

Research Article: New Research

Osteopontin Is a Blood Biomarker for Microglial Activation and Brain Injury in Experimental Hypoxic-Ischemic Encephalopathy

Plasma biomarker of hypoxic-ischemic brain injury

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1 **Osteopontin Is a Blood Biomarker for Microglial Activation and Brain**
2 **Injury in Experimental Hypoxic-Ischemic Encephalopathy**

3
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10 **Author contributions:**

11 Y.L., E.B.D., X.Z-B., S.C., and D.M.D, performed experiments and analyzed data. N.T.S., C-Y.K.
12 and Y-Y.S. designed experiments, analyzed data, and wrote the manuscript.
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33 of the Emory Neuroscience NINDS Core Facilities grant (P30NS055077).
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36 **ABSTRACT:**

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38 Clinical management of neonatal hypoxic-ischemic encephalopathy (HIE) suffers from the lack of
39 reliable surrogate marker tests. Proteomic analysis may identify such biomarkers in blood, but there
40 has been no proof-of-principle evidence to support this approach. Here we performed in-gel trypsin
41 digestion of plasma proteins from 4 groups of 10-day-old mice (untouched and 24 h after low-dose
42 lipopolysaccharide [LPS] exposure, hypoxia-ischemia [HI], or LPS/HI injury; n=3 for each group)
43 followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and bioinformatics
44 analysis to search for HI- and LPS/HI-associated brain injury biomarkers. This analysis suggested
45 the induction of plasma osteopontin (OPN) by HI and LPS/HI, but not by the sham and injury-free
46 LPS-exposure. Immunoblot confirmed post-HI induction of OPN protein in the brain and blood,
47 whereas the *Opn* mRNAs was induced in the brain, but not in blood. This disparity suggests brain-
48 derived plasma OPN after HI injury. Similarly, immunostaining showed the expression of OPN by
49 Iba1+ microglia/macrophages in HI-injured brains. Further, intracerebroventricular injection of LPS
50 activated microglia and up-regulated the plasma OPN protein. Importantly, the induction of plasma
51 OPN after HI is greater than those of matrix metalloproteinase 9 (MMP-9) and glial fibrillary acid
52 protein (GFAP). The plasma OPN levels at 48 h post-HI also parallel the severity of brain damage
53 at 7 d recovery. Together, these results suggest that the OPN may be a prognostic blood biomarker
54 in HIE by monitoring the brain microglial activation.

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60 **SIGNIFICANCE STATEMENT:**

61 There are no reliable blood biomarkers for neonatal brain injury in HIE to date, and the majority of
62 previous studies tested candidate biomarkers individually. Here we report, for the first time, proof-
63 of-principle evidence that mass spectrometry-based quantitative proteomic methods may efficiently
64 identify blood biomarkers in HIE. Using this method, we show that osteopontin (OPN), an integrin-
65 binding protein secreted by activated microglia, rises in blood and correlates with the severity of
66 brain damage in experimental HIE. These data suggest that OPN may be a blood biomarker in HIE.

67 **INTRODUCTION:**

68 HIE due to perinatal asphyxia is a major cause of neonatal mortality and neurological disabilities,
69 with an incidence of 1-2 per 1000 live births in the western world and more common in developing
70 countries. Hypothermia is the current best therapy of HIE, but there are no simple laboratory tests to
71 acutely monitor the brain damage, which has hindered the development of better treatments (Bennet
72 et al., 2010). Blood biomarkers are an attractive idea to overcome this obstacle, and previous studies
73 using a candidate approach have implicated MMP-9 and GFAP as potential blood biomarkers in HI
74 brain injury (Bembea et al., 2011; Bednarek et al., 2012). In contrast, quantitative proteomics has
75 not been utilized to identify plasma biomarkers in HIE, despite its success in the neurodegenerative
76 disease research (Donovan et al., 2013).

77 To test the applicability of quantitative proteomics for the discovery of HIE biomarkers, we
78 used in-gel trypsin digestion and LC-MS/MS to compare the plasma proteins isolated from 4 groups
79 of mouse neonates: unchallenged, low-dose LPS treated, the Vannucci HI- or LPS/HI-injured at 24
80 h recovery. The comparison with low-dose LPS-treated animals (which do not exhibit brain injury)
81 is to minimize LPS-induced, non-specific immune alterations in brain damage-related biomarkers in
82 LPS/HI injury. Here we report the proteomics-based discovery and validation of OPN as a plasma
83 biomarker for HIE.

84 OPN, also called Secreted Phosphoprotein 1 (SPP1), is a small integrin-binding ligand N-
85 linked glycoprotein (SIBLING) that mediates cell-cell and cell-matrix interactions (Bellahcene et
86 al., 2008). The basal OPN level in adult brains is low, but ischemia induces microglia/macrophages
87 to express OPN in the peri-infarct area (Ellison et al., 1998; Gliem et al., 2015). Previous studies
88 have shown OPN induction in rodent brains after neonatal HI, but whether there is increase of the
89 plasma OPN protein has not been evaluated (van Velthoven et al., 2011; Chen et al., 2011). Earlier
90 studies suggested protective functions of OPN in neonatal HI, but later studies failed to confirm this
91 finding (Albertsson et al., 2014; Bonestroo et al., 2015). Although the roles of OPN in HI-injured
92 brains remain uncertain, our results implicate plasma OPN as a prognostic biomarker for microglia
93 activation and brain damage in HIE.

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96 **MATERIALS AND METHODS:**

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98 **Animal Surgery**

99 The Vannucci model of neonatal HI with and without LPS pre-exposure (0.3 mg /kg, IP, 4 h prior to
100 HI), and intracerebroventricular (ICV) injection of LPS (1 μ g) was performed as described (Yang et
101 al., 2009; Yang et al., 2014). Briefly, 10-day-old C57BL/6 mice of both genders were subjected to
102 ligation of the right common carotid artery, and exposure to 10% O₂ for 40 min in glass chambers
103 submerged in a 37^oC waterbath 1 h later. These procedures were approved by the Institutional
104 Animal Care and Use Committee and conformed to the NIH Guide for Care and Use of Laboratory
105 Animals.

106

107 **Plasma preparation and proteomic analysis**

108 Blood, collected from rodent neonates into an Eppendorf tube containing EDTA, was centrifuged at
109 1000 g for 15 min. The supernatants were resolved by SDS-PAGE for proteomic analysis. Five gel
110 slices for each sample were excized based on molecular weight, individually digested with trypsin
111 (12.5 ng/ μ L), and the resulting peptides were analyzed independently by the reverse-phase liquid
112 chromatography coupled with tandem mass spectrometry (LC-MS/MS) on an Orbitrap-XL mass
113 spectrometer (Thermo Scientific, San Jose, CA) essentially as previously described (Dammer et al.,
114 2013; Dammer et al, 2015). The MS/MS spectra were searched against a concatenated target-decoy
115 mouse NCBI reference database using the SEQUEST Sorcerer algorithm (version 4.3.0, SAGE-N).
116 The peptides were classified by charge state and filtered by mass accuracy (\pm 10 ppm), and then
117 dynamically by increasing XCorr and Δ Cn values to reduce protein FDR to less than 1%. Peptide-
118 specific ion current intensities were extracted and compared using in-house software (Herskowitz et
119 al., 2011; Donovan et al., 2013). Accurate peptide mass and retention time was used to derive signal
120 intensity for every peptide across LC-MS runs for each sample. Only high-confidence differential
121 proteins [\log_2 (ratio) \geq \pm 0.89; signal-to-noise ratio $>$ 5 when compared to controls] with two or more
122 MS/MS-identified peptide counts were tabulated.

123

124 **Multiplex Assays Using Luminex**

125 The Luminex bead-based multiplex ELISA was designed following the manufacturer's instructions
126 (R&D Systems). Reactions were run on a Bio-Plex® Multiplex System, and the data were analyzed
127 using the Bio-Plex Manager Software.

128 **Quantitative RT-PCR**

129 RNA was isolated using the TRIzol® RNA isolation kit (Invitrogen) from rat and mouse peripheral
130 blood mononuclear cells, microglia SM826 cells, and neonatal brain tissues. Reverse transcription
131 was performed following the manufacturer's instructions (AB Applied System). Quantitative real-
132 time PCR was performed using a Bio-Rad CFX 96 system (Bio-Rad) and detected by SYBR Green
133 master mix (Bio-Rad) as previous described (Yang et al., 2014). The following *Opn* primers were
134 used for PCR reaction.

135 *Opn* (rat) GCTCTCAAGGTCATCCCAGTTG; TGTTTCCACGCTTGGTTCATC

136 *Opn* (mice) AGCCACAAGTTTCACAGCCACAAGG; CTGAGAAATGAGCAGTTAGTATTCCTGC

137 **Antibodies**

138 The following antibodies were used for immunoblotting or immunofluorescence labeling: anti-OPN
139 (R&D Systems, AF808), anti-Cystatin C (R&D Systems, AF1238), anti-GAPDH (EMD Millipore,
140 MAB374), anti-β-actin (Sigma-Aldrich, A5441), anti-MMP9 (Sigma-Aldrich, AV33090), anti-
141 GFAP (EMD Millipore, AB5804), anti-Iba1 (Wako, 019-1974), anti-transferrin (Abcam, ab9538),
142 anti-NG2 (Milipore, AB5320), and anti-NeuN (EMD Miliipore, MAB377). Secondary antibodies
143 for immunocytochemistry were conjugated to Alexa Fluor-488 and-594 (Invitrogen).

144

145 **Statistical analyses**

146 All values were expressed as mean ± SD. Significance of correlations was determined by Pearson's
147 correlation coefficient (GraphPad Prism). $P < 0.05$ was considered significant. Reporting of
148 significantly changed proteins identified by label free quantitative proteomics relied on $p < 0.01$
149 (CI=99%) and empirical false positive rate was determined as previously reported (Dammer et al.,
150 2015; Donovan et al., 2013), in a null experimental comparison of controls to be in the range of 10-
151 15%.

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154

155 **RESULTS:**156 **HI and LPS/HI insults, but not systemic LPS exposure, elevates the plasma OPN levels**

157 Plasma proteins of four groups of P11 C57BL/6 mice of both genders, unchallenged or at 24 h after
158 LPS exposure (0.3 mg/kg, IP), pure HI-, or LPS/HI-insult (n=3 for each group), were resolved by
159 SDS-PAGE followed by in-gel trypsin digestion and LC-MS/MS analysis (Fig 1A). TTC staining
160 confirmed cerebral infarct after HI and LPS/HI injury (pale color in Fig 1A), but not after low-dose
161 LPS-exposure. Label-free quantification was performed based on the ion current measurements of
162 identified peptides. By employing a fold change [$\log_2(\text{ratio}) \geq \pm 0.89$] and peptide spectral count (\geq
163 2) cut-off, we identified 16 proteins that were uniquely associated with HI and LPS/HI insults, but
164 not with LPS-exposure, compared with the unchallenged plasma samples (Fig. 1B, C). Among the
165 16 candidate biomarkers, OPN rises to 3.2-fold after HI and 2.5-fold after LPS/HI injury (Table 1).
166 Cystatin C/Stefin-3 (STFA3), elevated to 6.2-fold after HI and 3.2-fold after LPS/HI, is another
167 intriguing candidate since it was suggested to be a biomarker for renal injury in neonatal asphyxia
168 (Allegaert et al., 2015). Thus, we attempted to validate these two potential blood biomarkers, while
169 the prognostic value of the other candidates could be investigated in future studies.

170 To confirm the proteomic findings, we used immunoblotting to examine OPN and Cystatin
171 C in the blood and brain at 24 h following hypoxia, LPS-exposure, HI or LPS/HI insult (Fig 1D, E).
172 This analysis validated an increase of both OPN and Cystatin C in the blood after HI- and LPS/HI-
173 injury, but we chose OPN for further characterize OPN, because the basal level of plasma OPN is
174 very low, making its induction more unequivocal. Moreover, HI and LPS/HI induce robust OPN
175 expression in the ipsilateral cortex (Right, R* in Fig 1E), raising the possibility that plasma OPN
176 may derive from the HI-injured brain.

177 To test its specificity as a biomarker, we compared the amount of plasma OPN at 24 h after
178 intracerebroventricular injection of the phosphate saline (ICV-PBS), ligation of the right common
179 carotid artery (RCCAO), intraperitoneal injection of high-dose, 3 mg/kg LPS (IP-LPS[10x]), intra-
180 trachea injection of 0.3 mg/kg LPS (IT-LPS), cortical photothrombosis (thrombosis), and HI injury
181 in P10 mice (Fig 1F, the numbers of mice used for each condition are indicated). This analysis
182 showed that only thrombosis and HI caused significant induction of plasma OPN (** $p < 0.01$; ***
183 $p < 0.001$ compared to the basal level in unchallenged mice). These data suggest that plasma OPN
184 may monitor a common pathological process following cerebral ischemia and HI, such as microglia
185 activation, but it is not a sensitive marker for systemic immune activation.

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188 **HI-induced plasma OPN is derived from the brain microglia/macrophages**

189 To test the source of plasma OPN, we compared the *Opn* mRNA levels in the brain and peripheral
190 blood mononuclear cells (PBMCs) after HI insult in mouse and rat neonates. This analysis showed
191 20 to 35-fold increase of *Opn* mRNAs in the brain, but minimal changes in PBMCs in animals that
192 exhibited cerebral infarction at 24 h post-HI (Fig 2A and inserts). Our data are consistent with past
193 studies showing strong post-stroke induction of *Opn* mRNA in the rodent brain, but not in the blood
194 (Tang et al., 2002; Tang et al., 2003).

195 To determine OPN-expressing cells, we used immunostaining to examine HI-injured brains
196 and found strong OPN expression in Iba1+ microglia/macrophages, occasional OPN expression in
197 NG2+ glial progenitors, but not in GFAP+ astrocytes (Fig 2B), similar to previous reports (Ellison
198 et al., 1998; Gliem et al., 2015). Accordingly, in-vitro LPS stimulation triggers the expression of
199 OPN by microglial cells (Fig 2C). Moreover, ICV injection of LPS, a model of selective microglia
200 activation, induced the brain *Opn* mRNAs and the plasma OPN protein 24 h later (Fig 2D). These
201 results suggest that OPN is mainly produced by activated microglia and macrophages in HI-injured
202 brain, and subsequently transported to blood as a secreted phosphoprotein.

203

204 **Plasma OPN is a sensitive and predictive biomarker for brain injury in experimental HIE**

205 We also compared the protein levels of OPN, MMP-9 and GFAP in the brain versus blood after HI
206 in murine neonates. Immunoblotting suggested that the presence of multiple GFAP bands and the
207 increase of MMP-9 or OPN are all sensitive markers of HI injury in the brain (Fig 3A). In contrast,
208 only induction of OPN, but not MMP-9 or GFAP, was clearly detectable in the blood of these HI-
209 injured animals at 24 h recovery (Fig 3B). Moreover, multiplex ELISA (Luminex) showed strong
210 correlation between the plasma OPN levels and the OPN (Fig. 3C, $p = 0.0038$) and MMP-9 levels in
211 the brain (Fig 3D, $p = 0.0115$; $n=10$) at 24 h recovery. These results suggest that plasma OPN is a
212 monitoring biomarker for brain damage in experimental HI.

213 To examine the timing of plasma OPN induction after HI, we collected serial blood samples
214 at 6, 12, 24, 48, 72, and 96 h post-injury from a cohort of P7 rats for immunoblotting. This analysis
215 showed variable dynamics of OPN alterations in HI-injured animals, but strong induction (>5-fold)
216 only occurred after 24 h post-HI (Fig 3E, $n=5$). These data suggest that the rise of plasma OPN may
217 indicate the minimal onset time of perinatal asphyxia.

218 Finally, to test the prognostic value of plasma OPN for HI brain damage, we shortened the
219 duration of hypoxia (to 35 min) to produce more variable brain damage in the same cohort of mouse
220 pups (n=11), and examined the relationship between the severity of brain injury at 7 d post-HI and
221 the corresponding plasma OPN levels at 48 h (Fig 3F). We found that mice with mild brain injury
222 (n=5) exhibited low plasma OPN levels that were indistinguishable from the baseline at 48 h post-
223 HI, whereas animals with severe brain damage (n=6) showed higher plasma OPN levels from the
224 baseline at 48 h post-HI (Fig 3G). Yet, the 48 h plasma OPN levels between mice that would later
225 develop mild- or severe- brain damage were not significantly different due to outliers in each group.
226 These results suggest that plasma OPN levels have predictive value for the severity of HIE brain
227 injury, but multiple time-point monitoring or the use of additional biomarkers is needed to increase
228 the prognostic accuracy.
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231 **DISCUSSION:**

232 The objective of this study is to test the applicability of quantitative proteomic methods towards the
233 discovery of blood biomarkers for HI brain injury. Although therapeutic hypothermia (to 32-34⁰C)
234 starting within 6 hours after HI insult and continuing for 12-72 hours improve outcomes, 40-50% of
235 infants treated with hypothermia still die or develop significant neurological disabilities. Thus, there
236 is a need for better HIE therapies and reliable biomarkers to monitor the responses to treatments
237 (Bennet et al., 2010). A recent study indicated that magnetic resonance imaging performed at 44
238 weeks postmenstrual age or when clinically feasible is a reliable indicator of neurodevelopmental
239 outcomes at 6-7 years of age (Shankaran et al., 2015). Yet, simple laboratory assays using blood or
240 urine that can be performed more widely and acutely remain a highly useful tool in clinical care of
241 HIE. To this end, past research using a candidate approach has suggested several blood biomarkers
242 in HIE, such as GFAP and MMP-9 (Bembea et al., 2011; Bednarek et al., 2012). In contrast, an
243 open-ended proteomic strategy has not been utilized, despite its application in identifying the blood
244 biomarkers in obstetrics conditions (Klein et al., 2014). In preclinical study, only two-dimensional
245 differential gel electrophoresis has been used to compare the brain proteins after HI (Rosenkranz et
246 al., 2012). To our knowledge, the present study is the first report using quantitative proteomics to
247 identify blood biomarkers in experimental HI. Our results support the use of quantitative proteomics
248 for biomarker discovery and suggest osteopontin as a prognostic blood biomarker in HI brain injury.

249 In our experimental design, the control group of low-dose intraperitoneal LPS exposure
250 (which does not produce obvious brain damage) is designed to avoid non-lesion-associated immune
251 alterations in LPS/HI injury. Using mass spectrometry and statistical analysis of the plasma proteins
252 in four experimental groups, we uncovered 16 proteins that are associated with both HI and LPS/HI
253 brain injury. Among them, we selected OPN and Cystatin C for validation given their relevance to
254 neonatal asphyxia in the literature, and saved the other candidates for future study. Specifically, the
255 plasma Cystatin C level has been suggested to be a biomarker of renal injury in neonatal asphyxia
256 (Allegaert et al., 2014). Induction of the protein and mRNA of OPN was also reported in HI-injured
257 rodent neonatal brains (van Velthoven et al., 2011; Chen et al., 2011).

258 Our immunoblot results validated the increase of both OPN and Cystatin C in the blood after
259 HI and LPS/HI injury, but we decided to further characterize OPN given its low basal level (which
260 assists the detection of post-injury induction) and lesion-side specific induction in HI- and LPS/HI-
261 injured brains. These characteristics raise the possibility that post-HI plasma OPN may derive from

262 the injured brain, a scenario that is further supported by two observations. First, we showed that HI
263 induces the *Opn* mRNAs in the brain, but not in the blood, consistent with the previous finding of
264 brain-specific induction of *Opn* mRNAs in cerebral ischemia models (Tang et al., 2002; Tang et al,
265 2003). Second, cerebral ventricular injection of LPS (ICV-LPS)—a model of selective microglia
266 activation—was able to elevate the plasma OPN levels, whereas intraperitoneal injection of 10-fold,
267 3 mg/kg LPS (IP-LPS[10X])—a model to induce systemic immune responses—lacked this effect.

268 In addition, our results suggest that plasma OPN is more sensitive than MMP-9 and GFAP
269 as a biomarker of experimental HI brain injury. The higher sensitivity of OPN following HI injury
270 may relate to its intrinsic property as a secreted protein or the greater stability in blood (Lanteri et
271 al., 2012). We also showed that the plasma OPN induction typically occurs after 24 h of HI onset
272 and closely correlates with the brain MMP-9 level, and that high levels of plasma OPN at 48 h post-
273 HI correlate with severe brain injury at 7 d. Whether human HIE neonates show similar induction of
274 plasma OPN warrants further investigation. If a similar pattern is observed, monitoring the plasma
275 OPN levels in neonates may serve two purposes. First, the rise of plasma OPN may indicate the
276 minimal onset time of perinatal HI (approximately 24 h), which would suggest intrauterine HI stress
277 and poorer responses to hypothermia treatment. Second, the trajectory of plasma OPN alterations
278 may indicate the progression or recovery of brain injury in post-asphyxia neonates. Importantly, our
279 results do not exclude the possibility that plasma OPN may also derive from activated macrophages
280 outside the brain. Hence, differential diagnosis and multiple-tissue monitoring remain essential for
281 the interpretation of plasma OPN induction in neonatal care.

282 Mass spectrometry-based proteomics is a powerful strategy to determine disease-associated
283 biomarkers in biofluids, including blood, urine, and the cerebrospinal fluid. With this methodology,
284 no additional priori hypothesis of the biomarker is needed, and once a protein biomarker is found,
285 simple and relatively cheap laboratory tests can be developed for multiple time-point monitoring of
286 the disease progression even in less-developed areas in the world. Moreover, when applied to well-
287 controlled samples, quantitative proteomics is a very efficient method to uncover the differential
288 biomarkers. For example, the present study uses only 4 experimental groups and 3 mice per group,
289 but we were able to uncover OPN as a blood biomarker of HI brain injury by statistical analysis. By
290 adding comparison groups and enlarging the sample size, this quantitative proteomic strategy may
291 reveal additional blood biomarkers in HIE.

292 In summary, our findings support the application of proteomic analysis to identify biomarkers
293 in HIE. Moreover, the prognostic value of plasma OPN in HI brain injury warrants investigation in
294 human infants.
295
296

297 **FIGURE LEGEND:**

298 **Figure 1.** Proteomic analysis of plasma biomarker for murine neonatal HI brain injury.

299 **A**, Plasma proteins collected from unchallenged (UN), low-dose LPS (0.3 mg/kg, IP)-exposed, and
300 HI- or LPS/HI-injured P11 mice were separated by SDS-PAGE and analyzed as depicted (n=3 for
301 each). Also shown are representative TTC-stained brains in each group, showing cerebral infarction
302 (pale color) only in HI- or LPS/HI-injured animals. **B**, Venn diagram showing the number of unique
303 proteins in the depicted conditions when compared to the unchallenged animals. **C**, Heat-map and
304 hierarchical clustering of the 12 examined animals based on the signal intensity of HI- and LPS/HI-
305 associated proteins. Shown is the log₂ scale of fold change. **D, E**, Immunoblot showed specific OPN
306 induction in the plasma and the ipsilateral hemisphere (Right, R*) of mice after HI or LPS/HI insult.
307 In contrast, while Cystatin C showed an increased plasma level after HI- and LPS/HI-injury, it was
308 not elevated in the brain after these injuries. Asterisk: non-specific anti-Cystatin C band in the brain.
309 rOPN: recombinant mouse OPN used as positive controls. **F**, Immunoblotting showed that cerebral
310 stroke (thrombosis) also induced a high level of plasma OPN, whereas intracerebroventricular
311 injection of phosphate saline (ICV-PBS), unilateral carotid artery ligation (RCCAO), intraperitoneal
312 (IP) injection of 3 mg/kg LPS (IP-LPS[10X]), and intra-tracheal application of 0/3 mg/kg LPS (IT-
313 LPS) lacked this effect. The numbers of mouse pups examined in each condition are indicated. **:
314 p<0.01; ***: p<0.001 by *t*-test compared to the level in unchallenged animals.

315
316 **Table 1:** Plasma proteins associated with HI and LPS/HI brain injury at 24 h recovery in P10 mice.
317 Proteomics detection and statistical analysis was performed as described in the text. Sixteen high
318 confidence proteins changing in this category with two or more MS/MS-identified peptide feature
319 counts used for quantitation across the 12 samples are reported below.

320
321 **Figure 2.** The source and specificity of plasma OPN as a biomarker.

322 **A**, RT-PCR showed that HI triggered 20-30 fold increase of *Opn* mRNAs in the brain of P10 mice
323 and P7 rats at 24 h recovery, but only insignificant changes in PBMCs (n=4-5 for each group). **B**,
324 Immuno-labeling showed widespread OPN expression by Iba1+ microglia, occasional expression in
325 NG2+glial progenitors, but rare expression in GFAP+ astrocytes after HI injury (n > 4). Scale bar:
326 40 μm **C**, Application of LPS (1 ng/ml) to microglial SM826 cells in vitro led to induction of the
327 OPN protein. **D**, Similarly, ICV injection of LPS (1 μg) induced *Opn* mRNA in the brain, as well
328 as, the plasma OPN protein at 24 h recovery.

329

330 **Figure 3.** The sensitivity and prognostic value of plasma OPN as a biomarker for HI brain injury.

331 **A, B,** Comparison of OPN and two other candidate biomarkers (MMP-9 and GFAP) in the brain

332 and the brain after HI and LPS/HI injury. In brain extracts, elevation of MMP-9 or OPN expression

333 and multiple GFAP bands were all observed after injury, but in the plasma only OPN induction was

334 detectable at 24 h post-insult. **C, D,** Multiplex ELISA was used to compare the brain and the plasma

335 OPN or MMP-9 levels in P10 mice after HI. The plasma OPN levels showed strong correlation with

336 the brain OPN ($r = 0.82$) and the brain MMP-9 levels ($r = 0.75$, $n=10$) at 24 h after HI injury. **E,** HI-

337 injured P7 rats were used to assess the time course of plasma OPN induction after HI injury ($n= 5$).

338 Strong (> 5 -fold) induction of plasma OPN only occurred after 24 h post-HI. Asterisk: non-specific

339 signal or hyper-phosphorylated OPN. **F,** Comparison of the plasma OPN level at 48 h post-HI to the

340 severity of brain injury at 7 d. Shown are representative subjects for the low and high plasma OPN

341 groups Asterisk: the HI-injured hemisphere. **G,** Quantification showed significant elevation of the

342 plasma OPN levels in the severe damage group compared with unchallenged mice ($n=5$ and 6 per

343 group for mild and severe brain injury, respectively; $p=0.048$ by *t*-test).

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347 **REFERENCES:** (790 words)

348 Albertsson AM, Zhang X, Leavenworth J, Bi D, Nair S, Qiao L, Hagberg H, Mallard C, Cantor H,
349 Wang X (2014) The effect of osteopontin and osteopontin-derived peptides on preterm brain injury.
350 *Journal of Neuroinflammation* 11:197.

351
352 Allegaert K, Mekahli D, van den Anker J (2015) Cystatin C in newborns: a promising renal
353 biomarker in search for standardization and validation. *Journal of Maternal-Fetal and Neonatal*
354 *Medicine* 28:1833-1838.

355
356 Bednarek N, Svedin P, Garnotel R, Favrais G, Loron G, Schwendiman L, Hagberg H, Morville P,
357 Mallard C, Gressens P (2012) Increased MMP-9 and TIMP-1 in mouse neonatal brain and plasma
358 and in human neonatal plasma after hypoxia-ischemia: a potential marker of neonatal
359 encephalopathy. *Pediatric Research* 71:63-70.

360
361 Bellahcene A, Castronovo V, Ogbureke KU, Fisher LW, Fedarko NS (2008) Small integrin-binding
362 ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer. *Nature Reviews*
363 *Cancer* 8:212-226.

364
365 Bembea MM, Savage W, Strouse JJ, Schwartz JM, Graham E, Thompson CB, Everett A (2011)
366 Glial fibrillary acidic protein as a brain injury biomarker in children undergoing extracorporeal
367 membrane oxygenation. *Pediatric Critical Care Medicine* 12:572-579.

368
369 Bennet L, Booth L, Gunn AJ (2010) Potential biomarkers for hypoxic-ischemic encephalopathy.
370 *Seminars in Fetal and Neonatal Medicine* 15:253-260.

371
372 Bonestroo HJ, Nijboer CH, van Velthoven CT, van Bel F, Heijnen CJ (2015) The neonatal brain is
373 not protected by osteopontin peptide treatment after hypoxia-ischemia. *Developmental*
374 *Neuroscience* 37:142-152.

375
376 Chen W, Ma Q, Suzuki H, Hartman R, Tang J, Zhang JH (2011) Osteopontin reduced hypoxia-
377 ischemia neonatal brain injury by suppression of apoptosis in a rat pup model. *Stroke* 42:764-769.

378
379 Dammer EB, Duong DM, Diner I, Gearing M, Feng Y, Lah JJ, Levey AI, Seyfried NT (2013)
380 Neuron enriched nuclear proteome isolated from human brain. *Journal of Proteome Research*
381 12:3193-3206.

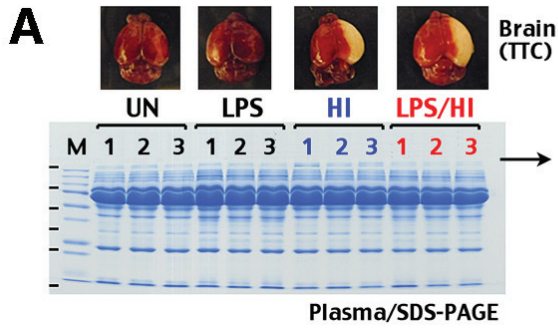
382
383 Dammer EB, Lee AK, Duong DM, Gearing M, Lah JJ, Levey AI, Seyfried NT (2015) Quantitative
384 phosphoproteomics of Alzheimer's disease reveals cross-talk between kinases and small heat shock
385 proteins. *Proteomics* 15:508-519.

386
387 Donovan LE, Dammer EB, Duong DM, Hanfelt JJ, Levey AI, Seyfried NT, Lah JJ (2013)
388 Exploring the potential of the platelet membrane proteome as a source of peripheral biomarkers for
389 Alzheimer's disease. *Alzheimer's Research and Therapy* 5:32.

390
391 Ellison JA, Velier JJ, Spera P, Jonak ZL, Wang X, Barone FC, Feuerstein GZ (1998) Osteopontin
392 and its integrin receptor alpha(v)beta3 are upregulated during formation of the glial scar after focal
393 stroke. *Stroke* 29:1698-1706

394

- 395 Gliem M, Krammes K, Liaw L, van Rooijen N, Hartung HP, Jander S (2015) Macrophage-derived
396 osteopontin induces reactive astrocyte polarization and promotes re-establishment of the blood brain
397 barrier after ischemic stroke. *Glia* 63: 2198-2207.
398
- 399 Gozal YM, Duong DM, Gearing M, Cheng D, Hanfelt JJ, Funderburk C, Peng J, Lah JJ, Levey AI
400 (2009) Proteomics analysis reveals novel components in the detergent-insoluble subproteome in
401 Alzheimer's disease. *Journal of Proteome Research* 8:5069-5079.
402
- 403 Herskowitz JH, Seyfried NT, Gearing M, Kahn RA, Peng J, Levey AI, Lah JJ (2011) Rho kinase II
404 phosphorylation of the lipoprotein receptor LR11/SORLA alters amyloid-beta production. *Journal*
405 *of Biological Chemistry* 286:6117-6127.
406
- 407 Klein J, Buffin-Meyer B, Mullen W, Carty DM, Delles C, Vlahou A, Mischak H, Decramer S,
408 Bascands JL, Schanstra JP (2014) Clinical proteomics in obstetrics and neonatology. *Expert Review*
409 *of Proteomics* 11:75-89.
410
- 411 Lanteri P, Lombardi G, Colombini A, Grasso D, Banfi G (2012) Stability of osteopontin in plasma
412 and serum. *Clinical Chemistry and Laboratory Medicine* 50:1979-1984.
413
- 414 Rosenkranz K, May C, Meier C, Marcus K (2012) Proteomic analysis of alterations induced by
415 perinatal hypoxic-ischemic brain injury. *Journal of Proteome Research* 11:5794-5803.
416
- 417 Shankaran S, McDonald SA, Laptook AR, Hintz SR, Barnes PD, Das A, Pappas A, Higgins RD,
418 Eunice Kennedy Shriver National Institute of Child H, Human Development Neonatal Research N
419 (2015) Neonatal Magnetic Resonance Imaging Pattern of Brain Injury as a Biomarker of Childhood
420 Outcomes following a Trial of Hypothermia for Neonatal Hypoxic-Ischemic Encephalopathy.
421 *Journal of Pediatrics* 167:987-993 e983.
422
- 423 Tang Y, Lu A, Aronow BJ, Wagner KR, Sharp FR (2002) Genomic responses of the brain to
424 ischemic stroke, intracerebral haemorrhage, kainate seizures, hypoglycemia, and hypoxia. *European*
425 *Journal of Neuroscience* 15:1937-1952.
426
- 427 Tang Y, Nee AC, Lu A, Ran R, Sharp FR (2003) Blood genomic expression profile for neuronal
428 injury. *Journal of Cerebral Blood Flow and Metabolism* 23:310-319.
429
- 430 van Velthoven CT, Heijnen CJ, van Bel F, Kavelaars A (2011) Osteopontin enhances endogenous
431 repair after neonatal hypoxic-ischemic brain injury. *Stroke* 42:2294-2301.
432
- 433 Yang D, Nemkul N, Shereen A, Jone A, Dunn RS, Lawrence DA, Lindquist D, Kuan CY (2009)
434 Therapeutic administration of plasminogen activator inhibitor-1 prevents hypoxic-ischemic brain
435 injury in newborns. *Journal of Neuroscience* 29:8669-8674.
436
- 437 Yang D, Sun YY, Bhaumik SK, Li Y, Baumann JM, Lin X, Zhang Y, Lin SH, Dunn RS, Liu CY,
438 Shie FS, Lee YH, Wills-Karp M, Chougnat CA, Kallapur SG, Lewkowich IP, Lindquist DM,
439 Murali-Krishna K, Kuan CY (2014) Blocking lymphocyte trafficking with FTY720 prevents
440 inflammation-sensitized hypoxic-ischemic brain injury in newborns. *Journal of Neuroscience*
441 34:16467-16481.
442
443



In-Gel Trypsin Digestion
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 Peptide Fractionation
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 Peptide ID & Quantification

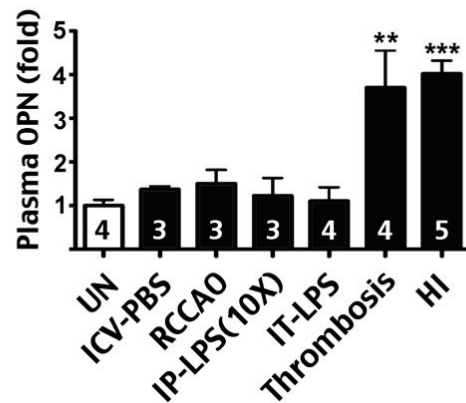
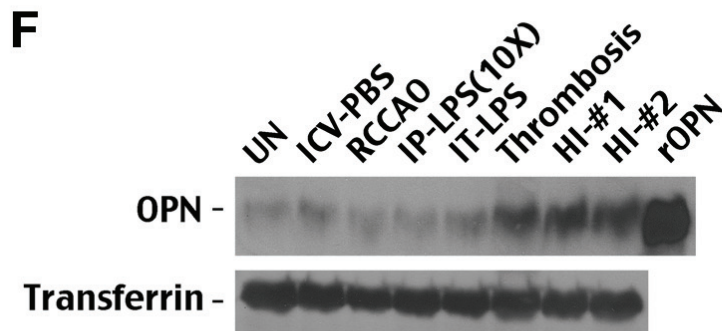
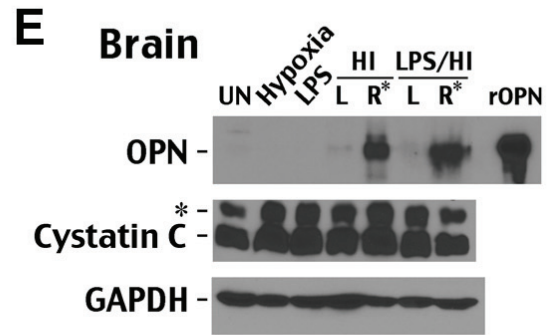
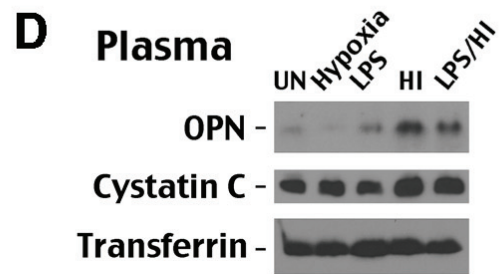
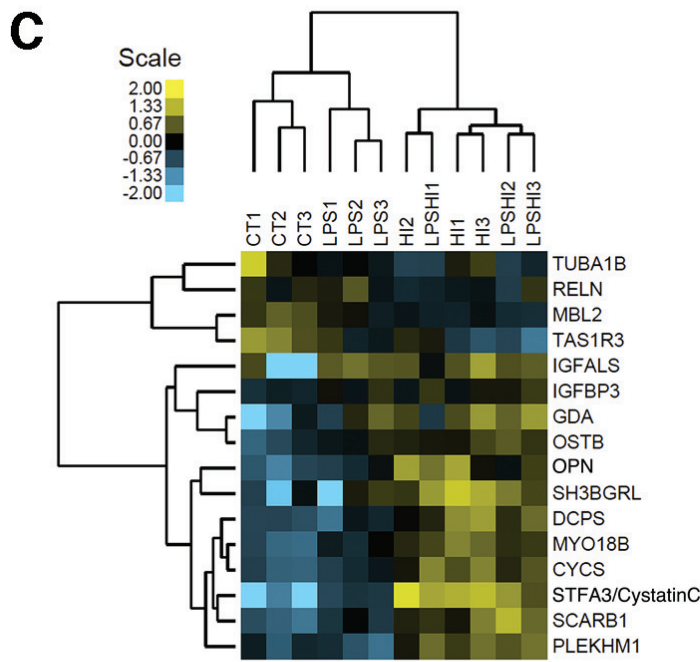
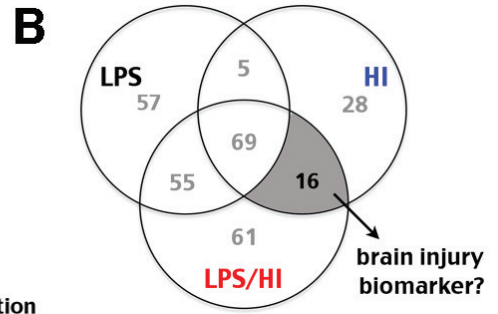


Table 1. Plasma proteins that are uniquely associated with HI and LPS/HI brain injury at 24 h recovery

Symbol	Description (all in <i>Mus musculus</i>)	Peptide Feature Counts	CT/CT log ₂ (ratio)	CT/CT SD, n=3	LPS/CT log ₂ (ratio)	LPS/CT SD, n=9	HI/CT log ₂ (ratio)	HI/CT SD, n=9	LPSHI/CT log ₂ (ratio)	LPSHI/CT SD, n=9
TUBA1B	Tubulin alpha-1B chain	58	-0.6	0.33	-0.78	0.5	-0.94	0.47	-1.11	0.41
RELN	Reelin precursor	18	0.01	0.22	-0.57	0.35	-0.96	0.27	-1.05	0.14
MBL2	Mannos-binding protein C precursor	86	0.23	0.28	-0.51	0.43	-1.17	0.3	-1.55	0.3
TAS1R3	Taste receptor typ1 1 member 3 precursor	5	-0.35	0.17	-0.77	0.43	-1.22	0.53	-1.38	0.56
IGFALS	Insulin-like growth factor-binding protein complex acid labile subunit precursor	171	-0.11	0.26	-0.56	0.18	-1.78	0.34	-2.06	0.31
IGFBP3	Insulin-like growth factor-binding protein 3 precursor	27	-0.19	0.16	0.13	0.16	-0.92	0.25	-1.28	0.35
GDA	Guanine deaminase	15	0.25	0.36	0.62	0.34	1.03	0.45	1.05	0.49
OSTB	Organic solute transporter subunit beta	8	0.42	0.14	0.79	0.29	0.9	0.31	1.05	0.33
OPN	Osteopontin	11	0.12	0.4	0.69	0.28	1.68	0.6	1.31	0.43
STFA3	Stefin-3 (Cystatin C)	14	-0.48	0.99	0.62	0.68	2.63	0.71	1.67	0.6
MYO18B	Myosin XVIIIb	2	-0.25	0.15	0.8	0.25	1.51	0.31	1.3	0.2
SH3BGRL	SH3 domain-binding glutamic acid-rich like protein	3	0.33	1.21	-0.09	1.98	1.86	0.84	1.7	0.76
DCPS	m7GpppX diphosphatase	12	0.2	0.35	0.34	0.45	1.55	0.61	1.47	0.32
PLEKHM1	Pleckstrin homology domain-containing family M member 1	2	-0.05	0.49	-0.12	0.45	0.92	0.42	1.06	0.44
CYCS	Cytochrome c, somatic	50	-0.35	0.14	0.64	0.21	1.59	0.23	1.44	0.28
SCARB1	Scavenger receptor class B member 1 isoform 1	2	-0.25	0.1	0.71	0.25	1.3	0.4	1.75	0.46

