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# Genetic Variation in the Mitochondrial Enzyme Carbamyl-Phosphate Synthetase I Predisposes Children to Increased Pulmonary Artery Pressure Following Surgical Repair of Congential Heart Defects: A Validated Genetic Association Study

**Jeffrey A Canter**[1], **Marshall L Summar**[1], **Heidi B Smith**[2], **Geraldine D Rice**[2], **Lynn D Hall**[1], **Marylyn D Ritchie**[1], **Alison A Motsinger**[2], **Karla G Christian**[3], **Davis C Drinkwater Jr**[3], **Frank G Scholl**[3], **Karrie L Dyer**[4], **Ann L Kavanaugh-McHugh**[4], and **Frederick E Barr**[2]

***1*** *Center for Human Genetics Research, Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN*

***2*** *Department of Pediatrics, Division of Critical Care Medicine, Vanderbilt Children's Hospital, Nashville, TN*

***3*** *Department of Cardiothoracic Surgery, Vanderbilt University Medical Center, Nashville, TN*

***4*** *Department of Pediatrics, Division of Cardiology, Vanderbilt Children's Hospital, Nashville TN*

## Abstract

Increased pulmonary artery pressure (PAP) can complicate the postoperative care of children undergoing surgical repair of congenital heart defects. Endogenous NO regulates PAP and is derived from arginine supplied by the urea cycle. The rate-limiting step in the urea cycle is catalyzed by a mitochondrial enzyme, carbamoyl-phosphate synthetase I (CPSI). A well-characterized polymorphism in the gene encoding CPSI (T1405N) has previously been implicated in neonatal pulmonary hypertension. A consecutive modeling cohort of children (N=131) with congenital heart defects requiring surgery was prospectively evaluated to determine key factors associated with increased postoperative PAP, defined as a mean PAP > 20mmHg for at least one hour during the 48 hours following surgery measured by an indwelling pulmonary artery catheter. Multiple Dimensionality Reduction (MDR) was used to both internally validate observations and develop optimal two-variable through five-variable models that were tested prospectively in a validation cohort (N= 41). Unconditional logistic regression analysis of the modeling cohort revealed that age (OR= 0.92, p=0.01), CPSI T1405N genotype (AC vs. AA: OR=4.08, p=0.04, CC vs. AA: OR=5.96, p=0.01), and Down syndrome (OR=5.25, p=0.04) were independent predictors of this complex phenotype. MDR predicted that the best two-variable model consisted of age and CPSI T1405N genotype (p<0.001). This two-variable model correctly predicted 73% of the outcomes from the validation cohort. A five-variable model that added race, gender and Down's syndrome was not significantly better than the two-variable model. In conclusion, the CPSI T1405N genotype appears to be an important new factor in predicting susceptibility to increased PAP following surgical repair of congenital cardiac defects in children.

Corresponding Author: Jeff Canter, MD MPH, Center for Human Genetics Research, 519 Light Hall, Vanderbilt University Medical Center, Nashville, TN 37212, Phone: (615) 343-8617, E-mail: jeff.canter@vanderbilt.edu, Fax: (615) 343-8619

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**Keywords**

Mitochondrial enzyme; CPS I; pulmonary artery pressure

## 1. Introduction

Congenital heart defects affect nearly 1% of all newborns (American Heart Association, 2005;Hoffman, 1995). Corrective or palliative surgery utilizing cardiopulmonary bypass is often performed during the first year of life (Aylin et al, 2004). Increased pulmonary artery pressure (PAP) can complicate the surgical repair of many of these cardiac defects by further compromising cardiac output in the immediate postoperative period (Russell, et al, 1998;Bandla et al, 1999;Steinhorn and Fineman, 1999;Gentles et al, 1997;Freeman et al, 1995;Nakajima et al, 1996;Adatia et al, 1996;Schulze-Neick, 2001). Invasive and expensive interventions, including mechanical ventilation with inhaled nitric oxide (NO), are often required to treat this complication (Russell, et al, 1998;Adatia et al, 1996;Journois et al, 1994;Miller et al, 2000; Yagahi et al, 1998; Zobel et al, 1998;Metzler and Beitzke, 1997).

Endogenous NO is critical for the maintenance of normal PAP (Steinhorn and Fineman, 1999;Schulze-Neick et al, 1999;Kirshbom et al, 1996). Endothelial cells generate NO from arginine, a precursor amino acid supplied by the urea cycle (Moncada and Higgs, 1993). We have previously demonstrated that infants undergoing surgical repair of congenital heart defects have significantly decreased serum arginine levels in the immediate postoperative period (Barr et al, 2003). This decrease in availability of a key substrate for NO following cardio-pulmonary bypass may increase the risk of postoperative pulmonary hypertension (Barr et al, 2003).

The rate limiting step in arginine production occurs inside the mitochondrion and is catalyzed by the enzyme, carbamyl-phosphate synthetase I (CPSI). This process is outlined in Figure 1 (Summar et al, 2003). The CPSI gene, located on human chromosome 2q35, consists of 38 exons and 37 introns that span more than 120 kilobases of genomic DNA (Summar et al, 2003). A specific single nucleotide polymorphism (SNP) designated as T1405N in this gene results in a threonine to asparagine amino acid substitution at an important cofactor binding site (Summar et al, 2003). We have previously shown that CPSI T1405N genotype affects plasma arginine levels (Pearson et al, 2001). In addition, the CPSI T1405N genotype distribution was significantly different in neonates who developed persistent pulmonary hypertension of the newborn (PPHN) compared to unaffected neonates (Pearson et al, 2001). Specifically, neonates with at least one copy of the A allele (Asparagine 1405 variant) appeared to be less likely to develop PPHN.

We hypothesized that the CPSI T1405N genotype would affect susceptibility to increased PAP in children undergoing surgical repair of congenital heart defects. This cohort study was initially constructed to determine if the T1405N polymorphism was an independent predictor of increased PAP measured by an indwelling pulmonary artery catheter during the postoperative period. A model incorporating key variables predicting this phenotype was then prospectively tested in a second validation patient cohort. Our objective was to determine potential clinical and genetic factors that predict the occurrence of increased PAP in this especially vulnerable population.

# 2. Methods

## 2.1 Ascertainment of Patient Populations

Two separate cohorts of children undergoing surgical correction of their congenital heart defects at Vanderbilt Children's Hospital were prospectively enrolled in this study. The first group was a continuous modeling cohort of 131 children enrolled between August 2000 and September 2002. The second group was a continuous validation cohort of 41 children enrolled between April 2003 and August 2004. Inclusion criteria were identical for both groups. Infants undergoing any of the following 5 surgical procedures for repair of congenital heart defects were eligible: atrioventricular septal defect (AVSD) repair, ventricular septal defect (VSD) repair, bidirectional Glenn procedure, modified Fontan procedure, and arterial switch procedure for transposition of the great vessels. Written consent was obtained from the parents of patients at the time of preoperative evaluation in accordance with a protocol and consent document approved by the Vanderbilt University Institutional Review Board. All surgical procedures were performed by one of two pediatric cardiac surgeons using similar cardiopulmonary bypass and cardioplegia preparations.

## 2.2 Definition of Increased Pulmonary Artery Pressure

All patients in this cohort had pulmonary artery pressure measured continuously during the postoperative period via a 3 French single lumen non-thermodilution pulmonary artery catheter (or 4 French double lumen central line in the superior vena cava in the case of patients undergoing a modified Glenn or Fontan procedure) inserted at the time of surgery. All patients had PA pressure monitoring performed for clinically indicated reasons at the discretion of the attending surgeon, no patient had a PA catheter placed solely for research purposes. Thermodilution catheters were not used due to risk of complications from large thermodilution catheters in infants. Using previously published perioperative criteria, increased PAP was defined as a sustained increase in mean pulmonary artery pressure (MPAP) of > 20mmHg for at least an hour in duration during the 48 hours following surgery (Kulick, 1998).

## 2.3 Genotyping of the CPSI T1405N Polymorphism

Three milliliters of arterial blood was obtained from patients preoperatively and stored in citrated tubes. The buffy coat was isolated and genomic DNA extracted from white blood cells utilizing a genomic DNA isolation kit (Promega Corp, Madison WI). Isolated DNA from patients and known controls was subjected to PCR utilizing previously described T1405N primers and optimal PCR conditions as previously described (Summar et al, 2003). PCR products were isolated by mutation detection enhancement (MDE) electrophoresis utilizing a MDE heteroduplex kit to clearly visualize single base substitutions (AT Biochem, Malvern, PA).

## 2.4 Statistical Analysis

Means for continuous variables were compared by means of the Student's t test for normally distributed variables. In this case, data are presented as means with standard deviations. If the assumption of a normal distribution could not be made for a given continuous variable, the Mann-Whitney U-test was used to compare results between PAP outcome categories. Categorical variables were compared using either the Chi-square test or Fisher's exact test. Multivariate logistic regression was used to estimate the adjusted odds ratio of the risk of individual CPSI genotypes after taking other factors into consideration. The level of significance in this study was set prior to analysis at 0.05. STATA 9.0 (STATA Corp., College Station, TX) statistical software was used to accomplish all of the analyses in this study. To determine whether the CPSI T1405N polymorphism was in Hardy-Weinberg equilibrium an analysis of the genotypes was performed by goodness-of-fit chi-square analysis with 2 degrees

of freedom. Expected values for the distribution of the T1405N polymorphism were based on allele frequencies from a sample of 400 unrelated adults from the same geographic area and with the same racial distribution as the children in this study (Summar et al, 2003). The mathematical model derived from unconditional logistic regression was then tested prospectively in the validation cohort. If the model's predicted probability was greater than 0.50 for the primary outcome in a given individual, then this was considered as a prediction of that outcome. The results of this model are presented as % accuracy of predicted values.

### 2.5 Multifactor Dimensionality Reduction (MDR) Analysis

Multifactor Dimensionality Reduction (MDR) was used in this analysis to both internally validate findings in the modeling cohort and to develop optimal two-variable through five-variable models for testing in the validation cohort (Hahn et al, 2003;Ritchie et al, 2001;Coffey et al, 2004;Cho et al 2004;Bastone et al, 2004;Moore, 2004;Moore and Williams, 2002;Ritchie et al, 2003). In the first step of Multifactor Dimensionality Reduction (MDR), the data is divided into a training set and an independent testing set for cross validation. Second, a set of *n* genetic and/or environmental factors is selected. These factors and their multi-factor classes are divided in *n*-dimensional space. Then the ratio of affecteds to unaffecteds is calculated within each multifactor class. Each multifactor cell class is then labeled "high risk" or "low risk" based on the ratio calculated, therefore reducing the *n*-dimensional space to one dimension with two levels. The collection of these multifactor classes composes the MDR model for a particular combination of factors. For each possible model size (one-variable, two-variable, etc.) a single MDR model is chosen that has the lowest number of misclassified individuals. In order to evaluate the predictive ability of the model, prediction error is calculated using 10-fold cross-validation. The result is a set of models, one for each model size considered. From these models, a final model is chosen based on minimization of prediction error and maximization of cross validation consistency (number of times a particular set of factors is identified across the cross validation subsets). The statistical significance of the final best model is determined through permutation testing. Permutation testing involves creating 1000 permuted datasets by randomizing the disease status labels. The entire MDR procedure is repeated for each, generating a distribution of prediction errors and cross validation consistencies that could be expected by chance alone. The significance of the final model is determined by comparing the prediction error and cross validation consistency of the final model to the distribution. A p-value is extracted for the model by its theoretical location in the distribution (Ritchie et al, 2001).

## 3. Results

### 3.1 Model Study Population

During the modeling study period, 131 consecutive children met inclusion criteria and were enrolled. Of these patients, 78 (60%) developed increased postoperative PAP. There were no significant differences between children with and without increased PAP with respect to gender, race or length of cardiopulmonary bypass. On univariate analysis, there were statistically significant differences between children with and without increased PAP with respect to age, weight, and Down's syndrome (Table 1). As expected, age at surgery and weight were highly correlated (r=0.87) and therefore weight was not included in subsequent analyses.

Patients with increased postoperative PAP tended to require increased duration of mechanical ventilation and increased utilization of inhaled nitric oxide (Table 1). There were also non-significant trends towards increased length of hospitalization and increased mortality in patients with increased postoperative PAP.

In the modeling cohort, the distribution of CPSI T1405N genotypes was different in patients who developed increased PAP compared to those who did not, p=0.031 (Table 2). The cohort N=131 was in Hardy-Weinberg equilibrium (p=0.70). Multivariate logistic regression analysis of the list of potential variables contributing to this phenotype revealed that T1405N genotype was an independent predictor of increased postoperative PAP (Table 3). Other variables that remained independent predictors included age at the time of surgery (OR= 0.92, 95% CI 0.87–0.98) and Down's syndrome (OR= 5.25, 95% CI 1.11–24.76) following adjustment for both CPSI T1405N status and cardiopulmonary bypass time.

MDR analysis of the modeling cohort concluded that a two-variable model incorporating age and CPSI T1405N genotype status had the highest cross-validation consistency and lowest prediction error (p<0.001). This is summarized in Figure 2. The equation for this model is presented in Figure 3. The next best model generated from the MDR analysis was a five-variable model consisting of age, CPSI T1405N genotype status, Down's syndrome, race and gender (p=0.049).

### 3.2 Validation Cohort

The validation cohort for this study consisted of 41 children at the same institution followed prospectively. These children had the same inclusion and exclusion criteria. They were collected and enrolled prior to the analysis outlined above. Using the two-variable model derived from the MDR analysis, age and CPSI T1405N genotype status, the model predicted with 73% accuracy the development of increased PAP. The model with age alone predicted the primary outcome with 66% accuracy. The five-variable model consisting of age, CPSI T1405N genotype status, Down's syndrome, race and gender predicted outcome in the validation cohort with 71% accuracy. The validation cohort had significantly fewer children with Down's syndrome develop increased PAP than the modeling cohort.

## 4. Discussion

This study of two consecutive cohorts of children undergoing surgical repair of congenital heart defects provided a special opportunity to further understand the clinical and genetic factors involved in the development of increased postoperative pulmonary artery pressure. Analysis of the initial modeling cohort again demonstrated the clinical relevance of the *CPSI* T1405N polymorphism in a phenotype where endogenous NO is critically important. Previously, this same polymorphism was implicated in neonatal pulmonary hypertension and hepatovenocclusive disease (HVOD) following bone marrow transplantation (Pearson et al, 2001,Kallianpur et al, 2005). The *CPSI* T1405N polymorphism is the result of C to A transversion at base 4332 in the 3′ region of the *CPSI* mRNA, changing the triplet code from the evolutionarily conserved ACC to AAC. This results in an amino acid change from threonine to asparagine at residue 1405 (referred to as T1405N) in the CPSI enzyme. Residue 1405 is located within the binding site of an essential CPSI cofactor, N-acetylglutamate (NAG) (Summar et al, 2001). CPSI is the key, rate-limiting enzyme that catalyzes the first step in the hepatic urea cycle. In addition to removal of waste nitrogen, the urea cycle also synthesizes arginine which is the main substrate for NO production by nitric oxide synthase. Preliminary enzyme-kinetic studies of recombinant carbamoyl-phosphate synthase in vitro showed that the Asn1405 variant was more efficient than the Thr1405 variant (Summar et al, 2004). This corresponds with the observation that neonates with the AA T1405N genotype (homozygous for the Asn1405 variant) had significantly higher levels of arginine and nitric oxide metabolites (Pearson et al, 2001). It is therefore plausible that the *CPSI* T1405N polymorphism may result in a significant change in CPSI enzymatic function.

In a previous study of infants undergoing congenital heart surgery, we found significantly decreased postoperative levels of arginine, citrulline (a urea cycle intermediate and precursor

of arginine) and nitric oxide metabolites (Barr, 2003). In that study, arginine levels fell by more than 50% and did not recover until 48 hours following surgery (Barr, 2003). In a related study, we demonstrated the effectiveness of oral citrulline in preserving preoperative levels of arginine and decreasing the risk of postoperative pulmonary hypertension in a similar population of children undergoing repair of congenital heart defects (Smith et al, 2006).

Previous reports have highlighted the complexity of this phenotype (Russell, et al, 1998;Bandla et al, 1999;Steinhorn and Fineman, 1999;Gentles et al, 1997;Freeman et al, 1995;Nakajima et al, 1996;Adatia et al, 1996;Schulze-Neick, 2001). In these previous reports, age at the time of surgery has been identified as an important factor in the development of elevated PAP, with younger children being at increased risk for this complication (Steinhorn and Fineman, 1999;Schulze-Neick et al, 2001). In this study, we identified a genetic factor, CPSI T1405N, that is also an independent risk factor for the development of increased PAP. Since many genetic association studies have been difficult to reproduce, we tested the model derived from our initial cohort on a subsequent validation cohort. Both cohorts were population-based in that all children were consecutively enrolled prior to their surgery to minimize selection bias. Additionally, we chose to target a single nucleotide polymorphism that had already been implicated in a similar phenotype thus limiting the potential effect of multiple comparisons. To further limit the possibility of a false-positive association, we utilized Multiple Dimensionality Reduction (MDR) in our model development (Hahn et al, 2003;Ritchie et al, 2001). Using this combination of cohorts and methods, we developed and validated a novel model, combining both clinical and genetic data, which contributes to the prediction of postoperative PAP.

The primary outcome variable in this study was a postoperative increase in mean PAP. We evaluated patients who had a 3 French single lumen non-thermodilution pulmonary artery catheter (or a central venous line in the superior vena cava for single ventricle patients) inserted at the end of surgery for clinically indicated reasons by the attending surgeon. We defined increased pulmonary artery pressure as a sustained increase in the mean PAP > 20 mmHg for at least 1 hour within 48 hours of surgery (Kulik, 1998). The threshold of a mean PAP>20 is an important outcome for several reasons (Steinhorn and Fineman, 1999;Kulik, 1998). In patients undergoing repair of single ventricle lesions, pulmonary blood flow is passive relying on low downstream pressures. Therefore, mean pulmonary pressures exceeding 20 to 25 mmHg would not be tolerated physiologically, leading to therapeutic intervention and reevaluation (Gentles et al, 1997;Metzler and Beitzke, 1997; Petrosian et al, 1999; Amodeo et al, 1997;Mosca et al, 2000). For patients undergoing repair of two ventricle lesions, in the immediate postoperative period mildly elevated pulmonary artery pressures may worsen right ventricular function. Finally, severe elevations in pulmonary artery pressure to systemic or near systemic levels elicit prompt therapeutic intervention with inhaled NO. For these reasons we utilized the above definition to capture patients with a mild to moderate increase in PAP. Unfortunately, without a thermodilution catheter, we were not able to measure pulmonary vascular resistance in these patients. Commercially available thermodilution catheters are too large to routinely place in small infants and children and because this was an observational study, we did not attempt to change the practice for postoperative pulmonary artery pressure monitoring from what is normally done by the cardiac surgeons.

While the model consisting of *CPSI* T1405N genotype and age at surgery was able to predict outcome in 73% of patients in the validation cohort, there are clearly many other factors involved in the development of postoperative PAP. Certainly other genetic, biochemical, and clinical factors will be identified that will increase predictive ability. Of particular interest will be the transport functions associated with the critically important portion of the urea cycle occurring inside the mitochondrion. Large, prospective, multi-centered studies with appropriate blinding will be necessary in order to begin to stratify for broad classifications of

congenital heart disease. Only when we have knowledge of these individual factors and how they interact will we be able to accurately predict the development of this complication. In summary, future predictive models of increased pulmonary artery pressure following surgery to correct congenital cardiac defects should incorporate *CPSI* T1405N genotype.

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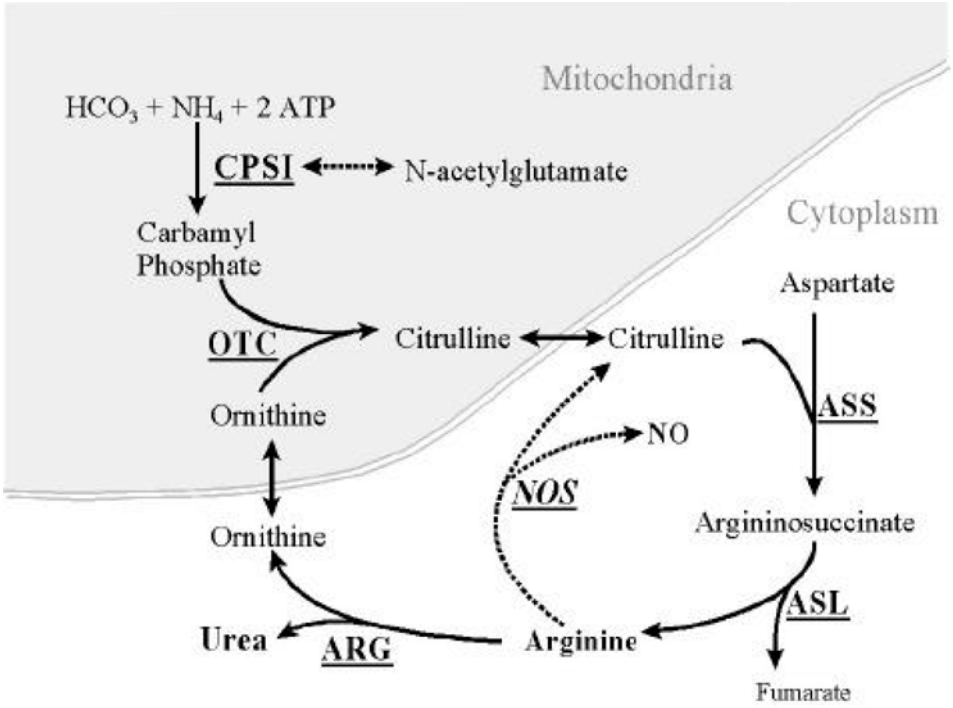


**Figure 1.**
The urea cycle. Carbamyl phosphate synthetase I is the rate limiting step in the urea cycle. The CPSI T1405N polymorphism is located in the N-acetylglutamate cofactor binding site. Note that arginine formed by the urea cycle serves as the substrate for NO.

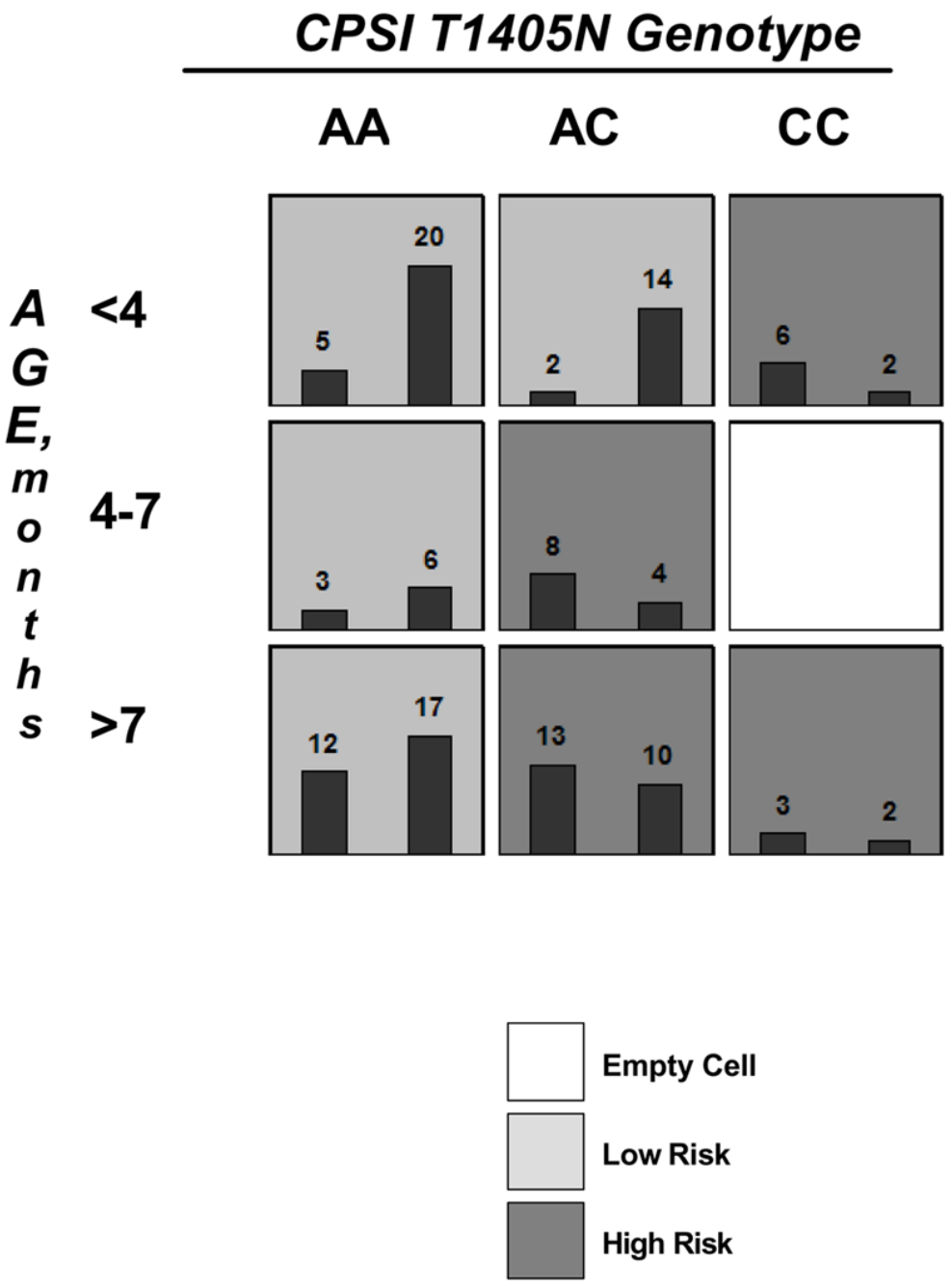


**Figure 2.**
MDR model demonstrating an interaction between CPSI 1405N genotype and patient age that predicted disease status. The average prediction error of the MDR model was only 32.5 % and the average prediction accuracy was 67.5 % ($p<0.001$). Light grey cells are low-risk, dark grey cells are high risk, and white cells contain no data points. Within each cell, the number of patients with increased PAP are shown in the left histogram while patients without increased PAP are shown by the right histogram.

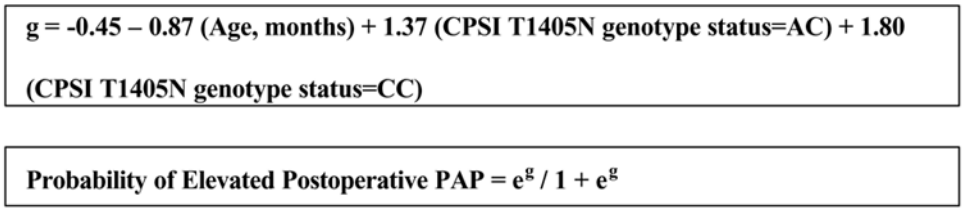


**Figure 3.**

The equation in the upper box represents the best two-variable model derived from the modeling cohort. The lower box represents the equation that transforms the modeling equation into the predicted probability of the outcome. This model was the best predictor of outcomes in the validation cohort with an accuracy of 73%.

## Table 1

**Demographic and Clinical Characteristics of Patients in the Modeling Cohort (N=131)**

The primary outcome variable in the modeling cohort was the postoperative development of increased pulmonary artery pressure (PAP). Of the 131 patients in the modeling cohort, 78 (60%) developed increased PAP. Age, weight, and Down's syndrome were independent risk factors for the development of increased postoperative PAP on univariate analysis. Weight was determined to highly correlated with age (r=0.87) and was not included in subsequent multivariate analyses. Race, gender, and length of cardiopulmonary bypass (CPB time) did not affect this outcome. Patients with increased postoperative PAP had longer duration of postoperative mechanical ventilation, and higher use of postoperative inhaled nitric oxide. Age, length of mechanical ventilation, and length of hospitalization are expressed as median and interquartile range, weight and cardiopulmonary bypass time are expressed as means ± standard deviation.

| Variable | Pulmonary Artery Pressure Following Surgery | | P-value |
|---|---|---|---|
| | Increased (N=78) | Not Increased (N= 53) | |
| Age, months | 4.5 (2.3–6.3) | 6.5 (5–11) | <0.01 |
| Race, | | | |
| %Caucasian | 77% | 85% | 0.96 |
| % African-American | 12% | 11% | |
| Gender, | | | |
| % Female | 38% | 38% | 0.96 |
| Weight, kg | 5.4 (±2.7) | 7.4 (±3.7) | <0.01 |
| Down's Syndrome, % | 22.5% | 3.6% | <0.01 |
| CPB Time, minutes | 110 (±42) | 100 (±32) | 0.12 |
| Duration of Mechanical Ventilation (hours) | 62 (22–142) | 13 (7–43.5) | <0.01 |
| Use of Inhaled Nitric Oxide | 31/78 (39.7%) | 6/53 (11.3%) | <0.01 |
| Length of Hospitalization (days) | 8 (6–15) | 7 (5–10) | 0.06 |
| Mortality | 8/78 (10.3%) | 2/53 (3.8%) | 0.20 |

## Table 2

**CPSI T1405N Genotype Distribution for Children Developing Increased Pulmonary Artery Pressure (PAP) Following Surgical Repair of Congenital Heart Defects**

The CPSI T1405N genotype distribution for children developing increased postoperative pulmonary artery pressure (PAP) following surgical repair of congenital heart anomalies is different between outcome groups (p=0.031) and supports our hypothesis that variation in this genotype may have a role in the development of postoperative elevation in PAP. Possible CPSI T1405N genotypes include homozygote (CC), heterozygote (AC), and homozygote (AA).

| Outcome Variable | CPSI Genotype CC | AC | AA | |
|---|---|---|---|---|
| Increased PAP, (%) | 44 (56%) | 30 (39%) | 4 (5%) | (N=78) |
| No increase in PAP, (%) | 20 (38%) | 24 (45%) | 9 (17%) | (N=53) |
| | | P-value=0.031 | | |

## Table 3

**Multivariate Logistic Regression Analysis Demonstrating That CPSI Genotype Independently Predicts Increased Postoperative Pulmonary Artery Pressure After Adjustment For Age, Cardiopulmonary Bypass Time and Down's Syndrome**

This table demonstrates the results of the multivariate analysis of the listed variables and their role in predicting the outcome. Note that CPSI genotype independently predicts the development on increased postoperative PAP after adjustment for age, cardiopulmonary bypass time (CPB time) and Down's syndrome. OR= odds ratio, CI= confidence interval. CPSI T1405N genotypes are compared as heterozygote (AC) vs homozygote (AA) and homozygote (CC) vs homozygote (AA).

| Variable | OR | 95% CI | P-value |
|---|---|---|---|
| Age | 0.92 | 0.87–0.98 | 0.01 |
| CPB Time | 1.00 | 0.99–1.01 | 0.53 |
| Down's Syndrome | 5.25 | 1.11–24.76 | 0.04 |
| CPSI T1405N Genotype | | | |
| AC vs. AA | 4.08 | 1.04–16.04 | 0.04 |
| CC vs. AA | 5.96 | 1.53–23.15 | 0.01 |