

NEONATAL PULMONARY HYPERTENSION

Urea-Cycle Intermediates, Nitric Oxide Production, and Carbamoyl-Phosphate Synthetase Function

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ABSTRACT

Background Endogenous production of nitric oxide is vital for the decrease in pulmonary vascular resistance that normally occurs after birth. The precursor of nitric oxide is arginine, a urea-cycle intermediate. We hypothesized that low concentrations of arginine would correlate with the presence of persistent pulmonary hypertension in newborns and that the supply of this precursor would be affected by a functional polymorphism (the substitution of asparagine for threonine at position 1405 [T1405N]) in carbamoyl-phosphate synthetase, which controls the rate-limiting step of the urea cycle.

Methods Plasma concentrations of amino acids and genotypes of the carbamoyl-phosphate synthetase variants were determined in 65 near-term neonates with respiratory distress. Plasma nitric oxide metabolites were measured in a subgroup of 10 patients. The results in infants with pulmonary hypertension, as assessed by echocardiography, were compared with those in infants without pulmonary hypertension. The frequencies of the carbamoyl-phosphate synthetase genotypes in the study population were assessed for Hardy-Weinberg equilibrium.

Results As compared with infants without pulmonary hypertension, infants with pulmonary hypertension had lower mean (\pm SD) plasma concentrations of arginine (20.2 ± 8.8 vs. 39.8 ± 17.0 μ mol per liter, $P < 0.001$) and nitric oxide metabolites (18.8 ± 12.7 vs. 47.2 ± 11.2 μ mol per liter, $P = 0.05$). As compared with the general population, the infants in the study had a significantly skewed distribution of the genotypes for the carbamoyl-phosphate synthetase variants at position 1405 ($P < 0.005$). None of the infants with pulmonary hypertension were homozygous for the T1405N polymorphism.

Conclusions Infants with persistent pulmonary hypertension have low plasma concentrations of arginine and nitric oxide metabolites. The simultaneous presence of diminished concentrations of precursors and breakdown products suggests that inadequate production of nitric oxide is involved in the pathogenesis of neonatal pulmonary hypertension. Our preliminary observations suggest that the genetically predetermined capacity of the urea cycle — in particular, the efficiency of carbamoyl-phosphate synthetase — may contribute to the availability of precursors for nitric oxide synthesis. (N Engl J Med 2001;344:1832-8.)
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FAILURE of the transition to a cardiorespiratory circulation at birth results in persistent pulmonary hypertension of the newborn. Characterized by elevated pulmonary vascular resistance with extrapulmonary right-to-left shunting of blood across the patent ductus arteriosus or the foramen ovale, persistent pulmonary hypertension can cause life-threatening hypoxemia in newborn infants. Transitional pulmonary hypertension occurs in 1.9 of every 1000 newborn infants and is associated with a mortality rate of 11 percent.¹

Administration of inhaled nitric oxide can be an effective treatment for persistent pulmonary hypertension in newborn infants.²⁻⁴ Endogenous nitric oxide is critical in the transition to a pulmonary circulation at birth^{5,6} and in the regulation of pulmonary vascular resistance.^{7,8} Endothelial cells generate nitric oxide from the precursor L-arginine,^{9,10} an amino acid supplied by the urea cycle. Theoretically, therefore, a link exists between nitric oxide production and the urea cycle.

At 36 weeks' gestation, the enzymes in the urea cycle function at only 40 to 90 percent of the levels found in adults.^{11,12} Carbamoyl-phosphate synthetase catalyzes the first, rate-determining step of the urea cycle (Fig. 1). Genetic variations in the activity of this enzyme affect the downstream availability of the urea-cycle intermediates. We recently described a C-to-A nucleotide transversion in exon 36 of the gene that encodes carbamoyl-phosphate synthetase, resulting in the substitution of asparagine (Asn) for threonine (Thr) at position 1405 (T1405N), which is in the critical N-acetylglutamate-binding domain.¹⁴ Previously, in adults given high-dose chemotherapy for bone marrow transplantation, we observed that patients with the Thr1405 variant of this enzyme had lower concentrations of citrulline and higher rates of hepatic veno-occlusive disease and death than those with the Asn1405 variant.¹⁴

We hypothesized that low concentrations of the urea-cycle intermediates and nitric oxide precursors arginine and citrulline would correlate with the presence of persistent pulmonary hypertension in neonates. We

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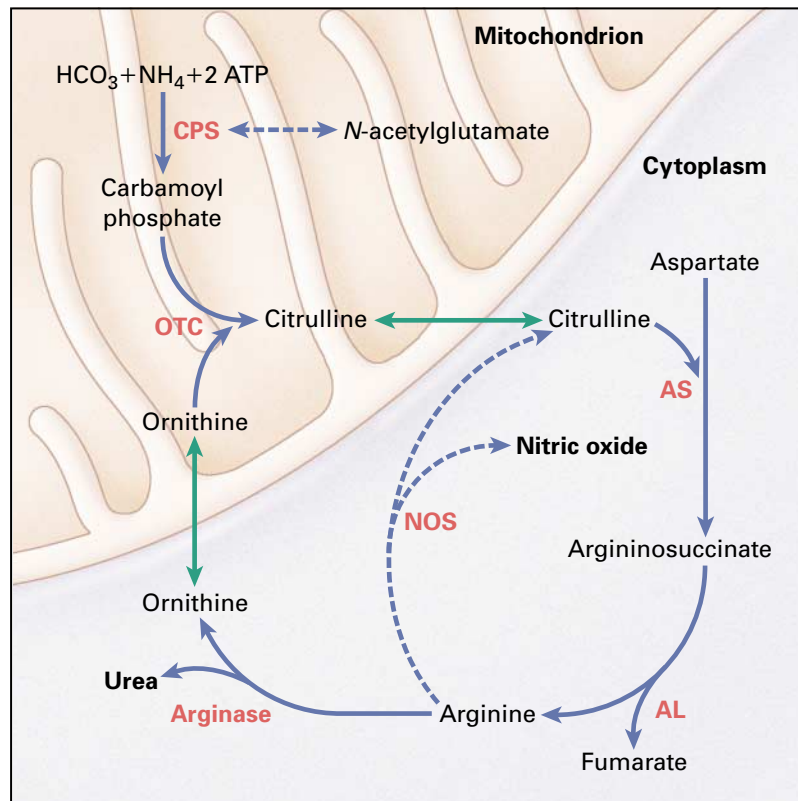


Figure 1. Enzymes and Intermediates in the Urea Cycle and Nitric Oxide Pathway.

The mitochondrial enzymes are present only in the urea cycle in the liver. The cytoplasmic enzymes argininosuccinate synthase (AS) and argininosuccinate lyase (AL) are also present in endothelial cells, where they recycle citrulline into arginine for high-output nitric oxide synthesis.¹³ CPS denotes carbamoyl-phosphate synthetase, NOS nitric oxide synthase, and OTC ornithine transcarbamylase.

further hypothesized that the distribution of the polymorphism at position 1405 in carbamoyl-phosphate synthetase would vary between infants with and those without persistent pulmonary hypertension.

METHODS

Patients

Between July 1, 1999, and June 30, 2000, neonates who were born at 35 weeks' gestation or later, weighed at least 2 kg at birth, and were admitted to Vanderbilt University Medical Center with a requirement for supplemental oxygen because of respiratory distress were evaluated for possible enrollment in this study. Infants with intrauterine growth restriction, infection confirmed by culture, congenital anomalies, or anatomical causes of pulmonary hypertension were excluded. At the time of enrollment (base line), when the infants were between 6 and 72 hours of age, 3 ml of blood was drawn from an umbilical-artery catheter before blood transfusion, enteral or parenteral protein intake, or inhaled nitric oxide administration. Approval from the institutional review board and written informed consent from the parents were obtained.

Infants were given a diagnosis of persistent pulmonary hypertension if they had sustained partial pressures of arterial oxygen below 100 mm Hg while breathing 100 percent supplemental oxygen, despite mechanical ventilation; a structurally normal heart; and an abnormally elevated pulmonary arterial pressure on echocardiography. An elevated pulmonary arterial pressure was considered

present if there was either right-to-left or bidirectional flow across the patent ductus arteriosus or foramen ovale (in 26 patients) or a systolic pulmonary arterial pressure greater than or equal to the systemic blood pressure according to Doppler measurement of the tricuspid-regurgitation jet (in 5 patients). Infants with respiratory distress who did not have pulmonary hypertension according to these criteria were designated as controls. In all the infants, the maximal oxygenation index was calculated (as the fraction of inspired oxygen multiplied by the mean airway pressure divided by the partial pressure of arterial oxygen, multiplied by 100).

Data on clinical characteristics were retrieved from medical records without knowledge of subsequent laboratory results. Laboratory personnel, pediatric cardiologists, and other caregivers were unaware of the infants' enrollment in the study. All the infants were treated according to institutional guidelines. Alveolar recruitment was achieved with either conventional mechanical ventilation or high-frequency oscillation; infants who subsequently had a partial pressure of arterial oxygen below 80 mm Hg while breathing 100 percent supplemental oxygen received inhaled nitric oxide. Infants without irreparable injuries who had a partial pressure of arterial oxygen below 50 mm Hg despite maximal medical therapy received extracorporeal membrane oxygenation.

Only one infant who fulfilled the criteria for entry into the study was excluded from subsequent analysis. This infant had no response to inhaled nitric oxide and died after extracorporeal membrane oxygenation was discontinued. Examination of a lung-biopsy specimen revealed alveolar-capillary dysplasia, an anatomical cause of pulmonary hypertension.

Laboratory Measurements

Analysis of amino acids was performed on protein-free extracts of fresh plasma. Amino acids were separated by cation-exchange chromatography (7300 amino acid analyzer, Beckmann, Palo Alto, Calif.). After conversion with triketohydrindene hydrate, primary amines were detected at 570 nm and secondary amines at 440 nm. Levels of all detectable amino acids were measured after calibration of the analyzer.

Plasma concentrations of nitric oxide metabolites were measured in a subgroup of 10 consecutive infants from whom samples of plasma were obtained and immediately frozen at -20°C . With the use of a colorimetric nonenzymatic assay (Oxford Biomedical Research, Oxford, Mich.), the plasma samples were deproteinized with zinc sulfate, and the nitrates in the samples were reduced to nitrite by incubation with cadmium beads. After centrifugation, the Griess reagents sulfanilamide and *N*-(1-naphthyl)ethylenediamine were added sequentially to the supernatants. Absorbances were measured at 540 nm; a standard curve plotted from the absorbances of diluted sodium nitrite standards was used to determine the concentrations of nitric oxide metabolites in the samples.¹⁵⁻¹⁷

Ammonia concentrations were measured in samples of fresh chilled plasma by means of dry-slide technology (Vitros 950 Analyzer, Ortho Clinical Diagnostics, Rochester, N.Y.). After the conversion of urea to ammonia by urease, concentrations of blood urea nitrogen were measured by identical methods.

Analysis of Polymorphisms in Carbamoyl-Phosphate Synthetase

Carbamoyl-phosphate synthetase genotypes were determined by single-strand conformation polymorphism analysis. Genomic DNA was isolated from preparations of whole blood (Qiagen, Valencia, Calif.). Oligonucleotide primers and the polymerase chain reaction were used to amplify a 253-bp fragment encompassing the C-to-A nucleotide transversion at position 4332 of exon 36 of the gene encoding carbamoyl-phosphate synthetase. After treatment with formamide, samples from the infants and previously sequenced control samples were subjected to electrophoresis for five hours at 4°C in a nondenaturing gel (FMC, Rockland, Me.) and then were stained with silver nitrate to detect DNA fragments.

An identical technique was used to detect two other carbamoyl-phosphate synthetase polymorphisms, one resulting from the substitution of alanine for threonine at position 344 (T344A) and the other from the deletion of CTT at position 118 of the complementary DNA (118delCTT). The polymorphism at position 344 lies in the nonfunctional glutaminase subdomain of carbamoyl-phosphate synthetase, whereas 118delCTT occurs in the 5' untranslated region of the gene. The distributions of these two polymorphisms were used as internal controls for other variations in the gene encoding carbamoyl-phosphate synthetase.

Statistical Analysis

Plasma concentrations of amino acids, nitric oxide metabolites, ammonia, and blood urea nitrogen in the infants with pulmonary hypertension were compared with those in the controls by means of the Wilcoxon rank-sum test to adjust for outlying values. All data are presented as means \pm SD. *P* values of 0.05 or less were considered to indicate statistical significance.

To determine whether the polymorphisms in carbamoyl-phosphate synthetase were in Hardy-Weinberg equilibrium, the frequencies of the alleles and of the genotypes were analyzed. The Hardy-Weinberg law states that $q^2 + 2pq + p^2 = 1$, where *p* and *q* are allele frequencies in a two-allele system. Analysis of the distributions of the carbamoyl-phosphate synthetase genotypes was performed by chi-square analysis with 2 degrees of freedom. Expected values were calculated with the allele frequencies found in a previously studied group of more than 400 unrelated adults who lived in the same geographic area and had the same distribution of ethnic backgrounds as the infants in this study.

RESULTS

Clinical Characteristics of the Patients

Sixty-five neonates with respiratory distress were enrolled in the study. Thirty-one of these infants (48 percent) had persistent pulmonary hypertension, whereas 34 (52 percent) did not have pulmonary hypertension and served as controls. There were no significant differences in the base-line characteristics of the two groups except in the maximal oxygenation index (Table 1). Among the infants with pulmonary hypertension, the primary diagnoses were perinatal asphyxia (in seven infants), idiopathic persistent pulmonary hypertension (in six), meconium aspiration syndrome (in six), hyaline membrane disease (in three), and other conditions (in nine). Among the controls, the primary diagnoses were transient tachypnea of the newborn (in seven infants), asphyxia (in six), meconium aspiration syndrome (in four), hyaline membrane disease (in four), pneumothorax (in four), and other conditions (in nine).

The infants with pulmonary hypertension had more severe illness than the controls, as indicated by their higher maximal oxygenation-index values ($P=0.001$) (Table 1). Fourteen of the infants with persistent pulmonary hypertension required treatment with inhaled nitric oxide, one required extracorporeal membrane oxygenation, and one died of severe perinatal asphyxia and multiorgan failure. All the remaining infants recovered from their illness and were discharged by 26 days of age.

Intermediates in the Urea Cycle and Nitric Oxide Pathway

The mean plasma concentration of arginine was significantly lower in the infants with pulmonary hypertension than in the controls (20.2 ± 8.8 vs. 39.8 ± 17.0

TABLE 1. BASE-LINE CHARACTERISTICS OF THE INFANTS WITH RESPIRATORY DISTRESS.*

VARIABLE	INFANTS WITH PULMONARY HYPERTENSION (N=31)	CONTROLS (N=34)
Birth weight — kg	3.3 \pm 0.7	3.3 \pm 0.6
Gestational age — wk	38.7 \pm 2	38.4 \pm 2
Male sex — no. (%)	19 (61)	26 (76)
Black race — no. (%)	7 (23)	6 (18)
Age at enrollment — hr	36 \pm 22	37 \pm 26
Requirement for mechanical ventilation — no. (%)	31 (100)	30 (88)
Maximal oxygenation index†	26 \pm 22‡	7 \pm 9

*Plus-minus values are means \pm SD.

†The oxygenation index was calculated as the fraction of inspired oxygen multiplied by the mean airway pressure divided by the partial pressure of arterial oxygen, multiplied by 100.

‡ $P=0.001$ for the comparison with the controls.

μmol per liter, $P < 0.001$) (Fig. 2). The mean plasma citrulline concentration tended to be lower in the infants with pulmonary hypertension than in the controls (8.3 ± 4.9 vs. 11.7 ± 8.1 μmol per liter, $P = 0.13$), but this difference was not significant. There were no significant differences between the two groups in the mean concentrations of any of 22 other individual amino acids analyzed or in the mean concentrations of total essential amino acids.¹⁸

Nitric oxide metabolites were measured in 10 of the enrolled infants (5 infants with pulmonary hypertension and 5 controls). The infants with pulmonary hypertension had significantly lower mean plasma concentrations of nitric oxide metabolites than the controls (18.8 ± 12.7 vs. 47.2 ± 11.2 μmol per liter, $P = 0.05$) (Fig. 2). However, the number of infants was too small to evaluate the relations between nitric oxide metabolites and amino acid concentrations or genotype.

Concentrations of ammonia in the infants with pulmonary hypertension tended to be slightly but not significantly higher than those in the controls (51.2 ± 16.7 vs. 45.7 ± 12.2 μmol per liter, $P = 0.14$). There was no significant difference between the two groups in blood urea nitrogen concentrations (12.1 ± 7.3 μmol per liter in those with pulmonary hypertension and 10.3 ± 5.7 μmol per liter in the controls, $P = 0.43$).

Analysis of Polymorphisms in Carbamoyl-Phosphate Synthetase

The distribution of the carbamoyl-phosphate synthetase genotypes for the polymorphism at position 1405 within the overall study population was significantly skewed from the expected distribution within the general population ($P < 0.005$ for the comparison with Hardy-Weinberg equilibrium) (Table 2). Although cross-product analysis of the group of infants with pulmonary hypertension as compared with the

control group revealed no significant difference in genotype distributions, none of the former group of infants were homozygous (AA) for the A-encoded Asn1405 variant. However, only three infants in the control group had the AA genotype, so our conclusion is based on a small number of infants.

When we examined arginine and citrulline concentrations in relation to the distribution of the carbamoyl-phosphate synthetase genotypes, we found that infants who were homozygous (CC) for the C-encoded Thr1405 enzyme had lower mean arginine and citrulline concentrations than infants with the AA genotype and hence the Asn1405 enzyme (Table 3). Only the difference in arginine concentrations was significant ($P = 0.01$). Heterozygous infants (those with the AC genotype) had intermediate concentrations of arginine and citrulline, which did not differ significantly from those in either homozygous group.

With regard to the T344A polymorphism and the 118delCTT polymorphism, located at less structurally and functionally critical regions in the carbamoyl-phosphate synthetase molecule than the T1405N polymorphism, the distributions of the homozygous and heterozygous genotypes were within the ranges predicted by the known distribution of these polymorphisms in the general population. There were no notable differences between the infants with pulmonary hypertension and the controls in the distribution of these genotypes, nor were there any correlations with the concentrations of urea-cycle intermediates. In addition, there was no evidence of significant linkage disequilibrium among the T1405N, T344A, and 118delCTT polymorphisms.

DISCUSSION

The cardiorespiratory transition that occurs in the initial hours and days after birth is regulated by multiple factors.¹⁹ One of the critical vasoactive mediators

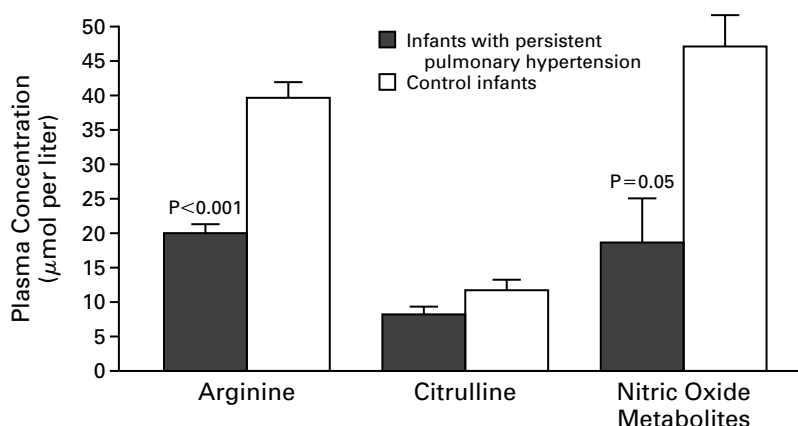


Figure 2. Mean (+SE) Concentrations of Nitric Oxide Precursors and Metabolites in the Infants with Persistent Pulmonary Hypertension and in the Control Infants.

P values are for the comparison with the concentrations in the control group.

TABLE 2. DISTRIBUTIONS OF CARBAMOYL-PHOSPHATE SYNTHETASE GENOTYPES.*

POPULATION	No. OF INFANTS	no. (%)		
		CC	AC	AA
General population	—	— (42)	— (46)	— (12)
Overall study population	65	17 (26)	45 (69)	3 (5)
Infants with pulmonary hypertension	31	8 (26)	23 (74)	0
Infants without pulmonary hypertension (controls)	34	9 (26)	22 (65)	3 (9)

*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.

involved in this transition is nitric oxide. We found that neonates with persistent pulmonary hypertension had significantly lower plasma concentrations of both arginine and nitric oxide metabolites than infants who had respiratory distress but did not fulfill the criteria for pulmonary arterial hypertension. However, our observations are preliminary because of the relatively small number of infants we were able to study.

A difference in plasma concentrations between the infants with pulmonary hypertension and the controls was found only for arginine and was not due to differences among the infants in nutritional status or respiratory mechanics. All the infants, regardless of whether they had pulmonary hypertension, were fasting and had the catabolic effects of acute illness. There was no significant difference between the two groups in the mean concentrations of total essential amino acids. The infants in both groups also had respiratory distress and required supplemental oxygen to control the potential effects of shear stress²⁰ and oxidative injury²¹ on the generation of endogenous nitric oxide by the pulmonary endothelium.

The coincident presence of low concentrations of nitric oxide metabolites and low plasma concentrations of arginine refutes the hypothesis of Vosatka et al.,²² who suggested that in infants with pulmonary hypertension there is excessive consumption of arginine during nitric oxide synthesis. Diminished levels of components of the L-arginine–nitric oxide pathway farther downstream — namely, plasma cyclic guanosine 5'-monophosphate²³ and urinary nitric oxide metabolites²⁴ — have also been reported in infants with persistent pulmonary hypertension. Our data, when combined with these observations, suggest that an arginine deficiency precedes and may lead to impaired nitric oxide synthesis in infants with persistent pulmonary hypertension.

In our study, the infants with pulmonary hypertension had plasma arginine concentrations well below the

TABLE 3. CONCENTRATIONS OF UREA-CYCLE INTERMEDIATES IN RELATION TO CARBAMOYL-PHOSPHATE SYNTHETASE GENOTYPES WITHIN THE STUDY POPULATION.*

INTERMEDIATE	CC (N=17)	AC (N=45)	AA (N=3)
	μmol per liter		
Arginine	21.2±7.7	29.0±18.3	35.7±7.6†
Citrulline	7.2±4.4	9.8±6.5	11.7±5.5

*Values are means ±SD. 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.

†P=0.01 for the comparison with the infants with the CC genotype.

concentrations known to down-regulate nitric oxide synthesis in vitro. These infants had an average extracellular arginine concentration of 20.2 μmol per liter, whereas the concentration of L-arginine needed to stimulate half-maximal synthesis of nitric oxide in cultured endothelial cells ranges from 37 to 73 μmol per liter.^{25,26}

Impaired nitric oxide production may be particularly poorly tolerated in newborn infants, in whom the demand for nitric oxide is high. Whereas in healthy adults the mean plasma concentration of nitric oxide metabolites is 19±7 μmol per liter,²⁷ in healthy infants it is 27.5±12.8 μmol per liter at birth and 53.8±14 μmol per liter by the fifth day of life.²⁸ In our study, the infants with pulmonary hypertension had plasma concentrations of nitric oxide metabolites of 18.8±12.7 μmol per liter at about 36 hours of age, whereas those whose pulmonary arterial pressure had decreased normally had concentrations of 47.2±11.2 μmol per liter.

Immature functioning of the urea cycle at birth results in higher steady-state concentrations of ammonia and lower production of urea-cycle intermediates in infants than in adults.^{11,12} In our overall study population, the average arginine and citrulline concentrations were 30.5 μmol per liter and 10 μmol per liter, respectively, as compared with averages of 74 μmol per liter and 27 μmol per liter in adults.²⁹ Further alterations in the concentrations of urea-cycle intermediates occur if there are genetic differences in the activity of carbamoyl-phosphate synthetase. In patients who have a deficiency of carbamoyl-phosphate synthetase, citrulline concentrations are almost undetectable, and administration of exogenous arginine as an essential amino acid is necessary.

More subtle changes in carbamoyl-phosphate synthetase also affect the availability of arginine and citrul-

line. Preliminary enzyme-kinetics studies of recombinant carbamoyl-phosphate synthetase in vitro showed that the Asn1405 variant was 30 to 40 percent more efficient than the Thr1405 variant (unpublished data). The infants with the AA genotype (i.e., those homozygous for the Asn1405 variant) had significantly higher arginine concentrations than the infants with the CC genotype (i.e., those homozygous for the Thr1405 variant). In infants, in whom the urea cycle is not fully developed, further decreases in arginine and citrulline production due to genetically determined variations in carbamoyl-phosphate synthetase function could affect the production of nitric oxide and the subsequent development of persistent pulmonary hypertension in neonates.

We hypothesized that infants with persistent pulmonary hypertension would have a different distribution of T1405N genotypes than infants with respiratory symptoms but no pulmonary hypertension. We expected the controls to have a distribution of carbamoyl-phosphate synthetase genotypes that was similar to that of the general population. Instead, we found that the overall study population of newborns with respiratory distress had a significantly skewed distribution of carbamoyl-phosphate synthetase genotypes for this functional polymorphism. However, only a small number of infants in the control group had the AA genotype. One possible explanation for the similarity between the group with pulmonary hypertension and the group without pulmonary hypertension is that all infants with transitional respiratory problems may have some degree of pulmonary hypertension^{30,31}; our definition of elevated pulmonary arterial pressure may merely have identified the most severely affected infants along this continuum. The finding that these ill infants had a skewed distribution of T1405N genotypes as compared with the general population supports the possibility that the functional status of carbamoyl-phosphate synthetase has a role in the cardiorespiratory transition at birth.

The absence of AA homozygosity among the infants with persistent pulmonary hypertension is noteworthy. The C-encoded Thr1405 variant of carbamoyl-phosphate synthetase is actually the evolutionarily conserved version, whereas the less frequent, A-encoded Asn1405 variant appears to be a relatively new, gain-of-function mutation (unpublished data). We speculate that persons with the AA genotype may have an advantage in terms of urea-cycle function and interrelated metabolic processes, especially under conditions of environmental stress.

Although the functional status of carbamoyl-phosphate synthetase appears to affect the neonatal transition, other key factors must regulate the metabolism of arginine and citrulline in endothelial cells. Of the infants with either the CC or AC genotype, those with persistent pulmonary hypertension had lower plasma concentrations of arginine and citrulline. The answer

may lie in other enzymes or transporters in the urea cycle and nitric oxide pathway; argininosuccinate synthetase is a particularly good candidate.³² The association that we detected between the Thr1405 variant of carbamoyl-phosphate synthetase and persistent pulmonary hypertension in newborns describes only one of many potential genetic vulnerabilities that interact to influence the physiologic response to the tremendous stress of birth. Insights gained from this interaction between genes and the environment may lead to new strategies to identify and treat newborns who are at highest risk for perinatal illness.

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