSAT) in the neonatal period in respect to gestational age. This was only possible because of the newly developed methods in the standardisation of hepcidin values across multiple retrospective studies in order to produce comparisons and averages. Secondly, it has unequivocally refuted our previous hypothesis that immature or growth-restricted neonates might have a lesser ability to trigger a hypoferremic defence and that this might explain their greater susceptibility to septicaemias. Consequently, we have shown that the premature and low birthweight neonates all exhibited a profound hypoferremia during the first 24 hours of life, with no detectable differences from the full-term, normal birthweight controls. The remarkable similarity in the hypoferremic response across all the study groups further accentuates the efficiency of the process and supports the likelihood that it occurs by the process of evolution. Thirdly, our results suggest that after a fast-acting, short-lived early postnatal hypoferremic response at birth, there is a period of hepcidin-resistance, resulting in slow and consistent iron efflux into the circulation over the next week of life. Further research is required to understand why neonatal iron homeostasis is unresponsive to high serum hepcidin concentrations after the hypoferremic period. While contemplating the use of iron-sequestration as a method of combatting antimicrobial resistant infections seems untimely, in light of our results and the threat we all face, the importance of scrutinising hypoferremia as a mechanism of protection has never been more evident.
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ANNEXES
## ANNEX 1.11 Collaborators and field team details and contributions

<table>
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<td>Supported with budget management and study administration.</td>
</tr>
<tr>
<td>Amulai Touray</td>
<td>Nutrition Theme Project Coordinator and Assistants, MRC Unit The Gambia</td>
<td>Supported with budget management and study administration.</td>
</tr>
<tr>
<td>Tolu Osunnuyi</td>
<td>Laboratory Managers, Keneba Field Station, MRC Unit The Gambia</td>
<td>Laboratory management for Keneba Laboratory were analysis was conducted for Chapters 5 and 6.</td>
</tr>
<tr>
<td>Ebrima Danso</td>
<td>Roche Cobas Integra 400+ Technician, Keneba Field Station, MRC Unit The Gambia</td>
<td>Provided technical support for Roche Cobas Integra 400+ seen in Chapter 5 and 6.</td>
</tr>
<tr>
<td>Patrick Okot</td>
<td>Database Manager, Keneba Field Station, MRC Unit The Gambia</td>
<td>Oversaw database design and maintenance for study.</td>
</tr>
<tr>
<td>Bakary Sonko</td>
<td>Database Manager, Fajara, MRC Unit The Gambia</td>
<td>Assistance in database maintenance for study.</td>
</tr>
<tr>
<td>Edrisa Sinjanka</td>
<td>Research Nurse Coordinator, Keneba Field Station, MRC Unit The Gambia</td>
<td>Oversaw nurse recruitment and medical training.</td>
</tr>
<tr>
<td>Name</td>
<td>Position</td>
<td>Responsibilities</td>
</tr>
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<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Musa Jarjou</td>
<td>Data Entry Manager, Keneba Field Station, MRC Unit The Gambia</td>
<td>Coordination of all data entry activities for study.</td>
</tr>
<tr>
<td>Natoma Jarra</td>
<td>Data Entry Clerk, Keneba Field Station, MRC Unit The Gambia</td>
<td>CRF Data entry for study.</td>
</tr>
<tr>
<td>Abubacarr Kandeh</td>
<td>Store Manager, Keneba Field Station, MRC Unit The Gambia</td>
<td>Assistance with local and international laboratory orders to Keneba Field Station.</td>
</tr>
<tr>
<td>Ebrima Jallow</td>
<td>Chief Driver, Keneba Field Station, MRC Unit The Gambia</td>
<td>Oversaw transportation of study samples and laboratory materials to Kiang Keneba.</td>
</tr>
<tr>
<td>Vivat Thomas-Njie</td>
<td>Coordinator of Clinical Trials Support Office, MRC Unit The Gambia</td>
<td>Advice with compliance with Good clinical Practice and ClinicalTrials.gov.</td>
</tr>
<tr>
<td>Haddy Manneh</td>
<td>Research SEN Nurses, Fajara, MRC Unit The Gambia</td>
<td>Provided all clinical care to study participants.</td>
</tr>
<tr>
<td>Hulaimatou</td>
<td>Provided all clinical care to study participants.</td>
<td>Conducted all sample and clinical data collection using study protocols.</td>
</tr>
<tr>
<td>Virginie Jones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morris Ndene</td>
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<tr>
<td>Abdou Camara</td>
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<td>Ebrima Faye</td>
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<td>James Mendy</td>
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<tr>
<td>Sherrifo Darboe</td>
<td></td>
<td></td>
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<tr>
<td>Sherrifo Jarju</td>
<td>Laboratory Technicians, MRC Unit The Gambia</td>
<td>Primary sample processing of all samples during night shifts.</td>
</tr>
<tr>
<td>Bubacarr JB Touray</td>
<td></td>
<td></td>
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<tr>
<td>Modou Jeng</td>
<td>Laboratory Technicians, Kanifing General Hospital</td>
<td>Primary sample processing of all samples during day shifts.</td>
</tr>
<tr>
<td>Tijan Bojang</td>
<td></td>
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<tr>
<td>Pa Ousman Nyang</td>
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<tr>
<td>Mariama Jallow</td>
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<tr>
<td>Omar MS Ceesay</td>
<td></td>
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</tr>
<tr>
<td>Zaith Babou</td>
<td>Study Driver, MRC Unit The Gambia</td>
<td>Driver for all community visits.</td>
</tr>
<tr>
<td>Pa Babou</td>
<td>Laboratory Manager, Kanifing General Hospital</td>
<td>Assistance in the running of the remote laboratory at Kanifing General Hospital.</td>
</tr>
<tr>
<td>Kebba Manneh</td>
<td>Chief Executive Officer of Kanifing General Hospital</td>
<td>Supervised all study activities at Kanifing General Hospital.</td>
</tr>
<tr>
<td>Haja Zainab Jalloh</td>
<td>Government SEN Nurses and Midwives, Kanifing General Hospital</td>
<td>Assistance in the clinical care of all study participants.</td>
</tr>
<tr>
<td>Binta M Jarju</td>
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<td>Diminga Mendy</td>
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<td>Fatou Cham</td>
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<td>Fatou Camara</td>
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<tr>
<td>Musu Ndura</td>
<td></td>
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<tr>
<td>Stella Yawson</td>
<td></td>
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<tr>
<td>Maley Camara</td>
<td></td>
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</tr>
<tr>
<td>Dr Mass Njie</td>
<td>Maternal Ward Clinicians, Kanifing General Hospital</td>
<td>Assistance in the clinical care of all study participants.</td>
</tr>
<tr>
<td>Dr Babading Daffeh</td>
<td></td>
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<tr>
<td>Name</td>
<td>Position</td>
<td>Responsibility</td>
</tr>
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<tr>
<td>Ousainatou Jallow Desmond Ayeh</td>
<td>Procurement Administrators, MRC Unit The Gambia</td>
<td>Assistance in procuring international and local study orders.</td>
</tr>
<tr>
<td>Honorary Jammeh</td>
<td>Study Orderly</td>
<td>Oversaw all non-medical study site cleaning and organisation.</td>
</tr>
<tr>
<td>Edward Demba</td>
<td>Laboratory Services Manager, MRC Unit The Gambia</td>
<td>Assistance in batch shipment of samples from Fajara to Keneba.</td>
</tr>
<tr>
<td>Yaya Giana Lamin Touray</td>
<td>Logistics Administrators, MRC Unit The Gambia</td>
<td>Assistance in importing study material and equipment.</td>
</tr>
<tr>
<td>Mariama Balajo</td>
<td>Human Resources Administrator, MRC Unit The Gambia</td>
<td>Assistance in recruitment of study staff.</td>
</tr>
</tbody>
</table>
## ANNEX 1.12 PhD Timeline

### A PhD Registration and Admin
1. Registration
2. Upgrading Report Writing
3. Upgrading Presentation

### B Ethics
1. Submission to LSHTM Ethics Committee
2. Submission to MRC ING SAM, MRC SCC and MRC/IGG Ethics Committee
3. Community Sensitisation
4. Open Day

### C Study Initiation
1. BMGF Grant Writing
2. Study Design
3. BMGF Funding Approval
4. Remote Laboratory Set Up
5. Clinical SOP Design
6. SSP Design
7. Procurement of Study Equipment
8. Data Analysis Plan
9. Database Design
10. Form Pilot Testing
11. Study Team Recruitment and Training

### D Clinical Section (Kanifing General Hospital - Kombo Area)
1. Recruitment of Subjects
2. Sample Collection
3. Primary Sample Analysis and Processing
4. Data Entry
5. Clinical Data Cleaning
6. Handover of Laboratory, Office and Ward Items (Kanifing General Hospital)

### E Laboratory Section (MRC Keneba - Klang)
1. Batch Sample Movement
2. Secondary Laboratory Analysis - Roche Cobas Integra 400+
3. Secondary Laboratory Analysis - Hepcidin ELISA
4. Secondary Laboratory Analysis - G6PD Deficiency Testing

### F Data Analysis Section
1. Data Analysis
2. Archiving Study Forms

### G Thesis Preparation
1. Methodology/Protocol Paper
2. Literature Review
3. Main Paper
4. Secondary Paper
5. Thesis Writing (Introduction, Discussion and Formatting)
Dear Dr Cerami

SCC 1525v1.1, Iron and Infection: Neonatal Nutritional Immunity (NeoInnate Study)

Thank you for submitting your revised proposal 16 February 2017 addressing the issues raised by the SCC at its meeting held on 6 February 2017.

I have looked at the modified proposal and I am satisfied with the changes you have made according to the SCC queries and recommendations. The proposal can now be forwarded to the Ethics Committee for further consideration at its meeting on 24 February 2017.

With best wishes

Professor Umberto D’Aessandro
Chair, Scientific Coordinating Committee

Documents submitted for review:
- Response letter – 14 February 2017
- SCC application form, version 1.1 – 16 February 2017
- Cover letter – 24 January 2017
- ICD, version 1.1 – 15 February 2017
- CVs: Carla Cerami; James Henry Cross
- Budget
ANNEX 1.14 The Gambia Government/MRC Joint Ethics Committee Letter

The Gambia Government/MRC Joint
ETHICS COMMITTEE

19 April 2017

Dr Carla Cerami
MRC Unit The Gambia, Keneba

Dear Dr Carla

SCC 1525v1.1, Iron and Infection: Neonatal Nutritional Immunity (NeoInnate Study)

Thank you for submitting your response letter dated 21 March 2017 addressing the issues raised by The Gambia Government/MRC Joint Ethics Committee at its meeting held on 24 February 2017.

I have also noted the revisions on the informed consent document as per our recommendations. This project has now received full Ethics Committee approval and may proceed.

With best wishes

Yours sincerely

Mr Malamin Sonko
Chairman, Gambia Government/MRC Joint Ethics Committee

Documents submitted for review:
- SCC letters – 9 February 2017/17 February 2017
- Response letters – 14 February 2017; 21 March 2017
- SCC application form, version 1.1 – 16 February 2017
- Cover letter – 24 January 2017
- ICD, version 1.2 – 21 March 2017
- CVs: Carla Cerami; James Henry Cross
- Budget
ANNEX 1.15 London School of Hygiene & Tropical Medicine Ethics Letter

London School of Hygiene & Tropical Medicine
Keswick Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636
www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Mr James Cross
LSHTM
22 September 2017

Dear James,

Study Title: Iron and Infection: Neonatal Nutritional Immunity (Neolmate)

LSHTM Ethics Ref: 14316

The Observational Committee reviewed the above application.

The documents reviewed were:

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<th>File Name</th>
<th>Date</th>
<th>Version</th>
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<td>SCCxxxx 22 Jan 2017 (Neolmate) - Appendix - Co-Investigator J.H.Cross CV</td>
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<tr>
<td>Investigator CV</td>
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<td>22/01/2017</td>
<td>1</td>
</tr>
<tr>
<td>Protocol / Proposal</td>
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<td>Protocol / Proposal</td>
<td>SCC1525v1.1 15 Feb 2017 (Neolmate) - Appendix - New Ballard Score Chart</td>
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<tr>
<td>Protocol / Proposal</td>
<td>SCC1525v1.1 15 Feb 2017 (Neolmate) - Appendix - Mock Consort Diagram</td>
<td>15/02/2017</td>
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<td>Information Sheet</td>
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<tr>
<td>Investigator CV</td>
<td>CV Andrew Prentice</td>
<td>04/07/2017</td>
<td>1</td>
</tr>
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</table>

Request for Clarification

The Committee would like to request further information or clarification before giving its final opinion of the research. The requested further information or clarification is set out below.

The Committee will delegate authority to confirm its final opinion on the application to the Chair.

Further information or clarification required

1) This applicant says they have upgraded, but gives the upgrade date as next month, 20 Sept. Can he clarify?

2) Abbrevations. Several non-standard abbreviations were used the application, which were not defined, including 'TSAT' (the primary study outcome) and 'DAG' (relating to the analysis). It would have been helpful if these had been spelled out or more clearly defined.

3) Blood draws. Please clarify when, and how frequently, blood samples will be drawn. In addition, please clarify the total amount of blood that will be drawn from pre-term, low birth weight, and full-term neonates over the duration of the study. Please provide a reference for the maximum allowable amount of blood to be drawn safely from neonates, particularly pre-term and low birth weight neonates.

4) Information sheet and consent form. The information sheet is extensive and the committee found that it was not well targeted to mothers or parents. Specifically, the number of blood draws and timing of these is it not entirely clear. Although the information sheet states the amount of blood that will be drawn...
ANNEX 1.16 Oral iron acutely elevates bacterial growth in human serum (FeVir Study)

RESEARCH PAPER COVER SHEET

Please note that a cover sheet must be completed for each research paper included within a thesis.

SECTION A – Student Details

Student ID Number          LSH158152  Title          Mr.
First Name(s)                James Henry
Surname/Family Name          Cross
Thesis Title                 Iron and Infection: Neonatal Iron Transition
Primary Supervisor          Dr Carla Cerami

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published? Nature Scientific Reports
When was the work published? 23rd November 2015

It was published during my registration and has been included as to provide further evidence that high levels of serum iron support the bacterial growth.

Have you retained the copyright for the work?* Yes  Was the work subject to academic peer review? Yes

*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.

Author Contributions
J.H.C., R.S.B., A.P. and C.C. designed the experiments. J.H.C. and A.T.J. performed experiments. J.H.C., R.S.B. and R.W. recruited patients; A.E., A.P. and C.C. analyzed data; A.P. and C.C. wrote the manuscript. All authors approved the final manuscript.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?

Please list the paper’s authors in the intended authorship order:

Stage of publication

Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

James H. Cross designed and performed the experiments. He also recruited patients and approved the final manuscript.

SECTION E

Student Signature

Date 08/11/2019

Supervisor Signature

Date 08/11/2019
Oral iron acutely elevates bacterial growth in human serum

James H. Cross1, Richard S. Bradbury1,2, Anthony J. Fulford1,3, Amadou T. Jallow1, Rita Wegmüller1, Andrew M. Prentice4,3 & Carla Cerami4

Iron deficiency is the most common nutrient deficiency worldwide and routine supplementation is standard policy for pregnant mothers and children in most low-income countries. However, iron lies at the center of host-pathogen competition for nutritional resources and recent trials of iron administration in African and Asian children have resulted in significant excesses of serious adverse events including hospitalizations and deaths. Increased rates of malaria, respiratory infections, severe diarrhea and febrile illnesses of unknown origin have all been reported, but the mechanisms are unclear. We here investigated the ex vivo growth characteristics of exemplar sentinel bacteria in adult sera collected before and 4 h after oral supplementation with 2 mg/kg iron as ferrous sulfate. *Escherichia coli*, *Yersinia enterocolitica* and *Salmonella enterica* serovar Typhimurium (all gram-negative bacteria) and *Staphylococcus epidermidis* (gram-positive) showed markedly elevated growth in serum collected after iron supplementation. Growth rates were very strongly correlated with transferrin saturation (p < 0.0001 in all cases). Growth of *Staphylococcus aureus*, which preferentially scavenges heme iron, was unaffected. These data suggest that even modest oral supplements with highly soluble (non-physiological) iron, as typically used in low-income settings, could promote bacteremia by accelerating early phase bacterial growth prior to the induction of immune defenses.

Iron deficiency (ID) remains the most pervasive nutritional deficiency worldwide. The prevalence of ID in mothers and young children frequently exceeds 50% in low-income countries. Insufficient iron impairs growth and cognitive development in childhood1,2.

Low cost iron supplements are effective for the treatment of ID and in countries with ID rates of >40%, the World Health Organization recommends universal iron supplementation of pregnant women and young children3–5. To overcome perceived limitations in the ability to absorb iron, supplements usually employ highly soluble forms of iron (ferrous sulfate or fumarate) given in rather large non-physiological bolus doses. The wisdom of these policies has long been questioned6,7 and has come under serious scrutiny starting in 2006 with the premature termination of a large trial in Pemba, Tanzania after significant increases in serious adverse outcomes (hospitalizations and deaths) in young children receiving iron-folate supplements were seen8. The emphasis was originally focused on malaria as the causative agent for the increases in morbidity and mortality during iron supplementation9 but subsequent trials have described excesses of other infections in groups randomized to iron or multiple micronutrients containing iron10–15. These findings have paralyzed iron supplementation policies.

The underlying mechanisms and the types of organisms responsible for these clinical and epidemiological observations remain unclear. Recent field studies suggest that oral iron supplementation in children increases susceptibility to bacterial infections, particularly diarrhea13,16, alters the gut microbiota16,17, and increases the virulence of many common bacterial enteropathogens18–20.
Numerous animal studies over many decades have shown that administration of iron in diverse forms accelerates the growth of peritoneally-injected pathogens, causing a septicemia with rapidly fatal outcomes\textsuperscript{21,22}. We here examine the possibility in humans that a simple oral dose of supplemental iron could promote bacterial growth in serum. We used a series of ex vivo bacterial growth assays with sentinel organisms that were selected on the basis of their varying modes of pathogenesis and abilities to scavenge iron from the host.

Results

Oral iron supplementation increases iron parameters in serum. To determine the effects of oral iron supplementation on bacterial growth in human serum, we enrolled 48 normal healthy non-anemic male subjects [mean ± SD: Hemoglobin (Hgb) = 14.5 ± 1.13 g/dL; Mean Corpuscular Volume (MCV) = 83.8 ± 5.5 fL; Ferritin = 62.8 ± 53.2 ng/mL]. Volunteers donated serum immediately before, and then four hours after, oral ingestion of 400 mg ferrous sulfate (containing the equivalent of 130 mg of elemental iron). Transferrin saturation (TSAT) increased from 42.1% (±12.5%, SD) to 75.7% (±18.1%, SD) and total serum iron increased from 30.3 μmol/L (±10.2 μmol/L, SD) to 53.0 μmol/L (±15.8 μmol/L, SD) four hours after iron supplementation.

Effects of oral iron supplementation on ex vivo bacterial growth in serum. We next measured the growth of the five species of sentinel bacteria in the baseline and post-dose sera. To account for the between-subject variance in the starting transferrin saturation levels, we used mixed statistical models to allow two nested higher levels of variation: patient and bleed (pre- and post-iron supplementation). This enabled us to independently analyze the effects of TSAT and iron supplementation.

\textit{S. aureus}, an organism with a strong preference for heme-derived iron\textsuperscript{23}, behaved differently from the other four bacteria (Fig. 1A). Iron supplementation had no impact on the general pattern of the growth curve (p = 0.3). Both the time to reach peak doubling time (p = 0.21), and the doubling time during the exponential growth phase (p = 0.78) were also unchanged by iron supplementation (Table 1). Growth did not correlate with TSAT (p = 0.08) (Table 2).

\textit{S. epidermidis} (Fig. 1B) demonstrated an initial delay in growth in comparison with the other species. Iron supplementation influenced the overall pattern of the growth curve (p < 0.0001). Specifically, iron supplementation reduced the lag phase, the time to reach peak doubling time (p = 0.001), and increased the doubling time during the exponential growth phase (p < 0.001) (Table 1). The very strong effect of iron on the overall increase in bacterial growth (X$^2$ = 55 (approximately), p < 0.0001) was equally explained using pre/post supplementation as a dichotomized variable or by TSAT (Table 2).

\textit{S. Typhimurium} (Fig. 2A) and \textit{E. coli} (Fig. 2B) both showed highly significant differences in their growth curves after iron supplementation (p < 0.0001). The doubling times post-iron supplementation were significantly shorter than pre-iron supplementation (p < 0.0001), but time to reach peak doubling time was unaffected by treatment (Table 1). Transferrin saturation had a very strong effect on overall
Analysis of Growth

<table>
<thead>
<tr>
<th></th>
<th>S. aureus</th>
<th>S. epidermidis</th>
<th>E. coli</th>
<th>S. Typhimurium</th>
<th>Y. enterocolitica</th>
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</thead>
<tbody>
<tr>
<td>Growth Curve</td>
<td>X² (5 df)</td>
<td>6.05</td>
<td>65.70</td>
<td>225.00</td>
<td>232.00</td>
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<td>p-value</td>
<td>0.30</td>
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<td>Time to Reach Peak</td>
<td>Mean pre (C95%)</td>
<td>3.34 (3.21, 3.47)</td>
<td>13.7 (11.8, 15.7)</td>
<td>1.86 (0.50, 3.21)</td>
<td>Not Applicable - No MAX</td>
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<td></td>
<td>Mean post (C95%)</td>
<td>3.25 (3.12, 3.38)</td>
<td>10.70 (10.0, 11.4)</td>
<td>2.56 (2.32, 2.79)</td>
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<td>z</td>
<td>−1.26</td>
<td>−3.24</td>
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<td>p-value</td>
<td>0.21</td>
<td>0.001</td>
<td>0.30</td>
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<td>Doubling Time</td>
<td>Mean pre (C95%)</td>
<td>1.74 (1.67, 1.81)</td>
<td>4.82 (4.2, 5.5)</td>
<td>2.14 (1.99, 2.29)</td>
<td>2.03 (1.81, 2.26)</td>
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<td>Mean post (C95%)</td>
<td>1.75 (1.68, 1.82)</td>
<td>3.36 (3.0, 3.7)</td>
<td>1.50 (1.44, 1.56)</td>
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<td>p-value</td>
<td>0.78</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
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</table>

Table 1. Statistical analysis of ex vivo bacterial growth assays. For each species of bacterium we compared its growth pre- and post-iron supplementation by examining differences in (1) Growth Curve, i.e. general pattern curve of the overall growth trajectories; (2) Time to Reach Peak Doubling Time, i.e. the time at which the rate of increase in log(OD₆₀₀) was at a maximum; and (3) Doubling Time During Exponential Growth Phase.

Hypothesis Testing

<table>
<thead>
<tr>
<th></th>
<th>S. aureus</th>
<th>S. epidermidis</th>
<th>E. coli</th>
<th>S. Typhimurium</th>
<th>Y. enterocolitica</th>
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<tr>
<td>(1) Iron supp. affects growth</td>
<td>X² (5 df)</td>
<td>5.92</td>
<td>55.70</td>
<td>221.00</td>
<td>213.00</td>
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<tr>
<td></td>
<td>p-value</td>
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<td>(2) Iron supp. affects growth independently of TSAT</td>
<td>X² (5 df)</td>
<td>8.85</td>
<td>7.53</td>
<td>35.10</td>
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<td>(4) TSAT affects growth independently of iron supplementation</td>
<td>X² (5 df)</td>
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Table 2. Statistical testing of the independent effects of iron supplementation and transferrin saturation (TSAT). Three models (mathematical equations) were fitted for each bacterial species: (a) iron supplementation × (t₁ t² t₃ t₄), i.e. iron supplementation, the time polynomials and their interactions; (b) TSAT × (t₁ t² t₃ t₄), i.e. TSAT, the time polynomials and their interactions; (c) (iron supplementation + TSAT) × (t₁ t² t₃ t₄), i.e. iron supplementation, TSAT and both their interactions with the time polynomials. We employed the likelihood ratio test to compare models for which the growth patterns were and were not dependent on TSAT or iron supplementation. The effects of iron supplementation and TSAT were derived from models (a) and (b) respectively and refer to the joint effects of the variable and the terms for its interaction with the time polynomials. The independent (conditional) effects of iron supplementation after controlling for TSAT, and TSAT after controlling for iron supplementation, are both derived from model (c) and again refer to the joint effects of the variable and the terms for its interaction with the time polynomials. For each bacterial species, each of the following four hypotheses were tested: (1) iron supplementation has an impact on bacterial growth; (2) iron supplementation has an impact on bacterial growth independently of TSAT; (3) TSAT has an impact on bacterial growth; and (4) TSAT has an impact on bacterial growth independently of iron supplementation. Significance was determined using a chi-squared test. Chi-squared and p-values are reported.

growth rates of both S. Typhimurium (X² = 348, p < 0.0001) and E. coli (X² = 300, p < 0.0001), comparable to, indeed a little larger than, that of iron supplementation in both S. Typhimurium (X² = 213, p < 0.0001) and E. coli (X² = 221, p < 0.0001). For E. coli, iron supplementation (X² = 35, p < 0.0001) and TSAT (X² = 69, p < 0.0001) each had significant effects on bacterial growth after controlling for the other. The same held true for S. Typhimurium growth where both iron supplementation (X² = 22, p = 0.0004) and TSAT (X² = 105, p < 0.0001) each had significant effects on bacterial growth after controlling for the other (Table 2). This is likely to be because, in addition to capturing the effect of supplementation, transferrin saturation also explains differences between individuals.
Recent molecular insights into human iron metabolism have challenged the basic pillars on which public health strategies involving highly soluble iron supplements have been developed. The prior belief that humans are constitutionally inefficient at absorbing iron, and hence require large non-physiological doses taken apart from food, is now overturned by the knowledge that hepcidin actively down-regulates iron acquisition especially in the presence of an infectious threat. The dual regulation of hepcidin by iron and infection (inflammation) underscores the threat posed by exogenous iron. An increase of hepcidin caused by an infection might have the evolutionary function of decreasing further iron uptake to achieve overwhelming numbers by the time adaptive immune defense mechanisms are up-regulated. For *Y. enterocolitica* (Fig. 2C), the maximum growth rate was difficult to locate. In fact, although the growth curves clearly differ significantly (*p* < 0.0001), the doubling times at one hour did not differ significantly. As was the case for *S. Typhimurium* and *E. coli*, TSAT had an impact on growth (*X^2_1 = 120*, *p* < 0.0001) as did iron supplementation (*X^2_2 = 108*, *p* < 0.0001). Additionally, iron supplementation (*X^2_3 = 33*, *p* < 0.0001) and TSAT (*X^2_4 = 37*, *p* < 0.0001) each had significant effects on bacterial growth after controlling for the other (Table 2).

**Discussion**

The biologically useful redox characteristics of the Fe(II) to Fe(III) transition place iron apart from other nutrients. Additionally, it lies at the epicenter of the host-pathogen battle for resource control. Host defense mechanisms to withhold iron from invading pathogens are some of the most evolutionarily conserved innate strategies against infection, but most bacterial species have evolved counter-acting strategies for pirating host iron including: (1) receptors that bind transferrin, lactoferrin or hemoglobin; and (2) low molecular weight siderophores that acquire iron from host proteins or from low molecular weight iron compounds.

The potential health threat posed by exogenous iron, repeatedly demonstrated in animal models, has tended to be overlooked in clinical settings. The recent iron trials with adverse outcomes in children have prompted new mechanistic studies providing experimental verification that oral iron adversely modifies the gut microbiome and increases the virulence of pathogenic enteric bacteria. In this study we focused on the issue of systemic, as opposed to enteric, bacterial infections building upon prior knowledge that iron can precipitate septicemias (for instance, based on the disastrous outcomes of intramuscular iron-dextran administration to Polynesian neonates).

The *ex vivo* assays we describe here show that customary oral supplementation with highly-soluble iron as ferrous sulfate can profoundly affect the growth dynamics of four of the five sentinel species we studied. This could potentially undermine a key component of innate immunity allowing such organisms to achieve overwhelming numbers by the time adaptive immune defense mechanisms are up-regulated. Note that the very strong correlations between TSAT and growth rates emphasizes the importance of this variable even in the presence of likely inter-individual differences in other iron-related (e.g. lipocalin-2, haptoglobin) and other (e.g. defensins) non-cellular defense mechanisms within the sera.

Recent molecular insights into human iron metabolism have challenged the basic pillars on which public health strategies involving highly soluble iron supplements have been developed. The prior belief that humans are constitutionally inefficient at absorbing iron, and hence require large non-physiological doses taken apart from food, is now overturned by the knowledge that hepcidin actively down-regulates iron acquisition especially in the presence of an infectious threat. The dual regulation of hepcidin by iron and infection (inflammation) underscores the threat posed by exogenous iron. An increase of hepcidin caused by an infection might have the evolutionary function of decreasing further iron uptake from the intestine to reduce circulating iron fuel for microorganisms. This suggests that we should not interfere via high dose iron supplements.
Transferrin saturation is homeostatically controlled with a normal range between 15–50% in males. Our data show that increasing TSAT from a mean of 42% to 76% profoundly stimulated bacterial growth with a continuous association across all levels of TSAT. The role of TSAT in mediating host susceptibility to infection has been known for almost half a century. However, neither the strength of this association, nor the ability of acute increases in TSAT following iron doses to so rapidly favor bacterial growth, have been previously appreciated. The dose level selected for the adults in this study was based on that most frequently used for young children with iron deficiency (2 mg/kg/day), however our subjects were iron replete. In iron deficient children, hepcidin would be down-regulated to allow maximal iron absorption and hence TSAT would be expected to rise even further. Although a higher percentage of increase can be expected, the TSAT baseline will be lower and may still end up lower than in iron replete men. In the event of accidental ingress of pathogens through a cut, abrasion or leaky gut, these high levels of TSAT could precipitate a fulminant bacteremia before other cognate immune defenses have time to respond.

Iron absorbed from a natural food matrix, or even when ferrous sulfate is given with food, is released much more slowly and causes minor deviations in TSAT and in non-transferrin bound iron (NTBI) which, according to the data presented here, would be much less likely to promote bacterial growth. In regions of the world where iron-rich foods are scarce, or too costly, supplementation with slow release nano-molecular formulations that mimic a food matrix may provide a safer option and might additionally reduce the adverse effects of unabsorbed iron on the gut microbiota. Field trials of such compounds are warranted. Additional safe approaches to supplement iron include the use of oral bovine lactoferrin which, according to the data presented here, would be much less likely to promote bacterial growth.

Materials and Methods

Subjects. Forty-eight male Gambian subjects (averaging 40y; range 21–64y) were recruited. Subjects had no history of fever, illness or anti-microbial use during the preceding seven days; were malaria rapid test negative and were non-anemic (Hemoglobin >12g/dL). All patients donated blood between 9 and 10am, on an empty stomach, immediately prior to and four hours after taking 400mg ferrous sulfate orally. Food was provided two hours after iron supplementation.

Informed consent was obtained from all subjects. This study was approved by the Gambian Government/MRC Joint Ethics Committee (SCC1312v2) and by the University of North Carolina Institutional Review Board (protocol #143044). All experiments were performed in accordance with the approved guidelines.

Biochemical Parameters. Complete blood counts were obtained using a Medonic M series (Boule Diagnostics Int AB, Stockholm, Sweden) hematology analyzer. Serum biochemical parameters including serum iron, transferrin, ferritin and transferrin saturation were obtained using a Cobas Integra 400 plus (Roche, Basel, Switzerland) biochemistry analyzer.

Bacterial Growth assays. Staphylococcus aureus (strain NCTC8325), Staphylococcus epidermidis (FDA strain PCI1200, ATCC12228), Salmonella enterica serovar Typhimurium (strain LT2, ATCC19585) and Escherichia coli (strain Crooks, ATCC8739) were grown overnight for 18 hours at 37°C in 5mL iron free minimal growth media, Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen). This was conducted in air with continuous shaking (250 rpm). A high-virulence, siderophore producing Yersinia enterocolitica (strain WA-314, ATCC51871) was grown in IMDM containing 10mM ethylene glycol tetraacetic acid (EGTA, Sigma) (pH7). All growth assays were run in triplicate in IMDM containing 50% heat-inactivated human serum. Bacterial growth was monitored by measuring the optical density at 620nm (OD620) hourly for 12 hours (Staphylococcus aureus, Salmonella enterica serovar Typhimurium, and Escherichia coli) and then at 20, 28, 36 hours (Staphylococcus epidermidis and Yersinia enterocolitica) using a Multiscan FC ELISA plate reader (Thermo Scientific).

Statistical analysis. We analyzed the growth assays with a mixed model using a quartic polynomial in Stata12 (StataCorp, College Station, TX). We used the logit of the mean of the three replicate OD readings at each time point as the response variable and modeled growth trajectories by fitting this to orthogonal polynomials (to degree 4) in time using mixed effects models with random intercept and coefficients due to patient and, nested within patient, bleed (pre and post). In effect, therefore, the growth trajectory for the $i$th blood sample was modeled by an equation of the form:

$$\log[OD_i(t)] = (\beta_0 + \tau_{ui}) + (\beta_1 + \tau_{ti})t + (\beta_2 + \tau_{2i})t^2 + (\beta_3 + \tau_{3i})t^3 + (\beta_4 + \tau_{4i})t^4 + \varepsilon_i$$

where $t$ is time since inoculation, the $\beta$s are estimated coefficients and the $\tau$s and $\varepsilon$s are the random effects.

For each species of bacterium, we compared its growth pre- and post-iron supplementation by examining differences in (1) Growth Curve i.e. general pattern of the overall growth trajectories; (2) Time to Reach Peak Doubling Time, i.e. the time at which the rate of increase in $\log[OD]$ was at a maximum and (iii) Doubling Time During Exponential Growth Phase, i.e. when the doubling time was at its greatest, or, if the lag phase was not detectable at 1 hour after inoculation. We estimated the timing of the maximum slope by setting the second derivative with respect to time of the deterministic component of the model to zero. We calculated the doubling time at $t$ as $\log(2)/(\text{slope of } \log(\text{OD}(t)))$. We employed
the likelihood ratio test to compare models for which the growth patterns were and were not dependent on the transferrin saturation or iron supplementation. We used the delta method to obtain the 95% confidence intervals for the time at which maximum slope occurred and the doubling time.

References


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Author Contributions
J.H.C., R.S.B., A.P. and C.C. designed the experiments. J.H.C. and A.T.J. performed experiments. J.H.C., R.S.B. and R.W. recruited patients; A.F., A.P. and C.C. analyzed data; A.P. and C.C. wrote the manuscript. All authors approved the final manuscript.

Additional Information
Competing financial interests: The authors declare no competing financial interests.


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ANNEX 3.8 New Ballard Score Sheet (Gestational Aging)

### The New Ballard Score

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#### Gestation by Dates

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- Hour

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#### Scoring

- Gest. Age by Maturity Rating
- Time of Exam
- Date
- Hour
- Age at Exam
- Hours

### Total Physical Maturity Score

#### Signature of Examiner

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#### References:

http://ballardscore.com/Pages/ScoreSheet.aspx