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Higher Activity of the Inducible Nitric Oxide Synthase Contributes to Very Early Onset Inflammatory Bowel Disease

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OBJECTIVES: The NOS2 gene encodes for the inducible nitric oxide synthase (iNOS), responsible for nitric oxide (NO) production, which contributes to antimicrobial and antipathogenic activities. Higher levels of both iNOS and NO-induced damage have been observed in inflammatory bowel disease (IBD) patients. NOS2 may have a role in a specific subset of IBD patients with severe and/or extensive colitis. Therefore, the aim of this study is to examine the role of NOS2 in such a subset, very early onset IBD (VEO-IBD).

METHODS: Seventeen tag single nucleotide polymorphisms (SNPs) in the NOS2 gene were successfully genotyped in VEO-IBD patients. Genetic associations were replicated in an independent VEO-IBD cohort. Functional analysis for iNOS activity was performed on the most significantly associated functional variant.

RESULTS: The NOS2 rs2297518 SNP was found to be associated in VEO-IBD in two independent cohorts. Upon combined analysis, a coding variant (S608L) showed the strongest association with VEO-IBD ($P_{combined} = 1.13 \times 10^{-6}$, OR (odds ratio) = 3.398 (95% CI (confidence interval) 2.02–5.717)) as well as associations with VEO-Crohn’s disease and VEO-ulcerative colitis (UC). This variant also showed an association with UC diagnosed between 11 and 17 years of age but not with adult-onset IBD (> 17 years). B-cell lymphoblastoid cell lines genotyped for the risk variant as well as Henle-407 cells transfected with a plasmid construct with the risk variant showed higher NO production. Colon biopsies of VEO-IBD patients showed higher immunohistochemical staining of nitrotyrosine, indicating more nitrosative stress and tissue damage.

CONCLUSIONS: These studies suggest the importance of iNOS in genetic susceptibility to younger IBD presentation due to higher NO production.

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Subject Category: Inflammatory Bowel Disease

INTRODUCTION
Very early onset inflammatory bowel disease (VEO-IBD), according to the Paris Modification to the Montreal classification, is described as IBD diagnosed in children under the age of 10 years.1 It is characterized by a higher tendency for disease manifestation in the colon, more disease extension, and a change in disease location over time.2–5

Pediatric genome-wide association studies (GWAS) have not focused on VEO-IBD;6,7 however, candidate gene studies have identified novel genetic variants that are associated exclusively with VEO-IBD indicating that very young children have unique genetic susceptibility (reviewed in ref. 8).

Although the pathogenesis of IBD is multi-factorial, genetic and functional studies have confirmed the critical role of the immune system in the pathogenesis of disease.9 Encoded by the NOS2 gene, inducible nitric oxide synthase (iNOS) produces nitric oxide (NO) in response to pro-inflammatory cytokines.10 Although NO is a weak free radical,11 it mediates cell-damaging and toxic effects by forming peroxynitrite, which contributes to DNA damage as well as protein damage by combining with tyrosine to form nitrotyrosine (NT).11,12

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These authors contributed equally to this work.

13International Early Onset Pediatrics IBD Cohort Study (www.NEOPICS.org).
Both iNOS expression and NT staining have previously been shown to be upregulated in the intestinal epithelium and inflamed colonic mucosa of IBD patients.\textsuperscript{10,11} Here, we report a genetic association with the \textit{NOS2} single nucleotide polymorphisms (SNPs) and VEO-IBD, VEO-Crohn’s disease (CD), and VEO-ulcerative colitis (UC). In addition, we found a strong age-biased association between the iNOS variant, rs2297518 (S608L), and VEO-IBD. Last, we explored the function of this SNP and found that it conferred higher NO production based on the risk allele.

**METHODS**

**SNP analysis and genotyping.** Eighteen tag SNPs providing complete genetic coverage of the \textit{NOS2} gene (chromosome 17, 26,083,792-26,127,555) were selected from the International HapMap Project (www.hapmap.org) Caucasian (CEU) phase II data Release 23a (minor allelic frequency > 1%). The Illumina GoldenGate Custom Chip (discovery cohort) as previously described\textsuperscript{13,14} and Taqman (for replication of the two most significant SNPs from the discovery cohort) were used at the Centre for Applied Genomics, Hospital for Sick Children.

**Subjects.** All VEO-IBD subjects had a confirmed diagnosis of IBD before the age of 10 based on the Paris classification.\textsuperscript{1} Phenotypic information and DNA samples were obtained from study subjects with approval of the institutional review ethics board for IBD genetic studies at the Hospital for Sick Children and Mount Sinai Hospital in Toronto. Replication cohorts had ethics board approval for genetic and phenotypic studies at the individual institutions. Written informed consent was obtained from all participants.

The discovery cohort consisted of total of 1,072 subjects including 159 VEO-IBD patients (91 VEO-CD and 68 VEO-UC) and 913 healthy controls recruited from the Hospital for Sick Children and Mt. Sinai Hospital in Toronto. The replication cohort consisted of 736 subjects including 153 VEO-IBD patients (50 VEO-CD and 53 VEO-UC), and 480 healthy controls. The affected subjects were recruited from NEOPICS sites (www.NEOPICS.org) and healthy controls were obtained from the Centre for Applied Genomics (Ontario Population Genomics Platform (plates used: 1–5; a complete description of this control population can be found at http://www.tcag.ca/cryo_population_control_DNA.html)). For the analysis of older IBD groups, 498 IBD subjects (351 CD and 147 UC) diagnosed between 11 and 17 years of age and 918 IBD subjects (419 CD and 499 UC) diagnosed after 17 years of age were included. To conduct systematic quality control on the raw genotyping data, we examined 770 SNPs genotyped for the initial cohort only (18 SNPs genotyped in the initial cohort). Detailed quality control has been previously reported in detail elsewhere.\textsuperscript{13,14} One \textit{NOS2} SNP, rs2297515, was excluded as it deviated significantly from Hardy–Weinberg equilibrium in the controls (\textit{P}<0.001). No other \textit{NOS2} SNP was excluded due to a genotype call rate of <95%, or due to sex discrepancies based on the heterozygosity rate from SNPs on chromosome X.

**Association analysis.** Association analyses of the discovery and replication cohorts were used to test associations of the 17 \textit{NOS2} SNPs with VEO-IBD, VEO-CD, and VEO-UC vs. healthy controls. Logistic regression analysis was applied for an additive model and Pearson \textit{X}\textsuperscript{2} tests were applied for dominant and recessive models. Models used to report \textit{P} values are described in this report. This analysis was done using the Goldenhelix (SVS 7.6.4) program. The combined cohort analysis pooled population data from both cohorts and analyzed using the same protocol as for the individual cohorts.

**Cell culture.** Genotyped B-lymphoblastoid cell lines were obtained from Coriell Cell Repositories and cultured in RPMI-1640X with 15% fetal bovine serum at 2 \times 10^5 cells/ml in an upright position at 5% CO\textsubscript{2} and 37 C. Supernatant was collected for Griess assay. In addition, Henle-407 cells transfected with the wild-type and S608L variant (designed with Agilent Technologies QuickChange II Site-Directed Mutagenesis Kit, according to the manufacturer’s instructions) of the \textit{NOS2}-pcDNA3 were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The Griess assay was performed using a Promega Griess Assay kit (Madison, WI, USA), according to the manufacturer’s instructions. Results were replicated. For the B-lymphoblastoid cell lines, absorption readings were taken at 570 nm as described by Uto et al.\textsuperscript{15} iNOS expression in B-lymphoblastoid cells was measured by western blot analysis using polyclonal Anti-Nitric Oxide Synthase 2 antibody (Boster Biological Technology, Pleasanton, CA, USA) and \textit{NOS2}-myc expression in transfected Henle-407 cells was measured using monoclonal Anti-Myc Tag antibody (Millipore, Billerica, MA, USA; clone 4A6). Briefly cells were grown in a 10-cm dish, washed twice with 1 \times phosphate-buffered saline and then lysed with standard protein lysis buffer (50 m\textsuperscript{m} Heps, pH 7.5, 150 m\textsuperscript{m} NaCl, 1.5 m\textsuperscript{m} MgCl\textsubscript{2}, 1 m\textsuperscript{m} ethylene glycol tetraacetic acid, 10% glycerol, 1% Triton X-100), supplemented with 1 m\textsuperscript{m} phenylmethysulfonl fluoride, 1 \mu g/ml aprotinin, 1 \mu g/ml leupeptin, 1 \mu g/ml pepstatin, and 1 m\textsuperscript{m} vanadate. A total of 50 \mu g of protein were used for western blot analysis.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded colonic biopsy samples from six VEO-IBD patients (three with major genotype and three with risk genotype) were obtained from the Department of Pathology at the Hospital for Sick Children with approval of the institutional review ethics board for IBD genetic studies at the Hospital for Sick Children. Location of biopsy samples and disease activity were matched between the two groups by a pathologist. Immunohistochemistry was performed with the SuperPicture 3rd Gen IHC Detection Kit from Invitrogen (Carlsbad, CA, USA). Briefly, paraffin sections were deparaffinized using Xylene and afterwards rehydrated with different percentages of ethanol. Antigen retrieval step was performed with high pressure-cooking, using 1 m\textsuperscript{m} EDTA, pH 9 with 0.05% Tween 20 as an antigen retrieval buffer. Primary antibody incubation was performed at 4°C overnight. Subsequently, the sections were washed three times with phosphate-buffered saline and incubated with peroxidase quenching solution for 5 min. The
sections were quickly rinsed in distilled water and washed two times with phosphate-buffered saline. HRP Polymer conjugate incubation was 30 min followed by three times washing with phosphate-buffered saline, each time for 5 min. 3,3'-Diaminobenzidine (DAB) chromogen was added to the sections for 1–5 min. The sections were counterstained with Haematoxylin and mounted with Entelan. NT staining was performed using the polyclonal Anti-Nitrotyrosine antibody from Millipore (anti-rabbit) at a dilution of 1:100.

Slides were scanned using a Zeiss Mirax Digital Slide scanner. Acquired images were deconvolved into grayscale DAB and haematoxylin channels using the Ruifrok and Johnston method as described. The staining intensity in the DAB channel for each patient sample and control was evaluated using ImageJ software. Briefly, a technician blinded to the patient diagnoses defined regions of interest representing intestinal epithelia. Within each region of interest, the NT-positive areas were defined as regions containing pixels with values > 50. These regions were determined algorithmically using the ImageJ ‘Threshold’ function. Regions of interest were converted to binary (where NT-positive regions were defined as 1.0; unstained regions were 0.0) and the fraction of NT-positive area to total area (within each region of interest) was determined. This method was repeated on multiple sections for each patient.

RESULTS

NOS2 association with VEO-IBD. Seventeen tag SNPs in the NOS2 gene were successfully genotyped in the VEO-IBD discovery cohort of 159 VEO-IBD subjects and 913 healthy control individuals (Table 1, Supplementary Table 1). We found that rs2297518 (S608L) was associated with VEO-IBD, VEO-CD, and VEO-UC (\(P_{IBD} = 6.2 \times 10^{-6}\); OR (odds ratio) (95% confidence interval) = 4.4 (2.3–8.4)) (Supplementary Tables 2 and 3). This variant was also significantly associated with IBD diagnosed under the age of 6 years (Supplementary Table 4), a more homogeneous phenotype of IBD patients with a predominance of pancolitis. In addition, rs1137933 was associated with VEO-IBD and VEO-UC (\(P = 7.43 \times 10^{-4}\); OR = 2.6 (1.5–4.6)). Both NOS2 variants showed a stronger association with VEO-IBD than adult IBD as observed here and in a previous study by Martin et al.

In an independent replication cohort, consisting of 153 VEO-IBD patients and 480 healthy controls, rs2297518 was also associated with VEO-IBD with a combined analysis of \(P_{combined} = 1.13 \times 10^{-6}\); OR = 3.4 (2.0–5.7). The association of rs1137933 with VEO-IBD did not replicate in this cohort; however, remained significant in the combined analysis (Table 2).

In the analysis of older children and adults with IBD (Supplementary Tables 5–10), NOS2 was associated with UC diagnosed between 11 and 17 years of age (A1b, Paris classification; rs2297518: \(P = 9.65 \times 10^{-3}\); OR = 3.3 (1.6–6.8); Supplementary Table 10) and not associated with adult-onset IBD (> 17 years age group). This analysis suggests that NOS2 variants are associated with VEO-IBD.

rs2297518 risk genotype is associated with higher production of NO. As B-lymphoblastoid cell lines have constitutive expression of iNOS, supernatants from B-lymphoblastoid cell lines genotyped for rs2297518, were collected. To measure the NO production, NO\(_2^\) levels, indicative of NO production, were measured using the Griess assay. Levels were compared among the three genotypes of rs2297518, homozygous for the risk allele (A/A), and heterozygous for both alleles (A/G). As shown in Figure 1a, cell lines homozygous for the risk allele had significantly higher production of NO (one-way ANOVA, \(F(2,8) = 24.39\); \(P = 0.0026\); Tukey’s HSD (honestly significant difference) test, \(P < 0.05\)) indicating that leukocytes in patients homozygous for the risk allele (iNOS S608L) have higher levels of NO production.

Table 1 Discovery cohort association analyses of 17 NOS2 SNPs with VEO-IBD

<table>
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<th>Chr.</th>
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<th>Position</th>
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<th>FA</th>
<th>(P)-value</th>
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FA, frequency affected; FU, frequency unaffected; L95 and U95, lower and upper 95th confidence interval; MAF, minor allelic frequency.

\(P\)-values are presented as uncorrected and recessive modeling.

Clinical and Translational Gastroenterology
As shown by the imputation analysis (Supplementary Table 13), B-lymphoblastoid cell lines genotyped for the rs2297518 SNP do not have a significant change in iNOS expression. Western blot for iNOS (Supplementary Figure 1a), although expression varied between cell lines, demonstrated no trend of expression changes among the three genotypes.

To explore the difference in the production of NO in human intestinal epithelial-derived cells, Henle-407 cells were used to study the difference in NO production between the major and risk variants of rs2297518. As shown in Figure 1b, cells transfected with the S608L variant of the iNOS-pcDNA3.1 construct showed higher constitutive production of NO than the wild-type variant ($P < 0.05$). This result further suggests that this coding variant alters iNOS protein function. Western blot for iNOS in transfected cells (Supplementary Figure 1b) confirmed that this difference was not due to an iNOS expression difference.

Patients with rs2297518 risk genotype show higher peroxynitrite levels in colonic biopsies. To assess any change in peroxynitrite levels in these patients, colonic biopsies from genotyped patients were stained with antibodies to NT, which is formed by nitrosylation of tyrosine residues due to peroxynitrite. Consistent with Griess assay results from the genotyped B-lymphoblastoid cell lines and transfected Henle-407 cells, VEO-IBD patients with the risk genotype of rs2297518 were shown to have higher levels of NT staining (Figure 2, Supplementary Figure 2). It is suggested that this is indicative of higher levels of nitrosative stress and damage in patients with the risk genotype. Although overall levels observed varied due to differences among individual patients and cases, NT staining was consistently more pronounced in both the epithelial (crypt cells and brush border) and leukocyte infiltrate.

**DISCUSSION**

iNOS is expressed in inflamed tissues and there is a known correlation between higher iNOS expression and colitis although the mechanism is unknown. Here we report that both rs2297518 and rs1137933 were found to be associated with VEO-IBD. Although previously reported in adults, this association was not replicated in recent adult-onset IBD GWAS and meta-analysis, where rs2297518 was not associated with either CD or UC, and rs1137933 was not included in the meta-analysis (http://medicine.yale.edu/intmed/ibdgc/index.aspx; Supplementary Tables 11 and 12).

### Table 2 Replication and combined cohort analyses of NOS2 SNP associated with VEO-IBD

<table>
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<th>SNP</th>
<th>Diagnosis</th>
<th>Replication analysis: VEO-IBD</th>
<th>Combined analysis: VEO-IBD</th>
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<td></td>
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<td>$P$-value</td>
<td>OR (95% CI)</td>
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<td>rs2297518</td>
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<td>VEO-IBD</td>
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<td>VEO-CD</td>
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<tr>
<td></td>
<td>VEO-UC</td>
<td>0.61</td>
<td>0.5 (0.7–4.5)</td>
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CD, Crohn’s disease; CI, confidence interval; IBD, inflammatory bowel disease; OR, odds ratio; SNP, single nucleotide polymorphism; UC, ulcerative colitis.

$P$-values are presented as uncorrected and recessive modeling.

**Figure 1** (a) Griess assay results from the B-lymphoblastoid cell culture. G is the non-risk allele and A is the risk-allele ($N = 9$). ANOVA testing ($F(2,8) = 24.39; P = 0.0026$) revealed statistically significant variance between the genotypes. Mean NO$_2$ production was not significantly different between the AG and GG genotypes; however, both genotypes were significantly different from the AA genotype (Tukey’s HSD test; $P < 0.05$). (b) Griess assay results from the transfected Henle-407 cells, comparing constitutive expression of wild-type and S608L variants of iNOS (Student’s $t$-test, $n = 6$, $P < 0.05$).

NO production, when normalized with protein concentration. As shown by the imputation analysis (Supplementary Table 13), B-lymphoblastoid cell lines genotyped for the
Furthermore, neither SNPs in LD ($r^2 < 0.8$) with rs2297518 (Supplementary Table 13) nor other NOS2 SNP were not found to be associated with either CD or UC in the meta-
analyses. However, an NOS2 SNP, rs2945412, was found to be associated with CD in the recent adult-onset IBD meta-
analyses indicating that common variants in NOS2 may also be associated with adult-onset IBD. Current estimates suggest that adult-onset IBD GWAS have explained $\approx 23$–$33\%$ of the inherited contribution to IBD risk. Therefore, there remains substantial ‘missing heritability’ that will not be fully explained by simply expanding the GWAS approach with larger numbers of patients. Our candidate gene approach using a specific extreme IBD phenotype, VEO-IBD in this case, may allow for the identification of novel susceptibility loci not found in GWAS and allow for further understanding of the genetic basis of IBD. Here we observed associations between rs2297518 and VEO-IBD, VEO-CD, and VEO-UC as well as an association with UC between 11 and 17 years of age. This age-biased association indicates the functional significance of this variant as well as the importance of iNOS activity in the susceptibility to younger disease presentation. However, there are limitations to the study including the small sample size compared with adult IBD genetic studies and variability in inflammation and iNOS staining in our patient population.

A number of inflammatory diseases have overlapping genetic susceptibility with IBD and rs2297518 (S608L) has also been implicated in the susceptibility of type 1 diabetes, severe asthma, and atrophic gastritis. Interestingly, the above diseases all have inflammatory or autoimmune phenotypes, indicating that an increase in iNOS activity may contribute to the pathogenesis of these diseases. The rs2297518 risk allele results in a S608L amino-acid change that is located in the catalytic domain of iNOS and Johannesen et al. suggest that this S608L substitution may affect the catalytic activity of iNOS, as it is close in proximity to the Flavin mononucleotide-binding region. This risk genotype of rs2297518 also resulted in higher iNOS activity in both genotyped B-lymphoblastoid cell lines as well as plasmid vector-transfected Henle-407 cells, as measured by the Griess assay. The higher iNOS activity observed was further supported by higher levels of NT staining in colonic biopsies of VEO-IBD patients with the risk genotype, suggesting that the higher NO production from this risk variant contributes to higher nitrosative stress and peroxynitrite damage in the intestines of IBD patients. As younger pediatric IBD patients (both CD and UC) often present with severe pancolitis and iNOS expression is correlated to the severity of colitis in UC patients, the higher tissue damage in these young children may explain the association of rs2297518 with VEO-IBD as compared with adolescent- and adult-onset IBD.

In an expression analysis from imputed genotypes (http:// www.sph.umich.edu/csg/liang/imputation), it was shown that
rs2297518 was associated with decreased ARG2 gene expression \( (P=6.0 \times 10^{-5}) \). Arg2 protein is an inhibitor of iNOS activity as it competes for l-arginine.\(^{27}\) With respect to gastrointestinal inflammation, it has been shown that Arg2 activity is induced in *Helicobacter pylori*-induced macrophages, which have reduced bacterial killing due to lower iNOS translation.\(^{28,29}\) As such, the higher iNOS activity observed in VEO-IBD patients with the rs2297518 risk allele may be due to the lower expression of the Arg2 that has been shown to be associated with the *NOS2* risk allele.

Internationally there is a documented rise in incidence of pediatric IBD, with 77.8% of studies investigating this trend reporting significant increases.\(^{30}\) In Ontario, this rise in pediatric IBD has been shown to be due to the specific increase in incidence of VEO-IBD, in contrast to the age group diagnosed between the ages of 11 and 17 years.\(^{31}\) Pediatric GWAS have not shown unique susceptibility loci compared with adult-onset IBD;\(^{6,7}\) however, these studies have focused on older children with the disease diagnosed between 11 and 17 years of age and mostly excluded VEO-IBD. VEO-IBD is considered a unique group of patients with a distinct phenotype and novel genetic susceptibility.\(^{8}\) This has been demonstrated in recent studies showing that rare and private mutation in a number of genes result in susceptibility to VEO-IBD including NCF2,\(^{14}\) IL10R,\(^{32,33}\) and ADAM17.\(^{34}\) As these examples have yielded the potential for personalized therapies for severe and complicated VEO-IBD cases, it is possible that VEO-IBD patients with higher activity of iNOS may benefit from advancements in therapeutic inhibition of NO production including specific iNOS inhibitors, such as N-[3-(aminomethyl) benzyl] acetamidine (1400W) and L-N6-(1-iminoethyl) lysine (L-NIL), which have been shown to have a protective effect in rat 2,4,6-trinitrobenzenesulfonic colitis models.\(^{11}\)

Thus, we propose that *NOS2* variants influencing higher activity of iNOS contribute to the genetic susceptibility to earlier onset of IBD due to NO-induced tissue damage and intestinal inflammation.

**CONFLICT OF INTEREST**

**Guarantor of the article:** Aleixo M. Muise, MD, PhD.  
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**Study Highlights**

**WHAT IS CURRENT KNOWLEDGE**

- Very early onset Inflammatory Bowel Disease (VEO-IBD) is characterized by more severe disease manifestation and behavior than in older patients.
- It is suggested that genetics have a larger role in VEO-IBD susceptibility; however, its genetics are not well characterized.
- iNOS-derived nitric oxide (NO) production is correlated with disease activity and tissue damage in IBD.

**WHAT IS NEW HERE**

- *NOS2*, which encodes iNOS, was strongly associated with VEO-IBD, VEO-CD, and VEO-UC susceptibility.
- This association was much stronger in VEO-IBD than in IBD diagnosed in older age groups and was replicated in an independent cohort.
- The risk genotype resulted in higher NO production and nitrotyrosine staining in colonic biopsies, indicative of higher nitrosative damage.

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