Association between the p.Thr1406Asn polymorphism of the carbamoyl-phosphate synthetase 1 gene and necrotizing enterocolitis: A prospective multicenter study

Rob M. Moonen1,2, Giacomo Cavallaro3, Maurice J. Huizing2, Gema E. González-Luis4, Fabio Mosca3 & Eduardo Villamor2

The p.Thr1406Asn (rs1047891) polymorphism of the carbamoyl-phosphate synthetase 1 (CPS1) gene has been linked to functional consequences affecting the downstream availability of the nitric oxide precursor L-arginine. L-arginine concentrations are decreased in preterm infants with necrotizing enterocolitis (NEC). In this multicenter prospective study, we investigated the association of the p.Thr1406Asn polymorphism with NEC in 477 preterm infants (36 cases of NEC) from 4 European neonatal intensive care units (Maastricht, Las Palmas de Gran Canaria, Mantova, and Milan). Allele and genotype frequencies of the p.Thr1406Asn polymorphism did not significantly differ between the infants with and without NEC. In contrast, the minor A-allele was significantly less frequent in the group of 64 infants with the combined outcome NEC or death before 34 weeks of corrected gestational age than in the infants without the outcome (0.20 vs. 0.31, P = 0.03). In addition, a significant negative association of the A-allele with the combined outcome NEC or death was found using the dominant (adjusted odds ratio, aOR: 0.54, 95% CI 0.29–0.99) and the additive (aOR 0.58, 95% CI 0.36–0.93) genetic models. In conclusion, our study provides further evidence that a functional variant of the CPS1 gene may contribute to NEC susceptibility.

Necrotizing enterocolitis (NEC) remains a significant cause of morbidity and mortality in neonatal intensive care units. Although several predisposing factors have been identified, the exact etiology of NEC is yet elusive. The combination of genetic predisposition, immaturity of gastrointestinal motility, digestive ability, intestinal barrier function, immune defense and microcirculatory regulation accompanied by a strong likelihood of abnormal microbial colonization in the intestine, leads to a confluence of predisposing factors1–9.

Nitric oxide (NO) has received increasing attention in the pathophysiology of NEC, as it participates in the regulation of intestinal blood flow and plays a key role in the maintenance of mucosal integrity, intestinal barrier function, and post-injury intestinal repair10–12. NO is generated by NO synthase (NOS) during the enzymatic conversion of L-arginine to L-citrulline. The NOS substrate L-arginine is an essential amino acid for young mammals13,14. Metabolic and molecular studies indicate that the underdevelopment of intestinal arginine synthesis may be primarily responsible for remarkably low plasma arginine concentrations in preterm neonates14,15. Several studies demonstrated that plasma arginine concentrations are even more decreased in preterm infants with NEC14,16–19. Moreover, data from two small randomized controlled studies16,20, pooled in a meta-analysis21, suggest that arginine supplementation reduces the incidence of NEC in preterm infants.

1Department of Pediatrics, Zuyderland Medical Center Heerlen, 6130 MB, The Netherlands. 2Department of Pediatrics, Maastricht University Medical Center (MUMC+), School for Oncology and Developmental Biology (GROW), Maastricht, 6202 AZ, The Netherlands. 3Neonatal Intensive Care Unit, Department of Clinical Sciences and Community Health, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, 20122, Italy. 4Department of Pediatrics, Hospital Universitario Materno-Infantil de Canarias, Las Palmas de Gran Canaria, 35016, Spain. Correspondence and requests for materials should be addressed to E.V. (email: e.villamor@mumc.nl)
Arginine is a urea cycle intermediate. The first step in the urea cycle occurs inside the mitochondrion and is catalyzed by the enzyme, carbamoyl–phosphate synthetase I (CPS1, EC 6.3.4.16)\(^2\). CPS1 deficiency in humans is a rare autosomal recessive inborn error of the urea cycle leading to hyperammonemia. Deficiency can be primary, due to mutations in the CPS1 gene (OMIM 608307, HGNC:2323) or secondary, due to the lack of the essential cofactor N-acetyl-L-glutamate\(^2\). In addition, a number of functional single nucleotide polymorphisms (SNPs) have been identified in the CPS1 gene\(^2\). One of those SNPs (pThr1406Asn also published as T1405N; rs1047891 formerly designated as rs7422339) has been linked to functional consequences affecting the downstream availability of urea-cycle intermediates, including L-arginine\(^2\). The SNP pThr1406Asn is a C-to-A nucleotide transversion (c.4217 C>A) in exon 36, which results in the substitution of asparagine (Asn) for threonine (Thr) in the critical N-acetylglutamate-binding domain\(^2\). The C-encoded Thr form of the polymorphism is considered the evolutionarily conserved version and the less frequent, A-encoded, Asn variant appears to be a relatively new, gain-of-function mutation\(^2\). It has been suggested that the A-allele may confer an advantage in terms of NO production, especially under conditions of environmental stress\(^2\). Previous studies demonstrated the association of the CPS1 pThr1406Asn genotype with clinical situations where endogenous NO production is critically important, such as persistent pulmonary hypertension of the newborn\(^2\), pulmonary hypertension following surgical repair of congenital heart defects\(^2\), and hepatic veno-occlusive disease after bone marrow transplantation\(^2\).

Several years ago, we reported in a retrospective series of 17 preterm infants with NEC and 34 controls that patients with NEC showed an underrepresentation of the A-encoded variant of the pThr1406Asn polymorphism of CPS1\(^2\). Those results suggested that the A-allele conferred protection against NEC and warranted confirmation using a prospective design and larger sample size. Herein we report the results of such study, involving 477 preterm infants (36 cases of NEC) from four neonatal intensive care units located in three different European countries (Spain, Italy, and the Netherlands). Since death in the first weeks of life is a competing outcome for NEC, we also analyzed the association of the pThr1406Asn polymorphism with the combined outcome NEC or death before 34 weeks of corrected gestational age (GA).

### Methods

#### Patients.

The study was approved by the Institutional Review Boards (IRBs) of the Maastricht University Medical Center (the Netherlands, registration number MEC 04–140), Hospital Universitario Materno-Infantil de Canarias (Las Palmas de Gran Canaria, Spain), Carlo Poma Hospital (Mantova, Italy) and Ospedale Maggiore Policlinico (Milan, Italy). The study was conducted according to institutional and IRB guidelines and regulations and registered in ClinicalTrials.gov Protocol Registration System (NCT00554866, ID 07-2-018, November 6, 2007). The manuscript was drafted according to the STROBE statement (http://www.strobe-statement.org/).

#### Samples and genotyping.

DNA was extracted using standard methods and stored at −20 °C until genotyping. A 214-bp fragment encompassing the pThr1406Asn polymorphism in exon 36 of the CPS1 gene was amplified using polymerase chain reaction (PCR). Primers used were (forward) GCM357 5′-TAAATGCAAGCTTGGCCAC-3′ and (reverse) GCM358 5′-GACCTTGAATCAAGTATTAGTGAAA-3′. The PCR mix consisted of 1X GeneAmp PCR Buffer...
**II (Perkin-Elmer, Branchburg, NJ), 0.2 mM deoxyribonucleoside triphosphate (Pharmacia Biotech, Bridgewater, NJ), 1.5 mM MgCl2 (Perkin-Elmer, Branchburg, NJ), 250 nM of both primers, and 0.025 U/μL of AmpliTaq Gold (Perkin-Elmer, Branchburg, NJ). Thermocycling conditions started with an initial denaturation of 10 min 95 °C, followed by 35 cycles of 95 °C (45 s), 55 °C (45 s), 72 °C (45 s), and ended with a final extension step of 10 min at 72 °C. The PCR product was purified and directly sequenced using the reverse primer.**

**Statistical Analysis.** Sample size was calculated based on data from our previous study27. Given an expected population incidence of NEC of 5%, expected frequencies for CC homozygosity of 0.7 (NEC group) and 0.4 (control group), alpha level = 0.05, and power of 0.8, 440 infants were needed to detect an odds ratio (OR) significantly different from 1.

Categorical variables were expressed as counts or percentages and compared using the chi-square test. Continuous variables were expressed as mean (SD) if they followed a normal distribution and compared using unpaired, two-sided t-test. If not normally distributed, continuous variables were expressed as median values (interquartile range, IQR; 25 th–75 th percentile) and compared using the Mann-Whitney U test. The Kolmogorov-Smirnov test was used to test for normal distribution of continuous data.

Differences in allelic frequencies and genotype distributions between the investigated populations, as well as Hardy–Weinberg equilibrium (HWE) for genotype distribution were assessed using a chi-square test. The Hardy–Weinberg law states that $q^2 + 2pq + p^2 = 1$, where p and q are allele frequencies in a two-allele system.

Logistic regression analysis was used to compute the ORs and their 95% confidence intervals (CI) for NEC and the combined outcome NEC or death before 34 weeks of corrected GA based on genotype after accounting for the covariates which were significantly different between the groups and are known risk factors for developing NEC.

Different genetic models were used to analyze the effect of the risk allele, including the general allelic (multiplicative or codominant model), dominant, recessive and additive models. Assuming a genetic penetrance parameter $\gamma$ ($\gamma > 1$), a multiplicative model indicates that the risk of disease is increased $\gamma$-fold with each additional copy of the risk allele; an additive model indicates that risk of disease is increased $\gamma$-fold for the genotype with one copy of the risk allele and $2\gamma$-fold for the genotype with two copies of the risk allele; a common recessive model indicates that two copies of the risk allele are required for a $\gamma$-fold increase in disease risk, and a common dominant model indicates that either one or two copies of the risk allele are required for a $\gamma$-fold increase in disease risk31. The major allele was considered as a reference and the interactions were tested in the different models by multivariable logistic regression model. All the statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corporation, Armonk, NY, USA) and conducted at the $P < 0.05$ level of significance.

**Results**

**Patient characteristics.** From 615 eligible infants, 477 (36 with NEC Bell stage II or greater) were included in the study (Fig. 1). Stage II NEC was present in 23 infants and stage III in 13 infants. In 21 cases surgery was required. The median age at the onset of NEC was 20 days (range 4–87, IQR 12–31). Nine of the cases of NEC occurred in Maastricht (5 stage II NEC, 4 stage III NEC), 16 in Las Palmas (9 stage II NEC, 7 stage III NEC), and 11 in Italy (9 stage II NEC, 2 stage III NEC). Single intestinal perforation was present in 5 infants (1 in Maastricht, 4 in Italy). Demographic and clinical characteristics of the infants with and without NEC are shown and compared in Table 1. Mean GA, mean BW and median Apgar score at 1 min of infants with NEC were significantly lower than in infants without NEC. In addition, infants with NEC showed a higher incidence of vaginal delivery, mechanical ventilation, BPD, hypotension, IVH, PVL, PDA, ROP and mortality. We adjusted for GA, BW, Apgar score at 1 min, mechanical ventilation, hypotension, and PDA in the subsequent logistic regression analysis.

The demographic and clinical characteristics of the infants with and without the combined outcome NEC or death before 34 weeks of corrected GA are shown and compared in Table 2. Mean GA, mean BW and median
|                          | NEC-yes (n = 36) | n data missing | NEC-no (n = 441) | n data missing | P value |
|--------------------------|------------------|----------------|------------------|----------------|---------|
| Birth weight (g)         | 844 (SD 216)     | 0              | 1016 (SD 266)    | 0              | 0.000   |
| Gestational age (wks)    | 26.7 (SD 1.9)    | 0              | 27.9 (SD 1.9)    | 0              | 0.000   |
| Male sex                 | 17 (47.2)        | 0              | 238 (54.0)       | 0              | 0.419   |
| Prenatal steroids        | 28 (80.0)        | 1              | 375 (88.2)       | 16             | 0.361   |
| Preeclampsia             | 3 (8.6)          | 1              | 68 (15.6)        | 5              | 0.264   |
| Chorioamnionitis         | 3 (8.3)          | 0              | 58 (13.3)        | 5              | 0.393   |
| PROM                     | 6 (16.7)         | 0              | 118 (27.2)       | 7              | 0.169   |
| Vaginal delivery         | 17 (58.6)        | 0              | 147 (34.2)       | 2              | 0.042   |
| Apgar (1 min)            | 5 [3–7]          | 1              | 6 [4–8]          | 5              | 0.026   |
| Apgar (5 min)            | 8 [6–9]          | 1              | 8 [7–9]          | 6              | 0.114   |
| RDS                      | 30 (83.3)        | 0              | 378 (85.9)       | 1              | 0.671   |
| Mechanical vent.         | 32 (88.9)        | 0              | 279 (64.0)       | 5              | 0.002   |
| BPD                      | 21 (58.3)        | 0              | 137 (31.3)       | 3              | 0.001   |
| Hypotension              | 26 (72.2)        | 0              | 175 (40.1)       | 5              | 0.000   |
| Sepsis                   | 23 (67.6)        | 2              | 227 (51.7)       | 2              | 0.073   |
| IVH                      | 16 (44.4)        | 0              | 110 (25.1)       | 3              | 0.012   |
| PVL                      | 4 (11.1)         | 0              | 14 (3.2)         | 4              | 0.017   |
| PDA                      | 25 (71.4)        | 1              | 233 (53.2)       | 3              | 0.037   |
| ROP                      | 13 (41.9)        | 5              | 85 (20.5)        | 27             | 0.006   |
| Mortality                | 8 (22.2)         | 0              | 42 (9.5)         | 0              | 0.017   |
| Death before 34 wks      | 5 (13.9)         | 0              | 29 (6.6)         | 0              | 0.101   |

Table 1. Baseline characteristics and neonatal complications in preterm infants with and without NEC. Results are expressed as mean (SD), median [interquartile range] or absolute numbers of patients (percentage). NEC: necrotizing enterocolitis (≥ stage II); PROM: prolonged rupture of membranes; RDS: respiratory distress syndrome; BPD: bronchopulmonary dysplasia; IVH: intraventricular hemorrhage (≥ grade 2); PVL: periventricular leukomalacia; PDA: patent ductus arteriosus; ROP: retinopathy of prematurity (≥ stage II).

|                          | NEC/death-yes (n = 65) | n data missing | NEC/death-no (n = 412) | n data missing | P value |
|--------------------------|------------------------|----------------|------------------------|----------------|---------|
| Birth weight (g)         | 818 (SD 230)           | 0              | 1032 (SD 261)          | 0              | 0.000   |
| Gestational age (wks)    | 26.4 (SD 2.0)          | 0              | 28.1 (SD 1.8)          | 0              | 0.000   |
| Male sex                 | 34 (52.3)              | 0              | 221 (53.6)             | 0              | 0.841   |
| Prenatal steroids        | 51 (81.0)              | 2              | 352 (88.7)             | 15             | 0.223   |
| Preeclampsia             | 6 (9.4)                | 1              | 65 (16.0)              | 5              | 0.170   |
| Chorioamnionitis         | 11 (17.2)              | 1              | 50 (12.3)              | 4              | 0.274   |
| PROM                     | 15 (23.8)              | 2              | 109 (26.8)             | 5              | 0.618   |
| Vaginal delivery         | 34 (52.3)              | 0              | 142 (34.6)             | 2              | 0.006   |
| Apgar (1 min)            | 5 [3–6]                | 1              | 6 [5–8]                | 6              | 0.000   |
| Apgar (5 min)            | 8 [6–9]                | 1              | 8 [7–9]                | 6              | 0.000   |
| RDS                      | 54 (83.1)              | 0              | 354 (86.1)             | 1              | 0.513   |
| Mechanical vent.         | 60 (92.3)              | 0              | 251 (61.7)             | 5              | 0.000   |
| BPD                      | 24 (37.5)              | 1              | 134 (32.7)             | 2              | 0.447   |
| Hypotension              | 52 (80.0)              | 0              | 149 (36.6)             | 5              | 0.000   |
| Sepsis                   | 38 (60.3)              | 2              | 212 (51.7)             | 2              | 0.202   |
| IVH                      | 31 (48.4)              | 1              | 95 (23.2)              | 2              | 0.000   |
| PVL                      | 5 (7.8)                | 1              | 13 (3.2)               | 3              | 0.072   |
| PDA                      | 45 (71.4)              | 2              | 213 (52.0)             | 2              | 0.004   |
| ROP                      | 13 (25.0)              | 13             | 85 (21.6)              | 19             | 0.581   |

Table 2. Baseline characteristics and neonatal complications in preterm infants with and without the combined outcome NEC or death before 34 wks of corrected gestational age. Results are expressed as mean (SD), median [interquartile range] or absolute numbers of patients (percentage). NEC: necrotizing enterocolitis (≥ stage II); PROM: prolonged rupture of membranes; RDS: respiratory distress syndrome; BPD: bronchopulmonary dysplasia; IVH: intraventricular hemorrhage (≥ grade 2); PVL: periventricular leukomalacia; PDA: patent ductus arteriosus; ROP: retinopathy of prematurity (≥ stage II).
The p.Thr1406Asn polymorphism is a known variant in the CPS1 gene. This study investigated the association of the p.Thr1406Asn polymorphism with NEC in very preterm infants. The minor allele frequency (MAF) of the p.Thr1406Asn polymorphism was 0.281 in the preterm population. A closer look at the recessive model for the combined outcome of NEC or death before 34 weeks of corrected GA revealed a statistically significant association (OR = 0.208, 95% CI: 0.11-0.39, P = 0.03).

Analysis of genotypes.

Allele and genotype frequencies were compared between infants with and without NEC. The minor allele frequency (MAF) for the p.Thr1406Asn polymorphism was 0.303 in the NEC group and 0.208 in the NEC-no group. After adjusting for birth weight, gestational age, and Apgar score, the minor A-allele of the p.Thr1406Asn polymorphism was associated with a reduced risk of NEC or death (aOR = 0.66, 95% CI: 0.36-1.19, P = 0.17).

Discussion

This study, one of the largest prospective investigations of a SNP with NEC, did not find a significant association between the p.Thr1406Asn polymorphism and NEC in very preterm infants. However, when the combined outcome of NEC or death was considered, a significant negative association was observed for the A-allele of the p.Thr1406Asn polymorphism. The association was found using the recessive and additive genetic models.
prospective cohort study, we observed that the A-allele was less frequent in the infants with NEC in a proportion close to reach statistical significance ($P = 0.09$). Moreover, only one infant with NEC was homozygous for the A-allele. Nevertheless, none of the genetic models could demonstrate a statistically significant association between the p.Thr1406Asn genotype and NEC.

An important issue in designing studies involving high-risk patients is the selection of an appropriate primary outcome when death is a competing outcome. In this situation, some patients will die before the outcome of interest can occur. For this reason, a composite outcome including death is often used when complications of prematurity such as BPD or NEC are studied. Since the onset of ‘classical’ NEC takes place around the end of the...
third week of life, we performed an additional analysis in which the infants with NEC were combined with the infants who died before 34 weeks of corrected GA. Interestingly, we observed that the minor A-allele was significantly less frequent among the infants with the combined outcome NEC or death. Moreover, the dominant and additive model demonstrated that the A variant of the pThr1406Asn polymorphism significantly decreased the risk of developing the combined outcome of NEC or death before 34 weeks of corrected GA. Thus, in other words, the minor A variant of the polymorphism might protect against this combined outcome.

In accordance with our results, it has been reported a protective role of the A-allele toward persistent pulmonary hypertension of the newborn, pulmonary hypertension following surgical repair of congenital heart defects, and hepatic veno-occlusive disease after bone marrow transplantation. Moreover, the haplotype formed by the SNPs of CPS1 rs715 and rs1047891 (pThr1406Asn) yield a protective association with decreased risk of coronary artery disease in women. As mentioned in the introduction, those findings led to the speculation that individuals with the A-allele may have an advantage in terms of availability of the NOS substrate L-arginine, especially under conditions of environmental stress. Accordingly, the serum levels of arginine were higher in term newborns carrying the AA genotype. However, activity reports on the in vitro activity of the pThr1406Asn variants are contradictory. Thus, natural (A-encoded) Asn1406 CPS has been reported to have higher enzymatic activity than the (C-encoded) Thr1406 variant but recombinant Asn1406 CPS showed inferior catalytic properties. In order to evaluate whether pThr1406Asn genotypes correlated with urea cycle intermediates levels in preterm infants, we measured the concentrations of arginine and citrulline in the first 128 infants included in the present cohort and we did not observe any significant differences. This lack of effect of CPS1 genotype on L-arginine concentrations has been also reported in adults. Nevertheless, one limitation of our study was that L-arginine levels were determined between 6 and 12 hours after birth, whereas NEC has its onset later in life. It can be speculated that the alterations in arginine levels related to the pThr1406Asn polymorphism may be only relevant under the stress conditions generated around the time of NEC onset. At that moment, being carrier of a genetic variant that potentially increases NO production (i.e., the A-allele) might be of critical relevance because NO is a key regulator intestinal blood flow, protector of the mucosa, and modulator of the inflammatory response. In addition, the infants homoyzgous for the C-allele might be more susceptible to NEC and arginine supplementation might be particularly indicated in this group.

One limitation of our study is that we did not collect information on the feeding practices. Human milk protects against NEC and, although the preterm formulas currently used have concentrations of arginine similar to the human milk, arginine intake might be different depending on the infants diet. Plasma arginine levels are likely to represent a balance between arginine intake, arginine synthesis, and the demands of protein synthesis and the multiple metabolic pathways for arginine utilization. Enteric arginine synthesis appears to be necessary to cover neonatal requirements, because mammalian milk is a relatively poor source of arginine, whereas its precursors proline and glutamine are abundant. In fact, proline is the major contributor to arginine synthesis in human preterm infants. CPS, ornithine aminotransferase, and argininosuccinate synthetase are key enzymes in the control of de novo intestinal synthesis of arginine, which are already expressed in the mid gestation human intestine. However, hypoargininemia often develops in preterm infants, in particular if they are maintained on total parenteral nutrition, and it has been suggested that the intestine only produces arginine if substrate is supplied through enteral nutrition. In situations of reduced availability of the substrate L-arginine or the cofactor BH₄, NOS enzymatic activity becomes uncoupled, resulting in the production of superoxide instead of NO. Therefore, besides resulting in a paucity of NO, the uncoupled enzyme will generate free radicals resulting in further intestinal damage. In addition, it should be taken into account that, besides NO, arginine is a substrate for synthesis of many biologically important molecules including agmatine, polyamines, and creatine. These metabolites have roles in energy metabolism, gene expression, apoptosis, and cell proliferation and differentiation, which are crucial in intestinal homeostasis. Our present results suggest that functional genetic variations in the CPS enzyme might be, at least partially, the link between hypoargininemia and NEC in preterm infants. Alternatively, recent experimental evidences highlighted the role of argininosuccinate lyase, another enzyme involved in the intestinal synthesis of arginine, in the pathogenesis of NEC.

**Concluding remarks.** NEC affects only a minority of preterm infants, which suggest an individual susceptibility toward the disease. Genetic polymorphisms might be an important factor in this individual susceptibility. Our study provides further evidence that a functional variant of the CPS1 gene may contribute to NEC susceptibility. Nevertheless, NEC is a complex multifactorial disease and an isolated genetic derangement may not be sufficient to account for the entire spectrum of its pathophysiology. Given the importance of prematurity, intestinal function, immune defense, inflammatory signaling, and microcirculatory regulation mechanisms, potential variations in many genes could protect or leave a host infant susceptible to NEC. Future studies investigating the association of multiple SNPs and NEC may allow for the development of a laboratory genetic test that could predict, when environmental factors are properly assessed, the risk/probability of preterm infants developing NEC and lead to more targeted therapies.

**References**

1. Neu, J. & Walker, W. A. Necrotizing enterocolitis. *N Engl J Med* 364, 255–264 (2011).
2. Neu, J. Necrotizing enterocolitis: the mystery goes on. *Neonatology* 106, 289–295 (2014).
3. Wu, S. F., Caplan, M. & Lin, H. C. Necrotizing enterocolitis: old problem with new hope. *Pediatr Neonatol* 53, 158–163 (2012).
4. Treszl, A., Tulassay, T. & Vasarhelyi, B. Genetic basis for necrotizing enterocolitis–risk factors and their relations to genetic polymorphisms. *Front Biosci* 11, 570–580 (2006).
5. Ng, P. C. Biomarkers of necrotizing enterocolitis. *Semin Fetal Neonatal Med* 19, 33–38 (2014).
6. Lim, J. C., Golden, J. M. & Ford, H. R. Pathogenesis of neonatal necrotizing enterocolitis. *Pediatr Surg Int* 31, 509–518 (2015).
7. Gordon, P. V., Swanson, J. R., Attridge, J. T. & Clark, R. Emerging trends in acquired neonatal intestinal disease: is it time to abandon Bell’s criteria? *J Perinatol* 27, 661–671 (2007).
8. Berman, L. & Moss, R. L. Necrotizing enterocolitis: an update. *Semin Fetal Neonatal Med* **16**, 145–150 (2011).
9. Lin, P. W., Nasr, T. R. & Stoll, B. J. Necrotizing enterocolitis: recent scientific advances in pathophysiology and prevention. *Semin Perinatol* **32**, 70–82 (2008).
10. Reber, K. M., Nankervis, C. A. & Nowicki, P. T. Newborn intestinal circulation. Physiology and pathophysiology. *Clin Perinatol* **29**, 23–39 (2002).
11. Upperman, J. S. et al. Mechanisms of nitric oxide–mediated intestinal barrier failure in necrotizing enterocolitis. *Semin Pediatr Surg* **14**, 159–166 (2005).
12. Rhoads, J. M. et al. Arginine stimulates intestinal cell migration through a focal adhesion kinase dependent mechanism. *Gut* **53**, 514–522 (2004).
13. Wu, G. Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**, 1–17 (2009).
14. Wu, G., Jäger, L. A., Bazer, F. W. & Rhoads, J. M. Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications. *J Nutr Biochem* **15**, 442–451 (2004).
15. Pearlson, D. L. et al. Neonatal pulmonary hypertension–urea-cycle intermediates, nitric oxide production, and carbamoyl-phosphate synthetase function. *N Engl J Med* **344**, 1832–1838 (2001).
16. Amin, H. J. et al. Arginine supplementation prevents necrotizing enterocolitis in the premature infant. *J Pediatr* **140**, 425–431 (2002).
17. Becker, R. M. et al. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. *J Pediatr* **137**, 785–793 (2000).
18. Zamora, S. A. et al. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. *J Pediatr* **131**, 226–232 (1997).
19. Richir, M. C. et al. Low plasma concentrations of arginine and asymmetric dimethylarginine in premature infants with necrotizing enterocolitis. *Br J Nutr* **97**, 908–911 (2007).
20. Polycarpou, E. et al. Enteral L-Arginine Supplementation for Prevention of Necrotizing Enterocolitis in Very Low Birth Weight Neonates A Double-Blind Randomized Pilot Study of Efficacy and Safety. *JPEN J Parenter Enteral Nutr*, 0148607112471561 (2013).
21. Mitchell, K. et al. Arginine supplementation in prevention of necrotizing enterocolitis in the premature infant: an updated systematic review. *BMC Pediatr* **14**, 1 (2014).
22. Martinez, A. I., Perez-Arellano, L., Pekkala, S., Barcelona, B. & Cervera, J. Genetic, structural and biochemical basis of carbamyl phosphate synthetase I deficiency. *Mol Genet Metab* **01**, 311–323 (2010).
23. Summar, M. L. et al. Environmentally determined genetic expression: clinical correlates with molecular variants of carbamyl phosphate synthetase I gene. *Gene* **311**, 51–57 (2003).
24. Summar, M. L. et al. Relationship between carbamoyl-phosphate synthetase genotype and systemic vascular function. *Hypertension* **43**, 186–191 (2004).
25. Canter, J. A. et al. Genetic variation in the mitochondrial enzyme carbamyl–phosphate synthetase I predisposes children to increased pulmonary artery pressure following surgical repair of congenital heart defects: a validated genetic association study. *Mitochondrion* **7**, 204–210 (2007).
26. Moonen, R. M. et al. Carbamylphosphate synthetase polymorphisms as a risk factor for necrotizing enterocolitis. *Pediatr Res* **62**, 188–190 (2007).
27. Moonen, R. M. et al. The T1405N carbamylphosphate synthetase polymorphism does not affect plasma arginine concentrations in preterm infants. *PloS one* **5**, e10792 (2010).
28. Jobe, A. H. & Bancalari, E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* **163**, 1723–1729 (2001).
29. Papile, L. A., Burstein, J., Burstein, R. & Koffler, H. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *J Pediatr* **92**, 529–534 (1978).
30. Clarke, G. M. et al. Basic statistical analysis in genetic case-control studies. *Nat Protoc* **6**, 121–133 (2011).
31. Das, A. et al. Methodological issues in the design and analyses of neonatal research studies: Experience of the NICHD Neonatal Research Network. *In Semin Perinatol* **40**, 374–384 (2006).
32. Yee, W. H. et al. Incidence and timing of presentation of necrotizing enterocolitis in preterm infants. *Pediatrics* **129**, e298–e304 (2012).
33. Llanos, A. R. et al. Epidemiology of necrotizing enterocolitis: a population-based study. *Pediatr Perinat Epidemiol* **16**, 342–349 (2002).
34. Hartila, J. A. et al. Genome-wide association study and targeted metabolomics identifies sex-specific association of CPS1 with coronary artery disease. *Nat Commun* **7**, 10558 (2016).
35. Ahuja, V. & Powers-Lee, S. Human carbamyl-phosphate synthetase: insight into N-acetylglutamate interaction and the functional effects of a common single nucleotide polymorphism. *J Inherit Metab Dis* **31**, 481–491 (2008).
36. Morgan, C. & Burgess, L. High protein intake does not prevent low plasma levels of conditionally essential amino acids in very preterm infants receiving parenteral nutrition. *JPEN J Parenter Enteral Nutr*, 0148607115594009 (2015).
37. Kohler, E. S. et al. The human neonatal small intestine has the potential for arginine synthesis; developmental changes in the expression of arginine-synthetizing and -catabolizing enzymes. *BMC Dev Biol* **8**, 107 (2008).
38. Tomlinson, C., Rafii, M., Sgro, M., Bull, R. O. & Pencharz, P. Arginine is synthesized from proline, not glutamate, in enterally fed human preterm neonates. *Pediatr Res* **69**, 46–50 (2011).
39. Sullivan, J. C. & Pollock, J. S. Coupled and uncoupled NOS: separate but equal? Uncoupled NOS in endothelial cells is a critical pathway for intracellular signaling. *J Cell Physiol* **188**, 571–579 (2001).
40. Premkumar, M. H. et al. Argininosuccinate lyase in enterocytes protects from development of necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol* **307**, G347–G354 (2014).
41. Franklin, A. L. et al. Are Immune Modulating Single Nucleotide Polymorphisms Associated with Necrotizing Enterocolitis? *Sci Rep* **5**, 18369 (2015).
42. Sampath, V. et al. SIGIRR Genetic Variants in Premature Infants With Necrotizing Enterocolitis. *Pediatrics* **135**, e1530–e1534, doi: 10.1542/peds.2014-3386 (2015).
43. Zhou, W. et al. Association of neonatal necrotizing enterocolitis with myeloid differentiation-2 and GM2 activator protein genetic polymorphisms. *Mol Med Rep* **12**, 974–980 (2015).
44. Henegar, E. et al. Genetic variants of the interleukin-18 promoter region (~607) influence the course of necrotising enterocolitis in very low birth weight neonates. *Eur J Pediatr* **161**, 410–411 (2002).
45. Bokodi, G., Derzsbach, L., Banyasz, I., Tulassay, T. & Varasheily, B. Association of interferon gamma T+874A and interleukin 12 p40 promoter CTCTAA/GC polymorphism with the need for respiratory support and perinatal complications in low birthweight neonates. *Arch Dis Child Fetal Neonatal Ed* **92**, F25–F29 (2007).
46. Prencipe, G. et al. Association between mannose-binding lectin gene polymorphisms and necrotizing enterocolitis in preterm infants. *J Pediatr Gastroenterol Nutr* **55**, 160–165 (2012).
47. Sampath, V. et al. The NFKB1 (g.-24519delATTG) variant is associated with necrotizing enterocolitis (NEC) in premature infants. *J Surg Res* **169**, e51–e57 (2011).
49. Treszl, A. et al. Lower prevalence of IL-4 receptor alpha-chain gene G variant in very-low-birth-weight infants with necrotizing enterocolitis. J Pediatr Surg 38, 1374–1378 (2003).

50. Henderson, G. et al. Cytokine gene polymorphisms in preterm infants with necrotising enterocolitis: genetic association study. Arch Dis Child Fetal Neonatal Ed 94, F124–F128 (2009).

51. Bányász, I. et al. Genetic polymorphisms for vascular endothelial growth factor in perinatal complications. Eur Cytokine Netw 17, 266–270 (2006).

Acknowledgements
This work was supported by a grant from “Fundación de Investigación Médica Mutua Madrileña”.

Author Contributions
R.M.M. participated in the design of the study, collection of data, analysis and interpretation of data, and drafted the first version of the manuscript. G.C., M.J.H., G.E.G.-L., and F.M. collected data, participated in the design of the study and the interpretation of the data, and helped to draft the manuscript. E.V. conceived the study, participated in the design of the study, collection of data, analysis and interpretation of data, and drafted the final version of the manuscript. All authors read and approved the final manuscript.

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

How to cite this article: Moonen, R. M. et al. Association between the p.Trh1406Asn polymorphism of the carbamoyl-phosphate synthetase 1 gene and necrotizing enterocolitis: A prospective multicenter study. Sci. Rep. 6, 36999; doi: 10.1038/srep36999 (2016).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2016