Necrotizing enterocolitis and high intestinal iron uptake due to genetic variants

Wolfgang Göpel[1], Josephine Drese[1], Tanja K. Rausch[2], Nele Twisselmann[1], Bettina Bohnhorst[3], Andreas Müller[4], Axel Franz[5], Andreas Ziegler[6], Christoph Härtel[1] and Egbert Herting[1]; for the German Neonatal Network[7]

BACKGROUND: Intestinal iron is a nutritional compound, which is essential for enteric microbiota. We evaluated the hypothesis that polymorphisms, which are known modifiers of intestinal iron uptake in adults, are associated with necrotizing enterocolitis (NEC) in preterm infants.

METHODS: Preterm infants (birth weight below 1,500 g) were studied. Single-nucleotide polymorphisms with known effects on serum iron levels (rs1800562, rs1799945, and rs855791) were determined using PCR. The effects of polymorphisms on NEC surgery were tested by Mendelian randomization. Outcome data were compared with χ^2-test, Fisher's exact test, *t*-test, and Cochran–Armitage test for trend and multiple logistic regression analysis.

RESULTS: Complete genotyping data were available for 11,166 infants. High serum iron levels due to rs855791 genotype were associated with a significantly reduced risk of NEC surgery (odds ratio (OR) 0.265; 95% confidence interval (CI) 0.11–0.65; adjusted $P = 0.011$). Carriers of the rs855791 A-allele not receiving prophylactic probiotics had a higher risk of NEC surgery (OR 1.12, 95% CI 1.08–1.70, nominal $P = 0.002$). Prophylactic treatment with probiotics was associated with a reduced risk of NEC surgery in carriers of the rs855791 A-Allele. No differences were found with regard to other short- or long-term outcome data.

CONCLUSION: Polymorphisms inducing lower intestinal iron uptake like the rs855791 A-allele might be an underestimated risk factor for NEC.

Iron is a nutritional compound that is important for growth of preterm infants, development of the nervous system and hematopoiesis, and is also essential for enteric microbiota. Human milk is low in iron; however, iron absorption in the gut of neonates and preterm infants is enhanced by lactoferrin, which is the main whey protein in human milk. Furthermore, binding to lactoferrin protects nutritional iron from being hijacked by bacteria in the relatively iron-poor environment of the human intestine (1). Recent research indicates that gut bacteria dysbiosis precedes necrotizing enterocolitis (NEC) (2) and that oral iron supplementation may adversely affect the gut microbiome by selectively favoring the growth of pathogenic strains (3).

Another unique feature of iron metabolism in humans is the lack of a natural route for excreting excess iron. Iron overload has been described not only in adults, but also in infants (4,5). Preterm infants, who are frequently supplemented with iron for long time periods, might be at a particular risk for iron overload, especially if they are carriers of variants with genetically determined high iron uptake.

In all populations, genetic variants influencing iron uptake are common. Large-scale studies involving more than 20,000 adults demonstrated a dose-dependent effect of the A allele of hemochromatosis gene (*HFE*) rs1800562, the G allele of *HFE* rs1799945, and the G allele of the transmembrane protease serin 6 gene (*TMPRSS6*) rs855791 on serum iron levels (6).

As genetic variants are randomly allocated at conception, iron uptake enhancement by the above-mentioned genetic variants is independent from postnatal nutrition, lactoferrin, iron supplementation, and other cofactors. We studied a large group of preterm infants with a birth weight below 1,500 g to describe short- and long-term effects of genetically altered iron uptake with special attention to the development of NEC.

METHODS

Patients were enrolled in the German Neonatal Network (GNN) between 2009 and 2014 and in the predecessor study of the GNN between 2003 and 2008. Infants were eligible for enrollment if their birth weight was <1,500 g and their gestational age was <37 weeks +0 days. Parents gave written informed consent. The study parts were approved by the local committee on research in human subjects of the University of Lübeck and the local ethical committees at the other study centers. Infants were selected for analysis if genotyping of all three polymorphisms was successful and information concerning NEC surgery was available.

Clinical data were collected at the study sites until discharge or death of the infant and were transferred to the study center (University of Lübeck). Maternal descent was categorized as "German", "other European", "Turkey, Middle East, and Northern Africa", "Asia", "Sub-Sahara Africa," and "Other or unknown". Small for gestational age status was defined as a birth weight below the 10th

[1]Department of Pediatrics, University of Lübeck, UKSH, Lübeck, Germany; [2]Institute for Medical Biometry and Statistics, University of Lübeck, Lübeck, Germany; [3]Department of Pediatric Pulmonology and Neonatology, Hannover Medical School, Hannover, Germany; [4]Department of Pediatrics, University of Bonn, Bonn, Germany; [5]Department of Neonatology, University Children Hospital, Tübingen, Germany; [6]StatSol, Moenring 2, Lübeck, Germany. Correspondence: Wolfgang Göpel (wolfgang.goepel@uksh.de)
[7]GNN-members and affiliations are listed before References.

Received 14 March 2017; accepted 6 August 2017; advance online publication 13 September 2017. doi:10.1038/pr.2017.195

percentile according to Voigt *et al.* (7). Treatment with iron was defined as any treatment with iron during the stay in the hospital. Erythropoietin treatment was defined as any prophylactic treatment with erythropoietin to avoid anemia of prematurity. Oral supplementation with *Lactobacillus acidophilus/Bifidobacterium infantis* probiotics was recorded as an optional variable until 2012 (written down as "additional medication") and in all infants thereafter (checkbox on case record forms). We did not include nutritional data (e.g., breastmilk vs. formula) in our analysis as nutrition was not recorded before 2013. Transfusion of blood was defined as any blood transfusion during the stay in the hospital. Intraventricular hemorrhage (IVH) was defined as any IVH according to Papile *et al.* (8). Sepsis was defined as clinical sepsis with positive blood culture. NEC requiring surgery was defined as clinical NEC with need for laparotomy with or without resection of the necrotic gut and macroscopic diagnosis of NEC made by the attending surgeon. Focal intestinal perforation (FIP) requiring surgery was defined as occurrence of spontaneous intestinal perforation with the need for laparotomy and macroscopic confirmation of isolated FIPs (without inflammatory component) rather than NEC made by the attending surgeon. Death was defined as death during the stay in the hospital.

Follow-up at 5 years was carried out only in infants who were enrolled in the GNN study. All infants were tested by a dedicated team from the study center (one physician, two study nurses, and one medical student) at participating sites. The follow-up team was not aware of clinical or genetic data of the participating children. Body length was determined with Harpenden Portable Stadiometer (Holtain, Crosswell, UK) and body weight with a calibrated scale (M300020, ADE, Hamburg, Germany). Cognitive function was tested with the "Wechsler Preschool and Primary Scale of Intelligence Third Edition (WPPSI-III)". Cerebral palsy was defined as gross motor function scale >1. Forced expiratory volume in one second (FEV1) (%) was assessed with "Easy on-PC" spirometry system (ndd, Zürich, Switzerland).

DNA samples from infants were obtained after birth during their stay in hospital using buccal swab or umbilical cord tissue and were transferred to the study center. DNA was extracted using a commercial DNA purification kit (Qiagen, Hilden, Germany). Genotypes were determined using the TaqMan 5′ nuclease assay (Applied Biosystems, Foster City, CA) and the 7900HT Real-Time PCR System. Context sequences and VIC/FAM labeling are provided in **Supplementary Table S1** online.

Mendelian randomization uses genetic variation as instrumental variables for an intermediate phenotype. As genotypes are randomly transmitted to children, the intermediate phenotype is unaffected by classical confounding. For this reason, demonstration that a genetic variation known to influence the intermediate phenotype level also modifies the disease risk represents an indirect evidence of causal association between phenotype and disease.

In our study two polymorphisms of the hemochromatosis gene (*HFE*) and one polymorphism of the transmembrane protease serin 6 gene (*TMPRSS6*) were used as instrumental variables for the intermediate phenotype "high iron uptake". All three polymorphisms are associated with increased serum iron levels in adults. In adults, each copy of the A allele of *HFE* rs1800562 increases the serum iron level by 0.37 SDs. The effect of the *HFE* rs1799945 G-allele and the *TMPRSS6* rs855791 G-allele is less pronounced with an increase in serum iron levels by 0.19 SD (8). The effect of each polymorphism on NEC was tested separately as in (ref. 6). Outcome data are reported accordingly. In addition to that, we calculated genetically estimated iron uptake by adding the effects of all three polymorphisms for each patient. The patients were pooled according to their intestinal iron as "high intestinal iron" (no iron uptake enhancing alleles), "intermediate intestinal iron" (serum iron +0.19 to +0.38 SD, i.e., one A-allele rs1800562 or one or two G-allele rs1799945 or rs855791), and "low intestinal iron" (high iron uptake, serum iron $>+0.5$ SD).

We tested the hypothesis that a genetic background of high intestinal iron uptake is associated with a lower risk of NEC treated with surgery. This was carried out with a Mendelian randomization approach, which was already described (6). As three genetic variants were tested, the significance level was set to <0.05 with adjustments for three tests by Bonferroni–Holm. All further P values are descriptive. Outcome data stratified to the TMPRSS6/HFE genotype and estimated serum iron levels were compared with χ^2-test, Fisher's exact test, t-test, and Cochran–Armitage test for trend and multiple logistic regression analysis. All P values are two-sided. Statistical analyses were conducted with SPSS (version 22, IBM, Armonk, NY, USA) and R (Version 3.3.1, Vienna, Austria).

RESULTS

We genotyped 12,342 preterm infants with a birth weight below 1,500 g. Genotyping was successful in 95.7% of the patients for rs855791, in 95% of the patients for rs1800562, and in 95.8% of the patients for rs1799945. Only patients with complete genotyping data for all three polymorphisms were analyzed ($n=11{,}166$). The frequency of NEC surgery was 2.5% (281/11,166). Mendelian randomization estimates for each polymorphism based on serum iron levels in adults (6) are given in **Figure 1**. The G-allele of rs855791 was

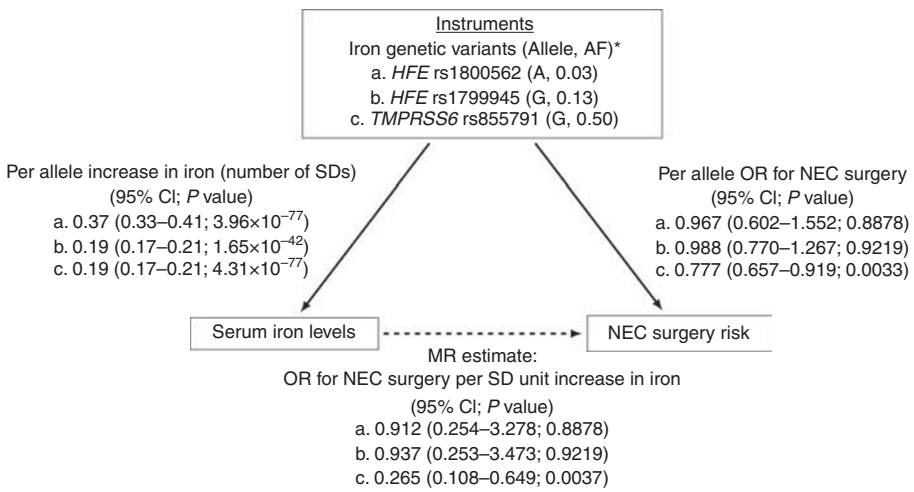

Figure 1. Graphical representation of the Mendelian randomization approach. Serum iron levels were transferred from adult data (ref. 6).

Table 1. Clinical data stratified to *TMPRSS6*-rs855791 genotype

rs855791 genotype	AA (low serum iron, high intestinal iron) $n = 2,139$	AG (intermediate serum iron, intermediate intestinal iron) $n = 5,384$	GG (high serum iron, low intestinal iron) $n = 3,589$	Total[a] $n = 11,166$
Gestational age (weeks)	28.7 ± 2.6	28.7 ± 2.7	28.7 ± 2.7	28.7 ± 2.7
Birth weight (g)	1,056 ± 302	1,069 ± 303	1,061 ± 303	1,064 ± 302
Male gender (%)	50.6	51.4	50.9	51.1
Multiple birth (%)	34.4	33.4	33.3	33.6
SGA (%)	18	17.7	18.2	17.9
EPO treatment (%)	13.2	13.3	13.3	13.3
Iron treatment (%)	85.5	85.6	86.6	85.9
Probiotics (%)	40.2	40.4	42.5	41.0
Blood transfusion (%)	40.7	40.2	39.8	40.2
Sepsis (%)	12.4	13.3	12.8	13.0
NEC (%)	**3.1**	**2.6**	**2.0**	**2.5**
FIP (%)	1.7	2.3	1.9	2.0
IVH (%)	17.3	17.2	16.2	16.9
ROP (%)	3.2	3.2	3.6	3.3
Death until discharge (%)	3.2	3.1	3.3	3.2

EPO, erythropoietin; FIH, focal intestinal perforation; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; ROP, retinopathy of prematurity; SGA, small for gestational age.
Data were compared with χ^2- and *t*-test. Significant differences ($P < 0.05$) are given in bold.
[a]Missing data: gestational age $n = 2$; SGA $n = 2$; EPO treatment $n = 38$, iron treatment $n = 3$, probiotics $n = 3$, blood transfusion $n = 16$, sepsis $n = 66$, surgery for FIP $n = 18$, IVH $n = 13$, ROP $n = 6$.

significantly associated with a reduced risk for NEC surgery (nominal $P = 0.004$ and adjusted $P = 0.011$). Clinical data stratified to the rs855791 genotype are given in **Table 1**. NEC rate was high (3.1 %) in the subgroup of infants with the AA genotype, leading to low iron uptake and consecutive higher intestinal iron levels. Infants with the heterozygous AG genotype had an intermediate NEC rate of 2.6%. Infants with the GG genotype (with high iron uptake and consecutive lower intestinal iron levels) had the lowest risk for NEC surgery (2.0%, odds ratio (OR) for AA vs. GG 0.62, 95% confidence interval (CI) 0.44–0.87; $P = 0.006$, Fisher's exact test). The respective data for the *HFE* polymorphisms rs1799945 and rs1800562 are given as **Supplementary Tables S2 And S3**. Few infants carried the rs1800562 AA genotype ($n = 15$). They had a significantly higher birth weight and a higher gestational age when compared with infants with the rs1800562 GG or AG genotype. No other significant differences were observed.

Frequencies of genotypes stratified to maternal descent are given in **Supplementary Table S4**. Although the iron uptake enhancing polymorphisms in the *HFE* gene (rs1800562 and rs1799945) were common in infants of German and European descent if compared with infants from Asia or Sub-Sahara Africa, the reverse was true for the *TMPRSS6* rs855791 G allele.

Although the possible maximum of iron-uptake-enhancing alleles in our study is 6, none of the infants carried more than four alleles. To give an estimate of the combined effect of all three polymorphisms, we multiplied the number of iron-enhancing alleles for each polymorphism by the iron serum SD values from adult data (6) and then added them for each patient. Overall, 1,473 infants (13.2%) did not carry one of the iron-uptake-enhancing alleles and were classified as "low iron uptake". Most infants ($n = 8,095$, 72.5%) carried a single rs1800562 allele or one or two rs1799945 or rs855791 alleles. Their serum iron level was estimated to be "intermediate" (+0.28 SD if compared with non-carriers). In all, 1,598 infants (14.3%) had two alleles (including at least one rs1800562) or – three to four iron-uptake-enhancing alleles. Their serum iron level was estimated to be +0.62 SD above infants without iron-uptake-enhancing alleles and was classified as "high". Genotypes and stratification are given in **Supplementary Table S5**; clinical data are given in **Supplementary Table S6**. The combined data of all three polymorphisms resulted in a more pronounced difference if compared with the rs855791 data. Rates for NEC surgery were reduced by ~ 50% in infants with high iron uptake if compared with infants without polymorphisms (3.2% vs. 1.8%, OR 0.54, 95% CI 0.34–0.87; $P = 0.01$, Fisher's exact test). This was also true if serum iron uptake variants were tested as a continuous variable in a multiple logistic regression analysis adjusted for gestational age, gender, multiple birth, maternal descent, small for gestational age status, treatment with probiotics, and participating study site (OR per additional estimated SD of serum iron: 0.44, 95% CI 0.22–0.88, $P = 0.02$). A sensitivity analysis was performed, and parameters were stable. In addition to iron uptake, only study site, gestational age (OR 0.66/week, 95% CI 0.62–0.69, $P < 0.001$), small for gestational

Table 2. NEC surgery rate in infants stratified to treatment with probiotics and *TMPRSS6* rs855791 genotype

rs855791 genotype	AA (low serum iron, high intestinal iron)	AG (intermediate serum iron, intermediate intestinal iron)	GG (high serum iron, low intestinal iron)	Total	P^a
No treatment with probiotics	3.6% 47/1,310	3.0% 97/3,208	1.9% 39/2,063	2.8% 183/6,581	0.002
Prophylactic treatment with probiotics	2.5% 22/882	2.0% 44/2,176	2.0% 32/1,524	2.1% 98/4,582	0.592

NEC, necrotizing enterocolitis.
[a]*P* from logistic regression for NEC surgery on genotype estimated separately for no treatment and treatment.

Table 3. Outcome data at 5 years stratified to the *TMPRSS6* rs855791 genotype

rs855791 genotype	AA (low serum iron, high intestinal iron), $n = 191$	AG (intermediate serum iron, intermediate intestinal iron), $n = 471$	GG (high serum iron, low intestinal iron), $n = 284$	Total, $n = 946$
Length (cm; mean ± SD)	111 ± 5.8	112 ± 5.3	111 ± 6.1	111 ± 5.7
Body weight (kg; mean ± SD)	17.6 ± 2.7	18.4 ± 3.2	18.1 ± 3.2	18.2 ± 3.1
FEV1 (%; mean ± SD)	91 ± 15	88 ± 17	89 ± 17	89 ± 19
Cerebral palsy (%)	7.6	5.0	5.1	5.6
WPPSI-IQ (mean ± SD)	96 ± 15	97 ± 15	96 ± 14	97 ± 14

FEV1, forced expiratory volume in one second; WPPSI-III, Wechsler Preschool and Primary Scale of Intelligence Third Edition.
Infants with AA genotype were compared with infants with GG genotype using χ^2-test and *t*-test. No significant differences ($P < 0.05$) were observed. FEV1% data are restricted to 750 infants and WPPSI-III scores are restricted to 862 infants.

age (OR 1.76, 95% CI 1.29–2.40, $P < 0.001$), and treatment with *L. acidophilus/B. infantis* (Infloran) probiotics (OR 0.64, 95% CI 0.47–0.87, $P = 0.005$) were significant predictors for NEC surgery. As treatment with probiotics is the only modifiable cofactor, we tested the effect of the rs855791 genotype stratified to probiotic treatment. In infants not receiving probiotics, risk for surgery-treated NEC was increased by 12% per A allele at rs855791 (OR 1.12, 95% CI: 1.08–1.70; nominal $P = 0.002$); however this association was not observed in infants who received prophylactic probiotics (OR 1.08, 95% CI: 0.88–1.33; nominal $P = 0.592$, Table 2).

Data concerning the time of NEC surgery were available for 135 of 281 infants (48%) since we started recording this parameter in 2011. The median time of NEC surgery was day 18 of life (interquartile range: days 10–31). No significant differences were observed between infants with different genotypes.

Five-year outcome data were available for 946 infants. No significant differences were observed if infants with low iron uptake (rs855791 AA genotype) were compared with infants with high iron uptake (rs855791 GG genotype, Table 3).

DISCUSSION

Here, we report that genetically determined low intestinal iron uptake (and consecutive higher intra-intestinal iron level) is associated with NEC in preterm infants. This finding is in line with a number of observational and experimental data supporting the hypothesis that high levels of intestinal iron might be a cofactor for the early development of gut dysbiosis and NEC.

Iron is essential for the growth of most bacterial species. A large-scale trial of oral iron supplementation was stopped because of excess morbidity and mortality due to infection in preschool children in a high malaria-transmission setting (9). Another recently published randomized controlled trial provides an explanation for this finding. It shows that oral iron supplementation adversely affects the gut microbiome by selectively favoring the growth of pathogens including *Clostridium perfringens* and *Escherichia coli* in Kenyan infants (3). These data indicate that intestinal iron levels might influence the development of local intestinal dysbiosis in the small intestine, preceding NEC (2).

The median time of NEC surgery in our study was day 18 of life, which is often before the point in time at which oral iron supplementation is introduced. Furthermore, randomized controlled trials of enteral iron supplementation have not reported adverse outcomes or increased NEC incidence in infants supplemented with iron (10–12).

Concerning the prophylactic effect of probiotics, our data suggest that certain probiotic species, such as the *L. acidophilus/B. infantis* preparation, which is commonly used in German neonatal intensive care units, might be able to reduce the iron supply for pathogenic strains in the small intestine. The combined use of both species might be of particular importance, as *Lactobacillus* species have little or no requirement for iron, but are rapidly overgrown if increasing amounts of iron are available for competing bacterial strains (13). Another iron-mediated protective effect of probiotics is suggested by recently published data from germ-free mice, indicating that intestinal cells favor iron storage after colonization with probiotics (14).

Formula nutrition of preterm infants is associated with an increased risk of NEC (15,16). The low lactoferrin content and relatively higher iron content of formula nutrition might be a double risk, as both factors will increase intestinal iron. Treatment with oral lactoferrin reduces the risk of NEC and sepsis in preterm infants (17). The molecular mechanisms of these protective effects are unknown, as lactoferrin is a multifunctional protein that enhances iron uptake but has additional antibacterial, antiviral, and anti-inflammatory activities (18).

Both low and high serum iron levels are described as risk factors for long-term neurocognitive dysfunction in the literature (19). Long-term outcome data of our study are reassuring with regard to the current standard of nutritional iron supplementation in preterm infants. However, 5-year follow-up in our study is yet limited to a relatively small group of 946 infants.

The three polymorphisms, which were evaluated in our study, are associated with serum iron and ferritin levels in adults (6,20). Although the polymorphisms are known determinants of iron status for years, the exact mechanism how these genetic variants enhance iron uptake is not clarified yet and pleiotrophic effects of these polymorphisms on other pathways cannot be excluded (21–23). A recent publication demonstrating that anemia is a risk factor for the development of NEC in preterm infants (24) is of particular importance with regard to pleiotrophic effects of the variants studied here, as the TMPRSS6-G-allele is associated with increased hemoglobin levels (25). However, effect sizes of single polymorphisms on complex traits like hemoglobin levels are rather small (25). A Mendelian randomization study targeting high and low hemoglobin levels and results of large-scale randomized trials of transfusion thresholds (NCT01393496) will probably be more informative with regard to the question whether anemia is a risk factor for NEC. No data concerning the interaction between specific alleles and infant iron or ferritin levels are published so far. Therefore, assumptions concerning serum iron and intestinal iron uptake in our study are entirely based on adult data, which is a major limitation of our study, at least with regard to short-term outcome data. Another limitation is the possible effect of the maternal genotype. Recently published data indicate that maternal *HFE* polymorphism influences umbilical cord blood lead levels. Therefore, iron transfer to the infants might also be influenced by the maternal genotype (26). Finally, the main determinant of iron uptake in our study was the *TMPRSS6* rs855791 polymorphism. As spurious association is always a matter of concern, our data with regard to NEC should be confirmed by additional studies of similar size and set-up.

In summary, polymorphisms inducing low intestinal iron uptake in adults were associated with an increased rate of NEC in preterm infants. No increased risk was observed in infants receiving *L. acidophilus/B. infantis* probiotics. Long-term outcome data of infants with high iron uptake did not differ in infants with low or intermediate uptake, supporting the current practice of oral iron supplementation in preterm infants, which is not associated with NEC. Our data indicate that polymorphisms reducing intestinal iron uptake in adults might be an underestimated risk factor for very early intestinal dysbiosis and NEC (6).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

ACKNOWLEDGMENTS

We thank all infants and their parents who participated. We also thank each neonatal unit and its staff for helping us with the study. We are grateful to Greco M. for providing the code for the Mendelian randomization analysis that they used in their own analysis.

STATEMENT OF FINANCIAL SUPPORT

This study was funded by the German Federal Ministry of Education and Research (BMBF 01ER0805 and BMBF 01ER1501). A.Z. acknowledges funding from the German Research Foundation (Research Unit Protect-Move, FOR 2488).

GERMAN NEONATAL NETWORK

Orlikowsky T. (Aachen); Wieg C. (Aschaffenburg); Rossi R. (Berlin); Weller U. (Bielefeld); Teig N. (Bochum); Hepping N. (Bonn); Körner T. (Bremen); Gerleve H. (Coesfeld); Roll C. (Datteln); Brune T. (Detmold); Heitmann F. (Dortmund); Rüdiger M. (Dresden); Höhn T. (Düsseldorf); Andree Berghäuser M. (Düsseldorf); Felderhoff-Müser U. (Essen); Reese J. (Eutin); Dördelmann M. (Flensburg); Ehlers S. (Frankfurt); Hentschel R. (Freiburg); Küster H. (Göttingen); Linnemann K. (Greifswald); Haase R. (Halle-Saale); v. d.Wense Axel (Hamburg); Schmidkte S. (Hamburg); Guthmann F. (Hannover); Jensen R. (Heide); Gortner L. (Homburg); Hillebrand G. (Itzehoe); Dawczynski K. (Jena); Müller D. (Kassel); Bendiks M. (Kiel); Kribs A. (Köln); Böckenholt K. (Köln); Gebauer C. (Leipzig); Eichhorn J.G. (Leverkusen); Böttger R. (Magdeburg); Schaible T. (Mannheim); Wintgens J. (Mönchengladbach); Hörnig-Franz I. (Münster); Urlichs F. (Münster); Seeliger S. (Neuburg/Donau); Schäfer S. (Nürnberg); Segerer H. (Regensburg); Olbertz D. (Rostock); Möller J. (Saarbrücken); Kannt O. (Schwerin); Hubert M. (Siegen); Vochem M. (Stuttgart); Heldmann M. (Wuppertal).

Disclosure: The authors declare no conflict of interest.

REFERENCES

1. Ganz T, Nemeth E.. Iron homeostasis in host defence and inflammation. Nat Rev Immunol 2015;15:500–10.
2. Warner BB, Deych E, Zhou Y, et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. Lancet 2016;387:1928–36.
3. Jaeggi T, Kortman GA, Moretti D, et al. Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. Gut 2015;64:731–42.
4. Lönnerdal B, Georgieff MK, Hemell O. Developmental physiology of iron absorption, homeostasis and metabolism in the healthy term infant. J Pediatr 2015;167:S8–14.
5. Dewey KG, Domellöf M, Cohen RJ, et al. Iron supplementation affects growth and morbidity of breast-fed infants: results of a randomized trial in Sweden and Honduras. J Nutr 2002;132:3249–55.
6. Pichler I, Del Greco M F, Gögele M, et al. Serum iron levels and the risk of Parkinson disease: a Mendelian randomization study. PLoS Med 2013;10:e1001462.
7. Voigt M, Rochow N, Guthmann F, et al. Birth weight percentile values for girls and boys under consideration of maternal height. Z Geburtshilfe Neonatol 2012;216:212–9.
8. Papile LA, Burstein J, Burstein R, et al. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. J Pediatr 1978;92:529–34.
9. Sazawal S, Black RE, Ramsan M, et al. Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and

mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 2006;367: 133–43.
10. Taylor TA, Kennedy KA. Randomized trial of iron supplementation versus routine iron intake in VLBW infants. Pediatrics 2013;131: e433–8.
11. Franz AR, Mihatsch WA, Sander S, Kron M, Pohlandt F. Prospective randomized trial of early versus late enteral iron supplementation in infants with a birth weight of less than 1301 grams. Pediatrics 2000;106: 700–6.
12. Joy R, Krishnamurthy S, Bethou A, Rajappa M, Ananthanarayanan PH, Bhat BV. Early versus late enteral prophylactic iron supplementation in preterm very low birth weight infants: a randomized controlled trial. Arch Dis Child Fetal Neonatal Ed 2014;99:F105–9.
13. Bailey JR, Probert CS, Cogan TA. Identification and characterisation of an iron-responsive candidate probiotic. PLoS ONE 2011;6:e26507.
14. Deschemin JC, Noordine ML, Remot A, et al. The microbiota shifts the iron sensing of intestinal cells. FASEB J 2016;30:252–61.
15. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr 2010;156: 562–7.
16. Spiegler J, Preuß M, Gebauer C, et al. Does breastmilk influence the development of bronchopulmonary dysplasia? J Pediatr 2016;169:76–80.
17. Pammi M, Abrams SA. Oral lactoferrin for the prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 2015: CD007137.
18. Lönnerdal B. Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas. Am J Clin Nutr 2014;99:712–7.
19. Amin SB, Myers G, Wang H. Association between neonatal iron overload and early human brain development in premature infants. Early Hum Dev 2012;88:583–7.
20. Sørensen E, Rigas AS, Thørner LW, et al. Genetic factors influencing ferritin levels in 14,126 blood donors: results from the Danish Blood Donor Study. Transfusion 2016;56:622–7.
21. Galesloot TE, Geurts-Moespot AJ, den Heijer M, et al. Associations of common variants in HFE and TMPRSS6 with iron parameters are independent of serum hepcidin in a general population: a replication study. J Med Genet 2013;50:593–8.
22. Nai A, Pagani A, Silvestri L, et al. TMPRSS6 rs855791 modulates hepcidin transcription *in vitro* and serum hepcidin levels in normal individuals. Blood 2011;118:4459–62.
23. Traglia M, Girelli D, Biino G, et al. Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. J Med Genet 2011;48:629–34.
24. Patel RM, Knezevic A, Shenvi N, et al. Association of red blood cell transfusion, anemia, and necrotizing enterocolitis in very low-birth-weight infants. JAMA 2016;315:889–97.
25. Van der Harst P, Zhang W, Mateo Leach I, et al. Seventy-five genetic loci influencing the human red blood cell. Nature 2012;492:369–75.
26. Karwowski MP, Just AC, Bellinger DC, et al. Maternal iron metabolism gene variants modify umbilical cord blood lead levels by gene-environment interaction: a birth cohort study. Environ Health 2014;13:77.