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Research Paper

Plasma metabolite score correlates with Hypoxia time in a newly born piglet model for asphyxia

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ABSTRACT

Hypoxic-ischemic encephalopathy (HIE) secondary to perinatal asphyxia is a leading cause of mortality and acquired long-term neurologic co-morbidities in the neonate. The most successful intervention for the treatment of moderate to severe HIE is moderate whole body hypothermia initiated within 6 h from birth. The objective and prompt identification of infants who are at risk of developing moderate to severe HIE in the critical first hours still remains a challenge. This work proposes a metabolite score calculated based on the relative intensities of three metabolites (choline, 6,8-dihydroxypurine and hypoxanthine) that showed maximum correlation with hypoxia time in a consolidated piglet model for neonatal hypoxia-ischemia. The metabolite score's performance as a biomarker for perinatal hypoxia and its usefulness for clinical grading and decision making have been assessed and compared to the performance of lactate which is currently considered the gold standard. For plasma samples withdrawn before and directly after a hypoxic insult, the metabolite score performed similar to lactate. However, it provided an enhanced predictive capacity at 2 h after resuscitation. The present study evidences the usefulness of the metabolite score for improving the early assessment of the severity of the hypoxic insult based on serial determinations in a minimally invasive biofluid. The applicability of the metabolite score for clinical diagnosis and patient stratification for hypothermia treatment has to be confirmed in multicenter trials involving newborns suffering from HIE.

1. Introduction

Perinatal asphyxia is characterized by intermittent periods of hypoxia ischemia that, if prolonged and intense enough, may cause irreversible damage to oxy-regulatory tissues such as brain [1]. The resulting hypoxic-ischemic injury evolves over time. Hence, the primary phase corresponding to tissue hypoxia is followed by a partial recovery upon reoxygenation/reperfusion (secondary phase). Along both these periods a precise sequence of pathophysiologic events leading to specific injuries is set in motion [2,3]. Hypoxic-ischemic encephalopathy (HIE) secondary to perinatal asphyxia is a leading cause of mortality and acquired long-term neurologic co-morbidities in both, the late preterm and term neonate with its overall incidence varying notably [4].

Clinical management of HIE patients is strongly affected by the perceived prognosis. To date, moderate whole body hypothermia is, together with air resuscitation, the most successful intervention for the treatment of moderate to severe HIE. Yet, the therapeutic window for initiating treatment is limited to 6 h from birth [4]. To make matters worse, the clinical severity of HIE varies over time after the insult, hampering an accurate assessment for diagnosis especially in the first hours after birth [1]. Currently, the diagnosis of an asphyctic process that evolves to HIE relies on prenatal clinical information (sentinel events), postnatal clinical evaluation including serial Apgar scores and neurological assessment, and cord blood gas analysis reflecting increased lactate levels and metabolic acidosis [5]. At a later time point amplitude-integrated (aEEG) or multichannel electroencephalography (mchEEG) and brain magnetic resonance imaging (MRI) further

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confirm the diagnosis and the degree of severity [1,2].

Metabolomic analysis of biofluids and tissues is becoming an increasingly popular field of research in neonatal medicine [6]. To date, literature reports a very limited number of studies on HIE involving human subjects. In a targeted LC-MS approach Walsh et al. [7] found changes in umbilical cord serum levels of acylcarnitines, glycerophospholipids and aminoacids in newborns with HIE. Reinke et al. studied umbilical cord serum from newborns suffering from asphyxia and HIE employing NMR [8] and established a correlation of their findings with clinical outcomes at 3 years of life in the same cohort [9].

Animal studies seeking for novel biomarkers capable of providing improved diagnostic power have been carried out [10,11]. Changes in retina and choroid tissues of piglets during hypoxia were studied [12,13]. With the aim of discovering early biomarkers, Solberg et al. [13] performed an untargeted metabolomic study in retinal tissue samples from a piglet model of perinatal asphyxia. After the hypoxic insult, elevated levels of the limiting intermediate compound in the major pathway of phosphatidyl-choline biosynthesis [14] (i.e. CDP-choline) were found with its concentrations correlating with the duration of retinal hypoxia. Supported by the observations in neuronal tissue, follow-up studies in minimal-invasively obtained biofluids were carried out revealing a set of 21 metabolites which showed significant changes in a liquid chromatography-time-of-flight-mass spectrometry (LC-TOF-MS) untargeted metabolomics study on plasma samples from piglets subjected to hypoxia and reoxygenation in comparison to a non-asphyxiated control group [15].

In this context, the present work proposes a metabolite score as an estimate of the duration and intensity of hypoxia based on LC-TOF-MS data from an untargeted metabolomics study on plasma samples from piglets subjected to hypoxia and reoxygenation conducted previously [15]. The metabolite score involves plasma metabolites that showed maximum correlation with the duration of hypoxia in a piglet model. With the introduction of the metabolite score we strive after a tool for a user-independent, accurate grading thereby aiding to stratify newborns suffering from HIE who are most likely to benefit from early, moderate therapeutic hypothermia and/or predicting outcomes.

2. Material and methods

2.1. Piglet model for neonatal hypoxia-ischemia

The animal study was carried out at Oslo University Hospital (Norway) and the Norwegian Council for Animal Research approved the experimental protocol (approval number 3399). Animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals by scientists certified by the Federation of European Laboratory Animals Science Association.

Fig. 1 illustrates the experimental study design. For details on the animal experiment, the reader is referred to Solberg et al. [15]. In short, 32 newborn Noroc (LyxLD) pigs aged between 12 h and 36 h, with hemoglobin (Hb) levels > 5 g/dL and in good general conditions were included in the study. Piglets were orally anesthetized, intubated, ventilated and surgically prepared [16]. After 60 min of stabilization, the piglets were randomized either to the intervention group (n=26) or the control group (n=6). Hypoxemia and subsequently hypoxia-ischemia were achieved by ventilation with a gas mixture of 8% O_2 in N_2 until either the mean arterial blood pressure (MABP) decreased to < 20 mm Hg or the base excess (BE) reached -20 mM L^{-1}. CO_2 was added during hypoxemia aiming at a $PaCO_2$ of 8.0–9.5 kPa (60–71.3 mmHg) in order to imitate perinatal asphyxia. After 30 min of reoxygenation, all animals were observed for 9 h receiving room air with continuous surveillance of blood pressure, saturation, pulse, temperature, blood gas measurements and lactate in whole blood samples taken directly from the arterial line and automatically drawn into an ABL 800 FLEX (Radiometer, Copenhagen, Denmark). At the end of the observation time, the animals were given an overdose of pentobarbital (150 mg/kg IV). The control group underwent the same procedures (i.e. anesthesia, surgery, ventilation and sample collection) and observation times, but was not exposed to hypoxia and reoxygenation.

2.2. Plasma sample collection, preparation and LC-TOFMS analysis

Blood samples were taken in ethylene-diamine-tetraacetic acid Vacutainer blood collection tubes before start of hypoxia (t_0), at the end of hypoxia (t_1) and 120 min after end of hypoxia (t_2) and at the corresponding time points for the control group (see Fig. 1). Plasma was obtained immediately after sampling by centrifugation of the whole blood samples at 2000*g* for 10 min at 4 °C. Plasma samples were stored at −80 °C until analysis.

After thawing plasma samples on ice, 150 µL of cold (4 °C) acetonitrile were added to 50 µL of plasma, followed by homogenizing on a Vortex mixer. Samples were centrifuged at a speed of 10000*g* at 4 °C during 10 min 25 µL of supernatant were added to 100 µL of IS solution (5 µM Phe-D_5 and 10 µM Meth-D_3 in H_2O, 0.1% v/v formic acid). 100 µL aliquots of sample extracts were transferred into 200 µL capped glass vials and placed in the refrigerated auto-sampler compartment.

Metabolomic profiling of the plasma extracts was performed on a 1200 RRLC Series Agilent chromatograph (Palo Alto, CA., USA) using a Zorbax SB-C8 (3×150 mm, 3.5 µm, Agilent) column coupled to a 5600-TripleTOF MS spectrometer (ABSciex, Framingham, MA, USA) operating in the positive ionization mode (ESI^+). Peak tables were generated employing the XCMS software [17]. Detailed information on the LC-TOFMS metabolic profiling can be found elsewhere [15].

2.3. Data analysis

Data analysis was carried out in Matlab 2015a (The Mathworks, Natick, MA, USA) using built-in as well as in-house written functions and the PLS Toolbox 8.0 from Eigenvector Research Inc. (Wenatchee, WA, USA). Data for Partial Least Squares regression (PLS) models were autoscaled and venetian blinds cross validation (CV) with 5 data splits was employed. Receiver Operating Characteristic (ROC) curves were calculated using the Biomarker Analysis module available on the MetaboAnalyst platform [18]. Missing values were estimated using the k-nearest neighbors algorithm. Data were used without further normalization, transformation or scaling.

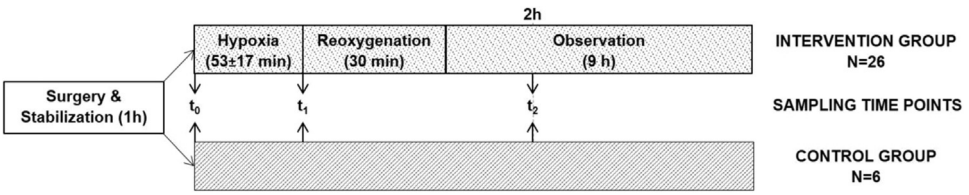

Fig. 1. Overview of the study design.