Increased interleukin-6 levels in neuron-derived plasma small extracellular vesicles in patients with aneurysmal subarachnoid hemorrhage

Niansheng Lai  
First Affiliated Hospital of Wannan Medical College

Yang Yao  
First Affiliated Hospital of Wannan Medical College

Feiyun Qin  
First Affiliated Hospital of Wannan Medical College

Tao Yu  
First Affiliated Hospital of Wannan Medical College

Dayong Xia  
First Affiliated Hospital of Wannan Medical College

Xintong Zhao  
First Affiliated Hospital of Wannan Medical College

Degang Wu  
First Affiliated Hospital of Wannan Medical College

Jiaqiang Liu  
First Affiliated Hospital of Wannan Medical College

Jinlong Yuan  
First Affiliated Hospital of Wannan Medical College

Xinggen Fang  
First Affiliated Hospital of Wannan Medical College

Zhenbao Li (zhenbaoli123@126.com)  
First Affiliated Hospital of Wannan Medical College

Research

Keywords: Subarachnoid hemorrhage, Small extracellular vesicles, Interleukin-6, Biomarker

Posted Date: February 29th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-15513/v1
Abstract

Background Aneurysmal subarachnoid hemorrhage (aSAH) is a severe type of stroke characterized by high rates of mortality and disability. Identifying circulating biomarkers is helpful to improve prognosis. In this study, for the first time, we identify circulating interleukin-6 (IL-6) levels in neuron-derived small extracellular vesicles (NDSEVs) as potential biomarkers in prognosis of aSAH.

Methods We extracted small extracellular vesicles from the plasma of aSAH patients and healthy controls and enriched them using sequential precipitation and anti-L1CAM antibody immunoabsorption. Subsequently, we determined IL-6 levels using an enzyme-linked immunosorbent assay (ELISA).

Results Plasma IL-6 NDSEVs showed distinct pattern differences between aSAH patients and healthy controls. There were significant correlations of IL-6 concentrations in plasma with severity in aSAH. The AUCs of IL-6 for distinguishing the severe aSAH patients from mild aSAH patients were 0.961. After multivariate logistic regression analysis, only age, acute hydrocephalus, and the levels of IL-6 NDSEVs enabled prediction of neurological outcome at 1 year. The IL-6 NDSEVs levels were greater and positively associated with disease and prognosis of aSAH patients.

Conclusions These data suggest a neuroinflammatory cascade in aSAH patients. IL-6 NDSEVs may be a biomarkers to monitor the progression of aSAH.

Background

Aneurysmal subarachnoid hemorrhage (aSAH) typically results from a ruptured aneurysm and is a clinical syndrome with 45% mortality and disability with morbidity of approximately 6–16 per 100000 individuals every year worldwide. It occurs at young ages and accounts for 5–7% of the total incidence of stroke[1, 2]. Major prognostic determinant include characteristics of the initial hemorrhage, which causes early brain injury (EBI) and early cerebral vasospasms, and may be associated with delayed cerebral ischemia (DCI) [3]. Recently, researchers have found that EBI after SAH may be a leading factor contributing to unfavorable outcomes[4, 5]. These findings suggest the importance of pathophysiologic mechanisms in the very early phase after aSAH, characterized by changes including microvascular filling defects, breakdown of ionic homeostasis, inflammation, and microarterial narrowing [6–8]. A reliable, early, economic and non-invasive approach is urgently in need to screen patients so as to improve prognosis.

Several studies reported small extracellular vesicles (sEVs), which are lipid membrane vesicles, cross blood-brain barrier (BBB) and specialize in long-distance intercellular communications, facilitating transfer of proteins, lipids, and nucleic acid for subsequent protein expression in target cells[9, 10]. sEVs also released by brain cells cross the BBB and can be detected in circulating blood[11–13]. Similarly, endothelial and peripheral cells secrete sEVs into the circulation. sEVs can be enriched from peripheral blood samples and can be used for detection of various proteins, lipids, and nucleic acids[14]. Together, these circulating sEVs potentially be ideal biomarkers to reflect the pathological progress of aSAH.
Several clinical studies have examined circulating sEVs contents, including functional proteins and various nucleic acid species as biomarkers for cerebral ischemia [15–19].

There is substantial evidence that the inflammatory response occurs early after SAH and contributes to the progression of SAH-induced EBI [4, 8, 20]. Potential biomarkers have been studied, including interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, IL-18, and tumor necrosis factor-alpha [21–23]. IL-6 is a proinflammatory cytokine, levels of which elevate in response to acute brain injury and other diseases [17, 24, 25]. Several studies have demonstrated that elevated IL-6 levels induce neuroinflammation and may be closely associated with the prognosis of aSAH [23, 26, 27].

Therefore, we hypothesized that aSAH leads to changes in expression levels of IL-6 in sEVs in the brain and that these NDSEVs are secreted into the circulation where they may serve as biomarkers for aSAH. In the present study, we extracted NDSEVs from the plasma of aSAH patients and healthy controls to determine the expression of IL-6 levels in NDSEVs and, ultimately, we detected IL-6 levels in NDSEVs of aSAH patients, which evaluated possible associations between the markers of early inflammatory response and disease progression.

Methods

Ethics

Study participants were recruited from the Department of Neurosurgery, The First Affiliated Hospital of Wannan Medical College, Wuhu City, China. The study was performed in accordance with the Declaration of Helsinki. Written informed consent was received from participants or valid proxies (family or a professional not directly involved in the study) prior to inclusion in the study. All experiments were approved by the Ethics Committee of the First Affiliated Hospital of Wannan Medical College.

Study design

The aSAH patients included in the present study were admitted from May 2016 to March 2017. We collected plasma from 117 patients with aSAH within the 24 h after SAH, out of a total of 224 patients with SAH during this period. The mean age ± SD was 59.91 ± 9.65 years (range, 40–83 y); there were 72 women and 45 men. Exclusion criteria were as follows: admission later than 24 h after onset of bleeding; non-aneurysmal SAH; liver, kidney, heart or lung insufficiency or infectious diseases; rebleeding after admission and poor prognosis upon admission without any intervention. Age-matched health persons in the fasting state were used as controls (n = 40).

Initial clinical status was accessed was using World Federation of Neurological Surgeons (WFNS) grading [28]. The amount of blood in computed tomography (CT) was assessed using the modified Fisher’ scale. Hydrocephalus and DCI were classified as present or absent based on CT scans or magnetic resonance imaging (MRI). All patients were treated with endovascular coiling. Neurologic outcome was assessed using the Modified Rankin Scale (mRS) at 1-year post-aSAH using a structured telephone
interview[29]. If no contact was obtained after this procedure, the patient was declared lost to follow-up. At the end of the follow-up period, patients with mRS scores of 0–2 were classified as having good outcomes, and those with mRS scores of 3–6 were classified as having poor outcomes. Tables 1 and 2 summarizes the basic characteristics of the population.

Sample processing

The blood samples were processed for plasma isolation within 2 h after withdrawal using BD EDTA-treated plasma separator tubes. Whole blood was centrifuged at 500 rpm and 4 °C for 10 min. The upper-layer was transferred into a RNase/DNase-free 1.5 mL EP tubes followed by further centrifugation at 3000 rpm and 4 °C for 10 min. Plasma was divided into aliquots and stored at -80 ºC for further analysis.

Isolation of NDSEVs from plasma samples

sEVs were isolated using miRCURY Exosome Kits (from Cell/Urine/CSF; Qiagen, Valencia, CA) following the manufacturer's protocol. Briefly, 400 μL of precipitation buffer B was added to 1 mL of CSF samples and mixed well for 60 minutes at 4 °C, followed by centrifugation at 10,000 g for 30 minutes at room temperature. The sEVs were harvested by removing the supernatant. The sEVs were resuspended in 100 μL of resuspension buffer. To enrich NDSEVs, total sEVs were harvested by immunoadsorption enrichment with mouse anti-human CD171 (L1CAM neural adhesion protein) biotinylated antibody (eBiosciences, San Diego, CA, USA), as described[11].

Then NDSEVs were lysed by 1% Triton X-100 lysis buffer (Beyotime, P0013) that contained protease and phosphatase inhibitor cocktails. The lysates were stored at -80 ºC.

Western blot analysis

Molecular weight maker (5 μl /lane; Thermos Scientific, Waltham, MA, USA) and protein samples (20 μg/lane) were separated using a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and electrophoretically transferred onto polyvinylidene difluoride membranes (PVDF, Immobilon-PSQ, Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature. Blots were incubated with primary antibodies in 5% BSA (in PBS + 0.1% Tween 20) overnight at 4 °C. The primary antibodies used were as follows: mouse anti-CD63 (1:1,000, Abcam), and rabbit anti-Alix (1:1,000, Abcam). Corresponding HRP-conjugated anti-rabbit, or anti-mouse (1:10,000, Pierce) secondary antibodies were incubated for 2 h at room temperature. Bands were visualized using an enhanced chemiluminescence (ECL) kit (Beyotime).

Transmission electron microscopy (TEM)

NDSEVs were adsorbed on carbon-coated nickel grids for 1 h, subsequently washed three times with PBS for 5 minutes and fixed with 2% formaldehyde for 10 min. Samples were contrasted using uranyl acetate and lead citrate (Sigma-Aldrich). After three washings in deionized water, grids were dried for several minutes and finally examined with a JEOL JEM-1400 transmission electron microscope at 80 kV.
**Enzyme-linked immunosorbent assay (ELISA)**

The concentrations of IL-6, and tetra-spanning exosome marker CD81 in NDSEVs were quantified using specific ELISA kits (Elabscience Biotechnology, China) according to the manufacturer’s instructions. The mean value for all determinations of CD81 in the patient cohort was set at 1.00 and relative values for each specimen were used to normalize their recovery. The final concentration of cytokines was measured using OD values. One laboratory technician measured all ELISAs without knowledge of the clinical information.

**Statistical analysis**

All data were analyzed using MedCalc version 15.0.0 (Medcalc Software bvba, Ostend, Belgium). Data were presented as mean ± standard deviation (SD). The Mann–Whitney U test was used to assess the differences between two groups and the Kruskal–Wallis test was used for differences between more than two groups. The correlations among the variables were calculated using Spearman rank correlation coefficient analysis. For comparison of levels of IL-6 over time between patients with poor and good outcome, a generalized linear relationship model with Spearman's correlation coefficient was used. Receiver operating characteristic (ROC) curves were constructed to determine the optimal thresholds of IL-6 for aSAH. A multivariable logistic regression model was analyzed to determine factors that predicted the mRS after adjusting for risk factors that reached P <0.1 in the univariate analyses. A P-value of <0.05 was considered significant.

**Results**

**Patient characteristics**

In this pilot study, a total of 117 aSAH patients and 40 health controls were recruited. One hundred and seven patients were excluded because of the reasons listed in Fig. 1. Plasma was taken at 24 h after SAH, and obtained in the fasting state in each healthy control. The detailed characteristics for these two groups are summarized in Table 2.

**NDSEV characterization**

The morphology of NDSEVs was evaluated using TEM. NDSEVs had a spherical shape with sizes of 30–100 nm surrounded by a membrane (Fig. 2A). There was no apparent difference in the size or shape of NDSEVs between the control and aSAH samples. Western blotting analysis showed that CD63 and Alix were expressed in NDSEVs (Fig. 2B).

**IL-6 in NDSEVs of aSAH patients and healthy controls**

The expression levels of IL-6 were determined in 117 aSAH patients and 40 healthy controls. The CD81-normalized levels of IL-6 in NDSEVs were significantly increased at 1-day post-aSAH when compared with healthy controls (aSAH: 365.71 ± 44.57 pg/ml; controls: 8.81±1.63 pg/ml; P <0.001) (Fig. 3).
Relationships of IL-6\textsubscript{NDSEVs} with aSAH severity

The WFNS grade is a tool used to assess the level of early brain injury after SAH. The modified Fisher’ scale was used to assess the amount of blood in CT images of aSAH patients. At the beginning of the acute period, the patients with a WFNS grade of I–III were classified as mild (mild aSAH), and those with a WFNS grades IV–V were classified as severe (severe aSAH). As illustrated in Fig. 4A, comparison of the severe aSAH patients with the mild showed that IL-6 levels were significantly greater (severe aSAH: 409.14 ± 27.06 pg/ml; mild aSAH: 338.56 ± 29.15 pg/ml) (P <0.001). To evaluate the utility of IL-6\textsubscript{NDSEVs} for discrimination of severe aSAH from mild aSAH, we performed ROC curve analysis and found that the area under curves (AUCs) of IL-6\textsubscript{NDSEVs} was 0.961 (95% CI: 0.909–0.988; P <0.001) (Fig.4B). The highest accuracy was at a cutoff expression value of 375.18, where the positive predictive value, negative predictive value, sensitivity, and specificity to identify severe aSAH were 91.1, 94.4, 91.11, and 94.44%, respectively.

To further investigate the relationships between the expression levels of IL-6\textsubscript{NDSEVs} and WFNS grade, Spearman’s correlation coefficient analysis was performed. The results revealed that in the aSAH patients, the level of IL-6\textsubscript{NDSEVs} ($\rho = 0.845; 95\% \text{ CI}: 0.753 \text{ to } 0.890; P <0.001$) was closely correlated with aSAH severity as scored by WFNS grade.

Relationships of IL-6\textsubscript{NDSEVs} with clinical outcomes of aSAH patients

It is important to determine the potential outcomes of aSAH patients at the earliest stage to optimize the treatment. Patients were divided into two groups according to their clinical outcomes. At the end of the follow-up period, patients with mRS scores 0–2 were classified as having good outcomes, and those with mRS scores 3–6 were classified as having poor outcomes. The mRS scores were lower in patients with lower expression of IL-6\textsubscript{NDSEVs} than in those with higher expression (P <0.001; Fig. 5A). On univariate analysis, age, aneurysm diameter, WFNS grade, modified Fisher score, acute hydrocephalus, DCI and IL-6\textsubscript{NDSEVs} levels were prognostic factors of 1-year post-SAH (P <0.1). In the multivariable logistic regression models, age, acute hydrocephalus and the levels of IL-6\textsubscript{NDSEVs} were significantly correlated with mRS score at 1 year post-SAH (P < 0.001; Table 3). Additionally, ROC curves revealed that the combined of IL-6\textsubscript{NDSEVs} and the clinical parameters were robust in discriminating poor outcomes, with an AUC value of 0.989 (95% CI: 0.950 to 1.00; P<0.001; Fig. 5B). Therefore, outcome prediction may be improved by determination of the expression of IL-6\textsubscript{NDSEVs}.

Discussion

In the present study, first, we found circulating vesicles in aSAH patients and healthy controls. Second, we found that the circulating IL-6\textsubscript{NDSEVs} showed distinct pattern differences between aSAH patients and healthy controls. Third, the circulating IL-6\textsubscript{NDSEVs} were higher in patients with higher WFNS grade than in
those with lower WFNS grade. Finally, the circulating IL-6 NDSEVs were associated with prognosis in aSAH patients.

Several inflammatory cytokines have been found to be linked to aSAH[21]. IL-6 plays an important role in brain injury, and associated with aSAH in numerous studies[23, 26, 30]. A previous study showed that IL-6 in cerebrospinal fluid (CSF) was a biomarker for predicting vasospasm after SAH[31]. Previous studies also showed that IL-6 levels in CSF were associated with prognosis in aSAH patients[21, 27, 30]. Elevated IL-6 levels in CSF may induce neuroinflammation and may be closely associated with the progression of delayed ischemic neurological deficits after SAH[25]. IL-6 and TNF-α in CSF could be important biomarkers for early diagnosis and disease monitoring in SAH patients[22]. Another study reported that CSF IL-6 was a biomarker for ventricular infection[32]. Yet another study showed that IL-6 in CSF could be a useful diagnostic tool for predicting shunt dependency in aSAH patients with acute hydrocephalus[33]. Serum IL-6 levels were elevated, and were associated with prognosis in aSAH patients[26, 34]. However, many previous studies also showed that determining the IL-6 levels in blood circulation may not be sufficient[30, 35]. Circulating IL-6 might directly reflect the situation of the brain, and peripheral factors might have little effect on cytokines in NDSEVs. For these reasons, the measurement of IL-6 levels in CNS-derived sEVs, as opposed to in blood or CSF, may be better reflect the actual role of IL-6 in aSAH. Therefore, in the present study, we determined whether IL-6 NDSEVs could serve as biomarkers between aSAH patients and healthy controls.

The important findings of our study were that IL-6 NDSEVs levels were elevated in aSAH patients, suggesting they might serve a biomarker for the prognosis of aSAH. IL-6 NDSEVs expression may be used to discriminate severe aSAH from mild aSAH. ROC curves with AUC values were constructed. The sensitivity and specificity shown in ROC curve were actually high for IL-6 NDSEVs levels. The age, acute hydrocephalus and IL-6 NDSEVs were good predictors of neurologic outcome, whereas aneurysm diameter, WFNS grade, DCI, or modified Fisher score were not. These results suggest that IL-6 may participate in the occurrence, development and repair progression of aSAH. Other CNS diseases as well may be associated with neuroinflammation. In fact, it has been reported that inflammatory cytokines in NDSEVs are elevated in traumatic brain injury[11]. Increased IL-6 Levels in astrocyte-derived exosomes could be useful to reveal neuroinflammation in sporadic amyotrophic lateral sclerosis patients[36]. For these reasons, IL-6 NDSEVs may not be suitable to help distinguish aSAH from other CNS diseases.

Although the present study aimed to investigate IL-6 NDSEVs levels as potential biomarkers correlated with disease severity and prognosis in aSAH patients, there were a few methodological limitations. First, these was a one single-center study with a wide age range, a small number of patients, and a diverse grade of severity of aSAH with WFNS grades I–V. Second, the samples were collected from patients after surgery and during drug therapy. The surgical treatment and drugs may induce changes in the expression levels of IL-6 NDSEVs.

**Conclusion**
IL-6_{NDSEVs} may be a reliable biomarker associated with EBI of aSAH. IL-6_{NDSEVs} could be another compelling predictor of clinical outcomes. The indicator may be an early outcome predictor for patients with aSAH, but not an independent one. Future studies are critical to determine whether IL-6_{NDSEVs} plays a regulatory role in disease progression of aSAH and to evaluate its potential as a therapeutic target.

**Abbreviations**

aSAH: Aneurysmal subarachnoid hemorrhage; AUCs: Area under curves; BBB: Blood-brain barrier; BCA: Bicinchoninic acid; CNS: Central nervous system; CSF: cerebrospinal fluid; CT: Computed tomography; CVs: Cerebral vasospasm; DCI: Delayed cerebral ischemia; EDTA: Ethylene Diamine Tetraacetic Acid; ELISA: Enzyme-linked immunosorbent assay; EBI: Early brain injury; ECL: Enhanced chemiluminescence; IL-6: Interleukin-6; mRS: Modified Rankin Scale; NDSEVs: Neuron-derived small extracellular vesicles; ROC: Receiver operating characteristic; SD: Standard deviation; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gels; TEM: Transmission electron microscopy; WFNS: World Federation Of Neurosurgical Societies.

**Declarations**

**Acknowledgements**

The authors thank all participants and cooperating clinicians, and the department of the Central Laboratory of First Affiliated Hospital of Wannan Medical College for the support of basic facilities and Jiaqi Zhang and Bingbing Zhang for assistance with the sample collection.

**Funding**

This work was supported by grants from the National Natural Science Foundation of China (no.81701357), Key Research and Development Program of Anhui province (no.201904a07020034), Funding of "Peak" Training Program for Scientific Research of First Affiliated Hospital of Wannan Medical College (no.GF2019G05), Anhui Province's (no.1708085QH181), Natural Science Research Project in Higher Education of Anhui Province (no.KJ2018A0253), and Science Research Project of Professional Personnel of First Affiliated Hospital of Wannan Medical College(no.YR201911).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

Niansheng Lai, Yang Yao, and Feiyun Qin conducted experiments and drafted the manuscript. Tao Yu, Dayong Xia, Xintong Zhao, Degang Wu, Jiaqiang Liu, and Jinlong Yuan contributed to the experiment
design and statistical analysis. Xinggen Fang and Zhenbao Li designed the experiments and revised the manuscript.

**Ethics approval and consent to participate**

Study participants were recruited from the Department of Neurosurgery at The First Affiliated Hospital of Wannan Medical College, Wuhu City, China. The study was performed in accordance with the Declaration of Helsinki. Written informed consent was received from participants or valid proxies (family or a professional not directly involved in the study). All experiments were approved by the Ethics Committee of the First Affiliated Hospital of Wannan Medical College.

**Consent for publication**

Not applicable.

**Competing interests**

The authors claim no conflict of interest.

**References**


Tables

Table 1. Characteristics and clinical data of study population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>aSAH group, n=117</th>
<th>Control group, n=40</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex; Male</td>
<td>45</td>
<td>16</td>
<td>0.85</td>
</tr>
<tr>
<td>Sex; Female</td>
<td>72</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Age; mean ± SD (years)</td>
<td>59.91± 9.65</td>
<td>57.03± 9.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Hypertension; Yes</td>
<td>49</td>
<td>15</td>
<td>0.71</td>
</tr>
<tr>
<td>Hypertension; No</td>
<td>68</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Smoking; Yes</td>
<td>40</td>
<td>11</td>
<td>0.56</td>
</tr>
<tr>
<td>Smoking; No</td>
<td>77</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>IL-6&lt;sub&gt;NDSEVs&lt;/sub&gt; ± SD(pg/mL)</td>
<td>365.71±44.57</td>
<td>8.81±1.63</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NDSEVs Neuron-derived small extracellular vesicles; SD Standard deviation

Table 2. Clinicopathological features of aSAH

<table>
<thead>
<tr>
<th>Parameters</th>
<th>mRS 0-2, n=78</th>
<th>mRS 3-6, n=39</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex; Male</td>
<td>30</td>
<td>15</td>
<td>1.00</td>
</tr>
<tr>
<td>Sex; Female</td>
<td>48</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Age; mean ± SD (years)</td>
<td>58.78±9.11</td>
<td>62.15±10.40</td>
<td>0.075</td>
</tr>
<tr>
<td>Hypertension; Yes</td>
<td>32</td>
<td>17</td>
<td>0.84</td>
</tr>
<tr>
<td>Hypertension; No</td>
<td>46</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Smoking; Yes</td>
<td>25</td>
<td>15</td>
<td>0.54</td>
</tr>
<tr>
<td>Smoking; No</td>
<td>53</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Diabetes; Yes</td>
<td>12</td>
<td>7</td>
<td>0.80</td>
</tr>
<tr>
<td>Diabetes; No</td>
<td>65</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Alcohol abuse; Yes</td>
<td>7</td>
<td>7</td>
<td>0.23</td>
</tr>
<tr>
<td>Alcohol abuse; No</td>
<td>71</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Aneurysm position</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACA + AComA</td>
<td>21</td>
<td>15</td>
<td>0.52</td>
</tr>
<tr>
<td>ICA + PComA</td>
<td>27</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>MCA</td>
<td>19</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Vertebrobasilar system</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Aneurysm diameter; mean ± SD(mm)</td>
<td>5.72±2.15</td>
<td>8.16±3.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WFNS Grade ; I</td>
<td>14</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WFNS Grade ; II</td>
<td>30</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>WFNS Grade ; III</td>
<td>19</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>WFNS Grade ; IV</td>
<td>15</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>WFNS Grade ; V</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Modified Fisher Score; I</td>
<td>5</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Modified Fisher Score; II</td>
<td>14</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Modified Fisher Score; III</td>
<td>39</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Modified Fisher Score; IV</td>
<td>20</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Acute hydrocephalus; Yes</td>
<td>11</td>
<td>18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acute hydrocephalus; No</td>
<td>67</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>DCI; Yes</td>
<td>12</td>
<td>23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DCI; No</td>
<td>66</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>IL-6&lt;sub&gt;NDSEVs&lt;/sub&gt; ± SD(pg/mL)</td>
<td>342.24±29.48</td>
<td>412.65±30.18</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

ACA Anterior cerebral artery; AComA Anterior communicant artery; DCI Delayed cerebral ischemia; ICA Internal carotid artery; MCA Middle cerebral artery; mRS Modified Rankin Scale; NDSEVs Neuron-derived plasma small extracellular vesicles; PComA Posterior communicant artery; SD Standard deviation; WFNS World Federation of Neurological Surgeons;
Table 3. Multivariable logistic analyses of risk factors in patients with aSAH at 1-year

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OR</th>
<th>95%CI</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per year</td>
<td>1.17</td>
<td>1.02 to 1.35</td>
<td>0.024</td>
</tr>
<tr>
<td>Aneurysm diameter &gt; 6.5mm</td>
<td>16.52</td>
<td>0.999 to 273.08</td>
<td>0.050</td>
</tr>
<tr>
<td>WFNS Grade</td>
<td>0.092</td>
<td>0.0075 to 1.13</td>
<td>0.062</td>
</tr>
<tr>
<td>Modified Fisher Score</td>
<td>0.14</td>
<td>0.012 to 1.58</td>
<td>0.11</td>
</tr>
<tr>
<td>Acute hydrocephalus</td>
<td>0.0043</td>
<td>0.0001 to 0.29</td>
<td>0.011</td>
</tr>
<tr>
<td>DCI</td>
<td>4.63</td>
<td>0.50 to 42.89</td>
<td>0.18</td>
</tr>
<tr>
<td>IL-6_{NDSEVs} levels</td>
<td>1.30</td>
<td>1.11 to 1.52</td>
<td>0.0013</td>
</tr>
</tbody>
</table>

The median of aneurysm diameter was 6.5 mm; CI confidence interval; OR odds ratio; WFNS World Federation of Neurological Surgeons; MFS Modified Fisher Score

Figures

Patients with SAH (n=224)

107 patients excluded
1. Admission more than 24h after aSAH (n=19).
2. Non-aneurysmal SAH (n=31).
3. Liver, kidney, heart or lung insufficiency or infectious diseases (n=18).
4. Rebleeding after admission (n=7).
5. Poor prognosis without any intervention (n=9).
6. Refusal of participation (n=12).
7. Loss of follow up or withdrawal of study (n=7).
8. Not enough sample volume (n=4).

117 patients finally included in this study

Patients with good outcome (n=78) Patients with poor outcome (n=39)
Figure 1

Inclusion criteria flowchart for the population investigated in this study.

Figure 2

Characterization of exosomes. (A) Transmission electron microscopy of isolated plasma exosomes. Bar, 100 nm. (B) Western blotting analysis of exosome markers (CD63 and Alix).
Figure 3

Expression of IL-6NDSEVs in aSAH patients and healthy controls. ***P <0.001.
Figure 4

(A) Relative levels of IL-6NDSEVs in aSAH patients with severe aSAH and those with mild aSAH. (B) ROC curves to distinguish severe from mild aSAH patients. ***P < 0.001.

Figure 5

(A) Relative levels of IL-6NDSEVs in aSAH patients with good outcome and those with poor outcome. ***P < 0.001.(B) ROC curves to distinguish SAH patients with poor outcomes.