Promoter Polymorphisms in the Plasma Glutathione Peroxidase (GPx-3) Gene
A Novel Risk Factor for Arterial Ischemic Stroke Among Young Adults and Children

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Background and Purpose—Plasma glutathione peroxidase (GPx-3)—deficiency increases extracellular oxidant stress, decreases bioavailable nitric oxide, and promotes platelet activation. The aim of this study is to identify polymorphisms in the GPx-3 gene, examine their relationship to arterial ischemic stroke (AIS) in a large series of children and young adults, and determine their functional molecular consequences.

Methods—We studied the GPx-3 gene promoter from 123 young adults with idiopathic AIS and 123 age- and gender-matched controls by single-stranded conformational polymorphism and sequencing analysis. A second, independent population with childhood stroke was used for a replication study. We identified 8 novel, strongly linked polymorphisms in the GPx-3 gene promoter that formed 2 main haplotypes (H1 and H2). The transcriptional activity of the 2 most prevalent haplotypes was studied with luciferase reporter gene constructs.

Results—The H2 haplotype was over-represented in both patient populations and associated with an independent increase in the risk of AIS in young adults (odds ratio = 2.07, 95% CI = 1.03 to 4.47; \( P = 0.034 \)) and children (odds ratio = 2.13, 95% CI = 1.23 to 4.90; \( P = 0.027 \)). In adults simultaneously exposed to vascular risk factors, the risk of AIS approximately doubled (odds ratio = 5.18, 95% CI = 1.82 to 15.03; \( P < 0.001 \)). Transcriptional activity of the H2 haplotype was lower than that of the H1 haplotype, especially after upregulation by hypoxia (normalized relative luminescence: 3.54 ± 0.32 versus 2.47 ± 0.26; \( P = 0.0083 \)).

Conclusion—These findings indicate that a novel GPx-3 promoter haplotype is an independent risk factor for AIS in children and young adults. This haplotype reduces the gene’s transcriptional activity, thereby compromising gene expression and plasma antioxidant and antithrombotic activities. (Stroke. 2007;38:41-49.)

Key Words: oxidative stress ■ platelets ■ thrombosis

Stroke is the second most common cause of death worldwide and the leading cause of long-term disability in developed countries. Almost 90% of the burden of cerebrovascular disease stems from arterial ischemic stroke (AIS). On average, 3% to 5% of AIS affects patients under the age of 45 years, yet prevalences of over 10% have been reported. The prevalence among children is lower than among adults, yet more than half of childhood stroke survivors develop some neurological or cognitive deficit, 30% have a recurrence, and 5% to 10% of affected children die. The most unique feature of stroke in the young is the heterogeneity of underlying etiologies that include nonatherosclerotic vascular pathies, cardioemobilism, and hematological and genetic causes. Despite extensive investigation, the etiology of this potentially devastating condition remains unknown in approximately one-third of young adults and children.

Plasma glutathione peroxidase (GPx-3) is a major antioxidant enzyme in plasma and, as a member of the selenocysteine-containing GPx family, scavenges hydrogen peroxide and organic (lipid) hydroperoxides produced during normal metabolism or after oxidative insult. Of the 5 known GPx isoforms, GPx-3 is the only one found in the extracellular space. It contributes to maintaining the vascular bioavailability of nitric oxide (NO), a major vasorelaxant and inhibitor of

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platelet function, as NO can be inactivated rapidly by reactive oxygen species (ROS). The reduction of oxidant stress by GPx-3 activity also protects against post-translational modifications of fibrinogen by ROS and NO-derived oxidants that increase its thrombogenicity.\(^9,10\) Consistent with this role, we recently described a novel, functional transcription start site of the GPx-3 gene and showed that GPx-3 gene transcription is regulated by oxygen tension and redox state.\(^11\)

Two studies of families with idiopathic childhood stroke have provided clinical evidence for the importance of GPx-3 in the modulation of NO bioavailability and thrombosis.\(^12,13\) The patients in these studies had hyperreactive platelets, and their plasma impaired the normal inhibition of platelet activation by NO. These findings were determined to be a consequence of a familial reduction in GPx-3 activity that correlated with decreased protein expression by immunoblot analysis.\(^11\) We, therefore, hypothesized that mutation(s) or polymorphism(s) in the plasma GPx-3 gene promoter may be responsible for the reduction in enzyme activity and predispose to a thrombotic disorder, thus constituting a genetic risk factor for thrombotic cerebrovascular disease. To address this hypothesis, we investigated the GPx-3 gene in a large series of young adults with AIS of unknown etiology by single-stranded conformational polymorphism (SSCP) and sequencing analysis, followed by risk analysis and functional characterization of the identified genetic variants. We then performed a replication study in a second, independent population with non-thrombotic conditions. The same matching criteria used for adults were applied to children. Written parental informed consent was obtained for all children. This study was approved by the local medical ethics committees of each institution, as well as the Institutional Review Boards at Boston University School of Medicine and Brigham and Women’s Hospital.

**Polymerase Chain Reactions**

Polymerase chain reactions (PCR) were performed in a mixture containing 100 to 200 ng of genomic DNA, 1 \(\mu\)mol/L of each primer, 200 \(\mu\)mol/L dNTP (Gibco), 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 to 3.5 mmol/L MgCl\(_2\) (see Table 1), 1 \(\mu\)mol/L dimethyl sulfoxide, and 0.5 U of high fidelity Taq polymerase (Roche Diagnostics Corp) in a final volume of 20 \(\mu\)L. The conditions for PCR amplifications were: initial denaturation at 95°C for 2 minutes, 35 cycles of 30 seconds at 95°C, 30 seconds at 52 to 58°C, and 30 seconds at 72°C, with a final extension at 72°C for 7 minutes. PCR reactions were performed in a Bio-Rad iCycler (Bio-Rad Laboratories). The annealing temperatures and MgCl\(_2\) concentrations were optimized for each primer pair, all of which are listed in supplemental Table I, available online at http://stroke.ahajournals.org.

**SSCP Analysis**

Nonradioactive SSCP analysis was performed on an Amersham Pharmacia PhastSystem. The PCR products were diluted 1:2 with denaturing loading buffer (98% formamide, 2% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 5 minutes, quenched on ice, and electrophoresed on precast nondenaturing acrylamide PhastGels buffered by native agarose strips. The gels used were either 20% homogeneous or 8% to 25% gradient gels. All gels were prerun for 100 Vh. Running conditions (time and temperature) were optimized for each primer pair and are shown in supplemental Table 1. DNA bands were visualized by automated silver staining with a PhastGel DNA Silver Staining Kit, involving consecutive washes in fixing solution (0.7% benzene sulfonic acid, 24% ethanol; 10 minutes at 50°C), staining solution (0.2% silver nitrate, 0.07% benzene sulfonic acid; 30 minutes at 50°C), distilled water (1 minute at 25°C), developing solution (9 minutes at 30°C), and preserving solution (8 minutes at 50°C). The PhastSystem, gels, silver staining kits, and buffer strips were purchased from Amersham Pharmacia.

**Determination of the \(-568 T\rightarrow C, -518 T\rightarrow C, \text{and } -65 T\rightarrow C\) Polymorphisms**

To genotype the \(-568 T\rightarrow C, -518 T\rightarrow C, \text{and } -65 T\rightarrow C\) promoter polymorphisms, 2 fragments were amplified, 1 containing both the \(-568 T\rightarrow C\) and \(-518 T\rightarrow C\) polymorphisms. Because the \(-518
1.5 kb of the promoter region and to generate PCR products were designed to yield partially overlapping fragments covering the oligonucleotide primers shown in supplemental Table I. Primers Amplification of genomic DNA was performed using the PCR with Screening of the promoter was screened by nonradioactive SSCP analysis, followed by automated sequencing of fragments with abnormal electrophoretic patterns (ABI Prism 3700 Automated DNA Analyzer). In addition, all samples were sequenced for the −568 T→C, −518 T→C, and −65 T→C polymorphisms.

Transient Transfections of \( \text{GPx-3} \) Reporter Constructs

Two firefly luciferase reporter gene constructs were generated with a pGL3-basic vector (Promega) containing 1272 bp of the \( \text{GPx-3} \) flanking region amplified from genomic DNA obtained from a control subject homozygous for the more common alleles (haplotype H1) and a patient homozygous for the less common risk alleles (haplotype H2). These constructs were transiently transfected into Caki-2 cells, a renal tubular carcinoma cell line that has previously been shown to express \( \text{GPx-3} \) in greater quantities than cell lines from other organs, because proximal renal tubular cells are the main source of \( \text{GPx-3} \) in vivo. Cells were cotransfected with a pRL-CMV vector (Promega), a plasmid expressing Renilla luciferase, to allow for normalization for transfection efficiency. The detailed methodology for generation of the reporter gene constructs, transient cell transfections, and luciferase assays is described elsewhere.

Determination of Prothrombotic Polymorphisms/Mutations

Factor V Leiden, the prothrombin G20210A mutation, and the \( \text{C677T} \) substitution in the methylenetetrahydrofolate reductase (\( \text{MTHFR} \)) gene were detected by PCR and restriction digestion, as shown elsewhere. These constructs were transiently transfected into Caki-2 cells, a renal tubular carcinoma cell line that has previously been shown to express \( \text{GPx-3} \) in greater quantities than cell lines from other organs, because proximal renal tubular cells are the main source of \( \text{GPx-3} \) in vivo. Cells were cotransfected with a pRL-CMV vector (Promega), a plasmid expressing Renilla luciferase, to allow for normalization for transfection efficiency. The detailed methodology for generation of the reporter gene constructs, transient cell transfections, and luciferase assays is described elsewhere.

Screening of the \( \text{GPx-3} \) Gene

Amplification of genomic DNA was performed using the PCR with the oligonucleotide primers shown in supplemental Table I. Primers were designed to yield partially overlapping fragments covering ∼1.5 kb of the promoter region and to generate PCR products ∼200 bp in size (with one exception: fragment P.9), because the sensitivity of SSCP is dependent on the size of the DNA fragment. The \( \text{GPx-3} \) promoter was screened by nonradioactive SSCP analysis, followed by automated sequencing of fragments with abnormal electrophoretic patterns (ABI Prism 3700 Automated DNA Analyzer). In addition, all samples were sequenced for the −568 T→C, −518 T→C, and −65 T→C polymorphisms.

T→C substitution does not affect any naturally occurring restriction sites, we designed a mutagenic antisense oligonucleotide primer to create selectively a restriction site 3 bases downstream from nucleotide −518: 5′-GAA AAC CCC ATT CTG GGT AGG GC (with \( \text{C} \) being the mutated base). The combination of the mutagenic primer with the −518T nucleotide created a restriction site for the endonuclease \( \text{Alw} \) I. Thus, when the −518T nucleotide was present, digestion of the PCR product with 5 U \( \text{Alw} \) I at 37°C for 2 hours yielded fragments of 213 bp and 82 bp in length. The −569 T→C substitution contained in the 260 bp fragment yielding fragments of 186 bp, 40 bp, and 27 bp in length; in the presence of the −518C nucleotide, only 2 fragments of 213 bp and 82 bp were generated. The −569 T→C substitution in the same fragment deletes a normally existing restriction site for the endonuclease \( \text{Bsa} \) I. Thus, in the presence of the −569T nucleotide was present, digestion of the product with 5 U \( \text{Bsa} \) I yielded 2 fragments: 169 bp and 84 bp in length.

The −65 T→C substitution is contained in the 260 bp fragment generated with the P.9 primer pair and deletes a normally existing restriction site for the endonuclease \( \text{Bsa} \) I. Thus, in the presence of the −65T nucleotide, digestion with \( \text{Bsa} \) I at 50°C for 2 hours yielded 2 fragments: 36 bp and 224 bp in length. All samples were electrophoresed on 2.5% ethidium bromide-stained agarose gels.

**TABLE 1. Characteristics of Study Subjects**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adults (n=123)</th>
<th>Controls (n=123)</th>
<th>( P ) Value</th>
<th>Patients (n=82)</th>
<th>Controls (n=82)</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>36.5±6.6</td>
<td>36.8±6.8</td>
<td>0.66</td>
<td>6.9±5.5</td>
<td>7.5±4.7</td>
<td>0.36</td>
</tr>
<tr>
<td>Male</td>
<td>57 (46.3%)</td>
<td>57 (46.3%)</td>
<td>1.0</td>
<td>47 (57.3%)</td>
<td>47 (57.3%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ethnic background</td>
<td></td>
<td></td>
<td>0.44</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>White</td>
<td>92 (74.8%)</td>
<td>100 (81.3%)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>29 (23.5%)</td>
<td>22 (17.9%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>2 (1.6%)</td>
<td>1 (0.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional vascular risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>50 (40.7%)</td>
<td>10 (8.1%)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>68 (55.3%)</td>
<td>45 (36.6%)</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>43 (35.0%)</td>
<td>35 (28.5%)</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>8 (6.5%)</td>
<td>3 (2.4%)</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No vascular risk factors</td>
<td>28 (22.7%)</td>
<td>52 (42.3%)</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormonal risk factors:¶§</td>
<td></td>
<td></td>
<td>0.087</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral contraceptive use</td>
<td>16 (24.2%)</td>
<td>10 (16.1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy</td>
<td>1 (1.5%)</td>
<td>0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Postpartum period</td>
<td>2 (3.0%)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inherited prothrombotic risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor V Leiden[( ^{\dagger} )]</td>
<td>5 (4.1%)</td>
<td>4 (3.3%)</td>
<td>0.99</td>
<td>5 (6.1%)</td>
<td>5 (6.1%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Prothrombin G20210A[( ^{\dagger} )]</td>
<td>5 (4.1%)</td>
<td>3 (2.4%)</td>
<td>0.72</td>
<td>3 (3.7%)</td>
<td>4 (4.9%)</td>
<td>0.99</td>
</tr>
<tr>
<td>MTHFR C677T**</td>
<td>19 (15.5%)</td>
<td>14 (11.4%)</td>
<td>0.35</td>
<td>18 (22.0%)</td>
<td>17 (20.7%)</td>
<td>0.85</td>
</tr>
</tbody>
</table>

*Some clinical characteristics of adults and children have been previously reported; †adult AIS patients vs controls; ‡childhood stroke patients vs controls; §values are mean±SD; ¶among 62 female controls (4 postmenopausal controls were excluded from this analysis) and 66 female AIS patients; |lin heterozygosity; ** in homozygosity.

\( T\rightarrow C \) substitution does not affect any naturally occurring restriction sites, we designed a mutagenic antisense oligonucleotide primer to create selectively a restriction site 3 bases downstream from nucleotide −518: 5′-GAA AAC CCC ATT CTG GGT AGG GC (with \( \text{C} \) being the mutated base). The combination of the mutagenic primer with the −518T nucleotide created a restriction site for the endonuclease \( \text{Alw} \) I. Thus, when the −518T nucleotide was present, digestion of the PCR product with 5 U \( \text{Alw} \) I at 37°C for 2 hours yielded fragments of 186 bp, 40 bp, and 27 bp in length; in the presence of the −518C nucleotide, only 2 fragments of 213 bp and 82 bp were generated. The −569 T→C substitution contained in the 260 bp fragment yielding fragments of 186 bp, 40 bp, and 27 bp in length; in the presence of the −518C nucleotide, only 2 fragments of 213 bp and 40 bp were generated. The −569 T→C substitution in the same fragment deletes a normally existing restriction site for the endonuclease \( \text{Bsa} \) I. Thus, when the −569T nucleotide was present, digestion of the product with 5 U \( \text{Bsa} \) I yielded 2 fragments: 169 bp and 84 bp in length.

The −65 T→C substitution is contained in the 260 bp fragment generated with the P.9 primer pair and deletes a normally existing restriction site for the endonuclease \( \text{Bsa} \) I. Thus, in the presence of the −65T nucleotide, digestion with \( \text{Bsa} \) I at 50°C for 2 hours yielded 2 fragments: 36 bp and 224 bp in length. All samples were electrophoresed on 2.5% ethidium bromide-stained agarose gels.
We were well matched for age, sex, and ethnic background. Adult AIS patients and controls profile of the study subjects. Differences in demographic characteristics and vascular risk factors between patients and controls were initially compared by univariate analysis using Student t test for age and the χ² test for all categorical variables. The Fisher exact test was used when data cell counts were sparse. Haplotypes were estimated from unphased genotypes by an expectation-maximization (EM) algorithm that assumes random mating and Hardy-Weinberg equilibrium. The EM algorithm carries out a series of iterations using observed data and estimated haplotype frequencies from the previous iteration to obtain expected diplotype frequencies, which are then used to update estimated haplotype frequencies. Iterations continue until convergence is reached. To perform this procedure, we used the HelixTree Genetics Analysis Software, version 2.4.0 (Golden Helix Inc). This program was also used to determine allele and genotype frequencies, haplotype frequencies, Hardy-Weinberg equilibrium, and linkage disequilibrium (LD) between polymorphism pairs. We used R as our measure of LD; this value translates the strength of association of 2 marker loci on the same chromosome without being strongly affected by sample size.

To test whether GPx-3 haplotypes were significantly associated with the risk of AIS, we assigned the most likely haplotype phase to individuals using the results of the EM procedure. For the GPx-3 haplotypes found to have a significant association with the risk of AIS by univariate analysis, we performed multivariate analysis at the diplotype level with logistic regression. Adjustments were made for age, gender, ethnicity, and vascular, hormonal, and inherited prothrombotic risk factors. The extent to which associations with the polymorphisms were modified by other risk factors was assessed through analyses stratified by these risk factors. All reported probability values were 2-sided. Statistical analyses were performed with SigmaStat version 3.0.1 (SPSS Inc).

Results
Arterial Ischemic Stroke in the Young
Table 1 shows the demographic characteristics and risk factor profile of the study subjects. Although all conventional vascular risk factors were more frequent among AIS patients, only hypertension and smoking reached statistically significant differences. Almost 80% of AIS patients had at least 1 vascular risk factor compared with less than half of the controls (P=0.0011). Hormonal risk factors trended toward a greater prevalence among women with AIS than female controls (28.7% versus 16.1%; P=0.087). The distribution of known inherited prothrombotic risk factors did not differ significantly between AIS patients and controls.

Analysis by SSCP of GPx-3 promoter fragments obtained from AIS patients and controls revealed abnormal electrophoretic patterns in 2 of the 9 fragments studied: fragments P.3 and P.7. Sequencing of these fragments identified 5 single base pair changes: −942 A→C, −927 T→C, and −861 A→T in fragment P.3; and −302 A→T and −284 T→A in fragment P.7. Numbering of the nucleotides was performed relative to the recently described, novel transcription start point, defined as +1.14 Sequencing further indicated that the nucleotide substitutions in these fragments were often found together on the same chromosome. Because these preliminary results suggested a highly polymorphic promoter with a high degree of LD, and because the sensitivity of SSCP is ~85% under ideal running conditions,17 we sequenced the entire GPx-3 5′flanking region (nt −1236 to +158) obtained from a patient homozygous for all of the above single nucleotide polymorphisms (SNPs) to search for base pair changes that may have been undetected by SSCP. Indeed, 3 additional single base pair transitions were identified: −568 T→C, −518 T→C, and −65 T→C, and these were subsequently assessed in all subjects.

All GPx-3 promoter polymorphisms were in Hardy-Weinberg equilibrium. The distribution of each of the SNPs in AIS patients and controls is shown in supplemental Table II, available online at http://stroke.ahajournals.org. The rare polymorphic alleles were significantly over-represented in AIS patients compared with controls. The LD map showed a high degree of linkage between polymorphism pairs, with R values ranging between 0.85 and 1, as shown in Figure 1. In fact, the 3 most 5′ SNPs (at positions −942, −927, −861), as
well as the −568, −518, and −302 polymorphisms, were in complete LD, forming 2 blocks which, in turn, were linked ($R^2=0.88$). An exception to these overall findings was the polymorphism at position −284, which did not have a significantly different distribution between patients and controls and was not linked to the other polymorphisms. Haplotype analysis revealed that of the 28 (n=256) possible combinations of these SNPs, only 8 haplotypes were identified, as shown in Table 2.

To assess a potential association of the GPx-3 promoter haplotypes with the risk of AIS among young adults, haplotype frequencies were imputed from the EM algorithm in the patient and control groups (Table 2). Overall, haplotypes H1 and H2 accounted for $\approx95%$ of the observed haplotypes in this population; owing to their low frequencies, haplotypes H3 to H8 were not included in further analyses. The frequency of the H2 haplotype was almost twice as high in AIS patients (13.1%) compared with controls (7.0%), yielding an odds ratio (OR) of 2.01 (95% CI=1.04 to 3.91; $P=0.025$). Heterozygous and homozygous carriers of the H2 haplotype (H1/H2 and H2/H2) were over-represented among AIS patients (23.6% versus 12.2%), increasing the risk of AIS over 2-fold when the H1H1 haplotype pair was used as reference (OR=2.26, 95% CI=1.07 to 4.81; $P=0.019$).

The findings from the univariate analyses were further investigated in a multiple logistic regression analysis that included age, sex, ethnicity, the vascular and inherited prothrombotic risk factors, and the GPx-3 H2 haplotype as covariates (Table 3). The association between the H2 haplotype and the risk of AIS remained unchanged, confirming this haplotype as an independent risk factor (OR=2.07, 95% CI=1.03 to 4.47; $P=0.034$). Hypertension showed the strongest association with AIS, increasing the susceptibility almost 8-fold (OR=7.67, 95% CI=3.22 to 18.22; $P<0.001$), whereas the risk estimate associated with smoking was 2.30 (95% CI=1.20 to 4.41; $P=0.012$).

Vascular risk factors, including smoking and hypertension, increase oxidant stress.20 To assess if these conditions modified the risk of AIS associated with the GPx-3 promoter polymorphisms, we analyzed the distribution of the H2 haplotype after stratification for smoking and hypertension (Table 4). Smoking alone was associated with an almost 2-fold higher risk of AIS ($P=0.044$), whereas smokers who were also carriers of the GPx-3 H2 haplotype had an OR for AIS of 4.22 (95% CI=1.48 to 12.42; $P=0.002$). All subjects who carried the H2 haplotype and were also hypertensive had strokes ($P<0.001$); therefore, no OR could be calculated for

### Table 2. GPx-3 Promoter Haplotype Frequencies and Pairs Among Study Subjects

<table>
<thead>
<tr>
<th>Haplotype pair</th>
<th>Controls (n=123)</th>
<th>Patients (n=123)</th>
<th>Controls (n=82)</th>
<th>Patients (n=82)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1/H1</td>
<td>89 (72.4%)</td>
<td>76 (61.8%)</td>
<td>60 (73.2%)</td>
<td>48 (58.5%)</td>
</tr>
<tr>
<td>H1/H2</td>
<td>14 (11.4%)</td>
<td>28 (22.8%)</td>
<td>12 (14.6%)</td>
<td>22 (26.8%)</td>
</tr>
<tr>
<td>H1/H3</td>
<td>12 (9.8%)</td>
<td>6 (4.9%)</td>
<td>6 (7.3%)</td>
<td>6 (7.3%)</td>
</tr>
<tr>
<td>H1/H4</td>
<td>2 (1.6%)</td>
<td>3 (2.4%)</td>
<td>2 (2.4%)</td>
<td>2 (2.4%)</td>
</tr>
<tr>
<td>H1/H5</td>
<td>1 (0.8%)</td>
<td>3 (2.4%)</td>
<td>1 (1.2%)</td>
<td>2 (2.4%)</td>
</tr>
<tr>
<td>H1/H6</td>
<td>1 (0.8%)</td>
<td>1 (0.8%)</td>
<td>0</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>H1/H7</td>
<td>0</td>
<td>1 (0.8%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H1/H8</td>
<td>0</td>
<td>1 (0.8%)</td>
<td>0</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>H2/H2</td>
<td>1 (0.8%)</td>
<td>1 (0.8%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H2/H3</td>
<td>2 (1.6%)</td>
<td>1 (0.8%)</td>
<td>1 (1.2%)</td>
<td>0</td>
</tr>
<tr>
<td>H2/H4</td>
<td>0</td>
<td>2 (1.6%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H3/H3</td>
<td>1 (0.8%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

The position of each polymorphism is based on the transcription start site being +1. Carriers of the GPx-3 promoter H1H1 haplotype pair were used as the reference group.
this group. Carriership of the H2 haplotype in the presence of at least one vascular risk factor yielded an over 5-fold increase in the risk of AIS (OR = 5.18, 95% CI = 1.82 to 15.03; P < 0.001), suggesting an interaction of this genetic variant with conventional vascular risk factors. Of note, the sample size in some of these subgroup analyses was very small, and, thus, the results must be interpreted with caution.

### Childhood Stroke

To validate the results found among young adults, we performed an independent replication study in a population with childhood stroke. The patient and control groups were well matched for age and sex, and all children were of white background (Table 1). The distribution of known inherited prothrombotic risk factors was essentially equivalent between groups.

The GPx-3 haplotypes identified among adults were determined in this pediatric population. As in adults, haplotypes H1 and H2 accounted for the majority of haplotypes observed. The GPx-3 H2 haplotype was more prevalent among affected children than among controls (13.4% versus 7.9%; Table 2) yielding a significantly increased carrier rate of the H2 haplotype (H1H2 and H2H2) among pediatric patients compared with controls (OR = 2.29, 95% CI = 1.04 to 5.51; P = 0.039). After adjustment for age, sex, and inherited prothrombotic risk factors, the H2 haplotype remained independently associated with the risk of childhood stroke with an OR of 2.13 (95% CI = 1.23 to 4.90; P = 0.027; Table 3).

### Ethnic Heterogeneity

In association studies conducted among subjects from different populations, the concern exists for spurious associations

---

### TABLE 3. Multiple Logistic Regression Model Incorporating the GPx-3 Promoter H2 Haplotype, Demographic Characteristics, and Vascular and Inherited Prothrombotic Risk Factors Of AIS Patients And Controls

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Adults</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPx-3 H2 haplotype</td>
<td>2.07</td>
<td>1.03–4.47</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.97</td>
<td>0.90–1.21</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>0.98</td>
<td>0.52–1.87</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td>1.41</td>
<td>0.63–3.16</td>
<td>0.40</td>
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</tr>
<tr>
<td>Hypertension</td>
<td>7.67</td>
<td>3.22–18.22</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>2.30</td>
<td>1.20–4.41</td>
<td>0.012</td>
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</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.57</td>
<td>0.32–7.83</td>
<td>0.58</td>
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<tr>
<td>Hyperlipidemia</td>
<td>1.06</td>
<td>0.52–2.16</td>
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<tr>
<td>Factor V Leiden</td>
<td>1.28</td>
<td>0.25–6.54</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Prothrombin G20210A</td>
<td>3.21</td>
<td>0.50–20.71</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>1.15</td>
<td>0.46–2.87</td>
<td>0.76</td>
<td></td>
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</tbody>
</table>

Carriers of the GPx-3 promoter H1H1 haplotype pair were used as the reference group.

### TABLE 4. Effect of the GPx-3 H2 Haplotype on the Risk of AIS, Stratified by Vascular Risk Factors

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Patients</th>
<th>Controls</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
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<td>No</td>
<td>10</td>
<td>8</td>
<td>1.94</td>
<td>0.63–6.06</td>
<td>0.20</td>
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<td>Yes</td>
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<td>33</td>
<td>1.89</td>
<td>1.01–3.69</td>
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<tr>
<td>Hypertension</td>
<td>19</td>
<td>7</td>
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<td>1.48–12.42</td>
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<tr>
<td>No</td>
<td>46</td>
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<tr>
<td>Yes</td>
<td>18</td>
<td>15</td>
<td>2.90</td>
<td>0.89–8.41</td>
<td>0.065</td>
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<tr>
<td>Any vascular risk factor</td>
<td>36</td>
<td>53</td>
<td>2.55</td>
<td>1.21–5.43</td>
<td>0.0075</td>
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</tbody>
</table>

*All ORs are relative to the reference category: no risk factor and H1H1 haplotype pair.*
secondary to ethnic variability in allele or haplotype frequencies. Indeed, among adult Brazilian controls, the frequencies of the H1 and H2 haplotypes differed between subjects of white and black descent (allele frequencies in whites: H1 84.5%, H2 7.9%; blacks: H1 75.0%, H2 9.1%), albeit not significantly (P = 0.13 for H1, P = 0.75 for H2). The risk conferred by the H2 haplotype is maintained in an ethnic variability in allele or haplotype frequencies. Indeed, among adult Brazilian controls, the frequencies of the H1 and H2 haplotypes differed between subjects of white and black descent (allele frequencies in whites: H1 84.5%, H2 7.9%; blacks: H1 75.0%, H2 9.1%), albeit not significantly (P = 0.13 for H1, P = 0.75 for H2). The risk conferred by the H2 haplotype is maintained in an ethnic variability in allele or haplotype frequencies. Indeed, among adult Brazilian controls, the frequencies of the H1 and H2 haplotypes differed between subjects of white and black descent (allele frequencies in whites: H1 84.5%, H2 7.9%; blacks: H1 75.0%, H2 9.1%), albeit not significantly (P = 0.13 for H1, P = 0.75 for H2). The risk conferred by the H2 haplotype is maintained in an ethnic variability in allele or haplotype frequencies. Indeed, among adult Brazilian controls, the frequencies of the H1 and H2 haplotypes differed between subjects of white and black descent (allele frequencies in whites: H1 84.5%, H2 7.9%; blacks: H1 75.0%, H2 9.1%), albeit not significantly (P = 0.13 for H1, P = 0.75 for H2). The risk conferred by the H2 haplotype is maintained in an ethnic variability in allele or haplotype frequencies. Indeed, among adult Brazilian controls, the frequencies of the H1 and H2 haplotypes differed between subjects of white and black descent (allele frequencies in whites: H1 84.5%, H2 7.9%; blacks: H1 75.0%, H2 9.1%), albeit not significantly (P = 0.13 for H1, P = 0.75 for H2). The risk conferred by the H2 haplotype is maintained in an 382 383 The polymorphisms at positions 942, 927, and 961, 384 as well as 568, 518, and 302, were in complete LD, 385 forming 2 blocks that were, in turn, linked. We can speculate 386 that this high degree of LD may be important for promoter 387 function. Recent investigations have argued that patterns of 388 LD in the human genome occur in block structure; a block is 389 characterized by a low level of recombination among contiguous 390 SNPs within it, marked by surrounding SNPs with a high level of recombination.31,32 Several algorithms have been proposed to determine block size and structure; how-
ever, the prevalence of the H1 and H2 haplotypes was sufficiently high in our study to obviate the need to use these methods. Haplotype studies are likely to represent an advantage over single SNP analyses because they exploit LD information to increase marker informativity, resulting in increased power to detect association.\textsuperscript{32–33} Furthermore, genetic information may relate to disease status as a functional set of SNPs, rather than any single SNP.\textsuperscript{34}

Thrombotic cerebrovascular disease is multifactorial in etiology and occurs as a complex interaction between environmental and genetic predisposing factors.\textsuperscript{35} In addition, most prothrombotic risk factors, such as factor V Leiden and the prothrombin G20210A mutation, are not major cardiovascular risk factors, but may assume increased importance in certain patient subgroups, such as children or patients with other conventional vascular risk factors.\textsuperscript{36} We hypothesized that the GPx-3 H2 haplotype might behave in a similar manner; because conventional vascular risk factors increase oxidant stress,\textsuperscript{20} an interaction between the GPx-3 H2 haplotype and environmental factors would be plausible. Indeed, the presence of vascular risk factors among carriers of the GPx-3 H2 haplotype more than doubled the risk of AIS when compared with patients who carried only the genetic or environmental risk factor alone. All subjects who carried the H2 haplotype and were also hypertensive had strokes, suggesting that this may be a particularly high-risk combination of factors.

We performed reporter gene studies with the 2 most common haplotypes in our population and found that the transcriptional activity of the H2 risk haplotype was lower than that of the H1 haplotype, especially under hypoxic conditions. The lower basal expression levels of the GPx-3 H2 haplotype and its compromised upregulation in hypoxia yield less ROS scavenging potential, thereby compromising antithrombotic and neuroprotective function. We have not determined in our study which specific polymorphism(s) is functionally relevant and responsible for the altered transcriptional response. Additional investigation including site-directed mutagenesis would be necessary to answer this question; however, the SNP at position −943 is located within an AP-1 site, suggesting that it may, at least in part, affect the GPx-3 promoter’s ability to respond to changes in redox state and oxidant stress. The AP-1 transcription factor is exquisitely redox-sensitive and regulates antioxidant and proinflammatory genes.\textsuperscript{37}

Some limitations of our study should be noted. First, our ability to correlate the GPx-3 haplotypes with GPx-3 activity levels in vivo was limited by the lack of availability of plasma samples from our study population. Second, we analyzed the effect of the GPx-3 haplotypes on AIS without taking into consideration specific pathogenic subtypes of AIS. Third, this study includes only survivors of thrombotic cerebrovascular disease, and the possibility of survival bias must be considered. Fourth, the sample sizes for some of the subanalyses, particularly the interaction studies, were small, and the associated risk estimates have to be interpreted with caution; the biological plausibility of the results, however, strengthens the validity of these results. Finally, given the ethnic heterogeneity of the study populations, the possibility that these findings are influenced by population stratification cannot be excluded. We did, indeed, find nonsignificant differences in haplotype frequencies between Brazilians of white and black descent and, therefore, chose patients and controls of similar ethnic distribution. In addition, we included ethnicity as an independent variable in the logistic regression analysis. The haplotype frequencies among pediatric controls was similar to that found in the adult whites, indicating that the distribution among whites in different countries may be more uniform.

Summary
In conclusion, we have identified novel GPx-3 promoter polymorphisms that form a risk haplotype associated with a significant increase in the risk of AIS among young individuals. These findings, in combination with prior work from our group, support a novel mechanism for thrombotic cerebrovascular disease that involves the antioxidant enzyme GPx-3 and its role in scavenging ROS that limit the bioavailability and antiplatelet effects of nitric oxide. This genetic variant interacts with conventional vascular risk factors to potentiate further the risk of AIS. Studies involving a larger number of subjects and patient populations with thrombotic disease in other vascular beds, as well as attempting to correlate the GPx-3 H2 haplotype with enzyme activity in plasma, are necessary to confirm these results and determine the generalizability of this defect.

Acknowledgments
Role of contributors: B.V. was responsible for the study design, screening of the GPx-3 gene, genotyping and haplotyping of study subjects, construction of GPx-3 vectors, data analysis and interpretation, and writing of the manuscript. R.J. performed genotyping, and hypoxia and luciferase studies. C.B. designed the primers for screening of the GPx-3 gene and participated in the screening of the GPx-3 gene. K.B. assisted with the statistical analysis and data interpretation, and helped in the preparation of the manuscript. G.K., P.S., and B.D. were involved with recruitment of study subjects and clinical data collection in Israel, Italy, and Brazil, respectively. J.A.-B. assisted with recruitment of study subjects in Brazil. F.O. assisted with genotyping. D.H. supervised the functional genomics studies. J.L. was responsible for the overall study and experimental design, study supervision, data analysis and interpretation, and writing and final critical review of the manuscript. The authors thank Professor Annamaria Laverda and Dr Chiara Gentilomo from the Pediatrics Department at Padua University for access to patients, and Ms Stephanie Tribuna for excellent editorial assistance.

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Disclosures
None.

References


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<td>300</td>
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<td>20%</td>
<td>4</td>
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<td>P.9</td>
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<td>GAAATCCAGCCCGCTCA</td>
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<td>58</td>
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Table S2. Genotypic distribution of GPx-3 promoter polymorphisms among study subjects

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<th>Patients (n=123)</th>
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<th>Patients (n=82)</th>
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<td>87 (70.7%)</td>
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<td>67 (81.7%)</td>
<td>57 (69.5%)</td>
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<sup>1</sup> AIS patient vs. controls;  <sup>2</sup> CVT patient vs. controls
<sup>1,2</sup> p-values were calculated assuming a dominant genetic model