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Polymorphisms in urea cycle enzyme genes are associated with persistent pulmonary hypertension of the newborn

Dinushan C. Kaluarachchi[1], Caitlin J. Smith[2], Jonathan M. Klein[1], Jeffrey C. Murray[1], John M. Dagle[1] and Kelli K. Ryckman[2]

BACKGROUND: Persistent pulmonary hypertension of the newborn (PPHN) is characterized by elevated pulmonary vascular resistance. Endogenous nitric oxide is critical for regulation of pulmonary vascular resistance. Nitric oxide is generated from L-arginine, supplied by the urea cycle (UC). We hypothesized that polymorphisms in UC enzyme genes and low concentrations of UC intermediates are associated with PPHN.

METHODS: We performed a family-based candidate gene analysis to study 48 single-nucleotide polymorphisms (SNPs) in six UC enzyme genes. Genotyping was carried out in 94 infants with PPHN and their parents. We also performed a case–control analysis of 32 cases with PPHN and 64 controls to identify an association between amino-acid levels on initial newborn screening and PPHN.

RESULTS: Three SNPs (rs41272673, rs4399666, and rs2287599) in carbamoyl phosphate synthase 1 gene (*CPS1*) showed a significant association with PPHN ($P = 0.02$). Tyrosine levels were significantly lower ($P = 0.003$) and phenylalanine levels were significantly higher ($P = 0.01$) in cases with PPHN. There was no difference in the arginine or citrulline levels between the two groups.

CONCLUSIONS: This study suggests an association ($P < 0.05$) between SNPs in *CPS1* and PPHN. These findings warrant further replication in larger cohorts of patients.

Persistent pulmonary hypertension of the newborn (PPHN) is characterized by sustained elevation of pulmonary vascular resistance. High pulmonary vascular resistance in the setting of normal or low systemic vascular resistance leads to extrapulmonary right-to-left shunting across the patent ductus arteriosus and/or foramen ovale. This can result in life-threatening hypoxemia, right ventricular failure, and even death, making PPHN a serious neonatal disease. PPHN results from failure of normal circulatory transition at birth. It occurs mostly in term or near-term infants and occurs in 1.9–2.8 per 1,000 live births (1–3). PPHN can be idiopathic or can result secondary to neonatal pulmonary diseases such as congenital diaphragmatic hernia, pulmonary hypoplasia, respiratory distress syndrome, pneumonia, and meconium aspiration syndrome (2).

Inhaled nitric oxide is the mainstay of current PPHN treatment. It is a potent selective pulmonary vasodilator. Endogenous nitric oxide has a critical role in the regulation of pulmonary vascular resistance and transition of pulmonary circulation at birth (4,5).

The main function of the urea cycle (UC) is to convert excess nitrogen in the form of ammonia to urea, which is excreted through the kidneys (6). The following five key enzymes make up the UC: carbamoyl phosphate synthase 1 (CPS1), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS1), argininosuccinate lyase (ASL), and arginase 1 (ARG1; Figure 1). An additional enzyme, N-acetylglutamate synthase (NAGS), provides CPS1 with its essential cofactor. Nitric oxide is endogenously synthesized from the precursor L-arginine by nitric oxide synthase (7). L-arginine is an amino acid supplied by the UC, and is the link between the UC and PPHN. Low L-arginine levels can theoretically decrease nitric oxide synthesis and lead to pulmonary hypertension.

Previous research has shown that polymorphisms in UC enzyme genes are associated with PPHN. In one study including 31 subjects, Pearson *et al.* discovered that the Thr1405 variant of *CPS1* was associated with PPHN (8). A more recent study including 36 subjects evaluated another form of pulmonary hypertension in neonates, pulmonary hypertension associated with bronchopulmonary dysplasia, and discovered an ARG1 single-nucleotide polymorphism (SNP) that may be protective against pulmonary hypertension (9). Previous studies have also shown that low plasma concentrations of arginine and nitric oxide metabolites are associated with PPHN (8,10).

In the current study, we hypothesize that polymorphisms in UC genes and low concentrations of specific UC intermediates are associated with PPHN. We performed a family-based candidate gene analysis to investigate the association between SNPs in UC enzyme genes and PPHN. We also performed a case–control study to identify the association between amino-acid levels and PPHN.

[1]Department of Pediatrics, University of Iowa, Iowa City, Iowa; [2]Department of Epidemiology, College of Public Health, University of Iowa, Iowa City, Iowa. Correspondence: Dinushan C. Kaluarachchi (dinushan-kaluarachchi@uiowa.com)

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METHODS

Study Population

Subjects for this study were identified from a neonatal registry of all high-risk infants treated at the University of Iowa Hospital and Clinics. All participants or their guardians provided signed informed consent for study enrollment in accordance with the protocols approved by the University of Iowa Institutional Review Board (IRB 200307031). Existing samples from patients with pulmonary hypertension were obtained from the neonatal intensive care unit (NICU) and Pediatric Repository, a biobank of data and DNA samples from neonates born at or transferred to the University of Iowa Hospital and Clinics (IRB 201411731). Patients with pulmonary hypertension and their families (parents and siblings), who did not have the opportunity to be enrolled in the NICU unit and Pediatric Repository, were contacted to provide samples for DNA extraction.

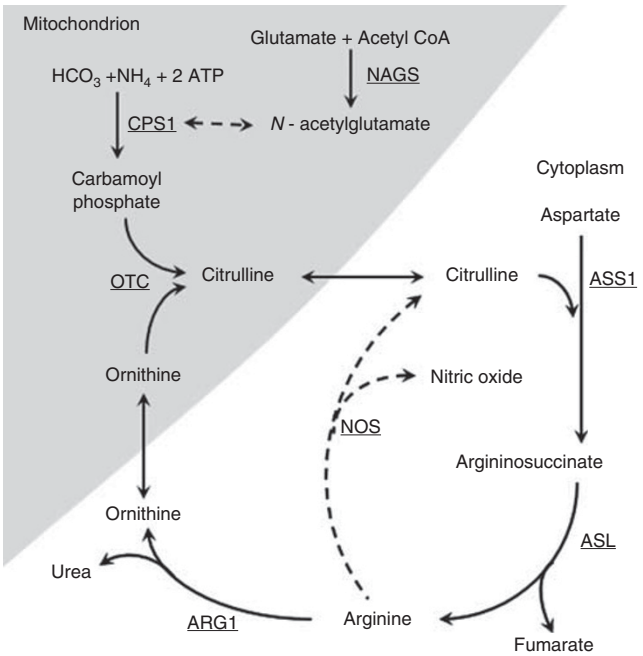

Figure 1. Urea cycle and nitric oxide pathway. NAGS, N-acetylglutamate synthase; CPS1, carbamoyl phosphate synthase 1; OTC, ornithine transcarbamylase; ASS1, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG1, arginase 1; NOS, nitric oxide synthase.

A total of 94 neonates diagnosed with and treated for PPHN between 1993 and 2010 and their families were identified for genotyping and analysis. Inclusion criteria were neonates with hypoxemic respiratory failure with the clinical diagnosis of pulmonary hypertension. Exclusion criteria were gestational age of <35 weeks, multiple major congenital anomalies, congenital diaphragmatic hernia, cyanotic heart disease, and/or the inability to obtain a DNA sample from the neonate and at least one parent.

Neonates with hypoxic respiratory failure were diagnosed with PPHN by the medical team using echocardiography, preductal/postductal oxygen saturation difference of $>10\%$, and/or a clinical response to inhaled nitric oxide. Echocardiographic findings consistent with PPHN included elevated pulmonary artery pressure as compared with systemic pressure, right-to-left or bidirectional shunting through patent ductus arteriosus, and/or patent foramen ovale. Approximately 30% of the study cohort had mild PPHN and were treated with only supplemental oxygen. Some neonates may have received enteral feeds and/or total parenteral nutrition.

DNA was extracted from venous blood, cord blood, buccal swabs, or saliva. Demographic and clinical information was collected through medical chart abstraction.

SNP Genotyping

We performed a family-based candidate gene analysis to study 48 SNPs (**Table 1**) in six UC enzyme genes (*CPS1, NAGS, ASS, ASL, ARG1,* and *OTC*). SNPs were selected according to known functional impact and according to a catalog of variants of UC enzyme genes by Mitchell *et al.* (6).

SNPs were genotyped using TaqMan probes (Applied Biosystems, Foster City, CA) and the Dynamic Array Integrated Fluidic Circuits (Fluidigm, San Francisco, CA). These genotyping assays included primers to amplify the region containing the SNP of interest and two TaqMan Minor Groove Binder probes that are specific to the polymorphic variant alleles at the site labeled with different fluorescent reporter dyes, FAM (5-carboxyfluorescein) and VIC (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein). All reactions were performed using standard conditions supplied by Fluidigm. Following thermocycling, fluorescence levels of the FAM and VIC dyes were measured using the EP1 Reader (Fluidigm) and genotypes were scored using the Fluidigm Genotyping Analysis software. Genotypes were entered into a laboratory database (Progeny, South Bend, IN) to generate data sets for analysis. Genotypes for SNPs were annotated according to alleles described in National Center for Biotechnology Information.

Haplotyping

Haplotype analysis of SNPs in the same gene or region was used to evaluate regional associations with PPHN. Haplotype analysis using sliding windows of two to six SNPs across the region was performed.

Table 1. List of genes and SNPs

Gene	Gene names	Chromosome	SNPs
NAGS	N-acetylglutamate synthase	17	rs228770, rs228771, rs228773, rs186636
CPS1	Carbamoyl-phosphate synthase 1	2	rs16844619, rs17824552, rs13020941, rs6435577, rs4399666, rs2287600, rs41272667, rs2287599, rs7607412, rs7572146, rs6724941, rs3213784, rs1047891, rs6748782, rs41272673, rs2216405
OTC	Ornithine transcarbamylase	X	rs5963409, rs5917574, rs5963417, rs5917591, rs5963427
ASS1	Argininosuccinate synthetase	9	rs11243372, rs7860909, rs1215985, rs11243414, rs10901072, rs2297599, rs652313, rs540140, rs1215940, rs1215970, rs12555797, rs10901080, rs666174, rs634432, rs7018779, rs10114424
ASL	Argininosuccinate lyase	7	rs160648, rs313829, rs2292938
ARG1	Arginase 1	6	rs2781667, rs2246012, rs3850245, rs34504481

SNP, single-nucleotide polymorphism.

Case–Control Study of Amino-Acid Levels

Data on the levels of nine amino acids including arginine and citrulline, measured by tandem mass spectrometry (MS/MS), were obtained from routine newborn screening from the State of Iowa Hygienic Laboratory. Newborn screening data for 32 of the 94 cases enrolled in this study were linked to their clinical medical record data. Sixty-two cases were excluded from the original 94 patients studied in the genotyping phase (38 were born before August of 2003 before the implementation of MS/MS to the Iowa newborn screening program; 24 were excluded as the initial newborn screening test was obtained either before 24 h from birth or after 72 h from birth). Data on analyte measurements were linked to the clinical medical record data. Data on the 64 unaffected controls matched for gender, gestational age, and year of birth were provided by the State of Iowa Hygienic Laboratory. These neonates were enrolled in the NICU and Pediatric Repository (IRB 201411731).

Approval for use of the newborn screening data was granted by the Iowa Department of Public Health Research and Ethics Review Committee.

Statistical Analysis

Statistical analyses of the genotype data were performed with a transmission disequilibrium test to determine nonrandom allele transmission from parents to offspring using the PLINK software (11). Haplotype analysis was also performed using PLINK software. A Bonferroni significance threshold of $P = 0.001$ (0.05/48 markers) was used to correct for multiple testing.

The case–control study analysis was performed with SAS version 9.3 (SAS Institute, Cary, NC). Demographic and clinical characteristics were compared between cohorts using χ^2-tests for categorical variables and Student's t-tests for continuous variables. A P value of < 0.05 was considered statistically significant for case–control study analysis.

RESULTS

Genotyping was completed in 94 cases and their families (total of 265 individuals). Seventy-seven of these were part of a complete triad (affected neonate and both parents) and seventeen of these were part of a dyad (affected neonate and one parent). The majority of the probands were male (65%) and Caucasian (88%). Average gestational age was 38.3 (±1.8) weeks and average birth weight was 3,532 g (±626). Nineteen (20%) of the infants were born before 37 weeks' gestation. Sixty-six infants (70%) were treated with inhaled nitric oxide and nine (10%) of them required extracorporeal membrane oxygenation. A summary of demographic and clinical characteristics is provided in **Table 2**.

Three SNPs in *CPS1* gene (rs41272673, rs4399666, and rs2287599) demonstrated a significant association with PPHN ($P = 0.02$; **Supplementary Table S1 online**). The haplotype analysis demonstrated a significant association for the CCACTA haplotype at rs2287599, rs7607412, rs7572146, rs6724941, rs3213784, and rs1047891 of *CPS1* ($P = 0.006$). None of the SNPs in the other five UC enzyme genes were associated with PPHN. None of the SNPs reached a formal level of significance when the conservative Bonferroni correction is applied for multiple observations.

Baseline characteristics of the case and control study groups were similar (**Table 3**). The mean tyrosine level was significantly lower in the cases with PPHN compared with the control group ($P = 0.003$). The mean phenylalanine level was significantly higher in the cases with PPHN ($P = 0.01$). There was no significant difference in the other metabolite levels between the two groups.

Table 2. Demographic and clinical characteristics of study population for genotyping

	Cases ($N = 94$), number (%) or mean (± SD)
Infant characteristics	
Male gender	61 (65)
Caucasian race	83 (88)
Birth weight (g)	3532 (±626)
Gestational age (weeks)	38.3 (±1.8)
Postdate (>40 weeks)	13 (17)
Premature (<37 weeks)	19 (20)
Apgar <7 at 5 min	18 (19)
Meconium aspiration syndrome	26 (28)
Pneumothorax	28 (30)
Pneumonia	19 (20)
Treated with iNO	66 (70)
Extracorporeal membrane oxygenation	9 (10)
Maternal characteristics	
Maternal age (years)	28.7 (±5.7)
Cesarean section	50 (53)
Complications of delivery	45 (48)
Cohort descriptors	
Triads (infant, mother, and father)	77 (82)
Inborn	24 (26)

iNO, inhaled nitric oxide.

DISCUSSION

PPHN is a major clinical problem in the neonatal intensive care units and can contribute significantly to morbidity and mortality in both term and near-term neonates (1,3,12–14). Various risk factors such as cesarean delivery, late-preterm- or post-term birth, large for gestational age, maternal black or Asian race, overweight, and maternal diabetes are associated with PPHN (15), but the exact etiology is unknown. Genetic polymorphism may increase susceptibility to PPHN (16,17). In the current study, we report an association ($P < 0.05$) between SNPs in the *CPS1* gene and PPHN.

Variants in UC enzyme genes have been associated with various cardiopulmonary diseases in children and adults. These cardiopulmonary diseases include asthma and bronchodilator response (18,19), myocardial infarction (20), hypertension (21), increased pulmonary artery pressure following surgical repair of congenital heart defects (22), hepatic veno occlusive diseases following bone marrow transplantation (23,24), and PPHN (8).

Table 3. Case–control analysis of amino-acid levels on initial newborn screening

	Cases ($N=32$) number (%) or mean (±SD)	Controls (N = 64) number (%) or mean (±SD)	*P* value
Male gender	20 (62.5)	40 (62.5)	1.00
Caucasian race	29 (90.6)	58 (90.6)	0.60
Birth weight (g)	3378 (586)	3257 (546)	0.32
Gestational age (weeks)	37.9 (1.7)	37.9 (1.7)	1.00
Age at initial newborn screening (h)	30.9 (8.7)	28.3 (6.1)	0.14
On TPN at the time of newborn screening	2 (6.25)	2 (3.1)	0.60
Ala	216.7 (±106.9)	196.1 (±74.8)	0.33
Arg	4.2 (±3.3)	4.5 (±2.4)	0.58
Cit	9.2 (±3.8)	9.9 (±2.6)	0.35
Glu	148.8 (±50.8)	148.1 (±38.5)	0.93
Leu	108.4 (±52.6)	100.9 (±31.6)	0.46
Met	23.6 (±14.4)	22.1 (±7.8)	0.56
Phe	67.3 (±21.1)	57.4 (±12.2)	0.01
Tyr	53.4 (±34.1)	78.7 (±41.8)	0.003
Val	88.7 (±39.6)	81.3 (±21.9)	0.33

TPN, total parenteral nutrition.
Metabolite levels are presented in μmol/l units.

In the current study, we performed a family-based candidate gene analysis including 94 neonates with PPHN and their parents. The family-based study design identifies risk alleles by examining nonrandom allele transmission from parents to offspring, avoiding issues associated with population stratification. Previous genetic studies on this topic have been conducted using case–control study designs. In genetic case–control studies, the frequency of alleles or genotypes is compared between the cases and controls. Genotype frequencies may vary among different ethnic populations. As a result, if case and control populations are not well-matched for ethnicity, the analysis can generate inaccurate results. The family-based study design avoids this potential confounding effect. We also studied 48 SNPs across all six UC enzyme genes—a significantly higher number of markers than previous studies on this topic.

We identified three new SNPs in *CSPI* gene (rs41272673, rs4399666, and rs2287599) associated with PPHN. SNPs rs41272673 and rs4399666 are intronic, whereas rs2287599 is a synonymous mutation. Twelve individual parents were heterozygous for rs41272673, representing 11 unique pedigrees. Seventy-seven individual parents were informative heterozygotes for rs4399666, representing 61 unique pedigrees. Seventy-five individual parents were informative heterozygotes for rs2287599, representing 60 unique pedigrees.

None of the SNPs have been previously reported to be associated with disease states. A previously reported *CPS1* T1405N polymorphism (rs1047891) by Pearson *et al.* (8) was not associated with PPHN in our cohort ($P=0.24$). We speculate that this difference could be because of the difference in the population or in the study methodology. Haplotype analysis identified the presence of CCACTA alleles at rs2287599, rs7607412, rs7572146, rs6724941, rs3213784, and rs1047891 of *CPS1* that was associated with PPHN ($P=0.006$). This effect was mostly mediated by the presence of CC alleles at rs2287599 and rs7607412. These SNPs met the nominal significance, but did not reach formal levels of significance when the Bonferroni correction was applied (threshold $P<0.001$). Given the exploratory nature of this study, these less stringent values are also of interest.

CPS1 encodes an enzyme located inside the mitochondrion that catalyzes the first committed step of the UC. As the rate-limiting enzyme of the UC, changes in CPS1 carry greater functional effect than the other enzymes in the pathway. The *CPS1* gene is located on human chromosome 2q35, and comprises 38 exons and 37 introns that span more than 120 kilobases (25). Genetic variants in the *CPS1* gene can affect the functional efficiency of the CPS1 enzyme, especially under environmental stress conditions. The *CPS1* T1405N polymorphism (rs1047891) has been studied widely and is associated with clinical outcomes under various environmental stresses such as increased pulmonary artery pressure following surgical repair of congenital heart defects (22), hepatic veno occlusive diseases following bone marrow transplantation (23), and PPHN (8). These associations are referred to as environmentally determined genetic expression effects (24). Even though the functional effects of rs41272673, rs4399666, and rs2287599 are not known presently, we can speculate that these three SNPs identified by our current study affect the functional efficiency of CPS1, limiting the substrate availability for nitric oxide. Increased demand for endogenous nitric oxide for decreasing pulmonary vascular resistance associated with neonatal stress following child birth, in the setting of decreased functional efficiency of CPS1, might bring out the PPHN phenotype.

Several studies have detected lower UC-intermediate levels including arginine and citrulline in the neonates with PPHN (8,10). Infants with PPHN had significantly low arginine and nitric oxide metabolite levels compared with infants without PPHN according to a study by Pearson *et al.* (8). The mean plasma citrulline concentration tended to be lower in the infants with pulmonary hypertension as well. Authors concluded that arginine deficiency precedes and may lead to impaired nitric oxide synthesis in infants with persistent pulmonary hypertension (8). In the current study, we did not find a difference in arginine or citrulline levels between the two groups, but the tyrosine level was significantly lower in the cases with PPHN. Nitration of tyrosine by nitrogen species such as peroxynitrite can result in nitrotyrosine (26,27). Nitrotyrosine, a biomarker of oxidative stress, has been found in elevated levels in patients with bronchopulmonary dysplasia (28). Patients with PPHN are managed with mechanical ventilation, high levels of inspired

oxygen, and inhaled nitric oxide. We speculate that low tyrosine levels are due to the formation of reactive oxygen species and nitrotyrosine in the setting of hyperoxic ventilation.

We also found that levels of phenylalanine were higher in cases with PPHN. Higher phenylalanine levels have been observed in preterm newborns with respiratory distress syndrome (29). Marginally higher levels of phenylalanine are also seen in preterm newborns with patent ductus arteriosus (29). Elevated phenylalanine levels could be due to impaired phenylalanine hydroxylase (PAH) activity. Depletion of 5,6,7,8-tetrahydrobiopterin (BH4), a cofactor for PAH, can impair the PAH activity (30). Oxidative stress due to the formation of reactive oxygen species can deplete 5,6,7,8-tetrahydrobiopterin, resulting in impaired PAH activity (30). Elevated phenylalanine levels and low tyrosine levels can be explained by insufficient conversion of phenylalanine into tyrosine due to impaired PAH activity. BH4 is also an important cofactor for nitric oxide synthase. Depletion of BH4, due to oxidation to 7,8-dihydrobiopterin, would lead to nitric oxide synthase uncoupling, generating superoxide instead of nitric oxide, leading to pulmonary hypertension (31,32).

Our study has several limitations. Our sample size is relatively small, even though we used a larger sample size compared with prior studies of a similar nature. The majority of the families were Caucasian, so the results may not be reflective of other ethnicities. The retrospective nature of the study is another limitation. We were unable to examine the relationship between CPS1 and amino-acid levels due to the limited number of individuals who had both genotypes and amino-acid metabolites. Nor were we able to assess whether the parents in our study had PPHN. Our use of a family-based study design is a strength allowing for the examination of transmission of alleles from the parent to affected offspring, an approach that had not been previously used to investigate UC enzyme genes. This study design avoids confounding by population stratification, unlike previous case–control studies. Our study also included a greater number of SNPs compared with previous studies.

In conclusion, this study suggests an association ($P<0.05$) between SNPs in the *CPS1* gene and PPHN. We identified three previously unreported SNPs in *CPS1* gene associated with PPHN. These polymorphisms might affect the functional efficiency of CPS1, leading to PPHN. There was no difference in the arginine and citrulline levels between cases with PPHN and unaffected controls. Tyrosine levels were significantly lower and phenylalanine levels were significantly higher in cases with PPHN. These findings provide guidance for future studies and need to be replicated in a large cohort of patients.

SUPPLEMENTARY MATERIAL

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

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