University of Cincinnati

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I, Opeolu Adeoye M.D., hereby submit this original work as part of the requirements for the degree of Master of Science in Clinical and Translational Research.

It is entitled:
Peripheral Leukocytes and Intracerebral Hemorrhage

Student’s name: Opeolu Adeoye M.D.

This work and its defense approved by:

Committee chair: Erin Nicole Haynes, DrPH
Peripheral Leukocytes and Intracerebral Hemorrhage

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by

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Abstract

About 70,000 people in the US die or suffer significant disability from spontaneous intracerebral hemorrhage (ICH) every year. No proven treatment is available that improves ICH outcomes. Since hematoma volume is the strongest predictor of ICH outcome, limiting hematoma expansion has been the primary goal of recent ICH clinical trials. Causes of secondary injury after the initial hemorrhage include hematoma expansion and cerebral edema. Identifying blood biomarkers associated with neurological deterioration and/or prognosis after ICH is a critical step for developing new therapies. Biomarkers that are mechanistically associated with hemostasis failure or cerebral edema would provide direction for exploring therapeutic targets, and identify patients in need of hemostatic or anti-inflammatory therapy.

Peripheral white blood cell (WBC) count has been associated with hemorrhage volume and functional outcomes after ICH. However, it is unknown whether elevated WBCs represent an acute phase response or whether there is a pathophysiologic role of peripheral WBCs in ICH. Neutrophils and monocytes are predominant WBCs and peripheral neutrophil and monocyte infiltration have been found to contribute to secondary injury in animal models of ICH. Circulating monocytes particularly are well recognized to express significant amounts of tissue factor, interact with platelets, and modulate inflammation as well as thrombosis and hemostasis.

We conducted the first human study to explore absolute monocyte counts in relation to ICH. We found that baseline peripheral monocyte count was independently associated with 30-day case-fatality in ICH patients who presented to the emergency department within 12 hours of symptom onset. Absolute monocyte count, a readily available and ubiquitous biomarker at presentation in ICH patients, may represent a novel prognostic tool after ICH.
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I. Background

Intracerebral hemorrhage (ICH) occurs in about 70,000 people a year in the United States and has a 30-day mortality of 32-50%. ICH represents 10-15% of strokes in the United States, Europe, and Australia, and 20-30% in Asian countries. The most important risk factor for ICH is age. Each advancing decade from 50-80 years represents a two-fold increase in ICH incidence. Hypertension is the most significant modifiable risk factor, occurring in 50-70% of patients. Hypertension is primarily associated with deep cerebral and brainstem ICH locations, and with lobar locations to a lesser extent. Hypertension also plays a proportionately larger role in younger compared to older patients.

The strongest contributors to this high mortality are the initial size of the hematoma, whether the hematoma continues to expand after presentation, and whether delayed cerebral edema occurs. Even among survivors, only 20% are independent at 6 months. Despite the biological and clinical relevance of hemostasis in preventing hematoma expansion, and inflammation and the immune response in mediating cerebral edema, there has been only limited clinical research on hemostasis and cerebral edema in ICH. Identifying blood biomarkers associated with failure of hemostasis and/or development of significant cerebral edema is a critical step for developing new therapies.

To date, no medical or surgical clinical trials have demonstrated a definitive functional outcome benefit after ICH occurs. Thus, ICH remains a challenging clinical and public health problem. This article discusses the natural history of acute ICH, the contribution of and interaction of the immune system with this natural history, and ongoing and future research that may enhance understanding of ICH pathophysiology in order to develop novel therapies that improve functional outcomes and reduce the overall societal burden of ICH.
Ia. Natural History of Acute ICH

Clinically, ICH presents with a focal neurological dysfunction that may be associated with headache, nausea, vomiting or other symptoms. After the initial presentation, early (within hours) neurological deterioration may occur due to hematoma expansion. Historically, the ‘hemorrhage’ in ICH was thought to be a static process. When it occurred, neurological deterioration was thought to be due to cerebral edema. A sentinel prospective study demonstrated that early neurological deterioration in ICH was in fact due to ongoing bleeding and hematoma expansion (Figure 1).

In that study of 103 ICH patients presenting within 3 hours of symptom onset, head CTs were obtained at baseline, an hour later and again at 20 hours. Twenty six percent of patients had significant (defined as >33%) increase in hematoma volume within an hour of the baseline CT and an additional 12% had further hematoma expansion at 20 hours. Hematoma expansion was significantly associated with neurological deterioration as measured by the Glasgow Coma Scale (GCS) and National Institutes of Health Stroke Scale (NIHSS). Subsequently, other large prospective studies have confirmed the dynamic changes in the hemorrhage early in ICH. A summary of prospective studies of hematoma expansion in ICH is presented in Table 1.
Table 1 – Prospective Studies of Hematoma Expansion in ICH

<table>
<thead>
<tr>
<th>Author</th>
<th>Brott †</th>
<th>Leira ‡</th>
<th>Mayer †</th>
<th>Lyden ‡</th>
<th>Anderson ‡</th>
<th>Mayer ‡, ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Patients</td>
<td>103</td>
<td>266</td>
<td>120</td>
<td>288</td>
<td>200</td>
<td>268</td>
</tr>
<tr>
<td>Maximum time from onset at presentation, hours</td>
<td>3</td>
<td>12</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Time to second CT, hours</td>
<td>20</td>
<td>48</td>
<td>24</td>
<td>72</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Definition of hematoma expansion</td>
<td>&gt;33%</td>
<td>&gt;33%</td>
<td>&gt;33%</td>
<td>&gt;33%</td>
<td>&gt;33% or 12.5mL</td>
<td>Per mL</td>
</tr>
<tr>
<td>Percent of patients with expansion</td>
<td>38</td>
<td>27</td>
<td>32</td>
<td>26</td>
<td>23</td>
<td>26</td>
</tr>
</tbody>
</table>

*Placebo or standard therapy arm of each trial
^Percent increase in mean volume

In addition to the volume of the parenchymal hematoma, the presence and expansion of intraventricular hemorrhage (IVH) is a powerful, independent predictor of ultimate outcome in ICH. IVH occurs in 45% of patients with spontaneous ICH and contributes to early neurological deterioration and outcomes.\(^17,18\) In a secondary analysis of a prospective study of 1033 patients with ICH, the presence of IVH was associated with a lower likelihood of favorable outcomes (15% versus 31%, p<0.00001).\(^17\) Another study showed that a >2mL increase in IVH from baseline to 24 hours had an Odds ratio of 4.21 (95%CI 1.06-16.63, p=0.0405) for predicting poor outcome.\(^18\) Thus, hematoma expansion within the brain parenchyma or into the ventricular system may have dire consequences for ICH outcomes.

Early deterioration in ICH may also be affected by cerebral edema. In a prospective observational cohort of 142 ICH patients, baseline and 24 hour absolute and relative (to the hematoma) edema volumes were measured.\(^19\) Baseline relative edema volume was found to be the strongest predictor of outcome and was associated with lesser odds of poor 3-month functional outcome. In contrast, patients with lesser amounts of baseline edema were more prone to develop increases in edema during the subsequent 24 hours.\(^19\) However, another
prospective study of 270 ICH patients described the evolution of perihematomal edema in the first 72 hours after ICH reported different results. Baseline, 24 hour and 72 hour CT scans were reviewed. Baseline perihematomal edema volume was highly correlated with baseline hematoma volume \( r^2 = 0.45 \). In multivariate analysis, both absolute (OR 1.85, 95CI 1.30-2.65, \( p = 0.001 \)) and relative (OR 1.48, 95CI 1.13-1.94, \( p = 0.004 \)) increases in perihematomal edema volume were associated with death or dependency at 90 days before adjusting for baseline hematoma volume. When additionally adjusted for baseline hematoma volume, neither absolute nor relative increases in perihematomal edema were associated with 90-day outcomes. The authors concluded that the degree of and growth in perihematomal edema are strongly related to the volume of the underlying hematoma and did not appear to have an independent effect on ultimate ICH outcome. Thus, the contribution of early changes in edema to overall ICH morbidity remains unclear.

Delayed edema may also occur from approximately three days to two weeks after ictus in ICH (Figure 2). Zazulia et al. studied the progression of mass effect or increased midline shift during hospitalization in 76 patients with supratentorial ICH. Early development of mass effect (within 48 hours) occurred in 13% (10/76) of cases and was associated with hematoma expansion; delayed mass effect (9-21 days) was thought to be independently related to edema and occurred in 9% (7/76) of cases. Sansing and colleagues studied 80 patients with supratentorial ICH. Peak edema volume was found to occur between days 5 and 6 after ICH onset. Edema growth was correlated with platelet count and the authors suggested that interactions between activated platelets and thrombin may contribute to edema development. Delayed mass effect is thought to be induced by red blood cell lysis and hemoglobin-induced neurotoxicity. Slow thrombin release over time has also been suggested as the basis for delayed edema.
there is no established means of predicting hematoma expansion, early edema or delayed edema in ICH, recently published and ongoing clinical trials are promising and suggest that targeted therapy to improve outcomes in ICH may be feasible in the near future.

Neuroprotective Therapies

As discussed above, secondary injury in ICH may result from cerebral edema, slow thrombin release, red blood cell lysis and hemoglobin-induced neurotoxicity. The multitude of processes that occur after ICH have been termed “neurohemoinflammation.” There is clinical interest in targeting these processes as a means of improving ICH outcomes. Two large trials of putative neuroprotective drugs have been published in ICH.15,23

The Glycine Antagonist in Neuroprotection (GAIN) International and GAIN Americas trials randomized ischemic stroke and ICH patients to gavestinel, a glycine-site antagonist, or placebo within 6 hours of symptom onset. Outcome was measured by the Barthel Index (BI) divided into 3 groups: 95 to 100 (independent), 60 to 90 (assisted independence), and 0 to 55 (dependent) or dead. In an analysis of the 571 included ICH patients, there was no significant difference in the trichotomized BI scores at 3 months between gavestinel and placebo (p=0.09).23

Figure 2 – Delayed cerebral edema with mass effect and midline shift after ICH
The Cerebral Hematoma And NXY Treatment trial (CHANT) explored the safety and potential for efficacy of NXY-059 (disufenton sodium), a free radical-trapping agent, in ICH patients presenting within 6 hours of onset. The odds ratio for improved 3-month mRS scores in the NXY-059 group was 1.01 (95% CI 0.75, 1.35). Overall, the drug had a good safety and tolerability profile, with no adverse effect on important clinical outcomes. However, given no signal of efficacy, the authors concluded that there was “no suggestion that NXY-059 benefits ICH patients.”

In addition to the studies described above, two smaller clinical trials have investigated citicoline, an intermediate compound in the synthetic pathway of structural phospholipids, as a putative neuroprotective agent in ICH. There are insufficient data to recommend citicoline for clinical use at this time. Overall, no neuroprotective therapy has been shown to be effective in ICH to date. However, a number of promising agents are progressing to early phase human trials. Table 2 summarizes potential neuroprotective drugs currently under study in early clinical trials of ICH.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Trial phase</th>
<th>Trial status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosuvastatin</td>
<td>II</td>
<td>Completed</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>II</td>
<td>Terminated</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>II</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>I</td>
<td>Completed</td>
</tr>
<tr>
<td>Albumin</td>
<td>II</td>
<td>Recruiting</td>
</tr>
<tr>
<td>High Dose Deferoxamine</td>
<td>II</td>
<td>Not started</td>
</tr>
<tr>
<td>Mild Hypothermia</td>
<td>I</td>
<td>Not started</td>
</tr>
</tbody>
</table>

*As reported on the ClinicalTrials.gov registry on 7 November 2012.

Ib. Immune Response and ICH

The pathophysiology of ICH remains poorly understood. Small vessel fibrinoid necrosis and rupture mediated by high blood pressure has been suggested as a possible etiology in the
initiation of acute ICH.\textsuperscript{26,27} It is well recognized that infiltrating white blood cells (WBC) play a role in secondary injury after ICH.\textsuperscript{26,29} Preclinical studies have found that leukocytes are present in and around the hematoma within hours after ICH.\textsuperscript{30} Neutrophils infiltrate the ICH first and may cause direct injury by releasing reactive oxygen species (ROS), inflammatory proteases and damage to the blood brain barrier.\textsuperscript{28,29} Neutrophils also facilitate the recruitment of monocytes to the ICH site, and neutrophil depletion has been associated with reduced monocyte infiltration and improved outcome in mouse models of ICH.\textsuperscript{31} Toll-like receptor (TLR) 4 mediates the recruitment of monocytes and inflammatory monocytes to the ICH site, and TLR 4 deficient mice were found to have improved outcome after ICH compared to wild type animals.\textsuperscript{32}

In clinical studies, the total peripheral WBC count has been associated with baseline hemorrhage volume, early neurological deterioration and functional outcomes after ICH.\textsuperscript{14,33-35} Neutrophils and monocytes are predominant WBCs and peripheral neutrophil and monocyte infiltration have been found to contribute to secondary injury in animal models of ICH.\textsuperscript{36,37} Monocytes in the blood stream are well recognized to play a central role in the pathogenesis of atherosclerosis and cardiovascular disease.\textsuperscript{38} Circulating monocytes also express significant amounts of tissue factor, interact with platelets, and modulate thrombosis and hemostasis.\textsuperscript{39-42} A recent clinical study found that increased expression of TLR 2 and TLR 4 in monocytes was associated with poor outcome after ICH.\textsuperscript{43} If peripheral monocyte and neutrophil counts, readily available biomarkers, are found to be strongly predictive of hematoma expansion, cerebral edema and/or ICH outcome it would lead to targeted research questions that may generate new treatments in ICH patients with elevated WBC counts.
II. Hypothesis, Aims, and Objectives

Objectives
To date, human studies of WBC association with ICH have examined total WBC count without differentiating cell types. We determined the association of peripheral WBC, monocyte and neutrophil counts with baseline ICH volume and 30-day case-fatality.

Hypothesis
In ICH patients aged 18 or older who present within 12 hours of symptom onset, total WBC, absolute monocyte and neutrophil counts at presentation and change in monocyte and neutrophil counts from presentation to 24 hours will be associated with presenting ICH volume and 30-day case-fatality.

Specific Aims

Aim #1 – Determine the association of presenting total WBC count, and monocyte and neutrophil counts with baseline ICH volume and 30-day case-fatality in order to advance current knowledge of the association of peripheral WBCs and the immune response after ICH with prognosis.

Aim #2 – Determine the association of change in total WBC, monocyte and neutrophil counts in the first 24 hours after symptom onset with baseline ICH volume and 30-day case-fatality in order to advance current knowledge of the natural history of changes in peripheral WBC expression in the acute phase of human ICH.
III. Research Methods

We analyzed a group of patients aged 18 or older with ICH previously identified as part of the Genetic and Environmental Risk Factors for Hemorrhagic Stroke (GERFHS) study from July 2008 to December, 2009. The institutional review board for each participating hospital system approved the GERFHS study. Cases were identified by retrospective review of primary and secondary ICD-9 codes 430 to 438.9 at all 16 acute care hospitals in the 5-county Greater Cincinnati/Northern Kentucky region. Study nurses also maintained active surveillance (“hot pursuit”) at several hospitals that treat most ICH in the area. All potential cases were abstracted by study nurses and reviewed in detail by study physicians. Exclusion criteria were previous ICH, traumatic ICH, hemorrhagic cerebral infarction, and hemorrhage associated with brain tumor, encephalitis, recent endarterectomy, and thrombolytic treatment of ischemic stroke. Patient demographics and putative risk factors for ICH were recorded by chart review. Full details of the GERFHS methodology have been published elsewhere.\textsuperscript{44}

For this project, we included patients previously enrolled in GERFHS who were seen at UC Health University Hospital and St. Elizabeth’s Hospital in Edgewood, KY. These hospitals were chosen because they receive the most ICH patients of all hospitals in the region.\textsuperscript{45} We performed additional chart review to determine baseline and 24 hour total WBC, absolute monocyte and absolute neutrophil counts. Absolute monocyte and neutrophil counts were calculated by multiplying the total WBC count by reported percent monocyte or neutrophil as appropriate. These values are routinely reported in hospital laboratories with the Complete Blood Count (CBC), a ubiquitous lab in hospitalized patients. While the initial chart review for GERFHS recorded WBC counts, the monocyte and neutrophil counts were not recorded. Demographics, ICH volumes, hematoma expansion and other clinical parameters of interest were previously determined as part of the GERFHS study.
For the analyses, monocyte, neutrophil and WBC counts and ICH volume were log transformed. The association of ICH volume with WBC counts was determined using linear regression. The association of WBC counts with 30-day case-fatality was determined using logistic regression.

IV. Results

We identified 186 ICH patients seen in the ED within 12 hours of symptom onset and with complete baseline data. Mean age was 67.3±14.8 years; 51% were male, and 22% black. Median [interquartile range] ICH volume was 12.8mL [4.9, 29.4]. Demographics and other clinical information are presented in Table 3.

<table>
<thead>
<tr>
<th>Table 3 - Demographics</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
</tr>
<tr>
<td>Sex (% male)</td>
</tr>
<tr>
<td>Race (% white)</td>
</tr>
</tbody>
</table>
| Location of stroke     | 104 (56%) Deep  
                        | 47 (25%) Lobar  
                        | 7 (4%) Brainstem  
                        | 27 (14.5%) Cerebellum  
                        | 1 (0.5%) Primary IVH |
| IVH (% any)            | 100 (53.8%) |
| Died within 30 days (%)| 74 (39.8%) |
| Discharge Modified Rankin Classification (% 0-2) | 16 (8.6%) |
| Baseline GCS (median [IQR]) | 14 [10, 15] |
| Baseline ICH volume (median [IQR]) | 12.8 [4.9, 29.4] |

After adjusting for patient age and initial hemoglobin, higher initial WBC count (p=0.0009) and higher neutrophil count (p=0.006) were significantly associated with larger baseline ICH volume, but monocyte count was not. After adjusting for patient age, GCS, the presence or absence of IVH, hemorrhage location, and ICH volume, the initial monocyte count was independently associated with greater odds of 30-day mortality (2.26-fold increased odds for every log-transformed unit of monocytes) (Table 4).
<table>
<thead>
<tr>
<th>Table 4 – Odds Ratios for 30-day Case-Fatality</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OR (CI)</strong></td>
</tr>
<tr>
<td>WBC (log)</td>
</tr>
<tr>
<td>Monocytes (log)</td>
</tr>
<tr>
<td>Neutrophils (log)</td>
</tr>
</tbody>
</table>

We examined whether the time from symptom onset to blood draw would impact the monocyte and neutrophil counts obtained. A longer time between symptom onset and initial blood draw was associated with lower monocytes (p=0.03) and higher neutrophils (p=0.01). We also examined how dynamic the changes in WBC were early in the course of ICH. A repeat CBC with available monocyte and neutrophil counts was obtained 6.1 ± 3.8 hours after the baseline labs in 73 patients. A longer time between initial and repeat blood draws was associated with greater changes in neutrophil count during this period (p=0.03), suggesting that neutrophils may continue to rise after initial blood draw, but no other time associated changes were observed. The early changes in these counts were not associated with baseline ICH volume or 30-day case-fatality.

V. Discussion

The main hypothesis for this project was that absolute monocyte and neutrophil counts at presentation and change in monocyte and neutrophil counts from presentation to 24 hours would be associated with presenting ICH volume and 30-day case-fatality in ICH patients who present within 12 hours of symptom onset. The basis for this hypothesis was that the absolute number of monocytes available early in the clinical course may allow for more monocytes to be recruited to the site of ICH and thereby contribute to secondary injury after ICH, as has been found in animal models of the disease.31 Our main finding was that higher monocyte count at presentation was independently associated with greater odds of 30-day case-fatality (2.26-fold increased odds for every log-transformed unit of monocytes). Coupled with the fact that we did
not find an association between presenting monocyte count and ICH volume, this suggests that the association of monocyte count with case-fatality may be due to the contribution of monocytes to secondary injury after ICH. This novel finding may enhance the understanding of the pathophysiology and clinical course of ICH and will lead to further investigations.

First, this finding should be confirmed in an independent data set. However, as has been mentioned, secondary injury after ICH may result from hematoma expansion (Figure 1) or cerebral edema (Figure 2). Thus, natural lines of inquiry from the main finding of this report include determining the association of monocyte count with hematoma expansion and neurological deterioration secondary to cerebral edema after ICH. Barring any obvious link of monocyte count to hematoma expansion and/or cerebral edema, mechanisms by which monocytes may contribute to poor outcome and case-fatality after ICH should be explored. Potential mechanisms include: increased availability of monocytes to attach to the vascular endothelium thereby damaging the BBB; increased availability of monocytes to bind to monocyte chemoattractant proteins (MCP) which are expressed in cerebral vessels and known to facilitate neuronal death and promote vessel injury after monocyte binding\textsuperscript{46,47}; and, total expression of TLRs 2 and 4 (among other pro-inflammatory molecules) contributing to cerebral edema and poor outcome.\textsuperscript{43}

As has been previously reported,\textsuperscript{14,33-35} we found that higher initial WBC count and higher neutrophil count were significantly associated with larger baseline ICH volume. In contrast to previous reports, these were not independently associated with outcome after we adjusted for monocyte counts. We did not find any associations between early changes in WBC, neutrophil or monocyte counts and ICH volume or case-fatality. However, we had a small sample size for examining this and only included very early repeat labs.
Overall, neutrophils have been primarily implicated as the inflammatory cells most likely to contribute to secondary injury after ICH, and monocytes have been thought to be recruited to the site of injury primarily as phagocytic cells aiming to limit injury mediated by neutrophils. Recent preclinical studies and now our clinical study suggest that monocytes may have an independent role in affecting ICH outcomes. Absolute monocyte count, a readily available and ubiquitous biomarker at presentation in ICH patients, may represent a novel prognostic tool after ICH.
VI. Bibliography


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