

Increased EEG delta frequency corresponds to chorioamnionitis-related brain injury

Antonio Wenceslao Danilo Gavilanes^{1,3}, Markus Gantert⁴, Eveline Strackx^{1,2,3}, Luc J. Zimmermann¹, Saskia Seeldrayers⁵, Johan S. H. Vles², Boris W. Kramer^{1,3}

¹Departments of Pediatrics, Neonatology, Maastricht University Medical Center, ²Child Neurology, Maastricht University Medical Center, in collaboration with the School for Oncology and Developmental Biology (GROW), Faculty of Health, Medicine and Life Sciences Maastricht University, The Netherlands, ³School of Mental Health and Neuroscience, Faculty of Health, Medicine and Life Sciences, Maastricht University and European Graduate School of Neuroscience (EURON), The Netherlands, ⁴Department of Obstetrics and Gynecology, Klinikum Osnabrück, Osnabrück, Germany and ⁵Animal Laboratory, Maastricht University Medical Center, Maastricht, The Netherlands

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1. Animals and surgical procedures
 - 3.2. Intrapartal EEG data sampling and off-line analysis
 - 3.3. Flow cytometric analysis
 - 3.4. Statistical analysis
4. Results
 - 4.1. CFAM amplitude
 - 4.2. CFAM frequency
 - 4.3. FACS activated microglia
 - 4.4. FACS apoptotic cell death
 - 4.5. CFAM vs. FACS variables
5. Discussion
 - 5.1. Clinical relevance of antenatal inflammation
 - 5.2. Inflammatory CNS impact
 - 5.3. CFAM registration
 - 5.4. EEG amplitude analysis
 - 5.5. EEG delta frequency analysis
 - 5.6. Methodological EEG drawbacks and study limitations
 - 5.7. Final remarks
6. Acknowledgements
6. References

1. ABSTRACT

We evaluated the impact of chorioamnionitis on the intrapartal EEG delta frequency in the non-anesthetized preterm sheep. 10 mg intra-amniotic LPS or saline were given 2 or 14 days before preterm birth at gestational day 125. Lambs were delivered by Caesarean section under local anesthesia. A 5-minute EEG depicted delta activity and amplitude, and the relationship between EEG delta activity and both the white matter (WM) and cortical microglial activation and apoptosis was analyzed. EEG delta activity was increased significantly in the 14-day LPS preterm fetuses compared to both preterm control and 2-day LPS animals ($p < 0.05$). No differences were seen between controls and the 2-day LPS fetuses. A direct association was demonstrated between EEG delta activity and both cortical microglial activation ($r = 0.645$, $p = 0.024$) and apoptosis ($r = 0.580$, $p = 0.048$), and between delta and WM activated microglia ($r = 0.742$, $p = 0.006$) and apoptosis ($r = 0.777$, $p = 0.003$). This study is the first to show a relationship between brain dysfunction and chorioamnionitis-related injury at birth.

2. INTRODUCTION

Chorioamnionitis and the resulting fetal inflammatory response syndrome are common in extremely preterm infants, affecting different organs like the lungs and the central nervous system (CNS) (1-3). Consequently, chorioamnionitis is a major predictor of both a complicated neonatal clinical course and a poor neurological long-term outcome (4-7). The identification of newborns previously exposed to antenatal inflammation remains difficult, especially if no clinical signs are present immediately after birth. The early detection of neuronal dysfunction in those newborns is of particular interest as it might have a diagnostic and prognostic value.

Previously we studied chorioamnionitis in the sheep model to investigate the link between antenatal inflammation and neurological injury (8, 9). In that study, we established for the first time that chorioamnionitis induces a global CNS inflammatory response and increased apoptosis of the white matter (WM) as well as the cortical and subcortical gray matter (GM) in the preterm fetal

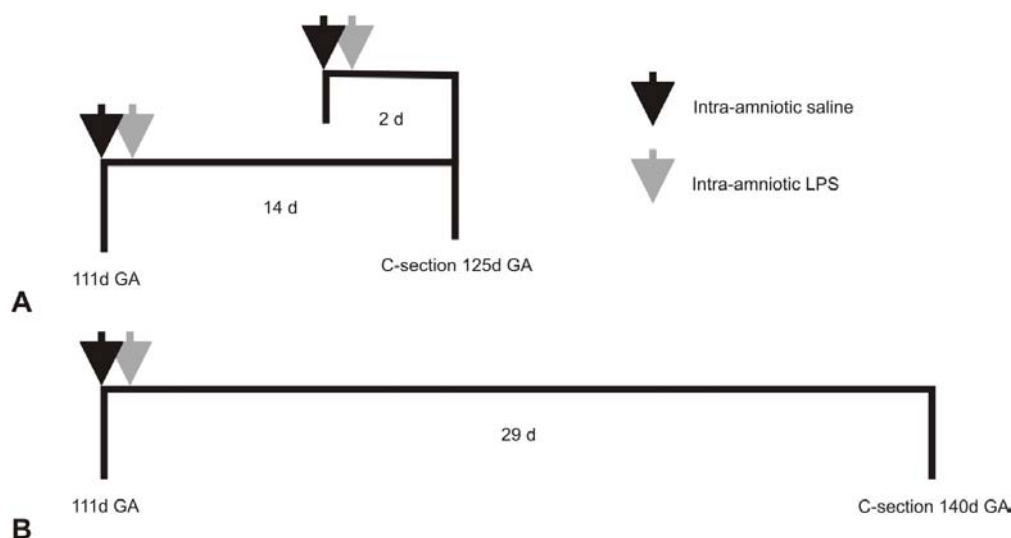


Figure 1. Schematic overview of the experimental design. Chorioamnionitis was induced by intra-amniotic LPS administration at 123d GA (2-day LPS, n=6) and at 111d GA (14-day LPS, n=5); (control saline, n=7). Ewes delivered preterm at 125d GA.

sheep. The brain was found to be more vulnerable than the cerebellum and the spinal cord, which may be due to their specific stage of maturation (8).

The present study further developed this model of antenatal inflammatory CNS injury by focusing on the effects on the peripartal EEG. We studied the effects of endotoxin induced chorioamnionitis at low gestational age (GA) by intra-amniotic lipopolysaccharide (LPS) administration and subsequent delivery at 125d GA (70% of full-term gestation). This specific gestational age corresponds to approximately 27 weeks of human CNS maturation, a vulnerable period of the human brain to develop WM disease (10). A 5-minute peripartal EEG was registered on a Cerebral Function Analysing Monitor (CFAM) as described elsewhere, providing detailed information about the amplitude and the frequencies of EEG activity (11). Interestingly, the well-established dominance of slow EEG frequency waves (delta frequency) in the immature brain supports its use as marker of neuronal function (12). The ewes did not receive general anesthesia for the Caesarean section, to avoid the alteration of the cerebral metabolism by systemic anesthesia during the CFAM registration, whereas the fetuses were maintained on placental circulation by the ex-utero intrapartum treatment (EXIT) procedure, assuring stable systemic hemodynamics. This allowed for a representative registration of early neuronal functional changes detected by CFAM. We hypothesized an LPS interval-related effect on the intrapartal EEG as well as a positive correlation between the EEG delta activity and the severity of CNS inflammation and injury in the LPS groups (8).

3. MATERIALS AND METHODS

3.1. Animals and surgical procedures

The study was performed according to the guidelines of the Animal Care Committee of the University of Maastricht, which approved the protocol. Time-mated

Texel ewes with singleton fetuses were randomly assigned to groups of five animals, to receive a single dose of 10 mg endotoxin (*Escherichia coli* 055:B5; Sigma Chemical, St. Louis, MO) resuspended in saline or the equivalent volume of saline for control by ultrasound guided intra-amniotic injections, as previously described (9). Chorioamnionitis was induced at a GA of 110 or 111d and at 123 d GA. Animals were sacrificed at the GA of 125d 2 days (n=6) or 14 days (n=5) after exposure to chorioamnionitis. The GA of 125d is comparable with a human GA of approximately 27 weeks (Figure 1).

Pregnant ewes delivered by Caesarean section under subcutaneous infiltration of 4% lidocaine. The fetal head was exteriorized while fetomaternal circulation was preserved by the EXIT procedure. Five minutes after delivery of the head the umbilical cord was clamped and cut, and the fetal sheep was fully delivered. Fetuses were sacrificed by a lethal injection of pentobarbital after EEG registration, while measurements of cord arterial blood gases were performed. Ewes were killed by a lethal injection of pentobarbital.

3.2. Intrapartal EEG data sampling and off-line analysis

EEG data were collected for 5 minutes immediately after delivery of the fetal head by a CFAM system, which was developed and used in the University Hospital of Maastricht (11). No systemic drugs were given before or during the recordings. Four shielded stainless-steel subcutaneous scalp electrodes (two parietal, one frontal and one reference electrode) were placed. The three-channel EEG (P3/F0 (left hemisphere), P4/F0 (right hemisphere) and P3/P4, international 10-20 system) registration was processed off-line. This includes weighted digital band filtering between 2 and 15 Hz, rectification, logarithmic amplitude compression, and smoothing of the signal. Intervals of two seconds were analyzed off-line by the computer yielding means EEG amplitude (bandwidth,

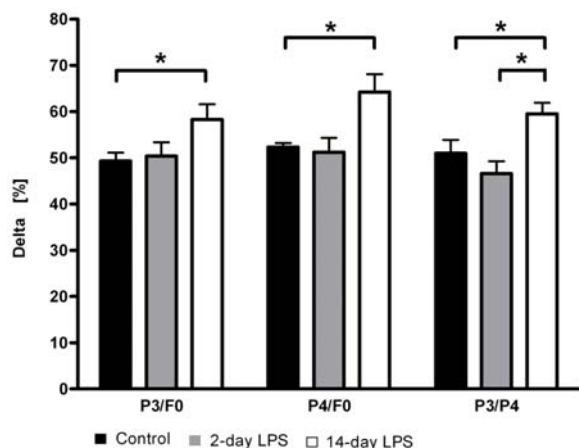


Figure 2. Delta frequency measured using a three-channel EEG (P3/F0 (left hemisphere), P4/F0 (right hemisphere) and P3/P4, international 10-20 system). Symmetrical enhancement of delta frequency [in %] in the 14-day LPS fetuses at 125d GA (control $n=7$; 2-day LPS, $n=6$; 14-day LPS, $n=5$). Data represents means \pm SEM (One-way ANOVA and post-hoc Bonferroni * $p < 0.05$)

minimal and maximal) and relative delta [1-3.5 Hz], theta [3.5-7.5 Hz], alpha [7.5-13 Hz] and beta [13-27Hz] spectral analysis. The power values for each frequency are expressed as a percentage of the mean total power, allowing comparisons across animals. Only the biparietal P3/P4 delta frequency was further analyzed and correlated with previously reported morphological data in the preterm groups (8). We used this original single-channel biparietal CFAM recording, because it represents the global electrocortical activity.

3.3. Flow cytometric analysis

WM and cortical samples of the preterm groups (125d GA) were run on a FACScalibur flow cytometry system (FACS, BD Biosciences, New Jersey, USA), equipped with an argon ion laser (488nm). Analysis was done using the Cell Quest Pro software (BD Biosciences, New Jersey, USA). AnnexinV / propidium iodide and OX42 staining, detecting apoptotic cells and activated microglia respectively, were reported by the authors (8). We correlated these data with the P3/P4 delta frequency percentage registered in those animals at birth (control, $n=3$; 2-day LPS, $n=5$; 14-day LPS, $n=4$).

3.4. Statistical analysis

Data were analyzed using a multivariate ANOVA (represented as mean \pm SEM). Significant effects were analyzed by post-hoc Bonferroni corrections. Pearson's correlation coefficients were computed to quantify the relationship between the EEG and the FACS variables. The accepted level of significance was $p < 0.05$. All calculations were done using Statistical Package for Social Sciences (SPSS 15.0 software).

4. RESULTS

There was no influence of gender, singleton/twin or weight on any of the analyzed variables. The cord

arterial blood pH was significantly decreased in the 14-day LPS animals ($p < 0.001$; control ($n = 3$) pH $7.39 (\pm 0.02)$ vs. 14-day LPS group ($n = 4$) pH $7.22 (\pm 0.04)$). A significant decrease in base excess (mmol/L) was shown in the 14-day LPS group compared to controls ($p < 0.001$; control ($n = 3$) $-2.4 (\pm 0.38)$, 14-day LPS group ($n = 4$) $-7.10 (\pm 0.83)$) (data not shown).

4.1. CFAM amplitude

Means for the maximal, minimal and bandwidth P3/P4 CFAM amplitude were unchanged in the 2 and 14-day LPS groups, compared with the control group (e.g. control preterm ($n=7$) maximal mean $5.75 \mu\text{volts} (\pm 0.28)$, 2-day LPS group ($n=6$) maximal mean $6.0 \mu\text{volts} (\pm 0.22)$ vs. 14-day LPS group ($n=5$) maximal mean $5.95 \mu\text{volts} (\pm 0.83)$). All amplitude recordings were symmetrical and seizures were not present (data not shown).

4.2. CFAM frequency

The percentage of delta frequency in the 14-day LPS group ($n=5$) was significantly increased compared to both 2-day LPS ($n=6$) and the saline treated groups ($n=7$) ($p < 0.05$; $59.50\% (\pm 2.15)$, $47.50\% (\pm 2.42)$ vs. $51.00\% (\pm 1.89)$, respectively) (Figure 2). Again asymmetrical spectral power recordings were not seen.

4.3. FACS activated microglia

Activated microglia were detected by OX42. Its percentage was very low in the saline group ($n=3$, $0.8\%-1.6\%$) as previously reported [9]. In the WM and cortical GM the percentage of activated microglia was significantly higher in the 2-day LPS group ($n=5$, $2.5\%-5\%$) than in the saline group. There was an even higher increase in the proportion of activated microglia in the 14-day LPS group ($n=4$, $3.2\%-6.8\%$) (data not shown).

4.4. FACS apoptotic cell death

The percentage of apoptotic cells was determined by AnnexinV+/PI- staining. It increased significantly in the 14-day LPS group ($n=4$, $28\%-46\%$) compared to the saline group ($n=3$, $12\%-23\%$) in both the WM and GM as previously reported [9]. In the cortex, again the percentage of apoptotic cells was higher in the 2-day LPS group ($n=5$).

4.5. CFAM vs. FACS variables

The Pearson correlation coefficient showed a significant positive correlation between delta frequency and the cortical activated microglia percentage ($r = 0.645$, $p = 0.024$) (Figure 3A). A significant positive correlation between the delta frequency and WM activated microglia percentage ($r = 0.742$, $p = 0.006$) was found as well (Figure 3B). Furthermore, a significant positive correlation was found between the relative delta frequency and the cortical apoptosis ($r = 0.580$, $p = 0.048$) (Figure 3C). Finally, a significant correlation was observed between delta frequency and WM apoptosis percentages ($r = 0.777$, $p = 0.003$) (Figure 3D).

5. DISCUSSION

The central finding of this study is that chorioamnionitis induced by intra-amniotic LPS causes preterm EEG alterations including a substantial

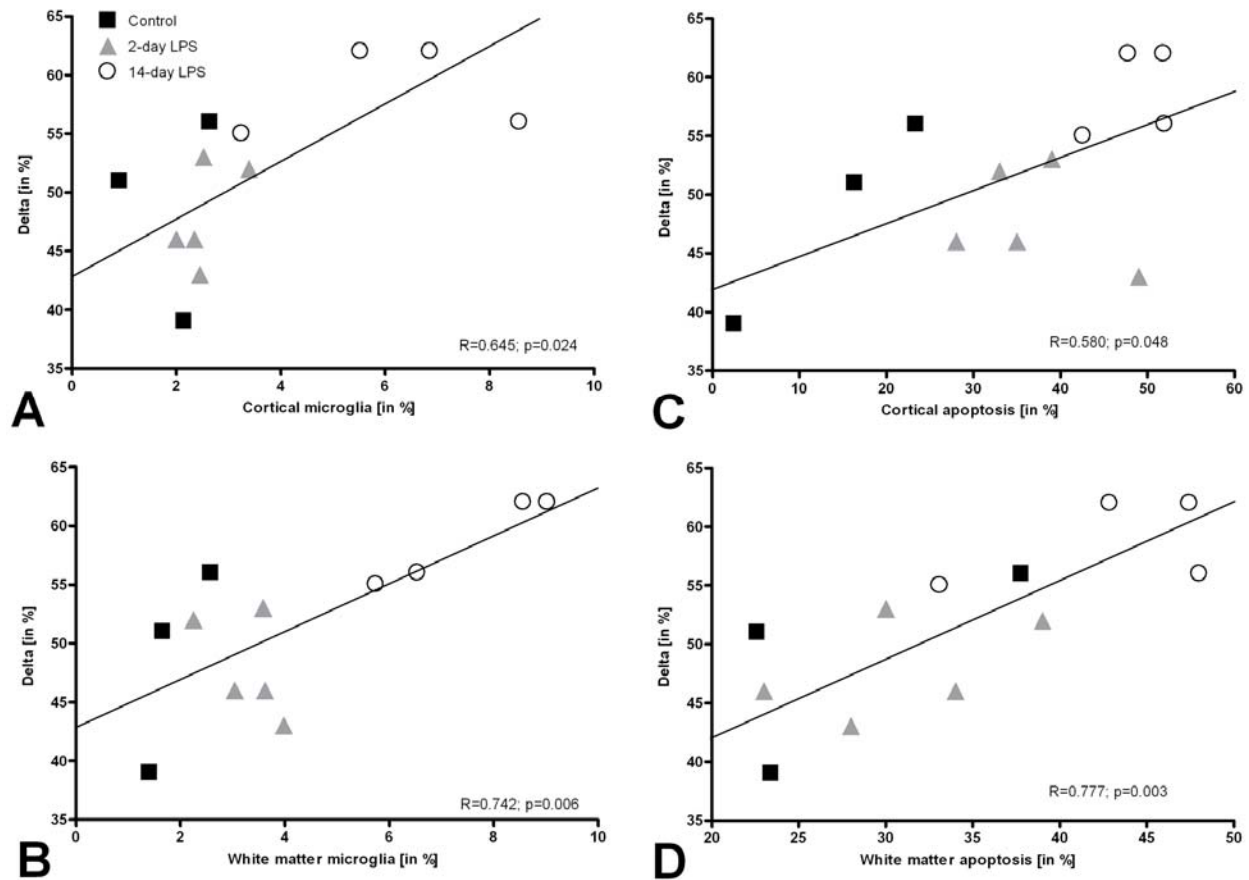


Figure 3. The Pearson correlation coefficient data. (A) Correlation between the delta frequency [in %] and cortical activated microglia [in %] in the preterm fetal sheep at a gestational age of 125 days ($R=0.645$, $p=0.024$). (B) Correlation between the delta frequency [in %] and white matter activated microglia [in %] in the preterm fetal sheep at a gestational age of 125 days ($R=0.742$, $p=0.006$). (C) Correlation between the delta frequency [in %] and cortical apoptosis [in %] in the preterm fetal sheep at a gestational age of 125 days ($R=0.580$, $p=0.048$). (D) Correlation between the delta frequency [in %] and white matter apoptosis [in %] in the preterm fetal sheep at a gestational age of 125 days ($R=0.645$, $p=0.024$) (Control $n=3$, 2-day LPS $n=5$ and 14-day LPS $n=4$).

enhancement of the delta frequency. In addition, preterm white matter and cortical microglial activation and apoptotic cell death significantly correlated with this functional variable. This finding was interval-dependent, meaning that the 14-day LPS fetuses were affected most. These findings support the concept that enhancement of the delta frequency may serve as a marker of inflammatory CNS injury as early as at birth.

5.1. Clinical relevance of antenatal inflammation

Preterm birth is by far the most important adverse outcome of pregnancy. It is inversely correlated with histological chorioamnionitis with an overall incidence of 30% prior to 34 weeks of gestation (1). In addition, preterm birth at an extremely early gestational age is associated with an even higher rate of histological chorioamnionitis (up to 80%) and funisitis (up to 25%) (2, 3). Furthermore, preterm newborns affected by systemic inflammation/infection show increased risk of neonatal morbidity and mortality as well as a higher risk to develop

chronic pathologies like lung disease and encephalopathy. Considering the impact of these deficits throughout the entire lifespan of an individual and bearing the social and economic costs in mind, a full understanding of the impact of antenatal inflammation on CNS development is crucial.

5.2. Inflammatory CNS impact

The effects of LPS are mediated through binding to the LPS binding receptor CD14, facilitating the activation of toll-like receptor 4 (TLR4). Interaction of LPS with TLR4 initiates a cascade of cellular events, including activation of the transcription factor nuclear factor - kappa B, which in turn leads to the transcription of pro-inflammatory cytokines (13-15). A prolonged fetal exposure to these pro-inflammatory cytokines may be caused by a sustained cellular inflammatory response in the amniotic fluid up to seven days after 10 mg IA LPS administration, as shown in this model by Kramer et al. (9). The route of these pro-inflammatory cytokines from the amniotic fluid to the brain, causing brain inflammation, is

not very well understood. In the brain, the native pro-inflammatory cytokines production by astrocytes and microglia as well as the systemic cytokine transfer can induce glutamatergic excitotoxicity and the release of nitric oxide and reactive oxygen species. These processes are known to cause mainly oligodendrocyte apoptosis and myelin degeneration (WM disease), which are involved in the pathogenesis of permanent injury to the developing brain (16). We recently found that besides WM disease, both cortical and subcortical gray matter injury contribute to the global CNS injury in the 125-day-old preterm fetuses (9). In the present study, we correlated part of the FACS data on brain WM and cortical activated microglia and apoptosis reported in that study with the EEG delta wave activity assessed in the same preterm animals at birth. By doing so, we linked, for the first time, representative data on both the CNS inflammation and secondary apoptotic changes with a functional marker (delta frequency) at birth.

5.3. CFAM registration

CFAM is a quantitative EEG registration in which the EEG signal is filtered and selectively amplified. EEG amplitude and relative spectral power analysis was assessed using the CFAM monitor developed in our institution (10). EEG measurements were performed over a period of 5 minutes.

5.4. EEG amplitude analysis

The background EEG amplitude registered by the CFAM has been widely described in several neonatal pathological conditions, and it is mainly used as a predictor of neurological outcome after hypoxic-ischemic encephalopathy in full-term infants (17). This study showed an unchanged CFAM amplitude in the fetuses exposed to chorioamnionitis compared to controls.

5.5. EEG delta frequency analysis

This study focused on the relative changes in the spectral power and analyzed the delta frequency for several reasons. First, the neonatal basic low frequency EEG predominantly consists of the slow delta band (12). Preterm control fetal sheep for instance, have a relative delta of 51.00% (± 1.89). Secondly, an increase in delta frequency activity is associated with low levels of consciousness, severe hypocarbia, seizures disorders, cerebral trauma, post-hypoxic encephalopathy and brain ischaemia (18). In the present work, delta frequency percentage increased markedly in the 14-day LPS preterm group. Third, increased low frequency EEG activity has been reported as the consequence of the sleep-promoting (somnogenic) effects of interleukins in rats, presumably by activation of the hypothalamic preoptic area, which is a primary sleep regulatory center (19, 20). Fourth, neonatal (intensive) care data on EEG spectral analysis have been reported by several authors. Inder et al. demonstrated that low spectral edge frequency (SEF), assessed one to three weeks postnatally in a heterogeneous group of preterm infants was a predictor of brain WM compromise at the corrected term age (21). However, Wong et al. could not reproduce these SEF findings in term infants suffering from hypoxic-ischemic encephalopathy, 17 to 96 hours postnatally (22).

Finally, Thaler et al. suggested that lower SEF might serve as a marker for fetal distress during episodes of variable decelerations (23).

Since its first report by Maynard in 1979, CFAM spectral analysis has been limited to the detection of neonatal seizures and to monitoring acute neurological pathologies in the older pediatric intensive care population (24-27). Experimental CFAM data in the preterm fetal sheep have been scarce and non conclusive (28, 29). Our previous CFAM studies demonstrated altered delta spectral changes in the newborn piglet subjected to either progressive hypotension or graded hypoxia (30, 31). Both studies showed that delta frequency augmentation correlated with impaired cerebral and systemic oxygenation and hemodynamics in the anesthetized newborn brain. There are no data from clinical or experimental studies demonstrating objectively antenatal inflammatory brain injury using the enhancement of delta frequency detected at birth.

The present study shows a significant positive correlation between cortical and WM microglia activation and apoptosis and delta frequency in the preterm LPS animals. These data demonstrate that chorioamnionitis affects the structural-functional organization of the preterm brain in a LPS interval-dependent manner.

5.6. Methodological EEG drawbacks and study limitations

PH, PaCO₂ and PaO₂ measurements in cord blood were only performed at the moment of delivery, whereas the CFAM registration was performed for 5 minutes. This made it impossible to measure other physiological variables, such as fetal heart rate, eye movements or breathing patterns. Additionally, this 5-minute timeframe made both the appropriate evaluation of EEG continuity and the classification of cyclic behavior of the fetal EEG by discriminating between high-voltage slow activity and low-voltage fast activity EEG impossible. However, limiting the time from uterine incision to delivery allowed a representative EEG amplitude and frequency registration. Given the fact that neither systemic anesthetics nor uterine relaxation were used during the EXIT procedure, an appropriate uteroplacental support was maintained.

Whether the differences between the groups may have been caused by 1) the length of intra-amniotic LPS exposure (2 vs. 14-day interval), 2) the stage of fetal neurodevelopment at the time of LPS exposure (E111d vs. E123d), or 3) both cannot be distinguished on the basis of the design of this study. However, we think it is likely that both the duration of exposure (up to 14-days) and the developmental stage (gestational age) at the time of LPS administration accounted for the given impact. Finally, despite the small number of animals examined, strong and significant correlations were seen between delta frequency and FACS variables (Figure 3A-B).

5.7. Final remarks

The duration of chorioamnionitis was positively correlated with the grade of apoptosis, microglial activation

and the shift to lower frequency EEG in the preterm fetuses. This findings are highly relevant as they for the first time document morphological and electrophysiological evidence of a CNS compromise at birth secondary to chorioamnionitis. Furthermore, it offers evidence of the potential diagnostic and prognostic value of EEG delta frequency by identifying those newborns who are at risk. The relationship between inflammatory brain injury and functional abnormalities as indicated by the significant enhanced EEG delta band, has to be further validated in human newborns.

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Send correspondence to: Antonio Wenceslao Danilo Gavilanes, Dept. of Pediatrics, Maastricht University Medical Center, PO Box 5800, 6200 AZ Maastricht, The Netherlands, Tel: 31-43 3876061, Fax: 31-43 3875246, E-mail: danilo.gavilanes@mumc.nl

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