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**WHY DO PREMATURE NEWBORN INFANTS DISPLAY ELEVATED BLOOD
ADENOSINE LEVELS?**

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ABSTRACT

Our preliminary data show high levels of adenosine in the blood of very low birth weight (VLBW) infants, positively correlating to their prematurity (i.e. body weight class). This prompted us to look for a mechanism promoting such impressive adenosine increase. We hypothesized a correlation with oxygen challenge. In fact, it is recognized that either oxygen lack or its excess contribute to the pathogenesis of the injuries of prematurity, such as retinopathy (ROP) and periventricular white matter lesions (PWMI). The optimal concentration of oxygen for resuscitation of VLBW infants is currently under revision.

We propose that the elevated adenosine blood concentrations of VLBW infants recognizes two sources. The first could be its activity-dependent release from unmyelinated brain axons. Adenosine in this respect would be an end-product of the hypometabolic VLBW newborn unmyelinated axon intensely firing in response to the environmental stimuli consequent to premature birth. Adenosine would be eventually found in the blood due to blood-brain barrier immaturity. In fact, adenosine is the primary activity-dependent signal promoting differentiation of premyelinating oligodendrocyte progenitor cells (OPC) into myelinating cells in the Central nervous system, while inhibiting their proliferation and inhibiting synaptic function.

The second, would be the ecto-cellular ATP synthesized by the endothelial cell plasmalemma exposed to ambient oxygen concentrations due to premature breathing, especially in lung. ATP would be rapidly transformed into adenosine by the ectonucleotidase activities such as NTPDase I (CD39), and NT5E (CD73). An ectopic extra-mitochondrial aerobic ATP synthetic ability was reported in many cell plasma-membranes, among which endothelial cells.

The potential implications of the cited hypotheses for the neonatology area would be great. The amount of oxygen administration for reviving of newborns would find a molecular basis for its assessment. VLBW infants may be regarded as those in which premature exposure to ambient oxygen concentrations and oxidative stress causes a premature functioning of the extra-

mitochondrial oxidative phosphorylation primarily in axons and endothelium. Adenosine may become a biomarker of prematurity risk, whose implications further studies may assess.

BACKGROUND

Most very low birth weight (VLBW) infants now survive [1] but some 10% of these will sustain neurological injuries leading to persistent disabilities. Morbidity of survivors among VLBW infants includes retinopathy of prematurity (ROP), necrotizing enterocolitis of newborn (NEC), broncho-pulmonary dysplasia (BPD), intraventricular hemorrhage (IVH), and periventricular white-matter injury (PWMI) [2]. The latter is the most common cause of brain injury in preterm infants and the leading cause of chronic neurological disability [3]. Lesions are a continuum from focal cystic necrotic to noncystic injuries, the latter now emerging as the predominant lesions. Since the 1960s (Banker and Larroche, 1962) unique VLBW infant white matter lesions have been described, consisting of coagulative necrosis known as periventricular leukomalacia (PVL). Recently, association of PVL with inflammation (O'Shea et al., 2013) and oxidative stress [4] was reported. Studies on titration of supplemental oxygen are suggestive of a primary role of hyperoxia in these lesions [5]. In fact, the optimal concentration of oxygen for resuscitation of preterm infants to limit both hyper- and hypo-oxygenation is still a matter of debate [6]. Limited premature ventilation was found to be less associated to systemic oxidative stress [7]. On the other hand, chronic hypoxemia was observed in extremely preterm infants, which 100% supplemental oxygen saturation could not amend [8]. Standard therapy is positive pressure ventilation to prevent alveolar atelectasis and methyl xanthine (essentially caffeine) administration [9].

Recently, to assess that initial resuscitation in air is preferable to 100% oxygen, markers of oxidative stress status *in vivo* were utilized, such as advanced oxidative protein products and isoprostanes (prostaglandin F₂-like compounds non-enzymatically formed *in vivo* from peroxidation of arachidonate) [10]. Among markers of oxidative stress, Adenosine (Ado) is emerging. Interestingly, Ado concentration was found significantly higher in the foetal circulation in patients with preeclampsia than in normal pregnancies [11]. Ado induces respiratory depression

[12], likely via their A(2A) receptors expressed by GABAergic neurons. It is also a potent immunosuppressor molecule [13] and a suppressor of synaptic function [14,15]. Ado produced inside the axons and released in case of axonal hypometabolism is a signal to premyelinating Schwann Cells and oligodendrocytes [16], promoting oligodendrocyte development [17]. It was shown that most extracellular Ado derives from extracellular adenosine 5'-triphosphate (eATP), [18], due to the action of the widely distributed ectonucleotidases NTPDase I (CD39), and NT5E (CD73) on eATP. Signals delivered by eATP and Ado are transduced by functional P1, P2Y and P2X receptor subtypes, respectively [19]. ATP can be generated by oxidative phosphorylation occurring exclusively inside the mitochondrion, occasionally released outside the cells due to death or lysis. Other mechanisms have been proposed, i.e. ATP-permeable release channels; non-conductive transporters of ATP [18] [20], release through pannexin hemichannels [21]. Also an ectopic ATP synthesis may be invoked, reported in many cell types, and some reports have shown that an ATP synthesis can occur at the surface of some cells.

The hypothesis

It is proposed that the trigger of Ado production is the premature exposure to atmospheric oxygen, i.e. the most prominent challenge for VLBW infants face upon birth. The source of blood Ado concentration can be both ecto- and endo-cellular. We hypothesize that a substantial part of Ado in the blood of very preterm infants derives from inside the hypometabolic brain axons. This fact is known [16,17], but in our opinion it needs careful reconsidering. The VLBW newborns are prematurely subject to multilevel sensory stimuli they were not supposed to experience. As yet unmyelinated axons involved in sensory neurotransmission display high firing in response to the environmental stimuli, extruding Ado. Brain accounts for a substantial percentage of the newborn body, and the blood-brain barrier is inefficient in the preterm infant. Further studies may tell whether the Ado concentration is high in the CSF. Considering its short blood half-life (about 15 sec) [22], Ado production must be massive to reach the observed blood concentrations. Another

Ado source in the systemic circulation should be postulated: this may be the metabolism of eATP produced by an extra-mitochondrial ectopic ATP synthesis in endothelial cells, due to the sequential activity of CD39 and CD73 [20]. Some of us have pioneered the hypothesis of a functional extra-mitochondrial aerobic ATP production, through the ectopic functional expression of respiratory chain complexes I to IV and of F₁F₀-ATP synthase (Ecto-ATP synthase) [23]. Oxygen availability in the lung endothelium would prematurely switch on the extra-mitochondrial oxidative phosphorylation (OXPHOS) machinery in endothelial cells. Notably, in both cases the abrupt increase in oxygen availability for the premature newborn (considering the hypoxic state of the fetus, living with an oxygen concentration ranging between 3 and 11%) would be the cause of the high production of adenosine.

Evaluation of the hypothesis

Posing that the premature axons reach inadequate energy charge for their metabolic needs, these will produce excess Ado. This can be bidirectionally transported across the membranes by the equilibrative nucleoside transporters (ENTs) or by concentrative nucleoside transporters (CNTs) [18]. In turn, Ado would promote myelination to sustain firing, acting as a potent neuron-glia transmitter to inhibit proliferation and stimulate differentiation of the OPCs [17]. Ado plays the same action potential-dependent signalling between axons and premyelinating Schwann Cells (SCs), which express functional adenosine and ATP receptors [16]. Moreover, Ado would play a double role, as it was shown to be able to efficiently suppress synaptic function [14,15], thereby relieving the hypometabolic neurons. OPCs express all four subtypes of adenosine receptors that are activated in response to action potential firing in Central NS [17,24,25]. Conversely, when energy charge is adequate, an excess ATP is released. Myelination is regulated by an electrical activity-dependent mechanism promoting or depressing it, which involves opposite roles for ATP and Ado signalling [26]. ATP is a signal inhibiting Schwann cells (SCs) myelination: nonsynaptic ATP release from dorsal root ganglion axons inhibits SCs proliferation [16].

Another source of Ado would be the eATP produced by the extra-mitochondrial OXPHOS in the endothelial cell plasma membrane. An extra-mitochondrial aerobic ATP synthesis was reported on the membranes of the HUVEC endothelial cells [27,28], and in other membranes [29–31]. In the immature lung of the VLWB newborn, atmospheric oxygen levels are bound to stimulate an ecto-ATP synthesis. ATP would then be metabolized to Ado by the sequential action of two ectocellular enzymes, namely CD39 and CD73 [20]. If this were the case, the generation of reactive oxygen species in the preterm infant would be concurrent rather than subsequent to the generation of Ado. Indeed, hypoxia/reoxygenation episodes in VLBW infants are the consequence of immature respiratory system [5], which can sustain a proinflammatory cascade and oxidative stress. A primary cause of mortality and long-term sequelae of VLBW infants is oxidative stress consequent to exposure to excessive oxygen concentrations. The premature operativity of the ectopic OXPHOS, due to the availability of oxygen, could produce both ATP and oxidative stress. In fact, an over functioning OXPHOS is a primary source of ROS [32]. Premyelinating axons are particularly sensitive to oxidative stress, recently implicated in OPC death [4]. The oligodendrocyte lineage in the human premature brain is a vulnerable cell population (reviewed in [33]). It has been shown that perinatal oxidative stress in the mouse causes a long-term decrease in oligodendrocyte number and impairs myelination. Intermittent hypoxemia episodes reproduced in neonatal mice were shown to replicate the phenotype of non-cystic WM injury [34]. Purines, especially eATP and its metabolite Ado are potent mediators of inflammation [35]. Ado and reactive oxygen species are among the principal chemical mediators involved in white matter injury. Endothelial cell oxidative damage is a primary cause of the fearsome intra-ventricular brain hemorrhage and neonatal respiratory distress syndrome (NRDS).

The present hypothesis involving an extra-mitochondrial OXPHOS by the endothelial cells, is different from current thinking, in that the importance of ectopic ATP production is still not universally recognized. This has generated an interesting dispute [36,37] about the implications of

myelin ability to aerobically synthesize ATP, both in CNS [38–40] and PNS [41], with a pivotal role of connexins [42].

These hypotheses might be tested in an animal model of prematurity.

Empirical data

Our preliminary pilot data, included in support of the proposed hypothesis, show that high concentrations of Ado are found in the blood of VLBW infants, with a peak after 15 days post-birth, positively correlating with the class of birth weight (Figure 1). The lower the weight, the higher the Ado concentration. We are presently studying a correlation among Ado levels and subsequent comorbidities such as PWMI (Ramenghi, unpublished data).

Consequences of the hypothesis and discussion

Ado released by the axons represents a signal for the oligodendrocyte progenitor cells (OPCs) to differentiate and start myelination, to maintain neurotransmission. However, the premature differentiation, consequence of the Ado signalling, implies a reduced OPC number and therefore a lower number of mature oligodendrocytes. This can disrupt the maturation of myelin causing subsequent lack of myelination and of fibers, which is in fact observed in VLWB infant brain. It has been suggested that a prerequisite to develop effective neuroprotective strategies for very preterm newborns requires understanding of glial behaviour [43]. Lack of myelin would contribute to axonal degeneration and associated periventricular lesions, due to lack of myelin-derived trophic support [43]. If such support is ATP supply, as recently confirmed [44]. Any damage to myelin would impair the correct coupling causing oxidative stress, and ATP depletion up to irreversible point at which axons and neurons lose their potential for survival.

A bias may even exist in the evaluation of new-borns, in that the more premature infants are those who will receive more care, and are therefore subjected to a more stimulating environment, which may rise Ado concentrations.

Posed that the target of injury mechanisms is the oligodendrocyte, all of the factors that predispose to PWMI such as cerebral white matter hypoxia, ischemia, adenosine and oxidative stress [2] can be better explained by the present hypothesis. Disruption of the maturation of myelin-forming OPCs may contribute to the pathogenesis of PWMI. It is anticipated that new strategies for directly preventing brain injury in premature infants can develop following the present unifying hypothesis. Moreover, it is interestingly reminiscent of lesions that are diagnostic in Multiple Sclerosis (MS) [45].

The importance of the present hypothesis lays in its possibility to foresee the susceptibility to the future disabling diseases which lead to long-term motor and cognitive impairment [46]. New preventative strategies for brain injury in premature infants may be imagined. For example, as our understanding of the pathogenesis of PWMI improves, it is anticipated that WM injury in the premature infant may be prevented. There will be an emphasis on hypoxia/hyperoxia, ischemia and their consequences as contributory factors to PWMI and postnatal WM hypomyelination, due to the unique vulnerabilities of developing oligodendrocytes, but more consideration is needed to upstream etiologies, such as the cited ectopic OXPHOS as a source of Ado. It will be interesting to examine the concentration of ADO in the CSF, to see if it locally higher than in blood or if undergoes variations correlated to pathological states.

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CONFLICT OF INTEREST STATEMENT: Authors declare no conflict of interest.

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CAPTIONS TO ILLUSTRATIONS

Figure 1. Blood Adenosine levels correlate to body weight. Median Blood adenosine (ADO) levels, in function of the classes of body weight: <1,500 grams (N=115); 1,501-2,500 grams (N=546); >2,500 grams (N=7388). Median levels were significantly different (p-value<0.001). Dried blood spot samples from newborns were collected on filter paper matrix at 3 days after delivery at Gaslini Pediatric Hospital, according to the newborn screening of congenital adenosine-deaminase deficiency, and analyzed by Tandem mass spectrometry [47].