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# Cord Blood Haptoglobin, Cerebral Palsy and Death in Infants of Women at Risk for Preterm Birth: A Secondary Analysis of a Randomised Controlled Trial☆

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ABSTRACT

*Background:* Antenatal exposure to intra-uterine inflammation results in precocious Haptoglobin (Hp) expression (switch-on status). We investigated the relationships between foetal Hp expression at birth with newborn and childhood outcomes.

*Methods:* We evaluated cord blood samples from 921 newborns of women at imminent risk for preterm delivery randomised to either placebo (n = 471, birth gestational age (GA) median [min-max]: 31 [24–41] weeks) or magnesium sulphate (n = 450, GA 31 [24–42] weeks]). Primary outcome was infant death by 1 year and/or cerebral palsy (CP) ≥2 years of corrected age. Adjusted odd ratios (aOR) for neonatal and childhood outcomes were calculated controlling for GA, birth weight, sex, and magnesium exposure.

*Findings:* Primary outcome occurred in 2.8% of offspring. Newborns were classified in three pre-defined categorisation groups by cord blood Hp switch status and IL-6 levels: inflammation-nonexposed (Category 1, n = 432, 47%), inflammation-exposed haptoglobinemic (Category 2, n = 449, 49%), and inflammation-exposed anhaptoglobinemic or hypohaptoglobinemic (Category 3, n = 40, 4%). Newborns, found anhaptoglobinemic or hypohaptoglobinemic (Category 3) had increased OR for intraventricular haemorrhage (IVH) and/or death (aOR: 7.0; 95% CI: 1.4–34.6, p = 0.02) and for CP and/or death (aOR: 6.27; 95% CI: 1.7–23.5, p = 0.006) compared with Category 2. Foetal ability to respond to inflammation by haptoglobinemia resulted in aOR similar to

☆ **Source of the work or study:** A Randomised, Controlled Trial of Magnesium Sulphate for the Prevention of Cerebral Palsy (the BEAM trial).

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inflammation-nonexposed newborns. Hp1-2 or Hp2-2 phenotypes protected against retinopathy of prematurity (aOR = 0.66; 95% CI 0.48–0.91, p = 0.01).

*Interpretation:* Foetal ability to switch-on Hp expression in response to inflammation was associated with reduction of IVH and/or death, and CP and/or death. Foetuses unable to mount such a response had an increased risk of adverse outcomes.

**Trial Registration**: clinicaltrials.gov Identifier: NCT00014989



**Research in Context**

*Evidence Before This Study*

It is well recognised that both prematurity and foetal exposure to intra-amniotic infection/inflammation represent key risk factors for short- and long-term adverse neonatal outcomes, including development of cerebral palsy. However, particularities of the foetal response to infection dictate that not all foetuses delivered preterm in the setting of inflammation will suffer adverse neonatal outcomes. Using proteomics, we previously discovered that antenatal exposure to intra-uterine inflammation results in precocious haptoglobin (Hp) expression (switch-on status) with circulating levels varying with foetal Hp phenotype. This finding occurred unexpectedly because prior to our study, Hp was considered near absent at birth, switching to adult levels within the first year of life. Subsequently, we found that Hp expression predicted better than the clinical diagnosis of presumed early-onset neonatal sepsis, neonatal morbidities including intra-ventricular haemorrhage and/or death. Lastly, we identified that in the setting of intra-amniotic infection, early delivery of premature neonates benefits a select subgroup of foetuses that have not yet progressed to Hp switch-on status. Completion of the Maternal Fetal Medicine Units Network (MFMU) Beneficial Effects of Antenatal Magnesium Sulfate (BEAM) trial, availability of archived cord blood samples, and its rigorous neonatal follow-up program was a unique opportunity to investigate the relationship between foetal Hp switch-on status at birth, and newborn and childhood outcomes in a large cohort of foetuses exposed to magnesium sulphate for neuroprotection.

*Added Value of This Study*

This study adds value to the existing evidence by reporting for the first time that anhaptoglobinemic or hypohaptoglobinemic babies have higher odds of intraventricular haemorrhage and/or death, and cerebral palsy at 24 months of age (corrected for GA), independent of neonatal gestational age at birth, birthweight, sex, or magnesium exposure.

*Implications of All the Available Evidence*

In neonates delivered in the setting of foetal inflammation, the ability of the foetus to promptly turn on expression of Hp may protect against cerebral palsy and/or death and intraventricular haemorrhage. Our study identified a group of foetuses with low Hp levels unable to mount such a response who have increased risks of adverse neurologic outcomes. Determination of functional hypo- or anhaptoglobinemia in the context of antenatal exposure to inflammation has the potential to identify a subgroup of particularly vulnerable infants where timely implementation of neuroprotective strategies may effectively minimise adverse outcomes. In the era of precision medicine, future clinical trials examining effectiveness of neuroprotective interventions in premature newborns should take into account the circumstances of preterm birth and endogenous Hp levels.

## 1. Introduction

Prematurity represents a key risk factor for short- and long-term adverse neonatal outcomes [1,2]. Out of numerous independent risk factors, preterm birth in the context of intra-uterine infection and early-onset neonatal sepsis represents a unique clinical entity due to the marked added risk for intraventricular haemorrhage (IVH), long-term neuro-developmental delay and cerebral palsy (CP) [1,3]. For many years, it was assumed that early-onset neonatal sepsis involved vertical transmission of live bacteria when preceded by intra-amniotic infection (IAI) or genital tract colonisation. However, particularities of the foetal response to infection indicate that it is prolonged exposure of a vulnerable foetus to inflammation that may lead to adverse outcomes even in the absence of a positive neonatal bacterial blood culture [4,5].

Proteomics has facilitated discovery of biomarkers to better understand the pathophysiology of early-onset neonatal sepsis with the goal of identifying as early as possible the newborns more likely to die or develop significant morbidity [6,7]. In prior studies, we demonstrated that antenatal exposure to IAI results in precocious "switch-on" of haptoglobin (Hp) expression in the cord blood of premature neonates [7]. Hp is an abundant plasma protein synthesised mostly by the liver and is a well-known acute phase reactant. Hp is a potent antioxidant with the ability to counter lipid peroxidation, twenty-fold more effectively than vitamin E [8]. A more indirect antioxidant effect of Hp is exerted through high affinity binding of free haemoglobin thereby inhibiting its oxidative activity [9].

Our previous discovery of elevated cord blood Hp in premature newborns with early-onset neonatal sepsis was unexpected because Hp was considered near absent at birth, gradually increasing to adult levels within the first year of life [10,11]. In our previously published study, we further asked whether the Hp switch-on pattern at birth could be used as a biomarker of early-onset neonatal sepsis to improve diagnosis and outcome prediction. We circumvented the absence of a "gold standard" diagnostic test for early-onset neonatal sepsis by using latent class analysis, a statistical method that has gained acceptance when a gold standard diagnostic test does not exist or is impractical [12]. Among the variables in latent class analysis model, Hp switch pattern had the highest discriminative power for early-onset neonatal sepsis while gestational age (GA) at birth, newborn sex, preterm prelabour rupture of membranes and Apgar scores were not significant and were excluded [7]. Among the 180 preterm newborns of this discovery cohort, Hp switch pattern alone drove category (originally called cluster) assignment for 97% of newborns. The remaining newborns (3%) whom all had switch-off Hp were further categorised based on cord blood interleukin (IL)-6. Using this combination of cord blood biomarkers, our algorithm was significantly better than the clinical diagnosis of presumed early-onset neonatal sepsis at predicting neonatal morbidities including IVH and/or death. Based on the above knowledge, we proposed that cord blood Hp switch pattern and phenotype may have the potential to improve the selection of newborns for prompt and targeted treatment at birth [7].

The *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Maternal-Fetal Medicine Units (MFMU) Network conducted a multicentre randomised controlled trial of magnesium sulphate [Beneficial Effects of Antepartum Magnesium (BEAM trial)] for the prevention of CP, which demonstrated that foetal exposure to

magnesium sulphate, prior to preterm birth did not reduce the risk for the primary outcome of moderate to severe CP and/or death [13]. Because the analysis revealed a reduced risk of CP among survivors, this led to the clinical recommendation of magnesium sulphate for imminent preterm birth. Two recent secondary analyses of the BEAM trial showed that among the offspring of women in the magnesium sulphate arm, neither cord blood magnesium nor cytokine levels were associated with CP and/or death [14,15]. This implies that the search for effective therapies to improve long-term neonatal outcomes of premature newborns needs to continue.

The MFMU BEAM trial and availability of archived cord blood samples offered a unique opportunity to investigate the relationships between foetal Hp expression patterns at birth and neurodevelopmental outcomes in a larger and different cohort than the one used for biomarker discovery and for development of our newborn categorisation algorithm. Furthermore, these relationships could be separately investigated for infants who were either exposed or not exposed to magnesium sulphate prior to birth.

## 2. Methods

### 2.1. Study Population

We performed a secondary analysis of all the available cord blood samples of babies delivered by mothers enrolled in the multicentre randomised clinical trial of magnesium sulphate for prevention of CP conducted by the *Eunice Kennedy Shriver* NICHD MFMU Network (ClinicalTrials.gov Identifier:NCT00014989) [13]. Inclusion criteria for the original clinical trial, were singletons or twins, GA 24–31 weeks, high risk for spontaneous preterm birth due to preterm premature rupture of membranes or advanced cervical dilatation of 4 to 8 cm in the setting of intact membranes, and delivery anticipated within 2–24 h. Exclusion criteria included: birth anticipated within <2 h, cervical dilatation >8 cm, preterm prelabour rupture of membranes at <22 weeks, foetal congenital anomalies or death, hypertension or preeclampsia, contraindications to magnesium sulphate and receipt of intravenous magnesium sulphate within the previous 12 h. Surviving infants underwent neurologic evaluation by an annually certified paediatrician or paediatric neurologist at 6, 12, and 24 months of age (corrected for GA). Infants with a normal neurologic examination at 1 year and who could walk 10 steps independently and had a bilateral pincer grasp were considered normal and did not undergo further physical examinations, although the scheduled neurodevelopmental examination was performed. Written informed consent was obtained from all participants.

In the original trial (December 1997–June 2007), group assignment was made according to a computer-generated random sequence, with stratification according to clinical centre and, in twin pregnancies, weeks of gestation (<28 or ≥28) [13]. Certified research nurses collected information on the mother's demographic features, medical history, and social history at enrolment in the study at the 20 participating sites across the United States, that were involved in the original trial. The nurses also obtained data on neonatal and maternal outcomes at delivery and at scheduled follow-up visits when the infant reached 6, 12, and 24 months of age (corrected for prematurity).

Diagnosis of CP was made by a certified paediatrician or paediatric neurologist using well-established criteria [13,16]. Children diagnosed with CP were further classified by the Gross Motor Function Classification System as having mild (level 1), moderate (levels 2 or 3), or severe CP (levels 4 or 5) [17]. Neurodevelopmental stages were assessed by a trained psychologist or psychometrist using the Bayley Scales of Infant Development II (BSID-II) test [15,18]. Components included a Mental Development Index (MDI) and a psychomotor development index (PDI). A score of<70 indicates significant impairment (>2 standard deviations below the mean). Diagnoses of IVH, retinopathy of prematurity (ROP), necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), patent ductus arteriosus, and proven neonatal sepsis were established based on well-recognised clinical criteria [13].

This analysis was restricted to singletons and twins with delivery outcome data that were live born and had umbilical cord serum available in the biorepository, and was considered exempt by the Institutional Review Boards of The Ohio State University and Nationwide Children's Hospital. There was no overlap between the BEAM trial and Hp discovery cohorts [7,13].

### 2.2. Primary and Secondary Outcomes of This Analysis

The primary outcome was an occurrence of the composite of infant death by 1 year of life and/or CP (all levels) at or beyond 2 years of corrected age. This primary outcome was identical with that of the main BEAM trial from which the samples originated. Secondary outcomes were diagnoses of IVH (grades 3–4), ROP, NEC, BPD, patent ductus arteriosus, proven neonatal sepsis, seizures, MDI < 70, and PDI < 70, again identical with the secondary outcomes of the BEAM trial.

### 2.3. Sample Size

Outcomes were assessed in 1041 patients in the magnesium sulphate randomisation arm and 1095 patients in the placebo arm of the original study [13]. The sample size for this study was based on available cord samples.

### 2.4. Umbilical Cord Blood Retrieval and Storage

Cord blood was collected immediately after delivery. Serum was separated within 45–120 min and frozen within 12 h of blood collection and stored at − 70 °C. A minimum of 50 μL serum were necessary to perform all analyses.

### 2.5. Immunoassay Methods for Hp, IL-6 and Western Blotting for Determination of Cord Blood Hp Switch-on Pattern and Hp Phenotype

Cord blood Hp and IL-6 concentrations were measured in all samples as previously reported [7]. In spike and recovery experiments, we confirmed that the presence of free haemoglobin in serum does not affect the measured Hp concentration.

The tetrameric Hp protein comprises two α- and two β-chains linked by disulphide bonds. In humans, Hp occurs in two co-dominant allelic forms, *Hp1* and *Hp2* that differ in the length of the α-chain [19]. The adult human population has three major Hp phenotypes (Hp1-1, Hp2-2 and the heterozygous Hp1-2), derived from variations in the α-chain with identical β-chains [20]. Anhaptoglobinemia and hypohaptoglobinemia (Hp0-0) are also clinically encountered in adults and could be either acquired or in rare instances the result of Hp allelic deletions [21]. Technical details for the immunoassays and Western blots uniquely performed part of this study are provided in the Appendix (p 2).

In our prior study, a cut-off in cord blood Hp immunoreactivity of 3370 ng/mL in our ELISA assay had ~100% sensitivity and specificity to segregate samples with switch-off from those with switch-on pattern on Western blots [7]. Because the limit of detection for clinical assays to detect anhaptoglobinemia in adults is 2 mg/dL [22], Western blots were performed on all cord blood samples measuring Hp ≥2000 ng/mL by ELISA (n = 507).

### 2.6. Cord Blood Magnesium and Other Biochemical Measures

Analysis of the magnesium concentration in cord serum was performed in a prior study with a minimum detectable magnesium concentration of 0.4 mEq/L [23]. Total protein concentration in cord serum was quantified using bicinchoninic acid assay (Sigma). Levels of free

haemoglobin were measured using leucomalachite green reagent as previously described [24].

### 2.7. Classification of Newborns

Newborns were classified into pre-defined categories based on our prior latent-class analysis algorithm [7] with the modification that exposed newborns based on switch-off Hp and high IL-6 were separated from those with Hp switch-on pattern. The 3 categories that emerged were as follows: **Category 1** (inflammation-nonexposed anhaptoglobinemic: Hp switch-off & IL-6 <100 pg/mL, n = 432); **Category 2** (inflammation-exposed haptoglobinemic: Hp switch-on independent of IL-6 level, n = 449); **Category 3** (inflammation-exposed anhaptoglobinemic or hypohaptoglobinemic: Hp switch-off & IL-6 ≥100 pg/mL, n = 40). Laboratory analyses and categorisation assignments were done by investigators blinded to clinical data and newborn outcomes. The analysis was conducted by the originally assigned groups. A representative Western blot with corresponding immunoreactivity values and assigned newborn categories is also shown in the Appendix (p 5).

### 2.8. Statistical Analyses

Quintile-to-quintile plots were used to assess variables for the Normal distribution. Comparisons using mother's data were tested using the Wilcoxon rank sums test for continuous variables and Chi-square or Fisher's Exact Test for categorical variables. We adjusted models using infant/child data for the correlation between twins using a Generalised Estimating Equation (GEE). Model fit for logistic models was assessed using the Hosmer and Lemeshow goodness-of-fit test and residual analysis was used to evaluate the fit of general linear models. Co-linearity among the variables was tested using the condition index. The log of HP and IL-6 were used in analysis and results from these models are back transformed. Adjusted odds ratios (aOR) and 95% confidence intervals are reported for major neonatal outcomes adjusted for GA, birth weight, and treatment group. A p value less than 0.05 was considered statistically significant. No imputation for missing data was performed. The SAS version 9.3. (SAS Institute, North Carolina) statistical software was used for analysis.

## 3. Results

### 3.1. Main Study

Of the 2241 women in the original trial (placebo: n = 1145; magnesium: n = 1096), 2215 had outcomes observed at delivery and delivered live born singletons or twins (placebo: n = 1133; magnesium: n = 1082) representing 2418 infants (placebo: n = 1244; magnesium: n = 1174) (Fig. 1) [13]. Umbilical cord serum was available for 921 of these neonates (placebo: n = 471, GA median [min-max]: 31 [24–41] weeks; magnesium sulphate: n = 450, GA 31 [24–42] weeks). In 920 neonates, data were available for the following secondary outcomes: ROP, NEC, BPD, patent ductus arteriosus, proven neonatal sepsis, and seizures; 30 were missing IVH. Fifty-five neonates were lost to follow-up after delivery leaving 866 children (placebo group: n = 444; magnesium group: n = 422) followed long-term to classify the primary outcome. In one baby born alive, the mother allowed the use of cord blood but withdrew consent for the long-term follow-up. MDI and PDI were classified in 766 and 770 children, respectively.

In Table 1, we present the demographic characteristics of the mothers and newborns separated by the treatment of the primary trial. The difference in numbers between mothers and newborns is accounted by 27 twin pregnancies with cord blood available for analysis [n = 15 (placebo group); n = 12 (magnesium group)]. There were no differences between the two treatment groups except for foetal sex, with a higher proportion of male newborns in the magnesium sulphate group (Chi square p = 0.038).

The frequency of the outcomes for the newborns with cord blood analysed as part of this study is presented in Table 2. Of the 921 samples available for this analysis, 24 samples were retrieved from newborns that developed CP or died (primary outcome of the analysis). Of the 13 deaths, 8 were with anomalies (2, placebo group; 6, magnesium group).

### 3.2. Cord Blood Hp, IL-6 and Magnesium Levels Based on Treatment Groups and Hp Switch Status

When data were analysed based on treatment group, there was no difference in cord blood Hp (placebo: mean [95% CI] 3297 [2551–4262] vs. magnesium: 3454 [2656–4494] ng/mL, Wilcoxon p = 0.803) and log IL-6 (placebo: 35.0 [27.6–44.4] vs. magnesium: 26.6 [21.3–33.2] pg/mL, Wilcoxon p = 0.083, back-transformed) levels. After our analysis for interactions, we determined that newborn's Hp phenotype or GA did not impact on these relationships. Cord blood magnesium levels were higher in the treatment group 2.55 [2.45–2.64] compared with placebo 1.56 [1.52–1.60] mg/dL (Wilcoxon p < 0.001).

Levels of individual cord blood analytes separated by Hp switch status are presented in the Appendix (p 2). We showed that newborns with Hp switch-on status had higher cord blood Hp and IL-6 concentrations (Wilcoxon p < 0.001). There was no difference in free haemoglobin and magnesium levels between newborns with switch-off and switch-on Hp status. A representative Western blot with samples from 11 newborns with their corresponding Hp and IL-6 immunoreactivity is shown in the Appendix (p 5). Among samples analysed in this study, a Hp cut-off of 3531 ng/mL distinguished between switch-off and switch-on samples with 96.9 [94.9–98.3] sensitivity and 97.4 [95.6–98.7] specificity, which is consistent with our prior discovery study [7].

### 3.3. Classification of Newborns

The newborn category groups were derived from the cord blood Hp switch status and IL-6 levels as exemplified in the Appendix (p 6). Of note, we found 4 hypohaptoglobinemic newborns (absent Hp-α band and weak Hp-β band on Western blot). Their IL-6 concentration was high (829 [419–2190] pg/mL) with Hp immunoassay levels (3771 [3601–5624] ng/mL) only slightly above the previously reported cut-off of 3370 ng/mL [7]. These newborns were thus classified in **Category 3** and ultimately as inflammation-exposed hypohaptoglobinemic. Newborn category allocation, Hp switch status, and Hp phenotype were equally distributed within the placebo and magnesium groups as presented in the Appendix (p 3). However, there was an association between phenotype and newborn category in both treatment groups (Appendix, p 4). Most of the inflammation-exposed newborns with Hp switch-on status (**Category 2**), displayed the Hp 2–2 phenotype and this was true for both placebo and magnesium groups.

### 3.4. Relationship Between Newborn Category, Hp Phenotype and Neonatal Outcomes

In Table 3, we present the relationships between newborn category group assignment and the primary and secondary outcomes of this analysis. Analysis of the primary outcome revealed that after adjustment for magnesium treatment, birth weight and GA at birth, newborns who were inflammation-exposed but anhaptoglobinemic or hypohaptoglobinemic (**Category 3**) had increased odds of CP or death when compared with inflammation-exposed haptoglobinemic babies (**Category 3** vs. **Category 2**, Hosmer and Lemeshow goodness-of-fit adjusted p = 0.006). A similar pattern was observed for the secondary outcome IVH and/or death (p = 0.016). **Category 3** newborns had a heightened risk for IVH and/or death when compared with non-exposed newborns (**Category 3** vs. **Category 1**, p = 0.016). The ability

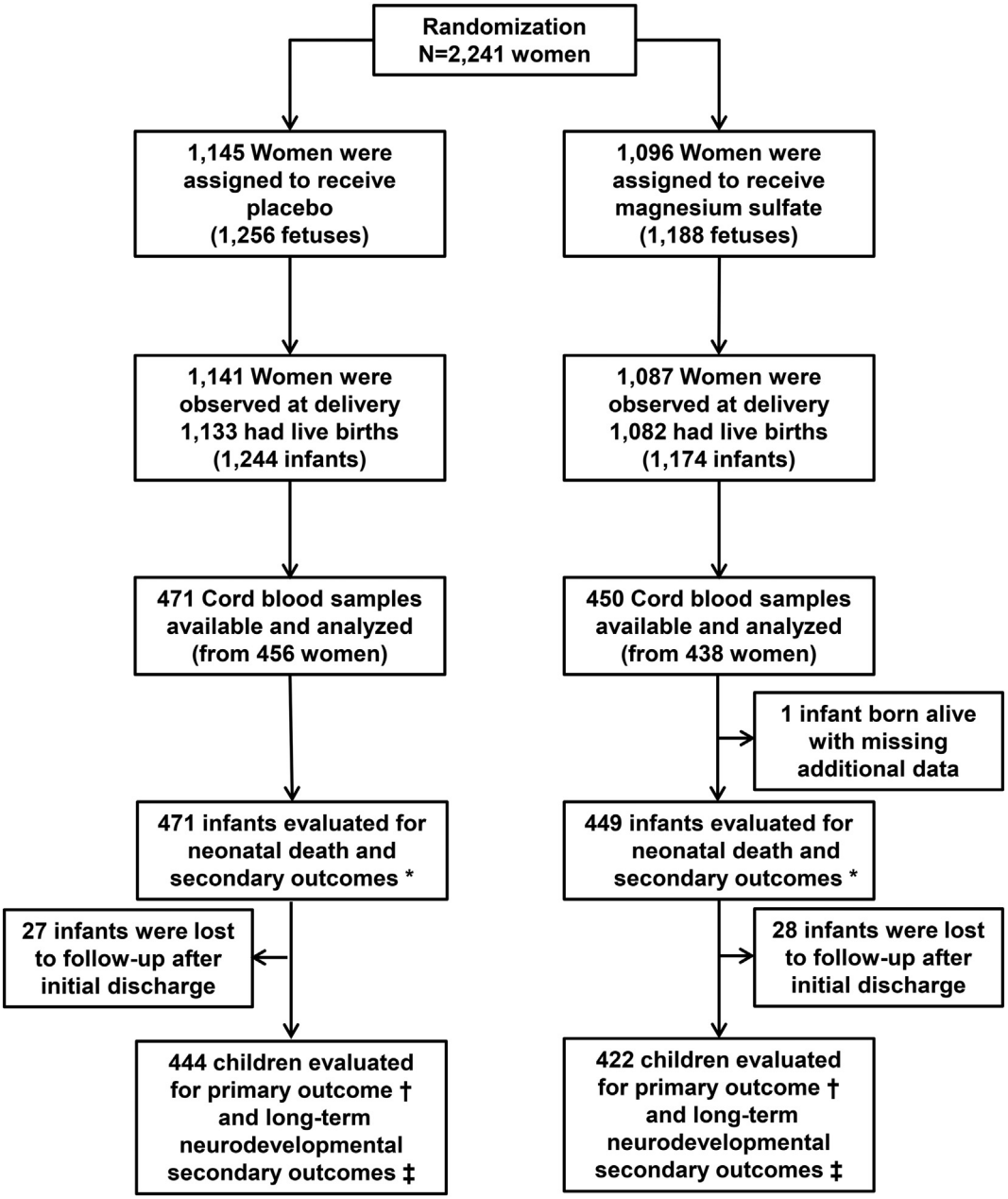


**Fig. 1.** CONSORT flow diagram with enrolment, randomisation and distribution of the mothers and infants whose cord blood samples were analysed in this study (n = 921). *Secondary outcomes: intraventricular haemorrhage (IVH) grades 3 or 4, retinopathy of prematurity, necrotizing enterocolitis, patent ductus arteriosum, proven neonatal sepsis, seizures and bronchopulmonary dysplasia; †Primary outcome: infant death by 1 year of life and/or CP (all levels) at or beyond 2 years of corrected age; ‡Secondary outcomes: seizures, mental developmental delay index <70, psychomotor delay index <70.

to express Hp in the context of inflammation protected the baby with odds of poor neonatal outcomes similar to that of non-exposed newborns (**Category 2** vs. **Category 1**, $p > 0.05$ for all).

Finally, when expressed, and after adjustment for birth weight, GA at delivery and cord blood magnesium level, Hp1-2 or 2-2 protected against ROP (aOR = 0.66; 95% CI 0.48–0.91, Hosmer and Lemeshow goodness-of-fit adjusted $p = 0.01$). No other outcome was associated with Hp phenotype.

## 4. Discussion

The current study underscores the potential protective role of Hp against major short- and long-term poor neonatal outcomes and may prove to be a valuable marker of neurologic damage in neonates exposed to inflammation in utero. Using the umbilical cord blood samples stored from the original NICHD MFMU BEAM trial, we confirmed an added value of IL-6 to identify exposed newborns with compromised Hp expression that are at a higher risk for adverse outcomes. We showed previously that antenatal cord blood Hp switch-on occurs independent of IL-6 level and detects most newborns exposed antenatally to IAI [3,7,25]. In our first biomarker discovery study [7], we noted a small number of newborns [that paradoxically maintained the switch-off Hp pattern (Hp0-0) despite high cord blood IL-6 (≥100 pg/mL)]. In anticipation that this observation will recapitulate in the analysis of the BEAM study cohort, for the current study, we a priori singled out these functionally hypo- or anhaptoglobinemic (Hp0-0) preterm infants exposed to IAI as **Category 3** newborns. The larger sample size afforded by the current study allowed us to hypothesise that these newborns represent a unique group with increased susceptibility to adverse outcomes perhaps related to the lack of Hp expression [7]. The current

**Table 1**
Baseline characteristics by treatment group.

| Characteristic | Placebo group | Magnesium group |
|---|---|---|
| *Mothers* | | |
| N | 456 | 438 |
| Maternal age, *years*[a] | 24 [15–44] | 25 [14–44] |
| Race/Ethnicity[b] | | |
| African American | 187 (41) | 177 (40) |
| Hispanic | 77 (17) | 75 (17) |
| Other | 192 (42) | 186 (43) |
| High school education[b] | 283 (62) | 290 (67) |
| Smoking[b] | 135 (30) | 115 (26) |
| Alcohol use[b] | 39 (9) | 43 (10) |
| Illicit drug use[b] | 44 (10) | 41 (9) |
| Antenatal steroids[b] | 444 (97) | 424 (97) |
| Steroids to delivery, *days*[a] | 6 [0–87] | 6 [0–115] |
| Antibiotics[b] | 433 (95) | 418 (95) |
| Clinical chorioamnionitis[b] | 46 (10) | 45 (10) |
| PPROM[b] | 405 (89) | 384 (88) |
| Rupture to delivery, *days*[a] | 7 (0–138) | 6 (0–116) |
| Indicated PTB[b] | 40 (9) | 46 (11) |
| Caesarean delivery[b] | 147 (32) | 148 (34) |
| Postpartum endometritis[b] | 22 (5) | 18 (4) |
| *Newborns*[c] | | |
| N | 471 | 450 |
| Male sex | 231 (49) | 252 (56)[†] |
| Gestational age at birth, *weeks* | 31 [24–41] | 31 [24–42] |
| Birth weight, *grams* | 1575 [580–4035] | 1589 [568–3752] |
| Twins (at least one analysed) | 55 (12) | 43 (10) |
| Twins (both analysed)* | 30 (6) | 24 (5) |
| Major congenital anomaly | 14 (3) | 21 (5) |
| 5-Min Apgar score < 7 | 47 (10) | 56 (12) |
| Need for resuscitation at birth | 383 (81) | 358 (80) |

*Abbreviations:* PPROM: preterm prelabour rupture of membranes; PTB: preterm birth.
[a] Data presented as median [min-max] and analysed using Wilcoxon rank sums test.
[b] Data presented as n (%) and analysed using Chi-square, unless noted with * (Fisher's Exact Test used).
[c] Adjusted for correlation between twins using Generalised Estimating Equations (GEE) with the Normal distribution for continuous variables and binomial for categorical variables. Data as median [min – max] for continuous variables and n (%) for categorical variables.
[†] $p < 0.05$ (Chi square test adjusted for correlation for twins).

dataset identified 40 of 921 newborns (4.3%) as **Category 3** members with an increased risk for the primary (CP and/or death) and secondary (IVH and/or death) composite outcomes, thus confirming our hypothesis.

Regardless of its aetiology, prompt determination of functional hypo- or anhaptoglobinemia in the context of antenatal exposure to inflammation has the potential to identify a subgroup of particularly vulnerable infants where timely implementation of available strategies (e.g. midline head positioning, deferoxamine iron chelation) may minimise adverse outcomes [26]. In the era of precision medicine, future clinical trials examining effectiveness of neuroprotective interventions in premature newborns should take into account the cause of preterm birth and endogenous Hp levels at birth that could be genetically determined. While follow-up genetic and expression studies have the potential to discriminate between genetic deficiency in Hp alleles and a dysregulation in Hp expression in response to developmental maturation or stimuli, concern remains as to the appropriate management of foetal and neonatal infection. Causes include defective Hp alleles as evidenced by anhaptoglobinemia frequencies ranging from 0.1% to 4% among different populations, including a 4% rate among blacks in the US [27]. Based on these numbers, it is probable that some neonates in the BEAM cohort had a genetic basis for the Hp0-0 phenotype despite being exposed to IAI. This is plausible given the high incidence in the original trial of preterm prelabour rupture of membranes (>85% both in magnesium and placebo groups) [13] which is frequently associated with IAI. Discrimination between anhaptoglobinemia and hypohaptoglobinemia has potential implications for management of subsequent pro-inflammatory responses to infection and inflammation, particularly among those exhibiting an adverse antenatal response. Conversely, prompt initiation of Hp expression in response to IAI (**Category 2**) may protect against foetal tissue damage through heme scavenging, antioxidant and anti-inflammatory properties to the extent that the rate of post-natal complications is not different from that of non-exposed neonates (**Category 1**). Mechanistic evidence in direct support of a role of Hp in preventing human adverse neonatal outcomes (i.e. IVH) comes from animal experiments. In a preterm rabbit pup IVH model, it was demonstrated that cell-free haemoglobin is causally involved in cerebellar damage, an effect reversed by intraventricular injection of Hp [28].

We observed an association between Hp variants and neonatal outcome. For every 1 unit increase in expressed Hp2, after adjusting for birth weight, GA at delivery and magnesium level, the odds of ROP decreased 0.66 (95% CI 0.48–0.91). Hp gene (chromosome 16) carries a common 1.7 kb copy number variant resulting in 2 variant alleles in humans, Hp1 and Hp2 [29]. This arrangement generates 3 protein isoforms, Hp1-1, Hp1-2, and Hp2-2, which differ remarkably in their efficiency to counteract oxidative stress. Our data is provocative because Hp2 provides inferior antioxidant protection compared with Hp1. Further investigation is warranted to provide a pathophysiologic explanation to this finding.

**Table 2**
Frequency of short- and long-term outcomes for infants with cord blood analysed part of this study separated by treatment group.

| Outcomes | Magnesium group | | Placebo group | | Relative risk (95% CI) | p value |
|---|---|---|---|---|---|---|
| | No. infants completing evaluation | Affected infants n (%) | No. infants completing evaluation | Affected infants n (%) | | |
| Primary composite outcome and components[a] | | | | | | |
| Cerebral palsy and/or death | 422 | 14 (3.3) | 444 | 10 (2.3) | 1.47 (0.66–3.28) | 0.34* |
| Cerebral palsy | 422 | 7 (1.7) | 444 | 6 (1.4) | 1.23 (0.42–3.62) | 0.71* |
| Secondary outcomes[a] | | | | | | |
| Retinopathy of prematurity | 449 | 60 (13.4) | 471 | 66 (14.0) | 0.95 (0.69–1.32) | 0.77* |
| Necrotizing enterocolitis | 449 | 26 (5.8) | 471 | 22 (4.7) | 1.24 (0.71–2.15) | 0.45* |
| Patent ductus arteriosus | 449 | 42 (9.4) | 471 | 44 (9.3) | 1.00 (0.67–1.50) | 0.99* |
| Bronchopulmonary dysplasia | 449 | 55 (12.3) | 471 | 54 (11.5) | 1.07 (0.75–1.52) | 0.71* |
| Intraventricular haemorrhage (≥grade 3) | 433 | 2 (0.5) | 457 | 2 (0.4) | 1.06 (0.15–7.5) | 1.00# |
| Mental developmental delay (<70) | 376 | 43 (11.4) | 390 | 31 (8.0) | 1.44 (0.93–2.23) | 0.10* |
| Psychomotor delay (<70) | 378 | 31 (8.2) | 392 | 33 (8.4) | 0.97 (0.61–1.56) | 0.91* |
| Proven sepsis | 449 | 39 (8.7) | 471 | 46 (9.8) | 0.89 (0.59–1.34) | 0.57* |
| Seizures | 449 | 2 (0.5) | 471 | 4 (0.9) | 0.52 (0.10–2.85) | 0.69# |
| Foetal congenital anomalies | | | | | | |
| Deaths with anomalies | 423 | 6 (1) | 444 | 2 (0.4) | 2.63 (0.59–11.64) | 0.18* |

*Chi square test; # Fisher's exact test. p values were adjusted for correlation between twins using Generalised Estimating Equations (GEE) and the binomial distribution.
[a] Primary and secondary outcomes were available only for the infants that completed the evaluations.

**Table 3**
Adjusted odds ratios for primary and secondary outcomes relative to newborn category allocation.

| Outcomes | Category 3 vs. Category 2 | | Category 3 vs. Category 1 | | Category 2 vs. Category 1 | |
|---|---|---|---|---|---|---|
| | aOR (95% CI) | p value[a] | aOR (95% CI) | p value[a] | aOR (95% CI) | p value[a] |
| Primary outcome | | | | | | |
| Cerebral palsy or death (n = 24) | 6.27 (1.67–23.48) | 0.006[b] | 3.73 (1.00–13.85) | 0.050 | 0.59 (0.24–1.47) | 0.259 |
| Secondary outcomes | | | | | | |
| Intraventricular haemorrhage (≥grade 3) and/or death (n = 17) | 7.04 (1.43–34.58) | 0.016[b] | 7.43 (1.46–37.74) | 0.016[b] | 1.06 (0.29–3.89) | 0.936 |
| Retinopathy of prematurity (n = 126) | 0.90 (0.27–3.00) | 0.866 | 0.54 (0.16–1.84) | 0.326 | 0.60 (0.35–1.05) | 0.071 |
| Necrotizing enterocolitis (n = 48) | 2.39 (0.76–7.48) | 0.136 | 1.90 (0.63–5.72) | 0.255 | 0.80 (0.42–1.50) | 0.482 |
| Bronchopulmonary dysplasia (n = 109) | 0.46 (0.10–2.02) | 0.301 | 0.38 (0.08–1.72) | 0.207 | 0.82 (0.46–1.46) | 0.505 |
| Proven sepsis (n = 85) | 1.05 (0.31–3.56) | 0.935 | 1.18 (0.33–4.19) | 0.797 | 1.12 (0.67–1.88) | 0.661 |
| Motor developmental index <70 (n = 74) | 0.70 (0.15–3.25) | 0.649 | 0.64 (0.14–3.01) | 0.576 | 0.92 (0.56–1.53) | 0.749 |
| Psychomotor index <70 (n = 64) | 1.10 (0.24–4.99) | 0.900 | 0.69 (0.15–3.07) | 0.623 | 0.62 (0.37–1.06) | 0.080 |

Category 1: inflammation-nonexposed newborns (n = 432).
Category 2: inflammation-exposed haptoglobinemic newborns (n = 449).
Category 3: inflammation-exposed anhaptoglobinemic or hypohaptoglobinemic (n = 40).
[a] Hosmer and Lemeshow goodness-of-fit test. p Values are adjusted for treatment group, birth weight, gestational age at birth and for correlation due to twins.
[b] p value <0.05.

Multiple studies have attempted to identify biomarker(s) for antenatal exposure to inflammation and early-onset neonatal sepsis, including C-reactive protein, procalcitonin and the pro-inflammatory cytokine IL-6, which peaks in response to infection before declining [30,31]. This phasic response makes most of the acute phase reactant proteins less reliable biomarkers. Mithal et al. measured elevated levels of three acute phase proteins including Hp in newborns with confirmed early-onset neonatal sepsis, although the Hp cut-off value in the multiplex platform was >3× higher than what we established for Hp switch-on profile [3,32]. Among the investigated newborns, only those with confirmed foetal inflammation had elevated Hp levels, supporting foetal inflammation as an antecedent to Hp expression and further demonstrating its value as a biomarker for antenatal exposure to an inflammatory stimulus.

Strengths of the current study include the ability to test the validity of Hp as a cord blood biomarker of neonatal exposure to inflammation before birth and its protective effect against a high risk of long-term consequences in a large cohort of premature neonates followed longitudinally up to 18 months of corrected age by investigators unaware of the antepartum course. Lastly, the data analysis was performed independent of our group, limiting the possibility of bias from idiosyncratic clinical diagnosis, or coding patterns. Our current analysis has several weaknesses. Due to circumstances beyond our control, there was a disproportionate reduction in samples from newborns with the most severe outcomes (e.g., IVH grades 3–4, moderate to severe CP, or death) from the original BEAM trial cohort [13]. Thus, the frequency of newborns predicted in **Category 3** in the analysed subset may underestimate the impact anhaptoglobinemia had on outcomes in the full original cohort.

## 5. Conclusion

In clinical data from 921 newborns enrolled in the MFMU Network BEAM trial, anhaptoglobinemia or hypohaptoglobinemia in the setting of in utero exposure to inflammation, was linked with higher odds of CP or death or IVH or death, while expression of Hp2 allele was related with a lower risk of ROP.

## Authors' Contribution

Catalin S. Buhimschi (C.S.B): formulated the hypothesis, wrote the original study proposal, designed the study, participated during data analysis, wrote the first version of the manuscript and critically revised it for important intellectual content. Irina A. Buhimschi (I.A.B.): formulated the hypothesis, wrote the original study proposal, designed the study, participated during data analysis, supervised or performed the laboratory experiments, wrote the first version of the manuscript and critically revised it for important intellectual content. Kathleen A. Jablonski (K.A.J): worked with C.S.B and I.A.B on formulation of the study design, performed data analysis, writing of the manuscript and revising it critically for important intellectual content. Dwight J. Rouse: protocol development and oversight as PI of the original BEAM trial, had substantial contributions to the conception or design of the work, data acquisition analysis, interpretation of data for the work performed under the original trial, critical revision of the current manuscript for important intellectual content. Michael W. Varner, Uma M. Reddy, Brian M. Mercer, Kenneth J. Leveno, Ronald J. Wapner, Yoram Sorokin, John M. Thorp, Jr., Susan M. Ramin, Fergal D. Malone, Marshall W. Carpenter, Mary J. O'Sullivan, Alan M. Peaceman, George R. Saade, Donald Dudley, and Steve N. Caritis had substantial contribution to protocol development, oversight, conception, design of the trial, data acquisition, analysis, interpretation of the original BEAM trial data, and critical revision of the current manuscript for important intellectual content. All authors have read and approved the final version of the paper.

## Conflict of Interests

Drs. Catalin S. Buhimschi and Irina A. Buhimschi report a patent filed by Yale University that relates to the work of the manuscript: Buhimschi CS, Buhimschi IA, Bhandari V. Markers for detection of complications resulting from in utero encounters. U.S. Patent Number 8,697,367. Filed: July 28, 2011; Issued April 15, 2014. In addition, Drs. Catalin Buhimschi, Irina Buhimschi, and Kathleen Jablonski report grants from NICHD during the conduct of this study. Dr. Ron Wapner reports consulting fees from Natera, Inc., Bioreference, and Illumina, and grants from Sequenom and Illumina with scopes outside that of the submitted work. The funding organisations had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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## Data Availability

Kathleen A. Jablonski, George Washington University Biostatistics Centre, Washington, DC had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The full trial protocol can be accessed upon written request to the George Washington University Biostatistics Centre, Washington, DC.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eclinm.2019.03.009.

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