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1 **Changes in S100B and troponin levels in a fetal sheep model of worsening acidosis.**

2

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6 ABBREVIATIONS

7 HIE: hypoxic-ischemic encephalopathy

8 HI: hypoxia-ischemia

9 FHR: fetal heart rate

10 c-TnT: cardiac troponin T

11 UCO : umbilical cord occlusion

12 ABSTRACT

13 Background - S100B and cardiac troponin T (c-TnT) are relevant biomarkers at birth of  
14 hypoxic-ischemic encephalopathy (HIE) and myocardial ischemia secondary to metabolic  
15 acidosis during labor, respectively. The purpose was to assess in-utero changes in S100B and  
16 c-TnT levels in an experimental model of labor-like acidosis.

17 Methods - Repeated umbilical cord occlusions (UCOs) in ten experiments were performed in  
18 mild (phase A, 1 UCO/5 mn), moderate (phase B, 1 UCO/3 mn), and severe (phase C, 1  
19 UCO/2 mn) period. The experiments were stopped if arterial pH reached 6.90.

20 Results - UCOs resulted in fetal acidosis with pH dropping to  $6.99 \pm 0.13$ . When compared  
21 to the baseline period fetal S100B increased between phases A and B ( $7\% \pm 4$  vs  $17\% \pm 13$ ,  $p$   
22  $= 0.030$ ) and between phases A and C ( $7\% \pm 4$  vs  $24\% \pm 8$ ,  $p < 0.001$ ). Fetal c-TnT serum  
23 levels increased during occlusions: 102 ng/L (58-119) in phase A, vs 119 ng/L (103-198) in  
24 phase B vs 169 ng / L (128-268) in phase C ( $p < 0.05$ , for all). When compared to the baseline  
25 control period, fetal  $\Delta$ cTnT was significantly modified throughout UCO series: 5.0% (-3; 45)  
26 in phase A, 51% (4; 263) in phase B, and 77% (56.5; 269) in phase C ( $p < 0.05$  for all).

27 Conclusions - S100B and c-TnT increased when fetal acidosis occurred, which reflects the  
28 potential neurological damage and fetal cardiovascular adaptation.

29

30

31 Key words: S100B, cardiac troponin T, acidosis, fetal sheep, biomarker, perinatal asphyxia

32 Introduction

33 Uterine contractions can reduce uteroplacental perfusion by up to 60% leading to progressive  
34 fetal hypoxia (1). At term, healthy fetuses are able to withstand this transient hypoxia by  
35 reducing oxygen consumption and redirecting the oxygenated blood flow to critical organs  
36 (heart, brain, and adrenals) through activation of the peripheral chemoreflex (2,3). Providing  
37 there is adequate time for fetoplacental reperfusion between contractions, this mechanism  
38 protects fetuses from severe and irreversible hypoxic injuries. However, some fetuses  
39 develop intrapartum metabolic acidemia which remains a leading cause for neonatal  
40 complications, such as stillbirth, hypoxic-ischemic encephalopathy (HIE) and cerebral palsy.  
41 In the past decade, tissue-specific biochemical damage markers, such as S100B and cardiac  
42 troponin T (c-TnT) have been examined in umbilical cord blood at birth (4,5). The calcium-  
43 binding S100B protein is mainly concentrated in the glial cells of the nervous system. S100B  
44 has been presented as the most promising neurobiomarker in neonates with birth asphyxia  
45 (6). The increase in S100B concentration in body fluids, such as cerebrospinal fluid, blood, or  
46 urine, is a reliable marker of brain damage following asphyxia in neonates (7). The advanced  
47 hypothesis is that in case of brain lesions, some of the S100B released by the affected tissue  
48 could spread through the blood-brain barrier into the systemic circulation. In neonates  
49 suffering from severe forms of HIE, S100B is elevated in umbilical cord blood samples at  
50 birth and is associated with the severity of disease and risk of further neurodevelopmental  
51 sequelae (8). Cardiac troponin T is commonly used as a myocardial ischemia marker in both  
52 adults and infants. Studies have shown that in perinatal asphyxia, it could also be a strong and  
53 useful predictor of myocardial injury in birth asphyxia and HIE (9). Costa et al. reported that  
54 asphyxiated neonates had higher c-TnT levels associated with decreased cardiac output when  
55 compared with control healthy newborns (10). Yet, the degree of prematurity, fetal weight  
56 and type of delivery may complicate the assessment (11).

57 The in-utero levels of these biomarkers in response to worsening acidosis have still not been  
58 described. The purpose of this study was to assess the changes in S100B and c-TnT levels in  
59 both fetal and maternal serums in an experimental model of severe labor-like acidosis.

60 Materials and methods

61 *Surgical preparation*

62 Near-term pregnant sheep (Ile de France, INRA, Val de Loire, France) with a gestational age  
63 of  $124 \pm 1$  days (term = 145 days) underwent our surgical procedure, as described previously  
64 (12,13). If an ewe had multiple fetuses, only one was instrumented and included in the  
65 experiment.

66 For anesthesia, ewes had a vascular infusion of 500 mL of Ringer lactate, then a  
67 premedication of 0.3 mL intravenous Sedaxylan (xylazine 20mg/ml; Dechra, The  
68 Netherlands). Induction was made with isoflurane 5% before intubation, and anesthesia was  
69 maintained with soflurane 2% (14). After maternal laparotomy and hysterotomy, catheters  
70 (4-Fr diameter; Arrow, Reading, PA, USA) were placed in the fetal axillary arteries. The  
71 catheter tips were advanced to the ascending aorta. Four electrocardiogram (ECG) electrodes  
72 (Mywire 10; Maquet, Rastatt, Germany) were placed on the fetal intercostal muscles to  
73 record fetal ECG. An inflatable silicone occluder (OC16; In Vivo Metric, Healdsburg, CA,  
74 USA) was placed around the umbilical cord and the volume of saline solution required to  
75 achieve complete occlusion was determined.

76 The ewes were acclimatized to the experiment room 1 hour a day in the presence of the main  
77 investigators in charge of carrying out the experiments.

78 The experimental protocol began 4 days after the surgery. After 1 hour of baseline period,  
79 repetitive umbilical cord occlusions (UCOs) were performed by injecting an isotonic solution  
80 into the occluder to obtain a total occlusion for 1 minute. The protocol was divided into three  
81 periods of 1 hour each, as described previously by Prout et al (15). During the mild phase,  
82 UCOs were repeated every 5 minutes (phase A). During the second phase (phase B), the  
83 rhythm was of one UCO every 3 minutes. Finally, the last period (phase C) consisted of one

84 UCO every 2 minutes. The protocol was stopped before the end of phase C if pH reached  
85 6.90 or below.

86 Euthanasia was performed either at the end of the experimental procedure, or in cases of  
87 spontaneous labor, in-utero fetal death, or death during surgery, by intravenous injection of 6  
88 mL/50 kg of T61 (1 mL contains embutramide 200mg + mebezonium 26.92 mg + tetracaine  
89 4.39 mg, MSD France, Courbevoie, France) (14).

#### 90 *Monitoring parameters and measurement techniques*

91 Arterial blood gas parameters were measured with the i-STAT 1 blood analyzer (i-STAT 1  
92 System; Abbott Point of Care, Inc, Princeton, NJ, USA) using CG4+ cartridges at specific  
93 time points. Hemodynamic data were recorded continuously throughout the experiment. The  
94 fetal arterial and intra-amniotic catheters were connected to pressure sensors (Pressure  
95 Monitoring Kit 1; Baxter, Guyancourt, France) that were connected to a blood pressure  
96 monitor (Monitor Merlin, Hewlett Packard, Palo Alto, CA, USA). The arterial blood pressure  
97 (ABP) was measured from the blood pressure phasic signal and referenced to the intra-  
98 amniotic pressure (IAP) (calculated ABP = observed ABP - observed IAP). Biochemical  
99 assays were performed from serum on a Cobas 8000 module e601 (Roche, Basel,  
100 Switzerland) using the electrochimiluminescence method. S100B and c-TnT were measured  
101 in both the mother (M S100B or M c-TnT) and fetus (F S100B or F c-TnT). Collected blood  
102 samples were centrifuged at 3500 g for 10 minutes and supernatant stored at - 80° C until  
103 assessment. Delta S100B and c-TnT were defined as the difference in percentage of increase  
104 or decrease compared to the baseline control period (F  $\Delta$  S100B, M  $\Delta$  S100B and F  $\Delta$  c-  
105 TnT). During the baseline period, every 20 minutes during each UCO phase and at the end of  
106 each UCO phase, a 5-minute period with no UCO was observed to assess fetal  
107 hemodynamics, mean arterial blood pressure (Mean ABP), fetal heart rate (FHR), and arterial  
108 blood gases. We analyzed the data from the baseline period and from the 5-minute intervals



109 in between each UCO phases where an additional blood sample was performed for S100B  
110 and c-TnT dosages.

### 111 *Statistics*

112 The gaussian variables are described in terms of mean and standard deviation and non-  
113 Gaussian variables in terms of median and interquartile range. The normality of the  
114 numerical variables was verified graphically and tested using the Shapiro-Wilk test.  
115 Parameter changes during the occlusion phases were evaluated using a mixed linear model  
116 with an unstructured covariance structure to account for the correlation between repeated  
117 measures and time and baseline data were included as fixed effects. Post-hoc comparisons  
118 between the three occlusion phases were performed using linear contrasts. The normality of  
119 the residues was verified graphically. In case of normality deviation (without possible log  
120 transformation), the comparisons between the three occlusion phases were performed using  
121 Wilcoxon's signed-rank test. Bilateral tests were performed with a significance level of 5%.  
122 Correlations between biomarker serum levels and acidemia parameters during occlusion  
123 phases (at all time of measures) were evaluated using linear mixed models by including sheep  
124 as a random effect and time as a fixed effect. Statistical analyses were performed using the  
125 SAS software (v. 9.4; SAS Institute, Cary, NC, USA).

### 126 *Ethical approval*

127 The use of anesthesia, surgical procedure, and experimental protocol were consistent with the  
128 recommendations of the Ministry of Higher Education and Research. This study was  
129 approved by the Animal Experimentation Ethics Committee (Comité d'Ethique en  
130 Expérimentation Animale, Avis N°2016121312148878).

131 Results

132 Fourteen ewes were instrumented. Four were excluded from our analysis: two fetuses died in  
133 utero at postoperative day 1, one delivery occurred at postoperative day 3 and one occluder  
134 ruptured during the first phase of UCO.

135 Ten experiments were performed. Nine ewes had a multiple gestation (Table 1). Five fetuses  
136 reached endpoint before the end of the experiment: one during phase B and the other four  
137 during phase C.

138 During the baseline control period, all measurements of FHR, mean ABP, and arterial blood  
139 gases were in the normal range (Table 2).

140 There was no significant difference between phases A, B, and C for heart rate and mean  
141 ABP. There was a significant difference in UCO ABP between phases A and C ( $54.7 \pm 6.2$  vs  
142  $36.1 \pm 17.2$ ,  $p = 0.009$ ) and between phases B and C ( $47.1 \pm 15.1$  vs  $36.1 \pm 17.2$ ,  $p = 0.006$ ).

143 Partial pressure of oxygen ( $\text{PaO}_2$ ) was not modified while partial pressure of carbon dioxide  
144 ( $\text{PaCO}_2$ ) increased significantly between phases A and C ( $53 \text{ mmHg} \pm 5.1$  vs  $69.9 \text{ mmHg} \pm$   
145  $16.7$ ,  $p = 0.006$ ). pH decreased significantly throughout UCO series:  $7.29$  ( $7.21$ - $7.37$ ) in  
146 phase A vs  $7.15$  ( $7.02$ - $7.28$ ) in phase B vs  $6.99$  ( $6.86$ - $7.12$ ) in phase C ( $p < 0.05$ , for all).

147 Lactates increased significantly between the 3 occlusion phases,  $5.88 \pm 2.35$  in phase A vs  
148  $11.14 \pm 3.93$  in phase B vs  $15.25 \pm 1.56$  in phase C ( $p < 0.05$  for all). Base excess also varied  
149 significantly from  $-1.2 \pm 6.3$  in phase A to  $-8.5 \pm 7.3$  in phase B to  $-14.8 \pm 5$  in phase C ( $p <$   
150  $0.05$ , for all).

151 Serum levels of fetal S100B increased progressively between phases A and B,  $11.5 \mu\text{g} \pm 3.8$   
152 vs  $12.9 \mu\text{g} \pm 4.4$ ,  $p < 0.001$ . We observed a significant difference in fetal S100B serum  
153 concentration when compared to baseline period ( $\Delta\text{S100B}$ ), with a progressive increase  
154 between phases A and B ( $7\% \pm 4$  vs  $17\% \pm 13$ ,  $p = 0.030$ ) and between phases A and C ( $7\%$   
155  $\pm 4$  vs  $24\% \pm 8$ ,  $p < 0.001$ ).

156 Fetal c-TnT serum levels increased during occlusions: 102 ng/L (58-119) in phase A, vs 119  
157 ng/L (103-198) in phase B vs 169 ng/L (128-268) in phase C ( $p < 0.05$ , for all). When  
158 compared to the baseline control period, fetal  $\Delta$ cTnT was significantly modified throughout  
159 OCT series: 5.0% (-3; 45) in phase A, 51% (4;263) in phase B, and 77% (56.5; 269) in phase  
160 C ( $p < 0.05$  for all). There was no difference observed in the maternal S100B and c-TnT  
161 dosages throughout the experiment.

162 Finally, we found a significant correlation between differences in biomarker serum levels  
163 throughout the experiment and acidemia parameters, such as pH, lactate, and base excess  
164 (Table 3).

165 Discussion

166 *Main findings*

167 Fetal hypoxia and its early detection during labor remains a major perinatal challenge. The  
168 aim is to reduce perinatal asphyxia and its complications, such as HIE. When reproducing a  
169 progressive and severe hypoxemia in near-term fetal sheep, we observed a significant rise in  
170 fetal S100B and c-TnT serum levels while pH decreases, and lactates rise.

171 *Interpretation*

172 HIE is defined as the clinical manifestation of impaired neonatal brain function following  
173 asphyxia due to an antenatal and/or perinatal adverse event (16). Various criteria (biological,  
174 clinical and sometimes electrophysiological) are used to define the severity of the  
175 neurological damage, many of them included in Sarnats' original classification (16). HIE  
176 pathogenesis is fairly complex and is still not understood fully. During HI insult, different  
177 intracellular mechanisms are activated, which in turn lead to focal or diffuse cell damage  
178 associated with a significant increase in neurobiomarkers, such as S100B (17). S100B is an  
179 acid calcium-binding protein specific to the central nervous system where it appears abundant  
180 in Schwann cells, glial cells, and neurons. Recently, a series of studies have found that the  
181 evaluation of S100B in different body fluids can help early diagnosis of neonates with  
182 perinatal asphyxia complicated with HIE (7). In particular, in cord blood samples at birth,  
183 S100B was significantly higher in infants with moderate or severe HIE. Increased  
184 concentrations were detected 48-72 hours before any clinical, laboratory, or ultrasound signs  
185 of brain damage. To our knowledge, the modification of S100B levels during a labor-like  
186 acidosis has not yet been evaluated. In our study, we observed an increase in S100B levels  
187 during the different phases of the experiment and this increase seems to be detectable as early  
188 as the onset of hypoxia, before pH drops below 7.00. In a different model of fetal hypoxia,  
189 Giussani et al. reported that, S100B concentrations increased significantly during acute

190 hypoxemia by  $24.9 \pm 2.9$  % (18). In human studies, the difference in S100B concentrations  
191 at birth is statistically significant between neonates with HIE and controls (8,19). The  
192 sensitivity of this neurobiomarker could be useful in daily practice as a second-line method  
193 for screening fetuses at risk of brain damage. Regarding S100B in maternal blood, an  
194 increased concentration has been reported within 3 hours of fetal hypoxic insult. Since no  
195 damage was seen in the maternal brain of the animals used, the authors concluded that some  
196 of the higher maternal S100B levels were of fetal origin, from across the placental barrier  
197 (20). In our study, we did not show any increase in maternal serum levels of S100B in  
198 response to severe hypoxia. The possibility of species-specific placental transport of the  
199 S100B may explain our differences from the literature.

200 During labor, several adaptive mechanisms such as heart rate decelerations or tachycardia,  
201 allow fetuses to withstand hypoxia (21,22). Following a perinatal HI insult, oxygen  
202 deprivation causes myocardial damage and neonates develop cardiovascular dysfunction as  
203 part of the multiorgan failure showcasing HIE (23,24). The cTnT concentration is higher in  
204 newborns developing HIE compared to those who developed no complications (9). In our  
205 study, we found that c-TnT increased early as the pH starts to drop below 7.15. These results  
206 reinforce the hypothesis of an anaerobic cardiac metabolism secondary to fetal hypoxia.  
207 Similarly, Sweetman et al. showed that neonates with severe neonatal hypoxic ischemia have  
208 significantly higher serum c-TnT concentrations than other asphyxiated groups (5). Troponin  
209 T may be a reliable marker of myocardial injury and may serve as an adjunct for early  
210 diagnosis of perinatal asphyxia.

#### 211 *Strengths and limitations*

212 This study provides data on progressive changes in fetal levels of S100B and c-TnT. There is  
213 a clinical interest in understanding how these markers behave during the equivalent of a very  
214 challenging labor. However, the small number of experiments and important variability of

215 results are obstacles to drawing strong conclusions. The baseline state of our fetuses prior to  
216 resuming the experiment was not comparable to previous reports, because PaO<sub>2</sub> was low and  
217 lactates elevated. We hypothesized that with the breed used and the high rate of multiple  
218 gestation, theb fetuses were not comparable to previous studies. The absence of a control  
219 group can be discussed, but the biomarker changes were compared to baseline values.  
220 Finally, a pH < 6.90 indicated the end of the experiment. Even if models that used pH as a cut  
221 off show minimal injury (15,25) compared to those that use hypotension (26), we observed  
222 mild to severe hypotension during phase C in our study.

223 Conclusion

224 S100B and c-TnT increased when fetal acidosis occurred, reflecting potential neurological  
225 damage and fetal cardiovascular adaptation. It will be interesting to correlate those changes to  
226 anatomopathology in a larger sample.

227 Competing interests

228 The authors have no conflicts of interest

229

230 Author contributions

231 Y.H., J.D.P, D.S. and L.G. conducted the experiments. Y.H., J.D.P. and L. L collected all

232 data. Y.H. and C.G. wrote the manuscript. E.D. conducted statistical analysis and wrote the

233 statistics methodology. J.D., P.M., V.H.D. and L.S. reviewed and corrected the manuscript.



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<b>Ewes</b>	<b>Number of fetuses</b>	<b>Weight of instrumented fetus</b>	<b>Study terminated due to low pH</b>	<b>Phase</b>
1	2	3805	<b>Yes</b>	C
2	3	2935	No	
3	2	3555	<b>Yes</b>	B
4	2	3850	<b>Yes</b>	C
5	2	3135	No	
6	2	3150	<b>Yes</b>	C
7	2	3380	No	
8	2	3245	<b>Yes</b>	C
9	2	3290	No	
10	1	3125	No	

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314

315 **Table 1. Description of our study group**

316 Weights are presented in grams

	Baseline	Umbilical Cord Occlusions Period			p		
	S	A	B	C	A vs B	A vs C	B vs C
FHR (bpm)	183.3 ± 10.6	172.2 ± 16.6	181.3 ± 23.1	176.4 ± 22.5	.17	.79	.45
Mean ABP (mmHg)	47.4 ± 10.8	55.3 ± 10.5	58.1 ± 8.0	59.7 ± 9.3	.35	.35	.52
UCO ABP (mmHg)	-	54.7 ± 6.2	47.1 ± 15.1	36.1 ± 17.2	.15	<b>.009</b>	<b>.006</b>
pH	7.39 ± 0.03	7.29 ± 0.08	7.15 ± 0.13	6.99 ± 0.13	<b>.001</b>	<b>&lt;.001</b>	<b>.002</b>
PaO <sub>2</sub> (mmHg)	13.6 ± 5.5	14.0 ± 3.8	15.2 ± 2.9	16.3 ± 2.7	.12	.078	.17
PaCO <sub>2</sub> (mmHg)	46.8 ± 6.2	53.0 ± 5.1	57.2 ± 7.0	69.9 ± 16.7	<b>.002</b>	<b>.006</b>	<b>.013</b>
Base excess (mEq/L)	3.1 ± 4.6	-1.2 ± 6.3	-8.5 ± 7.3	-14.8 ± 5.0	<b>&lt;.001</b>	<b>&lt;.001</b>	<b>&lt;.001</b>
Lactate (mmol/L)	2.51 ± 0.74	5.88 ± 2.35	11.14 ± 3.93	15.25 ± 1.56	<b>&lt;.001</b>	<b>&lt;.001</b>	<b>.008</b>
M S100B (μg/L)	0.44 ± 0.16	0.47 ± 0.14	0.43 ± 0.16	0.45 ± 0.19	.70	.67	.48
M Δ S100B (%)	-	-1.9 ± 2.7	-2.0 ± 5.6	-0.8 ± 0.13	.93	.98	.95
F S100B (μg/L)	10.7 ± 3.4	11.5 ± 3.8	12.7 ± 4.7	12.9 ± 4.4	<b>.032</b>	<b>.001</b>	.14
F Δ S100B (%)	-	7 ± 4	17 ± 13	24 ± 8	<b>.030</b>	<b>&lt;.001</b>	.18
M c-TnT (ng/L) <sup>1</sup>	8 (6 ; 14)	8.5 (6 ; 12)	9 (6 ; 14)	10.5 (6 ; 15)	.75	1	1
M Δ c-Tnt (%)	-	6.3 ± 7.9	3.3 ± 11.5	3.5 ± 15.7	.33	.87	.51
F c-TnT (ng/L) <sup>1</sup>	73 (49 ; 105)	102 (58 ; 119)	119 (103 ; 198)	169 (128 ; 268)	<b>.012</b>	<b>.008</b>	<b>.008</b>
F Δ c-TnT (%) <sup>1</sup>	-	5.0 (-3.0 ; 45.0)	51.0 (4.0 ; 263.0)	77.0 (56.5 ; 269.0)	<b>.012</b>	<b>.008</b>	<b>.008</b>

317 **Table 2. Hemodynamic, blood gas and biochemical measures**

318 Data are presented on average ± standard or median deviation (interquartile range).

319 <sup>1</sup> P-values were calculated using the Wilcoxon signed-rank test, p < .05 is significant (given in bold)

320 FHR: Fetal Heart Rate, bpm: beats per minutes, ABP: Arterial Blood Pressure, F: Fetal, M: Maternal, cTnT: Cardiac Troponin T; UCO:

321 Umbilical Cord Occlusion

	pH		Lactate		Base excess	
F Δ S100B	-0.049 ± 0.16	<b>0.007</b>	1.271 ± 0.295	<b>&lt;0.001</b>	-2.193 ± 0.559	<b>0.002</b>
F Δ c-TnT	-0.001 ± 0.001	<b>0.034</b>	0.016 ± 0.005	<b>0.004</b>	-0.024 ± 0.009	<b>0.021</b>

322

323 Table 3. Correlations between biomarker serum levels with acidemia parameters.

324 Data are presented on  $\beta \pm$  standard error.

325  $p < .05$  is significant (given in bold).

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