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RESEARCH ARTICLE

Adenosine Blood Level: A Biomarker of White Matter Damage in Very Low Birth Weight Infants

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Abstract: ***Background*:** Very low birth weight infants are at risk of developing periventricular white matter lesions. We previously reported high blood adenosine levels in premature infants and infants with low birth weight**.** We asked whether blood adenosine levels could be related to the vulnerability of the maturing white matter to develop lesions. The present study aims at finding a biomarker for the early detection of brain white matter lesions that can profoundly influence the neurodevelopmental outcome, whose pathophysiology is still unclear.

***Methods*:** Dried blood spots were prospectively collected for the newborn screening program and adenosine concentration measurements. Fifty-six newborns who tested four times for blood adenosine concentration (at days 3, 15, 30, and 40 post-birth) were included in the program. All infants underwent brain MRI at term equivalent age. Neurodevelopmental outcomes were studied with Griffiths Mental Development Scales (GMDS) at 12 ± 2 months corrected age.

Results: Blood adenosine concentration increased over time from a median of 0.75 µM at Day 3 to 1.46 µM at Day 40. Adenosine blood concentration >1.58 µM at Day 15 was significantly associated with brain white matter lesions at MRI (OR (95 % CI) of 50.0 (3.6-688.3), *p*-value < 0.001). A moderate negative correlation between adenosine at 15 days of life and GMDS at 12 ± 2 months corrected age was found.

Conclusion: These findings suggest a potential role for blood adenosine concentration as a biomarker of creberal white matter lesions in very low birth weight infants.

Keywords: Adenosine, biomarker, brain injury, brain MRI, periventricular white matter lesions, prematurity, very low weight at birth.

1. INTRODUCTION

Many very low birth weight (VLBW, weighing less than 1500 g) premature infants [1] are diagnosed with brain lesions *via* ultrasound and/or an MRI, later evolving into cerebral palsy or neurocognitive impairments [2-6]. The most common central nervous system (CNS) injuries in VLBW preterm infants are intraventricular (IVH) [7-10] and cerebellar (CBH) hemorrhage [4, 11], whose pathogenesis is multifactorial [8]. A further challenge remains in understanding the exact role of certain lesions in influencing the neurodevelopmental outcome, especially for minor forms of hemorrhage, as the diagnostic accuracy for these lesions without MRI is low. Moreover, the contribution of minor IVH in impairing the periventricular white matter development remains significant [12-16]. White matter lesions (WMLs) also represent an important group of diseases [4, 17, 18]. Among WMLs, a severe form of brain injury called periventricular leukomalacia (PVL) consists of multifocal areas of white

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matter cystic necrosis (c-PVL) near the lateral ventricles, resulting in white matter loss and dilation of the adjacent ventricles [2, 19-22]. PVL is rare, and a milder form, the punctate white matter lesion (PWML), exclusively diagnosed by MRI, is the predominant brain lesion in VLBW premature infants with an incidence of about 20 % [17, 23-26]. In turn, PWML is accompanied by mild impairment in the development of white matter tracts, affecting both neurobehavioral and cognitive development [2, 17]. Pathophysiology of WML remains unclear, but intrinsic vulnerability, inflammation, cerebral hyperoxia/hypoxia, and oxidative stress are the main risk factors reported in VLBW infants [6, 13, 27-32]. Early detection of WML in premature infants remains limited due to the lack of reliable biological markers. Diagnosis of WML is limited to later stages of the disease when radiological changes are visible in the US or MRI; therefore, there is a growing interest in discovering biomarkers for the early identification of infants at risk for WML and to understand its pathophysiology. Many factors and active molecules may affect the perinatal brain development that can be used as biomarkers [33]. We hypothesized that the purine ribonucleoside adenosine might be one of them. Our previous preliminary data showed that VLBW infants displayed high blood levels of adenosine, inversely correlated to their prematurity (*i.e.*, body weight class) [34]. The highest adenosine concentrations were found in infants with the lowest birth weight, with a peak 15 days post-birth during the newborn screening program for congenital diseases. Adenosine plays important physiological modulatory roles in cardiovascular, immune, and central nervous systems [35]. Adenosine is a neuron-glial transmitter inhibiting proliferation and stimulating differentiation of the oligodendrocyte precursor cells (OPC), promoting myelination in the central nervous system (CNS) [36]. However, during postnatal brain development, adenosine may exert deleterious effects, interfering with the maturation of the developing white matter [37]. Extracellular adenosine can be generated from adenosine triphosphate (ATP) hydrolysis or released from the cell through adenosine transporters [37, 38]. Its levels rapidly increase upon tissue ischemia and hypoxia [37], two conditions observed in preterm infants undergoing inflammation and oxidative stress due to increased oxygen availability and low antioxidant levels [21, 39, 40]. While several *in vitro* studies have been conducted to measure adenosine levels, few *in vivo* studies are found.

The present work aimed to measure the adenosine blood levels in a cohort of VLBW preterm infants to assess whether adenosine levels could be a biomarker of brain lesion risk.

2. MATERIALS AND METHODS

2.1. Patients

Inborn premature infants with a very low birth weight (VLBW), *i.e.*, with a birth weight lower than 1500 grams, admitted at the Neonatal Intensive Care Unit of Gaslini Pediatric Hospital, were subjects eligible for our study. Inclusion criteria also included performing cerebral MRI at TEA (between 39 and 41 weeks' post-menstrual age). The gestational age cutoff was less than 34 weeks. The exclusion criterion was the absence of cerebral MRI. VLWB premature infants born between February 2016 and July 2018 were enrolled, after applying the above-cited inclusion/exclusion criteria, the retrospective study group consisting of 56 VLBW premature infants.

The study was cleared by the Institution's Ethics Review Board for human studies, as it conformed to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The ethics committee (Comitato Etico Regione Lombardia) approved the study (no. 39354). Clinical and biochemical data were collected after obtaining signed, written informed parental consent.

2.2. Adenosine Assay

Blood adenosine levels were measured using the neonatal metabolic screening, a simple and reliable method routinely conducted in Italy. A correlation between elevated blood adenosine at 15 days of life and neurodevelopmental outcome at one-year corrected age was assessed. Data shed light on the possibility of conducting preventative interventions to delay or counteract brain damage. Blood samples, obtained by heel stick, drawn routinely in the context of a newborn screening program for congenital diseases, were blotted on filter paper (226, Ahlstrom, Helsinki, Finland), then dried. Dried blood spots (DBS) were processed for adenosine content, as described [41, 42] by Mass Spectrometry (a technique previously validated [42]) with slight modifications discussed below, at four time-points (day 3, T1; day 15, T2; day 30, T3; and day 40, T4), repeating the same procedure at each time point. Namely, spots of about 3.2 mm in diameter were cut from each DBS and extracted for 45 minutes at 45 ºC under mechanical stirring in a methanol solution (C_3OH/H_2O 60:40) containing internal adenosine standard labeled with stable isotopes. Five microliters of the extract were injected (Flow Injection) into the Tandem Mass Spectrometer LC-MS/MS (TQD-Waters) and quantified using mass/mass experiment type MRM (Multiple Reaction Monitoring) for specific transitions m/z of the molecular ions and the particular fragments. Finally, analytical results were processed with Neolynx software.

2.3. Cerebral Magnetic Resonance Imaging (MRI)

MRI was performed at term-equivalent age (TEA) on all infants enrolled in the study during post-discharge follow-up. Patients spontaneously breathed during the examination and were fed before MRI to achieve spontaneous sleep. The neuroradiologist decided wether there was a need for sedation to prevent head movements based on the infant's state of arousal and the quality of images after the first sequence. Hearing protection was used throughout. Heart rate and oxygen saturation were non-invasively monitored by pulse oximetry throughout the examination.

Cerebral MRI was performed using a 1.5 Tesla system (Intera Achieva; Philips, Best, and the Netherlands) with a dedicated head/spine pediatric coil. Our standard MRI protocol included 3-mm-thick axial T2- and T1-weighted images, 3-mm-thick coronal T2-weighted images, 3-mm-thick sagit-

tal T1-weighted images, axial diffusion-weighted images (DWI) (b value: 1,000 s/mm^2), and axial susceptibility-weighted images (SWI). WML was defined as increased T1 signal abnormalities and decreased T2 signal abnormalities in the cerebral white matter on MRI [2, 43]. IVH was evaluated as high intensity on T1-weighted images and low intensity on T2-weighted images. Minor forms of hemorrhage, detected as foci of signal loss on the SWI sequence, without continuity, suggestive of veins, were interpreted as hemosiderin depositions consistent with previous minor hemorrhage or blood deposition [12, 30]. CBH was also considered as hemosiderin depositions called punctate lesions in the early stages. The MRI evaluated a signal intensity within the posterior limb of the internal capsule (PLIC) as a marker of myelination; asymmetrical PLIC was considered as an early predictor of future hemiplegia [44, 45].

2.4. Griffiths Mental Developmental Scales

The neurodevelopmental outcome was assessed by Griffiths Mental Developmental Scales (GMDS) [46] at 12±2 months corrected age in 27 (84 %) out of the 56 patients sampled at 15 days of life. GMDS are widely used to assess the neurological development of infants and young children from birth to 8 years. A score below 80 is considered low; the normal range is above 90.

2.5. Statistical Analysis

The size of the study was determined based on the biological variability of adenosine blood in preterm newborns to allow the identification of statistical change with a probability of P=0.05 and power =80 %. Data were analysed with a consolidated workflow [47]. A non-parametric U-Mann-Whitney test was used to assess differences in adenosine levels between the patients who developed WML, PLIC, IVH, HD or CBH at each time point. The received operating characteristic (ROC) curve and Youden's index were used to assess diagnostic efficiency and the cutoff of each comparison, respectively. Odds ratios (OR), 95 % confidence intervals (CI), and *P*-values were tested to determine the association between Adenosine levels and WML, PLIC, IVH, HD, and CBH using a 2 x 2 contingency table and Fisher's exact test. A non-parametric Friedman test with Dunn's correction for multiple comparisons was used to compare relative time change of adenosine levels for each brain lesion group determined by MRI results. Pearson's correlation matrix was done to identify the linear correlation between adenosine levels and each continuous clinical variable. Data are expressed as the median and interquartile range (IQr), and results are considered significant at two-tailed *P*-values ≤ 0.05. All analyses were performed using Origin v9 and the previous version of package R available during the experiments.

3. RESULTS

3.1. Subjects and Demographic Data

Demographic and biochemical data of all subjects stratified according to the brain lesions determined through MRI, are summarized in Table **1**. Infants who developed IVH showed a lower birth weight (1123 grams IQR 907-1339 *vs* 946 grams IQR 721-1171; *p*-value <0.05) and gestational age (29 weeks IQR 27.2-30.8 *vs* 28 weeks IQR 25.8-30.2; *p*-value <0.05). A lower gestational age was also found in those subjects who showed pathological myelination of PLIC (29 weeks IQR 27-31 *vs* 27 weeks IQR 26.6-27.4; *p*-value <0.05). No other statistically significant differences in demographics were found relative to MRI results.

3.2. Adenosine Blood Levels

Globally, in our subjects, the median of adenosine blood concentration was 0.75 μM IQR 0.57-1.05 at T1 (3 day), whose level progressively and statistically increased (*p*-value <0.001) at the following time points (Fig. **1**). Stratifying subjects according to the brain lesions observed at MRI, we found that adenosine was statistically higher at T2 in subjects who developed WML (P=0.01) or pathological PLIC myelination (P=0.05) than in those who did not. The median/IQR μM values were 0.96, IQR 0.67-1.19 (WML-), 2.50 IQR 1.4-3.41 (WML+) (Fig. **2A**), 0.96, IQR 0.64-1.17 (pathological PLIC myelination-) and 2.44 IQR 1.47-3.09 (pathological PLIC myelination+).

Moreover, we found that adenosine was statistically higher at T1 and T4 in subjects who developed IVH (T1 P=0.05, T4 P=0.01) or HD (T1 P=0.05, T4 P=0.01) than in those who did not. The median/IQR μM values were at T1 0.73 IQR 0.57-0.89 (IVH-), 1.04 IQR 0.61-1.13 (IVH+) and 0.71 IQR 0.58-0.81 (HD-), 0.95 IQR 0.79-1.08 (HD+) and at T4 1.93 IQR 1.35-2.44 (IVH-), 3.42 IQR 2.61-7.02 (IVH+) (Fig. **2B**) and 1.93 IQR 1.35-2.44 (HD-), 3.42 IQR 2.61-7.02 (HD+) (Fig. **2C**).

ROC curve analysis revealed that adenosine levels at T2 were the best predictors for WML development, seen at MRI. In particular, the AUC, confidence interval (CI), and *P*-value for such discrimination were 0.87, 0.69-1 and P<0.001, with >1.58 μM as cutoff value (Fig. **2D**). The sensitivity, specificity, OR and its *P*-value were 83 % IC 36-99; 92 % IC 75-99; OR 5.88 (1.92-33.3); P=0.02, respectively. By contrast, no statistical association was found with the development of pathological PLIC myelination or other clinical variables (Fig. **3A-D**). The above analysis done for the other statistically significant differences of adenosine levels at T1 and T4, respectively did not find any statistical association with IVH or HD (Fig. **2B**, **C**). At T4, the AUC, confidence interval (CI), and *P*-value for the discrimination of IVH development were 0.81; 0.62-1 and P=0.02, with >2.1 μM as cutoff value (Fig. **2E**). Sensitivity, specificity, OR and *P*-value were 83 % IC 36-99; 66 % IC 44-83; OR 3.85 (1.49-16.67); P=0.047, respectively (Fig. **3B**). The same values were also found at T4 for infants who developed HD (Fig. **2F** and **3D**). The whole association between DBS adenosine levels and brain lesions, for each time point, is reported in Fig. (**3**).

There was no correlation between WML and other clinical complications associated with inflammation and oxidative stress. In particular, as far as post-hemorrhagic lesions are concerned, only 3/12 patients with IVH, 1/11 with CBH and 3/18 with HD, and 2/5 with alterations at PLIC myelination presented WML.

Table 1. Demographical data of infants stratified according to results findings at cerebral MRI performed with TEA.

-	WML		Pathological PLIC		IVH		HD		CBH	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Subject n	43	13	51	5	43	13	38	18	45	11
Male/Female, n	18/25	6/7	24/27	0/5	18/25	6/7	15/23	9/9	17/28	7/4
BW gr, median (IQR)	1075 (829-1321)	1104 (938-1270)	1100 (876-1324)	895 (682-1108)	1123 (907-1339)	946 (721-1171)	1126 (909-1343)	988 (758-1218)	1097 (872-1322)	1017 (772-1262)
GA weeks, median (IQR)	29 (27-31)	28 (27-29)	29 (27-31)	27 (27-27)	29 (27-31)	28 (26-30)	29 (27-31)	28 (26-30)	29 (27-31)	28 (26-31)
Pre-E, n (%)	9 (21)	2 (15)	10 (20)	1 (20)	10 (23)	1 (8)	9 (24)	2 (11)	10(22)	1 (9)
Chorioamnionitis, n (%)	3 (7)	1 (8)	4 (8)	0	3 (7)	1 (8)	3 (8)	1 (5)	3 (7)	1 (9)
PROM, n (%)	17(39)	3 (23)	20 (39)	0	16 (37)	4 (31)	14 (37)	6 (33)	17(38)	3 (27)
IUGR, n (%)	8 (19)	2 (15)	10 (20)	0	7 (16)	3 (23)	7 (18)	3 (17)	8 (17)	2 (18)
SGA n (%)	8 (19)	0	8 (16)	0	5 (12)	3 (23)	4 (10)	4 (22)	5 (11)	3 (27)
AGT, n (%)	30 (70)	7 (54)	13 (25)	3 (60)	30 (70)	7 (54)	28 (74)	9 (50)	32 (71)	5 (45)
Mode of birth, V/CS	0/35	2/11	10/41	0/5	7/36	3/10	7/31	3/15	9/36	1/10
Cord pH, median (IQR)	7.28 (7.2-7.4)	7.31 (7.2-7.4)	7.28 (7.2-7.4)	7.38 (7.3-7.5)	7.3 (7.2-7.4)	7.26 (7.2-7.4)	7.3 (7.2-7.4)	7.27 (7.2-7.4)	7.29 (7.2-7.4)	7.29 (7-7)
Cord - BE, median(IQR)	5.08 (0-10.2)	4.66 (1.7-7.7)	5.03 (0.1-9.9)	4.57 (4-5.2)	4.76 (0.1-9.5)	5.96 (0.6-11.4)	4.83 (-0.2-9.8)	5.4 (1.3-9.5)	5.21 (-0.1-10.5)	4.34 (2-7)
Hb gr/dl, median (IQR)	15.7 (13.6-17.8)	15.7 (14.3-17.1)	15.7 (13.7-17.7)	15.6 (13.3-17.9)	15.9 (13.8-18)	15.3 (13.8-16.8)	15.9 (14-17.8)	15.3 (13.1-17.5)	15.9 (14.1-17.7)	15 (13-18)
Adenosine, μM										
T1, median (IQR)	0.79 (0.58 - 1.03)	0.69 (0.58-0.80)	0.75 (0.58-1.0)	0.64 (0.49-1.08)	0.73 (0.57-0-89)	1.04 (0.61-1.13)	0.71 (0.58-0-71)	0.95 (0.59-1.08)	0.72(0.59-0-93)	0.9 (0.56-1.08)
T2, median (IQR)	0.96 (0.67-1.19)	2.5 (1.4-3.41)	0.96 (0.64-1.17)	2.44 (1.47-3.09)	0.94 (0.61-1.16)	2.44 (1.21-2.98)	0.92 (0.64-1.08)	2.35 (1.15-3.02)	0.93 (0.63-1.12)	2.28 (1.15-2.99)
T3, median (IQR)	1.45 (0.73-2.15)	2.95 (1.51-4.5)	1.41 (0.71-2.25)	2.84 (1.58-4.71)	1.43 (0.7-2.11)	2.91 (1.49-4.67)	1.42 (0.71-2.16)	2.90 (1.57-4.2)	1.42 (0.71-2.22)	2.89 (1.5-4.52)
T4, median (IQR)	1.94 (1.38-2.12)	3.40 (2.23-6.89)	1.92 (1.38-2.26)	3.24 (2.5-6.98)	1.93 (1.35-2.44)	3.42 (2.61-7.02)	1.93 (1.35-2.44)	3.42 (2.61-7.02)	1.92 (1.39-2.28)	3.45 (2.55-7.01)

Abbreviations: WML, white matter lesions; PLIC, posterior limb of internal capsule; IVH, intraventricular hemorrhage; HD, hemosiderin depositions; CBH, cerebellar hemorrhage; BW, birth weight; GA, gestational age; Pre-E, pre-eclampsia; PROM, premature rupture of membranes; IUGR, intrauterine growth restriction; SGA, small for gestational age; AGT, antenatal glucocorticoid treatment; V, vaginal delivery; CS, cesarean section; BE, base excess; Hb, hemoglobin; T1, 3 days; T2, 15 days; T3, 30 days; T4, 40 days.

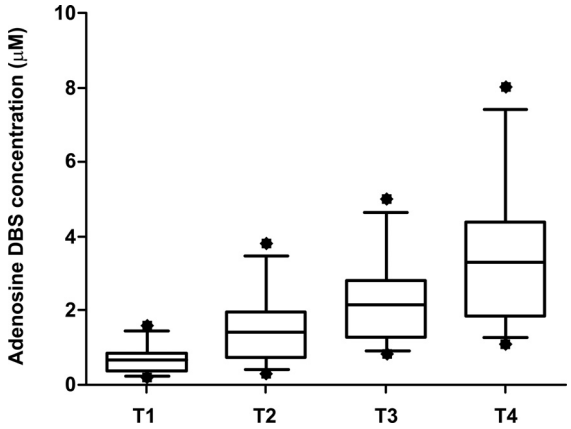

Fig. (1). Global Adenosine DBS concentration. Box plot show the concentration of Adenosine at the four different time points. The concentration median was significantly different (median, IQR and *P*-value were: T1=0.75 (0.36-0.87) μM; T2=1.44 (0.69-1.97) μM; T3=2.15 (1.25-2.79) μM; T4=1.46 (1.84-4.37) μM; *p*-value <0.001.

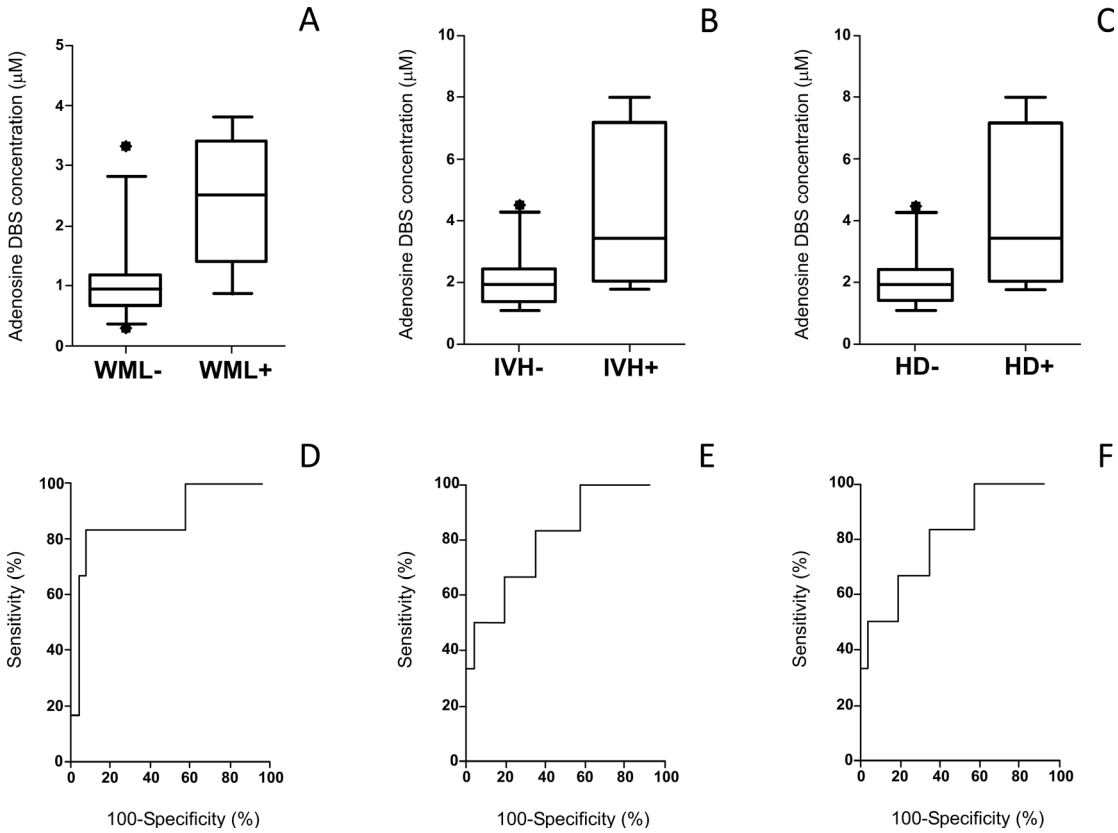

Fig. (2). Box plots and Received operating characteristic (ROC) curve analysis of Adenosine DBS levels. Box plots show the median and distribution of adenosine DBS levels in the entire cohort of subjects stratified for cerebral MRI results. Adenosine levels was statistically significant higher in subject who developed **A**) white matter lesions (WML) at T2, **B**) intraventricular hemorrhage (IVH) at T4 and **C**) hemosiderin depositions (HD) at T4 compared to those who did not. Received operating characteristic (ROC) curve for **D**) WML, **E**) IVH and **F**) HD and Youden's index were used respectively to assess diagnostic efficiency and their cutoff of positivity.

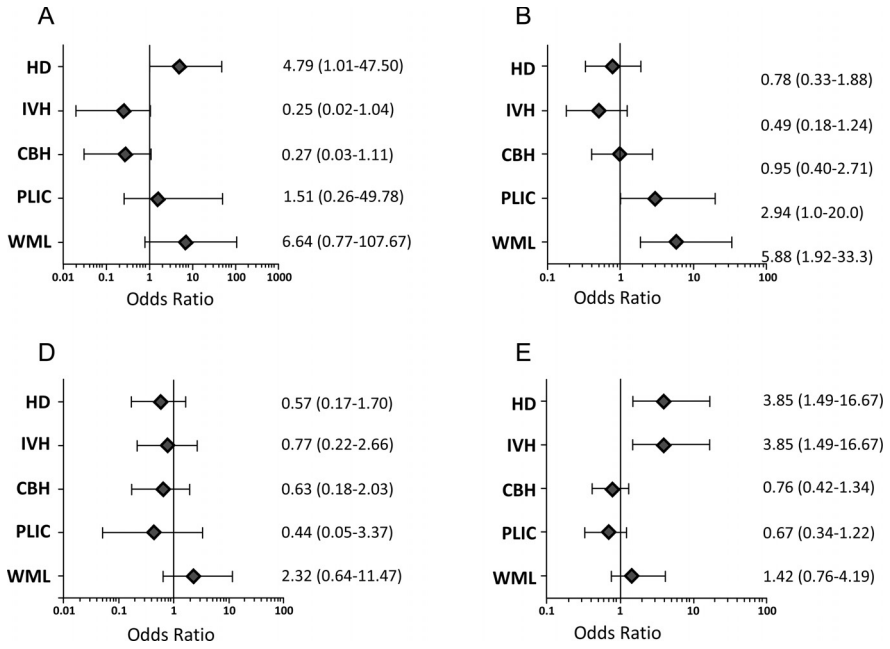

Fig. (3). Odds Ratio forest plot for Adenosine DBS levels and brain lesion at each time point. Forest plots show Odds Ratio and confidence intervals for the different brain lesion *i.e.* hemosiderin depositions (HD), intraventricular hemorrhage (IVH), cerebellar hemorrhage (CBH), pathological posterior limb of internal capsule (PLIC) and white matter lesions (WML) identified by mean of MRI at **A**) T1=3 days, **B**) T2=15 days, **C**) T3=30 days and **D**) T4=40 days.

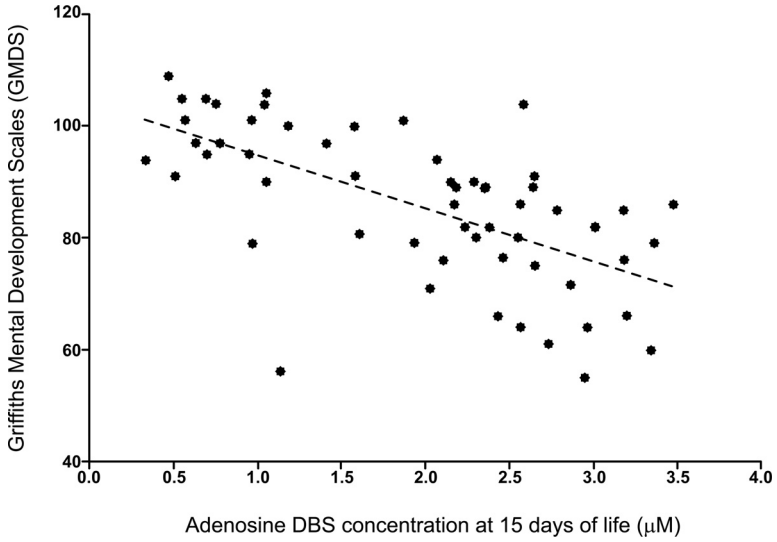

Fig. (4). Pearson Correlation between Adenosine DBS levels and Griffiths Mental Development Scales. Plots show a statistical significant negative linear correlation between adenosine DBS levels at 15 days of life and Griffiths Mental Development Scales (R=-0.56, P=0.05).

Stage V ROP (severe, *i.e.*, treated by photocoagulation) was found in half of our study population (28/56). These patients showed a positive association with WML (OR 4.63, CI 95 % 1.11-19.26; *p*-value <0.05) and pathological PLIC myelination (OR 2.22; CI 95 % 1.64-3.00 *p*-value <0.05). By contrast, BPD was identified in 32 % of our population (18/56): this complication was more frequent among patients with IVH showing a positive association (OR 4.63 CI 95 % 1.11-19.26; *p*-value <0.05). Moreover, we found that adenosine was statistically more abundant in subjects who developed both complications (BPD and IVH) compared to the other subjects. The median/IQR of adenosine μM values were 3.42, IQR 2.61-7.02 (BPD+ and IVH+) and 1.96, IQR 1.33-2.44 (other subjects).

No other significant associations between adenosine levels and brain lesions (WML, CBH, pathological PLIC myelination), visual (ROP) or pulmonary (BPD) complications were identified.

3.3. GDMS Test

Measurement of early child development was performed in 56 infants using the GMDS testing. Four out of 56 subjects (15 %) presented WML (3 patients presented PWML, and 1 patient was diagnosed with PVL). A moderate negative correlation between blood adenosine levels at 15 days of life and the GMDS score at 12 ± 2 months corrected age (Pearson's correlation coefficient: -0.52) was obtained (Fig. **4**). The further analysis showed a Pearson's correlation coefficient of -0.36 in infants with WML and a weaker correlation in the group without white matter lesions (Pearson's correlation coefficient -0.33).

4. DISCUSSION

The need for early identification of preterm infants at risk for developing neurological complications remains a challenge for clinicians. Adenosine blood levels are high in VLBW preterm infants. The highest adenosine concentration is at day 15 post-birth specifically in infants displaying the lowest birth weight, as reported previously [34]. The present study confirms that data, showing that adenosine blood concentration increases after delivery, with a peak at 15 days, significantly associated with subsequent detection of brain damages on MRI performed at TEA. High adenosine levels at 15 days of life positively correlated to the presence of WML and were associated with lower GMDS at 12 ± 2 months of corrected age, suggesting that it could represent a biomarker of brain development impairment risk. Adenosine levels at 15 days of life were a major predictor of WML incidence at TEA, with a sensitivity of 66.7 % and a specificity of 96.2 %.

The ultimate origin of the high adenosine levels in the VLBW blood is challenging. We had supposed that premature exposure to ambient oxygen concentrations and oxidative stress causes a premature functioning of the extramitochondrial oxidative phosphorylation primarily in the nervous and endothelial cells [34].

Which potential explanation may justify the elevated statistical significance (OR=5.8) of adenosine blood levels at Day 15 for predicting periventricular WML? Adenosine levels may be higher in the subjects that were more prone to oxidative stress damage from the ambient oxygen challenge, and therefore also to brain damage from genetic or environmental predisposing conditions.

Adenosine is produced by many cell types [48, 49]. Premature activation of unmyelinated axons typical of the immature brain during development may increase adenosine production in the extracellular space [36]. Adenosine clearance from the extracellular space on a time scale of seconds is mediated by multiple mechanisms, such as equilibrative nucleoside transporters [50], adenosine kinase, and adenosine deaminase [51, 52], the latter activity being defective in the premature, partly explaining plasma adenosine detection in the former [51]. Impairment in adenosine clearance may cause further adenosine accumulation, causing direct white matter toxicity [53].

On the other hand, considering that oxidative stress is implicated in the genesis of both inflammatory responses and WML [39, 54], we cannot exclude that adenosine levels in premature infants undergoing a hyperoxic challenge may represent the sign of oxidative stress [28, 55, 56], causing WML [57]. This would allow correlating adenosine blood levels to a systemic inflammatory state possibly responsible for the genesis of WML. Finally, inflammation associated with a hypoxic event may significantly increase vascular adenosine generation from extracellular ATP [58], promoting the expression of specific ectonucleotidases such as CD73 [55]. CD73 is responsible for the extracellular production of adenosine from AMP in the brain, and its expression is interestingly increased in the cortex and microglia of a low protein diet model of neuroinflammation [43]. In the same animal model, postnatal adenosine levels and A2a-receptor mRNA expression, which is implicated in the modulation of neuroinflammation, were increased in both cortex and microglial cells suggesting the involvement of adenosine in the process [43].

The elevated adenosine concentration at day 15 post-birth in those infants who developed brain WML appears intriguing, as it corresponds to a median of 31 weeks of life, *i.e*., the gestational age at high risk of developing white matter damages [9, 59, 60]. WML development has been related to the impairment of oligodendrocyte progenitor cells, the myelinating brain cells [36, 61]. Pre-oligodendrocytes (PreOLs) represent the predominant of the four developmental stages of the oligodendrocytes, corresponding to the stage at which the white matter vulnerability is higher [59, 62]. Adenosine may be produced by the overstimulated neurons as a signal to promote myelination [36]. Through A1A receptors localized on PreOLs, excess extracellular adenosine, would cause the cell premature maturation, reducing the absolute number of mature oligodendrocytes [36, 37]. PreOLs number reduction may eventually impair white matter maturation [63] (with abnormal PLIC appearance) as observed in association with WML, leading to altered white matter microstructure [17, 25]. Interestingly, the data confirms that vulnerability of white matter in premature infants is higher at certain gestational ages [9, 59, 60] and not inversely related to the level of prematurity, compared to intraventricular and cerebellar hemorrhage. The latter displays the highest incidence with the progressive reduction of gestational age and body weight [4, 27, 60, 64].

In addition, interruption of fetal supply of key nutrients, in particular lipids, not quantitatively and qualitatively balanced by appropriate postnatal nutrition, could accentuate white matter vulnerability to free radical insult [65]. Thus, a dysmyelinating process involving poor lipid supply could elicit further oxidative stress production, as previously reported [66, 67]. This oxidative stress may determine ATP extrusion to the extracellular space, rapidly metabolized to adenosine [49]. Glial cells can also release ATP under hypoxia or ischemia [3, 38, 68].

Adenosine blood values at 40 days (T4), in our study population were positively correlated with an increased frequency of IVH and hemosiderin deposition at the SWI sequence. In this case, the inflammation consequent to hemosiderin deposit after IVH may be the primary cause of the adenosine rise. Anyway, we cannot exclude the influence of chronic systemic inflammation.

The data appear consistent with the correlation between respiratory/ocular complications and the development of neurological sequels previously described [69, 70]. Among the subjects who presented ROP, a higher incidence of WML and pathological PLIC myelination was found in our population. Subjects with ocular and respiratory complications displayed higher adenosine values, especially at 40 days (T4), as opposed to those with a single complication. Interestingly, only patients with ROP treated by photocoagulation presented an alteration of PLIC myelination. The link between severe ROP and myelin alterations is intriguing in that an ectopic oxidative phosphorylation was described in both myelin and retinal rod outer segments [71, 72], where it was shown to cause the production of reactive oxygen species (ROS) [73]. The compound effect of the lipid environment immaturity and the oxygen challenge would impair the ectopic respiratory complexes expressed in the sheath, oxidative stress production and WML, due to lack of myelin trophic support. Neuronal death can, in turn, cause neuroinflammation and oxidative stress [74].

Different causes can explain increased circulating levels of adenosine in VLBW infants. An accelerated brain sensorial stimulation, out of the maternal womb, together with multi-drug exposure (such as caffeine, which may affect adenosine clearance), may promote adenosine production, in turn triggering/accelerating myelination, compared to a normal intrauterine program. This process may also be further compounded by the higher level of circulating oxygen, compared to intrauterine life, causing potential toxicity.

What interventions could be done to decrease the likelihood of WML in infants showing high levels of adenosine at 15 days of life is a matter of debate. Adenosine itself is supposed to play a neuroprotective role in neurological disorders, hemorrhage, and trauma [53]. However, adenosine's anti-inflammatory and neuroprotective abilities fail in some subjects, likely due to individual susceptibility. An antioxidant support therapy would also be beneficial. For example, a dietary integration with n-3 long-chain of polyunsaturated fatty acids, like Docosahexaenoic acid (DHA), is important for eye and brain development. Routine care for extremely preterm infants comprises very little supply of docosahexaenoic acids [75]. It was shown that maternal omega-3 fatty acid supplementation lengthens gestational age and increases offspring's birth weight [76].

The diagnostic limitations of this study are intrinsic to the unsolved problem of the change in adenosine levels and to the fact that it cannot be assayed in term infants, as in adults, in which its blood concentration ranges around zero, due to the multiple enzymes that restrict its presence in both the cytosol and the extracellular space, as stated above [51, 52]. In fact, those present some risk of neurodevelopmental disease, despite the lesser frequency. Adenosine levels in the different subgroups (early *vs* late preterm infants) may display other correlations with white matter damage, even

though susceptibility is strictly correlated to the gestational age [77-80].

To detect WML, we used a cerebral MRI with a standard strength magnet (1.5 Tesla). As shown recently, a stronger magnet (3 Tesla) [81] may be more precise for PWML detection, which could explain the low percentage of minimal WML in our study population. However, consistent developmental outcome studies of children bearing PWML are based on a standard 1.5 Tesla magnet [2, 17]. Moreover, it has been highlighted that slice thickness, more than the magnet strength, is the most important parameter able to identify the smallest cerebral white matter lesions [26].

Finally, the DBS used for adenosine concentration assay may differ as far as the haematocrit, drying, transport conditions [42], and leukocyte count [51] are concerned. However, in our laboratory, this is the only method validated for the neonatal screening of ADA-SCID [41, 42], and the sampling, conducted in the same way for all of the samples, has been standardised. In fact, the method we utilized detects plasma adenosine levels *in vivo* [82].

Another concern regards the influence of the Blood-Brain Barrier (BBB) on the ability of the assay to reflect the actual brain adenosine levels as it may hinder its transfer into the general circulation [83]. The existence of a BBB in the VLBW newborn is very controversial [83] and further compounded by the potential role of adenosine signaling in modulating its function. Other studies on animal models of prematurity have shown that the BBB is dysfunctional, displaying increased vascular permeability, neuroinflammation, and oxidative stress [84]. Accordingly, there is a lack of pericytes in premature infants, immaturity of basal lamina, and deficiency of a glial fibrillary acidic protein (GFAP) in the ensheathing astrocytes end-feet, together with an incomplete maturation of endothelial tight-junctions [85]. Nevertheless, we have recently shown in animal models that higher adenosine levels in the plasma correspond to white matter microglial activation [43], the final step of the plausible mechanisms associated with the development of WML of premature infants [18].

CONCLUSION

In conclusion, our findings show a correlation between adenosine blood levels and white matter brain lesions diagnosed at MRI in VLBW infants. In the same population, adenosine concentrations are associated with impaired neurological development at 12 months of age. Further prospective studies with expanded sample size and possible methodological improvements, like complex enzymatic stop solutions, may be needed to validate the potential role of adenosine as a biomarker and its use in clinical practice.

LIST OF ABBREVIATIONS

Ado = Adenosine

CBH = Cerebellar Hemorrhage

CNS = Central Nervous System

cPVL = Cystic Periventricular Leukomalacia

DBS = Dried Blood Spots

DWI = Diffusion-weighted Images

GMDS = Griffiths Mental Developmental Scales

IQR = Interquartile Range

IVH = Intraventricular Hemorrhage

MRI = Magnetic Resonance Imaging

PLIC = Posterior Limb of the Internal Capsule

Pre-OL = Pre-oligodendrocytes

PVL = Periventricular Leukomalacia

PWML = Punctate White Matter Lesions

ROC = Receiver-operating Characteristic

SD = Standard Deviation

SWI = Susceptibility-weighted Images

TEA = Term Equivalent Age

VLBW = Very Low Birth Weight

WML = White Matter Lesions

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The ethics committee (Comitato Etico Regione Lombardia) approved the study (no. 39354).

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. All the human procedures were followed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

CONSENT FOR PUBLICATION

Clinical and biochemical data were collected after obtaining signed written informed parental consent.

STANDARDS OF REPORTING

STROBE guidelines were followed.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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