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Original Article

Cerebrospinal fluid and plasma ascorbate concentrations following subarachnoid haemorrhage

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ABSTRACT

Background: Ascorbate, the biologically active form of vitamin C, is the primary neural anti-oxidant. Ascorbate concentrations have never been quantified following aneurysmal subarachnoid haemorrhage (aSAH).

Objective: To quantify plasma and cerebrospinal fluid (CSF) ascorbate concentrations in patients following SAH.

Design, Setting, Participants, Main Outcome Measures: Cohort study in which plasma and CSF ascorbate concentrations were measured longitudinally in 12 aSAH patients admitted to a quaternary referral intensive care unit and compared to one-off samples obtained from 20 pregnant women prior to delivery in a co-located obstetric hospital. Data are median [interquartile range] or median (95 % confidence intervals).

Results: Forty-eight plasma samples were obtained from the 12 aSAH patients (eight females, age 62 [53–68] years). Eight participants with extra-ventricular drains provided 31 paired CSF-plasma samples. Single plasma and CSF samples were obtained from 20 pregnant women (age 35 [31–37] years). Initial plasma and CSF ascorbate concentrations post aSAH were less than half those in pregnant controls (plasma: aSAH: 31 [25–39] μmol/L vs. comparator: 64 [59–77] μmol/L; $P < 0.001$ and CSF: 116 [80–142] μmol/L vs. 252 [240–288] μmol/L; $P < 0.001$). Post aSAH there was a gradual reduction in the CSF:plasma ascorbate ratio from ~4:1 to ~1:1. Six (50 %) patients developed vasospasm and CSF ascorbate concentrations were lower in these patients (vasospasm: 61 (25, 97) vs. no vasospasm: 110 (96, 125) μmol/L; $P = 0.01$).

Conclusion: Post aSAH there is a marked reduction in CSF ascorbate concentration that is most prominent in those who develop vasospasm.

1. Introduction

Ascorbate, the biologically active form of vitamin C, is an essential micronutrient with a normal plasma concentration of 30–80 μmol/L.[1] Through free radical scavenging, ascorbate is the primary antioxidant used and depleted in response to oxidative stress.[2] Brain tissue is highly susceptible to oxidative damage because of its high metabolic demand and high content of polyunsaturated fatty acids.[3] Ascorbate also has several non-antioxidant neural functions including modulation of dopamine and glutamate mediated neurotransmission, catecholamine synthesis, myelin sheath formation and maintenance of cerebrovascular endothelial integrity.[4] In health, ascorbate is actively transported into the central nervous system achieving concentrations that are up to 80 times greater in brain cells, and four times greater in cerebrospinal fluid (CSF) compared with plasma.[4]

Due to an overwhelming increase in free radicals and reactive oxygen species, plasma ascorbate concentrations decline rapidly in

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the setting of systemic critical illness, including sepsis, burns and major trauma.[5–7] Severe plasma ascorbate deficiency is also seen in isolated acute neurological insults, including stroke and traumatic brain injury, where the nadir in plasma ascorbate concentration correlates with worse functional outcomes.[8,9] However, no studies have examined plasma and CSF concentrations of ascorbate in patients with aneurysmal subarachnoid haemorrhage (aSAH).

Our objective was to longitudinally measure plasma and CSF concentrations in patients presenting with aSAH. To establish a contemporaneous comparator group, we measured plasma and CSF ascorbate concentrations in women having spinal anaesthesia in preparation for an elective caesarean section. This group was chosen as pregnant women at term have CSF concentrations of ascorbate that reside within the normal physiological range.[10] We hypothesized that in patients with aSAH, plasma and CSF ascorbate concentrations would reduce over time and that concentrations would be less in those that developed vasospasm.

2. Materials and methods

We conducted a prospective observational cohort study. The study protocol was approved by the Human Research Ethics Committee of Melbourne Health (2020.065), and conducted according to the principles of the Declaration of Helsinki and reported according to the STROBE statement.[11]

3. Participants

3.1. Patients with aneurysmal subarachnoid haemorrhage

Twelve patients aged 18–85 with aSAH were recruited from the intensive care unit (ICU) at the Royal Melbourne Hospital, a quaternary mixed medical/surgical and trauma ICU. Written informed consent was obtained from the patient or their medical treatment decision maker. Patients were excluded if they were not expected to survive 72 h.

3.2. Comparator group of pregnant women undergoing elective caesarean section

Twenty women undergoing elective caesarean section were recruited for the study from the Royal Women's Hospital, Melbourne. Exclusion criteria were a body mass index <18 kg/m^2, multi-foetal pregnancy, current smoker, hypertension, pre-eclampsia, gestational diabetes, hyperemesis and a past medical history of scurvy, malnutrition, ICU admission, SAH, diabetes mellitus or spina bifida. All pregnant participants provided written informed consent.

3.3. Protocol

Demographics, Acute Physiology and Chronic Health Evaluation II (APACHE II score), World Federation of Neurosurgeons Score (WFNS), interventions and ICU and hospital length of stay were recorded from patients with SAH. Blood samples (4 mL) were collected from existing catheters into chilled EDTA tubes three times per week (Monday, Wednesday, Friday) censored at hospital discharge or day 21 post-recruitment, whichever occurred first. In those patients in whom an extra-ventricular drain (EVD) was inserted for clinical need, fresh CSF from the preceding hour (4 mL) was collected aseptically from the collection chamber at the time of blood collection. Vasospasm was defined radiologically from computed tomography (CT) or digital subtraction angiography (DSA) performed on clinical grounds.

In pregnant participants we recorded demographics, foetal gestational age and medications including oral vitamin C supplements. A blood sample (4 mL) was collected at the time of peripheral cannula insertion on the day of delivery. A CSF sample (0.4 mL) was collected into a chilled EDTA tube upon accessing the subarachnoid space prior to delivery of spinal anaesthesia.

3.4. Plasma and CSF ascorbate concentrations

Blood and CSF were immediately centrifuged at 4 °C and 2740 XG for 10 min. Plasma or CSF were then diluted one part to four part methanol/water (90:10 v/v) and diethylenetriaminepentaacetic acid (DETAPAC, final concentration 250 μmol/L), mixed and left to stand for 10 min on ice to precipitate proteins. The solutions were then centrifuged at 4 °C and 2740 XG for 10 min and supernatant stored at −80 °C for subsequent analysis.[12,13] CSF and plasma ascorbate concentrations were measured at the Florey Institute of Neuroscience and Mental Health using CLARIOstar® fluorescent plate spectrophotometry (BMG Labtech) as previously desc ribed.[12,14] Samples were measured in duplicates, standards in triplicates and values were only accepted if the coefficient of variation (CV) was below 10 (mean CV was 1.9). Standard concentrations of L-ascorbic acid, Sigma (2, 3, 5, 7.5, 10, 20, 30, 40, 50, 70, 90, 110 and 130 μmol/L) were prepared freshly and the concentrations were determined to 4 decimal points by measuring absorbance using quartz cuvette and SPECTROstar Nano spectrometer (BMG Labtech). Sample ascorbate concentration was determined by using a 4-parameter fitting using MARS data analysis software supplied with the plate reader. Any sample with ascorbate concentration that fell above the maximum concentration (in case of CSF) was further diluted in 90 % methanol with DETAPAC before analysis.

3.5. Data analysis

3.5.1. Statistical analysis

As there were no pre-existing data in aSAH, sample size calculation was based on estimates. We assumed a CSF mean (standard deviation, SD) ascorbate concentration of approximately 300 (50) μmol/L in the pregnant cohort.[3] Accordingly, 12 patients with aSAH and 20 pregnant participants was estimated to have 90 % power to detect a mean reduction ≥60 μmol/L (≥20 % relative reduction) at an alpha of 0.05.

Continuous data are presented as median [inter-quartile range, IQR] or mean (standard deviation, SD) and categorical data as number (%). Baseline comparisons between groups for CSF and plasma relationships were performed by rank-sum test. The relationship between plasma and CSF ascorbate at baseline, and vitamin supplementation in the pregnant cohort, were examined using ordinary least-squares regression. Comparisons over time were estimated using random-effects (RE) generalized least squares (GLS) regression with robust standard errors to accommodate for within-patient correlation and presented as mean estimates with (95 % confidence interval, 95%CI).

Given the small study size, p-values are included for perspective.[15] No adjustment has been made for multiple comparisons and all analyses were performed in Stata 17 MP/4 (StataCorp LLC, College Station, Texas, United States).

4. Results

Between 15 March 2021 and 22 December 2021, we screened the ICU on weekdays for patients presenting with aSAH, though screening was partially interrupted between August 2021 and November 2021 due to COVID 19 related restrictions prohibiting research scientists attending the ICU and obstetric hospital.

Between 13 December 2021 and 14 February 2022, we screened the elective caesarean section list. aSAH patient eligibility and enrolment is summarized in Fig. 1. Demographic data and characteristics of the aSAH participants are summarized in Table 1. All aSAH patients were enterally fed. Summary statistics for plasma and CSF ascorbate levels are presented in Table 2.

5. Plasma and CSF ascorbate concentrations in pregnant women

There were 19 plasma samples and 20 CSF samples available for analysis. The median [IQR] plasma concentration of ascorbate was 64 [59–77] μmol/L (Fig. 2A). No participants had a plasma ascorbate concentration below the normal range (30 μmol/L). The median [IQR] CSF concentration of ascorbate was 252 [240–288] μmol/L (Fig. 2A). The minimum CSF ascorbate concentration was 202 μmol/L. The median [IQR] ratio of CSF to plasma ascorbate concentration was 4.1 [3.6–4.5] (Fig. 2B).

In pregnant women there was a weak positive correlation between plasma and CSF ascorbate concentrations ($R^2 = 0.23$; $P < 0.05$). There was no association between dose of daily vitamin C supplementation and either plasma or CSF ascorbate concentration.

6. Plasma ascorbate concentrations in patients with aSAH

Twelve participants with SAH were recruited and provided 48 plasma samples from day 0 to day 24 post the initial bleed. The mean (SD) time from aneurysm rupture to first plasma sample was 2.5 (1.5) days. Only one participant provided samples beyond 14 days. Plasma profiles of ascorbate concentration for days 1–14 post bleed are presented in Fig. 3. Seven participants (58 %) developed plasma concentrations consistent with hypovitaminosis C (<30 μmol/L) by mean (SD) 6 (2) days post bleed.

7. CSF ascorbate concentrations in patients with aSAH

Eight participants (66 %) had an EVD and provided 31 CSF samples from day 0 to day 24 post the initial bleed. The mean (SD) time from aneurysm rupture to first CSF sample was 2.2 (1.7) days. No patients with an EVD provided plasma samples prior to recruitment. Only one participant provided samples beyond 14 days. CSF concentrations of ascorbate decreased over time (Fig. 3). All SAH participants had an initial CSF ascorbate concentration below the minimum concentration observed in pregnant women 202 μmol/L).

8. Baseline plasma and CSF ascorbate concentrations between cohorts

Eight SAH participants (66 %) had an emergency EVD placement and provided paired samples of plasma and CSF. Initial median [IQR] plasma ascorbate concentrations were less in participants with SAH (SAH: 31 [25–39] μmol/L vs. pregnant comparator: 64 [59–77] μmol/L; $P < 0.001$) as were median CSF ascorbate concentrations (116 [80–142] μmol/L vs. 252 [240–288] μmol/L; $P < 0.001$) (Fig. 2A). The ratio of CSF to plasma ascorbate from the initial sample was numerically less in participants with aSAH but this did not reach statistical significance (CSF:Plasma ratio: SAH:

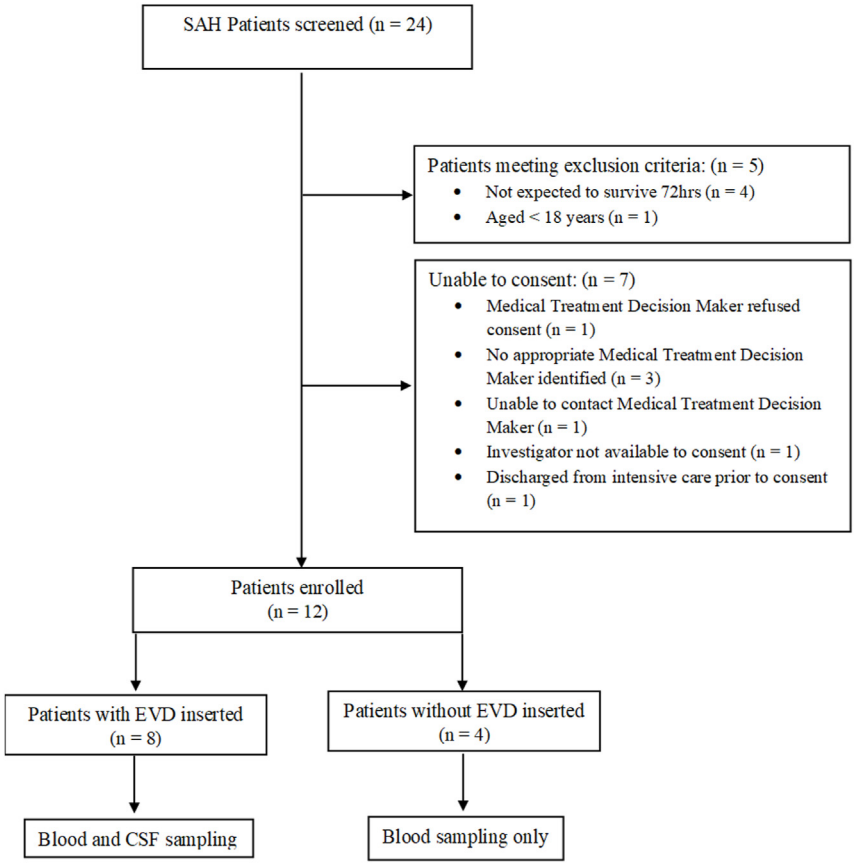

Fig. 1. CONSORT diagram. CSF, cerebrospinal fluid; EVD, external ventricular drain; SAH, subarachnoid haemorrhage.

Table 1
Descriptive details for aSAH cohort.

Covariate name	SAH participants
Age, med [IQR]	61.5 [53, 68]
Controls	34.5 [31, 37]
Male sex, n(%)	4/12 (33.3)
APACHE3 score, median [IQR]	60.0 [42.5, 63.5]
SOFA score, median [IQR]	6.5 [5.5, 8.5]
WFNS Grade, n(%)	12
Grade I	2 (16.7)
Grade II	1 (8.3)
Grade III	0
Grade IV	2 (16.7)
Grade V	7 (58.3)
Intraventricular haemorrhage, n(%)	10/12 (83)
Intracranial haemorrhage, n(%)	5/12 (42)
Interventions in ICU, n(%)	
Nimodipine	12/12 (100)
Vasoactive agent	12/12 (100)
Invasive ventilation	11/12 (91.7)
External ventricular drain	8/12 (66.7)
Angioplasty	1/12 (8.3)
Coil/Clip	5(42)/6(50)
Complications in ICU, n(%)	
Vasospasm	6/12 (50)
Cerebral infarct	5/12 (42)
Died	2/12 (16.7)

APACHE3, acute physiological and chronic health evaluation 3 score; ICU, intensive care unit; IQR, interquartile range; SAH, subarachnoid haemorrhage; WFNS, World Federation of Neurosurgical Societies.

3.3 [2.6–3.9] vs. pregnant comparator: 4.1 [3.6–4.5]; P = 0.06) (Fig. 1B). By 14 days post aneurysm rupture, CSF concentration of ascorbate had substantially reduced, with attenuation of CSF:Plasma ratio from 4:1 to 1:1 (Fig. 3).

9. CSF and plasma ascorbate concentrations in aSAH participants with and without vasospasm

Six (50 %) patients with aSAH developed vasospasm. Mean (95 % CI) CSF ascorbate concentrations were less in patients with vasospasm compared to those without vasospasm (61 (25, 97) vs. 110 (96, 125) μmol/L; P = 0.01), with no difference in the mean (95%CI) plasma ascorbate concentration (32 (25, 40) vs. 32 (25, 40)) μmol/L (Fig. 4). Accordingly, the ratio of CSF to plasma ascorbate concentration was markedly less in those participants with vasospasm (2.0 (0.96, 3.01) vs. 3.8 (3.2, 4.4); P < 0.01) (Fig. 4).

Table 2
Plasma and CSF ascorbate levels.

	aSAH group	Controls	P-value
Initial sample	n = 8/12	n = 19/20	
CSF (μmol/L)	116 [80, 142]	252 [240, 288]	< 0.001
Plasma (μmol/L)	31 [25, 39]	64 [59, 77]	< 0.001
CSF:plasma ratio	3.3 [2.6, 3.9]	4.1 [3.6, 4.5]	0.063
Mean of values in ICU			
CSF (μmol/L)	108 [72, 124]		
Plasma (μmol/L)	32 [28, 42]		
CSF:plasma ratio	3.2 [2.4, 3.8]		
Minimum value in ICU			
CSF (μmol/L)	87 [42, 124]		
Plasma (μmol/L)	24 [20, 34]		
CSF:plasma ratio	3.0 [2.0, 3.3]		
Maximum value in ICU			
CSF (μmol/L)	124 [85, 151]		
Plasma (μmol/L)	40 [31, 44]		
CSF:plasma ratio	3.3 [2.6, 3.9]		

CSF, cerebrospinal fluid; ICU, intensive care unit; SAH, subarachnoid haemorrhage. Data are median [IQR].

10. Discussion

10.1. Summary of main findings

We conducted this observational cohort study to compare plasma and CSF concentrations of ascorbate in patients with aSAH with those at term pregnancy. Our major findings are that: (i) more than half of patients developed plasma hypovitaminosis C within the two weeks post aneurysm rupture; (ii) plasma and CSF ascorbate levels were less in patients with aSAH from the day of first sampling compared to pregnant women, but with a preserved CSF to plasma ratio; and, (iii) the decrease in CSF ascorbate was more marked than the fall in plasma ascorbate with a progressive decline in the CSF to plasma ratio of ascorbate after the first week from aneurysm rupture. This was more pronounced in the setting of vasospasm and almost exclusively due to a reduction in CSF ascorbate concentrations. Finally, we also report CSF and plasma ascorbate levels in the healthy range in uncomplicated term pregnancy.

10.2. Relationship with other studies

Our findings, although novel, are consistent with previous observational data suggesting that oxidative stress is integral to the pathogenesis of subarachnoid haemorrhage, vasospasm and delayed cerebral ischemia.[16,17] Oxyhemoglobin released into the subarachnoid space is thought to generate free radicals and lipid peroxides which permeate the vessel wall, compromising production of the physiological vasodilators prostacyclin and nitric oxide, and directly injuring cerebrovascular endothelial and smooth muscle cells.[17–19] Ascorbate is the primary circulatory antioxidant and completely prevents detectable lipid peroxidation of low density lipoproteins and plasma lipids *in vitro*.[20]

Reactive oxygen species are difficult to measure in biological samples due to being present in low concentrations, having short biological half-lives, and being inherently unstable *in vitro*.[8] Accordingly, indirect indices of reactive oxygen species production have been employed to quantify oxidative stress post subarachnoid haemorrhage, including measurement of oxidatively modified macromolecules (lipid hydroperoxides) and antioxidants (vitamin C, vitamin E and uric acid).[16,17,21] In an observational cohort study, Polidori and colleagues compared plasma levels of lipid hydroperoxides and ascorbate between 25 patients with aSAH and 10 patients with lacunar strokes.[16] Patients with aSAH had significantly increased plasma lipid hydroperoxides and these elevated levels were associated with higher mortality. Similar to our findings, plasma ascorbate concentrations remained stable over time and tended to the low side of the normal range in the SAH population.[16] Plasma concentration of lipid hydroperoxides were significantly increased, and ascorbate concentrations significantly decreased, in patients who developed vasospasm compared with patients without vasospasm.[16] Kamezaki et al. reported CSF concentrations of lipid peroxides in 20 patients who underwent clipping for ruptured aSAH.[17] Increased levels of lipid peroxides in the CSF during the first week post aneurysmal rupture were predictive of both symptomatic vasospasm and poor outcome (severe disability or worse per the Glasgow Outcome Score).[17]

Intracisternal and intramuscular vitamin C as ascorbic acid have been shown to attenuate vasospasm in canine and rabbit models of SAH.[22–24] The only human data of vitamin C supplementation after aSAH comes from a single-centre Japanese case series of 217 patients.[25] Kodama et al. describe their local practice of routine use of cisternal irrigation therapy containing urokinase and vitamin C as ascorbic acid to prevent vasospasm following clipping of ruptured aneurysms.[25] They reported a low rate of vasospasm (2.8 %) with

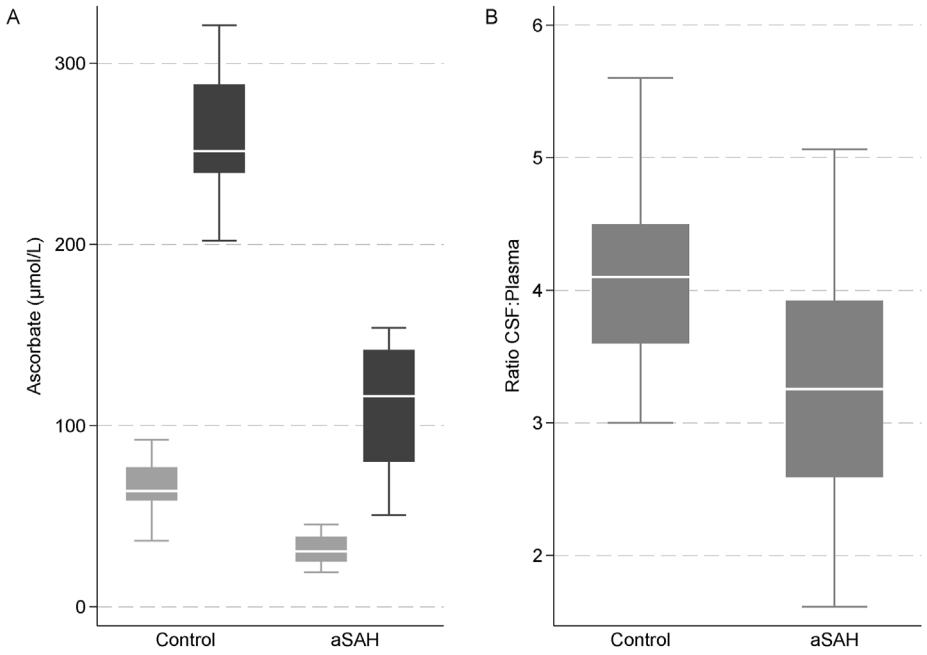

Fig. 2. Plasma and CSF ascorbate concentrations. (A): Plasma (light grey) and CSF (dark grey) concentrations of ascorbate in pregnant controls and aneurysmal SAH participants at the time of initial sampling (B): Ratio of CSF to plasma ascorbate concentrations in comparator group of pregnant women and aneurysmal SAH participants. Data are presented as centre marker = median and box limits = [IQR], with the upper and lower adjacent values (whiskers) being within 1.5x the IQR. SAH, subarachnoid haemorrhage; CSF, cerebrospinal fluid; IQR, interquartile range.

one patient (0.4 %) developing a permanent neurological deficit and no patients dying from complications of vasospasm.[25] Despite these intriguing data, the use of vitamin C, intracisternal or otherwise, in this group has not been subjected to a clinical trial.[26]

There has been one previous observational study reporting the concentration of ascorbate in the plasma and CSF of 21 pregnant women and ten healthy non-pregnant controls.[10] Both plasma and CSF ascorbate levels were higher in the pregnant cohort; mean (±SD) plasma (34 ± 16 vs. 19 ± 16 μmol/L, $p < 0.05$) and CSF (188 ± 31 vs. 158 ± 16 μmol/L, $p < 0.05$).[10] However, the mean plasma ascorbate concentration in the healthy cohort was below the threshold for hypovitaminosis C (<23 μmol/L) raising questions of the external validity of these findings.

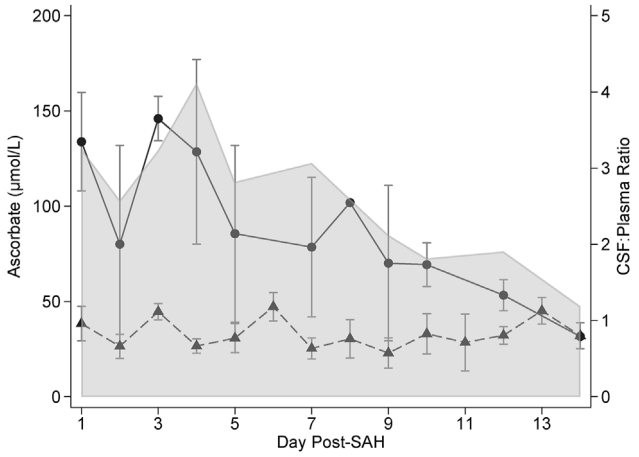

Fig. 3. Temporal profile of plasma and CSF ascorbate concentrations in patients with aSAH. Mean ascorbate concentration profiles (95%CI) over study day (1–14) in patients with aneurysmal SAH: CSF (= closed circles) and plasma (= closed triangles). CSF:plasma ratio is given by the shaded background. CSF, cerebrospinal fluid; SAH, subarachnoid haemorrhage.

10.3. The implications for future research or clinical practice

By measuring ascorbate in both the plasma and CSF, and comparing to a control population without neurological injury, we have been able to demonstrate that there is a disproportionate degree of hypovitaminosis C in the brain following SAH. We have demonstrated that this worsens over time, mirrors the known timing for clinical vasospasm, and is more pronounced in patients who develop such vasospasm. This provides mechanistic justification for ascorbate replacement or administration of supra-physiological supplementation to reduce the risk of vasospasm and improve neurological outcomes following SAH. The enteral route is inadequate to achieve supraphysiological plasma concentrations of ascorbate in the critically ill. Of note, all the patients with SAH were enterally fed with formulations containing vitamin C, reinforcing the requirement for administering ascorbate via the intravenous route.[27] Priorities for future research include determining whether supraphysiological concentrations of ascorbate in the CSF can be achieved with intravenous infusions and ultimately whether this may reduce the incidence of vasospasm and delayed cerebral ischemia.

10.4. The strengths and limitations of the study

This is the first study to report ascorbate concentrations in the CSF post aSAH. By collecting CSF over a maximum period of 21 days, we were able to demonstrate deterioration in ascorbate concentrations over time, report relationships with vasospasm and compare this to a population of controls without neurological injury. Several limitations should be acknowledged. Pregnant women were recruited as the comparator population as they routinely have the subarachnoid space accessed for delivery of spinal anaesthesia. Accordingly, there are inherent differences between the two groups including younger age, gender balance, routine use of vitamin C supplements and pregnancy status itself. In fact, female gender and younger age are associated with higher

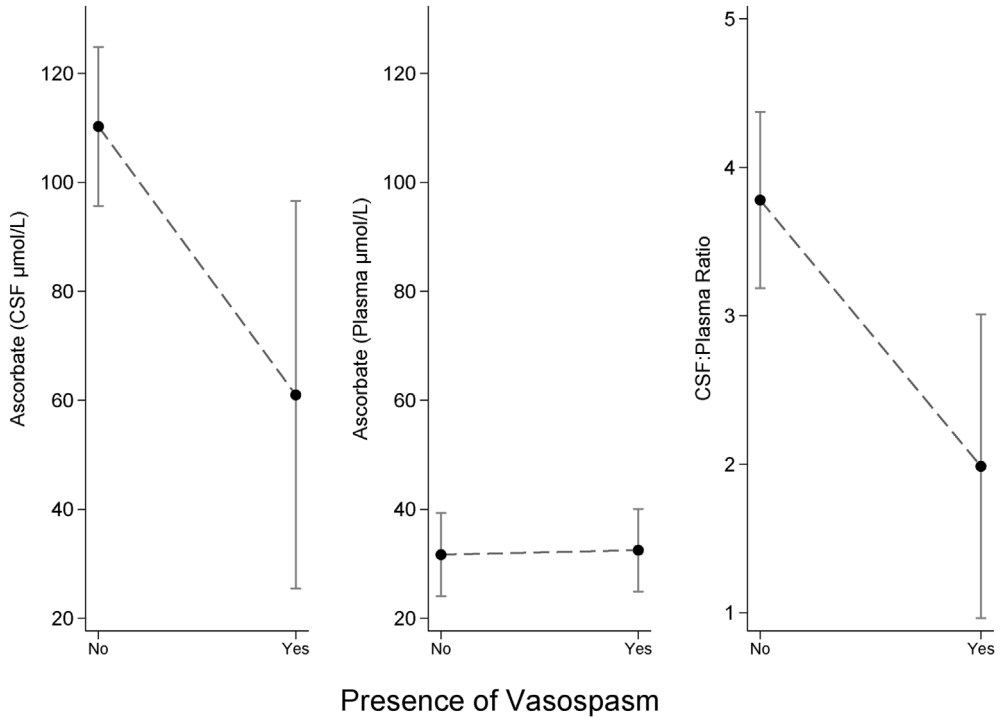

Fig. 4. Plasma and CSF ascorbate concentrations by vasospasm status. Mean (95%CI) CSF and plasma ascorbate concentrations, and the ratio of CSF:Plasma from all collected samples in those with and without vasospasm. Adjusted for repeated measures within subjects employing random effects regression with robust standard errors; P-values 0.012, NS and 0.003, respectively. CSF, cerebrospinal fluid; NS, not significant.

plasma ascorbate in health.[28] Pre-morbid plasma and CSF ascorbate concentrations are unknown in our SAH population, and we are unable to comment whether early samples reflect baseline hypovitaminosis C, an acute response to aneurysmal rupture or both. Due to the small sample size of aSAH participants with EVDs and sporadic sampling, we are restricted to binary comparisons in subgroup analyses and are unable to report temporal relationships between CSF concentrations and outcomes. While fresh CSF was collected and processed from the EVD within an hour of drainage we cannot exclude small decrements in the measured ascorbate concentration due to exposure to oxygen and light. Radiographical vasospasm can occur in the absence of a clinical correlate, and future research would be enhanced by using delayed cerebral ischemia rather than radiological vasospasm as a clinical endpoint. Finally, we did not collect long term neurological outcome, functional outcome, or quality of life data.

11. Conclusions

Median plasma and CSF ascorbate levels are lower in aSAH than in healthy pregnant women with half of the patients with aSAH developing hypovitaminosis C. Moreover, the ratio of CSF to plasma ascorbate concentration temporally decreases in the weeks post aSAH, almost entirely due to a reduction in CSF levels. Finally, this ratio is lowest in patients who develop vasospasm. These findings provide a biological rationale for future interventional studies of intravenous ascorbate supplementation in patients with aSAH.

Credit authorship contribution statement

Natasha Turner, Mark E Finnis, Yugeesh R Lankadeva, Yasmine Ali Abdelhamid, Adam M Deane and Mark P Plummer contributed to study conception and design, acquisition, analysis and interpretation of data, drafting and revising the manuscript for intellectual content and final approval of the version to be published. Brodie Farrow, Ashenafi H Betrie, Jeremy Sharman, and Patrick Tan contributed to acquisition of data for the work, revisiting the manuscript for critically important intellectual content. All authors met the authorship requirements outlined within the author guidelines and the final version of the manuscript was approved by all authors to be published.

Data sharing statement

The data that support the findings of this study are available on scientifically justified request from the corresponding author.

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Conflict of interest

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests: Yugeesh R Lankadeva has patent issued to Yugeesh R Lankadeva. Both Adam M Deane and Mark P Plummer are appointed as associate editors for CCR journal (voluntary role).

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ccrj.2023.10.003.

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