Quantitative analysis of HSV-1 shedding as a predictor of cerebral vasospasm severity in patients with subarachnoid hemorrhage

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Abstract. Cerebral vasospasm (CV) is a critical determinant of outcomes in patients with aneurysmal subarachnoid hemorrhage (aSAH). Despite advances in neurocritical care, modifiable risk factors for CV remain poorly understood, and identifying them could significantly enhance patient management and treatment strategies. The present study explored the potential link between the reactivation of herpes simplex virus type 1 (HSV-1), a common resident virus in cranial nerves, and CV severity. It was hypothesized that higher HSV-1 viral load in saliva may be associated with increased CV severity. Saliva samples were collected on days 4, 7, 10 and 14 post-aSAH, and HSV-1 DNA levels were measured using quantitative PCR. CV severity was assessed using the Lindegaard ratio (LR), with an LR >3 considered the diagnostic threshold for CV. A total of 36 patients were enrolled, and 139 saliva samples were collected. HSV-1 DNA was detected in 19.4% of samples (27/139), and 44% of patients (16/36) developed CV. HSV-1 seropositive patients made up 88.9% (32/36) of the cohort, with 50% exhibiting viral shedding during the study period. None of the HSV-1 seronegative patients (11.1%, 4/36) exhibited viral shedding or developed CV. Regression analysis showed a positive association between HSV-1 viral load and CV severity, with viral load explaining 27.8% of the variability (P=0.005). Age was also significant, with older patients experiencing less severe CV (P<0.001). Supervised machine learning identified viral load thresholds that aligned with standard LR values for moderate and severe CV. While the small sample size and observational design limit the generalizability of the results, these findings suggested that earlier detection and intervention for CV could be informed by assessing HSV-1 serostatus and monitoring viral activity through saliva samples or other non-invasive methods, highlighting the need for larger, controlled studies to validate these results.

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Introduction

Cerebral vasospasm (CV) is a severe and often life-threatening complication following aneurysmal subarachnoid hemorrhage (aSAH), characterized by the narrowing of cerebral arteries and leading to delayed ischemic neurological deficits (1). The pathophysiology of CV is multifactorial, and despite improvements in neurocritical care, its diagnosis remains challenging, and there are no quantitative methods to predict who will develop CV before it manifests. Current therapies, such as intra-arterial vasodilators, have limited efficacy and are often accompanied by systemic side effects, leaving an unmet clinical need (2-4). This lack of early diagnostic tools and predictive biomarkers, coupled with supportive treatments rather than curative interventions, limits effective management.

Neuroinflammation has been implicated in the pathogenesis of CV following aSAH, as the rupture of an aneurysm sets off a cascade of inflammatory responses that affect vascular tone and endothelial function (5-8). While inflammation is widely recognized (9-11), the sources and precise triggers of this inflammatory process are still under investigation. One potentially overlooked factor is the reactivation of neurotropic viruses, particularly herpes simplex virus type 1 (HSV-1), which infects a large portion of the adult population and can remain latent in cranial nerves (12-15). Reactivation of HSV-1, especially under conditions of physical or physiological stress exacerbates the inflammatory response (16,17); however, to the best of our knowledge, this connection has not been systematically explored in the context of CV.

HSV-1 latency in cranial autonomic and sensory nerves offers a plausible mechanism for its potential involvement in CV. Catecholamines, known to surge after aSAH (18-21), may initiate or promote viral reactivation via their effects on autonomic nerves, which harbor receptors for these stress-related neurotransmitters (9,10). Reactivation of the virus could contribute to local inflammation, particularly in areas with dense autonomic innervation, such as the cerebral arteries, which are supplied by the superior cervical ganglion, a key regulator of vasoconstriction (22-25). The resulting dynamic between viral reactivation and autonomic dysregulation may disrupt cerebrovascular function, tipping the balance toward vasospasm.

To explore this association, we conducted an exploratory study using HSV-1 viral load measurements in saliva as a proxy for reactivation in patients following aSAH. Protocols for monitoring HSV-1 shedding in saliva have been well documented and provide a reliable measure of viral load (26). The production of saliva is regulated by both sensory and autonomic nerves (27-30), which not only contribute to the salivary glands but also share innervation with structures involved in regulating vascular tone. Specifically, the trigeminal and superior cervical ganglia are key contributors to this dual role (31-33). Sensory nerves, like those from the trigeminal ganglion, regulate salivary flow, while autonomic nerves, particularly sympathetic fibers from the superior cervical ganglion, influence vascular dynamics. This overlapping innervation suggests that viral reactivation in these neural pathways could simultaneously affect both saliva production and cerebrovascular regulation. Given this anatomical and physiological connection, monitoring HSV-1 viral load in saliva offers a promising non-invasive method for assessing viral reactivation and may serve as an early biomarker for CV risk in patients following aSAH.

Patients and methods

Study design and participants. This prospective observational study was conducted at Harborview Medical Center (University of Washington, Seattle, WA, USA) between December 2020 and December 2022. Eligible participants were required to enroll within 4 days of aSAH and be capable of undergoing standard assessments for CV. Patients with intracranial hemorrhage not attributed to aSAH or those known to be taking antiviral medications within 2 weeks of arrival were excluded. Written informed consent was obtained from all participants or their legal proxy. All procedures were approved by the University of Washington Human Subjects Division (approval no. STUDY00010645; Seattle, WA, USA).

Participants were monitored for up to 14 days post-aSAH using daily neurological exams and transcranial Doppler ultrasonography (TCD). CV was diagnosed based on new neurological deficits not explainable by other causes, cross-sectional and/or catheter angiographic imaging, and evidence of elevated blood flow velocities on TCD. According to established clinical guidelines (2), a Lindegaard ratio (LR) >3, derived from TCD examinations comparing mean blood flow velocity in the middle cerebral artery to that of the ipsilateral extracranial internal carotid artery, was indicative of CV (2,34,35).

Biospecimen collection and analysis. Saliva samples were collected on days 4, 7, 10 and 14 (±1 day) post-aSAH using sterile Dacron swabs (Puritan Medical Products). Samples were stored in DNA preservation medium prior to analysis. HSV-1 DNA was extracted from saliva samples using the QIAamp DNA Mini Kit (cat. no. 51304; Qiagen GmbH), according to the manufacturer's protocol. To ensure specificity for HSV-1, targeted primers of the glycoprotein G gene (36) were used: Forward, 5'-CGCGAACAACAGTGTTAGCG-3' and reverse, 5'-ACGGTCGTCGCATCTGTCTT-3'. The PCR thermocycling conditions included an initial denaturation step at 95˚C for 10 min; followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 1 min and extension at 72˚C for 1 min; and a final extension step at 72˚C for 10 min. HSV-1 DNA was quantified in saliva samples using a quantitative fluorescent probe-based PCR assay (TaqMan® Gene Expression Assay; cat. No. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Per standard, a positive threshold for viral shedding was defined as 150 copies of HSV-1 DNA per ml of saliva (26). To determine HSV-1 serostatus, 7 ml blood was drawn from the peripheral IV line of the patients into a serum separator tube. The clinical laboratory at Harborview Medical Center separated the serum from the cells within 2 h. A 1-ml aliquot of the serum was then transferred to a standard transport tube, refrigerated and sent to an external laboratory for analysis within 2 weeks of collection. Specimens underwent HSV-1 Glycoprotein G-Specific Antibody, IgG testing by Chemiluminescent Immunoassay (test 0050292; ARUP Laboratories), following the laboratory standard protocol. An EU/ml index value exceeding 1.10 classified samples as positive (37,38). All personally identifiable information was removed to ensure participant anonymity and confidentiality.

Statistical analysis. Descriptive statistics were used to characterize the demographic and clinical profiles of the cohort. Categorical variables were analyzed using Pearson's χ^2 test or Fisher's exact test (where expected cell counts were <5), and continuous variables were analyzed using the Mann-Whitney U test. Linear regression analysis was applied to assess the relationship between HSV-1 viral shedding and CV severity across all shedding time points. While maximum viral load reduces the dataset to one value per subject, individual time point analysis preserves more data, allowing for greater statistical power. The model fit was evaluated using the coefficient of determination (R^2), and effect size was assessed using Cohen's f^2. Traditional multiple regression models were not ideal for this analysis due to the small sample size and the longitudinal nature of the data, where repeated measures were taken from the same subjects over time. Therefore, a Random Forest (RF) multiple regression analysis was performed to assess the relationship between viral load, statistically significant demographic variables and the LR. A model with 20 decision trees (n_estimators=20) was used to balance model complexity and minimize the risk of overfitting. The dataset was randomly divided into a training set (70%) and a test set (30%) for model evaluation. The model's performance was assessed using the R^2 score, which reflects the proportion of variance in the LR explained by the independent variables. To further account for repeated measures within subjects, generalized estimating

Table I. Demographic and clinical features.

Characteristic	Total cohort (n=36)	Vasospasm (n=16)	No vasospasm (n=20)	P-value
Mean age, years(SD)	59.5 (13.37)	53.6 (8.91)	64 (14.66)	0.02[b]
Female sex (%)	21 (58.31)	8 (50.00)	13 (65.00)	0.36[c]
Race and ethnicity (%)[a]				
American Indian or Alaska native	0	0	0	N/A
Asian	2 (5.56)	1 (6.25)	1 (5.00)	>0.99[d]
Black or African American	0	0	0	N/A
Hispanic or Latino/a	6 (16.66)	5 (31.25)	1 (5.00)	0.07[d]
Native Hawaiian or Pacific Islander	0	0	0	N/A
White	28 (77.78)	10 (62.50)	18 (90.00)	0.10[d]
Substance abuse disorder (%)	7 (19.44)	3 (18.75)	4 (20.00)	>0.99[d]
Smoking (%)	20 (55.56)	8 (50)	12 (60.00)	0.55[c]
New seizures (%)	13 (36.11)	5 (31.25)	8 (40.00)	0.59[c]
Aneurysm size (%)				
<7 mm	25 (69.44)	15 (93.75)	10 (50.00)	0.01[d]
7-10 mm	8 (22.22)	1 (6.25)	7 (35.00)	0.05[d]
>10 mm	3 (8.33)	0	3 (15.00)	0.24[d]
Fisher grade, median (IQR)	4 (2)	4 (1)	3 (2)	0.24[b]
Hunt-Hess scale, median (IQR)	3 (2)	3 (2)	3 (2)	>0.99[b]

[a]Self-reported by patient or proxy; [b]Mann-Whitney U test; [c]Pearson's $\chi 2$ test; [d]Fisher's exact test. IQR, interquartile range; SD, standard deviation.

equations (GEE) were employed, using an exchangeable correlation structure. A linear GEE model was fitted to assess the association between log-transformed viral load and LR over time. To account for non-linear effects, a quadratic GEE model was also fitted, incorporating both the log-transformed viral count and its squared term.

Finally, Classification and Regression Tree (CART) analysis was performed to identify viral load thresholds associated with increased CV risk. The CART model was constructed based on both average and maximum $\log_{10}$-transformed viral counts. Decision nodes represent thresholds for viral load that categorize patients as at moderate or high risk for developing CV, with LR >3 used as the criterion for defining CV. Statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc.), R version 4.0.5 (R Foundation for Statistical Computing) and Python version 3.8 (Python Software Foundation). $P<0.05$ was considered to indicate a statistically significant difference. This study conforms to the STROBE (39) guidelines for reporting observational research.

Results

Subjects. A total of 36 patients with acute aSAH were enrolled in this study (Table I). Of these, 44% (16/36) developed CV and 1 patient died during the course of the study. In total, 139 saliva samples were collected. The proportion of HSV-1 seropositive patients was 88.9% (32/36), with 19.4% (27/139) of all saliva samples testing positive for HSV-1 DNA. Of the seropositive patients, 50% exhibited viral shedding at some point during the study period. Seronegative patients (11.1%; 4/36) did not develop CV or viral shedding.

In the cohort, significant differences were observed between patients who developed CV and those who did not. Specifically, patients in the CV group were significantly younger, with a mean age of 53.6 years compared with 64.0 years in the non-CV group (P=0.02). Additionally, aneurysm size was notably smaller in the CV group, with 93.75% of these patients having <7-mm aneurysms, compared with 50% in the non-CV group (P<0.01). The higher proportion of Hispanic or Latino/a patients in the CV group (31.25% vs. 5%, P=0.07) and the lower proportion of aneurysms measuring 7-10 mm in the CV group (6.25% vs. 35%, P=0.05) were also notable, although these findings were on the threshold of statistical significance.

Viral shedding and CV severity. A significant positive association was identified between HSV-1 shedding and CV severity. Each $\log_{10}$ increase in viral DNA count corresponded with a 0.57 increase in the LR, indicating heightened CV severity. Linear regression analysis (Fig. 1) demonstrated that HSV-1 viral shedding explained 27.8% of the variability in CV severity among patients with aSAH (Cohen's $f^2=0.386$, P=0.005), indicating a large effect size. A multiple regression analysis was conducted to assess the association between HSV-1 viral shedding and CV severity, adjusting for age. After pairwise deletion in instances where no data were collected (due to patient death), the multiple regression analysis included 33 participants. The multiple RF regression achieved an R^2 of 0.640, explaining 64.0% of the variance in the LR. The predicted values ranged from 1.70 to 5.62, indicating a reasonable alignment with the actual values, despite the use of a smaller number of trees. The model effectively captured the relationship between viral count and CV severity (Fig. 2).

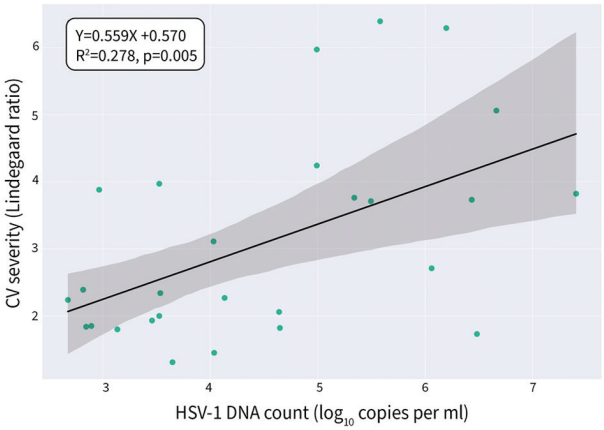

Figure 1. Scatterplot of HSV-1 viral load vs. CV severity. The scatterplot illustrates the relationship between HSV-1 DNA count (log_{10} copies/ml) and CV severity (Lindegaard ratio) in individual HSV-1 seropositive patients. A linear regression line is shown (y=0.559X + 0.570), indicating a significant positive association between viral load and CV severity. The regression model explains 27.8% of the variability in CV severity (R^2=0.278, P=0.005). CV, cerebral vasospasm; HSV-1, herpes simplex virus type 1.

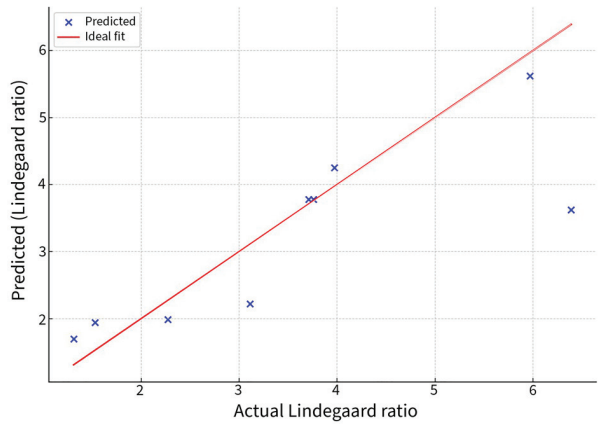

Figure 2. Actual vs. predicted LRs using RF multiple regression. The scatter plot compares the actual LR (x-axis) to the predicted LR values (y-axis) for the test set. The red line represents the ideal fit, where predicted values would closely align with the actual values. Predictions were generated using a RF model with 20 decision trees, achieving an R^2 score of 0.640. LR, Lindegaard ratio; RF, Random Forest.

Neurovascular dynamics. To evaluate the temporal relationship between HSV-1 viral shedding and the development of CV post-aSAH, the Mann-Whitney U test was used to compare the median viral loads (log_{10}-transformed) between patients with and without CV on days 4, 7, 10 and 14 post-aSAH (Fig. 3). Median values were chosen for this analysis due to their robustness in small datasets, as they are less affected by outliers and skewed distributions compared with mean values. The analysis revealed that median viral loads were higher in patients with CV, particularly at Day 10 post-aSAH (P=0.032).

To account for the correlated nature of repeated measures within participants over time, a linear GEE model was fitted (Table II). The model demonstrated a positive association between viral load and LR, showing that for every unit increase in log-transformed viral count, LR increased by 0.024 (95%

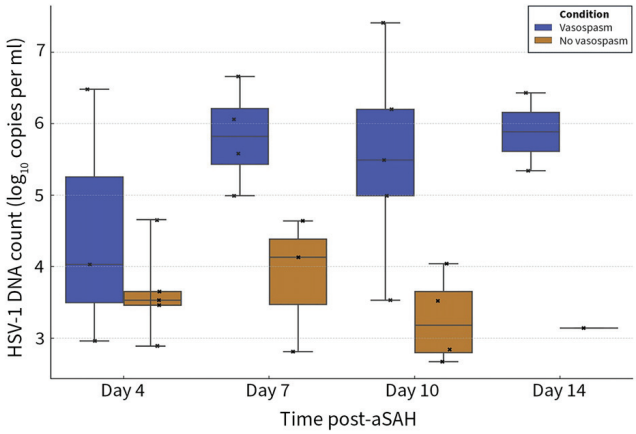

Figure 3. Temporal dynamics of HSV-1 shedding relative to CV in seropositive patients with aSAH. Box and whisker plots illustrate the distribution of HSV-1 DNA shedding (log_{10} count per ml) in the saliva of patients with CV (LR >3) and without CV (LR <3) at various post-aSAH time points. The plots reveal a trend of increasing viral shedding levels in patients with CV compared to those without, particularly noticeable on day 10, where a statistically significant difference was observed (P=0.032). Individual data points are denoted with X. Median (IQR) viral loads for patients with CV on days 4, 7, 10 and 14 were 4.03 (1.76), 5.82 (0.78), 5.49 (1.21) and 5.88 (0.55), respectively. For patients without CV, the median (IQR) viral loads were 3.53 (0.19), 4.13 (0.92), 3.18 (0.85) and 3.14 (0.00). Data presented for day 14 are for illustrative purposes, as only one value is included for the 'No vasospasm' group. aSAH, acute subarachnoid hemorrhage; CV, cerebral vasospasm; HSV-1, herpes simplex virus type 1; LR, Lindegaard ratio.

CI: 0.001-0.048), indicating a significant (P=0.039) but subtle effect. This effect started from an LR of 2.92 when the viral count was at the predicted baseline level.

The study further explored the non-linear dynamics between viral count and CV risk through a quadratic GEE model, incorporating both log-transformed viral count and its square. The analysis (Table II) revealed significant evidence for both the linear component (P<0.001, 95% CI: 0.084-0.259) and the quadratic deviation (P<0.001, 95% CI: 0.012-0.040), illustrating that while LR initially increased with viral count, this trend diminished at higher levels of viral shedding.

Supervised machine learning was employed to identify viral count thresholds associated with increased CV risk. CART analysis, using both average and maximum log_{10} HSV-1 viral counts, revealed significant thresholds (Fig. 4). The primary threshold, based on the average log_{10} viral count, identified 3.05 (1,123 count/ml, 95% CI: 2.85-3.25) as the point above which patients were moderately likely to develop CV. The secondary threshold, based on the maximum log_{10} viral count, identified 4.82 (65,966 count/ml, 95% CI: 4.60-5.04) as the point above which patients were highly likely to develop CV. These thresholds suggested progressive stages of CV risk, emphasizing a non-linear pattern, particularly at elevated levels of viral shedding.

Discussion

This study demonstrated a significant association between HSV-1 viral shedding and the severity of CV in patients with aSAH. HSV-1 DNA detected in saliva and specific viral load thresholds suggested that HSV-1 shedding could serve as a

Table II. Generalized estimating equations analysis for the association between herpes simplex virus type 1 shedding and cerebral vasospasm severity.

Term	Coefficient	Standard error	P-value	95% CI lower	95% CI upper
Linear model					
Intercept	2.921	0.192	<0.001	2.545	3.297
Log_{10}_Viral_Count	0.024	0.012	0.039	0.001	0.048
Quadratic model					
Intercept	1.796	0.314	<0.001	1.179	2.412
Log_{10}_Viral_Count	0.172	0.045	<0.001	0.084	0.259
Log_{10}_Viral_Count^2	0.026	0.007	<0.001	0.012	0.040

CI, confidence interval.

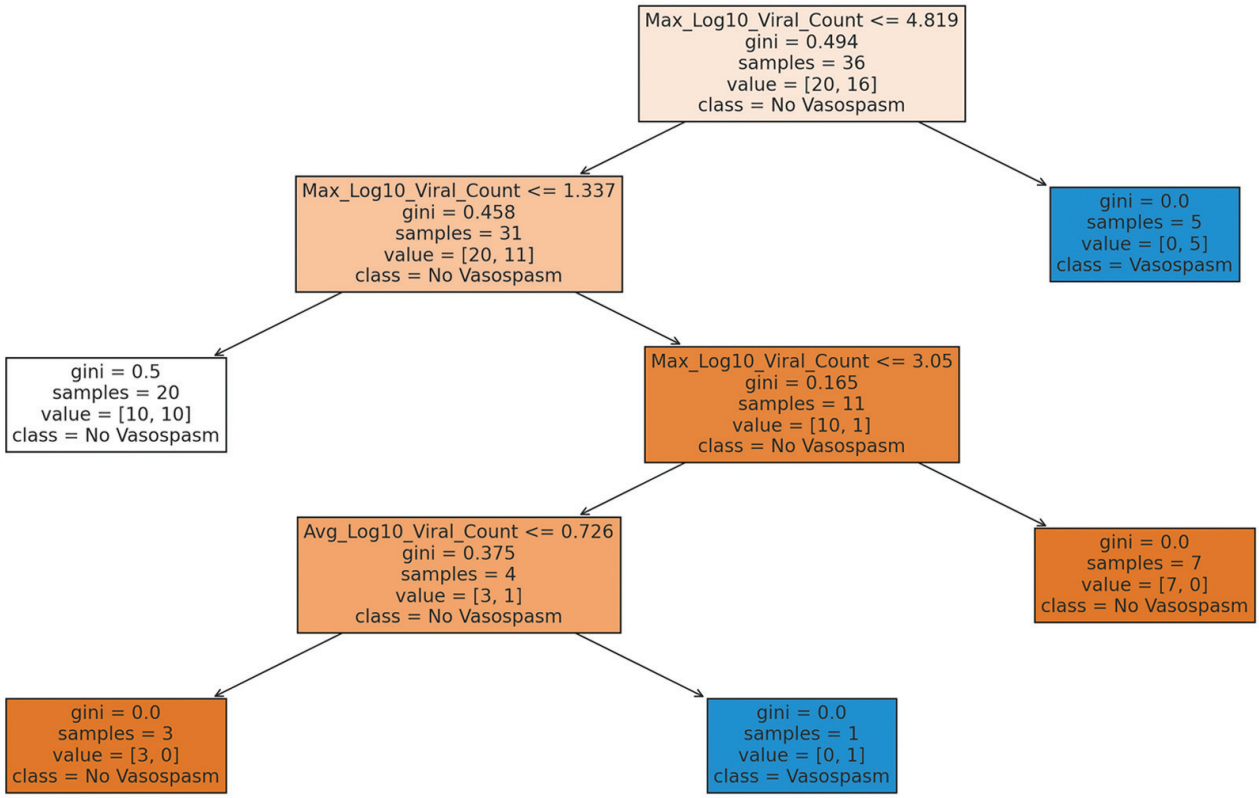

Figure 4. Decision tree for predicting CV risk based on HSV-1 viral counts. Combined decision tree illustrating the relationship between average and maximum log_{10} HSV-1 viral counts and the likelihood of CV in patients with acute subarachnoid hemorrhage. The tree was constructed using the Classification and Regression Tree analysis, with nodes representing decision points based on viral load thresholds. The class labels 'No vasospasm' and 'Vasospasm' indicate the presence or absence of CV, with a Lindegaard ratio >3 used as the threshold for vasospasm. This model identified critical viral load thresholds, highlighting both moderate and high-risk categories for CV development. CV, cerebral vasospasm; HSV-1, herpes simplex virus type 1.

predictive biomarker for CV, offering new possibilities for early intervention and monitoring to improve patient outcomes.

The small sample size and observational design of the study limit the generalizability of the findings and preclude definitive conclusions. Variability in viral load measurements may have been introduced by integrating specimen collection into routine care, where factors such as oral hygiene, salivary flow and timing of collection could have influenced results. While sympathomimetic pressors, commonly used in critical care, have not been directly linked to viral reactivation, their use may have indirectly affected viral detection and should be considered as a potential confounder. Moreover, the lack of demographic diversity within the study population may limit the robustness of the results across different patient groups. Although machine learning provides valuable insights by identifying thresholds and patterns not immediately apparent with traditional analysis, the small sample size limits the reliability of these models. Future research should aim to validate these findings in larger, multicenter trials to improve the robustness of viral load thresholds and temporal patterns.

Despite the potential of using salivary HSV-1 DNA as a biomarker, it is important to acknowledge its limitations. Future studies should consider complementary biomarkers to more comprehensively assess HSV-1 activity and its contribution to CV pathogenesis. Expanding the study population to include non-aSAH critically ill patients or more detailed comparisons between CV and non-CV groups could further elucidate the specific role HSV-1 serves in CV. Additionally, investigating other neurotropic viruses known to be resident in cranial nerves, such as varicella-zoster virus (13,40), could provide further insights into the infectious underpinnings of CV and broaden the scope of viral contributions to neurovascular disorders.

This study provides preliminary evidence of a significant association between HSV-1 viral shedding and CV severity in patients with aSAH. It introduces a quantifiable method for assessing CV risk in the absence of reliable predictive tools in current clinical practice. Early determination of HSV-1 serostatus and regular monitoring of viral load in patients with aSAH could enable earlier detection and more timely interventions, potentially using accessible and cost-effective antiviral therapies. Caution is warranted in interpreting these findings due to study limitations, and larger, controlled trials are necessary to confirm whether salivary HSV-1 viral load can serve as a biomarker for clinical use in this setting.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MW, MRL and EF confirm the authenticity of all the raw data. MW was responsible for project conception and design, data acquisition, analysis and manuscript drafting. MRL and EF contributed to data acquisition, analysis and manuscript drafting. JRZ and CMJ performed data analysis, interpretation and manuscript drafting. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This observational study was conducted in accordance with international and national regulations, including The Declaration of Helsinki and other relevant ethical principles. The research protocol was approved by the institutional review board of the University of Washington under the approval number STUDY00010645. Written informed consent was obtained from all participants or their proxy prior to their inclusion in the study.

Patient consent for publication

Not applicable.

Competing interests

Dr Levitt has unrestricted educational grants from Medtronic and Stryker, consulting agreements with Genomadix, and AIDoc, Stereotaxis, Aeaean Advisers and Metis Innovative; equity interest in Proprio, Stroke Diagnostics, Apertur, Fluid Biomed, Synchron and Hyperion Surgical; serves on the editorial board of Journal of NeuroInterventional Surgery; and is a member of the Data Safety Monitoring Board of Arsenal Medical. Dr Johnston has a consulting agreement with GSK and Assembly Biosciences; research funding is paid to the institution by GSK and Moderna. The remaining authors declare that they have no competing interests.

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