ADAMTS13 (A Disintegrin-like and Metalloprotease with Thrombospondin motifs), also known as von Willebrand factor (vWF) cleaving protease, is an established factor in blood coagulation and stroke. ADAMTS13 is a prime example for such a key component as it cleaves von Willebrand factor multimers, reduces platelet adhesion and aggregation, and downregulates thrombus formation and inflammation.

Methods and Results—We characterized the genetic architecture of ADAMTS13 through targeted next-generation sequencing of 48 affected children and their unaffected siblings and identified in total 241 variants (single nucleotide polymorphisms or insertions/deletions) in the ADAMTS13 gene. From these, based on significance in the sibship disequilibrium test \((P<0.05)\) or protein-altering properties, we selected 21 common variants covering the complete ADAMTS13 gene for genotyping in 270 trios and subsequent association analyses. Transmission disequilibrium testing was performed for affection status and ADAMTS13 activity levels using PLINK and FBAT, respectively. Ten single nucleotide polymorphisms were significantly associated with pediatric stroke \((P<0.05 \text{ to } P<0.001)\), 2 of which \((rs2285489 \text{ and } rs28793911)\) were also significantly associated with ADAMTS13 levels \((P=0.0004 \text{ and } P=0.0092)\). The resulting protective haplotype H1.1. \((T:U 95.5: 144.4; P=0.0016)\) is associated with increased ADAMTS13 levels \((age-adjusted P=0.0108)\). Haplotype association using a sliding window approach assigns this association to the ADAMTS13 von Willebrand factor–binding domain \((P=1.2 \times 10^{-4})\).

Conclusions—Our data provide a link between the genetic architecture of ADAMTS13, ADAMTS13 levels, and stroke susceptibility. Altogether, these studies render ADAMTS13 an attractive candidate for functional studies and may contribute to personalized diagnosis and treatment options in future. (Circ Cardiovasc Genet. 2016;9:357-367. DOI: 10.1161/CIRCGENETICS.115.001184.)

Key Words: genetics • genetic association studies • genetic variation • humans • stroke • thrombosis

Rare Variants in the ADAMTS13 Von Willebrand Factor–Binding Domain Contribute to Pediatric Stroke

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Background—Recently, we reported a gene network of ADAMTS (A Disintegrin-like and Metalloprotease with Thrombospondin motifs) genes as central component of the genetic risk contributing to pediatric stroke. ADAMTS13 is an established factor in blood coagulation and stroke. ADAMTS13 reduces adhesion and aggregation of platelets and downregulates thrombus formation and inflammation.

Methods and Results—We characterized the genetic architecture of ADAMTS13 through targeted next-generation sequencing of 48 affected children and their unaffected siblings and identified in total 241 variants (single nucleotide polymorphisms or insertions/deletions) in the ADAMTS13 gene. From these, based on significance in the sibship disequilibrium test \((P<0.05)\) or protein-altering properties, we selected 21 common variants covering the complete ADAMTS13 gene for genotyping in 270 trios and subsequent association analyses. Transmission disequilibrium testing was performed for affection status and ADAMTS13 activity levels using PLINK and FBAT, respectively. Ten single nucleotide polymorphisms were significantly associated with pediatric stroke \((P<0.05 \text{ to } P<0.001)\), 2 of which \((rs2285489 \text{ and } rs28793911)\) were also significantly associated with ADAMTS13 levels \((P=0.0004 \text{ and } P=0.0092)\). The resulting protective haplotype H1.1. \((T:U 95.5: 144.4; P=0.0016)\) is associated with increased ADAMTS13 levels \((age-adjusted P=0.0108)\). Haplotype association using a sliding window approach assigns this association to the ADAMTS13 von Willebrand factor–binding domain \((P=1.2 \times 10^{-4})\).

Conclusions—Our data provide a link between the genetic architecture of ADAMTS13, ADAMTS13 levels, and stroke susceptibility. Altogether, these studies render ADAMTS13 an attractive candidate for functional studies and may contribute to personalized diagnosis and treatment options in future. (Circ Cardiovasc Genet. 2016;9:357-367. DOI: 10.1161/CIRCGENETICS.115.001184.)

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Clinical Perspective on p 367
In 2012, we reported a genome-wide association study in 270 affected offspring trios, which demonstrates the association of several members of the ADAMTS gene family with pediatric stroke\(^1\) including a moderate association of single nucleotide polymorphisms (SNPs) in the vicinity of ADAMTS13. In parallel, we extended our studies to include the association of ADAMTS13 plasma levels as an intermediate phenotype and the risk for pediatric stroke\(^2\) and demonstrated that decreased ADAMTS13 activity is a risk factor (odds ratio, 6.70; 95% confidence interval, 2.58–17.38) for incident ischemic stroke in children without underlying disease.

In the present study, we characterized the genomic architecture underlying ADAMTS13 through a targeted next-generation sequencing approach in 48 pairs of patients with pediatric stroke and their unaffected siblings. Twenty-one ADAMTS13 variants identified in this discovery stage were subsequently genotyped in the above-mentioned cohort of 270 affected offspring trios and analyzed by transmission disequilibrium testing for association with stroke affection status and ADAMTS13 serum levels. By this, we establish a causal relationship between ADAMTS13 single nucleotide variants, ADAMTS13 levels, and pediatric stroke susceptibility.

### Methods

#### Subjects

**Ethics**
The present study was performed in accordance with the ethical standards laid down in the updated version of the 1964 Declaration of Helsinki and was approved by the medical ethics committee of the University of Münster, Germany. Written informed consent was obtained from all participants or their parent(s).

**Patients**

Enrollment and characterization of affected family trios have been described in detail in our initial genome-wide association study publication.\(^1\) In brief, all probands have been collected from 2000 to 2009 as part of a nation-wide survey. From an initial study sample comprising 300 consecutively enrolled families, 293 affected offspring trios (affected child with unaffected parents) were considered comprising 300 consecutively enrolled families, 293 affected offspring trios (affected child with unaffected parents) were considered eligible for genome-wide genotyping with Illumina HumanCNV370 arrays after assessment of DNA quality. Of these, 270 trios were available for statistical analysis after filtering for call rate (>97%), Mendelian inheritance error rate (<0.1%), and sex prediction match using the GenomeStudio Software.\(^1\) Potential population stratification is accounted for by choosing a family-based study design. ADAMTS13 levels were available for 195 of these 270 pediatric patients, but have not been collected for their parents. Standard laboratory protocols for the determination of hemostasis markers including the protocols for the ADAMTS13 ELISA are described in detail in our previous publication.\(^2\)

A subset of 48 independent pediatric probands together with 48 unaffected siblings was processed for targeted resequencing. Of these 48 affected pediatric probands, 41 are a part of the above-mentioned 270 trios. Probands were selected for sequencing if an unaffected sibling has been enrolled as control in our study and sufficient DNA material was available for both. An overview of probands characteristics for affected offspring trios and for selected discordant sib-pairs is given in Table I in the Data Supplement.

#### Targeted Resequencing

For subsequent sequencing, genomic DNA was isolated from EDTA blood samples using QIAamp DNA Blood Kit (Qiagen, Hilden, GFR); Genomic DNA samples of 48 sib-pairs were quantified using the Quant-IT PicoGreen dsDNA Assay Kit (Life Technologies, Darmstadt, GFR) and the Genesis RSP 100 (TeCN, Mainz, GFR) liquid-handling system. Library construction was performed starting with 1 μg of genomic DNA after the TruSeq DNA Sample Preparation Guide (Illumina, Eindhoven, NL) and the NimbleGen SeqCap EZ Choice Library short read User’s Guide (Roche, Penzberg, GFR) for in-solution hybrid selection according to the manufacturer’s instructions. The captured genomic region encompassing the entire ADAMTS13 gene plus surrounding regions contains the sequence from 133333412 to 133484533 base pairs on chromosome 9 (hg38). The prepared sequencing libraries were quality controlled and quantified using the HT DNA High Sensitivity Kit on a LapChip GX system (PerkinElmer, Rodgau, GFR). Library pools in equimolar concentrations were clustered and sequenced using TruSeq PE Cluster Kit v2 or v3 (Illumina) and TruSeq SBS Kit v2 and v3 (Illumina) in a paired-end mode (100 cycles) on a HiScanSQ sequencing system (Illumina).

#### Initial Quality Filtering

Sequencing reads were subject to quality control (trimming adapter sequences from the raw reads, discarding low-quality bases at the reads’ ends) using in-house developed software. After this quality filtering step, 78.9% of the read pairs were retained for further downstream analysis.

#### Read Mapping

The resulting high-quality reads (on average ≈382245 reads per sample) were mapped to the reference genome (hg19) using BWA software tool (version 0.6.2).\(^2\) More than 99% of the reads were successfully mapped resulting in a mean coverage of the target region of ≈227. Duplicates were defined as read pairs having identical outer mapping coordinates; Picard software tool (version 1.7.3) was used to remove them from further analysis. Reads were realigned around indels, and quality score recalibration was performed using GATK (version 2.0).\(^3\)

#### Variant Calling and Filtering

GATK tool UnifiedGenotyper was used to produce a raw variant set (SNPs and short indels). To filter false-positive variants from this set, hard filtering of the variants was performed.\(^4\)\(^\text{14}\)\(^\text{15}\) All variants with low-quality value (<50.0) were marked as potential errors. GATK VariantFiltration tool was used to exclude probable false-positive variant calls, specifically those showing high bias in mapping quality (MQRanksum < −12.5 for SNPs), ref/alt allele position within reads (ReadPosRankSum < −8.0 for SNPs, ReadPosRankSum < −12.0 for indels), strand bias (FisherStrand [FS] >6.0 for SNPs and FS >120 for indels). Further filtering criteria include low average quality (QualByDepth [QD] <2.0 for SNPs and QD <2.5 for indels), low mapping quality (<40.0), high evidence for >2 segregating haplotypes (HaploTypeScore >13.0 for SNPs), high evidence for inbreeding (inbreeding coefficient <0.6 for indels). Finally, both SNP and indel calls were filtered out when coverage depth was too low (<5).

Given a variant site, the depth of coverage and base qualities of the mapped reads vary across the sequenced samples; as a result, genotype calls for different samples differ in quality, as reported in Genotype Quality field in the variant call format file generated by GATK engine. To avoid usage of uncertain genotypes in the downstream analysis, we performed a further filtering step: genotypes with Genotype Quality value below a certain threshold were considered as unknown (no-call). For SNPs, this threshold value was set to 10, whereas indels were filtered at the Genotype Quality value of 20. Finally, we used the UCSC Liftover tool\(^\text{16}\) to remap genomic coordinates of identified variants to the genome assembly hg38.

#### Functional Annotation and Sibship Disequilibrium Test

To assess a potential functional effect of genomic variations, we used the SnpEff software tool (version 3.0)\(^\text{17}\) and Annovar (version...
To investigate which impact nonsynonymous SNPs have on the protein function, we used the Protein Variation Effect Analyzer (PROVEAN) web tool\textsuperscript{18} to assign PROVEAN and Sorting Intolerant From Tolerant (SIFT) scores, which indicate the likelihood of the genomic variant being damaging to the protein. For association analysis of detected variants, a family-based approach was implemented. As the affected and not affected individuals are related, using a standard case–control approach for association testing would lead to inflated false-positive rates. The sibship disequilibrium test\textsuperscript{19} allows to test for both association and linkage and does not require parental genotypes. For our analyses, we used a custom software implementation of sibship disequilibrium test.

### Haplotype Association With Sliding Window Approach

To test for haplotype association of ADAMTS13 variants, we used the sliding window approach as implemented in haplotype version of FBAT within the FBAT software tool.\textsuperscript{20} For each window size from a specified range (3–14 variants), we performed a haplotype association test by Monte Carlo testing using 10000 samples of all consecutive variants constituting a window. We have determined the most significantly associated haplotype for each window size, with windows of size 13 and 14 showing the smallest $P$ values.

### Validation of Variants Through Capillary Sequencing

Capillary sequencing has been applied for validation of next-generation sequencing (NGS) results for selected variants. Even though NGS has a high accuracy, there may be false-positive variant calls because of the immense number of variants. For variants showing nonconcordant results for NGS and capillary sequencing, we chose the results from capillary sequencing.

Capillary sequencing was performed through direct DNA sequencing using Big-Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) on a 3730 DNA Analyzer (Life Technologies). Variant regions were amplified using AmpliTaq Gold PCR Master Mix (Life Technologies) or Phusion High-Fidelity PCR Master Mix (NEB, Frankfurt, GFR), respectively. ExoSap-IT kit (Affymetrix, Santa Clara) was used for PCR product cleanup.

### Genotyping and Association Analyses in Full Pediatric Stroke Study Sample

Genotyping in 270 affected offspring trios of 22 SNPs selected by sequencing was performed with either predesigned or custom-made TaqMan SNP Genotyping Assays (Life Technologies) or KASP Genotyping Assays (LGC Limited, United Kingdom) on an ABI 7900HT sequence detector (Applied Biosystems, Darmstadt, GFR) using 2 ng of genomic DNA per SNP. Genotypes were generated by automatic calling using the SDS software version 2.3 (Applied Biosystems). The missing-genotype rate for all SNPs was <1.5% and minor allele frequencies was >1%. Hardy–Weinberg equilibrium for founders was tested using an exact test as implemented in PLINK software package version 1.07\textsuperscript{21} (http://pngu.mgh.harvard.edu/purcell/plink) and was met for all SNPs except for rs1055432 (Hardy–Weinberg equilibrium <0.01), which was removed from further analyses. The total set of 21 SNPs tested in the cohort of 270 affected offspring trios is listed in Table 1.

Association of pediatric stroke affection status was calculated using the transmission disequilibrium test as implemented in PLINK. No adjustment for covariates was applied. The Bonferroni method was used for correction for multiple testing. Association of haplotypes was calculated using the family trio association test implemented in Haploviev version 4.2.\textsuperscript{22} Quantitative ADAMTS13 activity levels available for affected children from 195 trios were tested for normal distribution by using the Kolmogorov–Smirnov test. No transformation of the data was necessary ($P=0.1723$). Association of family genotypes with quantitative ADAMTS13 levels was analyzed using a family-based association test as implemented in FBAT.\textsuperscript{23} FBAT offset was set to the mean of the quantitative trait (94.675). Because ADAMTS13 activity is age dependent, data were adjusted for age using linear regression. In addition, ADAMTS13 activity has also been adjusted for the covariates age, factor VIII activity, vWF antigen, and blood group. Covariates were selected according to the report by Lambers et al.\textsuperscript{24} The haplotype version of FBAT was applied to determine haplotypes associated with ADAMTS13 activity levels using the Monte Carlo method with 10000 permutation cycles. Adjustment for covariates was performed as described above.

### C-Alpha Test Statistic for Rare Variants

The C-alpha score test of Neyman and Scott\textsuperscript{25} has been proposed for association studies by Neale et al\textsuperscript{26} to test the observed distribution of rare variants in cases versus controls as a group. Under the null hypothesis of no association between the variants and the phenotype, C-alpha assumes that the distribution of counts (copies of an observed variant) should follow a binomial distribution. In opposite to many other collapsing tests, C-alpha does not assume that all the rare variants tested are damaging, i.e., have the same directionality. SNPs were selected if they are present in at least one of the affected individuals and having minor allele frequency <0.5%. Also, SNPs having at least 1 uncertain genotype (no-call) among all sequenced individuals were excluded. Thirty-six SNPs passed these criteria (Figure 1C; Table 3).

The C-alpha test statistic implemented in the R-package AssotesteR, deposited in CRAN (http://www.inside-r.org/packages/cran/AssotesteR), was used to compute the association of these 36 rare variants identified in the NGS analysis.

### Results

#### Targeted NGS and Validation

After performing variant calling and quality filtering as described in the Methods section of this article, we identified an overall 658 SNPs and 44 indels in the target region on chromosome 9. A total of 225 SNPs and 13 indels, of which 32 SNPs and 5 indels were not yet annotated, are located within the ADAMTS13 gene (Figure 1B; Tables II and III in the Data Supplement). Four of 11 nonsynonymous coding variants in ADAMTS13 showing either a PROVEAN score below −2.5 or an SIFT score <0.05 are predicted as damaging and are highlighted (Table 2).

From 26 SNPs validated by capillary sequencing within the 48 sip-pairs, 11 showed exactly the same results as in NGS, 8 were in >95% concordance with NGS results, 5 had nonconcordant results for NGS and capillary sequencing, and 3 SNPs failed in quality control of capillary sequencing, and 3 SNPs were additionally detected.

### Association of ADAMTS13 SNPs With Pediatric Stroke and ADAMTS13 Activity

Of 241 ADAMTS13 variants (228 SNPs and 13 indels) discovered in the NGS screening approach, 22 variants were forwarded to genotyping in the full pediatric stroke cohort comprising 270 father–mother–affected child trios as described previously.\textsuperscript{11} Selection criteria for these SNPs
were (1) significant \( P \) value in the sibship disequilibrium test, (2) predicted functional effect based on a PROVEAN score, (3) gene coverage and linkage disequilibrium, respectively, depending on availability of TaqMan assays. Of 21 SNPs passing the QC criteria (see Methods section of this article), 10 were significantly associated with pediatric stroke at a nominal \( P < 0.05 \) (Table 1), with rs28793911, rs671410, rs28680325 remaining significant when adjusting for multiple testing using Bonferroni correction. Of these SNPs, 9 are sufficient to tag the underlying genomic region using \( r^2 > 0.8 \) as a measure for linkage disequilibrium. In the corresponding haplotype analysis, a significant haplotype H1.1 was significantly identified for linkage disequilibrium-block 1 (\( P = 0.0016 \)) as was H2.1 for linkage disequilibrium-block 2 (\( P = 0.0014 \); Figure 2).

Subsequently, we tested the 21 SNPs for an association with ADAMTS13 activity in a subset of 195 trios where ADAMTS13 levels are available for the affected children. Two SNPs, rs2285489 and rs28793911, which are significantly associated with stroke affection status are also significantly associated with ADAMTS13 activity (rs2285489 (unadjusted/age adjusted): \( P = 0.0005/0.0004 \), rs28793911: \( P = 0.0115/P = 0.0092 \), implying that both SNPs directly influence ADAMTS13 activity (Table 1). When adjusting for covariates age, factor VIII activity, vWF antigen, and blood group, we lose some power because of missing phenotypes (160 individuals have been phenotyped for all covariates). Nevertheless, the association of rs2285489 holds at a \( P = 0.0085 \), whereas rs28793911 curtly misses significance at \( P = 0.0908 \) (Table 1). Interestingly, the undertransmitted haplotype H1.1. (T:U 95.5: 144.4; \( P = 0.0016 \) is also associated with an increased ADAMTS13 activity (\( P = 0.0108 \) age adjusted and \( P = 0.0633 \) adjusted for age, factor VIII activity, vWF antigen, and blood group; Figure 2), providing a link between ADAMTS13 variants and circulating ADAMTS13 levels or activity.

### Table 1. Transmission Disequilibrium Test With Pediatric Stroke and Quantitative ADAMTS13 Activity

| SNP       | BP         | A1 | A2 | MAF | T | U | OR (95% CI) | \( P \) Value | \( P \) Value (Age Adj) | \( P \) Value (Covar Adj.) |
|------------|------------|----|----|-----|---|---|-------------|---------------|------------------------|---------------------------|
| rs6597630  | 133414815  | T  | C  | 0.0769 | 42 | 32 | 1.31 (0.83–2.08) | 0.245 | 0.40187 | 0.436253 |
| rs34265876 | 133422087  | C  | T  | 0.0757 | 45 | 31 | 1.45 (0.92–2.29) | 0.1083 | 0.50616 | 0.600368 |
| rs34024143 | 133422462  | T  | C  | 0.1077 | 61 | 48 | 1.27 (0.87–1.86) | 0.2131 | 0.25778 | 0.26638 | 0.356536 |
| rs2285489  | 133424254  | T  | C  | 0.3745 | 100 | 145 | 0.69 (0.53–0.89) | 0.00404 | 0.00051 | 0.008527 |
| rs28571612 | 133425552  | A  | G  | 0.0776 | 44 | 37 | 1.19 (0.77–1.84) | 0.4367 | 0.29571 | 0.31093 | 0.323734 |
| rs3118667  | 133425943  | C  | T  | 0.4879 | 110 | 155 | 0.71 (0.56–0.91) | 0.0057 | 0.13848 | 0.1176 | 0.205714 |
| rs739469   | 133433609  | C  | G  | 0.1077 | 61 | 48 | 1.27 (0.87–1.86) | 0.2131 | 0.25778 | 0.26638 | 0.356536 |
| rs28793911 | 133434817  | T  | C  | 0.3567 | 93 | 143 | 0.65 (0.5–0.84) | 0.00114 | 0.01149 | 0.00918 | 0.09081 |
| rs3124770  | 133435084  | C  | G  | 0.0899 | 51 | 51 | 1 (0.68–1.47) | 1 | 0.19202 | 0.19708 | 0.749847 |
| rs2301612  | 133436862  | G  | C  | 0.4362 | 102 | 143 | 0.71 (0.55–0.92) | 0.00881 | 0.10743 | 0.086 | 0.44833 |
| rs28647808 | 133440409  | G  | C  | 0.0794 | 45 | 37 | 1.22 (0.79–1.88) | 0.377 | 0.35907 | 0.37597 | 0.310167 |
| rs28645493 | 133440617  | G  | C  | 0.0991 | 60 | 37 | 1.62 (1.08–2.44) | 0.01953 | 0.9738 | 0.95155 | 0.971316 |
| rs35267948 | 133441897  | -  | T  | 0.0699 | 33 | 32 | 1.03 (0.63–1.68) | 0.9013 | 0.72543 | 0.74497 | 0.650022 |
| rs28446901 | 133443675  | C  | G  | 0.2019 | 104 | 71 | 1.47 (1.08–1.98) | 0.01261 | 0.52661 | 0.52683 | 0.636677 |
| rs685523   | 133445787  | T  | C  | 0.1077 | 43 | 56 | 0.77 (0.52–1.14) | 0.1914 | 0.69275 | 0.71792 | 0.817146 |
| rs652600   | 133445896  | G  | A  | 0.2784 | 113 | 103 | 1.1 (0.84–1.43) | 0.4962 | 0.29323 | 0.23573 | 0.399658 |
| rs34063534 | 133446038  | T  | C  | 0.0467 | 24 | 24 | 1 (0.57–1.76) | 1 | 0.35852 | 0.36193 | 0.480948 |
| rs671410   | 133446558  | G  | T  | 0.4737 | 157 | 103 | 1.52 (1.19–1.95) | 0.00081 | 0.2899 | 0.24606 | 0.373182 |
| rs28680325 | 133451246  | A  | C  | 0.3343 | 95 | 148 | 0.64 (0.5–0.83) | 0.00067 | 0.06884 | 0.05563 | 0.18669 |
| rs34934621 | 133454478  | A  | G  | 0.0386 | 22 | 18 | 1.22 (0.66–2.28) | 0.5271 | 0.05256 | 0.05488 | 0.061129 |
| rs4962153  | 133458632  | A  | G  | 0.1548 | 83 | 53 | 1.57 (1.11–2.21) | 0.00101 | 0.56686 | 0.56498 | 0.562098 |

A1 indicates minor allele; A2, major allele; Age adj: ADAMTS13 (A Disintegrin-like and Metalloprotease with Thrombospondin motifs) activity adjusted for age of probands; BP, basepair position; Covar Adj, ADAMTS13 activity adjusted for covariates age, factor VIII activity, von Willebrand factor antigen, and blood group; ID, variant identifier; MAF, minor allele frequency; OR (95% CI), odds ratio (95% confidence interval); SNP, single nucleotide polymorphism; T, transmitted minor allele count in TDT; and U, untransmitted allele count.
Figure 1. Association of ADAMTS13 (A Disintegrin-like and Metalloprotease with Thrombospondin motifs) with pediatric stroke. A, Genome-wide association study in 270 pediatric stroke trios. Results are shown for chromosome 9, P values are calculated by transmission disequilibrium test. The region highlighted in green was resequenced for a subset of 48 patients with pediatric stroke and 48 unaffected siblings. B, Association results from next-generation sequencing in the pediatric stroke sample subset calculated by (Continued)
a group to overcome these obstacles and to guide downstream analyses of relevant nonsynonymous coding variants in relation to their location within ADAMTS13 protein domains. This test yielded a significant association with a combined $P$ value for all 36 rare variants of $P=5.73\times10^{-6}$.

Table 2. PROVEAN and SIFT Scores of Nonsynonymous SNPs in ADAMTS13 (A Disintegrin-Like and Metalloprotease With Thrombospondin Motifs) Gene

| ID            | BP          | Protein ID     | AA Pos | Domain | Ref | Alt | PROVEAN Score | PROVEAN Pred. | SIFT Score | SIFT Pred. |
|---------------|-------------|----------------|--------|--------|-----|-----|-------------|--------------|------------|------------|
| rs34024143    | 133422462   | NP_620595.1    | 7      | SP     | R   | W   | 0.15        | Neutral     | 0.078      | Tol.       |
| rs34024143    | 133422462   | NP_620596.2    | 7      | SP     | R   | W   | 0.01        | Neutral     | 0.086      | Tol.       |
| rs34024143    | 133422462   | NP_620594.1    | 7      | SP     | R   | W   | 0.48        | Neutral     | 0.09       | Tol.       |
| rs151048660   | 133433442   | NP_620595.1    | 355    | TSP1   | R   | H   | −0.09       | Neutral     | 0.262      | Tol.       |
| rs151048660   | 133433442   | NP_620596.2    | 386    | TSP1   | R   | H   | 0.04        | Neutral     | 0.266      | Tol.       |
| rs151048660   | 133433442   | NP_620594.1    | 386    | TSP1   | R   | H   | 0.29        | Neutral     | 0.282      | Tol.       |
| rs2301612     | 133436862   | NP_620595.1    | 417    | Cys    | Q   | E   | 1.46        | Neutral     | 1          | Tol.       |
| rs2301612     | 133436862   | NP_620596.2    | 448    | Cys    | Q   | E   | 1.61        | Neutral     | 1          | Tol.       |
| rs2301612     | 133436862   | NP_620594.1    | 448    | Cys    | Q   | E   | 1.81        | Neutral     | 1          | Tol.       |
| rs36220240    | 133436890   | NP_620595.1    | 426    | Cys    | P   | L   | −7.09       | Delet.       | 0.053      | Tol.       |
| rs36220240    | 133436890   | NP_620596.2    | 457    | Cys    | P   | L   | −7.26       | Delet.       | 0.043      | Dam.       |
| rs36220240    | 133436890   | NP_620594.1    | 457    | Cys    | P   | L   | −7.29       | Delet.       | 0.012      | Dam.       |
| rs11575933    | 133436943   | NP_620596      | 475    | Cys    | P   | S   | −0.6        | Neutral     | 0.209      | Tol.       |
| rs11575933    | 133436943   | NP_620595      | 475    | Cys    | P   | S   | −0.61       | Neutral     | 0.164      | Tol.       |
| rs11575933    | 133436943   | NP_620594      | 475    | Cys    | P   | S   | −0.58       | Neutral     | 0.406      | Tol.       |
| New           | 133437770   | NP_620595.1    | 455    | Cys    | M   | R   | −4.02       | Delet.       | 0.028      | Dam.       |
| New           | 133437770   | NP_620596.2    | 486    | Cys    | M   | R   | −4.12       | Delet.       | 0.03       | Dam.       |
| New           | 133437770   | NP_620594.1    | 486    | Cys    | M   | R   | −4.18       | Delet.       | 0.062      | Dam.       |
| rs28647808    | 133440409   | NP_620595.1    | 587    | Spacer | P   | A   | −5.39       | Delet.       | 0.092      | Dam.       |
| rs28647808    | 133440409   | NP_620596.2    | 618    | Spacer | P   | A   | −5.53       | Delet.       | 0.048      | Dam.       |
| rs28647808    | 133440409   | NP_620594.1    | 618    | Spacer | P   | A   | −5.48       | Delet.       | 0.05       | Dam.       |
| rs41314453    | 133442704   | NP_620596      | 732    | TSP1   | A   | V   | −1.74       | Neutral     | 0.374      | Tol.       |
| rs41314453    | 133442704   | NP_620595      | 701    | TSP1   | A   | V   | −1.61       | Neutral     | 0.366      | Tol.       |
| rs41314453    | 133442704   | NP_620594      | 732    | TSP1   | A   | V   | −1.8        | Neutral     | 0.331      | Tol.       |
| rs685523      | 133445787   | NP_620595.1    | 869    | TSP1   | A   | V   | 0.62        | Neutral     | 0.3        | Tol.       |
| rs685523      | 133445787   | NP_620596.2    | 900    | TSP1   | A   | V   | 0.69        | Neutral     | 0.297      | Tol.       |
| rs685523      | 133445787   | NP_620594.1    | 900    | TSP1   | A   | V   | 0.88        | Neutral     | 0.441      | Tol.       |
| rs28503257    | 133454467   | NP_620595.1    | 1002   | TSP1   | A   | T   | −1.36       | Neutral     | 0.189      | Tol.       |
| rs28503257    | 133454467   | NP_620596.2    | 1033   | TSP1   | A   | T   | −1.43       | Neutral     | 0.183      | Tol.       |
| rs28503257    | 133454467   | NP_620594.1    | 1033   | TSP1   | A   | T   | −1.79       | Neutral     | 0.184      | Tol.       |
| rs142572218   | 133454548   | NP_620595      | 1060   | TSP1   | R   | W   | −5.57       | Delet.       | 0.007      | Dam.       |
| rs142572218   | 133454548   | NP_620596      | 1029   | TSP1   | R   | W   | −5.44       | Delet.       | 0.004      | Dam.       |
| rs142572218   | 133454548   | NP_620594      | 1060   | TSP1   | R   | W   | −6.13       | Delet.       | 0.001      | Dam.       |

AA Pos indicates amino acid position; Alt, alternative; BP, basepair position; Cys, cysteine-rich domain; Delet./Dam./Tol., deleterious/damaging/tolerated effect of variant; ID, variant identifier; PROVEAN/SIFT Pred.: variant effect predicted by Protein Variation Effect Analyzer (PROVEAN)/Sorting Intolerant From Tolerant (SIFT); Ref, reference; SNPs, single nucleotide polymorphisms; SP, signal peptide; TSP1, thrombospondin 1 motif.
containing 13 consecutive variants assigns this association to the ADAMTS13 spacer- and cysteine-rich vWF-binding domain \( (P=2\times10^{-4}; \) Figure 3).}

**Discussion**

The current study establishes a causal relationship between ADAMTS13 single nucleotide variants, ADAMTS13 levels, and pediatric stroke susceptibility. Furthermore, it refines the described association of low ADAMTS13 levels and stroke in children, and assigns the association to at least 2 ADAMTS13 SNPs, rs2285489 and rs28793911, which are significantly associated with both, the affection status and ADAMTS13 activity in a large cohort of 270 father–mother–affected child trios. The consistent association of ADAMTS13 SNPs, including haplotype analysis and C-alpha test for rare variants identified through our NGS screening, further corroborates our previous, preliminary finding reported in the initial genome-wide association study.11 Our study is in accordance with previous reports on ADAMTS13 SNPs and stroke27 and cardiovascular disease in young patients.28 rs2285489, 1 of the 2 SNPs associated with pediatric stroke and ADAMTS13 levels, has recently been shown to be associated with aneurysmal subarachnoid hemorrhage in 183 patients and 680 controls29 linking ADAMTS13 to vascular fragility in the brain. rs2301612, a likely pathogenic variant located in exon 12, has been described to influence vWF-cleaving protease activity30 and has also been associated with ischemic stroke.7 Furthermore, our results assign 4 nonsynonymous SNPs, which likely exert deleterious effects on the domains required for vWF binding that is the cysteine-rich domain, the spacer and the TSP motif 7. Because binding of vWF to these domains is a prerequisite for vWF cleavage through the respective metalloprotease domain, the enrichment of rare, private, and potentially deleterious mutations in the vWF-binding domain in children affected with stroke points toward a mechanism of reduced vWF-cleaving activity and thus increased levels of circulating high molecular weight vWF. As a result, vWF-mediated platelet adhesion and aggregation on the vascular endothelium is elevated raising the chance of thrombus formation.

However, other mechanisms, which may influence regulation of ADAMTS13 mRNA expression and splicing through SNPs affecting the 3′ untranslated region/promoter region, splice donor–acceptor sites or synonymous changes modulating RNA structure as proposed by Edwards et al9 need to be considered as additional factors in the interindividual variation in ADAMTS13 activity. Our observation of rare mutations, which are distributed across the entire gene resulting in the significant C-alpha \( P=5.73\times10^{-6} \) compared with healthy controls from the 1000 Genomes project, supports this notion. In addition, our results clearly demonstrate that the observed variability in ADAMTS13 activity is at least, in part, heritable and regulated through variants in cis, which means in and surrounding the genomic sequence of the ADAMTS13 locus, as evidenced by our association of ADAMTS13 SNPs and activity.

Interestingly, we did not observe any of the mutations previously linked to TTP,31 supporting the perception that findings from monogenetic diseases, although phenotypically in part similar, cannot directly be translated to common, complex diseases. Pleiotropy on both, phenotype and genotype levels need to be taken in account, when investigating complex traits such as (microvascular) stroke.
Recently, in addition to patients with arterial thrombotic disorders such as cardiovascular diseases or stroke, Lotta et al.\(^3\) reported an excess of rare, coding SNPs in \textit{ADAMTS13} in patients affected with deep vein thrombosis (DVT), implying that variants in \textit{ADAMTS13} may also play a role in the genetic basis of DVT. For lack of a replication cohort of pediatric stroke—to our knowledge, there is no comparable stroke cohort available worldwide—we assessed 8 of the reported stroke-associated SNPs in the \textit{ADAMTS13} genomic region through genotyping as pseudoreplication in an independent pediatric study cohort for DVT consisting of 184 father–mother–affected child trios. Indeed, we find a weak association

| ID         | Chr | BP      | Ref | Alt | Function      | AF   |
|------------|-----|---------|-----|-----|---------------|------|
| rs141547732| 9   | 133414905| G   | A   | Intron        | 0.0053 |
| New        | 9   | 133415922| G   | A   | Intron        | 0.0053 |
| New        | 9   | 133415947| T   | C   | Intron        | 0.0053 |
| New        | 9   | 133416014| C   | T   | Intron        | 0.0053 |
| New        | 9   | 133416687| A   | C   | Intron        | 0.011 |
| rs191393358| 9   | 133416892| T   | C   | Intron        | 0.011 |
| New        | 9   | 133418368| G   | A   | Intron        | 0.0053 |
| New        | 9   | 133420669| T   | G   | Intron        | 0.011 |
| New        | 9   | 133421174| G   | A   | Intron        | 0.0053 |
| New        | 9   | 133422164| G   | A   | UTR\(_{5}^\prime\) | 0.011 |
| rs145823565| 9   | 133422693| G   | A   | Intron        | 0.0053 |
| rs182358533| 9   | 133422888| C   | T   | Intron        | 0.0053 |
| New        | 9   | 133427068| G   | A   | Intron        | 0.0053 |
| New        | 9   | 133427473| T   | C   | Intron        | 0.0053 |
| rs141987928| 9   | 133428001| T   | A   | Intron        | 0.021 |
| rs147632514| 9   | 133430662| G   | A   | Intron        | 0.011 |
| New        | 9   | 133432273| C   | A   | Intron        | 0.0053 |
| New        | 9   | 133433004| G   | A   | Intron        | 0.0053 |
| rs17724903 | 9   | 133433227| G   | A   | Intron        | 0.0053 |
| New        | 9   | 133433638| C   | T   | Intron        | 0.0053 |
| New        | 9   | 133434917| G   | A   | Intron        | 0.011 |
| New        | 9   | 133436603| G   | A   | Intron        | 0.0053 |
| New        | 9   | 133437770| T   | G   | Non-Syn (Cys-rich) | 0.011 |
| New        | 9   | 133438703| C   | T   | Intron        | 0.0053 |
| rs139184786| 9   | 133440289| G   | C   | Intron        | 0.0053 |
| New        | 9   | 133441071| G   | T   | Intron        | 0.0053 |
| New        | 9   | 133446098| A   | G   | Intron        | 0.011 |
| New        | 9   | 133447864| G   | C   | Intron        | 0.011 |
| New        | 9   | 133449443| C   | A   | Intron        | 0.0053 |
| New        | 9   | 133451774| C   | G   | Intron        | 0.016 |
| New        | 9   | 133451923| A   | G   | Intron        | 0.0053 |
| rs113402066| 9   | 133452769| C   | T   | Intron        | 0.0053 |
| New        | 9   | 133454715| C   | T   | Intron        | 0.011 |
| rs144029202| 9   | 133454834| G   | C   | Intron        | 0.011 |
| rs142868607| 9   | 133455814| T   | C   | Intron        | 0.011 |
| New        | 9   | 133458492| G   | T   | Intron        | 0.0053 |

AF indicates allele frequency of alternative allele; Alt, alternative allele; BP, basepair position; Chr, chromosome; Cys-rich, cysteine-rich protein domain; ID, variant identifier; Ref, reference allele; and UTR, untranslated region.
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for 2 SNPs with DVT (rs3118667: $P=0.0448$; odds ratio [95% confidence interval], 0.7404 [0.5514–0.9941]; rs4962153: $P=0.0076$; odds ratio [95% confidence interval], 1.683 [1.143–2.477]). Both SNPs do not pass the Bonferroni criterion for adjustment of multiple testing for 8 tests (rs3118667: $P$(corrected)=0.3581; rs4962153: $P$(corrected)=0.06074), but support the cumulative association found by Lotta et al indicating a common genetic component for both diseases. Especially rs4962153 seems to be a key factor for thrombotic and vascular diseases, as it has recently also been associated with cerebral aneurysms in a collective of 353 patients and 1055 healthy adults. However, the entire underlying relationships between high vWF and low ADAMTS13 levels and their relevance to thromboinflammatory and thrombotic events remain enigmatic and require large prospective studies.

We are aware that the present study bears the following limitations: first, we sequenced only 48 discordant sib-pairs (96 individuals) in the discovery stage and may, thus, have missed additional rare, private mutations of potential relevance to the phenotype. Additional mutations in functional domains or cis-regulating elements are likely discovered, as costs for NGS approaches drop and larger studies involving large-scale resequencing become feasible. Second, we lack an independent replication for the association of ADAMTS13 variants, the respective ADAMTS13 levels, and pediatric stroke, which is because of the unavailability of larger collections of comparable study samples. To our knowledge, our collection of father–mother–child trios affected with pediatric stroke is unique in size and phenotyping depth. However, in light of the existing literature on genetic variation in ADAMTS13, its association with ADAMTS13 levels and stroke susceptibility or outcome and its pseudoreplication in an independent DVT cohort, we are confident that our findings are true observations.

From the clinical perspective, understanding the link between variants in ADAMTS13 and circulating high molecular weight vWF levels and arterial thrombotic events is of tremendous importance. With its central role in the modulation of platelet adhesion and aggregation, its thrombus formation and inflammation, its function is crucial for preventing thrombosis in the microvasculature, as indicated by the occurrence of neurological deficits in TTP. Thus, increased levels or activity of ADAMTS13 may protect the brain from ischemia by regulating vWF–platelet interaction after reperfusion. Consequently, current activities are geared toward the introduction of recombinant ADAMTS13 concentrates as a possible treatment choice for familial TTP, acquired TTP, or other clinical conditions linked to ADAMTS13 activity such as sepsis, sickle-cell disease, and, last but not least, stroke. Considering that stroke is primarily a clinical diagnosis, an improved understanding of the interaction of ADAMTS13 and vWF provides valuable information for future treatment options, when genetic markers are included in the decision-making process. In fact, our strongest association with pediatric stroke is observed for undertransmitted, thus protective, ADAMTS13 alleles residing on a protective haplotype (designated H1.1), which is also significantly associated with increased ADAMTS13 activity, providing support for such an inclusion of genetic markers in the clinical setting. This is particularly relevant, when rare variants in functionally relevant sequences such as the ADAMTS13 promoter or interacting protein domains

Figure 3. $P$ values of the most significant haplotypes for each sliding window of size=13. The x-coordinate corresponds to the center of the window tested. Single nucleotide polymorphisms (SNPs) contributing to the most significant haplotype are indicated by arrows.
shift the dynamics of vWF cleavage through ADAMTS13, and thus directly affect hemostasis.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The past decade has provided us with remarkable insights into the genetic architecture of complex diseases, enhancing the translation of research into personalized medicine. Particularly, the use of genome-wide association studies and next-generation sequencing techniques provides us with novel clues on the molecular mechanisms underlying cardiovascular diseases. ADAMTS13, known as von Willebrand factor (vWF) cleaving protease, reduces adhesion and aggregation of platelets. Decreased or missing cleavage function of reduced or absent ADAMTS13 activity may constitute such a molecular disease mechanism via impaired degradation of ultralarge vWF multimers and excessive vWF-induced platelet aggregation on the endothelium. There is mounting evidence that genetic variants in ADAMTS13 gene are correlated with ADAMTS13 activity, pointing toward a mechanism of altered vWF-cleaving properties in the genetic predisposition to cardiovascular disease in general. This study establishes a causal relationship between genetic variants residing in the ADAMTS13 gene, circulating ADAMTS13 levels and the risk for stroke in children. Furthermore, it assigns 4 nonsynonymous single nucleotide polymorphisms to the domains required for vWF binding, which likely exert functional effects and directly influence circulating ADAMTS13 activity. Together with previous studies on cardiovascular disease and stroke in adults, these studies render ADAMTS13 an attractive candidate gene for functional studies, which may contribute to personalized diagnosis and treatment options in future. A study focus on this gene family offers the perspective to likely identify novel biomarkers and drug targets, leading to improved diagnostics and clinical care of patients affected by cardiovascular disease.

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