Validation of association of the apolipoprotein E ϵ 2 allele with neurodevelopmental dysfunction after cardiac surgery in neonates and infants

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Objective: Apolipoprotein E (*APOE*) genotype is a determinant of neurologic recovery after brain ischemia and traumatic brain injury. The *APOE* ϵ 2 allele has been associated with worse neurodevelopmental (ND) outcome after repair of congenital heart defects (CHD) in infancy. Replication of this finding in an independent cohort is essential to validate the observed genotype-phenotype association.

Methods: The association of *APOE* genotype with ND outcomes was assessed in a combined cohort of patients with single-ventricle CHD enrolled in the Single Ventricle Reconstruction and Infant Single Ventricle trials. ND outcome was assessed at 14 months using the Psychomotor Development Index (PDI) and Mental Development Index (MDI) of the Bayley Scales of Infant Development-II. Stepwise multivariable regression was performed to develop predictive models for PDI and MDI scores.

Results: Complete data were available for 298 of 435 patients. After adjustment for preoperative and postoperative covariates, the *APOE* ϵ 2 allele was associated with a lower PDI score ($P = .038$). Patients with the ϵ 2 allele had a PDI score approximately 6 points lower than those without the risk allele, explaining 1.04% of overall PDI variance, because the ϵ 2 allele was present in only 11% of the patients. There was a marginal effect of the ϵ 2 allele on MDI scores ($P = .058$).

Conclusions: These data validate the association of the *APOE* ϵ 2 allele with adverse early ND outcomes after cardiac surgery in infants, independent of patient and operative factors. Genetic variants that decrease neuroresilience and impair neuronal repair after brain injury are important risk factors for ND dysfunction after surgery for CHD. (J Thorac Cardiovasc Surg 2014;148:2560-8)

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Abbreviations and Acronyms

AIC	= Akaike's information criterion
ApoE	= apolipoprotein E
BSID-II	= Bayley Scales of Infant Development-II
CHD	= congenital heart defects
CNS	= central nervous system
CP	= cerebral palsy
CPB	= cardiopulmonary bypass
DHCA	= deep hypothermic circulatory arrest
HLHS	= hypoplastic left heart syndrome
ISV	= Infant Single Ventricle trial
LOS	= length of stay
MDI	= Mental Development Index
ND	= neurodevelopmental
PCR	= polymerase chain reaction
PDI	= Psychomotor Development Index
PHN	= Pediatric Heart Network
SVR	= Single Ventricle Reconstruction trial
TBI	= traumatic brain injury

See related commentary on pages 2568-9.

A dramatic reduction in mortality after surgical correction of congenital heart defects (CHD) in recent years has been accompanied by increasing recognition of adverse neurodevelopmental (ND) outcomes in some survivors.¹ Evaluation of children after neonatal repair of CHD demonstrates a pattern of ND sequelae characterized by cognitive impairment, speech and language abnormalities, impaired visual-spatial and visual-motor skills, attention deficit hyperactivity disorder, motor delays, and learning disabilities.²⁻⁴

Cerebral ischemia in the perioperative period has been proposed as a primary mechanism of central nervous system (CNS) injury. Management strategies during the operation, including type of support (deep hypothermic circulatory arrest [DHCA] or continuous cardiopulmonary bypass [CPB]), hemodilution, degree of cooling, and blood gas management, have been implicated as factors in postoperative ND dysfunction. These potential risk factors do not fully explain the frequency or pattern of ND dysfunction.⁵⁻⁸ There is significant interindividual variation in developmental outcome, even among children with the same cardiac defect, suggesting that other patient-specific factors may be important determinants of CNS injury.⁶

Genetic polymorphisms that increase susceptibility to neurologic injury are potentially important modifiers of ND outcome in children with CHD. Apolipoprotein E (*APOE*) genotype has been shown to have an important role as a determinant of neurologic recovery after CNS ischemia, intracerebral hemorrhage, and traumatic brain injury.⁹ A previous study demonstrated an association

between the *APOE* $\epsilon 2$ allele and postoperative ND disabilities in neonates and infants undergoing cardiac surgery with CPB.¹⁰ In that study, early ND outcomes were assessed with the Bayley Scales of Infant Development-II (BSID-II).

Most initial reports of genotype-phenotype associations are not subsequently validated. Ideally, validation should be performed in a similar population with similar environmental exposures. The phenotype assessed in the validation study should be the same as the initial report and similar measures used. In the current study, a combined cohort of patients from the Infant Single Ventricle (ISV) and Single Ventricle Reconstruction (SVR) trials was used to validate the association of the *APOE* $\epsilon 2$ allele with ND outcomes after surgery for CHD in infancy. For both the ISV and SVR trials, the study population consisted of infants with single-ventricle CHD who underwent surgery in the first year of life and in whom an ND evaluation was performed at 14 months of age using the BSID-II.^{7,8,11-13}

METHODS**Study Population**

The study population includes patients from both the ISV and SVR trials performed by the Pediatric Heart Network (PHN). The inclusion and exclusion criteria, study designs, and the participating centers for both studies have been described previously.^{12,13} The study populations contain many similarities that justify their grouping for the current study. In brief, inclusion criteria for the ISV study included (1) single-ventricle-type CHD (except pulmonary atresia with intact ventricular septum), (2) planned superior vena caval to pulmonary artery connection, and (3) absence of a genetic or medical condition that would affect growth. Inclusion criteria for the SVR trial included (1) a diagnosis of hypoplastic left heart syndrome (HLHS) or a related single, morphologic, right ventricular anomaly, (2) planned Norwood procedure, and (3) absence of a genetic or medical condition that would affect transplant-free survival. The primary outcomes and ND outcomes for both studies have been published previously.^{7,8,13} For the ISV trial, DNA was extracted from peripheral blood samples and testing of renin-angiotensin-aldosterone system pathway variants was performed.¹⁴ DNA samples were stored for future testing in the PHN biorepository in accordance with the informed consent process. Samples were sent to the University of Michigan for *APOE* genotyping. Participants in the ISV study who did not consent to future testing of their samples were not included in the current study. For the SVR trial, the participants gave consent for *APOE* genotyping. Samples were identified by the PHN Data Coordinating Center to verify that participants who were enrolled in both studies were only represented once in the current study.

Genotyping

Two methods were used to establish *APOE* genotype. The $\epsilon 2$ allele consists of a cysteine at positions 112 and 158; $\epsilon 3$ consists of a cysteine at position 112 and an arginine at position 158; $\epsilon 4$ consists of an arginine at both positions. These alleles result in 6 possible genotypes: 3 homozygous genotypes ($\epsilon 2/\epsilon 2$, $\epsilon 3/\epsilon 3$, and $\epsilon 4/\epsilon 4$) and 3 heterozygous genotypes ($\epsilon 2/\epsilon 3$, $\epsilon 2/\epsilon 4$, and $\epsilon 3/\epsilon 4$).

For the SVR participants, buccal swab epithelial cells were collected using CytoSoft cytology brushes (Medical Packaging Corporation, Camarillo, Calif) after obtaining informed consent. Genomic DNA was extracted using a PureGene kit (Gentra Systems, Inc, Minneapolis, Minn) according to the manufacturer's protocol. Genotype analysis for the presence of the single nucleotide polymorphisms that specify a cysteine and/or an arginine residue at codons 112 and 158 of the *APOE* gene, which define the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$

alleles, was performed using a TaqMan assay and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif) as described by Koch and colleagues.¹⁵ Reactions were carried out in 96-well microtiter plates in the ABI Prism 7000 Sequence Detection System. The assay volume of 22 μ L consisted of 11 μ L of 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), 75 nmol/L each forward and reverse primers, 25 nmol/L each allele specific probe, and 2 μ L of DNA (~30–100 ng). Amplification involved 40 cycles of denaturation at 95°C for 15 seconds and primer annealing and extension at 60°C for 1 minute. Patient samples were run along with controls representing all 3 possible genotypes (ie, Cys/Cys, Cys/Arg, and Arg/Arg) as well as a no-template control. Postamplification analysis of the genotypes at each codon was performed using the allelic discrimination analysis module of the ABI 7000 Sequence Detection System. The genotype of the patient was deduced from the combined results at amino acid positions 112 and 158. DNA samples were available for 398 SVR participants and genotyping was successful for 397. However, for 90 samples, there was no consent to use the DNA for secondary studies.

The ISV samples were amplified by polymerase chain reaction (PCR) and the amplicons were subjected to Sanger sequencing. PCR primers were designed to produce an amplicon containing the sequence differences that are associated with the *APOE* ϵ 2, ϵ 3, and ϵ 4 variants. PCR primers were selected using the LaserGene Primer Select Program (DNASTAR Inc, Madison, Wis). Forward (5'-CCG CCC CAT CCC AGC CCT TCT CC) and reverse (5'-TCC GGC TGC CCA TCT CCT CCA TCC) primers were selected. A 12- μ L PCR reaction was set up for each patient and control DNA sample. Each 12- μ L PCR reaction contained 1 μ L of 10–40 ng genomic patient or control DNA, 1 μ L of 1.5 pmol forward primer, 1 μ L of 1.5 pmol reverse primer, 3 μ L of DNase/RNase free water, and 6 μ L of HotStarTaq polymerase mixture (Qiagen, Inc, Valencia, Calif). PCR DNA amplification was performed on a thermal cycler (Bio-Rad MyCycler, Bio-Rad Life Sciences, Hercules, Calif) using 96-well plates. A touchdown PCR protocol was used as follows: initial denaturation at 94°C for 15 minutes, followed by 24 cycles with an annealing temperature decreasing 0.7°C per cycle, starting at 72°C for 30 seconds; denaturation at 94°C for 30 seconds, and extension at 72°C for 1 minute. An additional 32 cycles were added: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, with a final extension of 72°C for 10 minutes. Each PCR product was diluted with 40 μ L of distilled water and submitted for sequencing. Sanger sequencing was performed by the University of Michigan DNA Sequencing Core facility using an Applied Biosystems DNA Sequencer (Model 3730 XL). Each chromatogram was visually inspected using Applied Biosystems Sequence Scanner (v1.0) software to determine genotypes. Twenty-eight samples were genotyped using both methods with 100% concordance of genotypes with all genotype combinations represented. DNA was available for 160 patients and adequate genotypes were obtained in 150. Genotyping was unsuccessful in 10 patients because of low sample quantity or quality.

Neurodevelopmental Evaluation

In both trials, ND testing with the BSID-II was performed at 14 months by a designated study-site psychologist certified by the PHN's neuropsychological testing consultant (DCB). The BSID-II was administered in English or Spanish depending on the dominant language spoken at home. The BSID-II offers a standardized assessment of cognitive and motor development for children aged 1 to 42 months.¹⁶ It yields 2 scores: the Psychomotor Development Index (PDI) and the Mental Development Index (MDI). The PDI assesses control of gross muscle function, including crawling and walking, as well as fine muscle skills necessary for prehension, use of writing instruments, and imitation of hand movements. The MDI assesses memory, problem solving, early number concepts, generalization, vocalizations, and language and social skills. The mean $\pm$ standard deviation is 100 $\pm$ 15 in the normative population for both scores. Patients who were too impaired to complete neurodevelopmental testing were assigned a score of 50. The PDI score is usually more severely affected in infant survivors of cardiac surgery than the MDI score.^{5,7}

Statistical Analyses

All analyses were performed in R (<http://www.r-project.org/>). *APOE* genotypes were classified into 3 groups as follows: ϵ 2 (ϵ 2 ϵ 2 or ϵ 2 ϵ 3), ϵ 3 (ϵ 3 ϵ 3), or ϵ 4 (ϵ 4 ϵ 3 or ϵ 4 ϵ 4). Participants with ϵ 2 ϵ 4 genotype (n = 9) were excluded from the analyses because the alleles have opposing effects in adults and cannot be placed in either the ϵ 2 or ϵ 4 groups. Stepwise linear regression analysis was used to select from the numerous demographic, clinical, and genetic variables entering the model those that have the greatest contribution to the model as measured by Akaike's information criterion (AIC). The variables included in the stepwise regression model were surgical center, gender, a dummy variable for race with the white subgroup (the majority racial group) as the reference group, HLHS, gestational age, birth weight, presence of a genetic anomaly, post-Norwood length of stay (LOS) or LOS after neonatal palliative surgery, bypass time during palliative surgery, aortic crossclamp time, DHCA time, weight at stage 2 surgery, height at stage 2 surgery, head circumference at stage 2 surgery, post-stage 2 surgery LOS, weight/height/head circumference at 14 months, maternal education, and number of serious adverse events until 14 months of age. Model comparison was performed using AIC, beginning with a base model that included only surgical site and presence of genetic syndrome. Only specific demographic, clinical, and genetic variables that improved model prediction of the outcome of either MDI or PDI at 14 months were retained in each respective final regression model. A subgroup analysis was performed for nonsyndromic participants (n = 232) to ensure that *APOE* ϵ 2 genotype effects were consistent when excluding participants with chromosomal or other genetic anomalies.

RESULTS

The ISV trial randomized 230 patients. The SVR trial randomized 549 patients. There were 66 patients enrolled in both studies resulting in 713 unique participants for the combined cohort. There was no overlap with the original discovery cohort. In the ISV trial, 92% of survivors returned and had valid BSID-II scores.⁸ In the SVR trial, the follow-up rate for the BSID-II examination among transplant-free survivors was 86%.⁷ Overall, the ND evaluation was completed in 435 patients. Complete data, including *APOE* genotype, were available for 298 patients, who form the study cohort (Figure 1). The most common

FIGURE 1. The study cohort. ISV, Infant Single Ventricle trial; SVR, Single Ventricle Reconstruction trial; *APOE*, apolipoprotein E.

TABLE 1. Comparison between patients with complete and incomplete data

	Complete data (n = 298)	Incomplete data (n = 137)	P value
Patient variables			
Gender, n (%)			
Male	193 (64.8)	85 (62.0)	.59
Female	105 (35.2)	52 (38.0)	
Race/ethnicity, n (%)			
White	196 (65.8)	104 (75.9)	.035
Black	25 (8.4)	24 (17.5)	.084
Hispanic	64 (21.5)	9 (6.6)	5.32×10^{-5}
Other	13 (4.3)	0	
Hypoplastic left heart syndrome, n (%)	230 (77.2)	112 (81.8)	.32
Gestational age, wk $\pm$ SD	38.3 $\pm$ 1.49	38.4 $\pm$ 1.38	.52
Birth weight, kg $\pm$ SD	3.198 $\pm$ 0.516	3.162 $\pm$ 0.527	.51
Genetic or chromosomal syndrome, n (%)	47 (15.8)	31 (22.6)	.11
Maternal education, n (%)*			
Elementary school	8 (2.7)	1 (0.9)	.14
Junior high	4 (1.3)	1 (0.9)	
Some high school	23 (7.7)	8 (6.7)	
High school or GED equivalent	46 (15.4)	33 (27.7)	
Some college or 2-y vocational school	95 (31.9)	35 (29.4)	
4-y college	84 (28.2)	30 (25.2)	
Graduate degree	38 (12.8)	11 (9.2)	
APOE genotype			
ϵ 2 (ϵ 2/ ϵ 3 or ϵ 2/ ϵ 2)	33 (11.1)		
ϵ 3 (ϵ 3/ ϵ 3)	178 (59.7)		
ϵ 4 (ϵ 3/ ϵ 4 or ϵ 4/ ϵ 4)	87 (29.2)		
ϵ 2/ ϵ 4	0	9 (6.6)	
Stage 1			
Cardiopulmonary bypass time, min $\pm$ SD	130.4 $\pm$ 53.4	139.1 $\pm$ 51.4	.11
Crossclamp time, min $\pm$ SD	49.85 $\pm$ 25.1	53.6 $\pm$ 24.5	.15
DHCA use, n (%)	255 (85.6)	121 (88.3)	.45
Post-Norwood procedure LOS, d $\pm$ SD	30.8 $\pm$ 30.4	31.9 $\pm$ 29.3	.73
Stage 2			
Pre-stage 2 weight, kg $\pm$ SD	5.89 $\pm$ 1.07	5.92 $\pm$ 1.04	.79
Pre-stage 2 height, cm $\pm$ SD	61.6 $\pm$ 4.30	62.03 $\pm$ 4.24	.34
Pre-stage 2 head circumference, cm $\pm$ SD	39.9 $\pm$ 2.34	40.3 $\pm$ 2.20	.10
Post-stage 2 LOS, d $\pm$ SD	13.4 $\pm$ 23.0	12.4 $\pm$ 11.9	.55
Neurodevelopmental evaluation			
Weight at 14 mo, kg $\pm$ SD	9.22 $\pm$ 1.31	9.38 $\pm$ 1.28	.27
Height at 14 mo, cm $\pm$ SD	74.7 $\pm$ 3.76	75.2 $\pm$ 4.13	.20
Head circumference at 14 mo, cm $\pm$ SD	45.9 $\pm$ 1.98	45.7 $\pm$ 2.09	.46
Serious adverse events until 14 mo, n $\pm$ SD	0.71 $\pm$ 1.38	0.47 $\pm$ 0.89	.028
MDI score at 14 mo $\pm$ SD	90.6 $\pm$ 16.2	90.6 $\pm$ 17.3	.98
PDI score at 14 mo $\pm$ SD	76.9 $\pm$ 19.2	73.8 $\pm$ 18.7	.12

SD, Standard deviation; GED, General Educational Development; DHCA, deep hypothermic circulatory arrest; LOS, length of stay; MDI, Mental Development Index; PDI, Psychomotor Development Index; APOE, apolipoprotein E. *Information on maternal education collected on 119 patients in the incomplete data subset.

reason for missing data was lack of APOE genotype. The only significant differences in patient and management variables between patients with complete versus incomplete data were a greater proportion of Hispanic patients and a greater frequency of serious events in the group with complete data (Table 1). Overall, the cohort with complete data was predominately male (65%) and white (65%), very similar to the composition of the initial discovery cohort.^{5,17} The predominant diagnosis was HLHS (77%). The ϵ 2 allele

was present in 11% of the cohort. APOE genotype was in Hardy-Weinberg equilibrium, with a rejection cutoff of $P < 10^{-4}$. For the entire cohort, the median PDI was 76.9 (range, 50-120) and the median MDI was 90.6 (range, 50-132).

In the overall cohort, after adjustment for preoperative and postoperative covariates, the APOE ϵ 2 allele was associated with a lower PDI score ($P = .038$). Other significant predictors of the PDI score were study center, height at the

TABLE 2. Stepwise regression results for Bayley-II PDI score at 14 months of age (n = 298)

Variable	Beta coefficient $\pm$ SE	% PDI explained	P value
Site	—	10.87	.00037
Height at 14 mo (cm)	1.27 $\pm$ 0.303	8.46	3.28×10^{-5}
Post-Norwood LOS (d)	-0.137 $\pm$ 0.0394	5.34	.00062
Presence of genetic syndrome	-7.09 $\pm$ 2.54	1.93	.0057
Pre-stage 2 surgery head circumference (cm)	1.068 $\pm$ 0.458	1.18	.021
APOE ϵ 2 genotype	-6.50 $\pm$ 3.11	1.04	.038
Number of serious adverse events	-1.70 $\pm$ 0.972	0.89	.081
Gender (female)	2.98 $\pm$ 2.14	0.49	.16

SE, Standard error; PDI, Psychomotor Development Index; LOS, length of stay; APOE, apolipoprotein E.

time of ND evaluation, postoperative LOS during the Norwood hospitalization or neonatal palliation, presence of a genetic syndrome, head circumference at the time of stage 2 surgery, and the number of serious adverse events up to the neurodevelopmental evaluation (Table 2). The overall model explained 24.9% of the variability in the PDI scores. After removing the syndromic patients (n = 66), the association of the APOE ϵ 2 allele with the PDI score became less significant ($P = .06$). After covariate adjustment, patients with the APOE ϵ 2 allele would be predicted to have a PDI score that was approximately 6 points lower than those without the risk allele, explaining 1.04% of overall PDI variance.

For the overall cohort, there was a marginal effect of APOE ϵ 2 allele on MDI scores ($P = .058$). Significant predictors of the MDI score were study center, birth weight, postoperative LOS during the stage 2 hospitalization, presence of a genetic syndrome, race other than white, female gender, and head circumference at the time of ND

evaluation (Table 3). The overall model explained 31.4% of the variability in the MDI scores. After removing the syndromic patients (n = 66), the association of the APOE ϵ 2 allele with the MDI score became more significant ($P = .02$). The presence of the APOE ϵ 2 allele accounted for 0.84% of overall MDI variation. After covariate adjustment, nonsyndromic patients with the APOE ϵ 2 allele would be predicted to have a MDI score that was approximately 7 points lower than those without the risk allele.

DISCUSSION

These findings validate the previously reported association of the APOE ϵ 2 allele with early ND disability after cardiac surgery in neonates and infants.^{5,10} There are multiple reports of genotype-phenotype associations in many populations that have failed replication in independent studies.¹⁷⁻¹⁹ To our knowledge, this represents the first time that a genotype-phenotype association modifying postoperative outcomes after surgery for CHD has been replicated in an independent cohort. Replication of the initial finding is necessary to establish the validity of a genotype-phenotype association.¹⁷⁻¹⁹ The impact of APOE genotype on an individual patient's ND outcomes is significant. After adjustment for covariates, patients with the ϵ 2 allele are predicted to have PDI and MDI scores 6 to 7 points lower than those without the risk allele, similar to the magnitude of the effect in the discovery cohort.⁵ This is a significant decline and represents more than a 1/3 standard deviation decrease in the scores. However, APOE genotype explained only approximately 1% of the variation in MDI and PDI scores in the overall cohort, which is less than the variation explained by other factors such as site, postoperative LOS, growth, and presence of genetic syndromes. The small overall impact likely relates to the relatively low frequency of APOE ϵ 2, which was present in only 11% of the cohort.

ApoE-containing lipoproteins are the primary lipid transport vehicles in the CNS and have an important role in mobilization and redistribution of cholesterol and phospholipids during remodeling of neuronal membranes.⁹ The gene for the human APOE is located on chromosome 19 and codes for a 299 amino acid protein. There is increasing evidence that ApoE protein is important for neuronal repair. There are 3 common isoforms of ApoE protein (E2, E3, and E4), which are encoded by 3 alleles (ϵ 2, ϵ 3, and ϵ 4, respectively) and vary by single amino acid substitutions at 2 sites. The normal function of ApoE within neurons is believed to be maintenance of microtubular integrity and stabilization of the neuronal cytoskeleton.^{9,20} Mechanisms by which ApoE may modify CNS injury include protection against oxidative stress, modulation of the glial response to inflammation, and a direct neurotropic effect on injured neurons. ApoE synthesis is upregulated by astrocytes and oligodendrocytes after CNS injury or cerebral ischemia.

TABLE 3. Stepwise regression results for Bayley-II MDI score at 14 months of age (n = 298)

Variable	Beta coefficient $\pm$ SE	% MDI explained	P value
Site	—	15.46	1.18×10^{-7}
Post-Norwood LOS (d)	-0.0955 $\pm$ 0.0391	7.83	.015
Birth weight (g)	0.00623 $\pm$ 0.00172	4.19	.00035
Presence of genetic syndrome	-6.35 $\pm$ 2.20	2.58	.0043
Post-stage 2 LOS (d)	-0.215 $\pm$ 0.0815	1.79	.0909
Race other	-16.41 $\pm$ 5.46	1.70	.0029
Gender (female)	5.15 $\pm$ 1.89	1.09	.0068
Head circumference at 14 mo (cm)	0.966 $\pm$ 0.485	0.83	.048
APOE ϵ 2 genotype	-5.15 $\pm$ 2.70	0.84	.058
Maternal education	0.965 $\pm$ 0.686	0.46	.14

SE, Standard error; MDI, Mental Development Index; LOS, length of stay; APOE, apolipoprotein E.

Studies in *APOE* knockout mice have shown that ApoE deficiency worsens neuronal injury after cerebral ischemia and compromises the blood-brain barrier after CNS injury.^{9,20,21} A recent study using brain biopsies from patients with epilepsy found that neurons from patients with the *APOE* $\epsilon 4$ allele are less resilient to the chronic excitation of epilepsy.²² Neurons from patients with *APOE* $\epsilon 3\epsilon 3$ demonstrated more beneficial responses to hyperexcitability, neuroinflammation, and neuronal DNA damage. *APOE* genotype may also modulate white matter development. Westlye and colleagues²³ evaluated white matter microstructure in healthy adults. Carriers of either the $\epsilon 2$ or $\epsilon 4$ allele demonstrated regional decreases in fractional anisotropy. This finding, and the known role of ApoE in cholesterol transport, support a functional role for *APOE* in myelin-related processes in the brain. Trachtenberg and colleagues²⁴ assessed functional connectivity in cognitively normal adults using functional magnetic resonance imaging and found different patterns of connectivity based on *APOE* genotype. They speculate that *APOE* genotype has an intrinsic effect on the development and differentiation of functional networks in the brain.

In adults, strong associations have been validated between the *APOE* genotype and Alzheimer disease, as well as recovery after traumatic brain injury (TBI).^{20,25,26} In adults, the *APOE* $\epsilon 4$ allele is associated with an increased risk of Alzheimer disease and worse recovery after TBI. However, multiple studies have demonstrated that the effects of *APOE* genotype on recovery after brain injury are not the same in the immature developing brain as in the aging brain. In contrast to adults, the $\epsilon 2$ allele is associated with worse outcomes and the $\epsilon 4$ allele is protective after a variety of types of brain injury in infants and children. *APOE* genotype has been shown to modify the adverse effects of lead exposure in children. In a study of lead exposure by Wright and colleagues,²⁷ the *APOE* $\epsilon 4$ allele was associated with a 4.4 point higher score on the MDI. The negative effects of lead exposure on the MDI were 4-fold greater for $\epsilon 2$ and $\epsilon 3$ carriers compared with $\epsilon 4$ carriers. Chronic diarrhea with malnutrition is associated with deficits in cognition and executive function.²⁸⁻³⁰ In children who have multiple diarrheal episodes early in life, the $\epsilon 4$ allele is associated with better visual working memory and semantic fluency. However, Gelfand and colleagues³¹ found that the $\epsilon 4$ allele is associated with an increased risk of perinatal arterial ischemic stroke. In addition, the data on the impact of *APOE* genotype on the risk of cerebral palsy (CP) is mixed. Braga and colleagues³² reported a higher prevalence of *APOE* $\epsilon 2$ carriers in patients with CP and, in a study by Kuroda and colleagues,³³ *APOE* $\epsilon 2$ carriers had a 12-fold increased risk of CP and $\epsilon 4$ carriers a 5-fold increase. Wu and colleagues³⁴ also described an increased prevalence of the *APOE* $\epsilon 4$ allele in patients with CP; however, the

association was not significant after correction for multiple comparisons. A recent study from Australia which evaluated 35 candidate genes and the risk of CP found no association between *APOE* genotype and CP.³⁵

There are limitations to this study. Because this is a secondary analysis, data including *APOE* genotype, are incomplete for some patients. Nonetheless, our study has important strengths, which are necessary for validation of a genotype-phenotype association. Our validation cohort population was similar to the discovery cohort, had similar environmental exposures, and the same phenotype was assessed using the same instrument. Specifically, both the discovery and validation cohorts consist of neonates and infants with CHD undergoing surgery with CPB early in life. The ethnic makeup of the 2 cohorts is similar, reducing the risk of population stratification altering the results. Both the discovery and validation studies assessed the phenotype of early ND outcomes using the BSID-II, and the magnitude of the effect of the *APOE* $\epsilon 2$ allele on PDI and MDI scores was similar in both cohorts.

In summary, this study provides evidence confirming the previous report that the *APOE* $\epsilon 2$ allele is associated with worse early ND outcomes after cardiac surgery in neonates and infants. This *APOE* genotype–environment interaction demonstrates that genetic variants that impair neuroresilience and CNS recovery may explain some of the interindividual variation in developmental outcome after surgery for CHD. However, it would be premature to begin routine clinical testing of *APOE* genotype, as the impact is small and the knowledge would not alter clinical management. These findings support the need for studies to delineate the mechanisms by which *APOE* genotype modulates brain recovery and investigate potential therapies. Future studies of neurologic outcome and clinical trials of new neuroprotective strategies should include risk stratification for *APOE* genotype. In addition, further studies are needed to identify other genetic variants that modify the risk of brain injury and ND disability.

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Discussion

Dr Charles D. Fraser (Houston, Tex). I would really like to congratulate Dr Gaynor and colleagues for yet another important contribution to the search for understanding of the complexities of abnormal neurodevelopmental outcomes in children requiring cardiac surgery. This study presented on behalf of the Pediatric Heart Network Infant Single Ventricle Trial and the Single Ventricle Reconstruction Trial investigators sought to validate work that Dr Gaynor has previously conducted in a group of newborn infants with varying cardiac diagnoses in which the Apo-lipoprotein E E2 allele was associated with poorer neurodevelopmental outcomes as determined in 224 infants using the Bayley II Scale of Infant Development (BSID) evaluated by the Psychomotor Developmental Index (PDI) and the Mental Developmental Index (MDI).

The present study focuses on single-ventricle patients, predominantly children with hypoplastic left heart syndrome who survived to undergo the BSID evaluation at 14 months of life and whose APOE genotype had been assessed; from the initial combined cohort of 713 individuals; 298 patients were eligible for this study. And as Dr Gaynor has so elegantly presented, important findings include a median PDI of 76.9, normal being 100, and a median MDI of 90.6, also normal being 100.

For the entire cohort, the APOE E2 allele was associated with a statistically significant lower PDI score and a lower MDI score approaching significance. Then after removing clearly syndromic patients from the analysis, both PDI and MDI scores were significantly lower in children with the E2 allele. Of note, and part of my questioning below, the E2 allele was calculated to explain roughly 1% of the overall PDI and MDI variance, but the site of the surgery was most strongly associated with the observed variation accounting for between 10% and 15% of the variation.

So my questions are:

(1) Given these results, can genotyping for the E2 allele now be added to our armamentarium in counseling families about neurodevelopmental risk in their newborn infants facing cardiac surgery? And can this be extended into fetal life or should it be?

Dr Gaynor. Again, I think it is hard to know until we can actually do something about it. When we actually set up our original study, the Institutional Review Board told us we could not tell the families what gene we were testing or what the result was, because they were concerned that the epsilon 4 allele is the 1 associated with the risk of Alzheimer disease and they were concerned that we would be giving them news that would make them worry that we could do nothing about.

I think for clinical trials, yes, it is going to be something that will help us understand the variation. If we develop a therapeutic strat-

egy that can be based on the genotype, then it should definitely be incorporated into it. I do not think I would recommend it for routine care until we have a therapy based on it.

Dr Fraser. I do not want to dominate the discussion, but I have to have a corollary to that question though. So you are going to publish this and there are going to be parents who are going to find out this information and they are going to ask you, “Should my child have a genotype for this?” The answer would be “No”?

Dr Gaynor. I would tell them right now there is nothing we can do at the current time to change our therapy based on this genotype.

Dr Fraser. Question 2: you note the increasing evidence that APOE protein is important for neuronal repair and patients with abnormal APOE genotype are more susceptible to oxidative stress-related neuronal injury; is it not logical to conclude that perioperative strategies that mitigate the potential for neuronal injury should have benefit as modifiable risk factors for neurodevelopmental outcome because at present we are unable to modify the genotype?

Dr Gaynor. If we could find a strategy that might mitigate the risk, that would make sense. Currently, the best evidence we have is based on neural imaging techniques and early neurodevelopmental outcomes. Of all the strategies, continuous bypass, antegrade cerebral perfusion, deep hypothermic circulatory arrest, all are associated with somewhere between a 30% and 40% incidence of new injury based on early magnetic resonance imaging. That is from our institution, your institution, multiple other institutions.

In a recent large study from New Zealand, they looked at the risk of new injury and neurodevelopmental outcomes, and the degree of brain immaturity at the time of surgery was a more important predictor than the operative management strategies. In the SVR trial where patients were managed with both deep hypothermic circulatory arrest and antegrade cerebral perfusion, we could not identify a difference.

If we could identify an operative management strategy that we could show, then yes, it might make a difference. When we looked at different groups in our own cohort, we could not identify any difference in the effect whether or not they had circulatory arrest or continuous bypass.

Dr Fraser. Well, that is a good segue into my last question, and particularly provocative given the data that Dr Karamlou presented earlier. I find it fascinating and to my view the most important point of your study, frankly, that the strongest association is with the site of surgery. As per the above, given your previous work focusing on nonmodifiable risk factors, are you ready to update your viewpoint on the complex interplay of modifiable and nonmodifiable causes of injury?

To my reading of this study, although the E2 allele is associated with some significant level of increased susceptibility, the location of the child's surgery has a much stronger influence. My interpretation is that certain centers through the complex interplay of modifiable perioperative factors (surgery, perioperative care, cardiopulmonary bypass, and others unknown) are able to achieve better results in terms of brain protection and we do not fully understand why.

Dr Gaynor. I would agree. In the SVR paper we struggled with this. There are several potential things. We do not know if there are unmeasured differences in the patient populations between

the centers. To the best we could ascertain, the groups were similar.

There are clearly differences in operative strategies between the centers, but what we could measure in terms of bypass management, blood gas management, other things like that, none of those came out.

One of the other things that we did find and we were concerned about is that there may be some ascertainment by us between centers. There were some centers where it looked like there was a drift of scores over time, that the scores either got higher or lower over time. One of the flaws of the SVR strategy was that we did not have a retest of the evaluators for the Bayley scales. So there are a variety of reasons. There may be unmeasured factors. Everything that we looked at between the centers in terms of patient characteristics, operative management strategies, we could not identify a difference. We even did a subgroup analysis looking at Michigan, Wisconsin, CHOP, and Boston, which have very different strategies, and could not identify any difference in outcomes between those centers. So we tried to look at that to see if we could identify either a patient factor or a management strategy that would explain that, and we are still not sure and there is this lingering concern that some of it may be due to ascertainment bias.

Dr Fraser. Well, I just have to push on that a little bit. I could get it if it were a few-fold difference, but it is a 15-fold difference.

Dr Gaynor. I agree. The measured factors that we looked at, and there may be other factors that we did not, but in the subgroup analysis of the 4 largest centers contributing, we could not identify any real difference in outcomes. And where we could look to make sure the populations were the same and any impact of management strategies, we could not identify that. That leaves open, there is a hypothesis, there may be some differences; however, when you look again from your center, our center, neuroimaging, very different strategies, and yet we have very similar outcomes. I think it is an important question, but I do not think we have answered that yet.

Dr Hillel Laks (*Los Angeles, Calif*). It was a great paper. Is there any evidence for this APOE E2 allele, or in patients without that, that the age at which you are exposed to bypass or circulatory arrest makes a difference? In this particular analysis did the time of surgery with regard to the maturity of the brain make a difference?

Dr Gaynor. We included age at surgery as 1 of the covariates and it did not come out.

Dr Laks. And do you have data in terms of the maturity of the brain; when is the inflection point at which it might be safer to use these techniques and bypass?

Dr Gaynor. Well, it is hard to know. Based on early postnatal imaging before surgery, there is about a 4- to 5-week immaturity of the brain. If you think, we have a baby who is 37 or 38 weeks, their brain is probably something more along 32 to 33 weeks. There probably is a population of vulnerable white matter precursors at that point. And we know brain maturation is delayed in these kids. It suggests you would have to wait 5 to 6 weeks to allow the brain to come to a normal maturity.

Now, that is problematic in itself. You may have to keep the child in the ICU on prostaglandins. We have some data that was just presented at the American Heart Association that suggests delaying stage 1 past about 5 days of life increases the risk of white matter

injury. So I think it is a complex interplay. If they did not require neonatal surgery or did not have ductal-dependent pulmonary or systemic blood flow, it might be possible to delay until they are out of the neonatal period and the brain is matured. But many of these kids are ductal dependent for either systemic or pulmonary blood flow.

Dr Laks. But you could argue that an off-pump procedure, which would get a similar result, would be better if you could delay the open procedure for at least 5 weeks.

Dr Gaynor. It might be. But again, if you do a shunt off pump, you may introduce a steal. If you do the hybrid procedure for stage 1, there is concern that then cerebral blood flow is retrograde, up a

tiny arch, and it becomes an endartery. So there are competing factors, and I do not think the study has been done that would allow us to answer that question.

Dr Jennifer Hirsch-Romano (*Ann Arbor, Mich*). I just have 1 other question for you. This gets back to what Chuck Fraser brought up about the variation across sites. If you looked at the mortality across those sites, was that associated with worse neurodevelopmental outcomes at long-term follow-up?

Dr Gaynor. We tried to look at that thinking there might be a survivor bias, that some sites could get sicker kids through and they would have worse outcomes, and we could not identify anything like that.

EDITORIAL COMMENTARY

Validation accepted, but look at what else was revealed

Erle H. Austin III, MD

See related article on pages 2560-8.

Cardiac surgery for neonates and infants has evolved substantially in the past several decades with significant improvement in survival for all forms of congenital heart disease. Survival, of course, is only the first objective. Parents want their child to survive, but they also want their child to thrive after recovering from heart surgery. Those of us who care for these patients are paying an increasing amount of attention to neurodevelopmental outcomes.

One of several areas directed at improving neurodevelopmental outcomes is the identification of genetic markers that correlate with neurologic outcome. In 2003, Gaynor and colleagues¹ demonstrated a significant detrimental effect of the apolipoprotein E (APOE) $\epsilon 2$ allele on the neurodevelopmental outcome of patients aged 6 months or less who had undergone cardiac surgery. In this single-center prospective study, neurodevelopmental evaluation was performed using the Bayley Scales of Infant Development assessed at 12 months of age. The presence of the $\epsilon 2$ allele correlated with a 7-point decrease in the Psychomotor Developmental Index (PDI) compared with patients without

that allele. As the apolipoprotein E protein is considered an important factor in repairing injured neural tissue, the presence of the $\epsilon 2$ allele in these patients is believed to adversely affect the ability of the protein to perform such repair after neonatal and infant heart surgery.

As the authors of the accompanying report² indicate, reports of genotype-phenotype associations are rarely validated. The initial report was the product of much work and many resources at a center with a substantial volume of patients. Repeating that process for the sake of validation was unlikely. Fortunately, it was recognized that the Pediatric Heart Network had been independently studying similar patients in 2 multicenter trials (the Infant Single Ventricle [ISV] and the Single Ventricle Reconstruction [SVR] trials). Fortunately, APOE testing had been performed on the SVR patients and DNA had been extracted from blood samples of the ISV patients. Furthermore, in both trials, neurodevelopmental testing (Bayley Scales of Infant Development-II [BSID-II]) had been performed on most patients. Thus, the opportunity to validate or refute the findings of the initial report presented itself.

After appropriate selection of patients, the authors of the current report identified 298 patients with complete APOE and neurodevelopmental data. The presence of the $\epsilon 2$ allele imparted a 6-point decrease in PDI score, almost the same as the 7-point decrement in PDI determined in the primary report. A decrement in the Mental Developmental Index (MDI), the other portion of BSID-II, was also seen at a level just short of significance, but again, similar to what was found in the initial study. I think it is fair to say this genotype-phenotype association has been validated. The authors are to be congratulated for identifying this

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