Association of NOS3 tag polymorphisms with hypoxic-ischemic encephalopathy

**Aim** To test the association of NOS3 gene with hypoxic-ischemic encephalopathy (HIE).

**Methods** The study included 110 unrelated term or preterm born children (69 boys and 41 girls) with HIE and 128 term and preterm born children (60 boys and 68 girls) without any neurological problems after the second year of life. Children with perinatal HIE fulfilled the diagnostic criteria for perinatal asphyxia. All children were admitted to the Clinical Hospital Split between 1992 and 2008. We analyzed 6 tagging single nucleotide polymorphisms (SNP) within NOS3 gene (rs3918186, rs3918188, rs1800783, rs1808593, rs3918227, rs1799983), in addition to previously confirmed NOS3-associated SNP rs1800779. Genotyping was conducted using real-time polymerase chain reaction (PCR). Association analyses were performed according to allelic and genotypic distribution.

**Results** Allelic test did not show any SNP association with HIE. SNP rs1808593 showed genotype association (P = 0.008) and rs1800783-rs1800779 TG haplotype showed an association with HIE (P < 0.001). The study had 80% statistical power to detect (α = 0.05) an effect with odds ratio (OR) = 2.07 for rs3918186, OR = 1.69 for rs3918188, OR = 1.70 for rs1800783, OR = 1.80 for rs1808593, OR = 2.10 for rs3918227, OR = 1.68 for rs1800779, and OR = 1.76 for rs1799983, assuming an additive model.

**Conclusion** Despite the limited number of HIE patients, we observed genotypic and haplotype associations of NOS3 polymorphisms with HIE.
Hypoxic-ischemic encephalopathy (HIE) in the perinatal period is a very serious neurological problem in children. It causes long-term or permanent damage, such as cerebral palsy, epilepsy, certain forms of mental retardation, and cognitive and sensory disorders in a relatively large number of affected children (1,2). HIE occurs in premature and full-term children after intrauterine infection and/or chorioamnionitis, premature rupture of membranes, maternal trauma, shock, or other etiologies. Premature infants are a particularly vulnerable group for the occurrence of hemorrhagic and hypoxic-ischemic brain damage, and the incidence of these injuries is higher in children with lower gestational ages (1,3). All of these conditions impair cerebral blood flow, leading to ischemia and hypoxia and start a cascade of deleterious biochemical events that seriously and permanently injure the brain (2).

The main events during ischemia are an accumulation of free radicals, creation of excitatory neurotransmitters in selectively vulnerable areas, lack of adenosine triphosphate, and increase in intracellular calcium. Free radicals are highly reactive and cause damage of lipids, DNA, and proteins, which leads to neuronal death. One of the important radicals in this event is nitric oxide (2,4). Nitric oxide is a weak free radical formed during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). There are two isoforms of NOS: constitutive and inducible (NOS2). Constitutive NOS is active in vascular endothelial cells and is called endothelial nitric oxide synthase (NOS3), while the NOS which is active in the central and peripheral nervous system is called neuronal nitric oxide synthase (NOS1) (5).

The role of nitric oxide in the pathogenesis of ischemic brain damage is dual; protective and deleterious, depending on NOS isoform and the type of the cell that produces nitric oxide. It also depends on the term and intensity of oxidative stress (4). In an intense oxidative stress, nitric oxide produced by NOS1 leads to neuronal death, causing mitochondrial damage, loss of energy, and further disruption of calcium homeostasis (4). Nitric oxide produced by NOS3 has a protective role. Mild exposure to ischemia activates enzyme NOS3 and produces small amounts of nitric oxide with subsequent relaxation of blood vessels and vasodilatation. The nitric oxide produced by NOS3 plays a prominent role in maintaining cerebral blood flow and preventing neuronal injury (6). It has several different roles, such as inhibition of platelet aggregation, inhibition of platelet and leukocyte adhesion to endothelium, relaxation and inhibition of smooth blood vessel muscle cells proliferation, stimulation of angiogenesis, anti-inflammatory action, and prevention of oxidative damage (6). Because of the very important role of nitric oxide in these processes, impaired nitric oxide synthesis by NOS3 in vascular endothelial cells, may contribute to ischemic damage (7). Understanding this dual role of NOS and nitric oxide in the brain ischemia may lead us to prevention and treatment of ischemic damage (8).

Nitric oxide production in blood vessels is controlled by NOS3 gene located on the chromosome 7q36.1, which contains 26 coding exons and has genomic size of 21kb (6). It has been reported that NOS3 gene polymorphisms may lead to reduction or disruption of gene transcription, altering enzymatic function, and therefore cause susceptibility to ischemic brain diseases (9). So far, several gene polymorphisms of NOS3 have been found to be associated with cerebrovascular disease: Glu298Asp, also known as E298D, or G894T (rs1799983) in exon 7; a variable number of 27 bp nucleotide tandem repeat sequences (VNTR, 27pb) in intron 4; and T786C polymorphism (rs2070744) in the 5’t region of NOS3 (10). Another NOS3 gene polymorphism, -922A/G (rs1800779), showed an association with ischemic stroke (11). However, the results are still ambiguous, and functional consequences of NOS3 polymorphisms have not yet been completely clarified. There seems to be a correlation of NOS3 polymorphisms with the clinical manifestation of perinatal and neonatal diseases (12). Our study aims to clarify the role and impact of 7 NOS3 gene polymorphisms on the occurrence of perinatal HIE.

MATERIAL AND METHODS

Participants

The study included 110 unrelated full-term or preterm born children (69 boys and 41 girls) with hypoxic-ischemic injury and intracranial hemorrhage (HIE group). The control group consisted of 128 apparently healthy full-term and preterm born children (60 boys and 68 girls), without any neurological problems after the second year of life (control group), who were admitted to the Clinical Hospital Split between 1992 and 2008. All children with perinatal hypoxic-ischemic injury fulfilled the diagnostic criteria for perinatal asphyxia: fetal heart frequency during delivery <100 or >160 per min, and/or Apgar score at 5-minute ≤7, and/or blood gases pH<7.1. The clinical course of HIE was mild, moderate, or severe for all the affected children according to the classification (13). The HIE was confirmed with brain ultrasound. Routine cranial sonography was performed on preterm infants (gestational age less
than 37 weeks) on the first day of life and was repeated at least once during the first week and at 4th-6th week of age. The cranial sonography was performed on infants born at 37 weeks gestational age or later depending on clinical indications. Hypoxic-ischemic injury and intracranial hemorrhage were confirmed by magnetic resonance imaging at the age of 2 years. The HIE group consisted of 72.7% of patients with hypoxic-ischemic injury and of 27.3% of patients with hypoxic-ischemic injury accompanied with intracranial hemorrhages. The study was approved by the Ethics Committee of Clinical Hospital Center Split and School of Medicine Split, and informed consent was obtained from patients’ parents prior to the blood sampling.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the Perfect gDNA kit (Eppendorf, Hamburg, Germany). Six haplotype-tagging SNPs (rs3918186, rs3918188, rs1800783, rs1808593, rs3918227, rs1799983) were selected across the NOS3 gene using the Tagger software (http://www.broad.mit.edu/mpg/tagger/server.html) (14). The proportion of variation across the NOS3 gene region captured by tagSNPs was calculated based on the HapMap phase II using the same software (15). The SNP rs1800779 was also analyzed in this study because of its more common association with the development of HIE (16,17). Real-time polymerase chain reaction (PCR) analysis for 7 NOS3 polymorphisms was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and pre-developed TaqMan assay reagents. PCR was carried out according to the manufacturer’s protocol.

Statistical analysis

Prior to association analysis, we performed quality control of the obtained genotypes. We tested genotyping rate, Hardy-Weinberg equilibrium (HWE), and minor allele frequencies (MAF) for all samples using HaploView 4.1. MAFs were compared with the National Center for Biotechnology Information SNP database (NCBI dbSNP), MAFs for the Central European population (www.ncbi.nlm.nih.gov/projects/SNP/). Case-control single-point and multi-point association analyses were carried out using Haploview 4.1. Haplotypes frequencies were estimated using the expectation-maximization algorithm implemented in HaploView 4.1. P-value lower than 0.05 was considered significant. Calculations of 80% power study at α=0.05 were performed using Quanto. The results are expressed as OR. When $OR>1$, genotype confers sensitivity to the effects of exposure (18).

RESULTS

The control group of 128 participants consisted of 38.2% of full-term infants (≥37 gestation weeks), 26.6% of preterm infants of 32-36 gestation weeks, and 35.2% of preterm infants of <32 gestation weeks, while HIE group of 110 infants consisted of 18.2% of full-term infants, 34.5% of preterm infants of 32-36 gestation weeks, and 47.3% of preterm infants of <32 gestation weeks. Genotype call rate was 100% for all the investigated SNPs. Genotypes in cases and controls fit HWE, except for rs1808593, which slightly deviated from HWE in controls ($P=0.0023$). MAFs in controls were concordant with NCBI dbSNP frequencies for the Central European population. The 6 analyzed NOS3 tagSNPs – rs3918186, rs3918188, rs1800783, rs1808593, rs3918227, rs1799983 – were also analyzed in this study because of its more common association with the development of HIE (16,17). Real-time polymerase chain reaction (PCR) analysis for 7 NOS3 polymorphisms was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and pre-developed TaqMan assay reagents. PCR was carried out according to the manufacturer’s protocol.

Figure 1.

Linkage disequilibrium $r^2$ values of 7 NOS3 single nucleotide polymorphisms in the affected group of children with hypoxic-ischemic encephalopathy, calculated using HaploView.
frequently and GG genotype more frequently than in controls (Table 2). We also observed rs1800783-rs1800779 TG haplotype association with HIE ($\chi^2 = 11.769, P < 0.001$) (Table 3). Genotypic and allelic distribution did not correlate with gestation age in either HIE or control group.

Our study had 80% statistical power to detect ($\alpha = 0.05$) an effect with odds ratio (OR) = 2.07 for rs3918186, OR = 1.69 for rs3918188, OR = 1.70 for rs1800783, OR = 1.80 for rs1808593, OR = 2.10 for rs3918227, OR = 1.68 for rs1800779, and OR = 1.76 for rs1799983, assuming an additive model.

**DISCUSSION**

Our study did not demonstrate the association of any SNP with HIE. SNP rs1808593 showed genotype association and rs1800783-rs1800779 TG haplotype showed an association with HIE. Several recently published articles applied similar approach to the analysis of tagging polymorphisms but in correlation with other vascular disorders (19,20).

Nitric oxide produced in blood vessels is a well-known and important vasodilator with a pivotal role in maintaining vascular tone in both the cerebral and systemic circulation (4,11). Hypoxia-ischemia increases the influx of calcium in endothelial cells of blood vessels and activates NOS3, which leads to generation of nitric oxide, relaxation of smooth muscle cells, regulation of the blood flow through the tissue, and reduction of harmful effects of hypoxia and ischemia (4). A number of studies have investigated the association of polymorphisms in NOS3 gene with the occurrence of cerebral palsy, however only two studies have confirmed it (21,22). It seems that the polymorphism rs1799983 (-894G>T) located in the promotor region and the 5 tandem repeats in a 27-bp sequence in intron 4 of NOS3, 4a4b, are associated with variable plasma levels of nitric oxide (23,24). Also the rare C allele of the -786T>C (rs2070744) polymorphism in the 5'-flanking region of the gene has been shown to significantly reduce the activity of NOS3 promotor (25). We observed the associations of rs1800783 (-1474 T/A) - rs1800779 (-922 G>A) haplotype and perinatal HIE. The frequency of the rare rs1800783-rs1800779 TG haplotype in our study was 2% in cases and 4% in controls, suggesting that it might have a protective role against HIE. However, since it

### TABLE 1. Case-control results of 7 investigated NOS3 single nucleotide polymorphism (SNP) in hypoxic-ischemic encephalopathy group of 110 affected children and 128 controls

| SNP      | Minor allele | MAF*-cases | MAF-controls | $\chi^2$ | $P$ |
|----------|--------------|------------|--------------|---------|----|
| rs1800783 | A            | 0.368      | 0.406        | 0.722   | 0.3956 |
| rs1800779 | G            | 0.405      | 0.398        | 0.018   | 0.8922 |
| rs1799983 | T            | 0.290      | 0.300        | 0.055   | 0.8141 |
| rs3918227 | A            | 0.081      | 0.082        | 0.0     | 0.9933 |
| rs3918186 | T            | 0.113      | 0.117        | 0.015   | 0.9938 |
| rs3918188 | A            | 0.377      | 0.340        | 0.722   | 0.3955 |
| rs1808593 | G            | 0.227      | 0.281        | 1.809   | 0.1787 |

*MAF – minor allele frequencies.

### TABLE 2. Genotype analysis of 7 investigated NOS3 single nucleotide polymorphisms (SNP) with hypoxic-ischemic encephalopathy (HIE)

| SNP genotype | No. (%) of participants in | HIE group | control group | $\chi^2$ | $P$ |
|--------------|----------------------------|-----------|--------------|---------|----|
| rs1800783    |                            |           |              |         |    |
| TT           |                            | 43 (39.1) | 43 (33.6)    | 0.821   | 0.6633 |
| TA           |                            | 53 (48.2) | 66 (51.6)    |         |     |
| AA           |                            | 14 (12.7) | 19 (14.8)    |         |     |
| rs1800779    |                            |           |              |         |    |
| GG           |                            | 17 (15.5) | 18 (14.0)    | 0.063   | 0.968 |
| GA           |                            | 55 (50.0) | 66 (51.6)    |         |     |
| AA           |                            | 38 (34.5) | 44 (34.4)    |         |     |
| rs1799983    |                            |           |              |         |    |
| GG           |                            | 52 (47.3) | 60 (46.9)    | 0.28    | 0.869 |
| GT           |                            | 52 (47.3) | 59 (46.1)    |         |     |
| TT           |                            | 6 (5.4)   | 9 (7.0)      |         |     |
| rs3918227    |                            |           |              |         |    |
| CC           |                            | 93 (84.5) | 108 (84.4)   | 0.0159  | 0.9924 |
| CA           |                            | 16 (14.5) | 19 (14.8)    |         |     |
| AA           |                            | 1 (1.0)   | 1 (0.8)      |         |     |
| rs3918186    |                            |           |              |         |    |
| AA           |                            | 87 (79.1) | 101 (78.9)   | 0.081   | 0.96 |
| AT           |                            | 21 (19.1) | 24 (18.8)    |         |     |
| TT           |                            | 2 (1.8)   | 3 (2.3)      |         |     |
| rs3918188    |                            |           |              |         |    |
| CC           |                            | 42 (38.2) | 53 (41.4)    | 1.14    | 0.5729 |
| CA           |                            | 53 (48.2) | 63 (49.2)    |         |     |
| AA           |                            | 15 (13.6) | 12 (9.4)     |         |     |
| rs1808593    |                            |           |              |         |    |
| TT           |                            | 67 (60.9) | 59 (46.1)    | 9.625   | 0.0081 |
| TG           |                            | 36 (32.7) | 66 (51.6)    |         |     |
| GG           |                            | 7 (6.4)   | 3 (2.3)      |         |     |

### TABLE 3. Association analysis of NOS3 rs1808783-rs1800779 haplotypes with hypoxic-ischemic encephalopathy (HIE)

| Haplotype | HIE group frequencies | Control group frequencies | $\chi^2$ | $P$ |
|-----------|------------------------|---------------------------|---------|----|
| TA        | 0.586                  | 0.594                     | 0.027   | 0.08705 |
| AG        | 0.359                  | 0.398                     | 0.777   | 0.3781 |
| TG        | 0.021                  | 0.046                     | 0.000   | <0.001 |
is rare it is unlikely to have a major effect for disease development in the population. One possible explanation of the biological mechanism through which it (or a causal variant that it is tagging) may exert its function is that it influences mRNA levels of NOS3 gene. The polymorphisms rs1800783 and rs1800779 are located in the upstream and promoter region of the gene, which may influence mRNA transcription and reduce gene expression and may further lead to impaired production or reduced bioavailability of nitric oxide, causing susceptibility to severe clinical conditions in perinatal period (11,26). In fact, beneficial effects of inhaled nitric oxide in perinatology have been observed, especially in children with respiratory distress syndrome and bronchopulmonary dysplasia (27-29). Also, the treated infants had reduced incidence of brain injury and better neurodevelopmental outcome than the control group at the age of 2 years (30-32).

So far, two studies have shown a correlation of NOS3 -922A (rs1800779) polymorphism with cerebral palsy in preterm infants (16,17). The first study observed NOS3 A-922G heterozygous AG and homozygous GG genotypes more frequently in the affected children. They also showed an association of functional missense NOS3 polymorphism rs1799983 (–894 G/T or Glu298Asp) with cerebral palsy, however we did not replicate this finding in our study (16). The second study revealed the association between NOS3 promoter -922A (rs1800779) polymorphism and preterm birth among Caucasian children with cerebral palsy, but found no correlation for the second analyzed polymorphism, rs1799983 (–894 G/T) (Glu298Asp) of the same gene (17,33). In the present study, heterozygous TG genotype of rs1808593 SNP was found less frequently, whereas homozygous GG genotype was found more frequently in the group of HIE patients than in controls. However, genotypes for this SNP slightly deviated from HWE in controls, which may be the reason for the observed association. Therefore, the observed genotypic association can be an incidental finding. Two studies have associated NOS3 gene with hypoxic-ischemic brain damage, demonstrating the association of T-786 C and Glu298Asp polymorphisms (20,21).

Limitations of this study are a small sample size and potential population stratification due to possible relatedness among cases or controls in small populations (34). In light of this, future studies are needed to cover a larger part of Croatian population and provide the basis for further research in understanding of the influence of this gene in HIE development. They should also include other risk factors and clinical parameters.

In conclusion, despite the limited number of HIE patients, which reduced the statistical power of this study, we observed genotype and haplotype associations of NOS3 polymorphisms with HIE, obtaining evidence of the important role of this gene in susceptibility to hypoxic-ischemic perinatal damage. A complete comprehension of NOS3 genetic contribution to HIE development is vital for understanding the disease etiology and future treatment of the disease. New diagnostic, prevention, and therapeutic approaches might derive from this knowledge; for example, it might be useful to select children with high specific genetic risk for HIE development because low dose nitric oxide inhalation therapy may be a beneficial treatment. Today, such treatment is applied only in respiratory disorders (27,28) but it can also be used in the prevention hypoxic-ischemic brain damage.

Funding This study was supported by the Croatian Ministry of Science, Education and Sports (Genetic epidemiology of type 1 diabetes mellitus in Croatian population, project number 216-1080315-0293 and Genetic, clinical and demographic characteristics of the G-6-PD deficit in the Croatia, project number 216-0000000-3464).

Ethical approval Received from the Ethics Committee of Clinical Hospital Centre Split and School of Medicine Split.

Declaration of authorship RKŠ contributed to the development of the study. DP contributed to the preparation of the article, interpretation of data, and the final review. BR contributed to the development of the study. BL contributed to the development of the study. VK contributed to the development of the study. MT contributed to the development of the study. MP contributed to the development of the study. VB contributed to the development of the study. TZ contributed to the development of the study.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

References

1 Volpe JJ. Hypoxic-ischemic encephalopathy. In: Volpe JJ, editor. Neurology of the newborn. Philadelphia (PA): WB Saunders; 2008. p.400-82.
2 Perlman JM. Summary proceedings from the neurology group on hypoxic-ischemic encephalopathy. Pediatrics. 2006;117:528-33. Medline:16777819
3 Lai MC, Yang SN. Perinatal hypoxic-ischemic encephalopathy. J Biomed Biotechnol. 2011;2011:609813. Medline:21197402 doi:10.1155/2011/609813
4 Moro MA, Cardenas A, Hurtado O, Leza JC, Lizasoain I. Role of nitric oxide after brain ischaemia. Cell Calcium. 2004;36:265-75. Medline:15261482 doi:10.1016/j.ceca.2004.02.011
5 Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994;298:249-58. Medline:7510950
6 Albrecht EW, Stegeman CA, Heeringa P, Henning RH, van Goor H. Protective role of endothelial nitric oxide synthase. J Pathol. 2003;198:17. Medline:12474221 doi:10.1002/path.1250
7 ladecola C. Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci. 1997;20:132-9. Medline:9061868 doi:10.1016/S0166-2236(96)10074-6
8 Endres M, Laufs U, Joo JK, Moskowitz MA. Targeting enoS function. Heart. 2005;91:1275-7. Medline:16162616 doi:10.1136/hrt.2005.061325
9 Baier RJ. Genetics of perinatal brain injury in the preterm infant. J neuropathol Exp neurol. 2010;69:828-37. Medline:20613635 doi:10.1097/00004872-200210000-00002
10 Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol. 1976;33:696-705. Medline:9877669
11 Dani C, Bertini G. Inhaled nitric oxide for the treatment of preterm infants with respiratory distress syndrome. Neonatology. 2008;94:87-95. Medline:18332638 doi:10.1056/nEJMoa060442
12 Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol. 1976;33:696-705. Medline:9877669
13 Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol. 1976;33:696-705. Medline:9877669
14 Ta, Parkkonen M, et al. Genetic polymorphisms in nitric oxide synthase 3 gene are associated with ischemic stroke susceptibility in young black women. Stroke. 2005;36:1848-51. Medline:16100023 doi:10.1161/01.STR.0000177978.97428.53
15 International HapMap Consortium. The International HapMap Project. Nature. 2005;437:220-231. Medline:16085990 doi:10.1038/nature04225
16 Nelson KB, Dambrosia JM, Iovannisci DM, Cheng S, Grether JK, Lammer E. Genetic polymorphisms and cerebral palsy in very preterm infants. Pediatr Res. 2005;57:494-9. Medline:15718364 doi:10.1203/01.PDR.0000156477.00386.87
17 Gibson CS, MacLennan AH, Dekker GA, Goldwater PN, Dambrosia JM, Munro DJ, et al. Genetic polymorphisms and spontaneous preterm birth. Obstet Gynecol. 2007;109:384-91. Medline:17267840 doi:10.1097/01.AOG.0000252712.62241.1a
18 Gauderman WJ. Sample size requirements for matched case-control studies of gene-environment interaction. Stat Med. 2002;21:35-50. Medline:11782049 doi:10.1002/sim.973
19 McKnight AJ, Patterson CC, Sandholm N, Kliner J, Buckham TA, Parkkonen M, et al. Genetic polymorphisms in nitric oxide synthase 3 gene and implications for kidney disease: a meta-analysis. Am J Nephrol. 2010;32:476-81. Medline:20962522 doi:10.1159/000321340
20 Grontved A, Andersen LB, Franks PW, Verhage B, Wareham NJ, Ekelund U, et al. NOS3 variants, physical activity, and blood pressure in the European Youth Heart Study. Am J Hypertens. 2011;24:444-50. Medline:21252862 doi:10.1038/ajh.2010.265
21 Cho M, Hyun KS, Chung DC, Choi Y, Kim MJ, Chang JP. eNOS gene polymorphisms in perinatal hypoxic-ischemic encephalopathy. Korean Journal of Pathology. 2009;43:306-11. doi:10.4132/KoreanJPathol.2009.43.4.306
22 Vannemreddy P, Notarianni C, Yanamandra K, Napper D, Bocchini J. Is an endothelial nitric oxide synthase gene mutation a risk factor in the origin of intraventricular hemorrhage? Neuroporsch Focus. 2010;28:E11. Medline:20043715 doi:10.3171/2009.10.FOCUS09143
23 Wang XL, Mahaney MC, Sim AS, Wang J, Wang J, Blangero J, et al. Genetic contribution of the endothelial constitutive nitric oxide synthase gene to plasma nitric oxide levels. Arterioscler Thromb Vasc Biol. 1997;17:3147-53. Medline:9409304
24 Tsukada T, Yokoyama K, Arai T, Takemoto F, Hara S, Yamada A, et al. Evidence of association of the eNOS gene polymorphism with plasma NO metabolite levels in humans. Biochem Biophys Res Commun. 1998;245:190-3. Medline:9535806 doi:10.1016/j.bbr.1998.02.567
25 Veldman BA, Spiering W, Doevendans PA, Vervoort G, Kroon AA, de Leeuw PW, et al. The Glu298Asp polymorphism of the NOS 3 gene as a determinant of the baseline production of nitric oxide. J Hypertens. 2002;20:2023-7. Medline:12359981 doi:10.1097/00004872-200210000-00022
26 Presciutti S, Curcio M, Chillemi R, Barbuti S, Scatena F, Carli G, et al. Promoter polymorphisms of the NOS3 gene are associated with hypnotizability-dependent vascular response to noeticive stimulation. Neurosci Lett. 2009;467:252-5. Medline:19853644 doi:10.1016/j.neulet.2009.10.056
27 Dani C, Bertini G. Inhaled nitric oxide for the treatment of preterm infants with respiratory distress syndrome. Neonatology. 2008;94:87-95. Medline:18332638 doi:10.1159/000191719
28 Kinsella JP, Cutter GR, Walsh WF, Gerstmann DR, Bose CL, Hart C, et al. Early inhaled nitric oxide therapy in premature newborns with respiratory failure. N Engl J Med. 2006;355:354-64. Medline:16870914 doi:10.1056/NEJMoa060442
29 Mestan KK, Marks JD, Hecox K, Huo D, Schreiber MD. Neurodevelopmental outcomes of premature infants treated with inhaled nitric oxide. N Engl J Med. 2005;353:23-32. Medline:16000353 doi:10.1056/NEJMoa043514
30 Olivier P, Loron G, Fontaine RH, Pansiot J, Dalois J, Thi HP, et al. Nitric oxide plays a key role in myelination in the developing brain. J Neuropathol Exp Neurol. 2010;69:828-37. Medline:20613635 doi:10.1097/NEN.0b013e3181ea5203
31 Marks JD, Schreiber MD. Inhaled nitric oxide and neuroprotection in preterm infants. Clin Perinatol. 2008;35:793-807. Medline:19026341 doi:10.1016/j.clp.2008.07.015
32 Gibson CS, MacLennan AH, Dekker GA, Goldwater PN, Sullivan
TR, Munroe DJ, et al. Candidate genes and cerebral palsy: a population-based study. Pediatrics. 2008;122:1079-85. Medline:18977990 doi:10.1542/peds.2007-3758

33 Miller SP, Wu YW, Lee J, Lammer EJ, Iovannisci DM, Glidden DV, et al. Candidate gene polymorphisms do not differ between newborns with stroke and normal controls. Stroke. 2006;37:2678-83. Medline:17098620 doi:10.1161/01.STR.0000244810.91105.c9

34 Freedman ML, Reich D, Penney KL, McDonald GJ, Mignault AA, Patterson N, et al. Assessing the impact of population stratification on genetic association studies. Nat Genet. 2004;36:388-93. Medline:15052270 doi:10.1038/ng133