

# Genome-wide association meta-analysis of functional outcome after ischemic stroke

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## Abstract

### Objective

To discover common genetic variants associated with poststroke outcomes using a genome-wide association (GWA) study.

### Methods

The study comprised 6,165 patients with ischemic stroke from 12 studies in Europe, the United States, and Australia included in the GISCOME (Genetics of Ischaemic Stroke Functional Outcome) network. The primary outcome was modified Rankin Scale score after 60 to 190 days, evaluated as 2 dichotomous variables (0–2 vs 3–6 and 0–1 vs 2–6) and subsequently as an ordinal variable. GWA analyses were performed in each study independently and results were meta-analyzed. Analyses were adjusted for age, sex, stroke severity (baseline NIH Stroke Scale score), and ancestry. The significance level was  $p < 5 \times 10^{-8}$ .

### Results

We identified one genetic variant associated with functional outcome with genome-wide significance (modified Rankin Scale scores 0–2 vs 3–6,  $p = 5.3 \times 10^{-9}$ ). This intronic variant (rs1842681) in the *LOC105372028* gene is a previously reported trans-expression quantitative trait locus for *PPP1R21*, which encodes a regulatory subunit of protein phosphatase 1. This ubiquitous phosphatase is implicated in brain functions such as brain plasticity. Several variants detected in this study demonstrated suggestive association with outcome ( $p < 10^{-5}$ ), some of which are within or near genes with experimental evidence of influence on ischemic stroke volume and/or brain recovery (e.g., *NTN4*, *TEK*, and *PTCH1*).

### Conclusions

In this large GWA study on functional outcome after ischemic stroke, we report one significant variant and several variants with suggestive association to outcome 3 months after stroke onset with plausible mechanistic links to poststroke recovery. Future replication studies and exploration of potential functional mechanisms for identified genetic variants are warranted.

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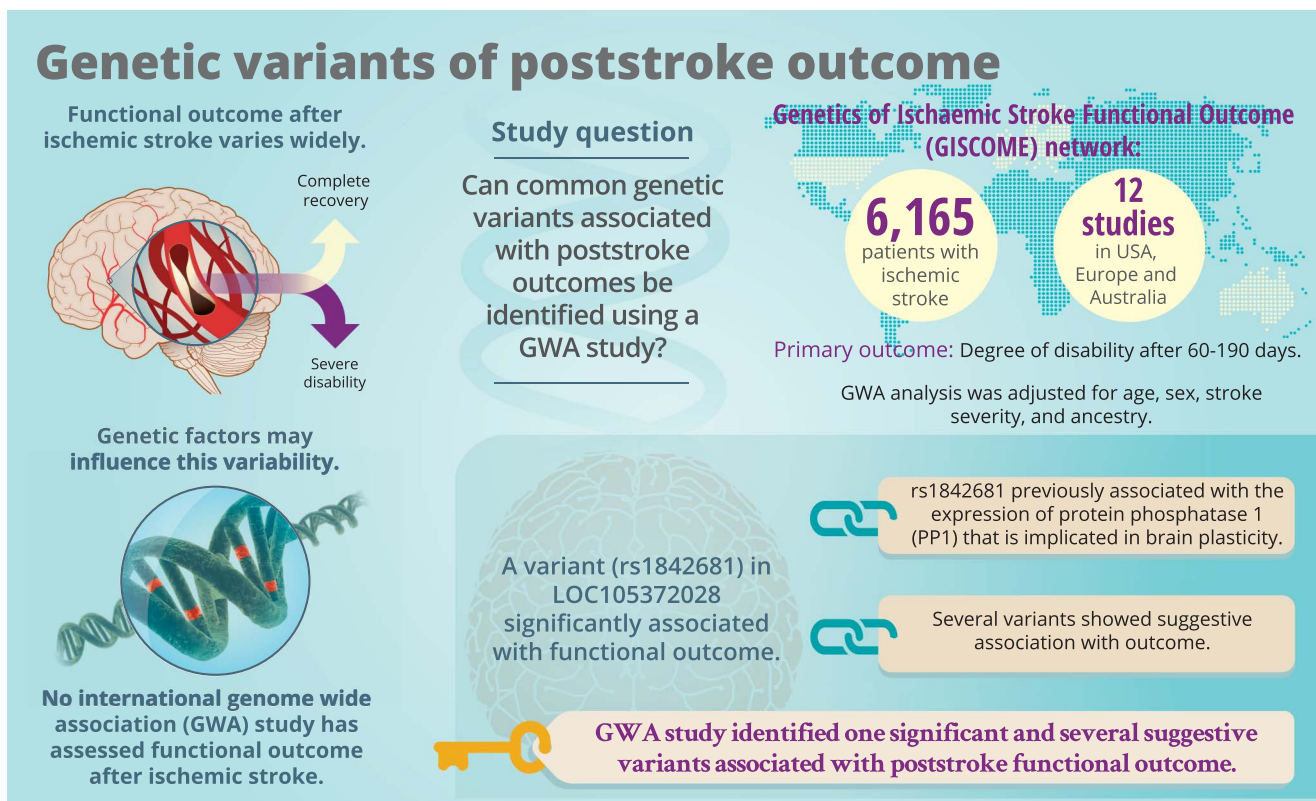
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## Glossary

EC = endothelial cell; eQTL = expression quantitative trait locus; GISCOME = Genetics of Ischaemic Stroke Functional Outcome; GTEx = Genotype-Tissue Expression; GWA = genome-wide association; HGVD = Human Genetic Variation Database; mRS = modified Rankin Scale; NIHSS = NIH Stroke Scale; OR = odds ratio; PP1 = protein phosphatase 1; SNP = single-nucleotide polymorphism.



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Functional outcomes after ischemic stroke have a wide range of interindividual variability, from complete recovery to persistent severe disability.<sup>1</sup> Although a large number of factors influence recovery after ischemic stroke, such as age and initial stroke severity, a discernible portion of this interindividual variation remains unexplained by clinical factors.<sup>1</sup> The unexplored mechanisms behind this variation represent potential keys in the search for a personalized poststroke management approach that would include accurate prognostic prediction as well as patient-tailored treatment, secondary prevention, and rehabilitation.

Genetic factors may account for part of the variability in stroke outcomes. Studies in humans and animals support a genetic influence on recovery after brain injury.<sup>2-4</sup> In addition, genetic variants identified through candidate gene studies have been reported to associate with functional outcome after stroke, e.g., within the brain-derived neurotrophic factor and cyclooxygenase-2 genes,<sup>5-7</sup> although these candidate gene study results have been inconsistent and need replication.<sup>1</sup> To

date, no genome-wide association (GWA) study has been published on functional outcome after overall ischemic stroke. Such a hypothesis-free approach may identify variants indicating novel pathways for poststroke pathophysiologic processes or recovery, and thereby suggest new targets for interventions and drug development.

The Genetics of Ischaemic Stroke Functional Outcome (GISCOME) network aims to identify genetic variants that may influence functional outcome after ischemic stroke.<sup>8</sup> Here, we present the results from a GWA study of functional outcome as assessed by the modified Rankin Scale (mRS) at 3 months after ischemic stroke.

## Methods

### Study population

The GISCOME study population and analysis plan have been previously described in detail.<sup>8</sup> In brief, the GISCOME study

population consists of patients with ischemic stroke of mainly European ancestry aged 18 years and older from 12 study locations in Europe, the United States, and Australia. After the publication of the GISCOME study protocol,<sup>8</sup> 2 additional sets of data from (1) the Sahlgrenska Academy Study on Ischemic Stroke, and (2) the Malmö Diet and Cancer Study have been added (data available from Dryad, supplemental Methods and tables e-1 and e-2, doi.org/10.5061/dryad.s38kf65). The GISCOME study includes centers with mRS and genotype data contributing to the International Stroke Genetics Consortium and the National Institute of Neurological Disorders and Stroke – Stroke Genetics Network (SiGN) study efforts studying genetics of stroke risk. We did not have any additional cohorts available at the time of this study for replication.

### Standard protocol approvals, registrations, and patient consents

All participants provided written informed consent to participate. For participants who were unable to communicate, consent was obtained from their next of kin. Local ethics committees approved the individual studies.

### Outcome

The mRS as close as possible to 90 days (60–190 days permitted) was selected to assess functional outcome, as described in the analysis plan.<sup>8</sup> The majority of the included studies ( $\approx 80\%$ ) assessed mRS at 3 months  $\pm$  2 weeks.<sup>8</sup> In most studies, this was done by face-to-face interviews. In 3 studies (Lund Stroke Register, Malmö Diet and Cancer Study, and parts of the Sahlgrenska Academy Study on Ischemic Stroke), data from the Swedish quality register for stroke (Riksstroke) were used to assess mRS by a validated translation algorithm.<sup>8,9</sup> This approach prevented a differentiation between the mRS scores 0, 1, and 2.

Based on our a priori analysis plan, we analyzed mRS as 2 dichotomous variables, (1) mRS 0–2 vs 3–6 and (2) mRS 0–1 vs 2–6, and also as the full ordinal scale variable. The mRS 0–2 vs 3–6 and ordinal scale analyses included a larger number of participants. Analyses of mRS 0–1 vs 2–6 were performed to explore whether a second dichotomization could identify any strong associations that were not picked up by the other analyses. In addition, we made an explorative effort to investigate potential associations with infarcts in subcortical and cortical locations separately. Since information about infarct location was not available, we divided the cases into small vessel disease (referred to as lacunar stroke) and other subtypes (referred to as nonlacunar stroke), according to subtype classifications in TOAST (Trial of ORG 10172 in Acute Stroke Treatment).<sup>10</sup> A total of 992 patients had lacunar stroke, 3,991 patients had nonlacunar stroke, and for 1,182 individuals, this information was missing.

### GWA analysis and meta-analysis

The methods for genotyping, imputation, and quality control of genotype data are described in data available from Dryad (supplemental Methods and tables e-1 and e-2, doi.org/10.

5061/dryad.s38kf65). Multivariable models were used for analyses of each outcome variable, under an additive genetic model. We aimed to explore genetic variants associated with functional outcome that were independent of stroke severity. In this primary model, results were adjusted for age, sex, ancestry (up to the first 5 principal components [data available from Dryad, table e-3]), and baseline stroke severity as assessed by the NIH Stroke Scale (NIHSS) at 0 to 10 days post stroke onset, with preference to as close to day 0–1 as possible.<sup>8</sup> In addition, models without adjustment for baseline stroke severity were performed for each outcome for comparison; however, unless otherwise stated, all results in this report refer to the primary model as described above.

All dichotomized analyses were performed with logistic regression using PLINK software version 1.90b4.6.<sup>11</sup> The full mRS was analyzed with ordinal logistic regression under a cumulative logit model using the MATLAB mnrfit algorithm (MATLAB Statistics and Machine Learning Toolbox Release 2016b; The MathWorks, Inc., Natick, MA). Under the proportional odds assumption, the effect of a predictor single-nucleotide polymorphism (SNP) is invariant of the choice of outcome categories. As a result, estimates from all study cohorts, including those that did not separate mRS 0–2, are comparable in ordinal regression. Furthermore, one unit change in the dosage of a variant with regression coefficient  $\beta$  suggests a change in the odds of an mRS score lower than or equal to  $x$  vs higher than  $x$  by a factor  $OR = e^{-\beta}$ , equally for all scores  $x$ . We tested deviations from proportional odds for all reported variants from the ordinal models. No significant deviations were found.

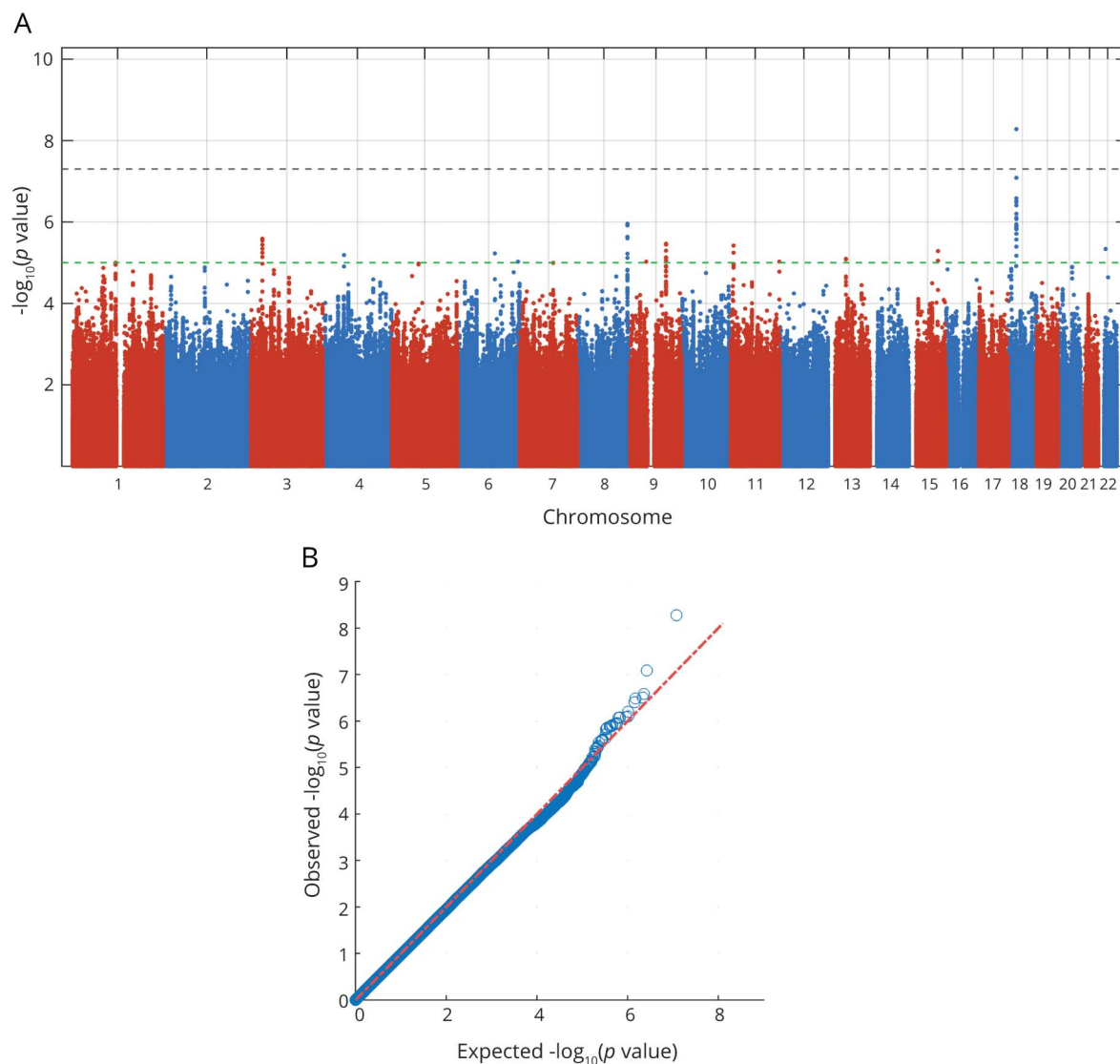
Inverse variance-weighted fixed-effects meta-analysis was performed using METAL software.<sup>12</sup> Variants with minor allele frequency  $< 0.01$  were excluded. Variants that were missing in  $> 50\%$  of cohorts were also excluded. After filtering, approximately 8.5 million SNPs were included in each of the final meta-analyses. Quantile-quantile plots are shown in figures 1 and 2 and in data available from Dryad (figures e-1 to e-4, doi.org/10.5061/dryad.s38kf65), and genomic inflation factors ( $\lambda$ ) are displayed in data available from Dryad (table e-4). There was no sign of population stratification based on these measures. Heterogeneity of SNP effects between studies was tested in the meta-analysis.

Markers with  $p$  values  $< 5 \times 10^{-8}$  were considered significant for association with outcome, while markers with  $p$  values  $< 1 \times 10^{-5}$  were considered suggestive. To facilitate comparison of the results from the dichotomized and ordinal analyses, we present all effect sizes as odds ratios (ORs) per copy of the minor allele; an OR  $> 1$  indicates a higher mRS score (worse outcome) per copy of the minor allele and an OR  $< 1$  indicates a lower mRS score.

### Investigation of expression quantitative trait loci

We explored associations of the markers with  $p$  values  $< 1 \times 10^{-5}$  and proxy SNPs ( $r^2 > 0.8$  in 1000 Genomes Phase 1

**Figure 1** Manhattan and quantile-quantile plots of analysis for associations with dichotomized mRS at 3 months



Outcome was measured as mRS 0–2 vs 3–6 at 3 months after ischemic stroke onset. Dotted lines show genome-wide significance (black,  $p < 5 \times 10^{-8}$ ) and suggestive association level (green,  $p < 10^{-5}$ ). Results are adjusted for age, sex, principal components, and baseline NIH Stroke Scale score. mRS = modified Rankin Scale.

European population) with expression quantitative trait loci (eQTLs) in publicly available datasets encompassing numerous tissues: Genotype-Tissue Expression (GTEx) V6,<sup>13</sup> GRASP2,<sup>14,15</sup> Human Genetic Variation Database (HGVD),<sup>16</sup> and BIOS.<sup>17</sup> For eQTLs,  $p < 10^{-4}$  was considered significant.

### Gene-based analysis

Gene-based tests were performed for each meta-analysis using VEGAS2 with linkage disequilibrium structure based on the European population.<sup>18</sup> All SNPs within  $\pm 10$  kbp from the untranslated regions 3' and 5' of each gene were included, to account for potential regulatory variants.<sup>18</sup> The number of genes included was approximately 23,000, which corresponds to a Bonferroni-corrected significance threshold of  $p < 2.2 \times 10^{-6}$ .

### Data availability

The datasets generated and analyzed during the current study are available on reasonable request.

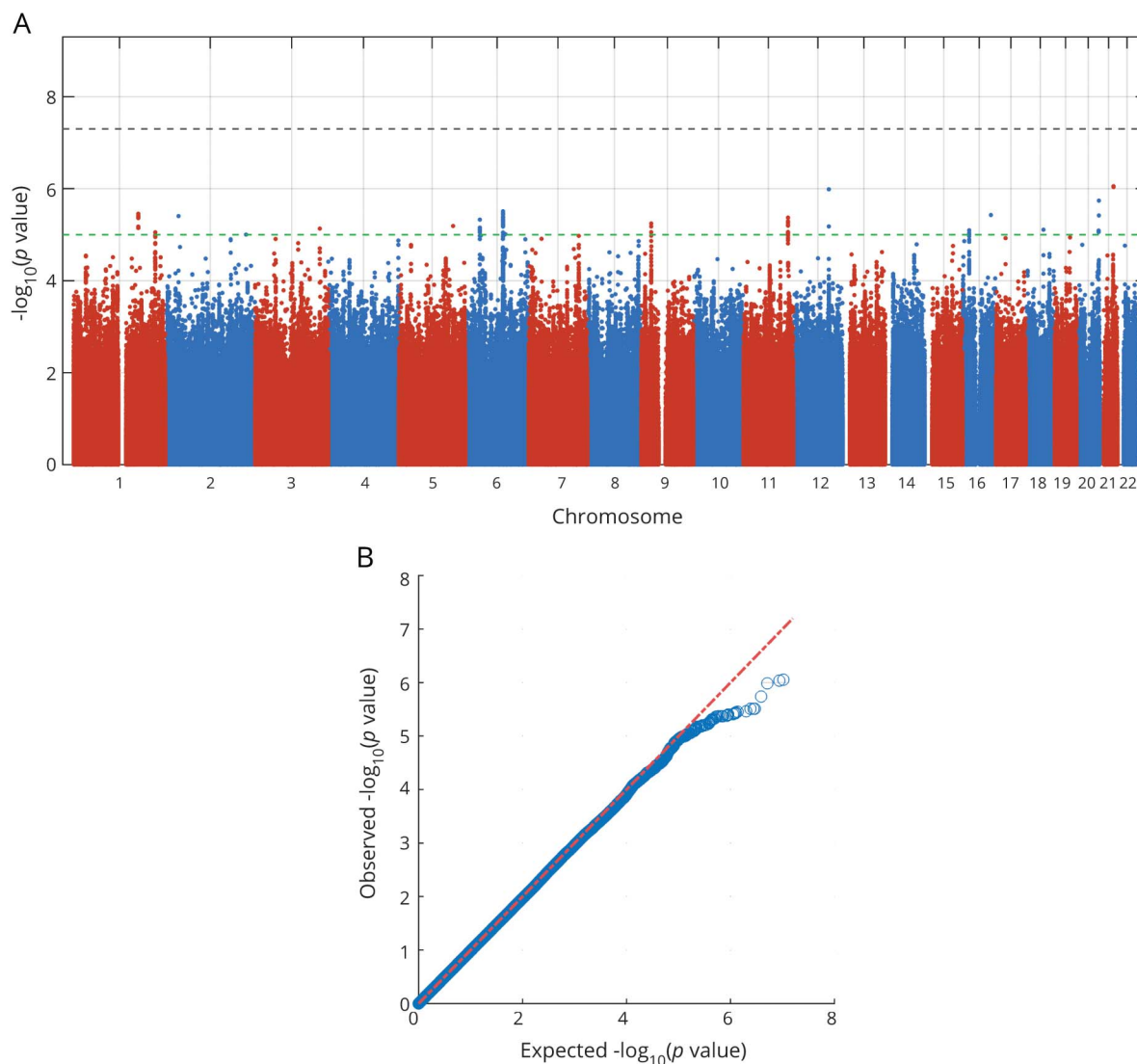
### Results

Characteristics such as age, sex, and stroke severity, as well as the numbers of included cases for each mRS score and in each outcome analysis, are displayed in table 1. The analyses of mRS 0–2 vs 3–6 and the ordinal analyses included 6,021 stroke cases, whereas analyses of mRS 0–1 vs 2–6 included 4,363 cases.

One common variant on chromosome 18q11.2 (rs1842681, minor allele frequency: 0.23) was associated at genome-wide



**Figure 2** Manhattan and quantile-quantile plots of analysis for associations with ordinal mRS at 3 months



Outcome was measured as ordinal mRS at 3 months after ischemic stroke onset. Dotted lines show genome-wide significance (black,  $p < 5 \times 10^{-8}$ ) and suggestive association level (green,  $p < 10^{-5}$ ). Results are adjusted for age, sex, principal components, and baseline NIH Stroke Scale score. mRS = modified Rankin Scale.

significance with outcome defined as mRS 0–2 vs 3–6 (OR for minor allele [A]: 1.40,  $p = 5.3 \times 10^{-9}$ ) (table 2, figures 1, 3, and 4). The effect was similar with and without adjustment for stroke severity (table 2), and the association was observed in the same direction, but with a somewhat lower effect size, for ordinal mRS (OR: 1.17,  $p = 1.5 \times 10^{-4}$ ) and mRS 0–1 vs 2–6 (OR: 1.12,  $p = 7.4 \times 10^{-2}$ ). In line with this, the distribution of the minor allele count for rs1842681 over mRS categories shows a threshold between mRS 2 and 3 (data available from Dryad, figure e-5, doi.org/10.5061/dryad.s38kf65). The variant is located in an intron of the gene *LOC105372028* (long noncoding RNA synonymous with *RP11-449D8.5* [Genome Reference Consortium Human Build 38/hg38]) and is in a putative binding site of several regulatory proteins (HaploReg, March 21, 2018). This variant has no eQTL reported in GTEx (June 1, 2018). However, in HGVD, it has a trans-eQTL for *KLRAQ1* (also

known as *PPP1R21*,  $p_{HGVD} = 1.67 \times 10^{-7}$ ). *PPP1R21* is expressed in the brain (GTEx, June 1, 2018).

No other SNP was significantly associated with mRS 0–2 vs 3–6, or with ordinal scale mRS (figures 1 and 2). The results for the lead SNPs of the top 10 independent loci for each outcome are displayed in data available from Dryad (tables e-5 and e-6, doi.org/10.5061/dryad.s38kf65). The results from analyses without severity adjustment are displayed in data available from Dryad (tables e-7 and e-8 and figures e-1, e-2, e-6, and e-7). There were no signs of heterogeneity for the effects of the reported SNPs. All  $I^2$  values were  $< 0.35$  and all  $p$  for heterogeneity  $> 0.08$ , except for one SNP (rs58448576) in the analysis of mRS 0–1 vs 2–6, which had an  $I^2$  of 0.48 and a  $p$  value of 0.03 (without adjustment for multiple testing). In the gene-based analyses, no gene reached the predefined threshold for significance (data available from Dryad, table e-9).

**Table 1** Characteristics of the study population and numbers of included patients for each mRS score and for each outcome (mRS 0–2 vs 3–6, 0–1 vs 2–6, and the full ordinal scale)

<b>Total no.</b>	6,165
<b>Sex, M/F, n</b>	3,497/2,668
<b>Age, y, median (IQR)</b>	70 (60–80)
<b>NIHSS (0–10 d), median (IQR)</b>	4 (2–8)
<b>mRS score at 3 mo, n</b>	
<b>0–2<sup>a</sup></b>	972
<b>0</b>	706
<b>1</b>	1,126
<b>2</b>	1,026
<b>3</b>	953
<b>4</b>	659
<b>5</b>	236
<b>6</b>	487
<b>Outcomes, n</b>	
<b>mRS 0–2 vs 3–6</b>	3,741 vs 2,280 <sup>b</sup>
<b>Ordinal mRS</b>	6,021 <sup>b</sup>
<b>mRS 0–1 vs 2–6</b>	1,796 vs 2,567 <sup>b</sup>

Abbreviations: IQR = interquartile range; mRS = modified Rankin Scale; NIHSS = NIH Stroke Scale.  
<sup>a</sup> Cases for which data from the Swedish quality register for stroke (Riksstroke) were used to assess mRS. This approach prevented differentiation among mRS 0, 1, and 2.  
<sup>b</sup> Numbers refer to the primary, fully adjusted model, including age, sex, principal components, and baseline NIHSS score. Models without adjustment for baseline NIHSS score included 3,830 vs 2,335 (mRS 0–2 vs 3–6), 6,165 (ordinal), and 1,832 vs 2,620 (mRS 0–1 vs 2–6) participants.

The analysis of mRS 0–1 vs 2–6 yielded no significant associations. These results are presented in data available from Dryad (tables e-12 and e-13 and figures e-3, e-4, and e-8, doi.org/10.5061/dryad.s38kf65).

### Suggestive associations with outcome

Thirty-three SNPs in 12 different loci (with at least 1 Mbp distance) were suggestively ( $p < 10^{-5}$ ) associated with mRS 0–2 vs 3–6 (excluding the SNPs in the significant locus on chromosome 18q11.2), and 75 SNPs in 17 different loci with ordinal mRS (figures 1 and 2). Of these 29 independent loci, the top SNPs of 16 loci have either significant eQTLs for and/or are located within or near (<100 kbp) genes that are expressed in the brain (GTEx, June 1, 2018). Five genes that were not reported to be expressed in brain tissue were expressed in arteries or lymphocytes (GTEx, June 1, 2018).

Among the suggestive associations, 3 are linked to genes with experimental evidence of influence on outcome from animal models of stroke and are discussed below.<sup>19–21</sup> First, rs2236406, an intron variant in the *PTCH1* gene, was identified in the mRS 0–2 vs 3–6 analysis (table 2, figures 3 and 4). The gene-based analysis also showed a suggestive association for *PTCH1* with mRS 0–2 vs 3–6 ( $p = 6.8 \times 10^{-5}$ ). Rs2236406 is an eQTL for long noncoding RNA RP11-43505.5 ( $p_{GTEx} = 5.7 \times 10^{-7}$ ), which overlaps the *PTCH1* gene. Second, in the ordinal analysis, a suggestive association with mRS was found for rs13299556 (table 2, figures 3 and 4). Associations at low  $p$  values were also found for this variant with dichotomous mRS, although not reaching the level for suggestive association (table 2). rs13299556 is an intron variant in the *PLAA* gene. There was no strong association for *PLAA* in the gene-based analysis (ordinal mRS;  $p = 0.072$ ). However, this variant, and variants in high linkage disequilibrium with it (figure 4), are reported as eQTLs for the nearby genes *TEK* and *LRRC19* ( $p_{GTEx} = 1.6 \times 10^{-6}$  for both). The  $p$  values from the gene-based analysis for

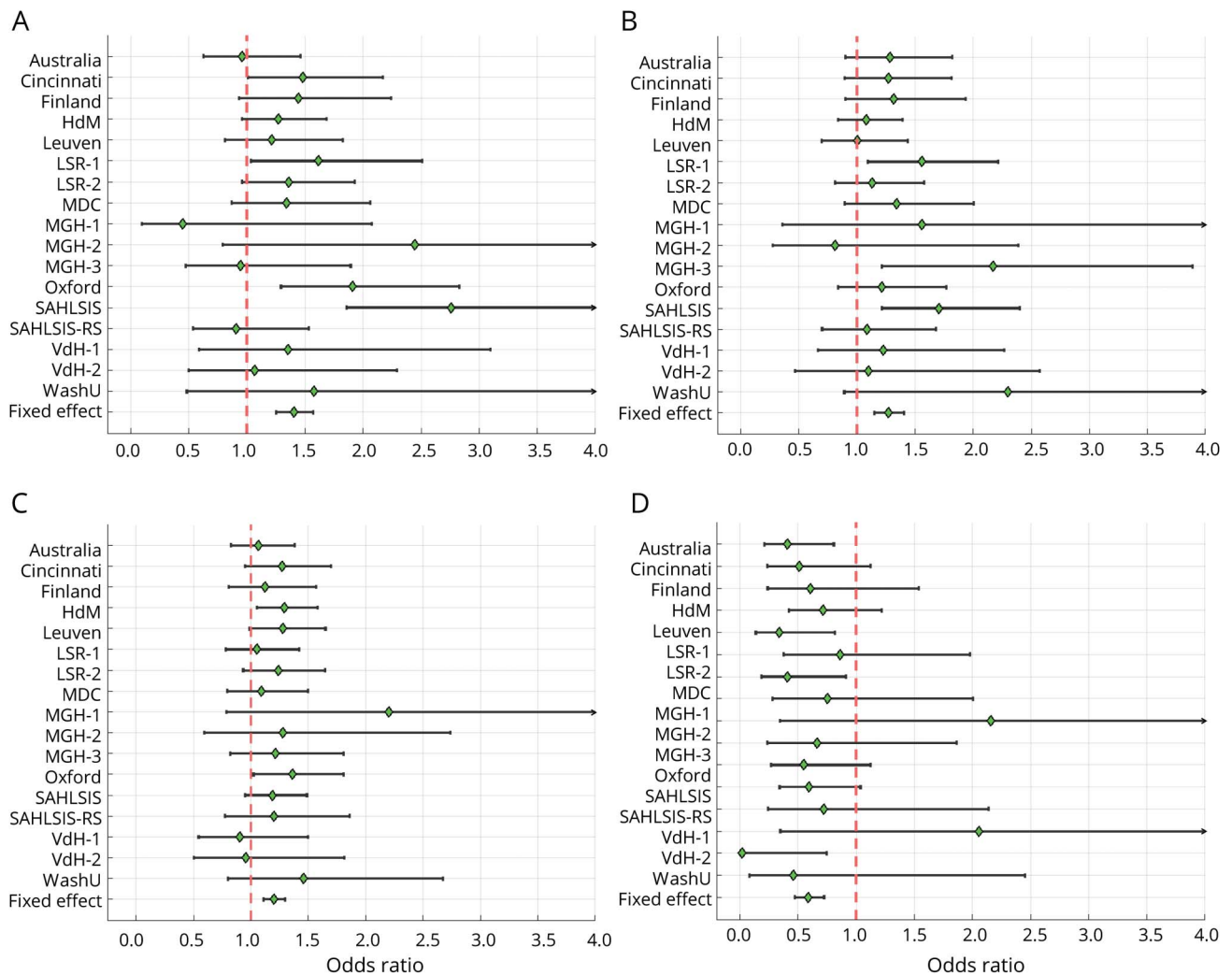
**Table 2** Comparison between analyses of mRS 0–2 vs 3–6 and ordinal scale mRS of selected genetic variants with either a significant association with outcome or a suggestive association and a link to a gene with experimental support for influence on poststroke outcome

Marker	MA	MAF	mRS 0–2 vs 3–6				Ordinal mRS			
			Primary		Not adjusted for NIHSS		Primary		Not adjusted for NIHSS	
			OR	$p$ Value	OR	$p$ Value	OR	$p$ Value	OR	$p$ Value
rs1842681	A	0.23	1.40	$5.27 \times 10^{-9,a}$	1.30	$1.46 \times 10^{-7}$	1.17	$1.53 \times 10^{-4}$	1.19	$2.98 \times 10^{-5}$
rs2236406	C	0.36	1.27	$3.43 \times 10^{-6,a}$	1.21	$1.98 \times 10^{-5}$	1.13	$9.11 \times 10^{-4}$	1.11	$3.96 \times 10^{-3}$
rs13299556	C	0.27	1.25	$6.25 \times 10^{-5}$	1.22	$2.93 \times 10^{-5}$	1.20	$5.69 \times 10^{-6}$	1.21	$7.59 \times 10^{-7,a}$
rs78734480	C	0.03	0.57	$4.79 \times 10^{-4}$	0.66	$2.34 \times 10^{-3}$	0.58	$1.03 \times 10^{-6,a}$	0.62	$5.00 \times 10^{-6}$

Abbreviations: MA = minor allele; MAF = minor allele frequency; mRS = modified Rankin Scale; NIHSS = NIH Stroke Scale; OR = odds ratio. ORs with effects above unity indicate a higher mRS (worse outcome) per copy of the minor allele. Primary model includes age, sex, principal components, and baseline NIHSS score.

<sup>a</sup> Indicates the outcome and model with the lowest  $p$  value for each marker.

**Figure 3** Forest plots for functional outcome at 3 months by study cohort



The plots show ORs and 95% confidence intervals for minor allele of (A) rs1842681 (mRS 0–2 vs 3–6), (B) rs2236406 (mRS 0–2 vs 3–6), (C) rs13299556 (ordinal mRS), and (D) rs78734480 (ordinal mRS). ORs with effects above unity indicate a higher mRS score (worse outcome) per copy of the minor allele. Results are adjusted for age, sex, principal components, and baseline NIH Stroke Scale score. For cohort abbreviations, see data available from Dryad (table e-1, doi.org/10.5061/dryad.s38kf65). mRS = modified Rankin Scale; OR = odds ratio.

those genes were 0.0019 and 0.084, respectively. The variant is also predicted to alter a putative regulatory motif sequence (HaploReg, March 21, 2018). Third, a suggestive association with ordinal mRS was found for an intron variant in *NTN4* (rs78734480; table 2, figures 3 and 4). This variant was also associated with dichotomous mRS at a low *p* value, although not below the cutoff for suggestive association (table 2).

### Analysis of lacunar and nonlacunar stroke

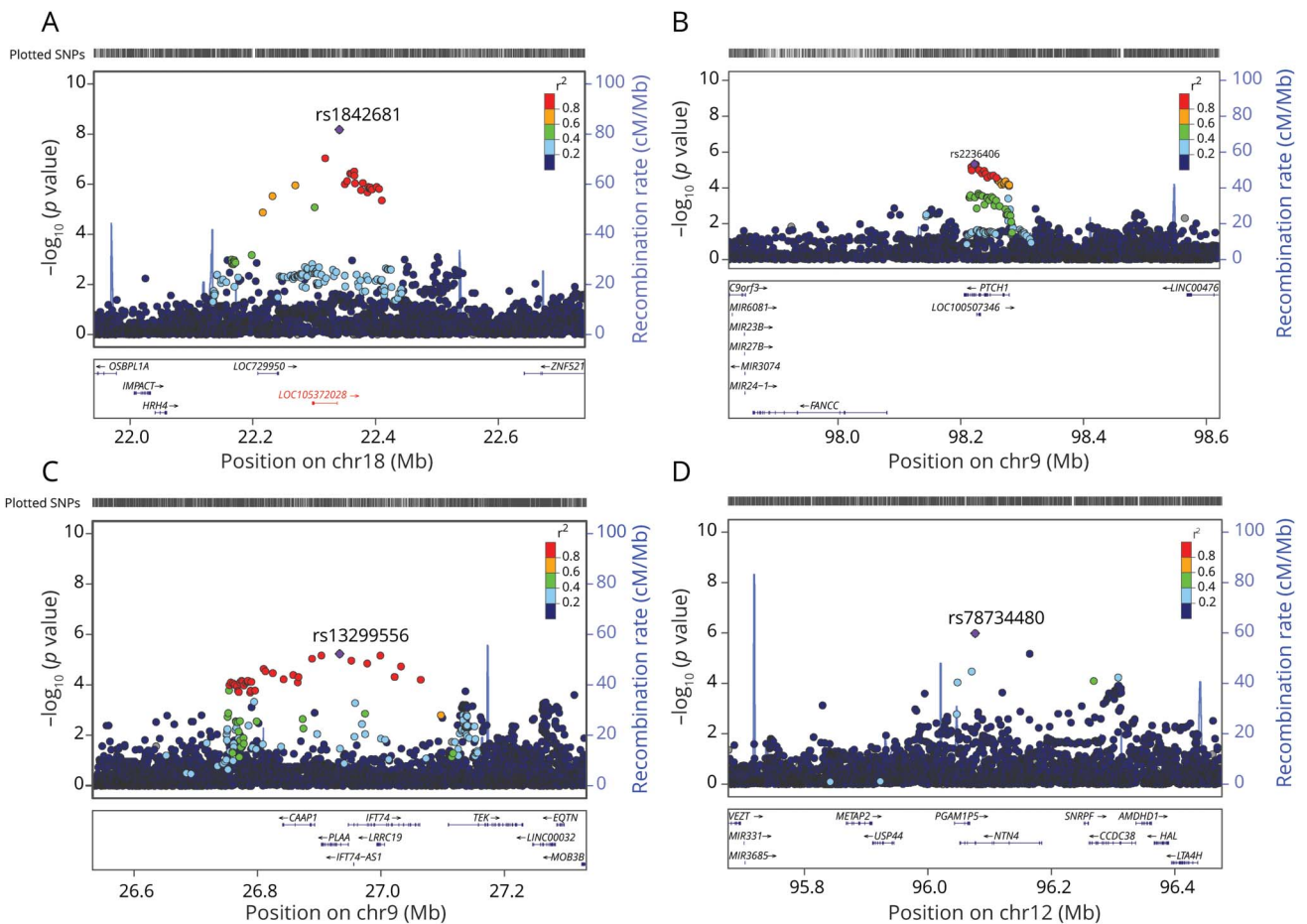
To evaluate whether any strong associations could be identified specifically in patients with lacunar stroke (small vessel disease strokes, *n* = 992), or in nonlacunar stroke (other etiologic subtypes including cortical infarcts, *n* = 3,991), we analyzed those groups separately. No genome-wide significant association was detected (all *p* values >6 × 10<sup>-7</sup>). The top 5 findings differed between these subgroups and are shown in data available from Dryad (tables e-10 and e-11, doi.org/10.5061/dryad.s38kf65).

## Discussion

This GWA study on functional outcome after ischemic stroke, including more than 6,000 patients, identified one significant locus and several suggestive variants related to genes with a potential mechanism for influencing stroke recovery and outcomes. Replication of these findings in independent datasets as they become available is the essential next step. The effect sizes of these genetic variants were generally small, and further studies should include even larger samples to identify additional variants associated with stroke outcome and to enable subgroup analyses.

The genome-wide significant SNP (rs1842681) for mRS 0–2 vs 3–6 is an intronic variant in the gene *LOC105372028* (long noncoding RNA), the function of which remains to be established. However, expression analyses show that the gene

**Figure 4** Regional association plots for outcome at 3 months after ischemic stroke onset



(A) Significant locus (rs1842681) showing association with mRS 0–2 vs 3–6, (B) rs2236406 (intron variant in the *PTCH1* gene) showing suggestive association with ordinal mRS, and (C) rs13299556 (intron variant in the *PLAA* gene with eQTL for *TEK*,  $p_{GTEx} = 1.6 \times 10^{-6}$ ) showing suggestive association with ordinal mRS. Results are adjusted for age, sex, principal components, and baseline NIH Stroke Scale score. *LOC105372028* (indicated in red, panel A) has been overlaid from the Genome Reference Consortium Human Build (GRCh) 38/hg38, as it was missing from GRCh37/hg19. The rs1842681 variant is intronic of the *LOC105372028* gene (chromosome 18: 24725781–24766645). Position for rs1842681 in GRCh38/hg38, 18:24761199. eQTL = expression quantitative trait locus; GTEx = Genotype-Tissue Expression; mRS = modified Rankin Scale; SNP = single-nucleotide polymorphism.

expression of *LOC105372028* is highest in brain tissue (National Center for Biotechnology Information, ncbi.nlm.nih.gov/gene/?term=LOC105372028) and rs1842681 is located in a putative regulatory element binding site (HaploReg, March 21, 2018). Furthermore, rs1842681 is a trans-eQTL for *KLRAQ1*, also known as *PPP1R21*, which encodes a regulatory subunit of protein phosphatase 1 (PP1). PP1 is a ubiquitous phosphatase implicated in many brain functions including learning and memory formation.<sup>22,23</sup> PP1 is also a key regulator of  $Ca^{2+}$ /calmodulin (CaM)-dependent protein kinase II (CaMKII) signaling, which is crucial for  $Ca^{2+}$ -mediated neuronal plasticity in the brain.<sup>24</sup> Thus, although speculative, rs1842681 may modulate expression of *PPP1R21*, which in turn could affect brain plasticity and thereby outcome post stroke, a hypothesis that requires validation through functional experiments. The association for rs1842681 with outcome was observed after adjusting for baseline NIHSS score, suggesting a mechanism that is independent of initial stroke severity. However, additional data from further studies are clearly

needed to corroborate this finding and clarify the potential role of this locus for functional outcome after stroke.

Twenty-nine independent loci were associated with ischemic stroke outcome at the predefined suggestive association level. Three of these loci are linked to genes with experimental evidence of influence on outcome from animal models of stroke.<sup>19–21</sup> First, the intronic variant in *PLAA* (rs13299556) was both one of the top findings in the ordinal analysis and associated with the dichotomized outcome at a low  $p$  value. It is located in a putative transcription factor binding site and is reported to be an eQTL for the expression of both *LRRRC19* and *TEK*. *LRRRC19* encodes a protein with a potential role in regulating neurite outgrowth,<sup>25</sup> and might thus influence stroke recovery. *TEK* encodes a tyrosine kinase predominantly expressed in endothelial cells (ECs). Focal upregulation of *TEK* has been demonstrated in capillaries at the border of infarcted myocardium, and expression is induced by hypoxia and proinflammatory cytokines in human



ECs in vitro.<sup>26</sup> *TEK* is activated by angiopoietin-1 and this promotes migration, sprouting, and survival of ECs.<sup>27,28</sup> Of note, results from a rodent ischemic stroke model suggest that upregulation of angiopoietin-1 and *TEK* improves stroke outcome,<sup>19</sup> and one clinical study reported an association between high plasma levels of angiopoietin-1 and poor outcome as assessed by mRS at 3 months after ischemic stroke.<sup>29</sup> Angiopoietin-2 is the antagonist of angiopoietin-1.<sup>30</sup> Angiopoietin-2 gain-of-function mice have enhanced blood-brain barrier permeability and increased brain infarct sizes upon permanent middle cerebral occlusion compared to wild-type mice, and both phenotypes were rescued by activation of *TEK* signaling.<sup>31</sup> The same study reported increased circulating serum concentrations of angiopoietin-2 in patients with acute ischemic stroke compared to controls.<sup>31</sup> Finally, in an ischemic stroke model, mice with type 2 diabetes mellitus showed increased angiopoietin-2 but decreased angiopoietin-1/*TEK* protein expression compared to wild-type mice, suggesting that the *TEK* signaling may be involved in diabetes-induced vascular damage post stroke.<sup>32</sup>

Second, an intronic variant in the *PTCH1* gene showed suggestive association with mRS 0–2 vs 3–6. *PTCH1* is involved in sonic hedgehog signaling, a pathway that for instance reduces oxidative stress on neurons and regulates ischemia-induced neuronal progenitor proliferation.<sup>33,34</sup> In rats with experimentally induced middle cerebral artery occlusion, inhibition of the sonic hedgehog signaling caused increased infarct size, and *PTCH1* was downregulated when sonic hedgehog signaling was inhibited.<sup>20,35</sup> The association between *PTCH1* and stroke outcome observed in the present study may thus potentially be explained by an increased infarct size. However, the effect of this variant was somewhat stronger when adjusting for baseline NIHSS score, which may suggest a potential influence also on recovery processes.

Third, a variant in *NTN4* showed suggestive association with ordinal mRS and was also associated with dichotomous mRS at a low *p* value. *NTN4* encodes a member of the netrin family of proteins expressed in brain tissue (ncbi.nlm.nih.gov/gene/59277) and with functions in processes with biologically plausible roles in stroke recovery such as angiogenesis, neurite growth, and migration.<sup>36,37</sup> In a mouse model, *NTN4* was upregulated in blood vessels and astrocytes in the ischemic core after stroke, and intracerebroventricular administration of *NTN4* led to improvements both in angiogenesis and in poststroke outcome, possibly through increased collateral blood flow leading to improved survival of neurons partially affected by ischemia.<sup>21</sup> In another study, *NTN4* contributed to thalamocortical axon branching in rodents and the expression was altered by neuronal activity in the cortex, implying that *NTN4* might act as a positive regulator for thalamocortical axon branching through activity-dependent expression.<sup>38</sup> Although highly speculative, this gene could thus have a role in the plastic process of axonal outgrowth and restoration of synaptic architecture post stroke.

Several additional variants with suggestive association to outcome in this study are linked to genes that may potentially be mechanistically involved in processes affecting stroke outcomes. For instance, *RUNX1* encodes a runt-related proangiogenic transcription factor with high expression in arteries (GTEx, June 1, 2018) that is upregulated after stroke.<sup>39</sup> In mice, expression of *RUNX1* is induced in putative neural stem or progenitor cells after brain injury and suggested to promote neuronal differentiation.<sup>40</sup> *TNR* encodes a protein that is involved in neuronal plasticity and is highly and exclusively expressed in the brain.<sup>41,42</sup> Examples of genes with a possible stroke subtype-specific significance among suggestive findings are *MTHFS*, which is involved in folate and homocysteine metabolism, pathways with potential influence on cerebral small vessel disease,<sup>43</sup> and *SCML4*, in which the variant rs74514008 was suggestively associated with ordinal mRS in the present study. *SCML4* has shown association with coronary artery disease in a recent GWA study, and subsequent functional characterization suggested a role in atherosclerosis.<sup>44</sup>

To further explore the SNPs associated with outcomes in different subgroups, such as certain stroke subtypes, patients with diabetes, or those receiving recanalization therapies would be of great interest. Mechanisms of neuron injury and recovery probably have similarities regardless of the etiology, but mechanism of recovery after cortical and subcortical stroke may be different. Therefore, we analyzed mRS 0–2 vs 3–6 in patients with lacunar and nonlacunar stroke separately. The top 5 independent loci differed between the 2 groups, but no genome-wide significant association was detected. Given the small sample sizes, especially for lacunar stroke, these analyses are clearly exploratory. Thus, in further studies with a higher number of patients, an important aim will be to further explore the genetic associations in specific subgroups.

The strengths of the present study include a relatively large sample size, well defined endpoints, extensive genetic data, and that analyses were adjusted for NIHSS score to assess the influence on outcome independent of baseline stroke severity. We performed dichotomous outcome analyses to identify genetic variants associated with being dependent or independent in activities of daily living, which reflects a clinically important difference in functional outcome. We also performed ordinal analysis, which aims to identify variants that have a similar effect across different degrees of functional outcome. To detect such variants, the ordinal model provides greater power.<sup>8</sup> Another strength of the ordinal approach is the ability to compensate for any differences in mRS assessment between studies.

There are also several limitations that should be considered. Although this study included more than 6,000 patients with ischemic stroke, only one significant locus was identified, and thus, the findings do not explain the assumed genetic variation for ischemic stroke outcomes. This implies that the effect sizes of individual SNPs on outcome of ischemic stroke are small and that our sample size might be insufficient even to detect

common genetic variants. Another important limitation is the explorative nature of the results, which clearly require future replication in independent cohorts as they become available. The use of the mRS as the primary outcome metrics is a clear limitation in that it is a crude measurement of outcome, especially when dichotomized. However, the mRS represents a well-established, formalized, and easily available measure of poststroke functional outcome, and the dichotomizations represent differences of great importance clinically and for the patient. The mRS scores were assessed at different time points ranging from 60 to 190 days, potentially diluting any detectable associations, as functional outcome status may vary over time. However, the majority of included studies assessed mRS at 3 months  $\pm$  2 weeks after stroke onset. We did not have information on premorbid mRS and this would have provided a valuable means to calculate the change in functional ability. Moreover, stroke severity was scored at different time points. However, in a majority of cases, NIHSS was scored early and in only 160 individuals later than day 3 after admission. As previously described,<sup>8</sup> we lack data on some important factors that influence outcomes such as acute therapies and rehabilitation, which is why these factors could not be accounted for in the analyses. However, data from the cohorts with available data on treatment with tissue plasminogen activator (about half of the cohorts) show that this treatment was given to 14% of the patients. We can also not exclude the possibility of selection bias since participation in the study required individual informed consent, and data entry also requested availability of functional status follow-up data. In line with this, our study sample mainly reflects milder strokes (median NIHSS score of 4, table 1), which may hamper the detection of factors influencing a greater dynamic range of recovery. Lastly, because our study population was of European ancestry, the results are not necessarily generalizable to other stroke populations. Further studies should ideally include a series of prespecified collection time points, specific clinical variables of importance for stroke outcome, and a battery of outcome metrics that assess several domains of recovery including motor impairment, aphasia, neglect, and cognitive impairment.

In this first large international GWA study on functional outcome after overall ischemic stroke, we report one significant variant and several variants with suggestive association to outcome at 3 months after stroke onset with plausible links to poststroke recovery. Future studies on common variants should include larger sample sizes, enabling subgroup analyses, as well as replication of the present results and elaboration of potential mechanisms.

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## Disclosure

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Continued



## Appendix (continued)

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