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Anne-Sophie Pulcrano-Nicolas, Alice Jacquens, Carole Proust, Frédéric Clarençon, Claire Perret, Eimad Shotar, Louis Puybasset, Wilfried Le Goff, Vincent Degos, David-Alexandre Trégouët, et al.

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Anne-Sophie Pulcrano-Nicolas, PhD[1,2]; Alice Jacquens, MD, MSc[3,4]; Carole Proust[5]; Frédéric Clarençon, MD, PhD[6,7]; Claire Perret, MSc[1,2]; Eimad Shotar, MD[7]; Louis Puybasset MD, PhD[3,4]; Wilfried Le Goff, PhD[1,2]; Vincent Degos MD, PhD[3,4*]; David-Alexandre Trégouët, PhD[5*]; Sophie Garnier, PhD[1,2]

[1] Sorbonne Université, UPMC, INSERM UMR-S 1166, F-75013, Paris, France

[2] ICAN Institute for Cardiometabolism and Nutrition, Paris, France

[3] Department of Anesthesia and Intensive Care, Pitié-Salpetrière Hospital, Assistance Publique-Hopitaux de Paris, Paris, France

[4] Université Paris 7, INSERM UMR 1141, Paris France

[5] INSERM UMR_S 1219, Bordeaux Population Health Research Center, University of Bordeaux, Bordeaux, France

[6] Sorbonne Université, UPMC, Groupe de Recherche Clinique Biosfast, Paris, France

[7] Department of Neuroradiology, Pitié-Salpêtrière Hospital, Paris France

* These authors equally contributed to the work

Corresponding Author:

David-Alexandre Trégouët, PhD

INSERM UMR_S 1219, Bordeaux Population Health Research Center,

146 rue Léo Saignat, 33076 Bordeaux, France.

Tel: 05 47 30 42 54

Email: david-alexandre.tregouet@inserm.fr

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Abstract

Objectives: To identify mRNA biomarkers of cerebral vasospasm in whole blood of patients suffering from subarachnoid hemorrhage (aSAH).

Methods: A prospective transcriptomic study for vasospasm was conducted in whole blood samples of 44 aSAH patients developing (VSP^+, n = 22) or not (VSP^-, n = 22) vasospasm. All patients' samples were profiled for 21,460 mRNA probes using the Illumina Human HT12v4.0 array. Differential statistical analysis was performed using linear mixed model.

Results: This study revealed that patients who developed vasospasm after aSAH presented with a significant ($p = 8.03 \times 10^{-6}$) increase of *Sphingosine-1-phosphate receptor 4* (*S1PR4)* mRNA level compared to patients who did not.

Conclusions: This result, consistent with previous experimental investigations conducted in animal models supporting the role of *S1PR4* and its ligand, S1P, in arterial-associated vasoconstriction, suggests that *S1PR4* could be used as a biomarker for cerebral vasospasm in aSAH patients.

Introduction

Cerebral vasospasm is one of the main severe delayed complications after aneurysmal subarachnoid hemorrhage (aSAH). It occurs in ~30% of aSAH patients and is associated with increased mortality/morbidity.[13] Vasospasm is characterized by a prolonged contraction of arterial smooth muscle cells, arterial lumen narrowing, cerebral hypoperfusion and subsequent potentially severe neurological deficit(s) secondary to delayed cerebral ischemia. Diagnosing cerebral vasospasm before it becomes clinically symptomatic is crucial as, when a patient becomes symptomatic and presents a neurological deficit, the vasospasm may have already been responsible for definitive sequelae and may not be responsive anymore to aggressive treatment. Many studies have suggested potential biomarkers (see [7]) to diagnose or predict the risk of cerebral vasospasm after aSAH, including S100B[9] and MMP9 [12]. None of these candidate biomarkers have yet been robustly validated in independent studies nor are routinely used in daily practice. Today, clinicians still lack robust tools to identify which patients will develop vasospasm secondary to aSAH. Additionally, the armamentarium to treat this delayed complication may sometime be inefficient, or even harmful. Patients admitted to Neuro-Intensive Care Units for aSAH are usually administered an invasive and aggressive treatment with severe side effects, which have been reported as frequently as in 15%-20% of aSAH patients.[10,5] A key element in the process of developing clinical prediction tools lies in the identification of disease's biomarkers that would hopefully be easily measurable and/or drug-targetable.

Assuming that the use of whole blood could be a good model tissue to discover vasospasm's biomarkers, we set up the VASOGENE cohort composed of aSAH patients prospectively followed-up for vasospasm and for which whole blood biobank was constituted.[8] Using a microRNA profiling strategy in VASOGENE samples, we recently identified hsa-miR-3177-3p as a new candidate marker for cerebral vasospasm.[8] In this work, we present the results of

a genome-wide search for whole blood gene mRNA levels that could associate with vasospasm and could later serve as biomarker for the disease and/or therapeutic target.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Study participants were patients with aSAH selected from the prospective VASOGENE study whose design has been previously reported.[8] All patients were hospitalized at the Neuro-Intensive care unit of the Pitié-Salpêtrière Hospital (Paris, France) within 48 hours of aneurysm rupture and treated by embolization or surgery within the first 96 hours. The VASOGENE study was carried out in accordance with the principles of the Helsinki Declaration, was approved by its local ethic committees and was declared in ClinicalTrial (https://www.clinicaltrials.gov/ct2/show/NCT01779713). All subjects or their legally authorized representatives signed written informed consent.

Readers are referred to supplement materials of [8] for extensive descriptions of the technical procedures and the study design of the VASOGENE study.

Study Design

The present work was conducted in a nested case-control sub-sample of the whole VASOGENE study that is composed of 89 aSAH patients aged ≥18 years and of Caucasian origin, excluding Africans, Hispanics, and Asians. All patients were followed up for at least 12 days and at each day a transcranial Doppler (TCD) was performed. In case of significant vasospasm depiction on TCD, a digital subtraction angiography (DSA) was performed to confirm vasospasm. Once detected by TCD and confirmed by DSA, vasospasm was defined as significant if patient had to be treated with intra-arterial pharmacological and/or mechanical dedicated therapy. From this sample of 89 aSAH patients, we selected 23 patients that developed significant vasospasm during the 12 days following aSAH (VSP^+) and for which daily mRNA data could be obtained. These 23 VSP^+ patients were then retrospectively

matched for age, sex and hemorrhage severity to 23 aSAH patients that did not develop significant vasospasm (VSP^-) during the 12 days period.

mRNA and miRNA preparation

Using an arterial catheter, blood samples were collected daily, on 2.5ml PAXgene blood RNA tubes, for a 12 days follow up period starting from the admission day in the neuro-intensive care unit. No heparin was present in the arterial line sample. After 4 hours at room temperature, tubes were then stored at -80°C. For the 23 VSP^+/VSP^- pairs, we extracted RNAs from biosamples collected at the admission day (D_0) and 3 days (D_{v3}) before the day VSP^+ patients experienced vasospasm (or the corresponding day for their matched VSP^- patient). RNAs were extracted and purified using the PAXgene blood RNA kit (Qiagen) that also collects miRNAs.

Gene expression profiling using mRNA microarray

Genome wide gene mRNA levels were profiled using the Illumina Human HT12v4.0 array (that includes ~47,000 probes) and processed using the GenomeStudio software. We selected for analysis only probes with detection p-values < 0.05 in at least 5% of the samples. Data were then normalized using the variance stabilization transformation (VST) and quantile normalization methodologies as implemented in the Lumi package.[4] Principal components analysis was performed to identify transcriptomic outliers. One VSP^+/VSP^- pair was thus excluded leaving 22 pairs for statistical differential analysis.

miRNA expression profiling using next-generation sequencing

Readers are invited to refer to Pulcrano-Nicolas et *al.*[8], for a comprehensive description of the experimental protocol and bioinformatics pipeline adopted for this miRNA sequencing profiling.

Statistical Methods

Associations of mRNA levels with vasospasm were tested using a linear mixed model taking into account the repeated measurements at D_0 and D_{v3} of a given aSAH patient as implemented in the *lme4* package available in R environment.[2] To identify mRNA levels whose changes over time may differ between VSP^+ and VSP^-, linear regression analyses were conducted where the difference in mRNA levels between D_0 and D_{v3} was used as the outcome. A Bonferroni threshold correcting for the number of tested mRNAs was used to declare study-wide statistical significance.

Note that D_0 and D_{v3} time points were overlapping for some patients who developed vasospasm earlier than 3 days after the hemorrhage, making the computation of the difference in mRNA levels between D_0 and D_{v3} feasible for only 17 VSP^-/VSP^+ pairs. For identified mRNA candidates, we looked for miRNAs correlates, available in 12 VSP^-/VSP^+ pairs, that could also associate with vasospasm.[8] All analyses were adjusted for age and sex.

Data Availability Statement

Normalized transcriptomic data are available in the European Genome-Phenome Archive platform under the acronym access code VASOGENE. Data access will be granted after request examination by Data Access Committee.

Results

Clinical characteristics of the study participants are shown in **Table 1**.

After quality controls, 21,460 probes corresponding to 14,889 distinct genes were considered to be highly expressed and kept for statistical association analyses.

Full results of the association scan for mRNA levels associated with vasospasm are given in **Supplementary Table 1**. The search for mRNA levels that differ between VSP^+ and VSP^- patients did not reveal any statistical association that reached the pre-specified threshold of 2.3×10^{-6} (~0.05 / 21,460). The strongest association reached $p = 5.3 \times 10^{-5}$ and was observed for *TP53INP1*.

By contrast, the search for probes with mean levels difference between D_0 and D_{v3} differing between VSP^+ and VSP^- patients revealed an interesting finding. Even though no association satisfied the Bonferroni correction, the Quantile-Quantile plot (**Figure 1**) summarizing these associations clearly shows that one probe demonstrated stronger statistical association than all the others. This probe (ILMN_1784737) maps to Sphingosine-1-Phosphate Receptor 4 (*S1PR4*) with $p = 8.03 \times 10^{-6}$. Full association results are provided in **Supplementary Table 2**. As shown in **Figure 2**, *S1PR4* mRNA level slightly increased over time in VSP^+ (+0.17 ± 0.25) while an opposite and more pronounced decrease (-0.29 ± 0.26) was observed in VSP^-.

In terms of prediction, the model including age, sex and the difference of *S1PR4* mRNA level over time was associated with an area under the receiving operating characteristic curve (AUC) of 0.927 [0.846-1] compared to 0.597 [0.399-0.794] for age and sex only. Of note, the mRNA levels of 10 candidate biomarkers previously proposed for vasospasm [7] were available in our mRNA study but none performed better than *S1PR4* (Supplementary Table 3), the strongest AUC being observed for endoglin (AUC = 0.75, p = 0.06).

We then sought for miRNAs, whose whole blood level changed between D_0 and $D_{v3,}$ and correlated with *S1PR4* mRNA levels, but did not find any (**Supplementary Table 4**).

Finally, in the subsample of VASOGENE patients with both mRNA and miRNA data (n_{pairs}= 12), the AUC of the prediction model integrating age, sex and *S1PR4* level difference was

0.896 [0.768 – 1]. This value increased, but not significantly (p = 0.12), to 0.931 [0.833 – 1] when the model additionally incorporated whole blood miRNA levels of hsa-miR-3177-3p we have previously shown to associate with vasospasm. [8]

Discussion

This study is so far the largest investigation for global gene mRNA levels in whole blood of aSAH patients followed for cerebral vasospasm. Having access to whole blood samples at the admission day in neuro-intensive care unit and 3 days before vasospasm enabled us to look for mRNAs differentially expressed between VSP^+ and VSP^- at both time points but also for mRNAs whose change over time differed between the two patient groups. While the former analysis did not reveal promising findings, the latter strongly suggested that patients with decreased *S1PR4* mRNA level after aSAH were at lower risk of vasospasm.

Several experimental arguments support *S1PR4*, that is mainly expressed in neurons at later developmental stages[1], as a good candidate biomarker for vasospasm. *S1PR4* belongs to the family of G-protein coupled receptors that are able to trigger the activation of specific signaling pathways following their binding to Sphingosine-1-phosphate (S1P), a sphingolipid mainly present in platelets. Activation of the S1P signaling was reported to play a role in multiple biological processes including immunity, inflammation[3] and could stimulate the production of spasmogenic substances.[11] Indeed, a study performed in a dog model for vasospasm showed that S1P exerts a vasoconstrictor activity in cerebral artery *via* the activation of the Rho-kinase pathway and an increase of Ca^{2+} in smooth muscle cells during the synthetic phase of the cells.[11] S1P has also been demonstrated to enhance myogenic tone in a mouse model of SAH.[14] In addition, another study performed in hypertensive rats demonstrated that S1PR4 prompted vasoconstriction of pulmonary vascular smooth muscle cells with Ca2+ increase after S1P injection.[6]

Given all these observations, we hypothesize that, following cerebral hemorrhage, platelets present in blood release S1P which then induces vasoconstriction through a S1PR4 related mechanism that needs to be deeply investigated but that could parallel that observed in the above-mentioned animal models. As the sphingolipid S1P was not measurable by the transcriptomic Illumina array, we could not assess its correlation with *S1PR4* mRNA level in our samples. Of note, as shown in **Supplementary Table 5**, none of the other *S1PR* genes expressed in whole blood (*S1PR1*, *S1PR3* and *S1PR5*) showed association with vasospasm.

Despite being the largest prospective cohort of aSAH patients followed up for vasospasm and profiled for mRNA whole blood level, this study suffers from several limitations. Its low sample size likely hampered our chance to detect study-wise statistical associations and decreased the general power of our study. Consequently, we cannot rule out that we have missed other key findings. We may also have missed some vasospasm-associated mRNAs due to the use, when the mRNA study was launched, of a microarray technology to measure gene expression while a next generation sequencing profiling would be nowadays more efficient. The main limitation of this work relates to the lack of formal replication of the association observed at *S1PR4*. Unfortunately, we are not aware of any similar prospective study that could be used to replicate this association. Further clinical and/or experimental works are definitively mandatory to definitively validate our finding. In particular, it would be important to assess how much our S1PR4 biomarker compares to clinical scores such as Fisher grade in predicting vasospasm in a standard clinical setting which was not possible to address here since VSP^+ and VSP^- patients were matched by design according to the hemorrhage severity. In addition, further clinical investigations would be needed to determine whether the measurement of *S1PR4* could be used to monitor the risk of vasospasm and whether a pharmaceutical approach aimed at controlling *S1PR4* regulation could help preventing from vasospasm.

Conclusions

In conclusion, our transcriptomic study identifies whole blood *S1PR4* mRNA level as a strong candidate biomarker for the risk of vasospasm in aSAH patients. The availability of S1PRs antagonists or other pharmaceutical therapies targeting S1PRs[1] open new avenues for developing therapeutic agent protecting against vasospasm's occurrence by modulating S1PR4 regulation.

Disclosures

Pr. Frederic Clarençon has a consultant or advisory relationship to disclose (Balt, Medtronic and Penumbra). Paid lectures. The other authors report no disclosures.

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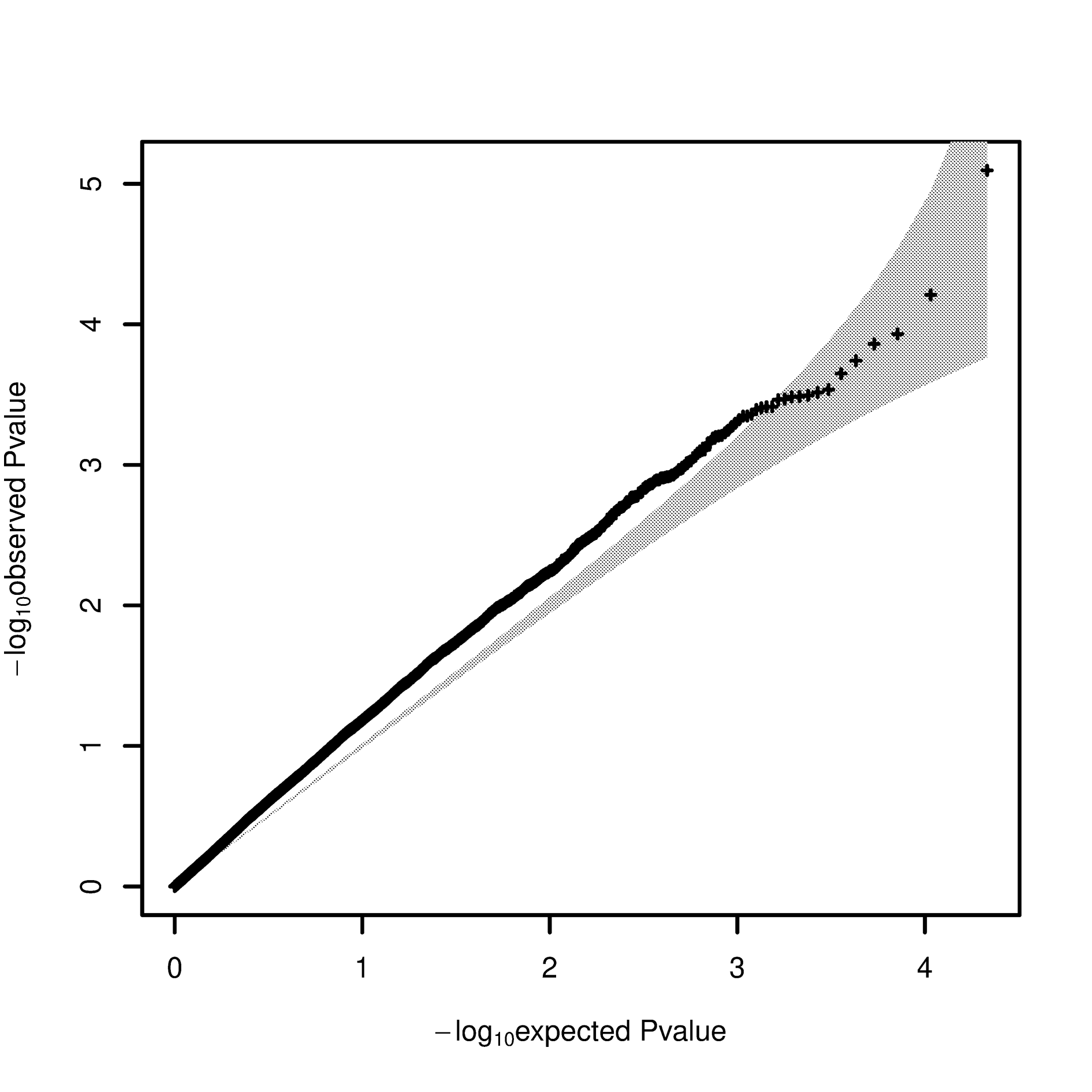
Table 1 Description of the clinical characteristics of the transcriptomic VASOGENE cohort

Figure 1 Quantile-Quantile plot summarizing the association of mRNA levels differences (D_{v3}-D_0) with the risk of vasospasm.

Figure 2 *S1PR4* mean differences expression levels separately in aSAH patients with (VSP^+) or without (VSP^-) vasospasm.


−log10observed Pvalue
0
1
2
3
4
5
−log10expected Pvalue
0
1
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4

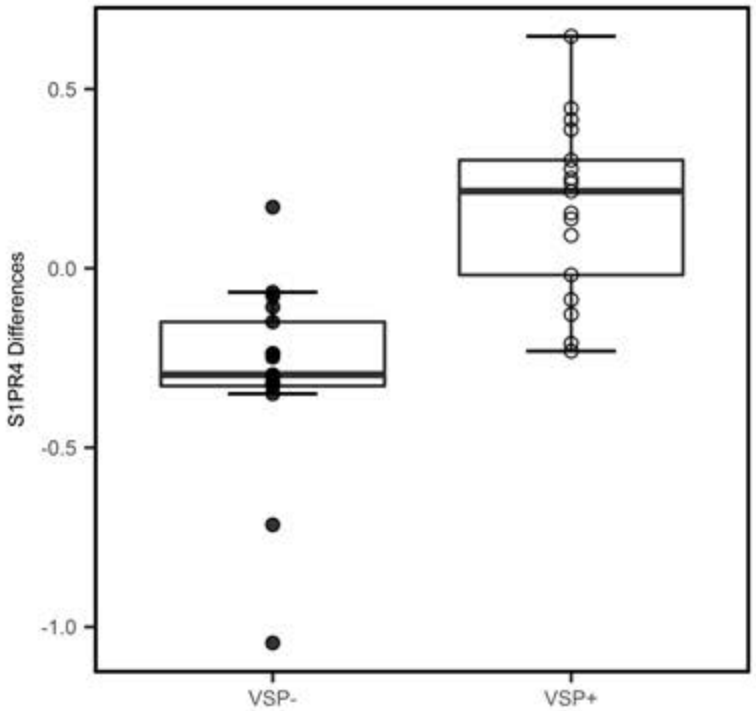
S1PR4 Differences
0.5
0.0
-0.5
-1.0
VSP-
VSP+

Table 1 Description of the transcriptomic VASOGENE cohort

	VSP+	VSP-	Pvalue[1]
	N =22	N=22	
Age	48.50 (10.80)	53.04 (12.52)	0.20
Female (%)	14 (63.63%)	14 (63.63%)	1.0
Smoker (%)	16 (72.73%)	11 (50%)	0.29
day of vasospasm onset			
3	2	-	
4	3	-	
5	4	-	
6	2	-	
7	4	-	
8	3	-	
9	2	-	
11	2	-	
Fisher score			0.91
1	2	2	
2	4	6	
3	5	4	
4	11	10	
WFNS[2]			0.11
1	9	12	
2	10	4	
3	0	3	
4	3	3	

Mis en forme : Droite, Droite : 0,76 cm

5	0	0	
GCS[3]>13	18	16	0.13

[1]Association test P-value derived from ANOVA and Chi-square test statistics for quantitative and qualitative data, respectively.

Shown data: mean (SD) for quantitative variables and count (%) for qualitative variables.

[2]World Federation of Neurological Surgeons score

[3]Glasgow Coma Scale