

Replication of Genetic Associations in the Inflammation, Complement, and Coagulation Pathways With Intraventricular Hemorrhage in LBW Preterm Neonates

KELLI K. RYCKMAN, JOHN M. DAGLE, KEEGAN KELSEY, ALLISON M. MOMANY, AND JEFFREY C. MURRAY

Department of Pediatrics [K.K.R., J.M.D., K.K., A.M.M., J.C.M.], Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242; Department of Molecular Biology and Genetics [K.K.], Cornell University, Ithaca, New York 14853

ABSTRACT: Intraventricular hemorrhage (IVH) is a significant morbidity seen in very LBW infants. Genes related to the inflammation, infection, complement, or coagulation pathways have been implicated as risk factors for IVH. We examined 10 candidate genes for associations with IVH in 271 preterm infants (64 with IVH grades I–IV and 207 without IVH) weighing <1500 g. The heterozygous genotype OR = 8.1, CI = 2.5–26.0, $p = 4 \times 10^{-4}$) and the A allele (OR = 7.3, CI = 2.4–22.5, $p = 1 \times 10^{-4}$) of the coagulation factor V (*FV*) Leiden mutation (rs6025) were associated with an increased risk of developing IVH grade I or II but not grade III or IV after correction for multiple testing with Bonferroni. Lack of association in the severe grades of IVH may be a result of lack of power to detect an effect given the small sample size ($n = 8$). However, this result is consistent with previous research that demonstrates that the heterozygous genotype of the *FV* mutation is associated with increased risk for the development of IVH but a decreased risk for the progression or extension to more severe grades of IVH. (*Pediatr Res* 70: 90–95, 2011)

Intraventricular hemorrhage (IVH), characterized as bleeding into the ventricular system of the developing brain, is one of the leading morbidities for very LBW (VLBW) preterm neonates (1,2). IVH ranges in severity from grade I to the most severe grade IV. The incidence for IVH grades I–IV is around 27% in neonates weighing <1500 g (1). Approximately, 45–85% of premature infants with the more severe grades of IVH (grades III–IV) incur cognitive disabilities such as CP and mental retardation, whereas infants with milder grades of IVH (grades I–II) are at risk for developmental delays (3,4). Risk factors for IVH include LBW, early GA, male gender, maternal smoking, preterm premature rupture of membranes (PPROM), chorioamnionitis, early onset sepsis, RDS, patent ductus arteriosus (PDA), and pneumothorax (2,5–8). However, many of these have not been consistently shown as risk factors for IVH and they do not entirely explain the etiology and pathogenesis of this complex disorder (9).

Recently, genetic factors have been implicated in the risk for developing IVH in both term and preterm infants (10–15). Twin studies suggest that shared genetic and environmental

risk factors explain 41.3% of the risk for developing IVH after controlling for gender, GA, and birth weight (16). Previous studies of genetic association with IVH have focused on genes related to either inflammation and infection or complement and coagulation. *IL1β*-511T (rs16944), *IL4*-590T (rs2243250), *IL6*-174C (rs1800795), and *TNF-α*-308 (rs1800629) all associated with IVH (10,17,18). In addition, *IL10*-1082A (rs1800896) is associated with an increased risk of periventricular leukomalacia (PVL), a condition that often occurs in conjunction with IVH (19,20). The coagulation factor V (*FV*) Leiden mutation (rs6025), a coagulation factor II (*FII*), prothrombin polymorphism (G20210A, rs1799963), and a coagulation factor XIII (*FXIII*) missense mutation (Val34Leu, rs5985) have been implicated in several studies as risk factors for the development of IVH (12,14,15,21,22). In addition, two genes integrin beta-3 (*ITGB3*) and estrogen receptor-alpha (*ESRI*) have been associated with IVH (13,23). However, many of these associations have not been tested in multiple independent populations or have not consistently replicated across studies.

To determine whether genetic associations previously identified replicate, we examined 10 candidate genes for association with IVH susceptibility: *IL1β*, *IL4*, *IL6*, *IL10*, *TNF*, *FII*, *FV*, *ITGB3*, and *ESRI*. We chose either the same single nucleotide polymorphism (SNP) previously associated with IVH or a SNP in high linkage disequilibrium (LD) with the associated SNP and tested associations in 271 preterm infants weighing <1500 g.

MATERIALS AND METHODS

Study population. Premature infants (delivery before 37 wk of gestation) admitted to the NICU at the University of Iowa Children's Hospital between 2000 and 2009 were recruited to examine preterm birth (PTB) and neonatal complications of prematurity. Blood or buccal swabs from infants and their parents have been collected and banked. Informed consent was obtained from participating families, and the study was approved by the University of Iowa Institutional Review Board (200506792) for sample recruitment and to access

Abbreviations: APGAR, appearance, pulse, grimace, activity, respiration; c-PVL, cystic periventricular leukomalacia; ESRI, estrogen receptor-alpha; FII, coagulation factor II; FV, coagulation factor V; HWE, Hardy-Weinberg equilibrium; ITGB3, integrin beta-3; IVH, intraventricular hemorrhage; LD, linkage disequilibrium; PDA, patent ductus arteriosus; PPROM, preterm premature rupture of membranes; PVL, periventricular leukomalacia; SNP, single nucleotide polymorphism; VLBW, very LBW

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Correspondence: Jeffrey C. Murray, M.D., Department of Pediatrics, University of Iowa, 500 Newton Road, 2182ML, Iowa City, IA 52242; e-mail: jeff-murray@uiowa.edu
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the associated clinical information necessary for this study. This population is a subset of one that has been described previously but evaluated for PTB with respect to the progesterone receptor and genes affecting cholesterol metabolism (24,25).

The first analysis consisted of 271 unrelated infants born <32 wk of gestation and weighing <1500 g. There were 48 sets of twins in this study, one twin was chosen from each pair. The twin with the most severe case of IVH was selected, if both twins had the same IVH status one twin was randomly selected. Cases were defined as infants with IVH grades I–IV, and controls were those without documented IVH. IVH grade was confirmed by ultrasonography with grade I defined as blood in the periventricular germinal matrix, grade II as blood in the ventricular system without ventricular dilatation, grade III as blood in the lateral ventricles with ventricular dilatation, and grade IV as blood in the ventricular system with parenchymal extension. We studied 207 infants without IVH, 28 with grade I, 10 with grade II, 16 with grade III, and 10 with grade IV. Demographics of this population are described in Table 1. A second phase of the study was performed after removing confounders that could potentially interfere with inflammation/infection and coagulation genetic associations. Exclusions included infants of women with heart disease (0.37%), bleeding disorder (1.1%), autoimmune disease (0.0%), thrombocytopenia (2.2%), gestational diabetes (3.0%), type I diabetes (0.74%), type II diabetes (1.1%), chronic hypertension (8.9%), preeclampsia (28.0%), eclampsia (1.1%), gestational hypertension (5.5%), hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome (7.7%), infants with congenital anomalies (3.0%), infants who were a twin (17.7%), and infants with one or both parents of non-Caucasian descent (23.2%). Race of infant was determined through self-reported questionnaire of the mother. The remaining subset included 103 infants, 81 without IVH, 10 with grade I, 4 with grade II, 8 with grade III, and none with grade IV.

DNA processing and genotyping. DNA was extracted from blood or buccal swabs (26). Ten SNPs were chosen for analysis with IVH; seven have been shown to associate with IVH previously (*IL10-1082* rs1800896, *TNF-308* rs1800629, *FII* G20210A rs1799963, *FV* G1691A rs6025, *FXIII* Val34Leu rs5985, *ITGB3* Leu33Pro rs5918, *ESR1* rs2234693) and three were in strong LD with SNPs previously reported to be associated with IVH (*IL1β-31* rs1143627 with *IL1β-511* rs16944 $r^2 = 0.96$; *IL6* rs2069832 with *IL6-174* rs1800795, $r^2 = 0.96$; and *IL4* rs2243270 with *IL4-590* rs2243250, $r^2 = 0.94$). LD was determined using the Caucasian population (CEU) from

Hapmap. Genotyping was performed using TaqMan (Applied Biosystems, Foster City, CA), as previously described (24). Allele scoring was done using the Sequence Detection Systems software (version 2.2, Applied Biosystems). The genotype data were uploaded into a Progeny database (Progeny Software, LLC, South Bend, IN), also containing phenotypic data, for subsequent statistical analysis.

Statistical analysis. Demographic characteristics were compared between infants without IVH ($n = 207$) and infants with IVH grades I–IV ($n = 64$) using the Wilcoxon rank sum test when comparing continuous traits and Fisher's exact test for dichotomous traits. The first dataset included 271 infants born <32 wk of gestation and weighing <1500 g. The second set included 103 infants after excluding the potential confounders described above. Markers were tested for deviations from Hardy-Weinberg Equilibrium (HWE) with Fisher's exact tests. Fisher's exact tests were also used to compare genotype and allele frequencies between the following groups; 1) infants without IVH and those with IVH grades I–IV; 2) infants without IVH and those with IVH grades I–II, and 3) infants without IVH and those with IVH grades III–IV. A Bonferroni significance level of $p < 5 \times 10^{-3}$ (0.05/10 independent tests) was used to correct for multiple testing. Logistic regression was performed on SNPs with significant ($p < 0.05$) genotype differences. The OR and CI using the Woolf test was calculated for SNPs with significant ($p < 0.05$) allele frequency differences. Statistical analysis was performed in Stata version 10.1 (Stata Corp, College Station, TX). In addition, logistic regression was performed after controlling for factors that differed by IVH status (Table 1), specifically appearance, pulse, grimace, activity, respiration (Apgar score) at 1 and 5 min, cystic PVL (c-PVL), twin status, and PDA. Power analysis was performed with PS Power (27).

RESULTS

Demographic data. Birth weight, GA, race, gender, retinopathy of prematurity (ROP), RDS, necrotizing enterocolitis, (NEC), sepsis, pneumothorax, smoking during pregnancy, diabetes, hypertension, PPRM, maternal preexisting conditions, thrombocytopenia, and congenital anomalies did not differ between infants with IVH compared to those without

Table 1. Demographic characteristics of 271 unrelated infants with ($n = 64$) and without ($n = 207$) IVH

Trait	No IVH ($n = 207$)	IVH ($n = 64$)	p^*
Birth weight ($n = 271$)	948 (416)	1,026 (485)	0.38
GA ($n = 271$)	28 (3)	27 (4)	0.34
APGAR 1 min ($n = 268$)	6 (3)	4 (4)	1.0×10^{-4}
APGAR 5 min ($n = 269$)	8 (2)	6 (3)	2.0×10^{-4}
Race ($n = 270$)			0.31
African-American	25 (12.1%)	7 (10.9%)	
Hispanic	12 (5.8%)	6 (9.4%)	
Caucasian	162 (78.6%)	46 (71.9%)	
Other	7 (3.4%)	5 (7.8%)	
Infant is a twin ($n = 271$)	28 (13.5%)	20 (31.3%)	2.0×10^{-3}
Gender—male ($n = 271$)	120 (58.0%)	38 (59.4%)	0.89
PVL ($n = 271$)	5 (2.4%)	11 (17.2%)	9.7×10^{-5}
ROP ($n = 262$)	66 (32.5%)	24 (40.7%)	0.28
PDA ($n = 271$)	74 (35.8%)	34 (53.1%)	0.02
RDS ($n = 271$)	174 (84.1%)	55 (85.9%)	0.84
NEC ($n = 271$)	17 (8.2%)	2 (3.1%)	0.26
Sepsis ($n = 115$)	62 (70.5%)	19 (70.4%)	1.00
Pneumothorax ($n = 270$)	12 (5.8%)	4 (6.3%)	1.00
Smoked during pregnancy ($n = 257$)	44 (22.0%)	46 (22.8%)	0.86
PPROM ($n = 271$)	40 (19.3%)	13 (20.3%)	0.86
Diabetes ($n = 271$)	10 (4.8%)	3 (4.7%)	1.00
Clinical chorioamnionitis ($n = 219$)	27 (15.7%)	6 (12.8%)	0.82
Hypertension/preeclampsia/eclampsia ($n = 271$)	75 (36.2%)	15 (23.4%)	0.07
Heart disease/bleeding disorder/autoimmune disease ($n = 65$)	3 (6.3%)	1 (5.9%)	1.00
Thrombocytopenia ($n = 31$)	3 (12.5%)	3 (42.8%)	0.11
Congenital anomaly ($n = 271$)	7 (3.4%)	1 (1.6%)	0.69

Median and interquartile range is given for continuous traits, and counts and percentages are given for dichotomous traits. Numbers of nonmissing observations are given for each trait.

* p values were calculated with Wilcoxon rank sum test for continuous traits and Fisher's exact test for dichotomous traits.

ROP, retinopathy of prematurity; NEC, necrotizing enterocolitis.

IVH (Table 1). As expected c-PVL was more frequent in infants with IVH (17.2%) compared to those without IVH (2.4%; $p = 9.7 \times 10^{-5}$). This association has been documented in previous reports (10). PDA was also more common in infants with IVH (53.1%) compared to those without IVH (35.8%; $p = 0.02$). In addition, as expected infants with IVH had lower Apgar scores at 1 and 5 min compared to those without IVH. There was also a higher incidence of twins among infants with IVH (31.3%) compared to those without (13.5%).

Genetic associations with IVH. All markers were in HWE in infants with and without IVH in the full dataset ($n = 271$). In the dataset after exclusion of potential confounders all the markers were in HWE with the exception of *ESR1* rs2234693 ($p = 0.03$) in infants with IVH. When comparing infants with and without IVH, we detected allele and genotype frequency differences for *IL1 β* rs1143627 and *FV* rs6025 in both the full dataset and the dataset with exclusions (Table 2). Infants with the CC genotype (OR = 3.1, CI = 1.3–7.5, $p = 0.01$), the CT genotype (OR = 2.2, CI = 1.1–4.7, $p = 0.04$), or the C allele (OR = 1.8, CI = 1.2–2.8, $p = 5 \times 10^{-3}$) of *IL1 β* rs1143627 were at increased risk for IVH compared to infants with the TT genotype or T allele. After excluding infants with potential confounders the association with IVH and the CT genotype of *IL1 β* rs1143627 remained (OR = 4.2, CI = 1.2–14.6, $p = 0.03$); however, the associations with the CC genotype ($p = 0.56$) and the C allele ($p = 0.23$) were no longer significant, possibly because of the decreased sample size and lack of

power to detect the effects (Table 2). After controlling for factors that significantly differed by IVH in Table 1; *i.e.* Apgar scores at 1 and 5 min, c-PVL, twin status, and PDA, the CT (OR = 2.8, CI = 1.2–6.8, $p = 0.02$) and CC (OR = 3.7, CI = 1.3–10.2, $p = 0.02$) genotypes were associated with increased risk for IVH in the full data and the CT genotype only (OR = 4.0, CI = 1.1–14.8, $p = 0.04$) was associated with increased risk for IVH in the data after exclusion criteria.

Infants heterozygous (OR = 4.9, CI = 1.6–14.8, $p = 5 \times 10^{-3}$) or with the A allele (OR = 4.6, CI = 1.6–13.6, $p = 2 \times 10^{-3}$) of the *FV* Leiden mutation were at increased risk for IVH. After excluding infants with potential confounders, infants heterozygous for the *FV* Leiden mutation (OR = 14.4, CI = 1.4–147.9, $p = 0.02$) or with the A allele (OR = 13.3, CI = 1.3–131.6, $p = 5 \times 10^{-3}$) were at increased risk for IVH. Only the association between *FV* rs6025 and IVH in the full dataset was significant after correction for multiple testing with Bonferroni. After controlling for Apgar scores at 1 and 5 min, c-PVL, twin status, and PDA, heterozygotes for the *FV* Leiden mutation were still at increased risk for IVH in the full data (OR = 4.0, CI = 1.1–14.9, $p = 0.04$) and after exclusion criteria (OR = 17.7, 1.3–244.7, $p = 0.03$).

Genetic associations with grade of IVH. *IL1 β* rs1143627 was significant or marginally significant for allele and genotype differences when comparing infants without IVH to those with IVH grades I–II or IVH grades III–IV (Table 3). Infants with the CT genotype (OR = 3.0, CI = 1.1–7.9, $p = 0.03$) or the C allele (OR = 1.7, CI = 0.99–3.0, $p = 0.05$) of *IL1 β*

Table 2. Genetic associations comparing premature infants (<32 wk gestation and <1500 g) with and without IVH

Full data ($n = 271$)		No IVH ($n = 207$)				IVH ($n = 64$)				p^* No IVH vs IVH	
Gene	rs# (A/B)	AA	AB	BB	F_A \dagger	AA	AB	BB	F_A \dagger	Genotype	Allele
<i>IL1β</i>	rs1143627 (C/T)	30	81	80	0.37	14	27	12	0.52	0.02	7.0×10^{-3}
<i>IL6</i>	rs2069832 (A/G)	18	70	81	0.31	6	21	23	0.33	0.92	0.81
<i>IL4</i>	rs2243270 (A/G)	94	52	10	0.77	31	11	2	0.83	0.50	0.25
<i>IL10</i>	rs1800896 (A/G)	54	103	42	0.53	16	35	11	0.54	0.79	0.92
<i>TNF</i>	rs1800629 (A/G)	5	45	142	0.14	0	19	39	0.16	0.23	0.66
<i>FII</i>	rs1799963 (A/G)	0	4	176	0.01	0	3	53	0.03	0.36	0.36
<i>FV</i>	rs6025 (G/A)	188	6	0	0.98	51	8	0	0.93	$5.0 \times 10^{-3}\ddagger$	6.0×10^{-3}
<i>FXIII</i>	Rs5985 (G/T)	49	29	5	0.77	19	4	1	0.88	0.18	0.11
<i>ITGB3</i>	rs5918 (C/T)	4	51	117	0.17	0	12	40	0.12	0.49	0.22
<i>ESR1</i>	rs2234693 (C/T)	44	93	48	0.49	14	26	16	0.48	0.88	0.91
Data with exclusions ($n = 103$)§		No IVH ($n = 81$)				IVH ($n = 22$)				p^* No IVH vs IVH	
<i>IL1β</i>	rs1143627 (C/T)	11	25	38	0.32	2	11	4	0.44	0.05	0.23
<i>IL6</i>	rs2069832 (A/G)	10	31	24	0.39	2	9	7	0.36	1.00	0.85
<i>IL4</i>	rs2243270 (A/G)	43	21	0	0.84	14	3	0	0.91	0.37	0.42
<i>IL10</i>	rs1800896 (A/G)	20	40	19	0.51	2	14	4	0.45	0.25	0.60
<i>TNF</i>	rs1800629 (A/G)	2	13	61	0.11	0	8	11	0.21	0.08	0.11
<i>FII</i>	rs1799963 (A/G)	0	2	70	0.01	0	2	17	0.05	0.19	0.19
<i>FV</i>	rs6025 (G/A)	77	1	0	0.99	16	3	0	0.92	0.02	0.02
<i>FXIII</i>	Rs5985 (G/T)	20	15	0	0.79	6	1	0	0.93	0.22	0.29
<i>ITGB3</i>	rs5918 (C/T)	2	25	43	0.21	0	8	9	0.24	0.73	0.82
<i>ESR1</i>	rs2234693 (C/T)	17	36	20	0.48	9	5	6	0.58	0.08	0.37

* p is calculated with Fisher's exact test comparing infants with no documented IVH to those with documented IVH grades I–IV.

\dagger F_A is the allele frequency of the A allele.

\ddagger Significant after correction for multiple testing with Bonferroni (threshold of 5×10^{-3}).

§ Infants of women with heart disease, bleeding disorder, autoimmune disease, thrombocytopenia, gestational diabetes, type I diabetes, type II diabetes, chronic hypertension, pre-eclampsia, eclampsia, gestational hypertension, HELLP syndrome, infants with congenital anomalies, infants who were a twin or triplet, and infants with one or both parents of non-Caucasian descent were excluded from analysis leaving a sample size of 103 infants.

Table 3. Genetic associations comparing premature infants (<32 weeks gestation and <1500 g) without IVH to those with grades I–II and those with grades III–IV

Full data (n = 271)		No IVH (n = 207)				IVH grades I and II (n = 38)				IVH grades III and IV (n = 26)				<i>p</i> * No IVH vs grades I and II		<i>p</i> * No IVH vs grades III and IV	
Gene	rs# (A/B)	AA	AB	BB	F_A†	AA	AB	BB	F_A†	AA	AB	BB	F_A†	Genotype	Allele	Genotype	Allele
<i>IL1β</i>	rs1143627 (C/T)	30	81	80	0.37	6	18	6	0.50	8	9	6	0.54	0.06	0.06	0.07	0.03
<i>IL6</i>	rs2069832 (A/G)	18	70	81	0.31	5	9	13	0.35	1	12	10	0.30	0.41	0.64	0.52	1.00
<i>IL4</i>	rs2243270 (A/G)	94	52	10	0.77	16	8	0	0.83	15	3	2	0.83	0.62	0.36	0.17	0.55
<i>IL10</i>	rs1800896 (A/G)	54	103	42	0.53	12	18	7	0.57	4	17	4	0.50	0.80	0.61	0.35	0.76
<i>TNF</i>	rs1800629 (A/G)	5	45	142	0.14	0	10	23	0.15	0	9	16	0.18	0.59	0.85	0.39	0.52
<i>FII</i>	rs1799963 (A/G)	0	4	176	0.01	0	1	33	0.01	0	2	20	0.05	0.58	0.58	0.13	0.13
<i>FV</i>	rs6025 (G/A)	188	6	0	0.98	27	7	0	0.90	24	1	0	0.98	1.0 × 10 ^{-3‡}	1.0 × 10 ^{-3‡}	0.58	0.58
<i>FXIII</i>	Rs5985 (G/T)	49	29	5	0.77	10	3	0	0.88	9	1	1	0.86	0.59	0.21	0.18	0.42
<i>ITGB3</i>	rs5918 (C/T)	4	51	117	0.17	0	7	23	0.12	0	5	17	0.11	0.75	0.35	0.77	0.40
<i>ESR1</i>	rs2234693 (C/T)	44	93	48	0.49	10	14	9	0.52	4	12	7	0.43	0.65	0.79	0.77	0.53
Data with exclusions (n = 103)		No IVH (n = 81)				IVH grades I and II (n = 14)				IVH grades III (n = 8)				<i>p</i> * No IVH vs grades I and II		<i>p</i> * No IVH vs grades III	
<i>IL1β</i>	rs1143627 (C/T)	11	25	38	0.32	1	7	3	0.41	1	4	1	0.50	0.15	0.47	0.19	0.21
<i>IL6</i>	rs2069832 (A/G)	10	31	24	0.39	2	5	4	0.41	0	4	3	0.29	1.00	1.00	0.75	0.57
<i>IL4</i>	rs2243270 (A/G)	43	21	0	0.84	7	3	0	0.85	7	0	0	1.00	1.00	1.00	0.10	0.13
<i>IL10</i>	rs1800896 (A/G)	20	40	19	0.51	2	8	3	0.46	0	6	1	0.43	0.80	0.83	0.26	0.78
<i>TNF</i>	rs1800629 (A/G)	2	13	61	0.11	0	4	7	0.18	0	4	4	0.25	0.41	0.31	0.11	0.12
<i>FII</i>	rs1799963 (A/G)	0	2	70	0.01	0	1	12	0.04	0	1	5	0.08	0.40	0.39	0.22	0.22
<i>FV</i>	rs6025 (G/A)	77	1	0	0.99	8	3	0	0.86	8	0	0	1.00	5.0 × 10 ^{-3‡}	6.0 × 10 ⁻³	1.00	1.00
<i>FXIII</i>	rs5985 (G/T)	20	15	0	0.79	3	1	0	0.88	3	0	0	1	0.63	1.00	0.26	0.59
<i>ITGB3</i>	rs5918 (C/T)	2	25	43	0.21	0	4	6	0.20	0	4	3	0.29	1.00	1.00	0.52	0.50
<i>ESR1</i>	rs2234693 (C/T)	17	36	20	0.48	6	5	2	0.65	3	0	4	0.43	0.29	0.14	0.02	0.79

* *p* is calculated with Fisher’s exact test comparing infants with no documented IVH with those with documented IVH grades I–II and grades III–IV. There were no infants with grade IV IVH after exclusions, so comparisons are only made with IVH grade III.

† F_A is the allele frequency of the A allele.

‡ Significant after correction for multiple testing with Bonferroni (threshold of 5 × 10⁻³).

§ Infants of women with heart disease, bleeding disorder, autoimmune disease, thrombocytopenia, gestational diabetes, type I diabetes, type II diabetes, chronic hypertension, pre-eclampsia, eclampsia, gestational hypertension, HELLP syndrome, infants with congenital anomalies, infants who were a twin or triplet, and infants with one or both parents of non-Caucasian descent were excluded from analysis leaving a sample size of 103 infants.

rs1143627 were at increased risk for IVH grades I–II compared to infants with the TT genotype or T allele. Infants with the CC genotype (OR = 3.6, CI = 1.1–11.1, *p* = 0.03) or the C allele (OR = 2.0, CI = 1.1–3.8, *p* = 0.02) of *IL1β* rs1143627 were at increased risk for IVH grades III–IV compared to infants with the TT genotype or T allele. These associations were not significant after correction for multiple testing with Bonferroni nor did they remain significant after excluding infants for potential confounders (Table 3). After controlling for Apgar scores at 1 and 5 min, c-PVL, twin status, and PDA, the CT (OR = 3.1, 1.1–8.8, *p* = 0.03) but not CC genotype (*p* = 0.1) increased risk for IVH grades I–II and the CC (OR = 6.5, 1.3–33.8, *p* = 0.03) but not CT (*p* = 0.2) genotype increased risk for IVH grades III–IV in the full data. These results did not remain significant (*p* > 0.05) in the data after exclusions.

When comparing infants with grades I–II IVH to those without IVH, there were allele and genotype frequency differences for *FV* rs6025 in both the full dataset and the dataset with exclusions (Table 3). Associations in both the full dataset and the dataset with exclusions were significant after correction for multiple testing with Bonferroni. Infants heterozygous (OR = 8.1, CI = 2.5–26.0, *p* = 4 × 10⁻⁴) or with the A allele (OR = 7.3, CI = 2.4–22.5, *p* = 1 × 10⁻⁴) for the *FV* Leiden mutation were at increased risk for IVH grades I–II. After excluding infants for potential confounders, the association

with IVH and both the heterozygous genotype (OR = 28.9, CI = 2.7–311.2, *p* = 6 × 10⁻³) and the A allele (OR = 24.5, CI = 2.4–247.2, *p* = 1 × 10⁻⁴) remained significant; however, there was no association in either dataset when comparing IVH grades III–IV to infants without IVH (*p* > 0.5). However, the power to detect the same effect in the A allele as observed in IVH grades I–II was 70% and therefore lack of association cannot be adequately established. After controlling for Apgar scores at 1 and 5 min, c-PVL, twin status, and PDA, heterozygotes for the *FV* Leiden mutation were still at increased risk for IVH grades I–II in the full data (OR = 5.5, CI = 1.5–20.0, *p* = 0.01) and after exclusion criteria (OR = 40.5, 2.0–840.1, *p* = 0.02).

DISCUSSION

Genetic studies of IVH have largely focused on genes involved in inflammation and infection, because this pathway is strongly implicated in the pathophysiology of perinatal brain injury, supported by animal models and studies in human preterm infants (10). We sought to replicate 10 candidate genes from the inflammation/infection and complement/coagulation pathways for association with IVH. We identified two genes (*IL1β* (rs16944) and *FV* (rs6025)) that associated with the risk for IVH in our cohort; thereby replicating previous studies.

Previously, the *IL1 β* -511 (rs16944) T allele was associated with an increased risk of IVH in 215 VLBW infants compared to the C allele (OR = 3.0, CI = 1.4–6.4, $p = 0.003$) (10). We validated this result by finding that the *IL1 β* -31 (rs1143627) C allele was associated with an increased risk of IVH ($p = 0.007$). This finding was significant or marginally significant for both IVH grades I–II and IVH grades III–IV. The C allele of *IL1 β* -31 is in strong LD ($r^2 = 0.96$) with the T allele of *IL1 β* -511. The *IL1 β* -31 C allele is associated with an increased production of IL1 β *in vivo* (28). There is substantive evidence that IL1 β is involved in the pathophysiology of perinatal brain injury. Injection of IL1 β causes brain injury in neonatal rats, and increased amniotic and/or cord blood levels of IL1 β are observed in infants with PVL and IVH (29–33). The exact mechanisms by which IL1 β is involved in IVH and perinatal injury is still not entirely clear; however, our research has identified that the same allele of *IL1 β* -31 (C allele) associated with increased levels of IL1 β is also associated with an increased risk of IVH.

Coagulation factors, specifically *FII*, *FV*, and *FXIII* have been considered as possible candidate genes for IVH because of likely interactions between thrombophilic factors and the pathology of IVH. It is hypothesized that increased fibrinolytic activity and decreased levels of clotting factors may contribute to the severity of intracranial bleeding that can occur in preterm infants (34,35). We found no significant associations with *FII* or *FXIII*; however, the *FV* Leiden mutation (rs6025) and IVH were strongly associated. The heterozygous genotype of rs6025 was previously shown to associate with an increased risk for IVH and to protect against the extension and/or progression to more severe grades of IVH (14,22). Our study supports these findings as the heterozygous genotype of the *FV* Leiden mutation was significantly associated in infants with IVH grades I–II ($p = 5 \times 10^{-3}$) but not IVH grades III–IV ($p = 1.0$). However, the lack of association detected in the severe grades of IVH must be interpreted with caution, because of the small sample size in the more severe grades of IVH ($n = 8$) and therefore lack of power (70%) to detect the same effect seen in IVH grades I–II (OR = 24.5). Moreover, it is to be noted that the effect size seen in the IVH grades I–II groups after stringent exclusions is likely inflated because of the small sample size; therefore, the power to detect an effect in the severe IVH group is likely lower. The *FV* Leiden variant has about a 5% allele frequency in the Caucasian population and is a glutamine to arginine replacement at amino acid position 506 that results in an increased risk for thrombosis (36). The associations with the *FV* Leiden mutation were the only results in our study that withstood Bonferroni correction for multiple testing and lends evidence to the hypothesis that the complement coagulation pathway is involved in the risk for the development of IVH.

Although several studies have found associations with the *FV* Leiden mutation (rs6025) (10,14,15,21,22) there have also been other studies that failed to find association (10,37). This could be due to ethnic heterogeneity, varied study design, or lack of an adequate control group for comparison. A strength of our study was the ability to compare very preterm (<32 wk of gestation) VLBW (<1500 g) infants with IVH to very

preterm VLBW infants without IVH, whereas other studies have compared infants with IVH to term infants without IVH and their associations are therefore confounded by GA and birth weight. However, one weakness of our study is a relatively small sample size and therefore associations that were not detected in our analysis may not be indicative of lack of association but rather because of the limited power to detect the effects. For example, for the genes for which no allelic association was detected (IL4, IL6, IL10, TNF, FII, FXIII, ITGB3, and ESR1), the power to detect these associations only reached 80% for effect sizes larger than an OR of 4.

However, this study does replicate two genes [*IL1 β* (rs16944) and *FV* (rs6025)] associated with the risk for IVH. IVH is a significant problem for very preterm VLBW infants. Although there is substantial research that has focused on understanding the etiology, mechanisms, and risk factors for IVH, little progress has been made in preventing this serious condition. It is important that continuing research focus on replicating previous findings and discovering new mechanisms and pathways for IVH. Identification of genetic risk factors can provide an opportunity to generate new therapeutic and preventative strategies in an era of personalized medicine.

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