Published in final edited form as:

Birth Defects Res B Dev Reprod Toxicol. 2013 October ; 98(5): 365–373. doi:10.1002/bdrb.21079.

Genetic Association Analyses of Nitric Oxide Synthase Genes and Neural Tube Defects Vary by Phenotype

Karen L. Soldano, Melanie E. Garrett, Heidi L. Cope, J. Michael Rusnak, Nathen J. Ellis, Kaitlyn L. Dunlap, Marcy C. Speer[†], Simon G. Gregory[‡], and Allison E. Ashley-Koch^{‡,*}

Center for Human Genetics, Duke University Medical Center, Durham, North Carolina

Abstract

Neural tube defects (NTDs) are caused by improper neural tube closure during the early stages of embryonic development. NTDs are hypothesized to have a complex genetic origin and numerous candidate genes have been proposed. The nitric oxide synthase 3 (NOS3) G594T polymorphism has been implicated in risk for spina bifida, and interactions between that single nucleotide polymorphism (SNP) and the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism have also been observed. To evaluate other genetic variation in the NO pathway in the development of NTDs, we examined all three NOS genes: NOS1, NOS2, and NOS3. Using 3109 Caucasian samples in 745 families, we evaluated association in the overall dataset and within specific phenotypic subsets. Haplotype tagging SNPs in the NOS genes were tested for genetic association with NTD subtypes, both for main effects as well as for the presence of interactions with the MTHFR C677T polymorphism. Nominal main effect associations were found with all subtypes, across all three NOS genes, and interactions were observed between SNPs in all three NOS genes and MTHFR C677T. Unlike the previous report, the most significant associations in our dataset were with cranial subtypes and the AG genotype of rs4795067 in NOS2 ($p = 0.0014$) and the interaction between the rs9658490 G allele in NOS1 and MTHFR 677TT genotype ($p = 0.0014$). Our data extend the previous findings by implicating a role for all three NOS genes, independently and through interactions with MTHFR, in risk not only for spina bifida, but all NTD subtypes.

Keywords

NOS1; NOS2; NOS3; MTHFR; neural tube defect; NTD; anencephaly; spina bifida

Introduction

Neural tube defects (NTDs) are the second most common birth defect, occurring in approximately 1 in 1000 live births of American Caucasians (Parker et al., 2010). NTDs represent a broad range of subtypes that are characterized by the location of the defect along

© 2013 Wiley Periodicals, Inc.

*Correspondence to: Allison E. Ashley-Koch, 905 S. LaSalle Street, DUMC 3445, Durham, NC 27710. allison.ashleykoch@duke.edu.

[†]Deceased.

[‡]These authors contributed equally to this article.

Additional Supporting Information may be found in the online version of this article.

the spinal cord, and whether the defect is an open lesion or a lesion closed and covered by skin. NTDs arise when the neural tube fails to close properly during early fetal development; this usually occurs within the third and fourth weeks of pregnancy and often before the pregnancy is detected. Improper closure at the posterior end of the neural tube results in defects to the lumbar–sacral (LS) and thoracic regions (T) of the spine, while defects at the anterior (or cranial) end result in cranial defects. The most common of all NTDs are spina bifida and anencephaly. Spina bifida is a general term that is used to describe multiple types of NTDs, primarily myelomeningocele and lipomyelomeningocele. Myelomeningocele is an open spinal defect resulting in protrusion of the meninges and spinal cord in the LS or T regions. Closed lesions usually result in less severe outcomes due to the lack of spinal cord exposure. One of the most severe NTDs, anencephaly, is an open cranial defect resulting in the absence of much of the brain, skull, and scalp, and is therefore incompatible with long-term survival.

Folic acid supplementation has reduced the incidence of NTDs and highlights the importance of folic acid in neural tube closure (MRC Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992; Committee on Genetics, 1999). In the folic acid pathway, the methylenetetrahydrofolate reductase (MTHFR) gene is responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which then converts homocysteine to methionine by the enzyme methionine synthase (MTR) (Fig. 1). Genetic association studies of these candidate genes have supported the link between NTDs and folate–homocysteine metabolism, most notably the MTHFR C677T nonsynonymous polymorphism (rs1801133). The TT genotype of this single nucleotide polymorphism (SNP) results in the substitution of an alanine to a valine at amino acid position 222. Individuals with this valine substitution have reduced MTHFR enzymatic activity and a less efficient conversion of homocysteine to methionine. It has been reported that the MTHFR 677TT genotype in mothers and/or offspring is associated with increased NTD risk (van der Put et al., 1997; White-head et al., 1995; Ou et al., 1996; van der Put et al., 1995; Shaw et al., 1998; Shields et al., 1999; Martinez de Villarreal et al., 2001; van der Put et al., 2001; Rampersaud et al., 2003; Relton et al., 2004b; Munoz et al., 2007; Carter et al., 2011; Yan et al., 2012), although this association has not been consistently replicated (Speer et al., 1997; Johnson et al., 1999; Stegmann et al., 1999; Barber et al., 2000; Davalos et al., 2000; Gonzalez-Herrera et al., 2002; Sadewa et al., 2002; Gutierrez Revilla et al., 2003; Nitsche et al., 2003; Relton et al., 2004a; Behunova et al., 2010). The disruption of MTR activity may also play a role in NTDs as MTR deficiency can also increase levels of plasma homocysteine. MTR can be inhibited by elevated levels of nitric oxide (NO), which interferes with the methyl transfer to homocysteine during methionine production (Danishpajoo et al., 2001).

NO is a cell signaling molecule that plays a role in many biologic processes by activating guanylyl cyclase and increasing cyclic guanosine monophosphate (GMP) from guanosine triphosphate (GTP) (Murad, 2004). NO synthase (NOS) is responsible for the synthesis of NO from L-arginine. Mammals have three distinct NOS isoforms: NOS1 (OMIM 163731), NOS2 (OMIM 163730), and NOS3 (OMIM 163729). NOS1, or neuronal NOS (nNOS), is involved in the production of NO in nervous tissue and is constitutively expressed in the brain and skeletal muscle; NOS2, or inducible NOS (iNOS), can be induced by endotoxins

and cytokines, and plays a role in immunologic/inflammatory response; and NOS3, also known as endothelial NOS (eNOS), is constitutively expressed in endothelial cells and vascular tissues (Michel and Feron, 1997; Bogdan, 2001). NO is present in the neural tube of chick embryos at neurulation (Traister et al., 2004) and it appears to regulate the balance between mitosis and programmed cell death (Plachta et al., 2003). The NO pathway has also been implicated in the process of proper neurulation of the neural tube through the folate–homocysteine pathway (Nachmany et al., 2006; Weil et al., 2004), thus making the NOS genes reasonable candidate genes for NTD risk.

The NOS3 G894T polymorphism (rs1799983), which results in the substitution of amino acid 298 from glutamic acid to asparagine, is a risk factor for elevated homocysteine levels in healthy nonsmoking adults with low serum folate (Brown et al., 2003). Based upon the established link between folate–homocysteine metabolism and NTDs, subsequent work has shown that the NOS3 894GT genotype is a risk factor for spina bifida (Brown et al., 2004). Further research on this SNP was unable to find an association between the NOS3 894GT genotype alone, however an interaction between the NOS3 894GT genotype and the maternal MTHFR 677TT genotype was found to be associated with increased spina bifida risk (van der Linden et al., 2007).

The overall evidence for involvement of NO with proper neural tube closure suggests there may be a role for more than one of the NOS genes. Therefore, we explored the association of all three NOS genes—NOS1, NOS2, and NOS3—as potential candidate genes for NTDs in a large family-based dataset of multiple NTD phenotypes. We also investigated potential interactions of these genes with the MTHFR C677T polymorphism.

Materials and Methods

Study Population

A total of 3109 Caucasian individuals in 745 NTD families were enrolled throughout the United States between 1994 and 2008 for the Hereditary Basis of Neural Tube Defects study conducted by the Center for Human Genetics at Duke University Medical Center. Ascertainment of participants occurred through a variety of sources including spina bifida clinics, support groups, and self-referral in response to advertisements. Blood and/or tissue samples were collected from individuals with various forms of NTDs, along with their parents and siblings. Family and medical histories were obtained through participant interviews. NTD diagnoses were confirmed through review of medical records, photos, and images including MRI, x-ray, and computed tomography (CT). Institutional Review Board (IRB) approval was obtained from Duke University Medical Center.

Since the heterogeneous nature of NTD phenotypes is likely to be caused by complex etiologies (Boyles et al., 2005), families were placed into one of four categories based on the phenotype of the proband: LIPO (lipomyelomeningocele), MYELO (myelomeningocele), CRAN (all types of cranial NTDs), or MISC (other NTD phenotypes). This was done in an effort to minimize any confounding effects that might have been caused by differing NTD subtypes. Individuals diagnosed with a meningocele with an overlying lipoma or fatty tissue resulting in a closed NTD phenotype were placed into the LIPO category. Those having an

open lesion meningocele NTD phenotype were categorized as MYELO. The MYELO category was further characterized by the location of the defect as either lumbar–sacral (LS) or thoracic (T). The CRAN category included probands with anencephaly, acrania, cranial meningocele, and encephalocele; CRAN was then evaluated in terms of BROAD (all cranial phenotypes) and NARROW (anencephaly and acrania only) classifications. The MISC category comprised families containing multiple affected individuals (each exhibiting different NTD phenotypes), as well as families with individuals not defined by the LIPO, MYELO, or CRAN categories (including phenotypes of tethered cord, spina bifida occulta, fatty filum terminale, split cord malformations, and other spinal defects). The number of affecteds, families, and total individuals represented in this study can be found in Table 1.

DNA Extraction Method

DNA was extracted from human blood and tissue samples using the AutoPure LS[®] DNA extractor with Puregene[®] system reagents (Qiagen, Germantown, MD) at the Duke Biorepository at the Center for Human Genetics. DNA samples were then plated into each respective well of a 384-well barcoded plate using a Biomek[®] FX Automated Liquid Handling Robot (Beckman Coulter, Brea, CA). These plates were subsequently used for SNP genotyping.

Genotyping

Haplotype tagging SNPs were chosen using Haploview (Barrett, 2009). SNPs were chosen using HapMap (www.hapmap.org) SNP genotype data for the Centre d'Etude du Polymorphisme Humain (CEPH) population (Utah residents with ancestry from northern and western Europe, CEU), with an $r^2 = 0.64$ and a minor allele frequency (MAF) >0.05; 27 SNPs were chosen in NOS1, 17 SNPs in NOS2, and nine SNPs in NOS3. Two MTHFR SNPs were selected based on previous reports (van der Put et al., 2001; Reyes-Engel et al., 2002) of association with NTDs (Supplementary Table S1).

Genotyping was performed using both custom and predesigned TaqMan[®] SNP Genotyping Assays (Applied Biosystems, Foster City, CA). PCR was performed according to manufacturer protocols on 3 ng of genomic DNA per sample using the GeneAmp[®] 9700 Dual 384- Well PCR system (Applied Biosystems, Foster City, CA) and subsequently scanned on an ABI PRISM[®] 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Within and across the plates were CEPH pairs and internal quality control (QC) replicates that were required to match 100%. Each SNP was required to have a minimum call frequency of 95% to be included in the analysis. SNPs were run on families with all the NTD phenotype populations (LIPO, MYELO, CRAN, and MISC). Analysis was performed only on those samples that met an overall performance of greater than 95%. Data were checked for Mendelian inconsistencies using PedCheck (O'Connell and Weeks, 1998) and any errors were resolved before data analysis.

Analysis Methods

Hardy–Weinberg equilibrium (HWE) was assessed for each SNP using Genetic Data Analysis (GDA; Zaykin et al., 1995). One affected and one unaffected individual were chosen from each family and the resulting groups were tested separately for deviations from

HWE (defined as p value <0.05). Additionally, pairwise linkage disequilibrium within each gene was determined using the Graphical Overview of Linkage Disequilibrium (GOLD) software (Abecasis and Cookson, 2000). To test for association in our cohort of families, we used the pedigree disequilibrium test (PDT; Martin et al., 2000), which provides substantial gains in power when extended pedigree data exist as opposed to nuclear families only. Both the allele test (PDT) and the genotype test (genoPDT) were run. To investigate potential interactions between NOS SNPs and MTHFR C677T predicting NTDs, we used generalized estimating equations implemented in additive regression models (GENMOD procedure, SAS version 9.2 [SAS Systems, Cary NC]). The generalized estimating equations approach controls for familial correlation among individuals of the same family. We restricted our analysis to Caucasian families to reduce population substructure ($n = 3109$ samples). A primary question of the NTD field is whether the same genetic risk factors contribute to all NTD phenotypes. Thus, to minimize potential genetic heterogeneity of NTD phenotypes, we analyzed all MYELO subjects together, as well as MYELO-LS and MYELO-T separately. Likewise, CRAN subjects were divided into NARROW (anencephaly and acrania) and BROAD (all cranial phenotypes) categories for analysis. We corrected for multiple testing using the method described by Li and Ji (Li and Ji, 2005), which constructs effective number of tests (M_{eff}) based on the LD structure of the SNPs. We then applied the Bonferroni method to generate the corrected threshold for significance testing based on the effective number of tests. The corrected significance level for association in our analysis was $0.05/M_{\text{eff}} = 0.0014$.

Results

A total of 53 haplotype tagging SNPs were selected for analysis in NOS1 (27 SNPs), NOS2 (17 SNPs), and NOS3 (9 SNPs). None of the SNPs deviated from HWE expectations in either the affected or unaffected individuals. LD plots displaying pairwise r^2 values for each NOS gene are shown in Figure 2. SNPs nominally associated with various NTD phenotypes are shown in Table 2 ($p < 0.05$).

The most significant SNP in NOS1 was rs816354 among those with the CRAN-NARROW phenotype (PDT $p = 0.0076$, genoPDT $p = 0.0083$) such that those with the T allele (TT genotype) were more likely to have anencephaly/acrania. This SNP was also associated with the CRAN-BROAD phenotype, but more marginally. Another NOS1 SNP, rs1552227, was associated with the MYELO-T phenotype (PDT $p = 0.0285$, genoPDT $p = 0.0076$) such that those with the C allele (CC genotype) were more likely to have a thoracic myelomeningocele.

The most significant association, both within the NOS2 gene and in the entire analysis, was between rs4795067 in NOS2 and the CRAN-BROAD phenotype (genoPDT $p = 0.0014$) such that individuals with the AG genotype were more likely to have cranial NTDs. This association met the multiple testing threshold. While rs4795067 was also nominally associated within the narrow cranial subset of anencephaly/acrania (genoPDT $p = 0.0114$), the strength of the association was reduced, perhaps due to reduced statistical power. rs2997515 in NOS2 was associated with all types of myelomeningocele (PDT $p = 0.004$, genoPDT $p = 0.005$) as well as just lumbar-sacral myelomeningoceles (PDT $p = 0.0025$,

genoPDT $p = 0.0059$). Both associations indicated that those individuals with the C allele (AC and CC genotypes) were more likely to have NTDs. Two additional NOS2 SNPs in LD with one another (rs1137933 and rs3794766, r^2 0.831) were associated with the LIPO phenotype (PDT $p = 0.006$ and $p = 0.0053$, respectively). Finally, rs2314810 in NOS2 was associated with the MISC phenotype (genoPDT $p = 0.0031$).

Associations in NOS3 were largely observed among those with the LIPO phenotype, the strongest of which being rs1549758 (genoPDT $p = 0.0077$) such that those with the CT genotype were more likely to have an NTD.

We also observed many nominally significant interactions ($p < 0.05$) between SNPs in the NOS genes and the MTHFR C677T polymorphism (shown in Table 3). The most significant interaction was between rs9658490 in NOS1 and MTHFR C677T predicting the CRAN-NARROW phenotype, such that those individuals with the rs9658490 G allele were 5.6 times more likely to have anencephaly/acrania than those with the alternate C allele, but only among those with the MTHFR 677TT genotype ($p = 0.0014$, meeting the multiple testing threshold). rs9658490 is in strong LD with another NOS1 SNP, rs4767523 (r^2 0.76), where a similar association was observed. The CRAN-BROAD phenotype was also associated with this interaction, though the strength of association was slightly weaker. Another NOS1 SNP, rs10507279, demonstrated a nominally significant interaction with MTHFR C677T predicting the CRAN-NARROW phenotype such that those individuals with the rs10507279 A allele were 2.1 times as likely to have anencephaly/acrania than those with the alternate G allele, but only among those with the MTHFR 677CC genotype ($p = 0.0079$). Finally, a NOS3 SNP, rs3918188, interacted significantly with MTHFR C677T to predict the CRAN-NARROW phenotype such that those individuals with the rs3918188 A allele are 4.8 times as likely to have anencephaly/acrania than those without it, but only among those with the MTHFR 677TT genotype ($p = 0.0085$). While the strongest interactions predicted cranial phenotypes, several nominally significant interactions were observed to be associated among the other NTD phenotypes as well.

Discussion

A haplotype tagging approach was employed to test the association of three NOS genes (NOS1, NOS2, and NOS3) with the occurrence of multiple NTD phenotypes. This approach allowed analysis of associations across the whole gene, rather than focus only on an individual polymorphism. Stratifying by NTD phenotypes proved valuable in teasing apart the various associations between the NOS SNPs and the different NTD phenotypes. Of the 53 total NOS SNPs genotyped, 21 SNPs were nominally associated ($p < 0.05$) with NTD phenotypes: 6 in NOS1, 9 in NOS2, and 6 in NOS3. The top five SNPs were associated with the occurrence of CRAN, MISC, MYELO, and LIPO NTDs, and represented all three NOS genes: rs4795067, rs2314810, rs2297515 (from NOS2), rs1552227 (NOS1), and rs1549758 (NOS3). Pairwise interactions between the NOS SNPs and MTHFR C677T were evaluated and identified 17 NOS SNPs (9 in NOS1, 6 in NOS2, and 2 in NOS3), which interacted significantly with the MTHFR C677T genotype. SNPs with the most significant interactions were rs9658490, rs4767523, rs10507279 (NOS1), and rs3918188 (NOS3); these interactions were associated with the CRAN phenotypes.

With the exception of rs1549758 (a synonymous mutation), the SNPs most significantly associated with our dataset, both for main effects associations (rs4795067, rs2314810, rs2297515, rs1552227) and for interactions with MTHFR C677T (rs9658490, rs4767523, rs10507279, and rs3918188), were located within the intronic regions of the three NOS genes. While it is unclear what effect these polymorphisms would have upon the gene itself, noncoding areas of the human genome can be regulatory in nature (Encode Project Consortium, 2007, 2012). Further studies would be needed to investigate what potential roles these NOS SNPs may have in gene regulation. Alternatively, these SNPs could be in LD with other functional polymorphisms that we did not genotype.

It is interesting to note that the only SNPs to meet multiple testing criteria were associated with the CRAN phenotypes. rs4795067 in NOS2 was associated with the CRAN-BROAD phenotype (genoPDT $p = 0.0014$), and the interaction between the rs9658490 G allele in NOS1 and MTHFR 677TT genotype, was associated with the CRAN-NARROW phenotype ($p = 0.0014$). These populations have not been well characterized in human genetic studies. That we see such strong associations in our dataset is suggestive of an underlying genetic cause in individuals with anencephaly that is different from the cause of other NTDs such as myelomeningocele and lipomyelomeningocele. This underscores the need to consider analyzing genetic associations separately within different NTD subsets rather than analyzing all NTD phenotypes together.

The NOS SNP that has been primarily investigated with NTDs is the NOS3 polymorphism G894T (rs1799983), because it is a functional polymorphism in amino acid 298 that results in a change from glutamic acid to asparagine. The most significant NOS3 SNP to be typed in our dataset was rs1549758 (genoPDT $p = 0.0077$) associated with the LIPO phenotype, not rs1799983 (genoPDT $p = 0.0392$). This NOS3 SNP results in the synonymous mutation of T774C in aspartic acid 258 and is actually in high LD with rs1799983 in our dataset ($r^2 = 0.881$). The differences between our study and the previous study could simply reflect differences in LD structure among the different study populations. Alternatively, the different associations could reflect differences in the manner in which each study defined the phenotype for analysis. For example, the original report was a case-control analysis, not a family-based study like ours, and the phenotype under investigation was spina bifida (Brown et al., 2004). We looked at four different phenotypic categories of NTDs, and the only one associated with rs1799983 (and rs1549758) was the LIPO phenotype. Here we saw only nominal significance with the GT risk genotype, perhaps due to a small affected sample size ($n = 77$). It is interesting to note that the LIPO dataset is made of individuals with a closed myelomeningocele, covered by a fatty tissue or lipoma. This phenotype is often grouped into the general description of spina bifida. Other haplotype tagging SNPs tested for NOS3 (rs1800779, rs1549758, rs3918227, rs1808593) were also associated with the LIPO phenotype, and rs7830 had a borderline association with the MYELO-LS phenotype, which we classify as an open lesion meningocele. As spina bifida is a general term that often encompasses both open and closed forms of meningocele, our results are broadly consistent with that of Brown and van der Linden (Brown et al., 2004; van der Linden et al., 2007) in that we see associations between SNPs in the NOS3 gene and LIPO and MYELO-LS phenotypes.

The significant associations between the various NOS SNPs and our stratified NTD phenotypes, as well as the significant interactions between the NOS SNPs and MTHFR C677T, highlight the putative relationship between all three NOS genes (NOS1, NOS2, and NOS3) and multiple NTD phenotypes (LIPO, MYELO, CRAN, and MISC). The mechanism by which this takes place is still unclear.

It has been hypothesized that NO may interfere with the methyl transfer to homocysteine during methionine production (Danishpajoo et al., 2001). Excess NO can inhibit MTR, resulting in elevated levels of plasma homocysteine. Though we previously examined MTR in our NTD dataset and did not find any associations (data not shown), it is possible that there are associations among genetic polymorphisms that we did not evaluate. Moreover, one of our limitations is that we do not have data recorded on clinical outcomes such as plasma homocysteine, plasma folate, NO levels, and vitamin B12. Associations between these metabolic outcomes and our genetic data would likely give us more information regarding the role of the NOS genes with regard to the folate, NO, and other metabolic pathways.

The role of NOS1 is in the production of NO in nervous tissue and has been found to have an effect on skeletal muscle; it has been largely studied with regard to neurotransmitter signaling, neurodegenerative diseases, and its effect on the peripheral nervous system. NOS1 has been investigated in diseases such as schizophrenia and bipolar disorder (Reif et al., 2006), implying that one possible role is in modulating several of the signaling cascades involved in psychiatric disorders (the serotonin transporter, dopaminergic, and glutamate system pathways). It has been associated with the increased risk of Duchenne muscular dystrophy through both mouse models (Thomas et al., 1998) and human studies (Sander et al., 2000) as well as having been investigated in a variety of diseases such as Alzheimer's, Parkinson's, asthma, heart function, and restless leg syndrome (Grasemann et al., 2000; Levecque et al., 2003; Danson et al., 2005; Galimberti et al., 2008; Winkelmann et al., 2008). NOS1 was associated with multiple subtypes in this study (CRAN, MYELO, LIPO, and ALL); there were also multiple subtypes with a NOS1/MTHFR C677T interaction (CRAN, MISC, and MYELO), most significantly rs9658490 ($p < 0.0014$). Whether NOS1 affects NTDs through a cell signaling pathway or through some other physiologic means would require further study.

Plachta et al. (2003) has shown that the NO pathways play a role in cell cycle progression. Endogenous NO was found to affect the number of neuroepithelial cells produced in the developing neural tube of the chick embryo. The short-term inhibition of NOS decreased cell proliferation in the neural tube by increasing mitosis and decreasing programmed cell death; however, long-term NOS inhibition limited cellular resources and increased programmed cell death. The sensitivity to the NO levels in the cell is suggestive of different methods of cell cycle control. The supplementation of folic acid was able to rescue cell cycle progression to normal levels, suggesting a role for the NO and folate pathways in the regulation of cell numbers of the developing neural tube.

NOS2 may affect NTDs through its role in the inflammatory and immune response. Maternal diabetes is associated with increased risk of NTDs in offspring (Lupo et al., 2012;

Sukanya et al., 2012). The function of NOS2 with respect to NTDs was explored in a diabetes study using mouse models (Sugimura et al., 2009). Immuno-histochemistry showed iNOS expression localized in the hindbrain and neural tube of the diabetic mice. In a case/control study of diabetic/nondiabetic mice, NOS2 (iNOS) was chemically inhibited in the diabetic mice, resulting in a decreased number of offspring with congenital malformations, primarily NTDs; iNOS knockout mice were also studied with similar results. Here, it was proposed that it was the increased accumulation of NO, resulting in excess peroxynitrite, that led to the development of NTDs by means of apoptosis.

There is growing evidence within the field of NTD research linking the development of NTDs with various ciliopathies (Wallingford, 2006). Cilia are microtubule-based projections found in most vertebrate cells. A recent article (Simet et al., 2013) has found that the molecular chaperone HSP90 interacts with NOS3 (eNOS) in regulating cellular beat frequency of the cilia in the lung, and during alcohol exposure cilia motility can become limited. While most associations with NTDs involve ciliogenesis (the process by which cilia are formed), if cilia motility is impaired, it is possible that the neural tube will not close properly. The genes primarily associated with ciliopathies are those involved in the planar cell polarity pathway. Further study is necessary to investigate whether NOS3 may be involved as well.

The greatest strength of our study was the large sample size. To our knowledge, this study represents one of the largest collections of NTD-affected individuals and their families. This larger dataset has likely allowed us to pick up some associations that might have otherwise been missed by other smaller datasets. Our population is also composed of a diverse array of NTD phenotypes, which has allowed us to test the association of the SNPs in the NOS1, NOS2, and NOS3 genes across a general class of NTDs, ALL, as well as by phenotypic classifications of CRAN, LIPO, MYELO, and MISC, thus allowing us to investigate how each NTD phenotype represents its own etiology. NOS3 was primarily associated with LIPO and MYELO-LS, whereas NOS1 was associated with ALL phenotypes, CRAN (BROAD and NARROW), LIPO and MYELO (LS and T). NOS2 also demonstrated associations with ALL phenotypes, CRAN (BROAD and NARROW), LIPO, MYELO (ALL and LS), as well as MISC. Our approach of phenotypic classification supports the hypothesis that each NTD may originate with different genes and environmental factors.

It is important to note that the association of ALL phenotypes was likely driven by the individuals in the MYELO category of phenotypes. Over half of the affected individuals in our study have a myelomeningocele phenotype (501/814). Only the NOS1 SNP rs11611788 and the NOS2 SNP rs17722851 were associated with ALL phenotypes, and both of these SNPs were also associated with the various MYELO phenotypes. It is likely that this overrepresentation of the MYELO population drove the association for these SNPs in the ALL category. Conversely, we may have missed some associations and interactions due to reduced power in the other phenotypes with lower representation in our dataset. Moreover, many of the main effects appear to be driven by the heterozygous group. This could be a true heterozygous effect, or alternatively, it could be due to reduced power in the homozygote group of the minor allele. Also, the power to detect interactions between the NOS SNPs and MTHFR C677T was hindered by small sample sizes, especially for the

cranial phenotypes. While the pattern of interactions observed is consistent within an LD block, several included the MTHFR CC or CT genotype instead of the TT risk genotype making interpretation difficult in light of the previous associations with the T allele representing risk. We also recognize that we employed multiple types of analyses and although we did apply a multiple testing correction, it did not sufficiently account for all comparisons and tests performed. For these reasons, we consider our findings preliminary and in need of replication in other datasets.

In summary, we have extended previous literature implicating a role for the NOS3 gene in spina bifida risk to include a possible role for all three NOS genes (NOS1, NOS2, and NOS3) across a variety of NTD phenotypes, particularly the cranial NTDs. We have also observed evidence for interaction with the MTHFR gene with these genes in contributing to NTD risk. Additional work will be required to fully elucidate the specific role of each gene and its interaction with the folate pathway in the etiology of NTDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the patients and their families for their participation in the study. The authors gratefully acknowledge support from NS039818. The authors would also like to thank the members of the NTD Collaborative Group: Joanna Aben, Children's Rehabilitation Service, Birmingham, Alabama; Arthur Aylsworth, Cynthia Powell, University of North Carolina, Chapel Hill, North Carolina; Joanne Mackey, Gordon Worley, Duke University Medical Center; Timothy Brei, Connie Buran, Indiana University School of Medicine, Indianapolis, Indiana; Joann Bodurtha and Kathleen Sawin, Medical College of Virginia, Richmond, Virginia; Mark S. Dias, Children's Hospital of Buffalo, Buffalo, New York; Philip Mack and Elli Meeropol, Shriner's Hospital, Springfield, Massachusetts; Nicole Lasarsky, Carolinas Medical Center, Charlotte, North Carolina; David McLone and Joy Ito, Children's Memorial Hospital, Chicago, Illinois; W. Jerry Oakes, University of Alabama, Birmingham, Alabama; Marion Walker and Paula Peterson, University of Utah, Salt Lake City, Utah; Bermans Iskandar, University of Wisconsin Hospitals, Madison, Wisconsin; Timothy George, Dell Children's Medical Center of Central Texas, Austin, Texas; Roger Stevenson, Greenwood Genetic Center, Greenwood, South Carolina. Further, we would like to thank Carol Haynes, Christina Markunas, Christopher Potocky, and Jacqueline Rimmner who generated early analysis of these data before the final version.

Grant sponsor: NIH; Grant number: NS039818.

References

- Abecasis GR, Cookson WO. GOLD—graphical overview of linkage disequilibrium. *Bioinformatics*. 2000; 16(2):182–183. [PubMed: 10842743]
- Barber R, Shalat S, Hendricks K, Joggerst B, Larsen R, Suarez L, Finnell R. Investigation of folate pathway gene polymorphisms and the incidence of neural tube defects in a Texas hispanic population. *Mol Genet Metab*. 2000; 70(1):45–52. [PubMed: 10833330]
- Barrett JC. Haploview: visualization and analysis of SNP genotype data. *Cold Spring Harb Protoc*. 2009; 2009(10) pdb ip71.
- Behunova J, Klimcakova L, Zavadilikova E, Potocekova D, Sykora P, Podracka L. Methylenetetrahydrofolate reductase gene polymorphisms and neural tube defects epidemiology in the Slovak population. *Birth Defects Res A Clin Mol Teratol*. 2010; 88(8):695–700. [PubMed: 20672355]
- Bogdan C. Nitric oxide and the regulation of gene expression. *Trends Cell Biol*. 2001; 11(2):66–75. [PubMed: 11166214]

- Boyles AL, Hammock P, Speer MC. Candidate gene analysis in human neural tube defects. *Am J Med Genet C Semin Med Genet.* 2005; 135C(1):9–23. [PubMed: 15816061]
- Brown KS, Kluijtmans LA, Young IS, Woodside J, Yarnell JW, McMaster D, Murray L, Evans AE, Boreham CA, McNulty H, Strain JJ, Mitchell LE, Whitehead AS. Genetic evidence that nitric oxide modulates homocysteine: the NOS3 894TT genotype is a risk factor for hyperhomocystenemia. *Arterioscler Thromb Vasc Biol.* 2003; 23(6):1014–1020. [PubMed: 12689917]
- Brown KS, Cook M, Hoess K, Whitehead AS, Mitchell LE. Evidence that the risk of spina bifida is influenced by genetic variation at the NOS3 locus. *Birth Defects Res A Clin Mol Teratol.* 2004; 70(3):101–106. [PubMed: 15039923]
- Carter TC, Pangilinan F, Troendle JF, Molloy AM, VanderMeer J, Mitchell A, Kirke PN, Conley MR, Shane B, Scott JM, Brody LC, Mills JL. Evaluation of 64 candidate single nucleotide polymorphisms as risk factors for neural tube defects in a large Irish study population. *Am J Med Genet A.* 2011; 155A(1):14–21. [PubMed: 21204206]
- Committee on Genetics. Folic acid for the prevention of neural tube defects. *American Academy of Pediatrics. Committee on Genetics. Pediatrics.* 1999; 104(2 Pt 1):325–327. [PubMed: 10429019]
- Czeizel AE, Dudas I. Prevention of the first occurrence of neural tube defects by periconceptional vitamin supplementation. *New Engl J Med.* 1992; 327(26):1832–1835. [PubMed: 1307234]
- Danishpajoo IO, Gudi T, Chen Y, Kharitonov VG, Sharma VS, Boss GR. Nitric oxide inhibits methionine synthase activity in vivo and disrupts carbon flow through the folate pathway. *J Biol Chem.* 2001; 276(29):27296–27303. [PubMed: 11371572]
- Danson EJ, Choate JK, Paterson DJ. Cardiac nitric oxide: emerging role for nNOS in regulating physiological function. *Pharmacol Ther.* 2005; 106(1):57–74. [PubMed: 15781122]
- Davalos IP, Olivares N, Castillo MT, Cantu JM, Ibarra B, Sandoval L, Moran MC, Gallegos MP, Chakraborty R, Rivas F. The C677T polymorphism of the methylenetetrahydrofolate reductase gene in Mexican mestizo neural-tube defect parents, control mestizo and native populations. *Ann Genet.* 2000; 43(2):89–92. [PubMed: 10998450]
- Encode Project Consortium. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature.* 2007; 447(7146):799–816. [PubMed: 17571346]
- Encode Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature.* 2012; 489(7414):57–74. [PubMed: 22955616]
- Galimberti D, Scarpini E, Venturelli E, Strobel A, Herterich S, Fenoglio C, Guidi I, Scalabrini D, Cortini F, Bresolin N, Lesch KP, Reif A. Association of a NOS1 promoter repeat with Alzheimer's disease. *Neurobiol Aging.* 2008; 29(9):1359–1365. [PubMed: 17418914]
- Gonzalez-Herrera L, Garcia-Escalante G, Castillo-Zapata I, Canto-Herrera J, Ceballos-Quintal J, Pinto-Escalante D, Diaz-Rubio F, Del Angel RM, Orozco-Orozco L. Frequency of the thermolabile variant C677T in the MTHFR gene and lack of association with neural tube defects in the state of Yucatan, Mexico. *Clin Genet.* 2002; 62(5):394–398. [PubMed: 12431255]
- Grasemann H, Yandava CN, Storm van's Gravesande K, Deykin A, Pillari A, Ma J, Sonna LA, Lilly C, Stampfer MJ, Israel E, Silverman EK, Drazen JM. A neuronal NO synthase (NOS1) gene polymorphism is associated with asthma. *Biochem Biophys Res Commun.* 2000; 272(2):391–394. [PubMed: 10833424]
- Gutierrez Revilla JI, Perez Hernandez F, Calvo Martin MT, Tamparillas Salvador M, Gracia Romero J. C677T and A1298C MTHFR polymorphisms in the etiology of neural tube defects in Spanish population. *Med Clin.* 2003; 120(12):441–445.
- Johnson WG, Stenroos ES, Heath SC, Chen Y, Carroll R, McKoy VV, Chatkupt S, Lehner T. Distribution of alleles of the methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism in familial spina bifida. *Am J Med Genet.* 1999; 87(5):407–412. [PubMed: 10594879]
- Leveque C, Elbaz A, Clavel J, Richard F, Vidal JS, Amouyel P, Tzourio C, Alperovitch A, Chartier-Harlin MC. Association between Parkinson's disease and polymorphisms in the nNOS and iNOS genes in a community-based case-control study. *Hum Mol Genet.* 2003; 12(1):79–86. [PubMed: 12490535]
- Li J, Ji L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity.* 2005; 95(3):221–227. [PubMed: 16077740]

- Lupo PJ, Canfield MA, Chapa C, Lu W, Agopian AJ, Mitchell LE, Shaw GM, Waller DK, Olshan AF, Finnell RH, Zhu H. Diabetes and obesity-related genes and the risk of neural tube defects in the national birth defects prevention study. *Am J Epidemiol.* 2012; 176(12):1101–1109. [PubMed: 23132673]
- Martin ER, Monks SA, Warren LL, Kaplan NL. A test for linkage and association in general pedigrees: the pedigree disequilibrium test. *Am J Hum Genet.* 2000; 67(1):146–154. [PubMed: 10825280]
- Martinez de Villarreal LE, Delgado-Enciso I, Valdez-Leal R, Ortiz-Lopez R, Rojas-Martinez A, Limon-Benavides C, Sanchez-Pena MA, Ancer-Rodriguez J, Barrera-Saldana HA, Villarreal-Perez JZ. Folate levels and N(5),N(10)-methylenetetrahydrofolate reductase genotype (MTHFR) in mothers of offspring with neural tube defects: a case-control study. *Arch Med Res.* 2001; 32(4): 277–282. [PubMed: 11440783]
- Michel T, Feron O. Nitric oxide synthases: which, where, how, and why? *J Clin Invest.* 1997; 100(9): 2146–2152. [PubMed: 9410890]
- MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. *Lancet.* 1991; 338(8760): 131–137. [PubMed: 1677062]
- Munoz JB, Lacasana M, Cavazos RG, Borja-Aburto VH, Galaviz-Hernandez C, Garduno CA. Methylenetetrahydrofolate reductase gene polymorphisms and the risk of anencephaly in Mexico. *Mol Hum Reprod.* 2007; 13(6):419–424. [PubMed: 17439956]
- Murad F. Discovery of some of the biological effects of nitric oxide and its role in cell signaling. *Biosci Rep.* 2004; 24(4–5):452–474. [PubMed: 16134022]
- Nachmany A, Gold V, Tsur A, Arad D, Weil M. Neural tube closure depends on nitric oxide synthase activity. *J Neurochem.* 2006; 96(1):247–253. [PubMed: 16300634]
- Nitsche F, Alliende MA, Santos JL, Perez F, Santa Maria L, Hertrampf E, Cortes F. Frequency of C677T polymorphism of 5, 10-methylenetetrahydrofolate reductase (MTHFR) in Chilean mothers of spina bifida cases and controls. *Rev Med Chil.* 2003; 131(12):1399–1404. [PubMed: 15022402]
- O'Connell JR, Weeks DE. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet.* 1998; 63(1):259–266. [PubMed: 9634505]
- Ou CY, Stevenson RE, Brown VK, Schwartz CE, Allen WP, Khoury MJ, Rozen R, Oakley GP Jr, Adams MJ Jr. 5,10 Methylenetetrahydrofolate reductase genetic polymorphism as a risk factor for neural tube defects. *Am J Med Genet.* 1996; 63(4):610–614. [PubMed: 8826441]
- Parker SE, Mai CT, Canfield MA, Rickard R, Wang Y, Meyer RE, Anderson P, Mason CA, Collins JS, Kirby RS, Correa A. National Birth Defects Prevention N. Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004–2006. *Birth Defects Res A Clin Mol Teratol.* 2010; 88(12):1008–1016. [PubMed: 20878909]
- Plachta N, Traister A, Weil M. Nitric oxide is involved in establishing the balance between cell cycle progression and cell death in the developing neural tube. *Exp Cell Res.* 2003; 288(2):354–362. [PubMed: 12915126]
- Rampersaud E, Melvin EC, Siegel D, Mehlretter L, Dickerson ME, George TM, Enterline D, Nye JS, Speer MC. Updated investigations of the role of methylenetetrahydrofolate reductase in human neural tube defects. *Clin Genet.* 2003; 63(3):210–214. [PubMed: 12694231]
- Reif A, Herterich S, Strobel A, Ehli AC, Saur D, Jacob CP, Wienker T, Topner T, Fritzen S, Walter U, Schmitt A, Fallgatter AJ, Lesch KP. A neuronal nitric oxide synthase (NOS-I) haplotype associated with schizophrenia modifies prefrontal cortex function. *Mol Psychiatr.* 2006; 11(3): 286–300.
- Relton CL, Wilding CS, Laffling AJ, Jonas PA, Burgess T, Binks K, Tawn EJ, Burn J. Low erythrocyte folate status and polymorphic variation in folate-related genes are associated with risk of neural tube defect pregnancy. *Mol Genet Metab.* 2004a; 81(4):273–281. [PubMed: 15059614]
- Relton CL, Wilding CS, Pearce MS, Laffling AJ, Jonas PA, Lynch SA, Tawn EJ, Burn J. Gene-gene interaction in folate-related genes and risk of neural tube defects in a UK population. *J Med Genet.* 2004b; 41(4):256–260. [PubMed: 15060097]
- Reyes-Engel A, Munoz E, Gaitan MJ, Fabre E, Gallo M, Dieguez JL, Ruiz M, Morell M. Implications on human fertility of the 677C→T and 1298A→C polymorphisms of the MTHFR gene:

- consequences of a possible genetic selection. *Mol Hum Reprod.* 2002; 8(10):952–957. [PubMed: 12356947]
- Sadewa AH, Sunarti, Sutomo R, Hayashi C, Lee MJ, Ayaki H, Sofro AS, Matsuo M, Nishio H. The C677T mutation in the methylenetetrahydrofolate reductase gene among the Indonesian Javanese population. *Kobe J Med Sci.* 2002; 48(5–6):137–144. [PubMed: 12594357]
- Sander M, Chavoshan B, Harris SA, Iannaccone ST, Stull JT, Thomas GD, Victor RG. Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. *Proc Natl Acad Sci USA.* 2000; 97(25):13818–13823. [PubMed: 11087833]
- Shaw GM, Rozen R, Finnell RH, Wasserman CR, Lammer EJ. Maternal vitamin use, genetic variation of infant methylenetetrahydrofolate reductase, and risk for spina bifida. *Am J Epidemiol.* 1998; 148(1):30–37. [PubMed: 9663401]
- Shields DC, Kirke PN, Mills JL, Ramsbottom D, Molloy AM, Burke H, Weir DG, Scott JM, Whitehead AS. The “thermolabile” variant of methylenetetrahydrofolate reductase and neural tube defects: an evaluation of genetic risk and the relative importance of the genotypes of the embryo and the mother. *Am J Hum Genet.* 1999; 64(4):1045–1055. [PubMed: 10090889]
- Simet SM, Pavlik JA, Sisson JH. Proteomic analysis of bovine axonemes exposed to acute alcohol: role of endothelial nitric oxide synthase and heat shock protein 90 in cilia stimulation. *Alcohol Clin Exp Res.* 2013; 37(4):609–615. [PubMed: 23078267]
- Speer MC, Worley G, Mackey JF, Melvin E, Oakes WJ, George TM. The thermolabile variant of methylenetetrahydrofolate reductase (MTHFR) is not a major risk factor for neural tube defect in American Caucasians. The NTD Collaborative Group. *Neurogenetics.* 1997; 1(2):149–150. [PubMed: 10732818]
- Stegmann K, Ziegler A, Ngo ET, Kohlschmidt N, Schroter B, Ermert A, Koch MC. Linkage disequilibrium of MTHFR genotypes 677C/T-1298A/C in the German population and association studies in probands with neural tube defects(NTD). *Am J Med Genet.* 1999; 87(1):23–29. [PubMed: 10528242]
- Sugimura Y, Murase T, Oyama K, Uchida A, Sato N, Hayasaka S, Kano Y, Takagishi Y, Hayashi Y, Oiso Y, Murata Y. Prevention of neural tube defects by loss of function of inducible nitric oxide synthase in fetuses of a mouse model of streptozotocin-induced diabetes. *Diabetologia.* 2009; 52(5):962–971. [PubMed: 19283362]
- Sukanya S, Bay BH, Tay SS, Dheen ST. Frontiers in research on maternal diabetes-induced neural tube defects: past, present and future. *World J Diabetes.* 2012; 3(12):196–200. [PubMed: 23301121]
- Thomas GD, Sander M, Lau KS, Huang PL, Stull JT, Victor RG. Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle. *Proc Natl Acad Sci USA.* 1998; 95(25):15090–15095. [PubMed: 9844020]
- Traister A, Abashidze S, Gold V, Yairi R, Michael E, Plachta N, McKinnell I, Patel K, Fainsod A, Weil M. BMP controls nitric oxide-mediated regulation of cell numbers in the developing neural tube. *Cell Death Differ.* 2004; 11(8):832–841. [PubMed: 15060574]
- van der Linden IJ, Heil SG, den Heijer M, Blom HJ. The 894G>T variant in the endothelial nitric oxide synthase gene and spina bifida risk. *J Hum Genet.* 2007; 52(6):516–520. [PubMed: 17479212]
- van der Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, van den Heuvel LP, Mariman EC, den Heyer M, Rozen R, Blom HJ. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet.* 1995; 346(8982):1070–1071. [PubMed: 7564788]
- van der Put NM, Eskes TK, Blom HJ. Is the common 677C->T mutation in the methylenetetrahydrofolate reductase gene a risk factor for neural tube defects? A meta-analysis. *QJM.* 1997; 90(2):111–115. [PubMed: 9068801]
- van der Put NM, van Straaten HW, Trijbels FJ, Blom HJ. Folate, homocysteine and neural tube defects: an overview. *Exp Biol Med (Maywood).* 2001; 226(4):243–270. [PubMed: 11368417]
- Wallingford JB. Planar cell polarity, ciliogenesis and neural tube defects. *Hum Mol Genet.* 2006; 15(Spec No 2):R227–R234. [PubMed: 16987888]

- Weil M, Abeles R, Nachmany A, Gold V, Michael E. Folic acid rescues nitric oxide-induced neural tube closure defects. *Cell Death Differ.* 2004; 11(3):361–363. [PubMed: 14685162]
- Whitehead AS, Gallagher P, Mills JL, Kirke PN, Burke H, Molloy AM, Weir DG, Shields DC, Scott JM. A genetic defect in 5,10 methylenetetrahydrofolate reductase in neural tube defects. *QJM.* 1995; 88(11):763–766. [PubMed: 8542260]
- Winkelmann J, Lichtner P, Schormair B, Uhr M, Hauk S, Stiasny-Kolster K, Trenkwalder C, Paulus W, Peglau I, Eisensehr I, Illig T, Wichmann HE, Pfister H, Golic J, Bettecken T, Putz B, Holsboer F, Meitinger T, Muller-Myhsok B. Variants in the neuronal nitric oxide synthase (nNOS, NOS1) gene are associated with restless legs syndrome. *Mov Disord.* 2008; 23(3):350–358. [PubMed: 18058820]
- Yan L, Zhao L, Long Y, Zou P, Ji G, Gu A, Zhao P. Association of the maternal MTHFR C677T polymorphism with susceptibility to neural tube defects in offsprings: evidence from 25 case-control studies. *PloS One.* 2012; 7(10):e41689. [PubMed: 23056169]
- Zaykin D, Zhivotovsky L, Weir BS. Exact tests for association between alleles at arbitrary numbers of loci. *Genetica.* 1995; 96(1–2):169–178. [PubMed: 7607454]

Fig. 1.
 The folate pathway: folate mediated one carbon metabolism.

Fig. 2.
Pairwise linkage disequilibrium (r^2) values for (A) NOS1, (B) NOS2, and (C) NOS3.

Table 1
Description of Phenotypes and Study Population

Phenotype category	Affecteds	Families	Total individuals
Cranial	121	120	447
Anencephaly/acrania	93		
Lipomyelomeningocele	77	78	288
Myelomeningocele	501	471	1959
Lumbar-sacral	428		
Thoracic	67		
Miscellaneous	115	76	415
ALL study participants	814	745	3109

Bold values represent the overall number of affected individuals in the four main categories of CRAN, LIPO, MYELO, and MISC.

Table 2
NOS SNPs and Significant Phenotypes with PDT and GenoPDT *p* Values <0.05

Gene	SNP	Minor allele	MAF	Phenotype category	Risk allele (genotype)	PDT <i>p</i> value	GenoPDT <i>p</i> value
NOS1	rs4767523	G	0.1019	CRAN-NARROW	G (G/T)	0.0412	0.0682
NOS1	rs816354	C	0.0789	CRAN-BROAD	T (T/T)	0.0116	0.0147
				CRAN-NARROW	T (T/T)	0.0076	0.0083
NOS1	rs11611788	G	0.0992	MYELO-LS	G (A/G)	0.0462	0.0641
				ALL	G (A/G)	0.0544	0.0182
NOS1	rs45654	C	0.4875	LIPO	T (T/T)	0.1336	0.0082
NOS1	rs1552227	T	0.2871	MYELO-T	C (C/C)	0.0285	0.0076
NOS1	rs3782221	A	0.2254	LIPO	G (G/G)	0.0407	0.0322
NOS2	rs9906835	G	0.4087	MISC	A (A/A)	0.0322	0.021
				MYELO-LS	G (A/G, G/G)	0.0321	0.0552
NOS2	rs1060826	A	0.3757	MYELO-ALL	A (A/A)	0.0488	0.1617
				MYELO-LS	A (A/A)	0.0415	0.1539
NOS2	rs2297515	C	0.1565	MYELO-ALL	C (A/C, C/C)	0.004	0.005
				MYELO-LS	C (A/C, C/C)	0.0025	0.0059
NOS2	rs2314810	C	0.0624	MISC	C (C/G)	0.0164	0.0031
NOS2	rs1137933	A	0.2240	LIPO	G (G/G)	0.006	0.0389
NOS2	rs4795067	G	0.3400	CRAN-BROAD	G (A/G)	0.0897	0.0014*
				CRAN-NARROW	G (A/G)	0.2858	0.0114
NOS2	rs17722851	A	0.1080	MYELO-ALL	A (A/T)	0.0511	0.0173
				MYELO-LS	A (A/T)	0.0413	0.0112
				ALL	A (A/T)	0.0337	0.0211
NOS2	rs3794766	T	0.2299	CRAN-NARROW	T (C/T)	0.1060	0.0386
				LIPO	C (C/C)	0.0053	0.0297
NOS2	rs10459953	C	0.3525	MYELO-LS	G (G/G)	0.0465	0.1322
NOS3	rs1800779	G	0.3650	LIPO	G (A/G)	0.2858	0.0117
NOS3	rs1549758	T	0.3105	LIPO	T (C/T)	0.2498	0.0077
NOS3	rs1799983	T	0.3223	LIPO	T (G/T)	0.1489	0.0392
NOS3	rs3918227	A	0.0980	LIPO	A (A/C)	0.0455	0.075

Gene	SNP	Minor allele	MAF	Phenotype category	Risk allele (genotype)	PDT <i>p</i> value	GenoPDT <i>p</i> value
NOS3	rs1808593	C	0.2472	LIPO	C (A/C)	0.2858	0.0348
NOS3	rs7830	A	0.3363	MYLEO-LS	C (C/C)	0.1088	0.049

* Significant at the multiple testing threshold of 0.0014.
MAF, minor allele frequency.

Table 3
Significant Interactions (*p* Values <0.05) between NOS SNPs and MTHFR C677T

Gene	SNP	Phenotype category	NOS risk allele	MTHFR C677T genotype	Odds ratio	95% Confidence interval	Comparison <i>p</i> value	Global interaction <i>p</i> value
NOS1	rs9658490	CRAN-BROAD	G	TT	3.701	1.17–11.67	0.0255	0.0111
		CRAN-NARROW	G	TT	5.6054	1.41–22.22	0.0142	0.0014*
NOS1	rs4767523	CRAN-BROAD	G	TT	4	1.07–14.97	0.0396	0.0097
		CRAN-NARROW	G	TT	6.3331	1.34–29.94	0.0199	0.0028
NOS1	rs2293054	CRAN-BROAD	A	CT	1.7088	1.06–2.75	0.0273	0.0116
			A	CC	0.5612	0.34–0.94	0.0279	0.0116
		CRAN-NARROW	A	CT	1.7864	1.07–2.99	0.0275	0.0154
NOS1	rs2139733	MISC	A	TT	2.686	1.35–5.34	0.0048	0.0231
NOS1	rs11612772	MISC	C	TT	2.7042	1.07–6.84	0.0358	0.0361
NOS1	rs12424669	MISC	T	TT	6.4267	1.76–23.47	0.0048	0.0127
NOS1	rs499776	MYELO-ALL	A	CC	0.7999	0.65–0.98	0.0334	0.0128
NOS1	rs10507279	CRAN-NARROW	A	CC	2.1209	1.03–4.36	0.0407	0.0079
NOS1	rs1123425	CRAN-NARROW	G	CC	0.5069	0.31–0.82	0.0057	0.0231
		MISC	G	CC	0.5807	0.36–0.93	0.0224	0.0396
NOS2	rs2297518	ALL	A	CC	1.2625	1.05–1.52	0.0129	0.0326
NOS2	rs11080344	MYELO-ALL	C	TT	0.6672	0.47–0.94	0.0205	0.0293
NOS2	rs1137933	MYELO-LS	A	CC	1.4548	1.13–1.88	0.0041	0.0377
		ALL	A	CT	0.8006	0.66–0.97	0.022	0.0290
			A	CC	1.3294	1.10–1.60	0.0029	0.0290
NOS2	rs8072199	MISC	T	CC	1.6526	1.13–2.42	0.0099	0.0199
NOS2	rs3794766	MYELO-LS	T	CC	1.465	1.14–1.89	0.003	0.0191
		ALL	T	CT	0.8034	0.67–0.97	0.0215	0.0275
			T	CC	1.3293	1.11–1.59	0.0022	0.0275
NOS2	rs10459953	MYELO-LS	C	CC	0.745	0.59–0.94	0.0122	0.0211
NOS3	rs3918186	CRAN-BROAD	A	CC	4.0683	1.71–9.7	0.0015	0.0155
NOS3	rs3918188	CRAN-BROAD	A	TT	4.8473	1.13–20.88	0.0341	0.0085
		MYELO-ALL	A	TT	1.5316	1.05–2.24	0.0281	0.0419
		ALL	A	TT	1.3702	1.01–1.88	0.0495	0.0201

* Significant at multiple testing threshold of 0.0014.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript