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# Carbamoyl Phosphate Synthetase Polymorphisms as a Risk Factor for Necrotizing Enterocolitis

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**ABSTRACT:** A C-to-A nucleotide transversion (T1405N) in the gene that encodes carbamoyl-phosphate synthetase 1 (CPS1) has been correlated with low plasma concentrations of L-arginine in neonates. As plasma L-arginine concentrations are decreased in premature infants with necrotizing enterocolitis (NEC), we hypothesized that the CPS1 T1405N polymorphism would correlate with the presence of NEC. We analyzed the CPS1 genotypes for the T1405N polymorphism in 17 preterm infants (≤30 wk and <1500 g) with established NEC, 34 preterm infants without NEC, and 25 healthy term infants. Distribution of genotypes did not differ between the NEC population (CC:AC:AA = 70.6%:23.5%:5.9%) and the preterm control group (CC:AC:AA = 41.2%:35.3%:23.5%; $p = 0.110$) or the term group (CC:AC:AA = 44%:48%:8%; $p = 0.228$). The C allele frequency was 82.4% in NEC and 58.8% in preterm control infants ($p = 0.018$) and analysis for linear trend demonstrated that incidence of NEC increased with the number of C alleles ($p = 0.037$). The CC genotype was associated with an increased risk of NEC in the preterm infants [odds ratio (OR) = 3.43, 95% confidence interval (CI): 1.01–11.49, $p = 0.048$), when compared with the grouped together AA/AC genotypes. These data suggest that the CPS1 T1405N polymorphism may be associated with the risk of NEC in preterm infants. **(*Pediatr Res* 62: 188–190, 2007)**

NEC remains a leading cause of morbidity and mortality in neonatal intensive care units. Although several predisposing factors have been identified, such as prematurity, enteral feeding, and infection, the pathogenesis of NEC remains elusive and accepted prevention and treatment strategies are lacking (1–8).

Over the past several years, nitric oxide (NO) has received considerable attention in the pathophysiology of NEC as it pertains to the regulation of intestinal blood flow and plays a role in the maintenance of mucosal integrity and intestinal barrier function as well as in postinjury intestinal reparation (7,9). NO is generated by NO synthase during the enzymatic conversion of L-arginine to L-citrulline. It has been suggested that the availability of L-arginine may be a factor limiting NO production, predisposing the immature gut to NEC. Several studies demonstrated that plasma arginine concentrations are decreased in premature infants with NEC (10–13). Moreover, arginine supplementation reduced the incidence of NEC (10). However, the cause of the relative arginine deficiency in premature infants with NEC remains unexplained.

Carbamoyl phosphate synthetase I (*CPS1*, OMIM 608307) is the rate-limiting enzyme catalyzing the first committed step of the urea cycle (14,15). A number of *CPS1* polymorphisms have been found that appear to result in functional consequences affecting the downstream availability of the urea-cycle intermediates, including L-arginine (14,15). A C-to-A nucleotide transversion at position 4332 of exon 36 of the *CPS1* gene (chromosome 2q35) has been described, resulting in the substitution of asparagine for threonine at position 1405 (T1405N), which is the critical *N*-acetylglutamate–binding domain of the enzyme (14,15). Pearson *et al.* (14) found that neonates (>35 wk of gestation) homozygous for the threonine 1405 variant of the CPS1 (CC genotype) had lower plasma L-arginine concentrations than neonates homozygous for the asparagine 1405 variant . In the present study, we hypothesized that the distribution of the polymorphism at position 1405 in the *CPS1* gene would vary between preterm infants with and those without NEC.

## METHODS

***Patients.*** We performed a retrospective case-control study based on a review of newborns infants with NEC hospitalized in the Neonatology Department of the University Hospital of Maastricht during the period January 1995 to March 2006. Data on clinical characteristics were retrieved from a computerized database. From a population of 719 newborns with gestational age ≤30 wk and birth weight <1500 g, 36 patients with the diagnosis of established NEC [stage II and III according to the criteria proposed by Bell *et al.* (1) and modified by Walsh and Kliegman (8)] were identified. Nine of the 36 patients had died, and in 10 cases the patients were not located or the parents refused to let their children participate in the study. Two controls were matched to each of the 17 NEC cases based on birth weight and gestational age. The incidence of other neonatal complications, such as infant respiratory distress syndrome, bronchopulmonary dysplasia, hypotension, sepsis, electrolyte disturbances, intraventricular hemorrhage/periventricular leukomalacia, convulsions, patent ductus arteriosus, and retinopathy of prematurity as well as duration of mechanical ventilation, the duration of supplemental oxygen, and the administration of pre- and postnatal steroids and indomethacin were compared between the two preterm groups. A second control group of 25 healthy term infants was formed to examine the distribution of the *CPS1* genotype in the local healthy population. All infants in this study were white. The study was approved by an Institutional Ethical Committee (dossier MEC 04-140) and written informed consent from the parents was obtained.

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**Abbreviations: CPS1,** carbamoyl phosphate synthetase 1; **NEC,** necrotizing enterocolitis

***Samples and Genotyping.*** Buccal cell samples for DNA testing were obtained with a sterile OmniSwab (Whatman) and collected in Eppendorf sterile PCR tubes. DNA was extracted using standard methods and stored at −20°C until genotyping. A 214-bp fragment encompassing the 4332 C>A polymorphism in exon 36 of the *CPS1* gene was amplified using polymerase chain reaction (PCR). Primers used were (forward) GCM357 5′-TAAATGCAGCTGTTTG CCAC-3′ and (reverse) GCM358 5′-GACTTG-CAATCAAGTAAGGTGAAA-3′. The PCR mix consisted of 1× GeneAmp PCR Buffer II (Perkin Elmer), 0.2 mM deoxyribonucleoside triphosphate (Pharmacia), 1.5 mM $MgCl_2$ (Perkin Elmer), 250 nM of both primers, and 0.025 U/μL of AmpliTaq Gold (Perkin Elmer). 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.*** Discrete variables are expressed as counts or percentages, and differences were compared using the $\chi^2$ test. Results for continuous variables are expressed as mean (SD) or, if variables were not normally distributed, as median (interquartile range) and compared with the *t* test or the Mann-Whitney *U* test, as appropriate.

Differences in allelic frequencies and genotype distributions between the investigated populations, as well as the presence of Hardy-Weinberg equilibrium, were tested by the $\chi^2$ test. The $\chi^2$ analysis was performed for linear trend conformity, according to the presence of zero, one, or two C alleles. OR and 95% CIs were calculated as a measure of association between genotype and the presence of NEC in preterm infants. To be able to correct for known risk factors for NEC (gestational age and birth weight), binary logistic regression analysis was performed. A $p$ value of $<0.05$ was considered statistically significant.

## RESULTS

***Patient characteristics.*** Seventeen preterm infants with NEC (Bell stage II or higher) and 34 preterm infants without NEC were enrolled in the study. There were no significant differences in baseline characteristics and neonatal complications between the two preterm populations (Table 1). In addition, there was no significant difference in administration of prenatal or postnatal steroids or indomethacin. Stage II NEC was present in nine infants and stage III in eight infants. Suspected NEC was present in three infants of the preterm control group. A second control group consisted of 25 healthy term infants (12 males) with a mean birth weight of 3330 g (SD = 464) and a mean gestational age of 39.4 wk (SD = 1.5).

***Analysis of genotypes and allelic frequencies.*** Genotype frequencies according to the study population are summarized in Table 2. The distribution of the *CPS1* genotypes for the polymorphism at position 1405 in each studied population fulfilled Hardy-Weinberg criteria. Genotype distributions (CC denotes homozygosity for the C-encoded Thr1405 variant, AA homozygosity for the A-encoded Asn1405 variant, and AC heterozygosity for this polymorphism at position 1405) of the term control population (CC:AC:AA = 44%:48%:8%) did not differ ($p = 0.836$) from those reported by Summar *et al.* (15) in 460 healthy adults in the U.S.A (CC:AC:AA = 42%:46%:12%). The preterm control population showed a higher proportion of the AA genotype (CC:AC:AA = 41.2%:35.3%:21.5%), but comparison with the term control population did not reach statistical significance ($p = 0.116$).

The distribution of genotypes in the NEC population (CC:AC:AA = 70.6%:23.5%:5.9%) was not significantly different when compared with the term control group ($p = 0.228$) or the preterm control group ($p = 0.110$). The frequency of the C allele was 82.4% in NEC infants and 58.8% in the preterm control group ($p = 0.018$), and the analysis for a linear trend regarding the presence of zero, one, or two C alleles demonstrated that the incidence of NEC increased with the number of C alleles ($p = 0.037$). In addition, preterm infants with the CC genotype presented a higher risk of NEC (unadjusted OR = 3.43, 95% CI: 1.01–11.49, $p = 0.048$) than grouped together homozygous and heterozygous carriers of the A-encoded allele (AA/AC genotype). In the logistic regression model, neither gestational age ($p = 0.952$) nor birth weight ($p = 0.650$) was significantly associated with NEC. However, when adjusted for birth weight and gestational age, the association between CC genotype and NEC did not remain significant (adjusted OR = 3.246, 95% CI: 0.91–11.52, $p = 0.069$).

Although the CC genotype was present in seven of the eight infants with stage III NEC and in five of the nine infants with stage II NEC, there was not a significant increased risk of stage III NEC *versus* stage II NEC in the carriers of the CC genotype (OR = 0.18, 95% CI: 0.02–1.71, $p = 0.149$) and linear trend analysis demonstrated that the presence of the C allele was not significantly increased in the stage III NEC patients ($p = 0.496$). None of the infants with stage II NEC and only one infant with stage III NEC presented the AA genotype.

**Table 1.** *Baseline characteristics and neonatal complications in the preterm populations*

| | NEC group ($n$ = 17) | Preterm control group ($n$ = 34) | $p$ |
|---|---|---|---|
| Birth weight, g | 912 (SD = 169) | 978 (SD = 297) | 0.315 |
| Gestational age, wk | 28.4 (26.9–29.4) | 28.6 (27.0–29.2) | 0.976 |
| Male sex | 8 (47) | 22 (65) | 0.227 |
| Apgar score | | | |
| After 1 min | 5 (4–9) | 5 (3–8) | 0.470 |
| After 5 min | 9 (7–9) | 8 (6–9) | 0.751 |
| Supplemental oxygen, d | 9 (1–12) | 11.5 (1–44) | 0.303 |
| Mechanical ventilation, d | 10 (3–19) | 4 (0–17) | 0.497 |
| IRDS | 10 (59) | 27 (79) | 0.120 |
| BPD | 5 (29) | 12 (35) | 0.674 |
| Hypotension | 8 (47) | 22 (65) | 0.227 |
| Sepsis | 15 (88) | 24 (71) | 0.161 |
| Electrolyte disturbances | 13 (76) | 25 (74) | 0.820 |
| IVH/PVL | 7 (41) | 12 (35) | 0.682 |
| Convulsions | 5 (29) | 4 (12) | 0.119 |
| PDA | 2 (12) | 7 (21) | 0.436 |
| ROP | 7 (41) | 6 (18) | 0.069 |

IRDS, infant respiratory distress syndrome; BPD, bronchopulmonary dysplasia; IVH/PVL, intraventricular hemorrhage/periventricular leukomalacia; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity.

Results are expressed as mean (SD), median (interquartile range), or absolute numbers of patients (percentage).

**Table 2.** *Distribution of the CPS1 genotypes for the polymorphism at position 1405*

| Population | No. of infants | CC | AC | AA |
|---|---|---|---|---|
| Summar *et al.* (15) | — | — (42) | — (46) | — (12) |
| Term control group | 25 | 11 (44.0) | 12 (48.0) | 2 (8.0) |
| Preterm control group | 34 | 14 (41.2) | 12 (35.3) | 8 (23.5) |
| NEC group | 17 | 12 (70.6) | 4 (23.5) | 1 (5.9) |

Results are expressed as absolute numbers of patients (percentage). CC denotes homozygosity for the C-encoded Thr1405 variant, AA homozygosity for the A-encoded Asn1405 variant, and AC heterozygosity for this polymorphism.

OR = 3.43 (95% CI: 1.01–11.49) for preterm with NEC versus preterm without NEC (CC *vs* AC + AA).

No significant difference in the prevalence of the CC genotype was found when comparing the NEC population with the term control population ($p = 0.089$, analysis for linear trend $p = 0.145$) or when comparing the preterm control population with the term control population ($p = 0.828$, analysis for linear trend $p = 0.338$).

## DISCUSSION

Identifying premature infants at increased risk of developing NEC remains an important but elusive objective. Prematurity is the only factor consistently found in epidemiologic studies to be an independent determinant of NEC (3,4,6,9). However, NEC affects only a minority of premature infants, which suggests an individual susceptibility toward the disease. Genetic polymorphisms might be an important factor in this individual susceptibility (16).

Using case-control methodology, we examined the relationship between a single nucleotide polymorphism (C-to-A nucleotide transversion in exon 36) of the gene that encodes CPS1, the rate-limiting enzyme in the urea cycle production of the NO precursor L-arginine, and the presence of NEC in preterm infants. We found that patients with NEC showed an overrepresentation of the C-encoded variant of *CPS1*. Linear trend analysis demonstrated the quantitative increase in C allele in NEC and homozygosity for C allele increased the risk of having NEC. Although the case and the controls were well matched and there was no significant association of gestational age or birth weight with NEC in the logistic regression model, when adjusted for these two known risk factors for NEC, the OR did not remain significant. Therefore, whether the CC genotype is an independent risk factor for NEC needs to be confirmed in studies involving larger populations.

Reduced serum concentrations of L-arginine have been reported in term neonates with the CC genotype (14) and in preterm infants with NEC (10–12). Although the explanation may lie in other enzymes or transporters in the urea cycle and NO pathway, our findings might represent a connection point between these previous findings and suggest a potential genetic vulnerability to NEC.

Endogenous synthesis of arginine is crucial for maintaining arginine homeostasis in neonates (13). Further, both metabolic and molecular studies indicate that the underdevelopment of intestinal arginine synthesis may be primarily responsible for hypoargininemia in preterm neonates (13). In premature infants, in whom the urea cycle is not fully developed (13,14), further decrease in arginine and citrulline production due to genetically determined variations in CPS1 function could affect the production of NO and the subsequent development of NEC. In this sense, the low prevalence of AA homozygosity among the infants with NEC is noteworthy. Similarly, Pearson *et al.* (14) reported a lack of AA homozygosity among neonates with pulmonary hypertension and Summar *et al.* (17) reported a reduced rate of postcardiac surgery–related pulmonary hypertension and hepatic veno-occlusive disease after bone marrow transplantation in patients with the AA genotype. They speculate that individuals with the AA genotype may have an advantage in terms of urea-cycle function and interrelated metabolic processes, especially under conditions of environmental stress.

NEC is a complex multifactorial disease, and an isolated genetic derangement may not be sufficient to account for the entire spectrum of its pathophysiology (9). Previous studies have associated NEC with genetic polymorphisms of interleukin (IL)-4 receptor $\alpha$ chain and IL-18 (16,18,19). Our findings provide the preliminary evidence that a single nucleotide polymorphism in the *CPS1* gene is associated with the risk of NEC. However, our study is limited by its retrospective nature, the relatively small size of the sample, its use of data from a single tertiary care institution, the lack of data of patients who died of NEC, and the lack of confirmation of the relationship between *CPS1* polymorphisms and levels of L-arginine in preterm infants. Therefore, our data should be confirmed in larger populations, and the relationships among *CPS1* polymorphisms, L-arginine, and the incidence of NEC warrant further investigation in a prospective cohort study.

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