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2017

Summanen , M , Seikku , L , Rahkonen , P , Stefanovic , V , Teramo , K , Andersson , S , Kaila , K & Rahkonen , L 2017 , ' Comparison of Umbilical Serum Copeptin Relative to Erythropoietin and S100B as Asphyxia Biomarkers at Birth ' , Neonatology , vol. 112 , no. 1 , pp. 60-66 . <https://doi.org/10.1159/000456063>

<http://hdl.handle.net/10138/310867>

<https://doi.org/10.1159/000456063>

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Comparison of umbilical serum copeptin relative to erythropoietin and S100B as asphyxia biomarkers at birth

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Short title: Birth asphyxia biomarkers

Keywords: neonate, birth asphyxia, arginine vasopressin (AVP), copeptin, erythropoietin, S100B

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32 **Abstract**

33

34 **Background:** Birth asphyxia, estimated to account for a million neonatal deaths annually, can cause
35 a wide variety of neurodevelopmental impairments. There is a need to develop new, swift methods to
36 identify those neonates who would benefit from neuroprotective treatments such as hypothermia.

37 **Objectives:** To examine the utility of cord serum copeptin, a stable byproduct of arginine vasopressin
38 release, as a biomarker of birth asphyxia based on a comparison with two biomarkers of hypoxia and
39 brain trauma, erythropoietin and S100B.

40 **Methods:** The study population consisted of 140 singleton, term neonates; 113 controls and 27 with
41 birth asphyxia (two/three criteria met: umbilical artery pH <7.10, base excess <-12 mmol/l, and 5-
42 minute Apgar score <7). All deliveries were planned vaginal, but 51 neonates were born by
43 emergency cesarean section. Copeptin, S100B, and erythropoietin levels in umbilical artery samples
44 were measured by immunoassays.

45 **Results:** Copeptin correlated in the entire study population more strongly with umbilical artery base
46 excess than S100B and erythropoietin, and only copeptin correlated with arterial pH. Furthermore,
47 only copeptin levels were significantly higher in cases of birth asphyxia, and in vaginally born
48 neonates they were found to increase as a function of labor duration. Copeptin was elevated in
49 neonates born via vacuum extraction, whereas erythropoietin levels showed a slight increase after
50 emergency cesarean section.

51 **Conclusions:** In this study population, S100B and erythropoietin were not valid biomarkers of birth
52 asphyxia. In contrast, our work suggests that copeptin has high potential to become a routinely used
53 biomarker for acute birth asphyxia and neonatal distress.

54 **Introduction**

55 Birth asphyxia is a severe clinical problem globally, which has been estimated to account for a million
56 of neonatal deaths annually [1]. The neurodevelopmental impairments in individuals who develop
57 neonatal hypoxic-ischemic encephalopathy (HIE) range from minor cognitive problems and
58 sensorimotor defects to cerebral palsy [1, 2]. Methods for identifying those neonates who would
59 benefit from neuroprotective treatments such as therapeutic hypothermia is a major challenge because
60 of the restricted time window of only a few hours for decision making. The increasing risk of HIE
61 associated with enhanced acidemia has been well established. The Apgar scores at 5 minutes are
62 known to correlate with the risk of neurological disability [3]. However, the sensitivity and specificity
63 of arterial cord blood pH values and Apgar scores with regard to outcome following HIE are low [2].
64 Thus, reliable biomarkers for predicting outcome after birth asphyxia are urgently needed.

65

66 A number of studies on blood-borne protein biomarkers in neonates have been published in recent
67 years [4-6]. Among these, erythropoietin (EPO) is a biomarker of chronic hypoxia [7], and high levels
68 of umbilical plasma EPO at birth are associated with an increased risk for adverse outcome [8]. S100B
69 is considered as a biomarker of brain cell damage [9], and its levels are known to rise at the early
70 phase of acute asphyxia [6]. In the present study, we examine the utility of cord serum copeptin, a
71 byproduct of arginine vasopressin (AVP) release, as a biomarker of birth asphyxia based on a
72 comparison with EPO and S100B. The rationale of this approach lies in the fact that a massive surge
73 of AVP release takes place during normal vaginal birth in response to activation of the fetal
74 hypothalamic–pituitary–adrenal (HPA) axis, which is further accentuated by various kinds of stress
75 factors such as infections, intra-uterine growth restriction, and acidosis/birth asphyxia [4, 5, 10, 11].
76 In contrast to AVP, copeptin is biochemically stable with a much longer half-life, and it is released
77 in an equimolar ratio to AVP, making it an ideal surrogate for AVP measurements [11, 12]. One of
78 the practical advantages of copeptin is that it is widely used as a biomarker of various
79 pathophysiological states like lower respiratory tract infections, septic shock and stroke in emergency
80 departments [13].

81

82 **Methods**

83 *Study design and patients*

The serum samples for this retrospective study were collected in the Department of Obstetrics and Gynecology, Helsinki University Hospital, Finland between May 2012 and April 2013. The study, approved by the local Ethics Committee of the Department of Obstetrics and Gynecology, Helsinki University Hospital, Finland (105/13/13/03/2012), consisted of 151 singleton births at or beyond 37+0 gestational weeks. Umbilical artery blood samples were collected immediately after birth from 72 neonates with suspected asphyxia, based on a 1-minute Apgar score <4 . The 1-minute Apgar score was used only for patient recruitment, to allow midwives to identify neonates suspected of having suffered from birth asphyxia. Umbilical artery cord blood samples from 79 control neonates with a 1-minute Apgar score ≥ 4 were collected during the five-day work week. Pregnancies complicated by maternal type 1 diabetes ($n=1$), preeclampsia ($n=3$), fetal growth restriction ($n=4$) and Rh immunization ($n=1$) were excluded. In addition, one neonate was excluded because of chromosomal anomaly and one had mitochondrial disease, leaving a total of 140 neonates for the final analysis. The neonates were divided into two groups, asphyxia and control, for data analysis. The neonate was considered to fulfill the diagnostic criteria for birth asphyxia if two of three criteria were met: umbilical artery pH <7.1 , umbilical artery base excess <-12 mmol/l, and a 5-minute Apgar score <7 [14, 15].

Gestational age was defined by fetal crown-rump length measurement at the first trimester ultrasound screening. Deliveries were all planned vaginal. Indications for emergency cesarean sections (ECS) were: fetal distress ($n=29$), labor dystocia ($n=10$), prolonged second stage of delivery ($n=2$), chorionamnionitis ($n=1$), fetal malpresentation ($n=3$), unsuccessful vacuum extraction ($n=4$), and umbilical cord prolapse ($n=2$). Data on maternal pregnancy characteristics and short-term perinatal outcome were collected from the hospital charts (table 1). Birth weight z-score was defined according to the Finnish population standardized for sex and gestational age [16].

Blood samples from the umbilical artery were used for measurements of pH, base excess (BE), pO_2 and pCO_2 (Radiometer ABL800 Flex blood gas analyzer, Copenhagen, Denmark). Serum samples from the umbilical artery were used for biomarker measurements.

Copeptin measurements

We used a sandwich enzyme-linked immunosorbent assay (ELISA, methodological details in the Supplementary material). The inter-assay variability was 5.8 %, which was estimated by calculating

the CV from eight duplicate samples run on two plates on two separate days. Fifteen serum samples were analyzed with both the ELISA and the BRAHMS copeptin Kryptor assay used in previous publications [4, 5], and a highly significant linear correlation (Pearson $r = 0.9793$, $p < 0.0001$) was found, covering the full range of values obtained using the Kryptor, 6.0 to 4637 pmol/l (median 381.2 pmol/l). This excellent linear correlation, with an R^2 of 0.9591 indicating a congruence of 96 % between the two methods, was used to convert the copeptin concentrations obtained with the ELISA to Kryptor concentrations. This allows for a direct comparison of our data with the already published copeptin results in neonates.

124

125 *S100B measurements*

Serum S100B was measured with electrochemiluminometric immunoassay using Modular e170 analyzer (Roche Diagnostics). The detection range for the S100B assay is 0.005 $\mu\text{g/l}$ and functional sensitivity less than 0.02 $\mu\text{g/l}$. The intra-assay coefficient of variation (CV) was less than 2.1 % and inter-assay variation better than 6.4 %.

130

131 *EPO measurements*

EPO was measured using a solid-phase chemiluminescent enzyme immunometric assay Immulite 2000 XPI analyser (Siemens Healthcare Diagnostics). The intra-assay CV was 3.6-6.8 %, while the total CV was 6.4-10.3 %. Detection limit was 1.0 IU/l, and functional sensitivity (CV 20 %) was 1.5 IU/l.

136

137 *Statistical analysis*

All three biomarkers were measured from each of the 140 samples included in this study. The following statistical analyses were performed with GraphPad Prism 6 or SPSS 22. Prior to calculating correlations and significances, statistical outliers ($p < 0.01$) for copeptin and S100B ($n=1$ for each) were excluded from the study population. Correlations between the pH, BE, S100B, EPO and copeptin values from all remaining samples were calculated with the Spearman correlation coefficient. Copeptin, S100B and EPO values between the study groups were compared using the Mann-Whitney U test or the Kruskal-Wallis test, and a receiver operating characteristic (ROC)-curve was drawn to determine the diagnostic accuracy of copeptin to birth asphyxia. The Chi-squared test or Fisher's exact test were used to determine significant differences in the study population.

147

148 **Results**

149 *Maternal and fetal/delivery characteristics*

150 The maternal and fetal/delivery characteristics are shown in table 1. There were no statistically
151 significant differences in any of the maternal or delivery characteristics between the two study groups.

152

153 *Dependence of biomarker levels on umbilical artery pH and BE*

154 The neonates' median pH, BE, pO₂ and pCO₂ values, which are routinely measured from umbilical
155 arterial cord blood at birth, are shown in table 1. The dependence of the three biomarkers on the blood
156 acid-base parameters are given in figure 1. Only copeptin levels showed a significant correlation with
157 umbilical artery pH ($r = -0.6219$, $p < 0.0001$, fig. 1a). All three biomarkers correlated with umbilical
158 artery BE (fig. 1d-f), with copeptin showing by far the highest correlation coefficient ($r = -0.6372$, p
159 < 0.0001).

160

161 *Biomarker levels and asphyxia*

162 Twenty-seven neonates in the study population belonged to the birth asphyxia group (see Methods
163 for present criteria). Copeptin levels were significantly higher among the neonates in the birth
164 asphyxia group compared to unaffected controls (mean 2450 pmol/l vs 1226 pmol/l, $p < 0.0001$, fig.
165 2c), whereas no differences in S100B (fig. 2a) or EPO (fig. 2b) levels were found. ROC-curve analysis
166 showed that copeptin concentrations discriminated with moderate accuracy between asphyxia, as
167 defined in this study, and controls: the area under the curve was 0.76 (95%-CI 0.69-0.86, fig. 2d). A
168 cut-off of 1522 pmol/l had a sensitivity of 77 % and a specificity of 70%.

169

170 *Biomarker levels and delivery mode*

171 All 140 deliveries were planned vaginal, but 51 neonates were ultimately born by ECS. Of the 89
172 vaginal deliveries, 33 were assisted with vacuum extraction. There were no differences in the delivery
173 mode between the asphyxia and control study groups (table 1). Copeptin levels were higher among
174 the neonates born via vacuum extraction as compared to ECS (mean 2021 pmol/l vs 1190 pmol/l, p
175 $= 0.003$, fig. 3a) or normal vaginal delivery, although the latter difference did not reach statistical
176 significance (mean 2021 pmol/l vs 1362 pmol/l, $p = 0.0522$, fig. 3a). No differences in S100B levels
177 were found between the groups (fig. 3b), whereas EPO levels were higher among neonates born via

178 ECS compared to normal vaginal delivery or vacuum extraction (means 713.7 U/l, 78.41 U/l, and
179 61.15 U/l, respectively; $p = 0.0002$ and $p = 0.001$, fig. 3c), which might reflect prenatal conditions
180 [7].

181

182 *Biomarker levels in relation to other variables*

183 S100B levels were higher in male neonates ($p = 0.0378$), but copeptin and EPO levels did not differ
184 based on the sex of the neonate. All biomarkers showed a correlation with the 5-minute Apgar score,
185 and copeptin and S100B correlated also with the 10-minute Apgar score (table 2). Copeptin and
186 S100B levels did not correlate with gestational age at birth, whereas EPO levels did ($r = 0.4513$, $p <$
187 0.0001). Only copeptin levels correlated significantly with birth weight ($r = -0.1713$, $p = 0.0438$).
188 Among the 89 neonates born vaginally, copeptin levels increased as a function of the total duration
189 of labor ($r = 0.3267$, $p = 0.0019$) and the duration of the second stage of labor ($r = 0.2787$, $p = 0.0086$;
190 table 2). EPO and S100B levels did not correlate with either of these variables.

191

192 **Discussion**

193 A wide spectrum of adaptive processes in respiratory, cardiovascular, and metabolic functions are
194 triggered at birth [4, 10]. Changes in biomarker concentrations during birth reflect, at least in part,
195 physiological fetal adaptive reactions, such as enhanced activation of the HPA-axis (see below), in
196 response to normal or complicated delivery. Distinct biomarkers have different profiles during fetal
197 and neonatal asphyxia [4, 7, 18]. The S100B level in plasma has been shown to rise at the early phase
198 of acute asphyxia [6, 18] and significantly higher S100B levels have been reported in asphyxiated
199 term neonates with intraventricular hemorrhage (IVH) or with HIE, compared to asphyxiated
200 neonates without IVH/HIE or to apparently healthy neonates [19]. EPO is a biomarker of chronic
201 hypoxia, and increased levels can be detected in fetal plasma and amniotic fluid in various
202 pathological pregnancies [7]. An association of high EPO levels during pregnancy and adverse acute
203 neonatal outcome, such as decreased umbilical cord pH, pO_2 and BE, and increased intensive care
204 unit admission, has also been reported [7, 20]. Furthermore, high levels of umbilical plasma EPO at
205 birth are associated with an increased risk for death or abnormal neurological outcome at two years
206 of age [8].

207

There are many *a priori* reasons why copeptin might turn out to be a highly useful biomarker of birth asphyxia, and a number of previous observations point in this direction [4, 5, 21]. During birth, the HPA axis shows massive activation, which results in the release of AVP, and this response is further enhanced by various types of stressors. Since AVP is highly unstable with a short half-life of 4-20 minutes [22], assays of this hypothalamic hormone itself are not suitable for clinical use. However, AVP is derived from a larger precursor peptide which contains copeptin, a stable C-terminal fragment with 39 amino acids. Copeptin is released in an equimolar ratio to AVP [23]. In line with the underlying HPA-based mechanisms, copeptin levels in cord blood increase in different stress situations, such as infections and hypoxia, both in term and preterm pregnancies [4, 5, 21]. High copeptin levels at birth are related to acute adverse neonatal outcomes such as IVH [21].

The single most important criterion for diagnosis of birth asphyxia is profound metabolic acidosis [14, 15]. Notably, copeptin levels increase along with decreasing umbilical cord blood pH and BE during normal birth [4], and they are even higher following birth asphyxia [5]. This means that the dynamics of enhanced copeptin plasma concentrations cover a wide range of levels of acidemia from normal to severely abnormal, i.e. those prevailing in birth asphyxia.

In line with the above considerations and data, we demonstrate here that copeptin levels are highly correlated with both arterial cord blood pH and BE (fig. 1). The strong and highly significant dependence of high copeptin levels on negative BE shown presently is of particular interest. In excellent agreement with the above data, copeptin levels turned out to be significantly higher in asphyxiated neonates vs controls in the present study. The sensitivity and specificity parameters in the ROC analysis were not as high as previously shown [5], which is attributable to the present inclusion criteria of the control group based on a 1-minute Apgar score of 4 or higher. In contrast, no difference was seen in S100B and EPO levels (fig. 2), most likely because of the relatively mild asphyxia criteria in the present work.

A further important finding in the present study is that copeptin levels increased as a function of the total duration of labor and on the duration of the second stage of labor, while EPO and S100B levels did not correlate with either variable. This result with copeptin is most likely explained by very recent observations that just a few contractions (most likely acting via transient periods of minor hypoxia

239 on the fetus) are sufficient to trigger detectable AVP/copeptin [24]. Given the cumulative nature of
240 the copeptin levels with a half-life of 30 minutes [25], the above dependence on labor duration is
241 readily explained. Thus, future work on sequential measurements of copeptin may turn out to be
242 valuable in enhancing the prognostic power of this biomarker.

243

244 It has been previously shown that significantly higher copeptin concentrations are observed after
245 vaginal delivery compared to delivery by primary cesarean section [4]. In the present study, the
246 patient cohort included only neonates born by ECS preceded by periods of labor contractions with
247 variable duration. Thus, it is not surprising that no significant difference in copeptin levels was
248 observed between neonates born vaginally or via ECS. However, copeptin levels were higher after
249 vaginal delivery assisted by vacuum extraction when compared to copeptin levels after ECS or normal
250 vaginal delivery, as reported before [4].

251

252 To summarize, our study indicates that copeptin has a high potential to become a routine biomarker
253 for neonatal distress and asphyxia. From a (patho)physiological point of view, its advantages are
254 based on the key role of AVP in the adaptations of the fetus to birth. From a practical point of view,
255 it is of much importance that serum copeptin is widely used as a biomarker in adults in emergency
256 departments [13], and therefore this approach can be readily extended to neonatal intensive care units.
257 Future studies are needed to determine whether copeptin concentrations at birth correlate with the
258 severity of HIE, and, more importantly, with long-term neurological outcome following birth
259 asphyxia.

260

261 **Acknowledgements**

262 The funding of work and materials for blood analyses was provided by Jane and Aatos Erkkö
263 Foundation (KK), Sigrid Juselius Foundation (KK) and the Academy of Finland (KK).

264

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330 **Figure legends**

331

332 **Fig. 1** Dependence of copeptin (a, d), S100B (b, e) and EPO levels (c, f) on pH (a-c) and base excess
333 (BE; d-f). P - and r - values for each pair are shown in the respective panels, regression lines were
334 drawn when the correlation was significant.

335

336 **Fig. 2** Comparisons between the birth asphyxia and control groups. There were no significant
337 differences in S100B (a) or EPO (b) levels between the groups. Copeptin (c) levels were significantly
338 higher in the asphyxia group compared to the control group. The medians and p-values of Mann-
339 Whitney U-test are shown (a-c). (d) ROC-curve for cord serum copeptin concentrations in relation to
340 birth asphyxia. The dotted lines indicate the optimal discriminative cut-off of 1522 pmol/l, resulting
341 in a sensitivity of 77 % and a specificity of 70 %.

342

343 **Fig. 3** Comparisons between biomarkers and delivery mode. Copeptin (a) levels were significantly
344 higher in neonates born via vacuum extraction compared to emergency cesarean section, whereas no
345 differences in S100B (b) levels were found between groups. EPO (c) levels were significantly higher
346 in neonates born via emergency cesarean section compared to the two other groups. The medians are
347 shown in each panel, and p - values of Kruskal-Wallis test are shown when the difference was
348 significant.

Table 1. Maternal and fetal/delivery characteristics of the study groups					
	Asphyxia (n = 27)		Control (n = 113)		p
Maternal characteristics					
Maternal age, years (mean)	30.9	<i>SD (5.9)</i>	31.1	<i>SD (5.5)</i>	0.815
Primiparity	15	<i>(55.6)</i>	62	<i>(54.9)</i>	1.000
<i>In vitro</i> fertilization	2	<i>(7.4)</i>	5	<i>(4.4)</i>	0.620
Smoking	4	<i>(14.8)</i>	9	<i>(8.0)</i>	0.277
Obesity (body mass index $\geq 30\text{kg/m}^2$)	2	<i>(7.4)</i>	17	<i>(15.0)</i>	0.530
Gestational diabetes	4	<i>(14.8)</i>	14	<i>(12.4)</i>	0.751
Chronic hypertension	1	<i>(3.7)</i>	3	<i>(2.7)</i>	0.580
Fetal/delivery characteristics					
Spontaneous vaginal delivery	7	<i>(25.9)</i>	49	<i>(43.4)</i>	0.257
Vacuum extraction	9	<i>(33.3)</i>	24	<i>(21.2)</i>	0.309
Emergency cesarean section	11	<i>(40.7)</i>	40	<i>(35.4)</i>	0.727
Gestational weeks at birth	41.1	<i>(40.0 - 41.6)</i>	40.7	<i>(39.7 - 41.7)</i>	0.372
Post-term births ($\geq\text{H42}^{+0}$)	5	<i>(18.5)</i>	22	<i>(19.5)</i>	1.000
Male	17	<i>(63.0)</i>	66	<i>(58.4)</i>	0.828
Birth weight (g)	3520	<i>(3260 - 3945)</i>	3656	<i>(3282 - 3991)</i>	0.499
Relative birth weight (SD)	-0.13	<i>(-1.02 - 0.62)</i>	0.02	<i>(-0.67 - 0.81)</i>	0.385
5 min Apgar score	6	<i>(4-7)</i>	8	<i>(6-9)</i>	<0.0001
10 min Apgar score	8	<i>(6-9)</i>	9	<i>(8-9.5)</i>	0.0009
Umbilical artery pH	7.03	<i>(6.97 - 7.08)</i>	7.21	<i>(7.14 - 7.30)</i>	<0.0001
Umbilical artery base excess	-12.7	<i>(-14.4 - -11.1)</i>	-5.80	<i>(-7.85 - -2.85)</i>	<0.0001
Umbilical artery pO ₂ (kPa)	1.9	<i>(1.1 - 2.8)</i>	2.4	<i>(1.7 - 3.3)</i>	0.029
Umbilical artery pCO ₂ (kPa)	10.1	<i>(9.2 - 12.7)</i>	7.8	<i>(6.7 - 9.2)</i>	<0.0001
Umbilical serum erythropoietin (U/l)	71.7	<i>(22.2 - 116.0)</i>	46.1	<i>(22.2 - 124.5)</i>	0.683
Umbilical serum S100B (µg/l)	0.33	<i>(0.19 - 0.63)</i>	0.31	<i>(0.24 - 0.45)</i>	0.712
Umbilical serum copeptin (pmol/l)	2279	<i>(1476-3144)</i>	973.7	<i>(320.9-1961)</i>	<0.0001
<i>Median (interquartile range) or number (percentage) are shown</i>					

Table 2. Biomarker correlations to other variables									
	Copeptin			S100B			EPO		
	n	r	p	n	r	p	n	r	p
5 min Apgar score	139	-0.2998	0.0004*	139	-0.2041	0.0159*	140	-0.2914	0.0005*
10 min Apgar score	139	-0.1678	0.0484*	139	-0.2048	0.0156*	140	-0.1380	0.1040
Gestational age	139	0.0644	0.4510	139	-0.0018	0.9836	140	0.4513	<0.0001*
Birth weight	139	-0.1713	0.0438*	139	-0.0235	0.7838	140	-0.0367	0.6672
Total labor duration	88	0.3267	0.0019*	89	0.1247	0.2444	89	0.0656	0.5414
Second stage labor duration	88	0.2787	0.0086*	89	0.1038	0.3332	89	0.0162	0.8802
<i>Significant correlations indicated by an asterisk.</i>									

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Fig. 1

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Fig. 2

Fig. 3