Free Fatty Acids and Delayed Cerebral Ischemia After Subarachnoid Hemorrhage

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Background and Purpose—The purpose of this study was to understand factors related to increases in serum free fatty acid (FFA) levels and association with delayed cerebral ischemia (DCI) after subarachnoid hemorrhage.

Methods—We performed serial measurement of systemic oxygen consumption by indirect calorimetry and FFA levels by liquid chromatography/mass spectrometry in the first 14 days after ictus in 50 consecutive patients with subarachnoid hemorrhage. Multivariable generalized estimating equation models identified associations with FFA levels in the first 14 days after SAH and Cox proportional hazards model used to identified associations with time to DCI.

Results—There were 187 measurements in 50 patients with subarachnoid hemorrhage (mean age, 56±14 years old; 66% women) with a median Hunt–Hess score of 3. Adjusting for Hunt–Hess grade and daily caloric intake, n-6 and n-3 FFA levels were both associated with oxygen consumption and the modified Fisher score. Fourteen (28%) patients developed DCI on median postbleed Day 7. The modified Fisher score (P=0.01), mean n-6:n-3 FFA ratio (P=0.02), and mean oxygen consumption level (P=0.04) were higher in patients who developed DCI. In a Cox proportional hazards model, the mean n-6:n-3 FFA ratio (P<0.001), younger age (P=0.05), and modified Fisher scale (P=0.004) were associated with time to DCI.

Conclusions—Injury severity and oxygen consumption hypermetabolism are associated with higher n-6 FFA levels and an increased n-6:n-3 FFA ratio is associated with DCI. This may indicate a role for interventions that modulate both oxygen consumption and FFA levels to reduce the occurrence of DCI. (Stroke. 2012;:00-00.)

Key Words: fatty acids • oxygen consumption • subarachnoid hemorrhage • vasospasm

Cerebrovascular vasospasm, which occurs most commonly between 4 and 14 days post-subarachnoid hemorrhage, results in delayed cerebral ischemia (DCI) in approximately 21% of patients with subarachnoid hemorrhage (SAH) and is a leading cause of long-term morbidity.1,2 Vasospasm is thought to be the end result of the activation of inflammatory cytokines that affect the reactivity and relaxation of smooth muscles in cerebral vessels. Despite advances in our knowledge of risk factors, prevention and treatment protocols have not significantly altered the incidence or sequelae of vasospasm.3

Acute brain injury results in a disturbance in the normal metabolic mechanisms due to sympathetic nervous system activation and systemic inflammatory response resulting in a metabolic state that can promote secondary complications. Studies in other critical illnesses have not only demonstrated the importance of the metabolic response and related sequelae, but have also begun to demonstrate the possible benefit of immune-modulating nutritional support.4,5

The production of serum free fatty acids may represent a common pathway by which hypermetabolism influences nutritional status and complications after SAH. The role of lipid peroxidation after SAH has been recognized in both laboratory and clinical settings.6 This process directly stimulates smooth muscle contraction by exerting cytotoxic effects on the vessel wall and by generating an inflammatory response involving metabolites of arachidonic acid, an n-6 free fatty acid (FFA).6,7

We recently demonstrated a direct relationship between systemic oxygen consumption (VO2) and inflammation after SAH and further found that acute elevation in VO2 was an independent predictor of delayed cerebral ischemia.8 Although many factors after acute brain injury can influence changes in VO2, an increase in lipid peroxidation may be the end result of the hypermetabolic state.

In this study, we sought to understand the relationship between systemic VO2 and levels of both n-6 and n-3 FFA levels after SAH. We hypothesized that higher levels of n-6 and n-3 FFAs would be related to higher systemic oxygen consumption and further that levels of n-6 FFAs would mediate the relationship between VO2 and DCI in the first 2 weeks after SAH.

1. D. Seres, MD; M. S. Elkind, MD, MS, Feb 19, 2017 1. Subarachnoid hemorrhage (SAH) is a leading cause of long-term morbidity.1,2

2. M. S. Elkind, MD, MS, Feb 19, 2017 2. Approximately 21% of patients with subarachnoid hemorrhage, results in delayed cerebral ischemia (DCI) in approximately 21% of patients with subarachnoid hemorrhage (SAH) and is a leading cause of long-term morbidity.1,2

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Methods

Patient Selection and Data Collection

This is an analysis of a consecutive group of patients that underwent analysis of serum FFAs in addition to comprehensive nutritional assessments (n = 50) of a previously reported prospective observational study of patients with aneurysmal SAH admitted to the neurological intensive care unit at Columbia University Medical Center. The criteria for study inclusion have been previously published. The clinical care for patients with SAH at Columbia University Medical Center has been described previously and conforms to guidelines set forth by the American Heart Association.

All study patients underwent serial assessments of FFA and inflammatory and metabolic parameters during the first 14 days after SAH. Each assessment was conducted once during 4 predefined time periods or phases: postbleed Day 0 to 3, postbleed Day 4 to 7, postbleed Day 8 to 10, and postbleed Day 11 to 14. All parameters were measured during the same 24-hour period within each phase. Data collection was considered complete in instances when patients died or were discharged from the hospital before completion of the 4 phases. The clinical intensive care unit team was blinded to all indirect calorimetry (IDC), FFA, and high-sensitivity C-reactive protein measurements.

SAH Data Collection

This study was conducted in parallel to data collection for our Subarachnoid Hemorrhage Outcomes Project (SHOP), which has been previously described in detail. Briefly, SHOP is a prospective outcomes database that since July 1996 has collected data regarding admission and in-hospital characteristics as well as long-term global outcome in all patients with SAH admitted to the neurological intensive care unit at Columbia University Medical Center. DCI was defined as either the presence of symptomatic vasospasm or the presence of an infarction on CT scan attributable to vasospasm. Symptomatic vasospasm was defined as clinical deterioration (ie, a new focal deficit, decrease in level of consciousness, or both) in the presence of confirmed vasospasm determined by CT angiography or cerebral angiography. Decreased level of consciousness was defined as a 2-point drop in the Glasgow Coma Score in a 24-hour period. All patients who experienced clinical deterioration underwent CT angiography to determine the presence of vasospasm and to rule out other causes of deterioration (eg, fever, hydrocephalus, rebleeding, cerebral edema) followed by medical and/or interventional therapy as indicated. All end points were classified according to a priori criteria and adjudicated weekly at a SHOP database meeting. The adjudication process involved a consensus agreement of each end point by neurocritical care faculty (N.B., K.I., J.C., S.A.M.) after a complete review of source documentation, imaging, and laboratory tests.

Laboratory Measurements

Serum samples were assayed for high-sensitivity C-reactive protein using an enzyme-linked immunoassay (BioCheck, Inc; normal range <3.0 mg/L). All other laboratory measures were measured daily as part of routine laboratory testing and recorded as part of the assessment for inflammation and infectious disease status.

FFA Measurement

Serum FFA measurements were performed by a liquid chromatography/mass spectrometry method. Serum samples extracted using a modified Folch protocol. Briefly, 3 mL of 2:1 chloroform:methanol (v:v) and 50 μL of 0.25 mmol/L deuterated palmitic acid in methanol as an internal standard was added to 100 μL of serum in a clean glass tube. The mixture was vortexed well and centrifuged at 3000 g for 10 minutes to separate phases. The lower chloroform phase was transferred to another clean glass tube using a Pasteur pipette. Two milliliters of chloroform was added to the remaining upper aqueous phase and mixed well and again centrifuged at 3000 g for 10 minutes to separate phases. The lower chloroform phases pooled and evaporated to dryness under nitrogen. The lipid extract was reconstituted in 5 mL of methanol and 500 μL of the redissolved lipid extract transferred to an autosampler vial (Waters, Milford, MA) for processing by liquid chromatography/mass spectrometry.

Liquid chromatography/mass spectrometry measurements of specific n-fatty acid concentrations were carried out on a Waters Xevo TQ MS ACQUITY UPLC system (Waters). The system was controlled by MassLynx Software 4.1 (Waters). Samples in liquid chromatography/mass spectrometry vials were maintained at 4°C in the autosampler until 5 μL was injected onto a Waters ACQUITY UPLC BEH C18 column (2.1×100 mm, 1.7-μm particle size; Waters) and a 2.1×5-mm guard column using the same packing material. The column was maintained at 40°C. The flow rate was 300 μL/min in a binary gradient mode initiated using 15% Solvent A (100% H2O) and 85% Solvent B (100% methanol). The concentration of Solvent B was increased linearly to 100% over 8 minutes and maintained at 100% until 10 minutes. The gradient was then re-equilibrated to 15% Solvent A and 85% Solvent B over 2 minutes before the next sample was injected. Negative electrospray ionization mass spectrometry using the selected ion recording mode was performed using the following parameters: capillary voltage, 3.8 kV; source temperature 150°C; desolation temperature 500°C; and desolvation gas flow, 1000 L/h. Specific n-6 and n-3 compounds extracted are listed in Table 1.

Oxygen Consumption Measurements

Details regarding the method by which we performed IDC studies have been previously published. Each IDC assessment was based on a steady state, which was defined as a 20- to 30-minute interval during which average minute VO2 and carbon dioxide production changed by ≤5% and <10%, respectively. Continuous measurements were averaged every 60 seconds throughout the entire IDC session.

Statistical Analysis

Continuous variables were assessed for normality. Normally distributed data were reported as a mean and SD. Nonparametric data were reported and analyzed as a median with 25% and 75% percentile values. Categorical variables were reported as count and proportions in each group. Generalized estimating equation analyses were performed with an identity link function and an exchangeable within-group correlation structure to determine the relationship between FFA and VO2 as well as factors that may predict either FFA or VO2 measurements. Multivariate models were built by entering in those factors found to have a probability value ≤0.1 on univariate analysis. Among similar variables that were highly intercorrelated, only the variable with the largest Wald χ2 value and smallest probability value in the generalized estimating equation analysis was used as a candidate variable in the final multivariable model. Factors that were found on univariate analysis to be associated (P < 0.1) with DCI were entered into a backward Wald Cox proportional hazards model to calculate adjusted hazards ratios and corresponding 95% CI for developing DCI. Poor outcome at 3 months posthemorrhage was defined as a modified Rankin Scale score ≥4. For all tests,
significance was set at \( P<0.05 \). All analyses were performed with SPSS Version 16.0 (Chicago, IL).

**Informed Consent**

Given the minimal risk of this study and use of residual blood and urine for laboratory assessments, this study was conducted with a waiver of consent when necessary. Data were linked with the SHOP database, which uses a tiered consent process, whereby consent was obtained from those patients who were able to provide consent at the time of injury. In neurologically impaired patients, family members were approached for assent to participation in the study. In cases in which capacity was regained, patients were directly approached for consent. This process of consent and the conduct of both studies were approved by the Institutional Review Board and were consistent with guiding principles for research involving humans.14

**Results**

**Baseline Characteristics**

Of the 65 patients with SAH admitted during the study period, 50 met inclusion criteria for study (mean age, 56±14 years old; 66% women). Six were excluded for arriving late, 5 for early withdrawal of care, and 4 for inability to perform IDC. The mean body mass index was 28±6 kg/m²; the median admission Hunt–Hess grade was 3 (interquartile range [IQR], 2–4) and the modified Fisher score was 3 (IQR, 3–4). Admission variables categorized by DCI status are shown in Table 2. Patients who developed DCI were more likely to have a poor outcome (modified Rankin Scale score ≥4) at 3 months than those who did not develop DCI (71% versus 36%, \( P=0.03 \)).

**IDC Measurements**

There were 187 measurements in 50 patients with a 14-day mean \( \text{VO}_2 \) of 240±70 mL/min per patient. There were 86 measurements done during mechanical ventilation with 46 of these measurements done at the time patients were on propofol (median dose, 30 \( \mu \)g/kg/min; IQR, 16–50 \( \mu \)g/kg/min). The median daily caloric intake during the day of each IDC measurement was 7.7 calories/kg (IQR, 1.2–16.6 calories/kg). The median high-sensitivity C-reactive protein and aneurysm size shown as median (25th percentile, 75th percentile).

**n-FFA Measurements**

The overall 14-day mean n-6 FFA and n-3 FFA levels were 206.3±79.3 \( \mu \)mol/L and 19.2±6.4 \( \mu \)mol/L, respectively, with a 14-day mean n-6:n-3 FFA ratio of 12.1±5.1. The use of propofol was not associated with a mean difference in level of n-6 FFA (247.6±84.5 \( \mu \)mol/L versus 187.4±22.7 \( \mu \)mol/L, \( P=0.2 \)), n-3 FFA (21.0±5.8 \( \mu \)mol/L versus 18.0±5.7 \( \mu \)mol/L, \( P=0.5 \)), and n-6:n-3 ratio (12.5±5.8 versus 11.5±4.5, \( P=0.5 \)). The amount of fat delivered in enteral nutrition and through propofol infusions did not correlate with n-6 FFA (Spearman \( \rho=0.1, P=0.5 \)) or n-3 FFA (Spearman \( \rho=0.03, P=0.7 \)) levels. In separate multivariate generalized estimating equation models, n-6 FFA levels and n-3 FFA levels were each found to be associated with \( \text{VO}_2 \), high-sensitivity C-reactive protein, and the modified Fisher scale (Table 3). The mean 14 day n-6 FFA, mean 14-day n-3 FFA, and mean n-6:n-3 FFA ratio were not associated with poor outcome (modified Rankin Scale score ≥4) 3 months after hemorrhage.

**Delayed Cerebral Ischemia**

Fourteen (28%) patients developed DCI on median postbleed Day 7 (IQR, 6–10). The mean \( \text{VO}_2 \) (284±50 mL/min versus 235±65, \( P=0.04 \)) as well as the mean n-6 levels (278±103 \( \mu \)mol/L versus 170±89 \( \mu \)mol/L, \( P=0.03 \)) and mean n-6:n-3 FFA ratio (15.7 versus 10±2, \( P=0.02 \)) were all higher in patients who developed DCI. There was no difference in the mean n-3 FFA levels between DCI and non-DCI patients (18±9 \( \mu \)mol/L versus 21±12 \( \mu \)mol/L, \( P=0.6 \)). The 14-day mean n-6:n-3 FFA ratio had a higher area under the curve for predicting DCI than the 14-day mean n-6 FFA level (Figure). An n-6:n3 FFA ratio of ≥8.8 was found to have a sensitivity of 93% and specificity of 80% for predicting DCI. The median time from n-FFA and \( \text{VO}_2 \)
measurement and diagnosis of DCI was 24 hours (range, 12–48 hours). In a backward Wald Cox proportional hazards model adjusting for VO₂ and sex, the modified Fisher score (\(P=0.004\)), younger age (\(P=0.05\)), and n-6:n-3 FFA ratio (\(P=0.001\)) predicted time to DCI (Table 4).

**Discussion**

We found that higher n-6 and n-3 FFA levels were associated with higher O₂ consumption and severity of initial hemorrhage.\(^\text{15}\) Additionally, an increased ratio of n-6:n-3 FFAs was independently associated with DCI after accounting for hemorrhage severity. These findings indicate that lipid peroxidation may mediate the relationship between hypermetabolism and DCI.

Lipid peroxidation after experimental SAH has been extensively studied, and metabolites of n-6 FFAs such as arachidonic acid have been implicated in the pathophysiology of vasospasm and related complications\(^\text{6}\) and has led to the development of a class of steroids that target lipid peroxidation. Tirilazad, a nonglucocorticoid 21 amino-steroid free radical scavenger with a mechanism of action believed to be an inhibition of iron-dependent lipid peroxidation was studied in several controlled trials after promising results in primate vasospasm models. The results from these multiple clinical trials demonstrated a consistent reduction in vasospasm and vasospasm-related complications, whereas there was an inconsistent effect on global outcomes.\(^\text{16,17}\) Despite these clinical therapeutic trials, few studies have focused on determinants of lipid peroxidation and serum levels of FFA in patients with SAH.

Similar to a study demonstrating FFA elevation in the cerebrospinal fluid of patients with SAH,\(^\text{7}\) we found that hemorrhage severity was associated with the extent of FFA elevation in the serum. However, our study is unique in the assessment of VO₂ and its potential role in the production of FFAs. Our study design precludes determining causality, but it is plausible that the hypermetabolic state was influenced by inflammatory status, leading to increased fat use and rise in serum FFA levels. In studies of nonbrain-injured critically ill patients, an inflammation-mediated hypermetabolic state has been linked to increases in FFAs with subsequent development of nutritional interventions designed to modulate the immune response and blunt downstream sequelae.\(^\text{4,18,19}\) We recently reported the strong relationship between high-sensitivity C-reactive protein and VO₂ after SAH and demonstrated the ability of rises in VO₂ to predict the occurrence of DCI.\(^\text{8}\) The current analyses point to the possibility that n-6 FFAs may mediate the relationship between VO₂ and DCI.

Our analysis found that the ratio of n-6:n-3 FFAs was more highly associated with DCI than just absolute levels of n-6 FFAs alone. The imbalance between these classes of FFAs may play a role in subsequent injury. Metabolic balance among different classes of fatty acids has been shown to be necessary for optimal cellular function. Moreover, n-6 FFAs and n-3 FFAs compete in metabolic pathways that impact cellular responses to physiological stress. The resultant competition for cyclo-oxygenases and lipoxygenases determines which types of eicosanoids will be synthesized and thus potentially influence inflammatory and vascular responses.

Because membrane n-6 and n-3 fatty acids are derived from the diet, tissue imbalances might be corrected by either

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**Table 3. Factors Associated With n-Free Fatty Acid Levels After SAH***

<table>
<thead>
<tr>
<th>Factor</th>
<th>Beta Coefficient</th>
<th>Wald χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-6 free fatty acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hunt–Hess grade</td>
<td>23.5</td>
<td>1.86</td>
<td>0.17</td>
</tr>
<tr>
<td>Modified Fisher Scale</td>
<td>93.0</td>
<td>6.73</td>
<td>0.01</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>0.65</td>
<td>3.95</td>
<td>0.04</td>
</tr>
<tr>
<td>VO₂, mL/min</td>
<td>2.0</td>
<td>11</td>
<td>0.001</td>
</tr>
<tr>
<td>Caloric intake, calories/kg</td>
<td>−0.84</td>
<td>0.22</td>
<td>0.64</td>
</tr>
</tbody>
</table>

| n-3 free fatty acid     |                  |         |      |
| Hunt–Hess grade         | 2.54             | 1.74    | 0.19 |
| Modified Fisher Scale   | 10.4             | 8.1     | 0.01 |
| hsCRP, mg/L             | 0.07             | 4.24    | 0.03 |
| VO₂, mL/min             | 0.26             | 15.67   | <0.001|
| Caloric intake, calories/kg | −0.02       | 0.01    | 0.91 |

SAH indicates subarachnoid hemorrhage; hsCRP, high-sensitivity C-reactive protein; VO₂, systemic oxygen consumption.

*Multivariate generalized estimating equation analyses for factors associated with n-6 and n-3 free fatty acid levels.

**Table 4. Cox Proportional Hazards Model Predicting Time to Delayed Cerebral Ischemia**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Hazards Ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-6-n-3 FFA ratio*</td>
<td>2.93</td>
<td>1.76–4.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Modified Fisher Scale</td>
<td>2.83</td>
<td>1.39–5.74</td>
<td>0.004</td>
</tr>
<tr>
<td>Age†</td>
<td>0.83</td>
<td>0.69–0.99</td>
<td>0.05</td>
</tr>
</tbody>
</table>

FFA indicates free fatty acid.

*Hazards ratio represents the risk increase for every standard deviation (5.1) increase in ratio.

†Hazards ratio represents the risk decrease for every 5-y increase in age.

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**Figure.** Receiver operating characteristic analysis predicting delayed cerebral ischemia.
decreasing n-6 fatty acid intake and/or by increasing n-3 fatty acid intake. As seen in our study, traditional enteral nutritional formulations do not impact FFA levels. The administration of n-3 fatty acids may help establish a causal link in patients with SAH by modulation of both responses given their competition for lipooxygenase and cyclo-oxygenase and resultant reduction and opposing effect on the inflammatory modulators, which are the metabolic products of arachidonic acid (n-6 FFA) when acted on by these enzymes. Formulations enriched with n-3 fatty acids have already been shown to modulate the inflammatory response and improve physiological profiles in patients with acute respiratory distress syndrome.4,20

In a recent prospective pilot randomized clinical trial of patients with SAH, eicosapentaenoic acid, a n-3 fatty acid, was orally administered at a daily dose of 1800 mg between Day 4 and Day 14 and compared with placebo in terms of the frequency of symptomatic vasospasm and cerebral infarction.21 Serum levels of eicosapentaenoic acid increased significantly and were associated with a decreased frequency of symptomatic vasospasm-related deterioration and infarcts.

Findings of this pilot study need further confirmation with additional data regarding VO₂ as well as eicosapentaenoic acid levels and n-6 FFA levels to better understand the mechanism by which n-3 FFAs may reduce the occurrence of DCI and improve outcome after SAH.

Our study has several strengths. First, all measurements and data collection were conducted prospectively with the clinical team blinded to results from IDC testing and FFA assessments. This eliminated any influence the knowledge of metabolic or inflammatory measurements may have had on diagnostic or therapeutic management, especially as it pertained to DCI. We carefully recorded and analyzed all pharmacological and physiological parameters that may have confounded the relationship between VO₂ and FFAs. Finally, we used, prospectively documented on the day of occurrence, a strict, validated definition for our primary end point, DCI,1 which has been previously validated as a strong predictor of outcome after SAH.

There are limitations to this study, however. Although we had 187 measurements, there were only 50 patients studied, and therefore our results may not fully describe the relationship between n-FFAs and nutritional intake. We believe that these results do provide preliminary evidence for the importance of the interrelationship among hypermetabolism, lipid peroxidation, and DCI after SAH and warrants further study. Serial measurements of FFAs and VO₂ have not been previously reported in patients with SAH and provide for novel analyses; however, we did not make these measurements daily, potentially biasing our analyses regarding their interrelationship and that with DCI. The median time difference between measurement and diagnosis was brief and therefore the effect, if any, of a time delay between measurement and DCI diagnosis should be minimal.

In summary, we believe there is evidence for an interrelationship between VO₂, the increase in n-6 FFAs and n-6/n-3 FFA ratio, and DCI after SAH. This suggests that immunomodulatory interventions using n-3 FFAs may reduce the incidence and impact of DCI after SAH and provide a rationale for further studies to test this approach.

**Acknowledgments**

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**Disclosures**

None.

**References**

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