Early biomarkers of brain development in preterm infants

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Vroege biomarkers van de hersenontwikkeling bij vroeggeborenen

(met een samenvatting in het Nederlands)

Biomarkers precoci di sviluppo cerebrale nei neonati pretermine

(con riassunto in Italiano)

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To Mia, and all the preterm infants I met in the last years,
the infinite courage and strength I perceived in such small human beings,
taught me to never give up

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GENERAL INTRODUCTION

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Introduction

Prematurity is the leading cause of death in the neonatal period and a high percentage of survivors will experience long term neurodevelopmental disabilities. Almost six percent of all preterm infants are born extremely preterm (born ≤28 weeks of gestation) with 25-50% of these at risk for altered brain development, and subsequent abnormal neurodevelopmental outcome. Magnetic resonance imaging (MRI) is currently used for the assessment of neonatal brain structure and development. However, MRI is expensive and does not allow bedside sequential assessment. Therefore, other early, non-invasive and risk free biomarkers for prediction of brain development and maturation are urgently needed. Electroencephalography (EEG) together with NIRS may be such a biomarker. Brain function can be assessed with EEG, which evaluates brain activity, while NIRS gives insights into cerebral blood flow autoregulation and brain oxygen consumption. Furthermore the possibility to use chemical biomarkers, such as markers of oxidative stress, to predict brain injury and establish the harmful role of oxygen, widely used for neonatal resuscitation, is not yet determined. Moreover, metabolomics is a promising technique for the production of early metabolic profiles of human diseases. However, its role in predicting abnormal brain development has so far not been investigated.

Biomarkers of brain function and oxygen consumption

Electroncephalography (EEG) and amplitude-integrated EEG (aEEG)

The neonatal electroencephalogram (EEG) is unique and characterized by specific patterns of electrical activity that mirror the rapid maturational changes of the developing brain. There are waveforms that are not present at any other time of life, furthermore sleep states are varied, change rapidly, and are different from those seen in infancy and adulthood. The EEG of the neonate is usually described in terms of background activity or pattern, which is the baseline activity of the brain at rest, during wakefulness or sleep and is characterized by the following features: continuity, amplitude, frequency, maturational characteristics, state differentiation, reactivity synchrony and symmetry. The latters are specific features of the multi channel EEG. Moreover, the EEG patterns of the full-term infant are very different from the ones of the preterm neonate. However, the features used to describe the background activity are similar. The EEG activity reflects brain function and is an important diagnostic tool for infants requiring neonatal intensive care, also because in this high risk population neurological symptoms may be very vague, or entirely absent. The EEG can be used for early detection of cerebral abnormalities, and also to give important predictive information on later neurodevelopmental outcome.[1] During the last trimester of fetal life there are rapid maturational

changes in the newborn brain which will take place in the extrauterine environment in case of preterm delivery. The brain weight increases fourfold from 28 weeks' to 40 weeks' conceptional age and changes from a very smooth surface at 26 weeks to a complex convoluted structure at term. At the same time, there are also many maturational changes that will also take place in neurons, synapses, and in the myelination of axons. The structural maturation of the brain is reflected in the gradual maturation of the EEG as gestation progresses.[2] The EEG of extremely premature infants reflects the immaturity of the fetal brain, with types of activity recorded that would be considered extremely abnormal in adult life and in a term infant. The most peculiar features of the very preterm EEG are long periods of quiescence or inactivity, interrupted only by bursts of high-voltage and large infra-slow potentials, containing rapid oscillations. These burst of activity are also called spontaneous activity transients (SATs) which were demonstrated to be a primary feature of the preterm infant EEG and are thought to be crucial for the establishment of precise neuronal connectivity. Consequently, aEEG and EEG could be useful for the assessment of the functional status of neuronal connections.[3] Furthermore the lengths of quiescent periods are directly proportional to the degree of prematurity and this normal background pattern is referred to as ''trace´ discontinu'' or discontinuous pattern. The intervals between bursts or SATs are called ''interburst intervals'' or interSAT intervals (ISI). As the neonate matures, the EEG becomes progressively more continuous, and the maximum accepted interburst interval decreases. Prolonged ISI in preterm infants have been associated with abnormal development at 2 years.[4] The preterm brain is highly vulnerable in the first postnatal weeks of life, and maintaining structural and functional brain integrity during this critical period is one of the main challenges in neonatal healthcare. The assessment of early brain activity is an important biomarker of functional brain development in preterm neonates.[3,5] A previous study showed that increased electrical brain activity, thus a higher number of SATs or less cortical electrical quiescence, i.e. shorter ISI, in the first postnatal days, correlated with faster brain volumetric growth up to term equivalent age.[6]

Amplitude-integrated EEG and cerebral function monitor (CFM) were developed by Dr. Douglas Maynard in the late 1960s for continuous EEG monitoring and his colleague, Dr. Pamela Prior, developed the clinical application, mainly used for adult patients.[7] The single channel aEEG is usually recorded using one pair of electrodes in the parietal zone (corresponding to P3 and P4 according to the international EEG 10–20 classification). The obtained signal is then amplified and passed through an asymmetrical band-pass filter that strongly prefers higher frequencies over lower ones and suppresses activity below 0,5-1 or 2 Hz and above 15 Hz in order to minimise artifacts such as: sweating, movements, muscle activity and electrical interference. Finally,x the signal is transformed using a semilogarithmic amplitude presentation, rectified, smoothed and time compressed and displayed on a semilogarithmic scale at slow speed (6 cm/h) at the cot-side. A second tracing continuously displays the original raw-EEG from one or two channels. The impedance is also continuously recorded to highlight the presence of artifacts due to high impedance. The band-width reflects variations in minimum

and maximum EEG amplitude, which are dependent on the maturity and severity of illness of the newborn infant.[8] Nowadays, aEEG is considered an excellent tool for continuous, non-invasive assessment of cerebral activity in newborns.[9] Routinely, aEEG tracings are classified based on the background pattern.[10] Recently, quantitative approaches for digital aEEG and raw EEG signals have been introduced.[11] These approaches classify spikes in cerebral activity as bursts or the equivalent SATs, which were already described as crucial for brain development.[12] This classification enables the calculation of the intervals between bursts, called inter SATs intervals (ISI) and the SATs per minute (SAT rate).[13] Studies in humans have already shown that both the SAT rate and ISI derived from the raw EEG, and quantitative aEEG parameters such as minimun amplitude EEG (min aEEG) and the % of time spent below 5μV, give valuable information about brain function of preterm infants during early phases of neonatal intensive care. In addition, aEEG variables have been associated with brain growth and development, and also with neurodevelopmental outcome.[3,6] Amplitude-integrated EEG is currently used for monitoring cerebral activity in preterm infants. aEEG background activity is more discontinuous, in agreement with the discontinuity of the full EEG. Normal values for aEEG background activity at different gestational ages with a specific scoring system have been published.[14,15] Similarly to the full EEG, sleep–wake cycling can be clearly identified in the aEEG from around 30 weeks gestation, but a cyclical pattern is already visible at 25–26 weeks gestational age. Some medications such as opioids, surfactant and benzodiazepines, can affect brain activity, modifying the background pattern.[16] Some studies demonstrated that early depression of background activity correlates with the severity of IVH, and the presence of burst suppression or very long ISI can predict a poor neurodevelopmental outcome at 2 years of age.[1,17] There are also many other factors such as the occurrence of bronchopulmonary dysplasia or sepsis, that can influence brain function or outcome. Thus, the prediction of outcome using aEEG in preterm infants is quite complex.[18,19] However, using new digital equipment, continuous display of interburst duration, clinicians might be able to use aEEG as a prognostic tool.[20] Additionally, the raw EEG can detect neonatal seizures using a seizure detection tool, useful for the early detection on brain injury. Furthermore, early aEEG maturity was associated with the total MRI maturation score at TEA in preterm infants.[21] The hypothesized correlation of brain functional and structural maturation creates an early potential interesting predictor of altered brain development.

Near-infrared spectroscopy (NIRS)

Bed-side, continuous and non-invasive neuromonitoring could provide valuable information about the occurrence of possible hemodynamic disturbances that are significantly associated with neurological morbidities and increased mortality in preterm infants.[22] Intraventricular hemorrhage and periventricular leukomalacia are responsible for neurodevelopmental impaiment in preterm newborns and are largely determined by the presence of an immature vasculature of the germinal matrix and periventricular white matter.[22] During the first days of

life, preterm infants are at increased risk of cerebral ischemia or hyperperfusion due to the impairment of cerebral blood flow autoregulation.[23] Near-infrared spectroscopy (NIRS) has been used, in the last years, for the assessment of cerebral vascular autoregulation.[24] NIRS technology is based on the possibility of near-infrared light to pass through biological tissues, such as the skin and the skull, and to measure the cerebral regional O_2 saturation ($crSO_2$).[23] The soft and thin tissues on a relatively large head make the application of NIRS the optimal and easy choice for neuromonitoring in the neonatal population.[22,25,26] High $crSO_2$ (and low cerebral fractional tissue oxygen extraction, cFTOE), both indicating cerebral hyperperfusion, were observed in infants who later developed high grade IVH.[27]

NIRS is useful for bedside, noninvasive and continuous monitoring of cerebral hemodynamics and oxygenation and can be used in conjuction with more expensive and not bedside tools such as arterial spin labeled perfusion (ASL) MRI, which provides a direct measurement of regional cerebral blood flow (CBF).[28]

Biochemical biomarkers of brain injury

Oxidative stress

Adapted from Tataranno ML, et al. *Clin Perinatol* 2015; 42: 529-539.

The preterm and term brain is particularly vulnerable to the oxidative stress (OS) insult because rapidly growing tissues are especially sensitive to the harmful effects of free radicals (FRs).[29] OS plays a pivotal role in the pathogenesis of brain injury, being the final common pathway for multiple converging events. OS may result from many different pathways including glutamate release and NMDA receptor activation, leading to excitotoxic processes; mitochondrial dysfunction; activation of enzymes, such as nitric oxide synthase (NOS); phagocyte activation; arachidonic acid cascade; Fenton reaction, driven by the release of non–protein bound iron (NPBI); and deficiency of the antioxidant system of the immature brain.[29,31] According to current knowledge, the pathophysiology of brain injury almost always involves multiple factors including hemodynamic, metabolic, nutritional, toxic, and infectious mechanisms, acting in the antenatal or postnatal period. The combination of these factors often triggers neuronal death processes.[32] The so-called “encephalopathy of prematurity” encompassing intraventricular hemorrhage (IVH) and periventricular leukomalacia is the major contributor to neonatal brain injury. Chronic placental inflammation and acute fetal and neonatal inflammation increase the risk of brain injury.[33] A characteristic feature of the fetal brain is the presence of many leptomeningeal anastomoses among major cerebral arteries, leading to the relative sparing of gray matter, whereas the telencephalic white matter, especially in the depths of the sulci, represents a border zone of blood supply between major cerebral arteries. Furthermore, the very high levels of polyunsaturated fatty acids in the neonatal

brain predispose to the generation of FRs and to OS injury. Polyunsaturated fatty acid are constituents of membrane lipids in the white matter and are highly susceptible to FRs damage. FRs attack on immature myelin leads to lipid peroxidation and lipid peroxides are themselves FRs.[34] The relative immaturity of the antioxidant system facilitates the exposure of fetuses and newborns to the damaging effects of OS. Particularly, superoxide dismutase, catalase, and glutathione peroxidase antioxidant enzyme systems are less active and are present in lower concentrations in the immature brain.[35] The presence, in the developing brain, of a transient increase in density and distribution of glutamate receptors could amplify brain injury caused by hypoxic-ischemic damage.[36] The discovery and validation of specific OS biomarkers of neonatal brain injury represents a key step forwards in the evolution of neonatal neuroprotection and is based on the measurement of a single, or a panel of biomarkers in biologic fluids and tissues, reflecting OS injury to neuronal cells.

Clinicians do not currently have access to biomarkers for early diagnosis or intervention in neonates with brain injury. Thus, the need to develop specific OS biomarkers to enable caregivers to make an early prediction of newborns at high risk for brain injury is now worthwhile. This will help to start preventative neuroprotective strategies, and to monitor the progression of the disease. Biomarkers can be considered as indicators of a disease process, but they can also give information about the worsening and progression of the disease. A reliable biomarker should be biologically plausible, with a high sensitivity and specificity, and should be measured with a reproducible and standardized methodology. Several biomarkers have been proposed for OS detection, but only a small number of them can be considered truly specific and reliable for brain injury; these include prostanoids and non-protein bound iron (NPBI).[29,37,38]

Prostanoids

Prostanoids are produced after non-enzymatic *in vivo* and *in vitro* peroxidation of polyunsaturated fatty acids. F2-isoprostanes (IsoPs) are first produced in phospholipids and then they are released into the bloodstream. The mechanism involved in their formation implies that FRs insult causes hydrogen abstraction from arachidonic acid and addition of molecular oxygen to form a peroxyl radical.[39] The following intermediates undergo double 5-exo-trig cyclization and addition of second molecular oxygen to form prostaglandin G2–like compounds, which are rapidly reduced to F2-IsoPs.[40] Prostanoids can derive from eicosapentanoic acid that is a direct precursor of docosahexaenoic acid (DHA), a primary structural component of the human brain and particularly of the cerebral cortex. Another source is the oxidation of adrenic acid,[37] highly concentrated in myelin within the brain white matter of primates. Prostanoids can be measured in plasma, tissues, cells, urine, cerebral spinal fluid, bile, and bronchoalveolar lavage fluid.[41]

Non Protein Bound Iron (NPBI)

The term NPBI was introduced to indicate a low-molecular-mass iron form, free from binding to plasma proteins. NPBI levels can be measured using high-performance liquid chromatography.[42] Iron toxicity is inversely proportional to the presence of ferritin, which is able to bind and detoxify ferrous ion, and directly proportional to the quantity of hydrogen peroxide to produce hydroxyl radicals through the Fenton reaction. Furthermore, lipid exposure to high concentration of NPBI leads to formation of IsoPs.[43,44]

Metabolomics

The metabolomics technique provides the quantitative analysis of an impressive amount of low molecular weight metabolites that are intermediates or final products of all the metabolic pathways in living organisms. Metabolomics can be applied to all biological fluids and the obtained prolifes are the result of the interaction between gene expression and the environment. This technique can be used to identify human diseases based on the variations in the metabolic profile.[45]

Metabolomic analytical techniques are nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS). Both allow the analysis of biological fluids or tissues and the extraction of latent metabolic information such as metabolic spectra, to enable the identification of biomarkers of human diseases.[46] Applications of metabolomics in the field of neonatology are very promising. Studies on intra-uterine growth retardation, prematurity, bronchiolitis, cytomegalovirus infection, diabetes, sepsis and inborn errors of metabolism showed important results and pave the way for future research.[47-55] Although all biological fluids can be used for metabolomic analysis, urine is particularly suited in the neonatal population due to its non-invasive method of collection and large availability.

Markers of brain development

Advanced MRI techniques

Conventional images such as T1- and T2-weighted images can be scored based on internationally accepted MRI scoring systems (Kidokoro, Woodward) with the possibility to predict long term neurodevelopmental outcome.[56,57] Using conventional images (MRI performed at 30 and 40 wks postnatal age) for automatic segmentations and parcellations[58-60] will enable to calculate quantitative descriptors characterising cortical development and brain volumes, which can give more insight into brain development of extremely preterm infants.

Technical advances in MRI have characterized patterns of injury to the developing brain, for example, using diffusion-weighted imaging (DWI), clinicians were able to early identify is-

chemic tissue in the neonatal brain. DWI allows correlation of the structural development of the brain with the functional development of the child. This particular technique, together with new image postprocessing tools capable to assess quantitative brain volume and surface changes, has produced more accurate neuroimaging correlates for later neurocognitive impairment. Diffusion tensor imaging (DTI) enables the assessment of WM integrity, and is particularly suitable for studying WM and brain maturation.[61] DTI assesses the Brownian motion of water in tissues, detecting microstructural changes which are not usually visible on conventional MRI.[62,63] Fractional anisotropy (FA) is derived from DTI and detects the direction of water diffusion in tissues depending on fiber coherency, axonal density, cell membranes and myelination.[55,56] Thus FA is a good tool to assess tissue microstructure integrity of the white matter. FA increases with brain maturation due to the structures' development and increased myelination and correlates with Bayley Scales of Infant Development.[61,64-66]

Aims and outline of the thesis

The general aim of this thesis is to describe the use of the above mentioned early biomarkers, or the combination of biomarkers, and its interpretation in a clinical setting, to understand and predict of brain development and brain injury in preterm infants.

PART 1 | THE USE OF BIOMARKERS — CLINICAL INTERPRETATION

Chapter 2 describes how early aEEG is affected by the use of morphine in extremely preterm infants. **Chapter 3** studies how oxidative stress is affected by resuscitation with either 100% oxygen or room air. **Chapter 4** examines the relation of oxidative stress and the histology of the placenta in preterm infants.

PART 2 | COMBINING BIOMARKERS

In **Chapter 5** the combination of NIRS and aEEG/EEG was used to study oxygen utilization in the early neonatal period. **Chapter 6** uses serial MRIs in combination with aEEG/EEG to evaluate the changes in brain morphology and microstructure, in relation to early brain activity, in extremely preterm infants. In **Chapter 7** the role of the biochemical urinary metabolic profile and MRI are used to predict brain injury in preterm infants in the first postanatal days.

PART 3 | DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

In **Chapter 8** a summary of the findings of the present thesis is presented. Implications and future directions are discussed. In **Chapter 9** a summary in Dutch and Italian is provided.

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part one

THE USE OF BIOMARKERS
CLINICAL INTERPRETATION

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The effect of morphine on early brain activity in extremely preterm infants

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Submitted

Background and aim | Amplitude-integrated electroencephalography (aEEG) provides a continuos and bedside assessment of brain activity in preterm infants. Several studies report the effect of sedative medication, widely used in neonatal intensive care units, on aEEG/EEG background pattern. Our aim was to study the effect of morphine and its cumulative dose on aEEG quantitative measures in extremely preterm infants, recorded over the first three days after birth.

Methods | 174 preterm infants were enrolled in 3 European tertiary NICUs (mean GA 26±1wks) and monitored during the first 72 hours after birth with continuous 2 channel aEEG (BrainZ, Natus, Seattle). Six epochs of aEEG recordings were selected at 4-6h, 10-12h, 20-24h, 32-36h, 44-48h, 68-72h, taking the periods of the cross-sectional signal with most continuous activity and without ictal discharges. Minimum amplitude of aEEG (min aEEG), percentage of time amplitude <5 μV (% of time <5μV),) spontaneous activity transients (SAT rate i.e. SAT/min) and interSAT interval (ISI) were calculated for each epoch using an in-house developed program (SignalBase®, Utrecht, NL). For infants who received morphine during one or more epochs by clinical indication, the cumulative morphine dosage was calculated as a sum of mg/kg per day over the first three days after birth. A multivariable mixed model and multivariable linear regression model were respectively chosen to check the association of morphine administration (yes/no) and morphine cumulative dose with aEEG/EEG measures. Multivariable analyses were adjusted for cerebellar hemorrhage, intraventricular hemorrhage (all grades), mechanical ventilation and center.

Results | Eighty-nine newborn infants (51.1%), out of the 174, received morphine during the study period. Morphine administration and cumulative dose (min 0.07, max 1.7 mg/kg over the study period) had a significant effect on all quantitative aEEG/EEG measures, causing depression of early brain activity (longer ISI, reduced SAT rate, decreased min aEEG and increased % of time <5 μV) in all epochs. Both morphine administration and dosage showed a significant negative association with SAT rate (coeff resp:-1.38;-1.70; p value <0.01) and a significant positive association with ISI (coeff resp: 2.90; 5.64; p value <0.01). A negative association was found between morphine administration and cumulative dose and min aEEG (coeff resp:-0.78;-1.14; p value <0.01) while a positive association was observed for the % of time<5 μV (coeff resp:14.80; 15.18; p value <0.01). A significant effect of GA and postnatal age (PNA) on both aEEG/EEG measures was observed.

Conclusion | Our findings suggest that morphine administration and cumulative dose are strongly associated with a reduction in brain activity in extremely preterm infants, thus the administration of sedative drugs should be taken into account when interpreting aEEG/EEG. Both acute and long-term consequences of morphine on brain development should be investigated to optimize preterm neurodevelopmental outcome.

Introduction

Extremely preterm infants spend the last trimester of gestation in the NICU environment. This period is crucial for brain development, with the formation of the majority of sulci and gyri, with a 5-fold increase in brain volume, but especially the growth of long-range brain connections, which are important for the higher cognitive function development.[1,2] aEEG is a non-invasive bedside tool increasingly used for neuro-monitoring in preterm infants. Studies support the idea that the quality of the early brain activity, measured with EEG and amplitude-integrated EEG (aEEG), is important for shaping neuronal connectivity during prematurity.[3] Particularly, the presence and number of spontaneous activity transients (SATs), which are represented by burst of activity, are considered to guide the development of brain connections.[3,4] Benders and colleagues demonstrated that increased brain activity (thus an increased number of SATs) in the first three postnatal days correlated with a faster growth of total brain volume and subcortical gray matter at term equivalent age in a group of preterm infants; on the contrary, a less active brain showed decreased growth in volume.[5]

A few studies demonstrated that early aEEG patterns can be predictive of both short and long term outcome[6,7] however, more studies including a higher number of infants are needed to confirm these findings.[8] Especially the presence of very low-voltage inter SAT (bursts) intervals (ISI, thus periods of brain inactivity) with duration of more than two hours, was associated with a poor outcome.[7] In this context, the exposure of preterm infants to factors which are able to influence and modulate brain electrical activity can potentially affect later brain development. Sedative medications and morphine can depress aEEG/EEG activity[9-11] with a loss of its predictive value for long-term neurodevelopmental outcome.[7] However, so far, no studies investigated the specific effect of morphine and its cumulative dose on quantitative aEEG/EEG measures such as SATs and periods of brain inactivity (ISI) in a large cohort of extremely preterm infants.

Thus, our aim was to study the effect of morphine administration and morphine cumulative dose on aEEG/EEG quantitative measures, recorded over the first three days of life in a large cohort of extremely preterm infants.

Materials and methods

Patients

A total of 204 extremely preterm infants (<28 weeks of gestational age, min 23+1 wks; max 27+6 wks) admitted to three European tertiary neonatal intensive care units (NICUs) were enrolled in the present study: 106 at the Wilhelmina Children's Hospital in Utrecht, 42 at the Skåne University Hospital in Lund and 26 at the Sahlgrenska University Hospital in Go-

thenburg. Written parenteral consent was obtained from parents of infants included in the NEOBRAIN study, while the others waived the need for written informed consent, according to national legislation, since amplitude-integrated EEG/EEG monitoring was part of standard clinical care in those units. Patients data were anonymized in each center prior to analysis and the medical ethical review committee gave permission for the use of the clinical data for research purposes. Exclusion criteria were the presence of chromosomal or congenital abnormalities and neuromonitoring with other devices than the BrainZ device (BRM2, BRM3 BrainZ, Natus, Seatlle, USA). Furthermore, 25 infants were excluded since they had either incomplete or bad quality aEEG/EEG data during the first 72 hours after birth, four infants did not have morphine data available, one infant received remifentanyl instead of morphine for intubation, thus the final cohort consisted of 174 infants. All included infants, receiving morphine in bolus or infusion during the first three days after birth, were eligible for morphine administration due to clinical reasons such as sedation, intubation, mechanical ventilation and analgesia. Perinatal data and data about the development of intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), patent ductus arteriosus (PDA) both surgically and medically treated, and bronchopulmonary dysplasia (BPD) were also collected. BPD was defined as oxygen dependence at 36 weeks of postconceptional age.[12] Germinal matrix-intraventricular hemorrhage (GMH-IVH) was diagnosed according to the classification of Papile et al.[13] The first cranial ultrasound was routinely performed within 24 hours after birth and serially repeated until term equivalent age.[14] All infants underwent a magnetic resonance imaging (MRI) at term equivalent age, according to the protocol of the NeoBrain consortium, and images were assessed for brain injury and maturation (white matter, cortical and deep grey matter and cerebellum injury).[15]

Amplitude-integrated EEG acquisition

AmplitudeEEG/EEG bedside monitoring at a sampling rate of 256 Hz was started as soon as possible after birth and generally, within 3 hours after birth and continued for 72 hours. Subcutaneous needle electrodes or hydrogel electrodes were used, with a central reference electrode. The analysis was performed on the cross-sectional signal (P3-P4), known to be predictive of neurodevelopmental outcome in preterm infants.[7] All Infants were monitored with BrainZ monitors (BrainZ, Natus, Seattle) and infants monitored with other devices than BrainZ were excluded from the study due to technical reasons (impedance, inhomogeneity of data, and differences in filters).

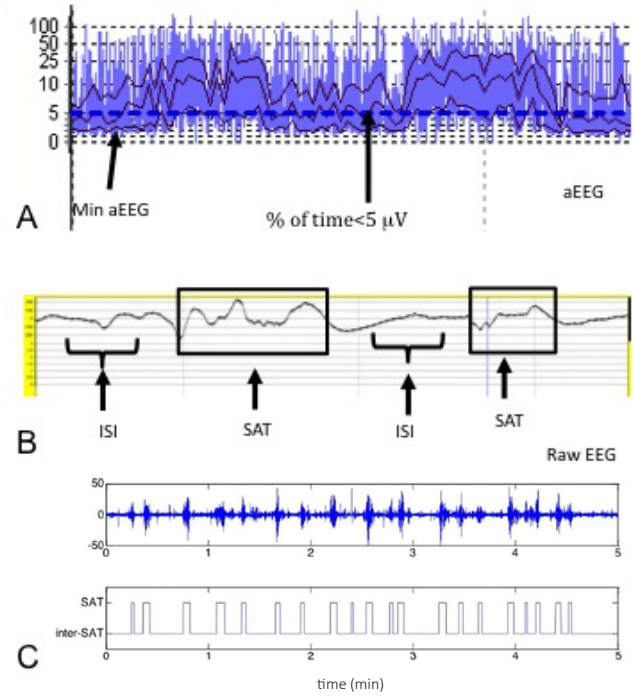
aEEG/EEG analysis

SignalBase software (SignalBase® v7.8, University Medical Center Utrecht, The Netherlands) was used to analyze the raw EEG data. Firstly, all the aEEG/EEG records were visually assessed in order to avoid artifacts, caretaking events, periods with high impedance, and ictal discharges in the selected epochs. Six specific postnatal time-points were chosen: 4-6h, 10-

12h, 20-24h, 32-36h, 44-48h and 68-72h. At all time points, an epoch of 1 hour was manually selected. The aim of the first assessment was to select the best hour (less artifacts, the best brain activity) per time-point.

A quantitative analysis was performed on the raw EEG using the same software program to obtain the number of SATs per minute (SAT rate) (rounded to whole numbers) and the inter SAT interval (ISI, i.e. time between SAT) in seconds, both derived from the raw EEG using a nonlinear energy operator (NLEO) (http://iopscience.iop.org/0967-3334/31/11/N02). Finally as aEEG measures, the percentage of time spent below 5 microvolts (% of time<5μV) and the minimum amplitude of the aEEG (min aEEG) (the mean lower border of aEEG during each study period) were obtained from the aEEG records. All the aEEG/EEG measures are explained and shown in Figure 1.

Figure 1 | A. aEEG trace. The lower black line indicates the minimum amplitude EEG (min aEEG). The blue dot-line indicates the 5 μV threshold. Using SignalBase we measured the percentage of time spent below this threshold (%of time < 5 μV). B. curly brackets indicate periods of brain inactivity or flat EEG trace corresponding to interSAT intervals (ISI). Boxes are periods of brain EEG activity and corresponds to SATs, the number of SATs per minute (SAT rate) was obtained using SignalBase program. C. This is an example of how our in house developed program (Signal-Base) was able to select SATs from the raw EEG.



Morphine data

Morphine was always administered on clinical indication i.e. sedation, intubation procedure, mechanical ventilation, or analgesia. Morphine administration (yes/no) was marked for each study period. Furthermore, for babies who received morphine during one or more epochs, the total morphine dosage was calculated as the sum of mg/kg/day over the first 3 days after birth. Data were obtained by patients charts.

Statistics

Data were analyzed using R Statistics (version 2.15.3, www.r-project.org) and SPSS (IBM SPSS Statistics v 21.0). Clinical characteristics were summarized as mean ± standard deviations (SD), median with IQR, percentages and absolute frequencies. Correlations between aEEG/EEG quantitative measures and clinical variables were first visualized in dots-plots and checked using the Spearman correlation test (2-tailed).

Univariate linear regression models were used to select clinical variables with a significant effect on the aEEG. Afterwards, a multivariable mixed model was used to check the association between aEEG/EEG measures over the six selected epochs and morphine administration (yes/no). Multivariable linear model was used to investigate the effect of morphine cumulative dose (total amount over the first 3 days after birth) on aEEG/EEG variables (mean over the six epochs). All analyses were adjusted for GA, postnatal age (PNA), morphine yes/no (or cumulative dose), any cerebellar hemorrhage, IVH (any grade, yes/no), mechanical ventilation, center. Possible interactions between the different clinical variables were also taken into account. A p value <0.05 was considered significant. Finally, in order to support our hypothesis about the morphine effect on EEG, we selected seven neonates who received morphine during the first day but not on day 2 and 3 and we compared their aEEG/EEG trend to seven random GA matched controls (no morphine).

Results

The analysis showed some inter-center differences (Table 1). Eighty-nine neonates out of 174 received morphine during the study period. The mean gestational age and birth-weight were significantly lower in infants from Lund and Gothenburg compared to Utrecht hospital but there was no difference among groups from the three hospitals in the number of morphine treated neonates or in the morphine cumulative dose. Clinical characteristics of the total population are shown in Table 2.

Table 1 | Population characteristics per center.

Characteristic		Utrecht (n = 106)	Lund (n = 42)	Gothenburg (n = 26)
GA mean (SD)		26.5 (1.0)*	25.3 (1.2)	25.8 (1.2)
Morphine yes n (%)		51 (48)	20 (47)	18 (69)
BW mean (SD)		901 (172)°	771 (169)	837 (172)
Male gender n (%)		61 (57.5)	23 (54.8)	14 (53.8)
Cesarean section n (%)		50 (47.1)°	32 (76.2)	13 (50)
Umbilical cord pH mean (SD)		7.27 (0.12)	7.34 (0.04)	7.31 (0.06)
Apgar score 1 min median (IR)		5 (2)	5 (2)	5 (2)
Apgar score 5 min median (IR)		7 (1)	7 (2)	7 (2)
pH in cord blood mean (SD)		7.28 (0.1)	7.34 (0.04)	7.31 (0.06)
IVH grade I-II n (%)		22 (20.8)	6 (14.3)	6 (23,1)
IVH grade III-PHVD n (%)		11 (10.4)	1 (2.4)	1 (3.8)
PDA n (%)	Medical treatment	44 (41.5)	13 (31)	11 (26.2)
	Surgery	13 (12.3)*	15 (35.7)	10 (34.6)
NEC n (%)		5 (4.7)	2 (4.8)	2 (7.7)
BPD n (%)		37 (34.9)^	13 (31)	15 (57,7)
Sepsis n (%)		33 (31.1)°	4 (9.5)	6 (23.1)
Cerebellum hemorrhage at 40 wks PNA n (%)		9 (8,4)*	8 (19)	9 (34.6)
Mechanical ventilation n (%)		66 (62.3)	34 (81)	17 (65.4)

* p <0.05 between Utrecht vs the other hospitals, ° p <0.05 Utrecht vs Lund; ^ p <0.05 Utrecht vs Gothenburg. Abbreviations: GA=gestational age. BW=birth weight. IVH=intraventricular hemorrhage. PHVD= post-hemorrhagic ventricular dilatation. PDA=patent ductus arteriosus. NEC= necrotizing enterocolitis. BPD= bronchopulmonary dysplasia. PNA= postnatal age. SD=standard deviation. IR=interquartile range.

Table 2 | Clinical characteristics and differences between groups.

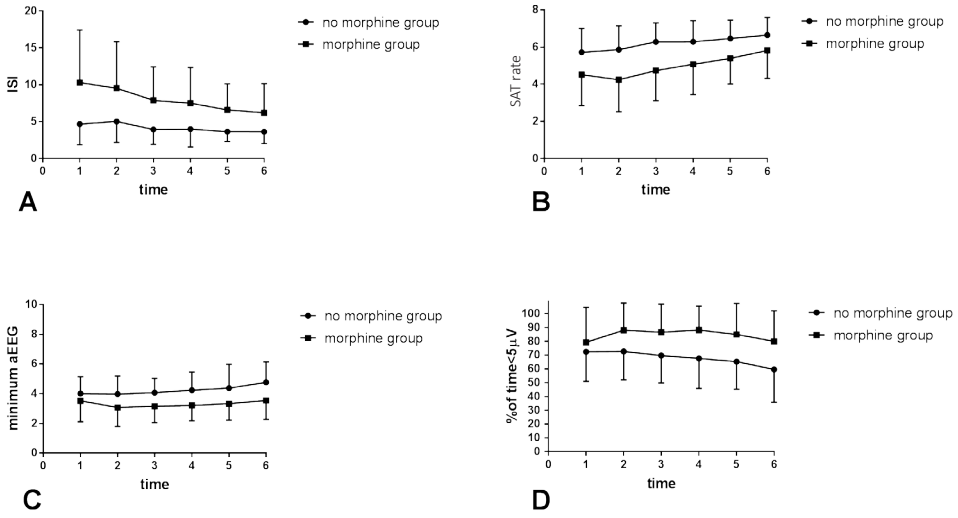
		Total population (n = 174)	No morphine group (n = 85)	Morphine group (n = 89)
GA mean (SD)		26.10 (1.22)	26.32 (1.14)*	25.98 (1.20)*
BW mean (SD)		857 (178)	896 (174)*	831 (172)*
Gender	Male n (%)	97 (55.7)	41 (48.2)*	56 (62.9)*
Cesarean section n (%)		94 (54)	45 (52.9)	53 (59.6)
Umbilical cord pH mean (SD)		7.30 (0.09)	7.31(0.08)	7.30(0.10)
Apgar score 5 min median (IR)		7 (2)	7 (2)	7 (2)
IVH grade I-II n (%)		34 (19.5)	14 (16.5)*	20 (22.5)*
IVH grade III-PHVD n (%)		13 (7.4)	3 (3.6)*	10 (11.2)
Cerebellum hemorrhage at 40 wks PMA n (%)		26 (14.9)	11 (12.9)	15 (16.9)
Mechanical ventilation n (%)		117 (67.2)	31 (36.5)*	86 (96.6)*
PDA n (%)	Medical treatment	43 (24.7)	21 (24.7)	22 (25.9)
	Surgery	37 (21.3)	13 (15.3)*	24 (27)*
Sepsis n (%)		43 (24.7)	22 (25.9)	21 (23.6)

* p value <0.05 between morphine and no morphine group. Abbreviations: GA=gestational age. BW=birth weight. IVH=intraventricular hemorrhage. PHVD= post-hemorrhagic ventricular dilatation. PDA=patent ductus arteriosus. PNA= postnatal age. SD=standard deviation. IR=interquartile range.

Effect of morphine use on rawEEG and aEEG measures

Patients receiving morphine showed significantly longer ISIs and decreased SAT rates in all epochs (Figure 2 resp. A and B). The mixed model showed a significant positive association between morphine administration and ISI in all epochs. Particularly, significantly prolonged periods of interburst interval were observed in morphine treated patients, both during and after administration (Table 3). The mixed model also showed a negative effect of gestational and PNA on ISI, thus infants with higher GA and PNA had a significantly shorter ISI. Furthermore, a negative association was found between morphine administration and SAT rate in all epochs, demonstrating a significantly decreased number of SAT/min (i.e. decreased brain activity) in the morphine group (Table 3). In accordance to the previous model, SAT rate was also positively influenced by gestational and PNA, indicating that patients with higher GA and PNA had a significantly higher number of SAT/min (Table 3).

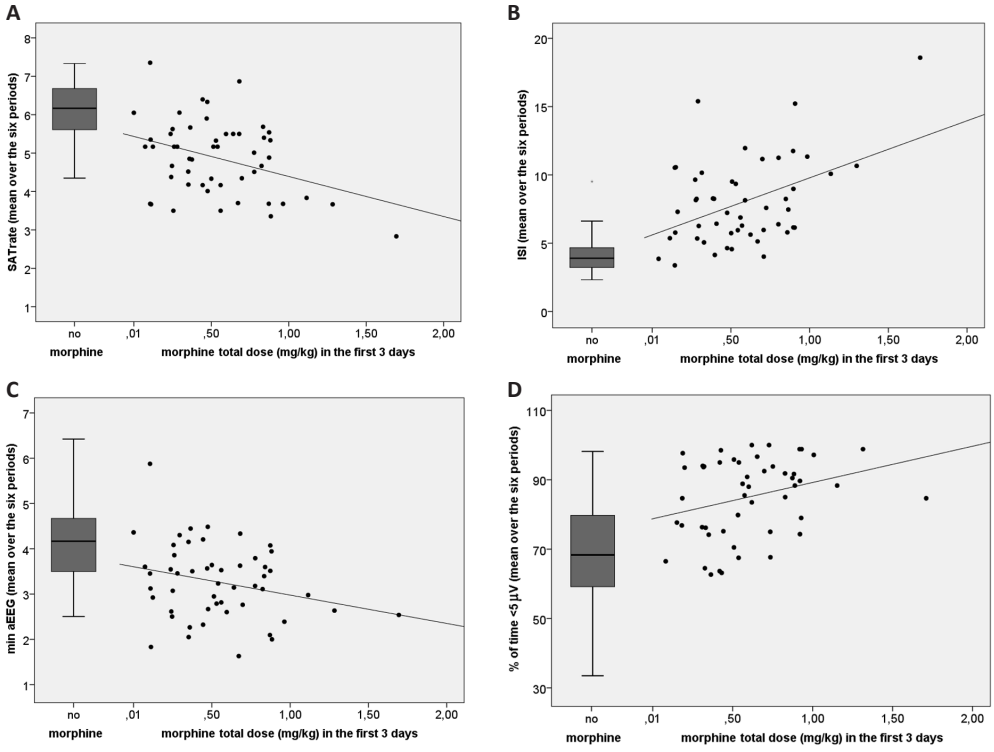
Figure 2 | Morphine effect on rawEEG (A and B) and aEEG (C and D) at six time points.



Abbreviations: ISI, inter SAT interval in seconds; SAT rate, number of spontaneous activity transients/min.

Furthermore, min aEEG was significantly lower and the % of time <5µV higher in the morphine group patients (Figure 2 resp. C and D). The mixed model showed a significant negative association between morphine administration and min aEEG, while a positive effect of GA and PNA was observed (Table 3). The percentage of time spent below 5 µV (period of depressed brain activity) was significantly increased by morphine administration, while patients with higher GA and PNA spent significantly less time below 5 µV.

Figure 3 | Correlation between morphine cumulative dose and rawEEG (A–B) / aEEG (C–D).



Abbreviations: ISI, inter SAT interval in seconds; SAT rate, number of spontaneous activity transients/min; min aEEG: minimum amplitude aEEG.

Morphine dose effect on aEEG/EEG measures

The cumulative dose of morphine was significantly and negatively correlated with SAT rate (Figure 3 A). In addition, high morphine doses were positively correlated with ISI (Figure 3 B). The aEEG analysis showed that the morphine dose was negatively correlated with min aEEG and positively with % of time <5µV (Figure 3 C and D respectively). In the multivariable linear regression analysis GA had a significant positive effect on SAT rate, while the cumulative dose of morphine had a significant negative effect (Table 4). In contrast, GA and morphine showed a negative and positive association with ISI, respectively. GA showed a significant positive association wth min aEEG, while morphine a negative effect (Table 4). The % of time <5 µV was significantly and negatively affected by GA and positively by morphine cumulative dose (Table 4). Neither the presence of IVH, cerebellum hemorrhage nor mechanical ventilation showed any significant effect on aEEG/EEG measures.

Table 3 | Multivariable mixed model between morphine administration and raw EEG/aEEG measures.

Raw EEG measures		β coeff	CI 95%	*P* value
ISI	Time (PMA, hours)	-0.488	-0.719;-0.257	<0.001
	GA (wks)	-0.822	-1.322;-0.321	0.001
	Morphine (yes/no)	2.900	2.008; 3.791	<0.001
	Interaction time:GA	-0.017	0.008; 0.025	<0.001
	IVH (yes/no), cerebellar hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	-	-	NS
SAT rate	Time (PMA, hours)	0.160	0.081; 0.240	<0.001
	GA (wks)	0.417	0.132; 0.564	0.001
	Morphine (yes/no)	-1.386	-1.878;-0.952	<0.001
	Interaction time:GA	-0.005	-0.008;-0.002	<0.001
	IVH (yes/no), cerebellum hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	-	-	NS
aEEG measures				
Min aEEG	Time (PMA, hours)	0.008	0.005;0.011	<0.001
	GA (wks)	0.152	0.042;0.261	0.007
	Morphine (yes/no)	-0.782	-1.051;-0.782	<0.001
	IVH (yes/no), cerebellum hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	-	-	NS
% of time <5 μV	Time (PMA, hours)	-0.121	-0.176;-0.067	<0.001
	GA (wks)	-2.818	-4.601;-1.036	0.002
	Morphine (yes/no)	14.802	10.413; 19.192	<0.001
	IVH, cerebellum hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	-	-	NS

Abbreviations: GA: gestational age. PMA=post menstrual age. IVH=intraventricular hemorrhage.

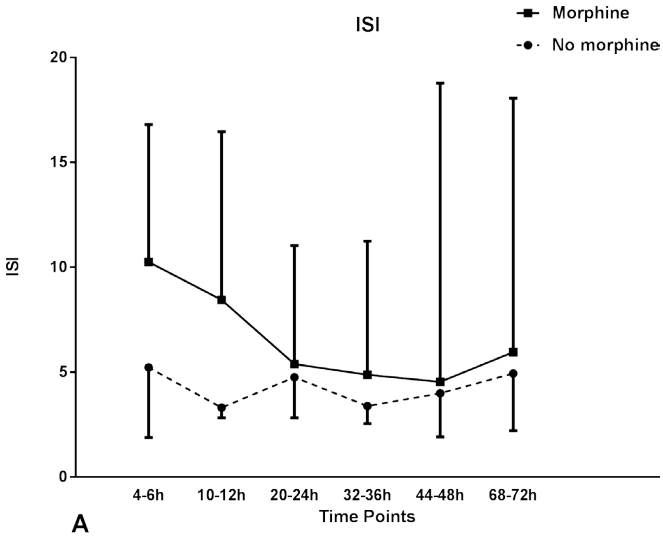
In seven infants receiving morphine only during the first day (24 h) but not on days 2 and 3, a clear reduction in SAT rate and a prolonged ISI during the first 24 h was observed compared to the GA-matched controls (no morphine patients) (Figure 4 A and B). On day 2 and 3 their aEEG/EEG activity recovered to values similar to the control group for both SAT rate and ISI (Figure 4 A and B).

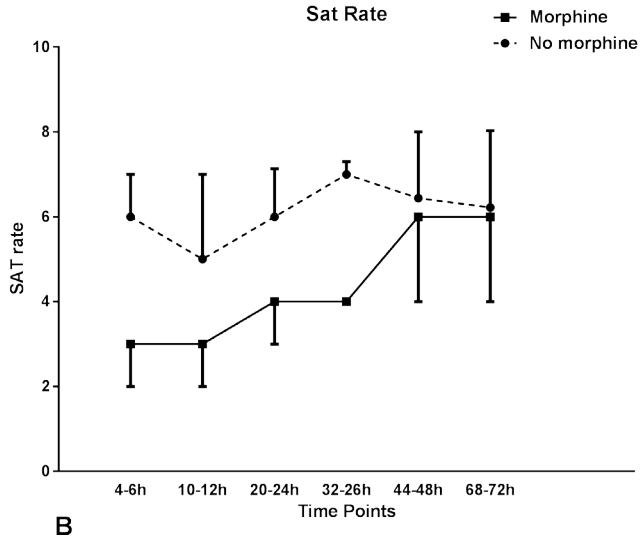
Table 4 | Multivariable linear regression: morphine cumulative dose and raw EEG/aEEG measures.

Raw EEG Measures		β coeff	CI 95%	*P* value
ISI	GA (wks)	-0.603	-1.025;-0.182	0.005
	Morphine (cumulative dose mg/Kg)	5.649	4.032;7.267	<0.001
	IVH (yes/no), cerebellum hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	-	-	NS
	Interaction morphine:GA	-	-	NS
SAT rate	GA (wks)	0.390	0.142; 0.638	0.003
	Morphine (cumulative dose mg/Kg)	-1,704	-2.307;-1.100	<0.001
	IVH (yes/no), cerebellum hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	-	-	NS
	Interaction morphine:GA	-	-	NS
aEEG measures				
Min aEEG	GA (wks)	0.368	0.234;0.503	<0.001
	Morphine (cumulative dose mg/Kg)	-1.144	-1.713;-0.576	<0.001
	IVH (yes/no), cerebellum hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	-	-	NS
	Interaction morphine:GA	-	-	NS
% of time<5μV	GA (wks)	-5.473	-7.691;-3.254	<0.001
	Morphine (cumulative dose mg/Kg)	15.185	7.254;23.116	<0.001
	IVH (yes/no), cerebellum hemorrhages	-	-	NS
	Mechanical ventilation (yes/no)	7.121	0.909;13.333	0.025
	Interaction morphine:GA	-	-	NS

Abbreviations: GA: gestational age. IVH=intraventricular hemorrhage.

Figure 4 | Line-plot illustrating the differences between 7 patients who received morphine only on day 1, but not on day 2 and 3 (only in the first 24 hours) and 7 random GA-matched control infants.





Vertical lines represent the interquartile ranges, while dots and squares represent the median in the morphine and no-morphine patients respectively. Infants receiving morphine clearly had more depressed brain activity (longer ISI [Fig 4 A] and lower SAT rate [Fig 4 B]) in the first 24 hours but when morphine was stopped, their values went back to values similar to the control group. Abbreviations: ISI, inter SAT interval in seconds; SAT rate, number of spontaneous activity transients/min.

Discussion

This study investigated the effect of morphine administration on aEEG/EEG quantitative measures, recorded over the first three days after birth, a crucial transition period for extremely preterm infants. Morphine administration was significantly associated with both aEEG and raw EEG depression of early brain activity (SAT rate, min aEEG) in all epochs and with an increase in length of inactivity (IBI and % of time <5μV). Furthermore, the total amount of morphine increased cerebral depression by prolonging ISI and the % of time < 5 μV. The cumulative morphine dose also decreased brain activity with a significant reduction of SAT rate, i.e. periods of bursts of activity and min aEEG i.e. the mean lower border of amplitude EEG trace.

To our knowledge, this is the first study investigating the effect of morphine and its cumulative dose on aEEG/EEG using a new quantitative approach in a large cohort of extremely preterm infants. The results confirm previous data from a smaller randomized cohort of very preterm infants, demonstrating prolonged cerebral depression after morphine administration.[9] The quantitative approach has many advantages since it is more objective and free from bias than visual aEEG/EEG assessment although it requires a relatively artifact-free recording. In previous studies, a strong association of quantitative aEEG/EEG measures with GA and with PNA during the first 3 days was observed.[7, 11,16,17] These findings support the hypothesis that extrauterine life and GA independently influence aEEG/EEG activity.[18]

Our study showed that morphine administration, independently from GA and PNA, negatively influenced aEEG/EEG quantitative measures, leading to a more depressed brain activity. Our analysis was adjusted for confounding factors, such as perinatal morbidities, which are known to affect brain activity. Amplitude EEG/EEG changes, in relation to morphine administration, are likely linked to morphine-induced disturbances in brain mechanisms which are also dose-dependent, resulting in a change in EEG graphoelements.[10] Opioids administration can cause a reduction in neuronal density and dendritic length, as well as increased apoptosis in rodents and *in vivo* models of human microglia and neurons.[19,20] From a neurophysiological perspective, the main result of morphine administration on the neonatal aEEG/EEG is a suppression of the background pattern.[11] The suppression of the EEG background can be caused by the use of medications, such as morphine but also through the anatomical disconnection of the thalamus from the cerebral cortex.[21] The activation of μ opioid receptors, which are predominant in the neonate and are mainly located in layers 4 and 1 of the neocortex, may cause the functional thalami-cerebral cortex "disconnection".[22] The increase in potassium conduction may be the underlying mechanism for electro-cortical suppression.[21]

Studies on preterm infants demonstrated that early aEEG patterns can predict both short and long term outcome already at 48 h after birth.[6,7] However, not all studies looking at the predictive value of the aEEG/EEG on preterm outcome, took the role of medication and the possible effect on brain activity into account.

The neurodepressive effect of morphine was described by other authors, mainly focusing on the background aEEG pattern, thus on a qualitative aEEG analysis.[11] In the paper by Natalucci and colleagues, morphine was mainly associated with the total maturity score and the cycling subscore, but not with the min and max amplitude,[11] the quantitative aEEG variables taken into account. The results of the present study, together with those other studies[9,11] strongly suggest that the administration of sedative drugs should be considered when interpreting aEEG/EEG since its predictive value may be affected by the use of morphine.

Recent experimental studies have shown that early brain activity is crucial for neuronal survival and the development of brain networks, in particular increased activity correlates with volumetric brain growth and, on the contrary, reduced cortical activity negatively influenced subsequent brain growth.[5] Thus, a balanced approach is important for the administration of sedatives and neuro-depressive drugs in order to provide the minimum effective dose for analgesia.[9] Morphine is a commonly used as a sedative drug in preterm neonates requiring mechanical ventilation.[23] The use of sedatives is fundamental in neonatal intensive care units, since invasive procedures and the consequent pain and stress, are negatively associated with brain development,[24,25] even if there are insufficient data recommending the routine use of opioids for pain management.[26]

In this perspective, our results show the effect of morphine cumulative dose on aEEG/EEG, with a negative influence of high morphine dose on brain activity. This supports the importance of adequate and constant pain assessment in order to adjust sedative drugs dose in this high risk population.[27]

An important implication of sedation in extremely preterm infant is to evaluate both acute and long term effects of morphine on brain activity and development in order to optimize preterm neurodevelopmental outcome. Some studies suggest that morphine use in preterm neonates may potentially affect brain development, leading to both short and long term neurodevelopmental adverse effects.[26] When looking at long term outcome, studies performed in rodents showed that morphine can participate in modifying processes of maturation and proliferation of specific brain regions,[28-31] resulting in long-term sequelae. Particularly, Baijic and colleagues found a significant increase in apoptotic neurons in the cortex and amygdala of rats, regions known to be important for respectively sensory and emotional memory processing.[31]

However, the mechanism of morphine-induced apoptosis was not clearly identified. One study in mice showed that exposure to high concentration of morphine was associated with Purkinje cell death and impaired cerebellar development[32] A study by Bekheet and colleagues found a decreased diameter of Purkinje cells and decreased thickness of both molecular and granular layers of the cerebellum in morphine treated albino rats.[33]

There are not enough data to support a consensus on the role of morphine on short and long term outcome of preterm infants. A study by Steinhorn and colleagues showed that a low-dose of morphine analgesia was associated with early alterations in cerebral structure and short-term neurobehavioral impairment that, however, did not persist at 7 years of age.[34] In a long-term follow-up of a small group of infants from the NEOPAIN study, there was an adverse effect of neonatal morphine on the visual analysis domain of intelligence quotient at 5 years, but no differences were observed in the overall intelligence quotient.[35] However, the morphine treated children of the same cohort, had smaller head circumference, impaired short-term memory, and social problems.[36] A recent study found that a 10-fold increase in morphine exposure was associated with a 5.5% decrease in cerebellar volume in 136 serially scanned extremely preterm infants with morphine being independently associated with impaired cerebellar growth and poorer neurodevelopmental outcomes at 18 months of corrected age.[37]

Since there is still no consensus, both acute and long-term effects of morphine on brain activity and development (brain volumes, cortical development) should be further investigated.

This study has some limitations: first we could not take into account in our analysis the exact timing of morphine administration for each patient because those data were not available in our records. Furthermore, it may be argued that newborns from the morphine group had a lower GA and were sicker compared to the others, thus the underlying condition may have a greater impact on aEEG/EEG tracings than the medication itself. However, our analysis was adjusted for all those confounding factors and morphine was still significantly associated to the aEEG/EEG measurements.

Overall, this study showed that morphine administration and dosage were strongly associated with a reduction in brain activity and increase in brain inactivity in the first three days of life in extremely preterm infants. Thus, the administration of sedative drugs should be considered when interpreting the aEEG since its predictive value may be affected by such drugs. Furthermore, as already described by other authors, preterm infant cerebral activity increases with increasing maturity (PMA) and is influenced by GA. Finally, both acute and long-term effects of morphine on brain activity and development (brain volumes, cortical development) should be investigated in more detail to optimize preterm neurodevelopmental outcome.

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3

Resuscitating preterm infants with 100% oxygen is associated with higher oxidative stress than room air

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Aim | The starting fraction of inspired oxygen for preterm resuscitation is a matter of debate, and the use of room air in full-term asphyxiated infants reduces oxidative stress. This study compared oxidative stress in preterm infants randomised for resuscitation with either 100% oxygen or room air titrated to internationally recommended levels of preductal oxygen saturations.

Methods | Blood was collected at birth, two and 12 hours of age from 119 infants <32 weeks of gestation randomised to resuscitation with either 100% oxygen (n = 60) or room air (n = 59). Oxidative stress markers, including advanced oxidative protein products (AOPP) and isoprostanes (IsoP), were measured with high-performance liquid chromatography and mass spectrometry.

Results | Significantly higher levels of AOPP were found at 12 hours in the 100% oxygen group ($p < 0.05$). Increases between two- and 12-hour AOPP ($p = 0.004$) and IsoP ($p = 0.032$) concentrations were significantly higher in the 100% oxygen group.

Conclusion | Initial resuscitation with room air versus 100% oxygen was associated with lower protein oxidation at 12 hour and a lower magnitude of increase in AOPP and IsoP levels between two and 12 hours of life. Correlations with clinical outcomes will be vital to optimise the use of oxygen in preterm resuscitation.

Introduction

For more than 100 years, health professionals have used 100% oxygen freely for neonatal delivery room resuscitation on the premise that newborn depression was largely caused by hypoxia.[1,2] Today, it is well known that oxygen, while essential for life, may also generate toxic free radicals species that have the potential to cause cellular and organ damage.[1] Consequently, the exact starting fraction of inspired oxygen (FiO_2) for delivery room resuscitation has become a matter of great debate over the last few years. There is robust evidence that using room air instead of 100% oxygen to resuscitate full-term, asphyxiated infants with mature pulmonary systems is feasible and reduces oxidative stress and mortality. Nevertheless, the need for resuscitation in term infants is often completely different to that of preterm infants, who usually need some amount of oxygen because of pulmonary immaturity.[3,4] The evidence for using less, or indeed more, oxygen to resuscitate preterm infants who have both decreased antioxidant defences[5] and pulmonary immaturity, continues to be unclear. Studies on a small number of patients have demonstrated that using less oxygen for preterm resuscitation is feasible, but information about long-term clinical outcomes[3,4] remain unsubstantiated.[6,7] In addition, the use of room air for a prolonged period, such as more than two or three minutes, leads to hypoxia. The lack of consensus was demonstrated by a survey of neonatal practitioners. Many clinicians continued to use 100% oxygen because they were still uncertain about the consequences of using less oxygen but also because the change from using 100% oxygen implies a high cost to provide blenders and oximeters. This high cost could be prohibitive in lower-resourced countries.[8]

Further information on the effects of using less, or indeed more, oxygen to resuscitate this already high-risk population of infants is required. Oxidative stress markers are often used to demonstrate physiological reactions to high or low oxygen exposure. In this study, we report on preliminary oxidative marker results obtained from a subset of infants <32 weeks of completed gestation who were randomised at birth to start resuscitation with either 100% oxygen or room air as part of a larger clinical study called Targeted Oxygen for the Resuscitation of Preterm infants and their Developmental Outcomes, TO2RPIDO.[9] Instead of being left on a set FiO_2 for a specific time, the amount of oxygen given to infants in this study was adjusted according to preductal peripheral oxygen saturation (SpO_2) derived from recommendations of current international resuscitation guidelines.[10,11] We hypothesised that infants who were initially resuscitated with 100% oxygen would have higher levels of oxidative markers than those resuscitated with room air.

Patients and methods

The TO2RPIDO study, recruited infants in Australia, Malaysia and Qatar between December 2009 to June 2014.[9] The trial was still recruiting patients when this study was conducted so that this present analysis of oxidative stress markers were not presented in relation to clinical outcomes. The current study reports on a subset of 119 infants <32 weeks of completed gestation, where 60 were assigned to 100% oxygen and 59 to room air.

These infants were randomly and consecutively recruited in the two Australian neonatal intensive care units (NICUs): The Royal Hospital for Women and the John Hunter Children's Hospital, Newcastle, both in New South Wales, Australia. Randomisation was computer generated and allocated to the specific centres and stratified by gestation in order to prevent inadvertent over-enrolment into extremes of gestational age. Infants were grouped into blocks of 10 of less than 276 weeks and 28-316 weeks' of completed gestation.

Resuscitation procedures

Prior to commencement of the study, 100% oxygen was standard of care for delivery room resuscitation for all infants in participating hospitals. Randomisation was performed prior to birth to either 100% oxygen or room air. In this study FiO_2 was titrated according to set pre-ductal SpO_2 readings obtained with the Masimo Rad 7® oximeter, CA, USA (12) as recommended by international guidelines.[11] It is worthy to note that these guidelines do not make specific starting FiO_2 recommendations for preterm infants, stating lack of current evidence. FiO_2 was increased or decreased by 10% increments to meet these targets. 100% oxygen was given to any infant whose SpO_2 remained <65% at five minutes after birth despite FiO_2 adjustment, whose heart rate was <100 beats per minute despite adequate ventilation, who required cardiac compression and if requested by the attending practitioner. FiO_2 titration continued to admission in the NICU. All the infants received standard clinical care after NICU admission.

Oxidative marker analysis

NPBI was chosen as marker of potential oxidative stress because it indicates increased susceptibility to oxidative damage especially in *in vivo* studies.[13] Isoprostanes are chemically stable *in vitro* and *in vivo* and are specific and reliable markers of lipid peroxidation. They are thus reliable markers of in situ oxidative injury.[21] AOPP, markers of protein oxidation, remain stable during sample storage both at −20° C and −80° C for six months, allowing for batched analysis of progressive specimens.[17] AOPP was chosen as proteins are the major targets of free radicals, being present in abundance in cells, plasma and most tissues.[17]

NPBI plasma levels were measured using partial modifications of the methods described by Kime et al.[13,14] The system was operated isocratically at a pressure of approximately 115 bar and flow of 0.75 ml/min. The detection wavelength was 450 nm with a reference wavelength at 620 nm. A low affinity ligand, disodium nitryloacetic acid, was first used to complex all low molecular weight iron and all iron that was non-specifically bound to serum proteins such as albumin.

A two-steps filtration process was used. Firstly, filtration was performed with a 100-kDa molecular weight cut-off Whatman ultracentrifuge filter followed by filtration with a filter set at a 20 kDa cut-off. The filtrate was analysed by direct injection into a reverse-phase liquid chromatography system, utilising pre-column derivation with the high-affinity iron chelator, the 3-hydroxyl-1-propyl-2-methyl-pyridin-4-one hydrochloride. AOPP were measured using spectrophotometry on a microplate reader. The instruments were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide. In test wells, 200 µl of plasma diluted 1:5 in phosphate buffered saline was distributed on a 96-well micro-titer plate, and 20 µl of acetic acid was added. In standard wells, 10 µl of 1.6 M potassium iodide was added to 200 µl of chloramine-T solution, 0-100 µmol /L, followed by 20 µl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader against a blank containing 200 µl of phosphate buffered saline, 10 µl of potassium iodide, and 20 µl of acetic acid. Since the absorbance of chloramine-T at 340 nm is linear up to 100µmol/L, AOPP concentrations were expressed as µmol/l chloramine-T equivalents. Isoprostanes were detected using the tandem mass spectrometer run in multiple reaction monitoring with the electrospray source operating in negative ion mode, and by exploiting the transitions m/z 353.3 > 193.2 for F2-isoprostanes and 357.3 > 197.2 for the internal standard d4-8-iso prostaglandin 2α. The samples were stored at −80°C until analysis, adding five µL of the internal standard solution at four ng/mL and 50 µL of methanol to 50 µL of plasma. After a gentle mix and settling for five minutes, the mixture was vortex-mixed for 30 seconds. Proteins were separated by centrifugation at 13,000g for five minutes. The quantity of 80 µL of the supernatant were diluted with 80 µL of an aqueous solution of acetic acid at 0.6%. A clear extract was obtained by a further centrifugation and the final injection of 100 µL represented 25 µL of the original specimen. External calibration points were prepared by adding to 200 µL-aliquot of water 10 µL of the working solutions of F2-isoprostanes with the concentrations ranging from 0.25 up to 25 ng/mL in order to obtain resulting aqueous solutions from 12.5 pg/mL up to 1250 pg/mL.[15-17] Changes, such as an increase or a decrease, between the two time points, two and 12 hours, for AOPP, NBPI and isoprostanes were considered to evaluate the increase in oxidative stress biomarkers few hours after birth. Time zero, meaning biomarkers measured in cord blood, was excluded from this analysis because at this time point oxidative stress will more likely reflect prenatal factors rather than post delivery interventions.[17]

Statistical analysis

Results are presented as mean and standard deviations, unless otherwise stated. ANOVA for repeated measurements was used to assess interactions between groups and time. When a significant interaction was found, the effect between time and groups was assessed using separate paired and unpaired t-tests. The changes in biomarkers concentration between two time points, two and 12-hours, were computed as a difference, delta, for each biomarker such as AOPP, NPBI and isoprostanes in the two groups. Statistics were performed using SPSS for Windows, version 20, Chicago inc. Differences were considered significant if p was <0.05. Bonferroni correction was also applied.

Ethics approval

The Chair of the TO2RPIDO study data and safety monitoring committee advised that publication of these interim laboratory results would not compromise the validity of the main study. The primary outcome of the main study was mortality and the secondary outcomes were the detection of major disabilities at two years and the development of bronchopulmonary dysplasia at 36 weeks' of completed gestation.[9] Approval for this study was obtained from The Hunter New England Human Research Ethics Committee (09/03/18/5.07). Signed informed consent was obtained from parents/guardians at least six hours prior to delivery. Investigators involved in recruitment of patients were blinded to the laboratory and statistical analysis of the oxidative markers.

Results

Population demographics are detailed in Table 1. Two room air infants were switched over to 100% oxygen during resuscitation. SpO_2 levels at one and five minutes were significantly higher in 100% oxygen infants at one and five minutes-of-age (Figure 1). The partial pressure of oxygen in the blood was significantly higher in the 100% oxygen group on admission to the NICUs.

Oxidative markers results

All infants showed a trend towards a decrease in all oxidative stress biomarkers between zero and two hours but an increase by 12-hours (Figure 2). Significantly higher levels of AOPP were found at 12-hours in the 100% oxygen group when to those resuscitated with room air (Table 2 and Figure 2). The levels of isoprostanes and NPBI, did not differ between the two groups.

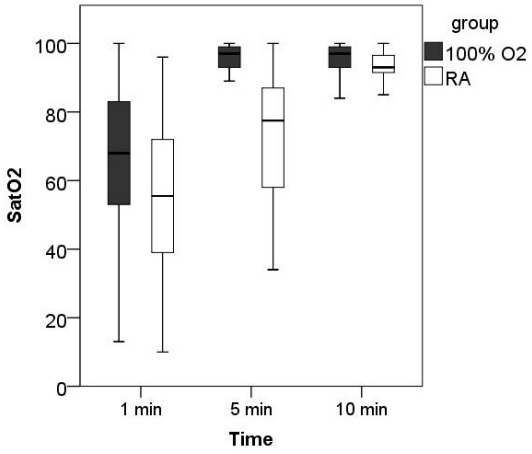
Table 1 | Clinical characteristics of the infants.

	100% oxygen N = 60			Room air N = 59			
	Mean ± SD	**Minimum**	**Maximum**	**Mean ± SD**	**Minimum**	**Maximum**	***P* value#**
Gestational age (weeks)	27.8 ±1.7	24	31	28.1 ±1.8	24	31	0.462
Birth weight (grams)	1070.6 ±339.7	260	1840	1066.8 ±378.4	140	1850	0.958
pH	7.27±0.10	7.00	7.50	7.25±0.18	6.80	7.90	0.707
PaO_2 (mmHg)*	52±28	12	129	34±13	6	67	0.011*
$PaCO_2$ (mmHg)	54±15	29	88	50±16	26	91	0.457
HCO_3^- (mEq/l)	23.8±3.7	17.0	35.0	21.6±4.8	6.0	28.0	0.099
BE (mol/l)	-2.1±6.4	-11.8	21.0	-5.8±5.6	-25.6	0.9	0.038*
Apgar score 1 min	6±2	1	9	6±2	2	9	0.503
Apgar score 5 min	8±1	4	10	8±1	4	10	0.101
Apgar score 10 min	9±1	7	10	9±1	6	10	0.235
	Median	IQR		Median	IQR		
SpO_2 1st min (%)	68	53-83		55	39-72		0.017*
SpO_2 5th min (%)	97	91-99		77	57-87		0.000*
SpO_2 10th min (%)	97	93-99		93	91-97		0.614

unpaired t-test between the two groups; * p value< 0.005. Abbreviations: SD: standard deviation, IQR: inter-quartile range, PaO_2: peripheral partial pressure of oxygen, $PaCO_2$: partial pressure of carbon dioxide, HCO_3^-: bicarbonate, BE: Base Excess; SpO_2: peripheral oxygen saturation. * first arterial blood gas on admission to the neonatal nursery

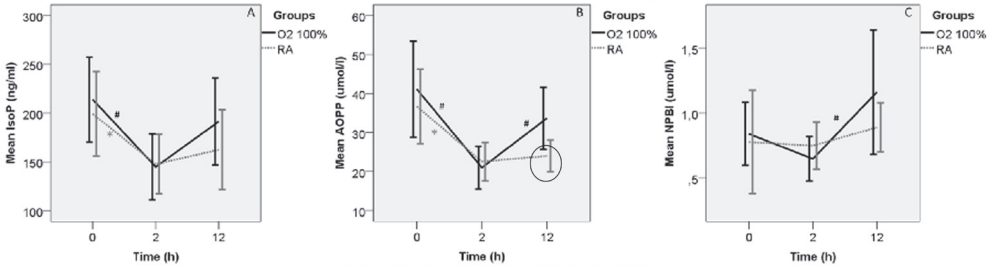
Differences in AOPP and isoprostanes levels between two and 12-hours were significantly higher in the 100% oxygen group when compared to the room air group (Table 3 and Figure 3). NPBI changes, however, were not different between the groups but showed a non-significant trend towards an increase in both groups.

Figure 1 | Sat O2 at 1, 5 and 10 minutes in the two groups.



SpO_2, peripheral oxygen saturation

Figure 2 | Lines graph showing the trend of oxidative stress biomarkers over time within each group.



Vertical lines represent means and 95% CI of each biomarker foreach time-point. * and # show p value, respectively, for RA and 100% oxygen group. (A) *RA group: IsoP (0–2 hours) p = 0.017; # 100% oxygen: IsoP (0–2 hours) p = 0.003. (B) *RA: AOPP (0–2 hours) p = 0.03; # 100% O2 group: AOPP (0–2 hours) p = 0.002 and # 100% O2 group: AOPP (2–12 hours) p = 0.001. (C) # 100% O2 group: NP BI (2–12 hours) p = 0.022. Significantly higher levels of AOPP were found at 12 hours in 100% O2 compared to RA group (p value = 0.027). CI, confidence interval; RA, room air; IsoP, isoprostanes; AOPP, advanced oxidative protein products; NPBI, non-protein-bound iron; O2, oxygen; h, hours

Table 2 | Biomarker results in 100% oxygen and room air groups.

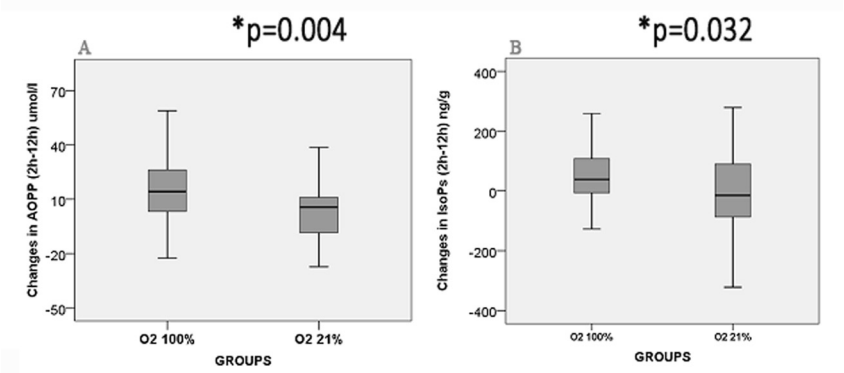
	100% oxygen (n = 60)			Room air (n= 59)			
	Median	**IQR lower**	**IQR upper**	**Median**	**IQR lower**	**IQR upper**	***P* value#**
Isoprostanes (ng/ml) time 0	172.50	115.27	272.75	193.55	106.77	263.50	0.630
Isoprostanes (ng/ml) time 2h	129.80	62.10	192.30	124.80	76.70	202.00	0.898
Isoprostanes (ng/ml) time 12h	178.10	93.27	238.52	128.60	66.35	263.00	0.336
AOPP (µmol/l) time 0	27.30	12.95	60.93	27.05	17.27	57.75	0.570
AOPP (µmol/l) time 2h	15.77	12.44	25.54	16.21	11.44	27.79	0.670
AOPP (µmol/l) time 12h	28.67	19.38	42.48	19.94	16.02	32.75	0.027*
NPBI (µmol/l) time 0	0.86	0.12	1.22	0.69	0.18	0.89	0.787
NPBI (µmol/l) time 2h	0.71	0.14	0.94	0.70	0.24	1.14	0.419
NPBI (µmol/l) time 12h	0.90	0.44	1.30	0.98	0.35	1.23	0.267

unpaired t-test between the two groups , * p value< 0.005. Abbreviations IQR: interquartile range, h: hours, AOPP: Advanced oxidative protein products, NPBI: Non protein bound iron

Discussion

Preterm newborns are particularly susceptible to oxidative stress. This is due in part to the rapid environment change from the womb that is poor in oxygen to a relatively rich oxygen habitat but also to the relative deficiency of antioxidant protection. The release of NPBI in response to hyperoxia, to hypoxic-ischaemic conditions and to inflammation, for example, is exaggerated. In our study a significantly higher level of biomarkers of protein injury was found at 12-hours in infants who were resuscitated with 100% oxygen compared to those with room air group but there were no differences in markers of lipid injury such as isoprostanes or levels of oxidative stress markers, as indicated by NPBI. AOPP is a reliable biomarker of protein damage secondary to oxidative stress as they are mainly formed by oxidation of sulphydryl groups.[17] Plasma proteins are critical targets for oxidants and the detection of AOPP in biologic fluid can be used to detect and to estimate the degree of oxidant-mediated protein damage. AOPP are the terminal products of protein exposure to free radicals and they do not have oxidant properties but may act as inflammatory mediators that trigger the oxidative burst of neutrophiles, monocytes and T-lymphocytes, thus leading to a phagocyte-derived oxidative stress.[17] Biochemical characterisation also reveals that AOPPs are carried by oxidised plasma proteins, especially albumin, both in its monomeric form or in aggregates.[17]

Figure 3 | Changes between 2 and 12 hours after birth in AOPP (A) and IsoP (B) in 100% oxygen and room air groups.



Abbreviations: h, hours; AOPP, advanced oxidative protein products; IsoP, isoprostanes; O2, oxygen.

Table 3 | Biomarker results in 100% oxygen and room air groups.

	100%			Room air			
	Mean (SD)	**Min**	**Max**	**Mean (SD)**	**Min**	**Max**	***P* value†**
Changes in IsoP (ng/g) (time 12–2 hours)	75.92 (138.02)	-127.20	437.70	-1.33 (146.53)	-322.00	279.00	0.032*
Changes in AOPP (lmol/L) (time 12–2 hours)	16.98 (23.80)	-22.50	92.42	1.49 (17.70)	-52.29	38.70	0.004*
Changes in NPBI (lmol/L) (time 12–2 hours)	0.69 (1.50)	-0.93	6.56	0.13 (0.74)	-1.16	2.01	0.059

SD, standard deviation; Min, minimum value; Max, maximum values, h, hours; O2, oxygen; IsoP, isoprostanes; AOPP, advanced oxidative protein products; NPBI, non-protein-bound iron. * *P* value < 0.005. † Unpaired t-test between the two groups.

Free radicals attack proteins, modifying proline and basic amino acid residues, generating many different products, such as carbonyl moieties, protein-protein cross-linkages, and oxidation of the protein backbone. This later results in protein fragmentation and loss of function.[17] One advantage of AOPP analysis is due to the relatively long half-life of these oxidised proteins and to their stability also *in vivo*. Higher AOPP concentrations in the 100% oxygen group may therefore reflect the central role of protein protection against oxidative injury.[21-24]

Protein peroxidation usually occurs earlier than lipid peroxidation and whether prolonging blood sampling beyond 12-hours would have elucidated changes in other biomarkers is uncertain.[17] There was, however, an ethical obligation to restrict blood collection to infants who required blood tests for clinical reasons. Few infants required routine blood tests after 12-hours-of-age in this population. In addition blood sampling was restricted due to the risk of perpetuating sampling aenemia. The exploration of other specimens such as urine, may provide a more easily accessible long-term method of assessing continuing oxidative stress.

Infants who were resuscitated with 100% oxygen had greater increases in all oxidative stress markers between two and 12-hours-of-age. This suggests that the use of 100% oxygen, even in a targeted approach, may cause lipid as well as protein damage. NPBI is a marker of pro-oxidant status as well as systemic hypoxia. It can indicate increased susceptibility to oxidative damage and is specifically applicable to *in vitro* and *in vivo* studies while isoprostanes are specific and reliable markers of lipid peroxidation, specifically derived from oxidation of arachidonic acid.[13,15] Isoprostanes are chemically stable *in vitro* and *in vivo*, thus they are considered good markers for the assessment of possible in situ oxidative injury.[21] Isoprostanes have vasoactive, inflammatory, and mitogenic properties[21,27,28] and may mediate and/or be directly responsible for a number of physiological and/or pathological processes. Isoprostanes levels are increased in pregnancies complicated by preeclampsia,[29] in the amniotic fluid of infants with fetal growth restriction,[30] in small for gestational age infants and in infants at risk of significant postnatal morbidities such as bronchopulmonary dysplasia, intracranial haemorrhage, necrotising enterocolitis, and retinopathy of prematurity. The lack of difference in isoprostanes and NPBI levels in our study, however, may have been due to the targeted approach where infants were provided with the amount of oxygen they required to achieve target SpO_2 levels. This is in contrast with previous studies, where infants were kept on set oxygen levels for a specified amount of time regardless of SpO_2 and that rapidly led to either hyper or hypoxia.[8,20]

The Benefits of Oxygen Saturation Targeting study II[25] and the Study to Understand Prognoses and Preferences for Outcomes and Risks of Treatments,[26] called respectively BOOST II and SUPPORT study, were able to demonstrate that targeting postnatal SpO_2 below 90% in infants below 28 weeks' of completed gestation resulted in an increased risk of death, a finding that was in contrast with previous expert opinion. This finding required the analysis of more than 3,000 patients, a significantly higher number than that currently used to guide international recommendations.[10,11] Pure oxygen had been freely used in delivery room resuscitation of newborn infants for more than two centuries[2,5] but recent evidence, primarily derived from studies on asphyxiated full-term infants, suggested that using less oxygen, such as room air, was a feasible alternative and that this practice might even decrease oxidative stress and improve short and long-term outcomes.[18,19] Even though there was, and there still remains, little evidence[6,7] about the clinical impact of this practice on the premature population, who often require some degree of oxygen supplementation due to pulmonary immaturity, international resuscitation guidelines were changed from as early as 2005 to recommend the use

of less oxygen for the resuscitation of all newborn infants.[10,11] Significantly more information regarding the impact of these relatively new recommendations on a high-risk and already vulnerable population are therefore urgently required. In this study, we mainly focused on oxidative stress markers demonstrating that using 100% oxygen in a targeted approach, targeting specific SpO_2 levels, increased AOPP levels at 12-hours-of-age and whether these changes were reflective of significant clinical outcomes could not be elucidated in the present report since the recruitment of patients of the TO2RPIDO trial was on-going when the laboratory analysis was conducted.

Conclusions

In conclusion, our study found that using room air to start preterm neonatal resuscitation resulted in lower AOPP levels at 12-hours compared to 100% oxygen and in lower changes of AOPP, NPBI and isoprostanes over time. Whether these findings were related to clinically important outcomes still needs to be elucidated in a study on a significantly larger population of infants. However, due to the widespread changes in practice that has the potential to affect more than 13 million newborn infants each year, comprehensive and well-designed studies in this question are urgently required.

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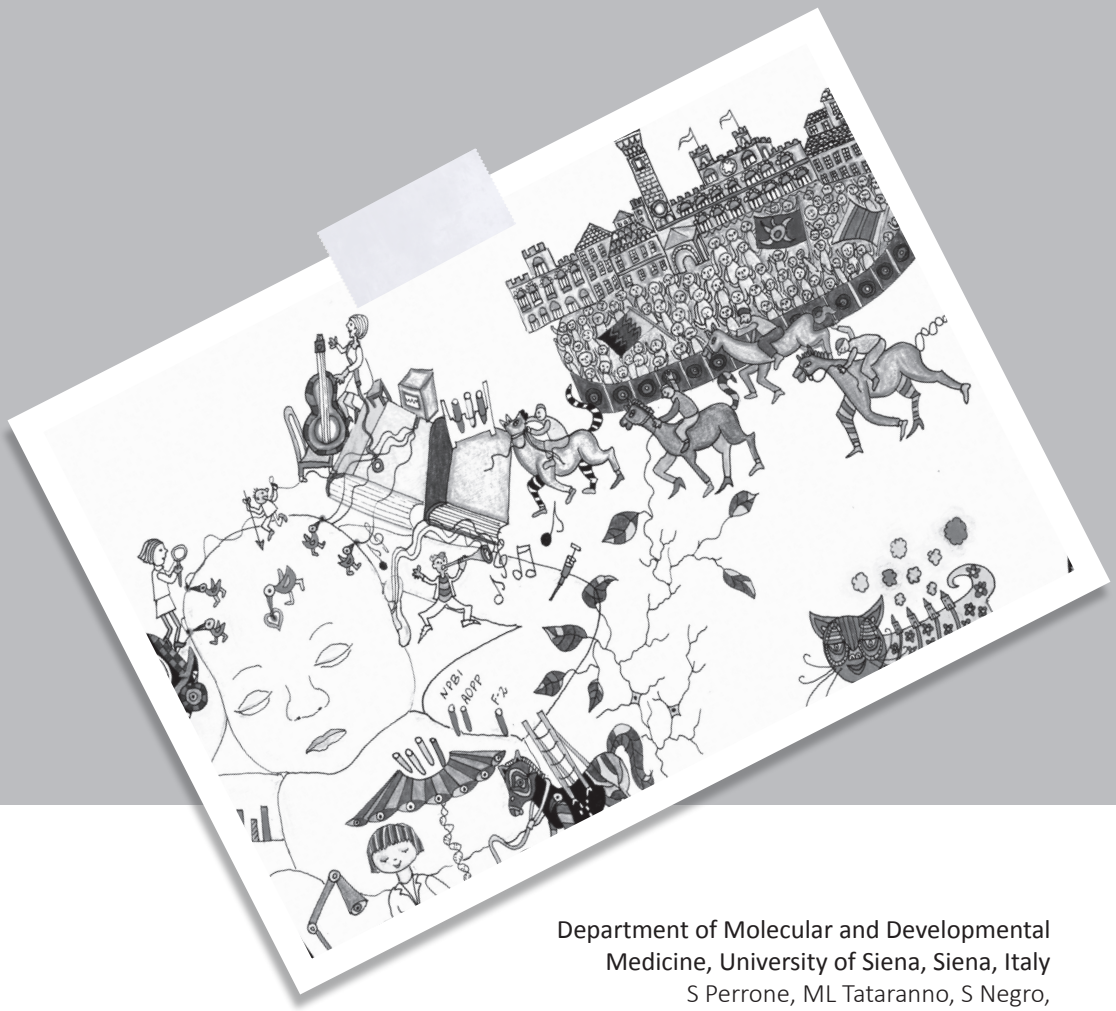

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Placental histological examination and the relationship with oxidative stress in preterm infants

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Background | Prenatal conditions of enhanced oxidative stress (OS) linked to inflammation or hypoxia have been associated with impaired fetal growth and preterm delivery. Little is known regarding biomarkers of OS in the cord blood of preterm infants and placental histological patterns.

Objectives | To test the hypothesis that placental lesions indicating chorioamnionitis (CA) or vascular underperfusion (VU) are associated with increased OS in the offspring.

Methods | 120 neonates born below 29^{+6} weeks of gestational age (GA) were enrolled. Histological characteristics of placentas from their mothers were classified as normal (CTRL group), histological CA (HCA) and vascular underperfusion (VU). Serum concentrations of isoprostanes (IsoPs), non-protein bound iron (NPBI) and advanced oxidative protein products (AOPP), were determined in cord blood.

Results | IsoPs, NPBI and AOPP were significantly increased in HCA group compared to CTRL group. The multivariable regression model, adjusted for GA, maternal age, parity, maternal diabetes, maternal obesity and presence/absence of fetal growth restriction (FGR), showed a significant association between the presence of HCA and increased OS biomarkers levels in cord blood (IsoPs: $p = 0.006$; NPBI: $p = 0.014$; AOPP: $p = 0.007$). Placental VU lesions were significantly associated with higher umbilical IsoPs, NPBI and AOPP levels (IsoPs: $p = 0.008$; NPBI: $p = 0.002$; AOPP: $p = 0.040$). In the cases of placental VU lesions associations were also found between high AOPP levels and low GA ($p = 0.002$) and the presence of fetal growth restriction ($p = 0.014$).

Conclusions | Placental lesions indicating inflammation or impaired perfusion are associated with higher cord blood levels of OS biomarkers explaining the fetal susceptibility to oxidative injury and the need of antioxidant protection.

Introduction

Placenta is a diary of intrauterine life. Many studies support the thesis that placental diseases such as infection or inflammation are responsible of preterm birth in up to 50% of cases,[1,2] even if only a partial correlation was found between clinical and placental finding and gestational age was the main determinant for neonatal outcome.[3] Particularly, prenatal conditions of enhanced oxidative stress (OS) linked to inflammation or hypoxia, have been associated with impaired fetal growth and preterm delivery. During hypoxia, several pathways involving intracellular calcium release and activation of nitric oxide synthase lead to increased free radicals (FR) generation.

During inflammation, the direct activation of inflammatory cells, especially granulocytes, releases a large amount of oxygen radicals and proteases, that contribute to killing bacteria but also enhance OS. OS-induced damage plays an important role in several pathological processes that are involved in fetal-neonatal pathology.

Histological chorioamnionitis (HCA) is responsible for approximately 60-70% of pregnancies complicated by preterm prelabor rupture of membranes (pPROM). In our previous studies we reported that OS was associated with pPROM through the presence of OS markers in the amniotic fluid samples.[4,5] Higher concentrations of OS markers in umbilical cord blood were also observed in pregnancies that are complicated by preeclampsia, fetal growth restriction (FGR), and subsequent development of necrotizing enterocolitis, maternal obesity and gestational diabetes.[4,6-8] Histological analysis of placenta is an useful tool for detecting the etiology and recurrent risk of pregnancy disorders. There is a paucity of information regarding the relationship between placental histological patterns and OS markers in cord blood of preterm infants. Moreover, it still remains unclear whether the presence of HCA or placental vascular underperfusion lesions, the most common risk factors for pPROM and preterm birth, are related to increased OS markers in the umbilical cord.

In this study we tested the hypothesis that placental lesions indicating HCA or vascular underperfusion (VU) are associated with increased levels of OS biomarkers in cord blood.

Material and methods

Samples collection and population

Between November 2012 and December 2014, a prospective cohort study of 120 preterm babies and respective placentas were recruited (for more clinical perinatal details see Tables 1 and 2) at the Department of Molecular and Developmental Medicine, University Hospital of Siena, Italy. The study protocol was approved by the local ethic board. Informed consent was obtained from the babies' parents before the enrollment.

Inclusion criteria were: GA < 32 weeks, inborn and single pregnancies. Exclusion criteria were: congenital malformations, inborn errors of metabolism, infants outborn, multiple pregnancies. The study was prospectively oriented. Babies were consecutively enrolled. Immediately after delivery, blood samples were obtained from the vein of a double-clamped umbilical cords using a tube containing butylated hydroxytoluene (BHT) to prevent any undesirable chain peroxidation. After blood centrifugation, the serum was aliquoted and stored at -80°C until the samples were assayed. After expulsion, the placentas were fixed in formalin. Tissue samples from the placenta, were processed and embedded in paraffin. Placenta samples were taken at specific loci: from a minimum of 2 sections of the umbilical cord, at both fetal and placental side, a membrane roll, from insertion of the umbilical cord and two slides of normal placentas, including decidua and chorionic plate, and additional slides if macroscopical abnormalities were present. Tissue sections were stained with hematoxylin and eosin for standard histological examination.

Histologic examination of fetal adnexa was performed by a single pathologist, who was blind to cord blood data and to the clinical status of the women and newborns. The population was divided in three groups according to placenta histological pattern: HCA, VU and control group.

Control group was defined as any preterm labours not associated with identified morphological alterations despite accurate placenta histological examination.

At birth, newborns weighing below the 10th percentile than expected for their gestational age were considered small for gestational age (SGA).[9]

Umbilical cord blood IsoPs, NPBI and AOPPP concentrations

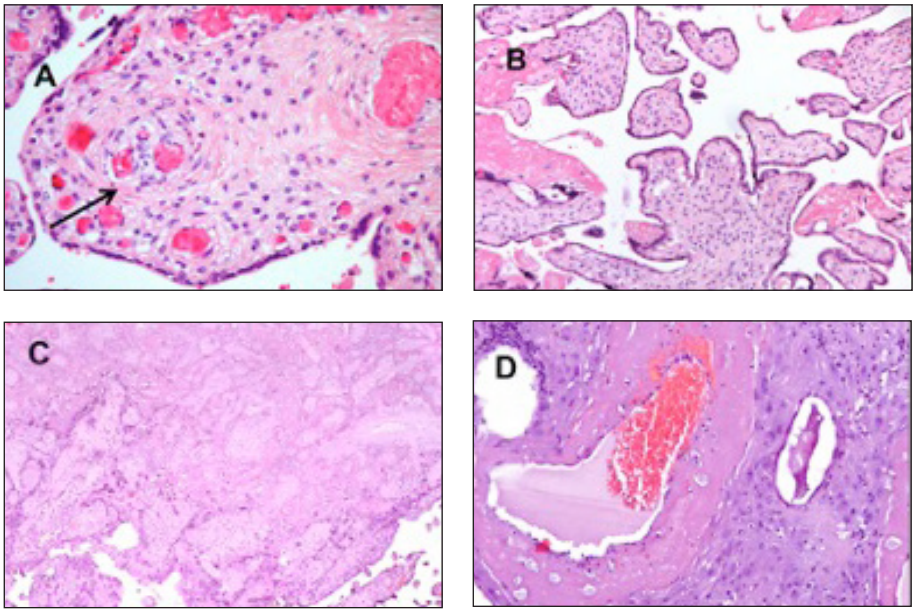
Concentrations of F2-Isoprostanes (IsoPs, pg/ml), Non-Protein Bound Iron (NPBI, mmol/l) and Advanced Oxidative Protein Products (AOPP, mmol/l), were determined in cord blood. IsoPs were measured with an LC-MS/MS based method previously reported by Casetta B. et al.[10] NPBI was measured by HPLC-DAD [11]. AOPP was measured by a colorimetric technique.[12] IsoPs are prostaglandin prostaglandin-like compounds which come from *in vivo* and *in vitro* peroxidation of arachidonic acid. Particularly F2-IsoPs are initially formed in phospholipids and then released into the blood. These prostanoids are less reactive and more stable than other peroxidation products such as aldehydes or peroxyl radicals so they can be easily found in plasma and urine. NPBI is the iron which is free of high affinity binding to transferrin. Asphyxia and acidosis can increase the release of NPBI predisposing the newborn to OS through the Fenton and Haber-Weiss reactions, leading to aggressive oxidants generation. Newborns are particularly susceptible to NPBI-induced oxidative damage. AOPP were chosen since proteins are critical targets for oxidants, the detection of AOPP can be useful to detect the degree of protein damage mediated by OS. AOPP are terminal products of protein exposure to FR but they do not have oxidant properties and are produced from the oxidation of proteins, especially albumin.

Histological placental patterns

Histologic examination of fetal adnexa included at least 3 full-thickness placental areas and sections of the umbilical cord at 3 different levels. A diagnosis of histologic chorioamnionitis (HCA) was made on hematoxylin and eosinestained sections in the presence of at least 10 polymorphonuclear leukocytes per field in 10 nonadjacent 400-power fields, as previously described.[13-15] The presence of even a few polymorphonuclear leukocytes within the umbilical cord vessels was equated with HCA. Vascular underperfusion lesions were defined following the standardized criteria described by Redline et al.[16,17]

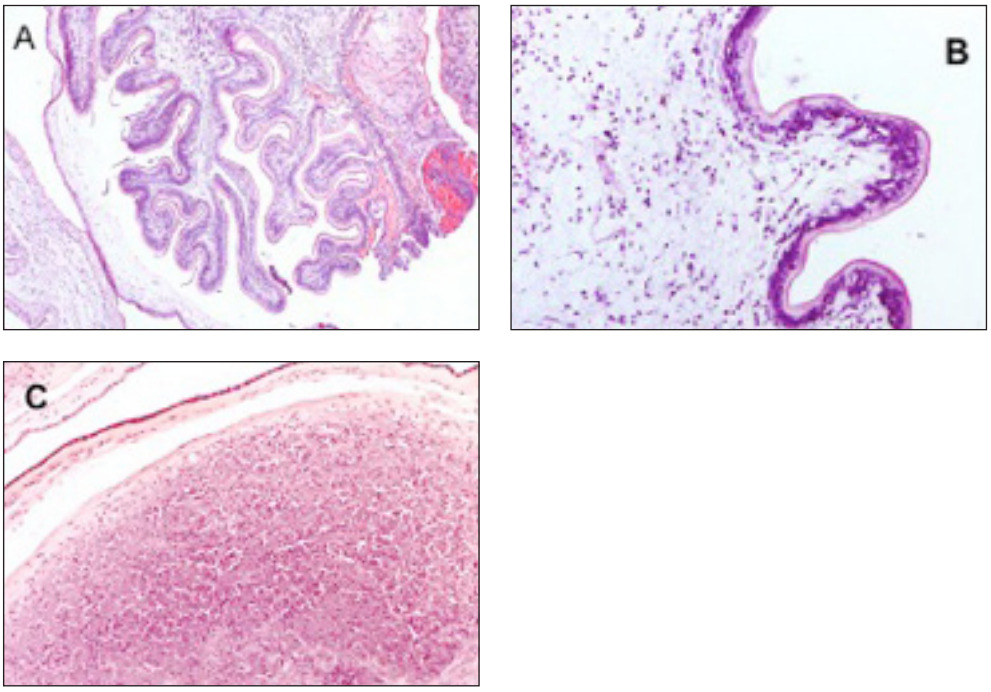
We classified 3 groups based on presence of vascular lesions or villous changes in relative absence of inflammation, (vascular underperfusion, VU group, n = 48; Figure 1), and evidence of infection and inflammation (histological chorioamnionitis, HCA group, n = 41; Figure 2), at histological analysis of placenta. Detailed histological findings of placental lesions associated to HCA or VU are reported in Table 3. A third group was used as control (CTRL group, n = 31), because no placental morphological alterations were identified despite accurately placenta histological examination.

Figure 1 | Different representative aspects of vascular underperfusion.



A, thrombosis and recanalization of a stem villous vessel, original magnification 20×; B, avascular villi, original magnification 10×; C, recent placenta infarct, original magnification 4×; D, acute atherosis of a decidual spiral artery in preeclampsia, original magnification 20×.

Figure 2 | Chorioamnionitis.



A: diffuse, severe inflammatory infiltrate in the amniotic membranes, original magnification 4×; B: higher magnification of A, original magnification 20×; C: the inflammatory infiltrate is confined to the chorion and maternal decidua, while the amnion is spared by granulocytes, original magnification 10×.

Statistical analysis

Data elaboration was carried out separately for each biomarker, using logarithmic (ln) transformation for variables which were not normally distributed. ANOVA univariate analysis with Bonferroni correction and a multivariate regression model were performed for the analysis of OS data. The analyses were adjusted for confounding factors. All p-values were from two-sided tests, and all statistical analyses were performed using SPSS 23.0 for Mac OS X (SPSS Inc., Chicago, IL, USA).

Results

Population

Clinical characteristics of mothers and newborns are shown respectively in Tables 1 and 2. Seven mothers in the CTRL group had gestational diabetes but only diet treatment was needed to control glycemia levels. Furthermore, in the CTRL group, nine mothers had cervical incontinence, three had uterine malformations, six presented p-PROM, ten showed low sociodemographic and psychological status. Two of the three mothers in CTRL group and two of the six mothers in the VU group with autoimmune diseases had autoimmune thyroiditis.

Table 1 | Baseline characteristics of mothers.

		CTRL (n = 31)	VU (n = 48)	HCA (n = 41)	*P* value
Maternal age (years) mean ± SD		33 ± 7	36 ± 6	32 ± 6	Ns
Parity median (IR)		1 (0;2)	1 (0;2)	1 (0;2)	Ns
Gestational Diabetes % (n)		22 (7)	6 (3)	2 (1)	Ns
Maternal Obesity % (n)		19.4 (6)	4.2 (2)	7.3 (3)	Ns
Type of conception % (n)					
	Spontaneous	61.3 (19)	35.4 (17)	68.3 (28)	0.007
	IVF	22.6 (7)	29.2 (14)	7.3 (3)	
Autoimmune diseases % (n)		9.7 (3)	12.5 (6)	0 (0)	0.041
Pre-eclampsia % (n)		6.5 (2)	10.4 (5)	0 (0)	Ns
pPROM % (n)		19.4 (6)	6.3 (3)	19.5 (8)	Ns
Positive vaginal swab % (n)		12.9 (4)	8.3 (4)	29.3 (12)	0.009
Stained AF % (n)		0 (0)	0 (0)	2.4 (1)	Ns
Positive placenta culture % (n)		0 (0)	0 (0)	14.6 (6)	0.001
Type of delivery % (n)					
	SVD	6.5 (2)	8.4 (4)	26.8 (11)	0.028
	CS	6.5 (2)	10.5 (5)	9.6 (4)	
	ECS	87 (27)	81.1 (39)	63.6 (26)	

Abbreviations: SD, standard deviation; CTRL, control group, VU, vascular underperfusion group; HCA, chorioamnionitis group; IR, interquartile range; IVF, in vitro fertilization; pPROM, preterm prelabour rupture of membranes; AF, amniotic fluid; SVD, standard vaginal delivery; CS, elective cesarean section (planned procedure before the onset of labour); ECS, emergency cesarean section (CS occurring outside of the planned procedures).

Table 2 | Baseline characteristics of neonatal population.

		CTRL (n = 31)	VU (n = 48)	HCA (n = 41)	*P* value
GA, mean ± SD (weeks)		29 ± 2	29 ± 3	27 ± 3	<0.0001
BW, mean ± SD (grams)		1203 ± 203	1159 ± 431	944 ± 331	<0.0001
Vein cord blood pH, mean ± SD		7.03 ± 0.18	7.00 ± 0.15	7.01 ± 0.15	NS
Vein cord blood BE, mean ± SD		6.1 ± 3.2	6.9 ± 5.8	7.2 ± 4.1	NS
Apgar score 1 min, median (IR)		7 (4;8)	7 (2;8)	4 (2;7)	NS
Apgar score 5 min, median (IR)		8 (7;9)	9 (7;9)	7 (6;9)	NS
Days of O_2 therapy, median (IR)		8 (5;21)	6 (3;50)	18 (3;51)	NS
SGA, % (n)		9.7 (3)	25 (12)	26.8 (11)	NS
NEC, % (n)		3.2 (1)	4.2 (2)	58.5 (24)	NS
BPD, % (n)		19.4 (6)	25 (12)	26.8 (11)	NS
PDA, % (n)		61.3 (19)	58.3 (28)	58.5 (24)	NS
Conservative treatment, % (n)		29 (9)	18.8 (9)	31.7 (13)	
Surgical treatment, % (n)		6.5 (2)	14.6 (7)	7.3 (3)	
ROP, % (n)	Grade I	0 (0)	4.2 (2)	9.8 (4)	NS
	Grade II	3.2 (1)	12.5 (6)	12.2 (5)	
IVH, % (n)	Grade I	12.9 (4)	6.3 (3)	9.8 (4)	0.0025
	Grade II	19.4 (6)	10.4 (5)	19.5 (8)	
	Grade III	6.5 (2)	14.6 (7)	34.1 (14)	
	Grade IV	0 (0)	2.1(1)	0 (0)	
Hydocephalus, % (n)		6.5 (2)	2.1 (1)	4.9 (2)	NS
PVL, % (n)		3.2 (1)	18.8 (9)	14.6 (6)	NS
Mortality, % (n)		3.2 (1)	6.3 (3)	26.8 (11)	0.002
Need of primary intubation, % (n)		64.5 (20)	47.9 (23)	78 (32)	0.011

Abbreviations: CTRL, control group; VU, vascular underperfusion group; HCA, chorioamnionitis group; GA, gestational age; SD, standard deviation, BW, birth weight; IR, interquartile range; BE, base excess; SGA, small for gestational age; NEC, necrotizing enterocolitis; BPD, bronchopulmonary dysplasia; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity; IVH, intraventricular hemorrhage; PVL, periventricular leukomalacia.

Table 3 | Histological findings of placental lesions associated to HCA or VU.

Histologic chorioamnionitis (HCA)	Vascular underperfusion (VU)
Acute subchorionitis/chorionitis	Villus changes
Acute chorioamnionitis	Villous infarcts
Necrotizing chorioamnionitis	Increased syncytial knots
Subchorionic microabscesses	Villous agglutination
Umbilical phlebitis/chorionic vasculitis	Increased intervillous fibrin
Umbilical arteritis	Decreased placental weight/increased fetoplacental weight ratio
	Distal villous hypoplasia
	Decidual hemorrhage in the relative absence of inflammation
Concentric umbilical perivasculitis	Vascular lesions
("necrotizing funisitis")	Persistent muscularization of basal plate arteries
	Acute atherosis of basal plate arteries and/or decidual arterioles
	Fetal thrombotic vasculopathy
	Thrombi, large fetal vessels
	Fibromuscular sclerosis
	Mural hypertrophy of decidual arterioles

Figure 3 | Cord blood levels of IsoPs in CTRL, HCA and VU groups.

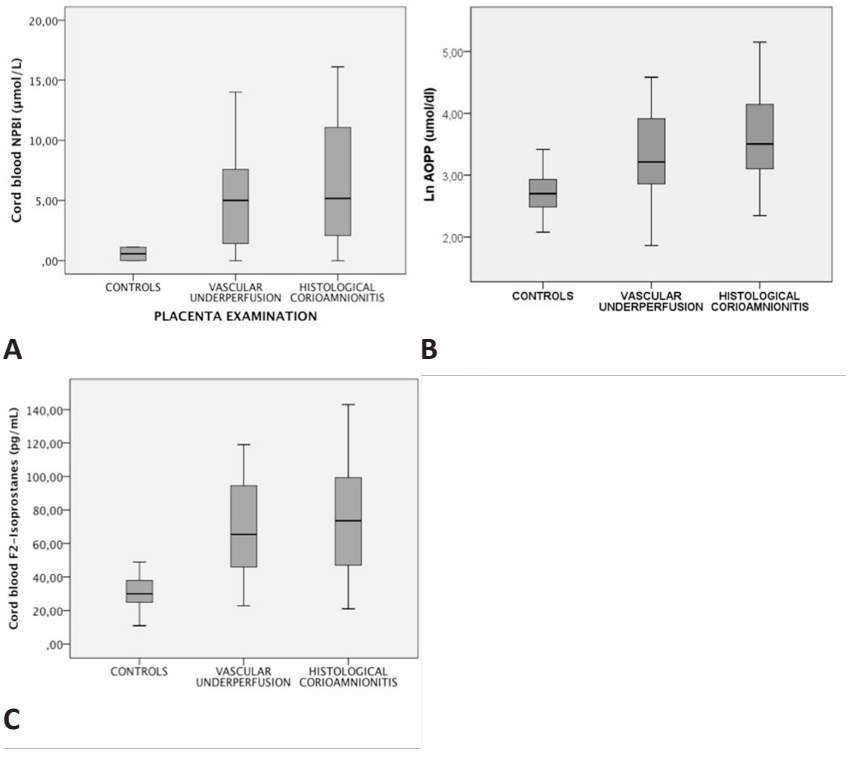


Figure 4 | Relationship between the presence of histological CA and OS biomarkers adjusted for GA, maternal age, parity, maternal diabetes, maternal obesity and FGR.

Dependent variable: NPBI	***p***	**B**	**95% CI**	
			lower	upper
HCA	0.014	6.72	1.46	11.99
Dependent variable: F2-IsoPs	***p***	**B**	**95% CI**	
			lower	upper
HCA	0.006	41.45	13.16	69.74
Dependent variable: AOPP (ln)	***p***	**B**	**95% CI**	
			lower	upper
HCA	0.002	1.05	0.43	1.67

Coefficient B with corresponding confidence interval (CI) is displayed when the factor was part of the remaining model (backward regression analysis), as also the p-value.

Finally, two mothers in VU group presented with systemic lupus erythematosus and two of them showed celiac disease. No signs of histological placental injury was observed in any cases of the CTRL group. Acute intrapartum events, such as cord prolapse, uterine rupture, sudden and sustained fetal bradycardia, shoulder dystocia or complicated breech extraction were not observed in the enrolled population.

Oxidative stress analysis

Higher IsoPs levels were found in HCA group and in VU group than CTRL group (respectively: p = 0.003, p = 0.022) NPBI and AOPP (ln) were significantly increased in HCA group compared to the CTRL group (NPBI: 7.34 ± 6.84 vs 0.94 ± 1.24, p = 0.007; AOPP ln: 3.64 ± 0.78 vs 2.71 ± 0.41, p = 0.007), Figure 3. The multivariable regression model, showed a significant association between the presence of HCA and increased level of OS biomarkers, Figure 4. The analysis was adjusted for GA, maternal age, parity, maternal diabetes, maternal obesity and presence/absence of FGR, Figure 5. The presence of VU placenta lesions was significant associated with higher IsoPs, NPBI and AOPP levels in cord blood. A significant association was also observed between high AOPP levels and low GA and between high AOPP levels and presence of FGR in placentas with VU lesions.

Figure 5 | Relationship between the presence of vascular underperfusion and OS biomarkers adjusted for GA, maternal age, parity, maternal diabetes, maternal obesity and FGR.

Dependent variable: NPBI	***p***	**B**	**95% CI**	
			lower	upper
VU	0.001	4.84	2.27	7.41
Dependent variable: F2-IsoPs	***p***	**B**	**95% CI**	
			lower	upper
VU	0.008	32.98	9.37	56.59
Dependent variable: AOPP (ln)	***p***	**B**	**95% CI**	
			lower	upper
VU	0.006	0.60	0.19	1.01
GA	0.004	-0.16	-0.26	-0.05
FGR	0.015	0.54	0.12	0.96

Coefficient B with corresponding confidence interval (CI) is displayed when the factor was part of the remaining model (backward regression analysis), as also the p-value.

Discussion

Placenta plays a key role in developmental plasticity. Changing developmental signals or placental adaptation occurred in response to an altered maternal environment may be the general underlying mechanisms that link altered placental function to fetal programming.[18] Fetal programming occurs when the normal pattern of fetal development is disrupted by an abnormal stimulus or insult applied to a critical point in intrauterine life and ultimately leads to chronic metabolic diseases. Pregnancies complicated by conditions such as hypoxia, infections and diabetes are associated with alterations in placental vasculogenesis, trophoblast expression of transporters and hormone production contributing in alteration of fetal development.[19] Chorioamnionitis is an acute inflammation of the membranes and chorion of the placenta usually associated with significant maternal and fetal adverse outcomes.[20,21] Moreover, intrauterine infection has been implicated in the pathogenesis of adverse neonatal sequelae, including long-term disabilities.[22,23]

The OS injury arises from the lack of a proper antioxidant system or when FR excessive production occurs in case of hypoxia, hyperoxia, ischemia or inflammation/infection.[24,25] There are evidences that OS is implicated in placenta development as well as in the pathophysiology of human pregnancy complications ranging from miscarriage to pre-eclampsia, intra- uterine growth restriction and pPROM.[4,5,26] Nevertheless, OS role in intra-amniotic infection has not yet been clarified. The total oxidative status assessed in vagina washing fluid has been found significantly higher in women with pPROM and HCA compared to patients with pPROM without HCA development.[27] Furthermore, an experimental study showed evidence of fetal responses to OS in systemic and alveolar compartments in fetal lambs exposed to intra-amniotic endotoxin in a CA model.[25]

In this study, three different oxidative stress markers, reflecting the potentiality of OS injury (NPBI), the intensity of lipid peroxidation (IsoPs) and the intensity of protein oxidation (AOPP), were used. We found that all of these markers were detectable in umbilical cord blood from pregnancies complicated by HCA or VU lesions. This finding is in concordance with our previous results in amniotic fluid[4] and with data obtained from cord blood of preterm newborns with clinical signs of perinatal hypoxia.[12,28] NPBI, IsoPs, AOPP concentrations may be influenced by acute intrapartum events that lead to hypoxia or ischemia. This role cannot be excluded in our newborns with acidosis despite cord prolapse, uterine rupture, sudden and sustained fetal bradycardia, shoulder dystocia or complicated breech extraction did not occurred in our population. Furthermore there were no statistical differences in cord blood pH and BE among the three groups. The association between cord blood OS and HCA and VU placental lesions supports the hypothesis that umbilical cord blood and amniotic fluid OS markers are useful as reliable markers of HCA and VU lesions in pregnancies complicated by preterm birth. We postulate that OS builds up during the course of pregnancy in response to various maternal stimuli becoming the common final end-point underlying condition of hypoxia/infectious complications and adverse intrauterine environment. Fetal exposure to enhanced OS during pregnancies may contribute to altered placental function, fetal growth structure and preterm delivery. In this contest, OS may be the general underlying mechanism that links adverse intrauterine environment with fetal programming. The next observation from this study is that umbilical cord blood IsoPs, NPBI and AOPP concentrations positively correlate with the HCA and VU lesions independently from gestational age at delivery. In the present study infants with histological signs of chorioamnionitis has a significantly lower GA compared to the other groups and some studied showed that the antioxidant enzyme defenses do not mature until very late in gestation,[29] thus we decided to adjust our model also for GA.

Our results strongly support the hypothesis that, instead of reflecting increasing pregnancy demands throughout advanced pregnancy, umbilical cord blood OS markers reflects cellular OS and damage related to placental lesions indicating inflammation or impaired perfusion.

The present study has some limitations. First, we had no data about Doppler ultrasound, thus we could not evaluate the association of Doppler ultrasound with specific types of placental lesion. Second, we could not detect biomarkers of OS in placenta or in amniotic fluid but only in cord blood, thus we had not faced with all the different etiologies of OS in cord blood. One of the strengths of this study is that we evaluated three umbilical cord blood markers that cover different aspects of oxidative stress in a very homogenous group of women suffering of preterm delivery. Second, there are no previous references in the literature describing the association between umbilical cord blood markers of OS with respect to histological patterns of placenta. Third we evaluated the concentrations of NPBI and products of OS-induced damage such as lipids and protein peroxidation products, which reliable indicate the extent of OS. Together these data suggest that hypoxia and inflammation may regulate placental development/function through OS, with plausible effects on fetal programming. Data also indicate increased neonatal susceptibility to oxidative injury mediated by placental HCA and VU, suggesting the need of antioxidant protection during pregnancies and immediately after birth.

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part two

COMBINING BIOMARKERS IN CLINICAL PRACTICE

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5

Early oxygen utilization and brain activity in preterm infants

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Background | The combined monitoring of oxygen supply and delivery using Near-InfraRed spectroscopy (NIRS) and cerebral activity using amplitude-integrated EEG (aEEG) could yield new insights into brain metabolism and detect potentially vulnerable conditions soon after birth. The relationship between NIRS and quantitative aEEG/EEG parameters has not yet been investigated.

Aim | Our aim was to study the association between oxygen utilization during the first 6 h after birth and simultaneously continuously monitored brain activity measured by aEEG/EEG.

Methods | Forty-four hemodynamically stable babies with a GA < 28 weeks, with good quality NIRS and aEEG/EEG data available and who did not receive morphine were included in the study. aEEG and NIRS monitoring started at NICU admission. The relation between regional cerebral oxygen saturation (rScO2) and cerebral fractional tissue oxygen extraction (cFTOE), and quantitative measurements of brain activity such as number of spontaneous activity transients (SAT) per minute (SAT rate), the interval in seconds (i.e. time) between SATs (ISI) and the minimum amplitude of the EEG in μV (min aEEG) were evaluated.

Results | rScO2 was negatively associated with SAT rate (β=-3.45 [CI=-5.76--1.15], p=0.004) and positively associated with ISI (β=1.45 [CI=0.44-2.45], p=0.006). cFTOE was positively associated with SAT rate (β=0.034 [CI=0.009-0.059], p=0.008) and negatively associated with ISI (β=-0.015 [CI=-0.026--0.004], p=0.007).

Conclusions | Oxygen delivery and utilization, as indicated by rScO2 and cFTOE, are directly related to functional brain activity, expressed by SAT rate and ISI during the first hours after birth, showing an increase in oxygen extraction in preterm infants with increased early electro-cerebral activity. NIRS monitored oxygenation may be a useful biomarker of brain vulnerability in high-risk infants.

Introduction

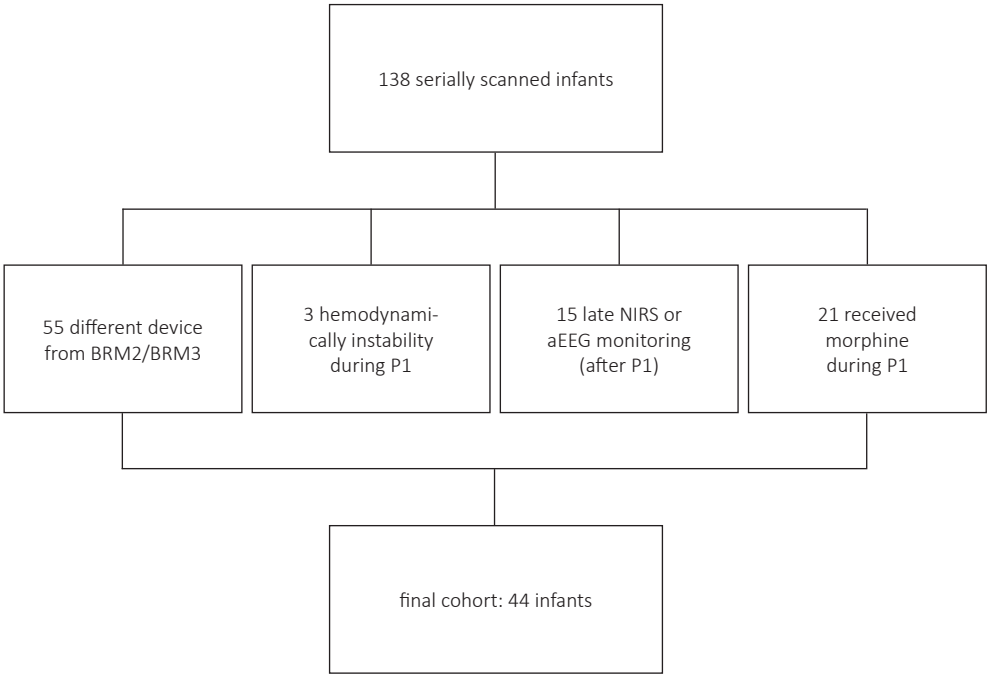
Neuro-monitoring tools such as Near-InfraRed Spectroscopy (NIRS) and amplitude integrated EEG (aEEG) are becoming part of daily clinical care in many neonatal intensive care units (NICUs).[1] NIRS utilizes infrared light to monitor regional cerebral oxygen saturation ($rScO_2$), in a mixed venous-capillary-arterial compartment.[2] NIRS can therefore be used to estimate cerebral oxygenation, and also as a surrogate for cerebral perfusion.[2] In addition, when combined with arterial oxygen saturation (SpO_2), the cerebral fractional tissue oxygen extraction (cFTOE) can be calculated ($[SpO_2\text{-}rScO_2]/SpO_2$), which can be used as an estimator of oxygen utilization of the brain.[2,3] On the other hand, aEEG is an excellent tool for continuous, non-invasive assessment of cerebral activity.[1] Routinely, aEEG tracings are classified based on the background pattern.[4] Recently, quantitative approaches for digital aEEG and raw EEG signals have been introduced.[5] These approaches classify spikes in cerebral activity as bursts or the equivalent spontaneous activity transients (SATs), which are likely to be crucial for brain development.[6,7] This classification enables the calculation of the intervals between bursts, called inter SATs intervals (ISI) and the SATs per minute (SAT rate).[5,8] Studies in humans have already shown that both the SAT rate and ISI, and quantitative aEEG parameters give valuable information about brain function of preterm infants during early phases of neonatal intensive care.[8-12] In addition, especially these aEEG variables have been shown to be associated with brain growth and development, and also with neurodevelopmental outcome.[5,8-10,13] Hence, the simultaneous assessment of NIRS and (a)EEG couples monitoring of oxygen supply and delivery to cerebral activity and could therefore yield new insights into brain metabolism and detect potentially vulnerable situations. In the past, studies on the combination of NIRS with aEEG/EEG monitoring, showed that a higher cFTOE was associated with a narrower aEEG bandwidth suggesting more oxygen utilization to meet higher metabolic demand in case of a more mature aEEG/EEG.[14] However, the relationship between NIRS and quantitative EEG parameters such as SAT rate or ISI has not yet been investigated. Therefore, the aim of the current study was to compare the pattern of oxygen delivery and utilization, as determined by NIRS, to the simultaneously acquired quantification of the cross-cerebral digital aEEG/EEG signal during the first 6 h after birth.

Material and methods

Patients

In this observational study, patients were selected from a larger longitudinal MRI study cohort of preterm infants with a GA < 28 weeks. MRI's have been performed as standard clinical care at the Wilhelmina Children's hospital between 2008 and 2013. Those babies did only receive serial MRI if they were clinically stable (n= 138; Figure 1).

Figure 1 | Flow chart of study population selection.



P1 indicates 0-6 h after birth (study period).

Since we were interested in evaluating the physiological correlation between NIRS and aEEG/EEG parameters, only babies with good quality NIRS and aEEG/EEG data available were included in the study (Figure 1). Permission from the medical ethical review committee of the University Medical Center Utrecht (MERC UMC Utrecht) for the MRI study was obtained. Patient data were anonymized prior to analysis. Since this was a retrospective study, using NIRS and aEEG/EEG monitoring as part of standard clinical care, no written consent or specific ethical approval was required. The MERC UMC Utrecht waived the need for parental consent for the use of medical data. Additional exclusion criteria were: chromosomal or congenital abnormalities, hemodynamic instability, and administration of morphine or other sedative drugs during the selected period.[15] Morphine administration before or during the study period was an exclusion criteria because it has been shown to cause suppression of the aEEG/EEG activity.[15,16] Likewise, infants who were hemodynamically instable such as infants with arterial blood pressure less than 10th percentile for birth weight or treated with inotropes during the study period were excluded, as inotropes can alter cerebral blood flow while presumably not affecting cerebral oxygen consumption.[17] Furthermore, neonates were excluded if they had

either incomplete NIRS or aEEG/EEG data (minimum 3 hours of monitoring) during the first 6 hours after birth: 44 infants were eligible for the analysis (Figure 1). In all enrolled neonates, NIRS and aEEG monitoring started within 3 hours after birth. One hour of registration of the NIRS and aEEG/EEG signal was chosen between 4-6h postpartum and subsequently the relationship between NIRS and aEEG/EEG was evaluated. A small window was chosen, since the first hours after birth brain perfusion and metabolism undergo large changes to adapt to extra-uterine life.[18] Germinal matrix-intraventricular hemorrhage (GMH-IVH) was diagnosed according to the classification of Papile et al.[19] The first cranial ultrasound was routinely performed at admission, within 6 hours after birth and serially repeated till term equivalent age.[20]

NIRS

A 2-wavelength (730 and 810 nm) near-infrared spectrometer (INVOS 4100-5100; Covidien, Mansfield, MA) was used. A transducer (small adult SomaSensor SAFB-SM; Covidien, Mansfield, MA) containing a light-emitting diode and 2 distant sensors (30 and 40mm) was positioned on the fronto-parietal side of the infant's head and fixated with an elastic bandage to prevent displacement.[2] $rScO_2$ was calculated from the differential signals obtained from these 2 sensors, expressed as the predominantly venous weighted percentage of oxygenated hemoglobin (oxygenated hemoglobin/total hemoglobin [oxygenated hemoglobin + deoxygenated hemoglobin]).[2,21] The $rScO_2$ provides an absolute measure, and has the advantage over measuring oxygenated and deoxygenated hemoglobin of being less sensitive to patient movements. The $rScO_2$ was recorded simultaneously with heart rate, arterial blood pressure, and arterial saturation on the right hand (SpO_2). SpO_2 was measured using Philips Intellivue MP70 patient monitor containing Nelcor technology. To investigate the balance between oxygen delivery and oxygen consumption, cFTOE was also calculated as $(SaO_2\text{-}rScO_2)/SaO_2$ using an algorithm in the program. An increase in this parameter reflects increased oxygen extraction by brain tissue, whereas a decrease suggests less utilization or increased delivery of oxygen.[3]

aEEG monitoring

Two-channel rawEEG and aEEG tracings were obtained simultaneously with the NIRS signal in all neonates using BrainZ cerebral function monitors (BRM2 or BRM3, Natus CA, Seattle, USA). Only the cross-sectional signal was used for analysis by subcutaneous needle electrodes in P3-P4 position with a central reference electrode to measure impedance. Needle electrodes were preferred, since they are more suitable for stable long-term recording. In addition, they usually have lower impedance compared to gel electrodes and less handling is required to maintain a good impedance following insertion of the needles. The P3-P4 cross-sectional signal was chosen since it has been proven to be a good predictor for neurodevelopmental outcome.[8]

Data analysis

In house developed software (SignalBase®; version: 7.8; University Medical Center Utrecht, Utrecht, The Netherlands) was used to perform the simultaneous post-processing of the NIRS and EEG data. In each patient a 1 hour epoch (P1) was manually selected between 4 and 6 hours after birth. The recorded aEEG/EEGs were assessed visually to identify marked artifacts, periods of high impedance, and events that were annotated by the nurses (e.g. care, blood sampling). Based on the aEEG, periods were chosen with a more continuous activity, including both active and quiet sleep, and free from suspected electrical discharges and artifacts. The NIRS data was evaluated in a similar way, resulting in the selection of 1h-epochs with "clean" NIRS and EEG/aEEG data. For the EEG/aEEG the following variables were calculated: number of SATs per minute (SAT rate) (rounded to the nearest whole number) also called "bursts",[6,22] the interval in seconds (i.e. time) between SATs (ISI) (also called "interburst intervals"),[6] both derived from the raw EEG, and the minimum amplitude of the aEEG signal in μV (min aEEG).

The quantification of SATs was done using a nonlinear energy operator (NLEO) contained in the SignalBase software.[5] EEG data were recorded at a sampling rate of 256 Hz. In the present cohort 7 patients were registered with BRM 2 and 37 with BRM 3 monitors. These devices had a filter setting of 2Hz and 0.5 Hz respectively. This difference implies a lower sensitivity of BRM2 monitors for low frequencies. To evaluate the possible bias of the different filter settings, 10 patients with BRM3 monitoring were randomly selected and re-sampled with a 2Hz filter. Results were then compared and no significant differences were found between aEEG/EEG variables during P1. The moments of intubation and surfactant administrations were avoided, since this was marked in the events.[16,23] For the cFTOE calculation the involved signals are the arterial oxygen saturation and the regional oxygen saturation (NIRS). Both signals are first smoothed. This is done by applying two sub sequential filters. For each signal: the first filter is an averaging filter with a Chunk width of 50 seconds and a sample rate equal to the sample rate of its source ('rectangular moving average') and the second filter has a Chunk time of 36 seconds. The sample rate of the second filter is equal to the sample rate of the resulting signal. This will result in a Low pass filter of-6dB at 0.01 Hz and <-30 dB for averaging >0.02 Hz. The smoothed signals are then re-sampled with a re-sampling time of 1 second.

Statistical analysis

Clinical data are summarized as mean ± standard deviations (SD), percentages and absolute frequencies where appropriate. The association between NIRS (i.e. rScO2 and cFTOE) and aEEG/EEG parameters (i.e. SAT rate, ISI, min aEEG) was first visualized in dot plots. Correlations were checked using the Spearman correlation test (2-tailed). Afterwards a multivariable linear regression analysis was performed, adjusting for GA, arterial pCO2 and hemoglobin. A p value <.05 was considered statistically significant.

Table 1 | Baseline clinical characteristics of studied infants.

Clinical characteristics of the population (n=44)		
GA, mean (SD) wks		26.4 (1.0)
BW, mean (SD) g		892 (155)
Gender		
	Male , n (%)	21 (47.7)
	Female, n (%)	23 (52.3)
Apgar score		
	1 min, median (IR)	5 (3-6)
	5 min, median (IR)	8(7-8)
	10 min, median (IR)	8 (8-9)
GMH-IVH at any point in time		
	None, n (%)	37 (84.1)
	Grade I-II, n (%)	3 (6.8)
	Grade III, n (%)	4 (9.1)
GMH-IVH during the first day of life		
	None, n (%)	42 (95.5)
	Grade I-II, n (%)	0 (0)
	Grade III, n (%)	2 (4.5)
Initial respiratory support		
	SIMV or HVO, n (%)	23 (52.3)
	CPAP, n (%)	21 (47.7)
Surfactant at any point in time, n (%)		30 (68.2)
Blood pressure		
	Systolic, mean (SD) mmHg	41 (6)
	Diastolic, mean (SD) mmHg	25 (6)
	Mean blood pressure, mean (SD) mmHg	30 (2)
Aterial blood gas analysis in P1		
	pH, mean (SD)	7.31 (0.05)
	pCO_2, mean (SD) mmHg	43.8 (6.6)
	Hb, mean (SD) mmol/l	9.5 (1.3)

SD, standard deviation; IR, interquartile range; GA, gestational age; BW, birth weight; GMH-IVH, germinal matrix hemorrhage-intraventricular hemorrhage (according to Papile et al.[19]; SIMV, synchronized intermittent mandatory ventilation; HFO, high frequency oscillatory ventilation; CPAP, continuous positive airway pressure; all values are referred to P1: 0-6h after birth unless otherwise stated.

Results

Population

The selected epochs had a mean duration of (mean ±SD) 50 ± 8 min, this was manually selected between 4 and 6 h after birth and the $rScO_2$, cFTOE and aEEG/EEG variables were computed. Simultaneously measured arterial blood pressure was always within normal values (mean blood pressure during the selected period: 32 ± 6 mm Hg). Data about arterial hemoglobin and pCO_2 during the study period were also collected. None of the infants had hemoglobin values lower that 7 mmol/l (maximum value 12.5 mmol/l) during the study period (Table 1). Minimum and maximum value of pCO_2 were respectively 30 and 60 mmHg. Seven infants developed GMH-IVH at any point in time, but only two of the seven infants showed a GMH-IVH during the first ultrasound examination (within 6 hours after birth), showing a grade III (Table 1). None of the patients was reported to have an abnormal resistance index at cerebral ultrasound. None of the babies experienced arterial oxygen saturation values lower than 88% or seizures during the selected period. None of the patients were small for GA or was intrauterine growth restricted (p<3).

The relationship between NIRS monitored $rScO_2$ and cFTOE and aEEG/EEG measurements

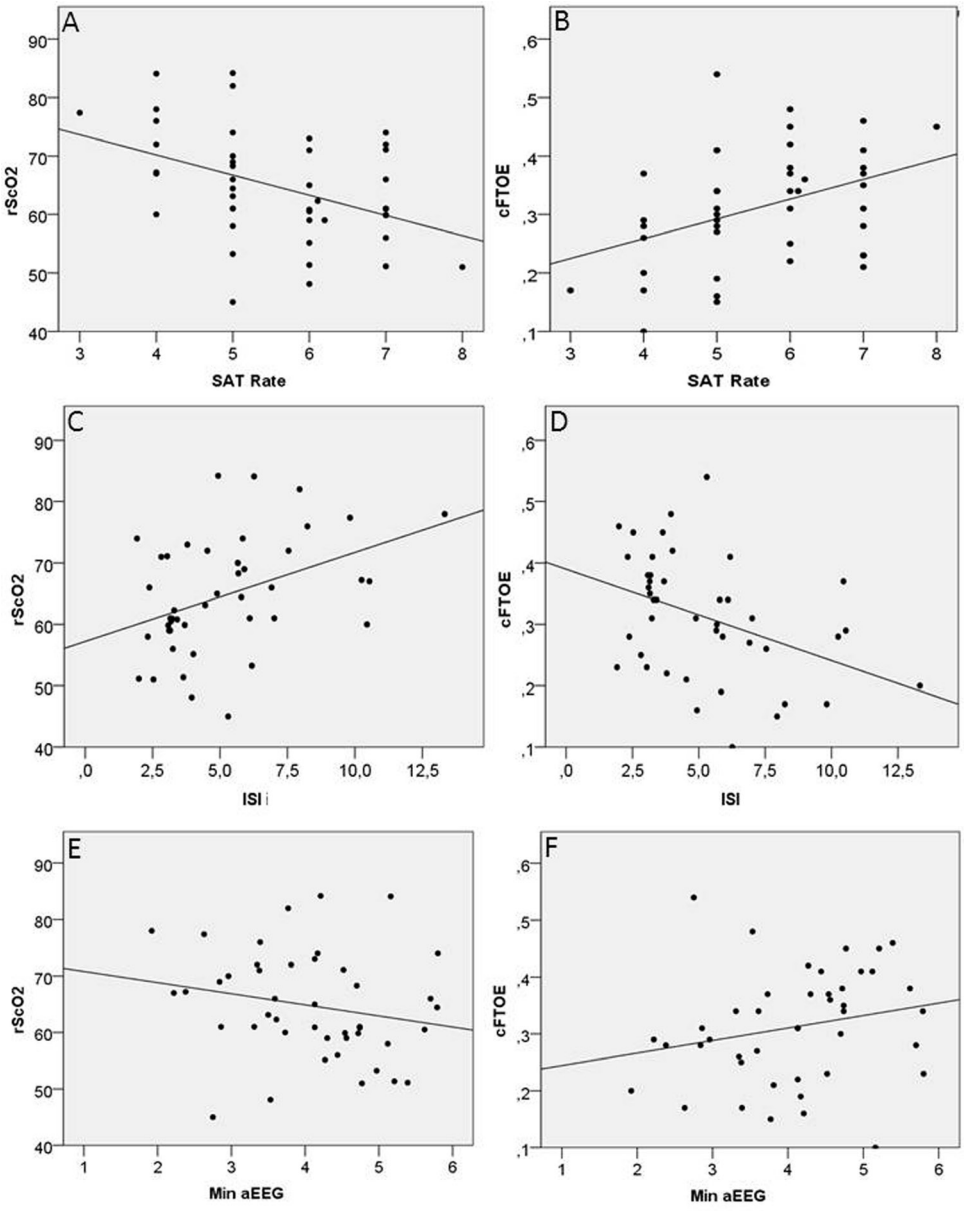
Brain activity significantly changed with GA, with higher SAT rate in infants with a higher GA (Table 2). The rScO2 did not change with GA and was negatively associated with SAT rate and min aEEG (p<0.01 and p<0.05 respectively) and positively with ISI (p<0.01) (Figure 2 panels A, E, and C respectively). cFTOE showed a significant positive association with SAT rate (p<0.01) and min aEEG (p<0.05) and a negative association with ISI (respectively: p<0.01; Figure 2 panels B, F and D resp).

Table 2 | Mean NIRS and aEEG/EEG values during the study period.

NIRS and aEEG/EEG variables	Total population
$rScO_2$, mean (SD) %	65 (9)
cFTOE, mean (SD)	0.31 (0.09)
SAT rate, mean (SD) min	5.5 (1.1)*
ISI, mean (SD) sec	5.1 (2.6)
Min aEEG, mean (SD) μV	4 (1)

*SAT rate was significantly related to GA (r=0.31, p <.05 in relation to GA)

Figure 2 | Scatter-plots showing the relation between NIRS and aEEG/EEG variables during the study period (P1).



In particular $rScO_2$ was significantly associated to SATrate (A) ($p<0.01$), ISI (C) ($p<0.01$) and min aEEG (E) ($p<0.05$) and cFTOE to SAT rate (B) ($p<0.01$), ISI (D) ($p<0.01$) and min aEEG (F) ($p<0.05$) in P1. P1 indicates 0-6 h after birth (study period).

Table 3 | Multivariable linear regression analysis.

	$rScO_2$	cFTOE	GA	pCO_2	Hb
	B [CI]	B [CI]	B [CI]	B [CI]	B [CI]
SAT rate	-3.45* [-5.76;-1.15]	0.034* [0.009;0.059]	88 [-1.90;3.67]	-0. 24 [-0.635;1.153]	1. 34 [-0.647;3.331]
ISI	1.71* [0.70;2.72]	-0.018* [-0.028;-0.007]	-15 [-2.91;2.59]	-0. 39 [-0.802;0.005]	1. 49 [-0.429;3,421]
Min aEEG	-1. 97 [-4.85;0.91]	0. 02 [0.008;0.052]	-17 [-3.15;2.80]	0. 28 [-0.724;0.149]	1. 42 [-0.834;3.679]

*p<0.01 adjusted for GA, pCO_2, hemoglobin (Hb); CI, 95% confidence interval.

In the multivariable analysis, correcting for GA, arterial pCO_2 and hemoglobin the $rScO_2$ and cFTOE were independently related to SAT rate and ISI. In particular $rScO_2$ was negatively associated with SAT rate (β=-3.45 [CI=-5.76--1.15], p=0.004) and positively related to ISI (β=1.45 [CI=0.44-2.45], p=0.006). In addition cFTOE was found to be positively associated with SAT rate (β=0.034 [CI=0.009-0.059], p=0.008) and negatively associated with ISI (β=-0.015 [CI=-0.026--0.004], p=0.007) (Table 3). When repeating the analysis excluding the 7 infants who showed a GMH-IVH at any point in time (excluding also the two babies who showed a GMH-IVH within 6 hours after birth) the results did not change. Hemoglobin and pCO_2 were not significantly associated to NIRS variables.

Discussion

Our study suggests a higher metabolism, as indicated by lower $rScO_2$ and increased cFTOE, during increased brain activity, as indicated by SAT rate independently of GA, hemoglobin and pCO_2 values. Consistently with these findings, O_2 delivery ($rScO_2$) was higher and O_2 extraction (cFTOE) was lower during decreased brain activity (ISI). These results are in agreement with previous studies showing an increased cFTOE with more mature electro-cortical activity.[14] However, our results extend previous findings because we used a new objective quantitative digital measurement of early brain activity based on automatic detection of SAT rate and ISI, overcoming all the subjective aEEG/EEG measurements.[5] To our knowledge, this is the first study focusing on the relation between NIRS and quantitative digital aEEG measurements in the first 6 hours after birth. During this transitional period, brain perfusion and metabolism undergo large changes to adapt to extra-uterine life.[18] Ter Horst and colleagues speculated that the higher cFTOE reflects higher cerebral oxygen extraction and consumption.[14] However, the increase in cFTOE can also be related to impaired cerebral blood flow

(CBF) and decreased oxygen delivery but in the latter case we would have likely observed suppression of brain electrical activity.

In contrast to our findings, ter Horst and colleagues did not find any relationship between aEEG/EEG and $rScO_2$. We speculate that the negative association between $rScO_2$ and increased brain activity (SAT rate) was due to a higher oxygen use caused by an increased metabolism demonstrated also by the increased cFTOE and the related higher electro-cerebral activity. Yoxall and colleagues reported that increased metabolism is accompanied by an increase in cerebral oxygen consumption and consequently by an increase in CBF as part of the so called neurovascular coupling.[24] Recently, a significant association between superior vena cava flow and aEEG at 12 h after birth was reported. Interestingly, infants with low superior vena cava flow had significantly lower min aEEG at 12 h as compared with those with normal flow.[25] Thus, hemodynamic changes and especially changes in CBF that occur immediately after birth may affect cerebral circulation and also neuronal activity as shown in our results.

Kissack and colleagues demonstrated that hemodynamic responses to neuronal activation are not fully developed in the neonatal brain, compared with the adult brain. In extremely preterm infants there is no correlation between CBF and spontaneous changes in the cerebral metabolic rate of oxygen during the first 48 h after birth; instead, cFTOE changes rather than CBF to meet changes in oxygen requirement.[26,27] On the other hand, some old studies demonstrated that basically the decrease in $rScO_2$ and the related increase in O_2 extraction (cFTOE) means a higher metabolism, which is reported to happen with increased fetal age, straight forward physiology.[28] Another study from Arichi T et al. comparing the BOLD signal response in preterm infants, term infants, and healthy adults showed decreased response time and increased signal amplitude with increasing postnatal age, suggesting that in young infants the increase in cerebral oxygen consumption may be relatively greater than the corresponding increase in CBF during functional activation.[29]

We did not find any association between pCO_2 or hemoglobin and NIRS variables. This suggests that within normal ranges, they do not highly influence oxygen delivery and extraction in preterm infants.

We found that SAT rate increased with increasing GA already within a few hours of extrauterine life followed by a simultaneous increase in FTOE and a decrease in $rScO_2$. Previous studies, mainly focusing on weekly aEEG recordings, have already shown the maturational effect of GA on brain activity.[30-32]

An example of how this combined monitoring of NIRS and aEEG/EEG can be a useful indication for clinicians to focus on preserving oxygen supply is represented by GMH-IVH in preterm infants. In the presence of a GMH-IVH the background activity of the EEG/aEEG is depressed during the first days after birth, and the extent of the depression correlates with the degree of GMH-IVH.[33,34] Recently Alderliesten et al demonstrated that higher $rScO_2$ and lower cFTOE

values were observed before brain injury became apparent and these changes were highly indicative for subsequent development of a severe GMH-IVH.[35,36] Thus, the combined monitoring of cerebral oxygen delivery/utilization and brain activity can be useful for early identification of infants at risk of developing a GMH-IVH and these changes in continuous monitoring are seen before the injury became visible on ultrasound examination.[35,37] In our study only 7 babies developed a GMH-IVH and none of them showed parenchymal involvement, thus we could not perform a separate analysis for this group of patients, but the relation between NIRS and aEEG was still present also after excluding those patients.

A possible limitation of the study is that NIRS signal was recorded in the fronto-parietal area while aEEG was recorded between P3-P4 electrodes. These areas are separated by 2 to 3 centimeters. The aim of the current study was to compare a measure of global brain activity to a measure of global brain perfusion/oxygenation. The P3-P4 (a)EEG electrodes positions are known to be representative for measuring global brain activity and no major differences were found between seizures detection with cross-sectional P3-P4 electrodes and two-channel aEEG.[38,39] Furthermore frontal-parietal NIRS has been shown to correlate with measures of global cerebral perfusion.[40] Moreover, indices of cerebral oxygenation as measured by NIRS in preterm infants are quite comparable between regions.[41]

Furthermore NIRS is known to be influenced by different compartments (arterial, capillary, venous), which can be affected by declivity. In our NICU incubators are set at similar declivity for every patient. Regarding head position in particular, the NIRS sensor was always placed on the front parietal side on which the infant was not lying at the moment. So the placement in terms declivity/head position has been very uniform among the infants in this study. Furthermore in 2010 Ancora and colleagues showed that hemodynamic changes after posture variations depend on GA and no statistically significant differences were found in CFTOE and $rScO_2$ in hemodinamically stable extremely preterm newborns with different postures.[42]

Finally we found that oxygen consumption increases considerably with increasing activity anyway we would not suggest to sedate those babies more, since that would decrease brain activity, which is essential for brain development.[43]

Conclusions

In conclusion, our study shows how oxygen utilization, as indicated by rScO2 and cFTOE, is directly related to parameters quantifying brain activity, as indicated by SAT rate and ISI in the immediate neonatal period. This combination of NIRS and aEEG simultaneous monitoring and consequently of rScO2/cFTOE and electrocerebral activity may be a noninvasive useful biomarker of brain function in high-risk, hemodynamically stable infants and could therefore yield insight in brain metabolism and detect potentially vulnerable conditions.

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6

Changes in brain morphology and microstructure in relation to early brain activity in extremely preterm infants

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Objective | to investigate the relation of early brain activity with structural (growth of the cortex and cerebellum) and white matter microstructural brain development.

Methods | Thirty-three preterm neonates (gestational age 26±1 weeks) without major brain abnormalities were continuously monitored with electroencephalography (EEG) during the first 48h of life. Rate of spontaneous activity transients per minute (SAT rate) and interSAT interval (ISI) in seconds per minute were calculated. Infants underwent brain MRI around 30 (mean 30.5, min:29.3-max:32.0) and 40 (41.1; 40.0-41.8) weeks postmenstrual age. Increase in cerebellar volume, cortical gray matter volume, gyrification index, fractional anisotropy (FA) of posterior limb of the internal capsule (PLIC) and corpus callosum (CC) were measured.

Results | SAT rate was positively associated with cerebellar growth ($p = 0.01$), with volumetric growth of the cortex ($p= 0.027$), increase in gyrification ($p= 0.043$) and increase in FA of the CC ($p= 0.037$). ISI was negatively associated with cerebellar growth ($p= 0.002$).

Conclusions | Increased early brain activity is associated with cerebellar and cortical growth, structures with rapid development during preterm life. Higher brain activity is related to FA microstructural changes in the CC, a region responsible for inter-hemispheric connections. This study underlines the importance of brain activity for (micro)structural brain development.

Introduction

The preterm brain is highly vulnerable in the first postnatal weeks of life, and maintaining structural and functional brain integrity during this critical period is one of the main challenges in neonatal healthcare. The assessment of early brain activity is an important biomarker of functional brain development in preterm neonates.[1,2] In early life, myelination of the thalamo-cortical axons is guided by spontaneous electrical signals, and electroencephalography (EEG) is a useful tool for the assessment of the functional status of these connections. The filtered and time compressed amplitude-integrated EEG (aEEG) is a bedside tool available for continuous monitoring of brain function in the neonatal unit. Increased brain activity on aEEG is recognized as bursts of high voltage with rapid oscillations, interrupting a period of inactivity.[3,4] These high voltage bursts are known as spontaneous activity transients (SAT) and are thought to be crucial for the establishment of brain networks.[1] SATs have unique and specific characteristics and can be used as representation of activity in different brain areas and they are necessary for a normal brain. In somatosensory regions SATs are associated with limb movements in mammalian fetuses and infants.[5] In the primary visual cortex, SATs are the expression of spontaneous retinal waves and are abolished by eye removal.[6] In humans, visual SATs abruptly disappear around 35 weeks gestation.[7] A previous study in our center showed that increased electrical brain activity, or less cortical electrical quiescence, in the first postnatal days, correlated with faster volumetric growth of different brain structures up to term equivalent age.[8] The hypothesized correlation of brain functional and structural maturation creates an early potential interesting predictor of altered brain development.

However, the effect of early brain activity on microstructural brain development has not been established. Fractional anisotropy (FA) is related to fiber coherence, axonal density and degree of white matter organization. FA of the white matter typically increases with brain maturation and correlates with long-term neurodevelopment.[9,10]

White matter, cerebral cortex and cerebellum are the largest and most vulnerable brain structures in extremely preterm neonates. Furthermore, a developmental relation is suggested between the cerebral cortex and the white matter,[11] as well as between the cerebral cortex and the cerebellum.[12] The aim of this study was to examine structural development of the cerebral cortex and cerebellum of the preterm brain, as well as the microstructural development of white matter, in relation to early brain activity.

Material and methods

Study population

All neonates with a gestational age (GA) at birth below 28 weeks, born between May 2008 and March 2013, and admitted to the Neonatal Intensive Care Unit of the Wilhelmina Children Hospital (Utrecht, The Netherlands), received according to clinical protocol aEEG monitoring during the first three days of life, and MRI of the brain around 30 weeks postmenstrual age (PMA) and again around term equivalent age (TEA). Infants with congenital malformations, genetic disorders or metabolic diseases were excluded. Infants with severe brain injury (n=20) were also excluded, as severe brain injury is known to affect the reliability of MRI analysis. Morphine administration is also known to interfere with early brain activity.[13] Assessing this effect was beyond the scope of this study, and therefore neonates receiving morphine during aEEG registration were excluded as well (n=10). The total number of in- and exclusions can be found in Supplemental Figure S1. The medical ethical review committee gave permission for use of the clinical data for research purposes.

Clinical parameters

Clinical parameters were obtained by chart review. Birth weight (BW) z-scores were computed using the Dutch population reference data.[14] Bronchopulmonary dysplasia (BPD) was considered present in case of oxygen dependency at 36 weeks PMA. Hypoglycemia was defined as a whole-blood glucose level <2,5 mmol/L. Hypotension was defined as mean arterial blood pressure (mmHg) of less than the GA (in weeks) at that time. Arterial blood pressure was measured using an indwelling arterial catheter. Severe brain injury (an exclusion criterion) was defined as the presence of an intraventricular hemorrhage (IVH) grade III or IV (according to Papile et al.),[15] post-hemorrhagic ventricular dilatation (ventricular index >97th percentile),[16] periventricular leukomalacia grade II or III,[17] or a large cerebellar hemorrhage (>3mm). White matter injury (WMI) was assessed by two neonatologists (L.d.V. and M.B.) using the Kidokoro scoring system.[18]

aEEG acquisition

Bedside aEEG at a sampling rate of 256 Hz was started as soon as possible after birth and continued for at least 48 hours. Subcutaneous needle electrodes were used with a central reference electrode measuring the impedance. The P3-P4 cross-sectional signal was chosen, and is known to be predictive of neurodevelopment in preterm infants.[19] Due to technical reasons (impedance, inhomogeneity of data, differences in filters) only infants monitored with BrainZ monitors (BRM2/BRM3, BrainZ, Natus, Seattle) were enrolled (Supplemental Figure S1).

EEG post-registration analysis

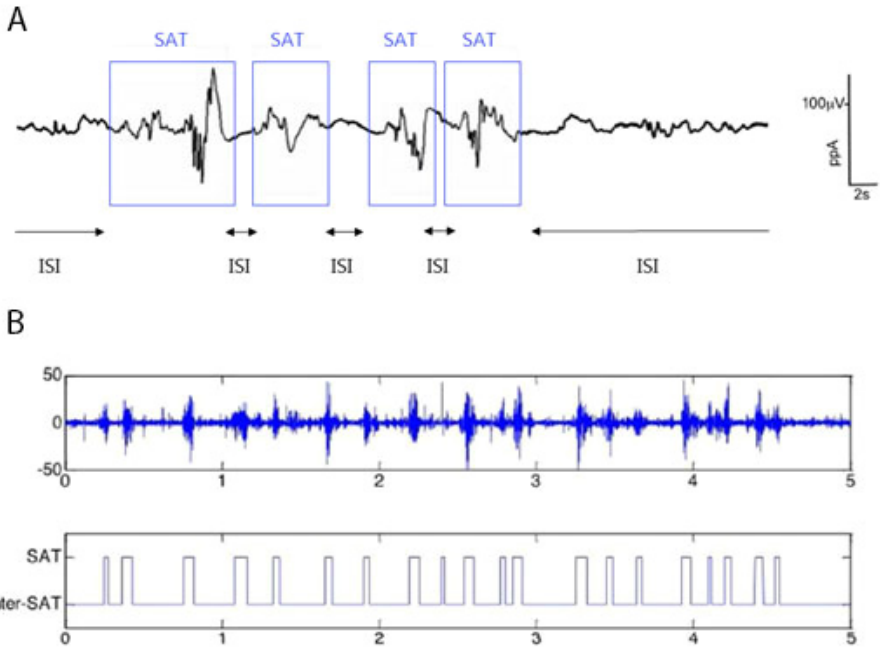
In house developed software (SignalBase® v7.8, University Medical Center Utrecht, The Netherlands) was used to process the raw EEG data. In all patients 3 epochs of 1 hour were manually selected at the aEEG at three specific postnatal time-points: 20-24h (T1), 32-36h (T2), and 44-48h (T3). The aEEG records were visually assessed to identify artifacts, caretaking events and periods with high impedance, in order to select the best hour per epoch.

Quantitative analysis with the same software program obtained the number of SATs per minute (SAT rate) (rounded to whole number) and the inter SAT interval (ISI, i.e. time between SAT) in seconds per minute, both derived from the raw EEG. SAT and ISI, so-called event based EEG measures, are explained in Figure 1 (A and B). The quantification of SAT and ISI was done using a nonlinear energy operator (NLEO) (http://iopscience.iop.org/0967-3334/31/11/N02). In the present cohort, 7 patients were registered with BRM 2 and 26 patients with BRM 3 monitors. These devices had a filter setting of 2 Hz and 0.5 Hz respectively, which implied a lower sensitivity of BRM2 monitors for low frequencies. Ten patients monitored with BRM3 were randomly selected and re-sampled with a 2Hz filter. Results were compared with the 0.5Hz filter and no significant differences were found in EEG parameters.

MRI acquisition

MRI was performed on a 3T MR system (Achieva, Philips Medical Systems, Best, the Netherlands). At 30 weeks PMA, an MRI compatible incubator was used to reduce patients discomfort and maintain temperature (Dräger MR Incubator, Lübeck, Germany and Nomag® IC 3.0, Lammers Medical Technology GmbH, Lübeck, Germany, with a dedicated neonatal head coil). At TEA, infants were positioned within a vacuum pillow in a SENSE head coil. The protocol included T2-weighted imaging in the coronal plane (turbo spin echo, at 30 weeks: repetition time 10085 ms; echo time 120ms; slice thickness 2 mm, in-plane spatial resolution 0.35 x 0.35 mm2; at TEA: repetition time 4847-6293 ms; echo time 120-150 ms; slice thickness 1.2 mm, in-plane spatial resolution 0.35 x 0.35 mm^2, full brain coverage) and diffusion tensor imaging (DTI) in the axial plane (single-shot spin-echo, repetition time 5685 ms, echo time 70 ms, field of view 180x146 mm2, 32 diffusion-weighted images with b-value of 800 s/mm2 and 1 non-diffusion-weighted image; matrix size 128x102 mm, slice thickness 2mm, full brain coverage). During the whole examination, a neonatologist was present. Oxygen saturation, respiratory and heart rate of the infants were monitored. If necessary infants were sedated using oral chloral hydrate 30 mg/kg at 30 weeks PMA and 50-60 mg/kg at TEA. All infants received double-layer hearing protection using Minimuffs (Natus Medical Incorporated, San Carlos, CA) and Earmuffs (EM's 4 Kids, Brisbane, Australia).

Figure 1 | Event-based EEG measures.



(A) is an example of a 30 seconds EEG record of one infant, showing several spontaneous activity transients (SAT) with the inter SAT intervals in between (ISI). (B) shows an epoch of 5 minutes EEG with below detected SAT and ISI.

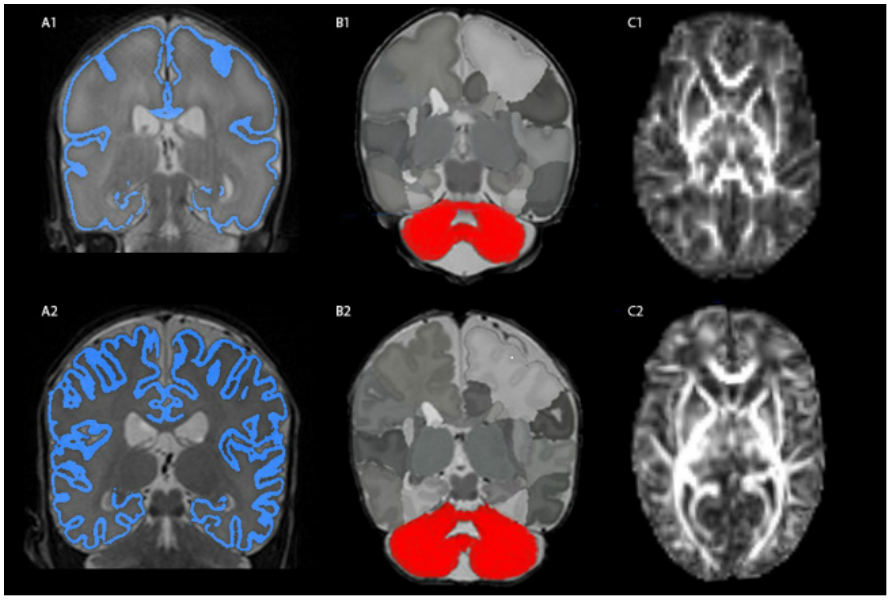
MR image analysis

T2-weighted scans were used to segment the cerebellum with an automated segmentation method,[20] which allowed computation of cerebellar volume. This method has shown reliable results among infants scanned between 28 and 44 weeks PMA. Results of all segmentations were visually inspected and small corrections were performed if deemed necessary. Examples are provided in Figure 2B.

To enable analysis of the cortex, another automated segmentation method was used for the segmentation of the cortical gray matter using T2 weighted images.[21] This method allowed computation of cortical gray matter volume (cm^3) –characterizing cortical growth– and gyrification index –characterizing cortical maturation.[22] Gyrification index was measured as the ratio between inner cortical surface and a smooth convex hull around the white matter (i.e. a simulated cortex around the brain without any folds). Example results of cortical gray matter segmentations are shown in Figure 2A.

Quality assessment and analysis of DTI images were performed with the diffusion MRI toolbox ExploreDTI ® (http://www.exploredti.com/). Data were corrected for eddy current induced geometric distortions and subject motion as described previously.[23] In this procedure, the required reorientation of the B-matrix was performed and the tensor model was fitted to the data with the REKINDLE approach.[23,24] The FA values of the posterior limb of the internal capsule (PLIC) and corpus callosum (CC) were computed by registering the images to a neonatal atlas[25] using both affine and elastic transformation (Figure 2C).

Figure 2 | Examples of image analysis results.



The top row shows the 30 weeks examples, the bottom row shows the 40 weeks examples. A1 and A2 are examples of the method of Moeskops et al.(16) used for the cortical quantification analysis. B1 and B2 are examples of the method of Makropoulos et al.(14) used to obtain the cerebellar volume. C1 and C2 are examples of the DTI registration (http://www.exploredti.com/) used to obtain the FA of the posterior limb of the internal capsule and corpus callosum.

Statistical analysis

Statistical analysis was performed using IBM SPSS v 21.0 (Chicago, IL). The time effect of EEG variables over the 3 epochs (T1-T2-T3) was checked through a linear regression model, and in the absence of time dependency, the three epochs were averaged to a single value of SAT rate and ISI. All structural brain volumes at 30 weeks and at TEA were corrected for total brain volume. Growth of the structural brain volumes and increase of gyrification index and FA parameters were measured as growth/increase per 10 weeks. For these calculations the absolute difference between the measurement at TEA and at 30 weeks was taken, divided by the period (in weeks) between the 2 scans and multiplied by 10 with the following formula: [(vol 40-vol 30)/wks between scans]*10, as already described by Kersbergen et al.[26] All data were corrected for postmenstrual age at the time of the MRI. First, a univariable regression analysis was performed to determine which factors had the largest effect on MRI parameters (results shown in Supplemental Table S1). Due to the small sample size, only the most influential variables were included in the multivariable linear regression analysis. A univariable linear regression analysis was used to determine the effect of clinical confounders on EEG variables (results shown in Supplemental Table S2). The multivariable regression model included GA, BW z-score and WMI score and was built to evaluate the associations between quantitative measures of early brain activity and increase of the MRI measures. A separate multivariable model was built per EEG parameter and MRI parameter. A p-value below 0.05 was considered statistically significant.

Results

Population

In total, 33 infants were eligible for the study, all with good quality aEEG and serial MRI. Clinical characteristics are presented in Table 1. Early MRI was performed at a mean PMA of 30.5, (range 29.3-32.0) weeks and TEA MRI at a mean PMA of 41.1 (range 40.0-41.8) weeks. Growth of the structural and microstructural brain measurements between 30 and 40 weeks is shown in figure 3. None of the enrolled patients presented with clinical or laboratory signs of perinatal asphyxia. None of the infants were mechanically ventilated during EEG monitoring. One infant received phenobarbital at the dose of 10 mg/kg on day 2 because of suspicion of electrical discharges. However, no severe brain injury was observed in this patient. None of the other included infans received any other medication influencing brain activity such as benzodiazepines and barbiturates.

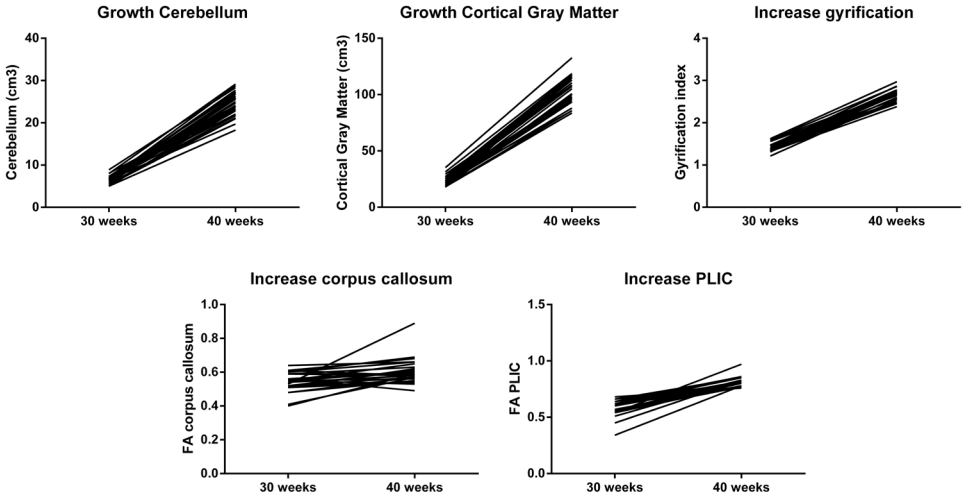
In infants with clinical signs of hypotension, needing inotropes, the mean dopamine dose was 5 µg/kg/min, SD 2,3, range 5-12 µg/kg/min. The univariable linear regression analysis on the effect of clinical confounders on EEG variables showed that BW z-score was significantly and nevatively associated with ISI (Supplemental Table S2).

Table 1 | Baseline characteristics of the study population.

Baseline characteristics	N=33
Gestational age (weeks) mean (SD)	26.0 (1.0)
Birth weight mean grams (SD)	916 (157)
SGA (<p10) n (%)	2 (6)
Male/Female n (%)	12/21 (36/64)
Apgar score 1 min median (IQR)	6 (3-7)
Apgar score 5 min median (IQR)	8 (7-9)
pH arterial/venous cord blood mean (SD)	7.27 (0.08)
Hypoglycemia (<2.5 mmol/l)	
Once n (%)	4 (12.1)
Repeated episodes n (%)	1 (3)
Hypotension (MABP<GA)	
Fluids n (%)	4 (12.1)
Inotropes n (%)	12 (36.4)
GMH-IVH grade I-II n (%)	3 (9)
Patent ductus arteriosus	
Conservatively treated n (%)	16 (49)
Surgically treated n (%)	1 (3)
Necrotizing enterocolitis n (%)	1 (3)
Bronchopulmonary dysplasia n (%)	5 (15)
Culture proven sepsis n (%)	14 (42)
Punctate cerebellar hemorrhage at TEA n (%)	3 (9)
Mild or Moderate WMI n (%)	23 (77)
Weight at 30 wks scan mean grams (SD)	1183 (190)
HC at 30 wks scan mean cm (SD)	27 (2)
Weight at 40 wks scan mean grams (SD)	3421 (388)
HC at 40 wks scan mean cm (SD)	35 (1)

IVH: intraventricular hemorrhage; TEA: term equivalent age; WMI: white matter injury. MAPB: mean arterial blood pressure. HC: head circumference

Figure 3 | Structural and microstructural brain growth.



Increase between 30 and 40 weeks for all 33 individuals are shown for cerebellar volume, cortical gray matter (cGM) volume, gyrification index (GI), corpus callosum (CC) fractional anisotropy (FA) and posterior limb of the internal capsule (PLIC) FA. For each variable mean (SD) and range (;) is provided: cerebellum 30wks (cm3): 6.48 (0.87) (5.00;8.90), 40 wks: 24.56 (2.63) (18.28;29.16). cGM 30wks (cm3): 22.84 (3.91) (17.78;35.18), 40 wks: 104.57 (11.82) (83.65;132.69). GI 30wks: 1.40 (0.09) (1.21;1.62), 40 wks: 2.66 (0.13) (2.38;2.97). FA CC 30wks: 0.53 (0.06) (0.40;0.64), 40 wks: 0.59 (0.08) (0.36;0.89). FA PLIC 30wks: 0.57 (0.07) (0.34;0.68), 40 wks: 0.80 (0.04) (0.69;0.97). All the values shown were corrected age at scan.

Early brain activity and structural brain growth

A significant positive association between SAT rate and cerebellar growth was found with conversely a significant negative association between ISI and cerebellar growth (Table 2, Figure 4). Other clinical variables did not show an effect on cerebellar growth in the multivariable analyses. SAT rate was also positively correlated with growth of cortical gray matter and increase in gyrification index, while ISI did not show a relation.

A clear example of the clinical relevance of the present study is that an increase of one SAT/minute in the first 48 hours after birth will bring an increase of around 0.2 cm^3 to the cerebellar volume and of around 5 cm^3 of cortical grey matter volume. An increase of one second in ISI will result in a decrease of around 0.14 cm^3 in cerebellar volume.

Table 2 | EEG parameters and structural brain development.

	Cerebellum		Cortical gray matter			
	Volumetric growth		Volumetric growth		Gyrification	
	B (CI)	p-value	B (CI)	p-value	B (CI)	p-value
SAT	0.234 (0.060;0.408)	0.010	4.706 (0.566;8.847)	0.027	0.050 (0.002;0.099)	0.043
BW z-score	-0.008 (-0.255;0.239)	0.945	0.702 (-4.598;6.002)	0.787	-0.079 (-0.140;-0.018)	0.014
WMI score	0.040 (-0.093;0.173)	0.540	-1.134 (-4.498;2.231)	0.493	-0.032 (-0.070;0.006)	0.095
GA	0.022 (-0.152;0.195)	0.801	1.356 (-2.836;5.548)	0.666	0.022 (-0.029;0.073)	0.374
ISI	-0.149 (-0.237;-0.062)	0.002	-1.735 (-4.049;0.578)	0.135	-0.019 (-0.047;0.009)	0.178
BW z-score	-0.100 (-0.310;0.109)	0.334	0.011 (-6.886;6.907)	0.997	-0.066 (-0.132;-0.001)	0.048
WMI score	-0.020 (-0.150;0.110)	0.753	-1.134 (-4.498;2.231)	0.493	-0.022 (-0.060;0.016)	0.238
GA	0.020 (-0.162;0.202)	0.827	0.626 (-4.184;5.435)	0.790	0.021 (-0.033;0.074)	0.426

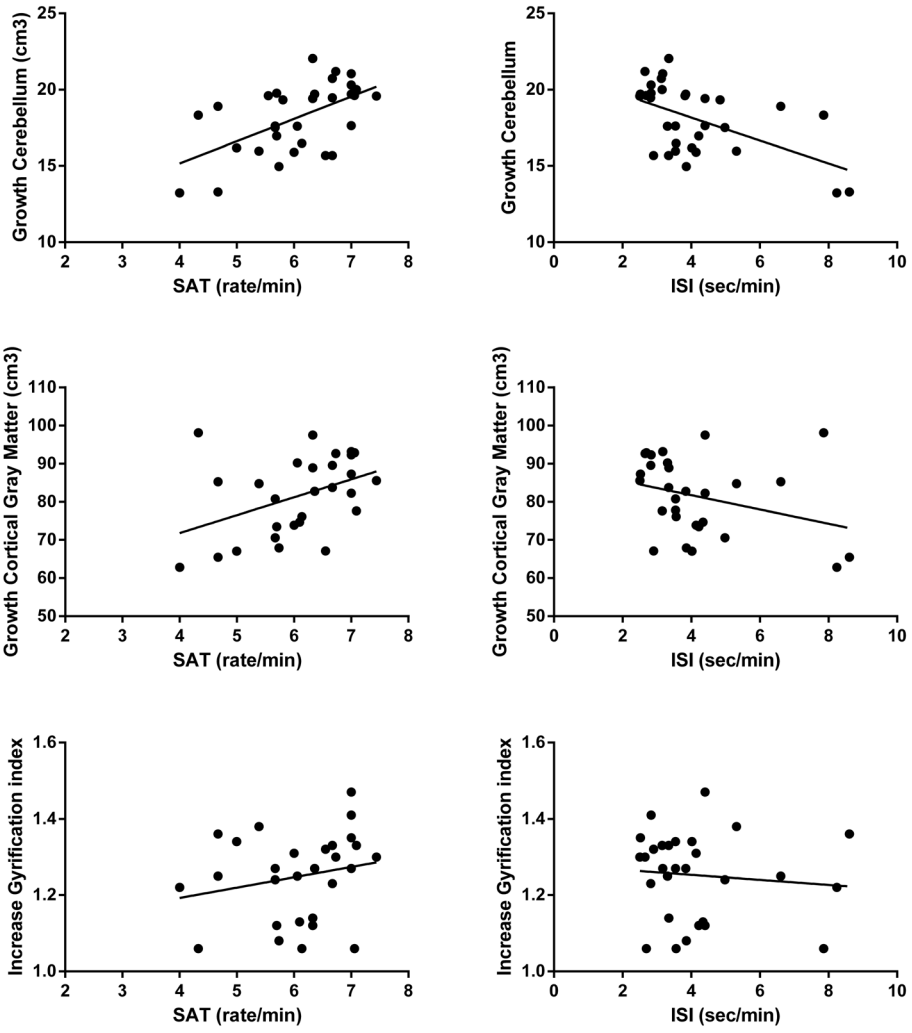
Rows are showing the EEG parameters (SAT and ISI) and the other clinical parameters included in the multivariable analyses. Separate multivariable analyses were performed for ISI en SAT. Columns are showing the growth of cerebellar volume, cortical gray matter volume and gyrification index. B-value with confidence interval (CI) and p-value are shown. SAT: spontaneous activity transient. ISI: inter-SAT-interval. BW: birth weight. GA: gestational age. WMI: white matter injury. ns: not significant. Volumetric growth is calculated per 10 wks.

Early brain activity and microstructural brain growth

The multivariable analysis showed a positive association between SAT rate and increase in FA of the CC (Table 3). Thus, an increase in one SAT per minute, will result in an increased FA value of the CC of around 0.03.

No association between ISI and the FA of the CC was seen. Also, no associations were found between EEG-measurements (SAT and ISI) and increase in FA of the PLIC.

Figure 4 | EEG and structural brain growth.



Associations of EEG parameters (spontaneous activity transient (SAT) and inter-SAT-interval (ISI)) with cerebellar volume, cortical gray matter volume and gyrification index are shown. Coefficients and confidence intervals corrected for confounding factors are shown in table 2, therefore R and p values are not included in this figure. EEG variables mean (SD) and range (;) is provided: SAT (rate/min): 6.0 (0.8) (4.0;7.4), ISI (sec/min): 4.1 (1.6) (2.5;8.6).

Table 3 | EEG parameters and microstructural brain development.

	Corpus Callosum		PLIC	
	Increase FA		Increase FA	
	B (CI)	p-value	B (CI)	p-value
SAT	0.027 (0.020;0.053)	0.037	-0.013 (-0.067;0.042)	0.608
BW z-score	0.004 (-0.051;0.059)	0.882	-0.054 (-0.120;0.012)	0.098
WMI score	-0.012 (-0.052;0.027)	0.501	-0.105 (-0.184;-0.027)	0.013
GA	0.021 (-0.010;0.052)	0.168	0.053 (-0.012;0.117)	0.098
ISI	-0.008 (-0.022;0.005)	0.207	0.009 (-0.018;0.037)	0.466
BW z-score	0.006 (-0.052;0.065)	0.811	-0.054 (-0.120;0.012)	0.098
WMI score	-0.009 (-0.051;0.033)	0.639	-0.105 (-0.184;-0.027)	0.013
GA	0.030 (-0.001;0.060)	0.053	0.053 (-0.012;0.117)	0.098

Rows are showing the EEG parameters (SAT and ISI) and the other clinical parameters included in the multivariable analyses. Separate multivariable analyses were performed for ISI en SAT. Columns are showing increase of FA in the corpus callosal and PLIC. B-value with confidence interval (CI) and p-value are shown. FA: fractional anisotropy. PLIC: posterior limb of the internal capsule. SAT: spontaneous activity transient. ISI: inter-SAT-interval. BW: birth weight. GA: gestational age. WMI: white matter injury. ns: not significant. Microstructural brain development was calculated as growth per 10 wks.

Discussion

This study shows increased early brain activity (expressed by higher SAT rate and lower ISI) to be associated with increased growth of the cerebral cortex and cerebellum as also with changes in corpus callosal FA, in a relatively healthy cohort of extremely preterm infants. These results underline the importance of early life neuro-monitoring in extremely preterm neonates in order to identify those at risk of altered brain development.

This study confirms the importance of SATs for cortical gray matter growth, as presented in a previous paper (8), and adds the importance of early spontaneous activity to cortical maturation (i.e. gyrification). SATs are the most notable feature of the preterm EEG, characterized by nested activity at multiple frequencies, including also very low frequency activity (0.1-0.5 Hz) that is filtered by conventional EEG.[1] In healthy human infants, SATs disappear around 35 weeks of PNA.[27] The association between early brain activity (i.e. SAT rate and ISI) and morphological maturation of the cortex between 30 and 40 weeks, is thought to be a result of the complex interplay between structural and functional processes in this period of brain network development.[28] EEG findings are the result of cortical network activity, changing with

maturation of the brain during the last part of gestation.[29] From 24 weeks onward afferent thalamo-cortical axons grow from the thalamus into the subplate where accumulation and waiting takes place before migration to the cortex. Migration from the subplate into the cortical plate induces synaptogenesis of the thalamo-cortical axons, which corresponds to the appearance of SATs at EEG.[30,31] It is known that SATs are first focal and concentrated in the sensory cortex, and become more widespread later during development.[1] Onset of synchronicity between hemispheres can be seen around 30 weeks of gestation, whereas more precise and consistent synchrony appears only from around 35 weeks of conceptional age and it is not completely dependent on callosal connections.[32] This study shows increased SAT rate to be associated with cortical gray matter maturation, which is supported by other studies finding more complex EEG patterns with increased gyrification.[33] A recent study, comparing subjects with no or minimal brain injury to those with moderate/severe brain injury, showed that the latter have lower burst frequency and longer IBI and includes data from both rodent and human preterm subjects. In the rodent model of the same study, diminished brain activity was associated with a delayed neuronal morphological development, demonstrating that spontaneous brain activity (SATs) is fundamental for normal maturation and development of cortical circuits and neurons. These data suggest that early brain injury impairs activity-dependent maturation of neuronal circuits during a highly sensitive period of development.[34] We were able to show that increased cerebellar growth is associated with early brain activity, whereas in contrast low SAT rate and prolonged ISI are associated with poor cerebellar growth. An approximately three-times increase in cerebellar volume from 28 to 40 weeks of gestation has been documented which makes the cerebellum, more than the cortical gray matter, the brain structure showing the largest relative growth in this period.[26] This suggests cerebellar and cortical expansion to be most vulnerable to disturbances (in brain activity) in this early period of life of preterm infants. Several studies have suggested a developmental relation between the cerebellum and cortical gray matter in infants born preterm where the cortex and cerebellum appear to be connected in a closed circuit with bidirectional feedback, supported by recent studies describing reduced contralateral cortical gray matter growth in case of prematurity related cerebellar injury.[35-37] Both cerebellar and cortical alterations are associated with neurodevelopmental sequelae in the domains of cognition and motor development.[35-37] This study, suggesting developmental dependency upon early brain activity of the cerebellum and the cortex, puts forward that cortical alterations might already find their origin in the first period of life in extremely preterm neonates. Although, it can not be excluded that the importance of brain activity and subsequent brain development, is a surrogate of the infant's well-being, such as optimal cardiovascular and nutrition conditions, therefore optimal brain growth.

In this study, we showed prolonged ISI in early life to be associated with decreased cerebellar growth between 30 and 40 weeks. Prolonged ISI (and thus decreased cortical activity) has been associated with acidosis and adverse long-term neurodevelopmental outcome at 2

years of corrected age in preterm infants.[19,38] Furthermore some studies demonstrated that the survival of developing neurons is strongly dependent on the growing spontaneous activity in the early neuronal networks.[39,40] Thus, deprivation of neurons of this activity, reflected by prolonged ISI, may be linked to cell apoptosis and reduced dendritic pruning.[41,42] Based on our study we can hypothesize that prolonged ISI in early life has more effect on cerebellar growth than on cortical gray matter development.[43]

To the best of our knowledge, we have shown for the first time that increased early brain activity was associated with microstructural changes in the corpus callosum, as determined by faster increase in FA. This confirms the hypothesis of a link between early brain function and white matter microstructural maturation. White matter FA increases with brain maturation as a result of increasing myelination, fiber coherence and axonal density. White matter organization is known to be correlated with long-term neurodevelopmental outcome in this vulnerable group of neonates.[44,45] Being the largest white matter fiber bundle, the corpus callosum is responsible for the majority of the cortico-cortical, inter-hemispheric connections.[46] Reduced FA of the corpus callosum might reflect altered myelination as well as axonal damage, resulting in decreased interhemispheric processing. The PLIC is one of the WM structures with the largest increase in FA over the period of 30 to 40 weeks postmenstrual age.[47] White matter organization of the PLIC, predictive of motor development and vulnerable to injury in preterm infants,[44] did not show an association with early brain activity in this study. This could maybe be explained by the fact that neonatal morbidity has a stronger effect on the process of maturation of the PLIC, than the effect found by early brain activity.

This study examined a relatively healthy cohort of preterm infants by excluding those who died, were unstable for MRI, received morphine or had severe brain injury.

The main problem of these neonates is extreme prematurity, and many factors can influence later brain development. This study aimed to take into account as less severe preterm problems as possible by excluding infants with severe brain injury. In this group of vulnerable infants an association between early cortical network activity and brain (micro)structural development is suggested.

The first limitation of this study is the small population, leading to limited statistical strength. The used analysis methods require high quality EEG and MRI data, resulting in a limited number of infants eligible for inclusion. However, selection bias does not seem to play a role as baseline characteristics between included and excluded patients (based on image quality) were comparable. The small sample size results in a limited number of included relevant co-variables in the analysis. Thus, the analysis could not be corrected for all confounders on aEEG and could not optimally be controlled for all known variables associated with brain growth. Furthermore the small sample size yields the possibility to underscore the effect of other confounders.

Conclusions

This study demonstrates the association of early brain activity to structural and microstructural brain growth in a cohort of relatively healthy extremely preterm infants. Higher SAT rate and decreased ISI are signs of increased early cortical network activity and appear to be associated with increased growth of cerebellum and cortical gray matter, as also cortical maturation. Cerebellum and cortex undergo the most rapid growth in the last part of gestation and are connected in a closed neuronal system. A change in FA of the corpus callosum was also shown to be associated with higher spontaneous brain activity in early preterm life. This study underlines the importance of early brain activity for improved (micro)structural brain development in extremely preterm infants and the need for neuro-monitoring in these vulnerable newborns.

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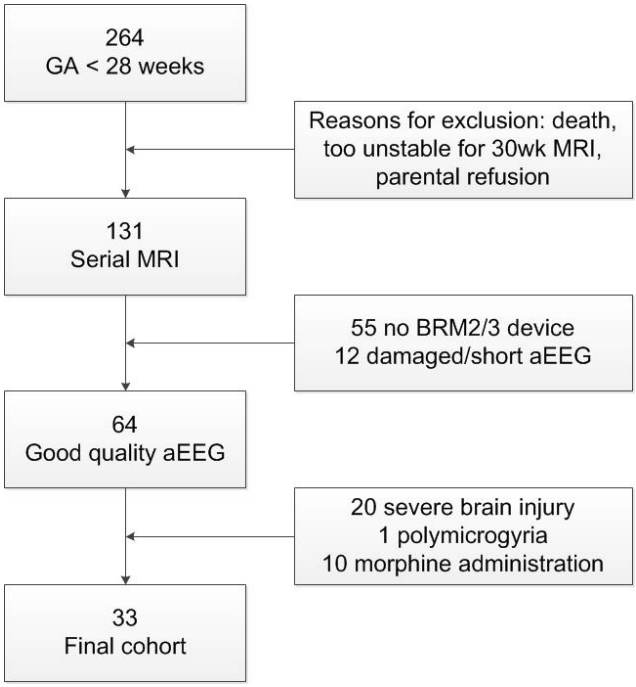
Supplemental Figure 1 | Flowchart of patients.



Supplemental Table 1 | EEG parameters and structural/microstructural brain development (univariable analysis).

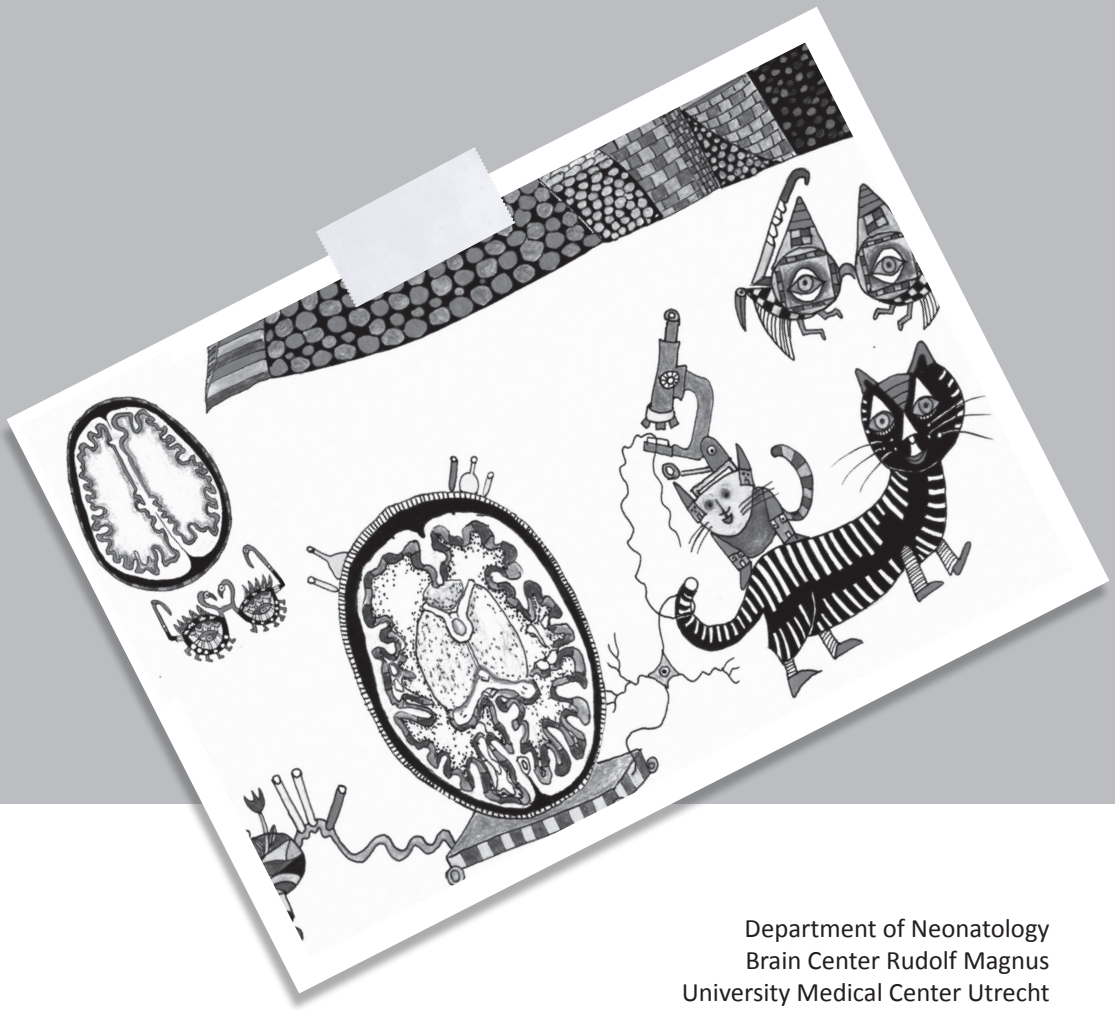
	Cerebellum		Cortical gray matter				Increase in FA			
	Volumetric growth		Volumetric growth		Gyrification		CC		PLIC	
	B (CI)	p-value	B (CI)	p-value	B (CI)	p-value	B (CI)	p value	B (CI)	p value
SAT	0.244 (0.076;0.413)	0.006	0.393 (0.132;0.654)	0.005	0.028 (-0.021;0.077)	0.052	0.025 (-0.012;0.062)	0.170	-0.027 (-0.065;0.011)	0.152
ISI	-0.153 (-0.240;-0.067)	0.001	-0.173 (-0.317;-0.029)	0.021	-0.007 (-0.034;0.019)	0.576	-0.015 (-0.035;0.005)	0.133	0.014 (-0.007;0.035)	0.173
GA	0.049 (-0.139;0.237)	0.095	-0.161 (-0.128;0.450)	0.262	0.013 (-0.036;0.062)	0.581	0.016 (-0.027;0.060)	0.445	-0.007 (-0.053;0.038)	0.738
BW z-score	0.079 (-0.137;0.295)	0.462	0.315 (0.007;0.624)	0.046	-0.048 (-0.102;0.007)	0.085	0.026 (-0.042;0.094)	0.426	-0.009 (-0.081;0.063)	0.798
Sepsis*	-0.049 (-0.387;0.289)	0.786	0.101 (-0.417;0.620)	0.692	-0.006 (-0.094;0.082)	0.885	-0.013 (-0.088;0.063)	0.731	0.006 (-0.072;0.084)	0.877
NEC*	-0.001 (-0.168;0.166)	0.987	0.139 (-0.109;0.387)	0.261	0.027 (-0.014;0.068)	0.181	0.003(-0.030;0.036)	0.863	-0.006 (-0.040;0.028)	0.707
BPD*	-0.035 (-0.492;0.422)	0.877	-0.279 (-0.967;0.409)	0.413	0.034 (-0.081;0.149)	0.550	-0.030 (-0.119;0.060)	0.502	0.014 (-0.079;0.108)	0.755
Surgery*	-0.138 (-0.822;0.547)	0.684	-0.377 (-1.407;0.653)	0.459	0.081 (-0.088;0.251)	0.335	0.105 (-0.074;0.284)	0.237	-0.088 (-0.275;0.099)	0.341
IVH*	-0.146 (-0.283;0.576)	0.492	-0.065 (-1.105;0.974)	0.899	-0.041 (-0.213;0.131)	0.632	-0.045 (-0.229;0.139)	0.615	-0.038 (-0.228;0.153)	0.687
WMI score	0.025 (-0.119;0.169)	0.429	-0.079 (-0.310;0.152)	0.487	-0.009(-0.047;0.030)	0.652	0.037 (0.010;0.064)	0.010	0.218 (-0.017;0.453)	0.067

Rows are showing the clinical parameters and the EEG parameters (SAT and ISI) analyzed in the univariable analyses. Columns are showing the growth of cerebellar volume, cortical gray matter volume, gyrification index and the increase in FA of the PLIC (posterior limbs of the internal capsule) and CC (corpus callosum). B-value with confidence interval (CI) and p-value are shown. SAT: spontaneous activity transient. ISI: inter-SAT-interval. BW: birth weight. GA: gestational age. NEC: necrotizing enterocolitis (any grade). BPD: bronchopulmonary dysplasia. IVH: intraventricular hemorrhage (any grade). WMI: white matter injury. *yes/no variable

Supplemental Table 2 | Clinical factors in relation to EEG parameters.

	SAT		ISI	
	B (CI)	**p-value**	**B (CI)**	**p-value**
GA	0.124 (-0.218;0.466)	0.465	-0.349 (-0.971;0.273)	0.262
BW z-score	0.352. (-0.033;0.737)	0.071	-0.847 (-1.529;-0.164)	0.017
hypoglycemia	0.468 (-1.237;2.173)	0.579	-0.084 (-2.943;2.776)	0.953
hypotension	0.220 (-0.457;0.898)	0.511	-0.580 (-1.821;0.660)	0.347
Cerebellar hemorrhage at TEA	-0.325 (-0.766;0.115)	0.142	0.525 (-0.295;1.345)	0.201
WMI score	0.059 (-0.231;0.350)	0.679	-0.175 (-0.696;0.347)	0.499

Rows are showing the clinical parameters included in the univariable analyses in relation to the EEG parameters (SAT and ISI) . B-value with confidence interval (CI) and p-value are shown. SAT: spontaneous activity transient. ISI: inter-SAT-interval. BW: birth weight. GA: gestational age. TEA: term equivalent age. WMI: white matter injury.



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Predictive role of urinary metabolic profile for abnormal MRI score in preterm neonates

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Submitted

Background | Early identification of neonates at risk for brain injury is of outmost importance. Urinary metabolomics are potential, non-invasive biomarkers of brain disease.

Objective | To study the urinary metabolic profile at 2 and 10 days after birth in preterm neonates with and without severe MRI abnormalities at term equivalent age.

Methods | 30 preterm infants with a GA <28 wks (mean 26.6; SD 1.0) were consecutively enrolled. Urine samples were collected at 2 and 10 days after birth and analyzed using proton magnetic resonance spectroscopy (^{1}H-NMR). A 3T MRI was performed at TEA and images were scored for white matter (WM), cortical grey matter (cGM), deep GM (dGM) and cerebellar abnormalities, using the Kidokoro MRI score (1). Infants were divided in two groups: one with normal MRI or mild abnormalities and one with moderately to severely abnormal MRI score. Receiver-operating characteristic (ROC) curves analyses were performed to distinguish infants with moderately/severely abnormal MRI score (i.e. total WM score ≥5 and/or total cGM score≥2).

Results | The principal component analysis (PCA) did not show significant clustering between normal/mild abnormal MRI score and moderate/severe MRI abnormalities for all regions (cGM, WM, dGM and cerebellum) at both time points, 2 days and at 10 days after birth. The ROC curves distinguished neonates at both 2 and 10 days after birth who later developed a markedly less mature cGM score from the others [2 d: AUC 0.72, specificity (SP) 65%, sensitivity (SE) 75% and 10 d: AUC 0.80, SP 78%, SE 80%] and moderately to severely abnormal WM score [2 d: AUC 0.71, specificity (SP) 80%, sensitivity (SE) 72% and 10 d: AUC 0.69, SP 64%, SE 89%].

Conclusions | ^{1}H-NMR urinary spectra of extremely preterm infants at 2 and 10 d were able to discriminate different metabolic profiles in patients with moderately to severely abnormal cGM score and WM score at TEA. Urine spectra are promising for early identification of neonates at high risk of brain damage and allow to better understand the multifactorial pathogenesis of altered neonatal brain development.

Introduction

In developed countries approximately 1.5 % of neonates are born extremely preterm, and despite the high rate of survival, the percentage of moderate or severe neurodevelopmental disability remains high, depending on gestational age and neonatal morbidity.[1,2] Thus, early identification of neonates at risk for brain injury is of outmost importance to start appropriate intervention, but despite the large number of investigations aiming to early identify those high risk infants, prediction of long term-outcome is still challenging. Magnetic resonance imaging (MRI) is an additional tool to estimate the wide spectrum of preterm brain injury and, when performed at term equivalent age (TEA), is able to predict adverse neurodevelopmental outcome.[3] In particular, white matter (WM), cortical grey matter (cGM) and cerebellum are the largest and most vulnerable brain structures in extremely preterm neonates.[4] Among all, cerebral WM is the most commonly involved site of injury in preterm infants.[5] During the last years there were many attempts to define a comprehensive and objective MRI scoring system in order to define the severity of injury, and to correlate this score with long term outcome. In 2013, Kidokoro and colleagues developed an MRI scoring system, providing a reliable brain injury classification that can be used at term equivalent age (TEA).[6] Together with MRI, the use of biomarkers might be useful to predict, diagnose and monitor brain injury and development. In this context, a new promising tool for disease prediction is proton magnetic resonance spectroscopy (^{1}H-NMR) in biological fluids and, particularly, in urine.[7] Urinary metabolomics can be a potential, non-invasive source of biomarkers of brain disease. Urine, differently from blood, does not undergo homeostatic mechanisms and may reflect the body status, being informative for some diseases.[7,8] Urinary metabolomics was found to be a good diagnostic tool, with high sensitivity and specificity for psychiatric disorders such as major depression, bipolar disorder and autism spectrum disorders.[9-11] Furthermore some studies showed the possible use of urinary metabolomics in the early diagnosis of cerebrovascular diseases such as stroke[12] and neurodegenerative diseases (Alzheimer's and Parkinson's disease, multiple sclerosis and transmissible spongiform encephalopathy).[13-15] Interestingly, a study by Ottens and colleagues demonstrated that urinary metabolomics was reliable in identifying patients with traumatic brain injury and in monitoring the evolution of rehabilitation.[16]

Our aim was to study the urinary metabolic profile at 2 and 10 days after birth in preterm neonates with and without moderately to severely abnormal MRI score at TEA.

Material and methods

Population

Sixty-five extremely preterm neonates with gestational age (GA) <28 wks, consecutively born from July 2012 till June 2013 at the Wilhelmina Children Hospital in Utrecht, were eligible for the study. Infants were excluded if they had congenital malformations or if there was a clinical suspicion of genetic or metabolic disorders. Written informed consent was obtained from all parents. The study was approved by the hospital medical ethics committee. All infants were scanned around TEA according to clinical protocol. Eight neonates died in the neonatal period, thus were excluded from the study since MRI at TEA was not available. Twenty-five neonates were excluded since parents did not give consent for enrollment. One infant did not have available MRI at TEA and one more infant turned out to have a metabolic disorder, thus they were both excluded from the study. A total of thirty infants were finally included in the study. Furthermore, clinical parameters and data on the occurrence of prematurity-related diseases such as intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), patent ductus arteriosus (PDA) and bronchopulmonary dysplasia (BPD) and other clinical data were collected from patients charts. All infants received the same parenteral nutrition and the daily steps in oral feeding increase were done, following the local protocol for enteral and parenteral nutrition of the Wilhelmina Children's Hospital/UMC Utrecht, depending on the tolerance of the neonate. Breast milk or formula milk, when breast milk was not available, were used for enteral nutrition. Parenteral nutrition was introduced on day one after birth, with a solution of glucose and amino acids (Primene 10%). Lipids (Intralipid 20%) were added between day one and two after birth. Proteins and lipids intake were progressively increased, to a maximum of 3 g/kg proteins on day three to four after birth, and 2.5 g/kg lipids between day four and five after birth.

Urine samples and storage

Urine samples were collected at 2 (between 44 and 50 hours) and 10 (between 238 and 242 hours) days after birth. Samples were non-invasively collected by the nurses, using a small plastic bag from which 2 ml of urine was withdrawn. Each sample was assigned with a patient anonymized number and sampling time. Samples were frozen at -80°C soon after sampling and stored at the Wilhelmina Children's Hospital/UMC in Utrecht. All urine samples were shipped in dry ice to the Nuclear Magnetic Resonance (^{1}H-NMR) laboratory of the University of Siena for the ^{1}H-NMR analysis.

^{1}H-NMR analysis

Urine NMR measurements were performed on a Bruker DRX 600 MHz Avance Spectrometer with a Selective Inverse Probe (SEI) equipped with Z gradient coil. Spectra were acquired at a constant temperature of 298.0 ± 0.1 K by using a 90° pulses. Furthermore, 10 seconds delay was included in the pulse sequence to allow T1 relaxation. In fact, T1 values (in the range 1.5–2.8 s) of the analyzed metabolites are such that a 10 s delay allows full recovery of longitudinal magnetization after a 90° pulse, as verified by constant integral values for D1 ≥ 5s. A 0.3 Hz line broadening function was applied before Fourier transformation. A saturation pulse of 2 s duration was applied at the water resonance to suppress the water signal. 32 K data points per scan were used and 128 transients were accumulated. Each urine sample was first centrifuged at 2000 rpm for 5 min and analyzed afterwards. Sample (550 µl) plus 50 µl of a TSP-d4 20 mM solution, were measured into a 0.5 mm (outer diameter) MR tube. All spectra were first run at their own physiological pH; we use this first spectrum only for an overview of the contained metabolites, than we adjust the pH at 2.50 ± 0.02 in the same MR tube, with a microelectrode and we run a second spectrum. The chemical shift of ionizable fluids is highly dependent on the pH. At a pH of 2.50, all chemical shift values are reproducible within ±0.01 ppm.[9] Moreover, under the described conditions, the methyl signals of creatine and creatinine are clearly separated (3.05 ppm for the methyl signal of creatine and 3.13 ppm for creatinine) and the methyl signal of lactic acid (1.41 ppm) is not overlapped by the methyl resonance of threonine (1.33 ppm). The pH was adjusted using a minimal volume of HCl, starting from a 3 M and ending with a 0.05 M and directly frozen at-80°C.

MRI acquisition

The brain MRI was performed on a 3T MR system (Achieva, Philips Medical Systems, Best, The Netherlands) at TEA. Infants were positioned within a vacuum pillow in a SENSE head coil. The protocol included T2-weighted imaging in the coronal plane (repetition time 4847-6293 ms; echo time 120-150 ms; slice thickness 1.2 mm, in-plane spatial resolution 0.35 x 0.35 mm2, full brain coverage) and coronal T1-weighted imaging (repetition time 9.5 ms; echo time 4.6 ms; slice thickness 1.2 mm, full brain coverage). An experienced neonatologist was present during the examination. Oxygen saturation, respiratory and heart rate of the infants were continuously monitored. If necessary, infants were sedated using oral chloral hydrate using a dose of 50-60 mg/kg through the gastric tube. All infants received double-layer hearing protection using Minimuffs (Natus Medical Incorporated, San Carlos, CA, USA) and Earmuffs (EM's 4 Kids, Brisbane, Australia).

MRI scoring system

All MRI images were scored for the presence of brain abnormalities by two experienced neonatologists (L.d.V. and M.B.) using the Kidokoro scoring system.[6] According to the scoring system, white matter (WM), cortical grey matter (cGM), deep GM (dGM) and cerebellar abnormalities were evaluated. The score class for each region (WM, cGM, DGM, cerebellum) was calculated, obtaining the following classes: normal, mildly abnormal, moderately and severely abnormal classes, according to Kidokoro et al.[6] Scorings were performed using OsiriX (32-bit version, www.osirix-viewer.com), which allowed free conversion to all planes.

Statistical analysis

All statistical analyses were carried out on pre-processed data. Unit area normalization, Pareto scaling and mean centering have been performed on each spectra before analysis. In order to analyze the differences in metabolic profile that are connected to the different newborn status, we used the two-step analytical procedure (often used in metabolomics): first, we use an unsupervised technique, Principal Components Analysis (PCA), to find trajectories and clustering; second, we model the system by using a classification technique, in this case, partial least square discriminant analysis (PLSDA).

Finally, using PLSDA, the discriminant performance of spectra has been checked, and receiver-operating characteristic (ROC) curves were obtained, in order to verify the ability to distinguish infants with later moderately/severely abnormal MRI score from the infants with normal/mild MRI scores, as explained in the MRI scoring system paragraph.[6] ROC curves were built using the whole spectrum of metabolites obtained from the ^{1}H-NMR analysis.

Data were analyzed using R program (R Core Team (2016)). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org).

Results

Clinical characteristics are presented in Table 1. One newborn showed clinical and biochemical signs of mild perinatal asphyxia. None of the infants had an arterial cord blood pH below 7.00. MRIs were performed at a mean post-natal age of 40.9 (SD 0.4) wks. MRI scores are presented in Table 2. None the patients showed focal cGM abnormalities or dGM volume reduction. The principal component analysis (PCA) did not show significant clustering between normal/mild abnormal MRI score and moderate/severe MRI abnormalities for all regions (cGM, WM, dGM and cerebellum) at both time points, 2 days and at 10 days after birth. However, ^{1}H-NMR urinary metabolic profile analysis showed that lactate and leucine

at 2 days were significantly higher in patients with moderately/severely abnormal WM score, compared with infants with normal or mildly abnormal WM score (see Table 3). Threonine, lactate and glycine were significantly increased at 2 days in patients with moderately to severely abnormal cGM score. Acetate signal was significantly increased at 10 days in infants with moderate/severe abnormal WM MRI score, while carnitine and glycine were significantly higher at the same time point in newborns with moderate/severe cGM score. 3-OH butyrate, alanine and N-acetylated compounds were decreased at 10 days in infants with cGM moderate/severe abnormalities.

Table 1 | Clinical characteristics of the population.

Baseline characteristics		**N = 30**
GA (wks), mean (SD)		26.6 (1.0)
BW (gr), mean (SD)		911 (178)
Gender, male (%)		15 (50)
Apgar score 1st min, median (IQR)		5 (3;7)
Apgar score 5st min, median (IQR)		8 (7;8)
Arterial umbilical cord pH, mean (SD)		7.24 (0.09)
Arterial umbilical cord BE mmol/l, mean (SD)		-5.1 (4.0)
Arterial umbilical cord lactate mmol/l, mean (SD)		5,0 (2,9)
PDA surgery, n (%)		2 (6.7)
BPD, n (%)		7 (23.3)
Late-onset sepsis, n (%)		4 (13.3)
IUGR		2 (6.7)
IVH n (%)	Grade 1-2	6 (20)
	Grade 3-4	3 (10)

Abbreviations: GA: gestational age, BW: birth weight, SD: standard deviation, BE: base excess, PDA: patent ductus arteriosus, BPD: bronchopulmonary dysplasia, IVH: intraventricular hemorrhage, IQR: interquartile range.

Table 2 | MRI classes, using the Kidokoro MRI score, in the study group.

Global MRI classes (N = 30)	
Normal	3 (10)
Mildly abnormal	19 (63.3)
Moderately abnormal	6 (20)
Severe	2 (6.7)

Data are n (%). *Table continues on next page*

Table 2 *(continued)* | MRI classes, using the Kidokoro MRI score, in the study group.

MRI WM score sub-items	N=30	MRI WM score classes	N=30
Cystic lesions n (%)	4 (13.3)	Normal	4 (13.3)
Focal unilateral	1 (3.3)	Mild abnormalities	16 (53.4)
Extensive unilateral	3 (10)	Moderate	4 (13.3)
Myelination		Severe	6 (20)
Only PLIC	27 (90)		
Ventricular dilatation			
Both sides <7.5mm	22 (73.4)		
One side 7.5-10mm	4 (13.3)		
Both sides 7.5-10 or 1 side >10	4 (13.3)		
cGM score sub-items		**cGM score classes**	
Signal abnormalities	0 (0)	Normal	8 (26.6)
Gyral maturation		Mild abnormalities	10 (33.3)
Delay < 2 wks	11 (36.7)	Moderate	5 (16.7)
2-4 wks	18 (60)	Severe	7 (23.3)
>4 wks	1 (3.3)		

Increased IHD			
IHD <4mm	17 (56.6)		
4-5 mm	6 (20)		
5-6 mm	2 (6.7)		
>6 mm	5 (16.7)		
dGM score sub-items		**dGM score classes**	
Signal abnormalities		Normal	28 (93.4)
Focal unilateral	1 (3.3)	Mild abnormalities	1 (3.3)
Focal bilateral	1 (3.3)	Moderate	1 (3.3)
Volume reduction	0 (0)	Severe	0 (0)
Cerebellum score sub-items		**Cerebellum classes**	
Punctate unilateral	6 (20)	Normal	3 (10)
Punctate bilateral	1 (3.3)	Mild abnormalities	19 (63.3)
Volume reduction		Moderate	6 (20)
cTCD >50 mm	24 (80)	Severe	2 (6.7)
47-50 mm	5 (16.7)		
<47 mm	1 (3.3)		

Data are n (%). Abbreviations: WM: white matter, cGM: cortical grey matter, dGM: deep gray matter, PLIC: posterior limb of the internal capsule, CR: corona radiate, IHD: inter-hemispheric distance, cTCD: coronal transverse-cerebellar diameter

Table 3 | Summary of altered metabolites extract from PCA analysis of urine average ^{1}H-NMR spectra.

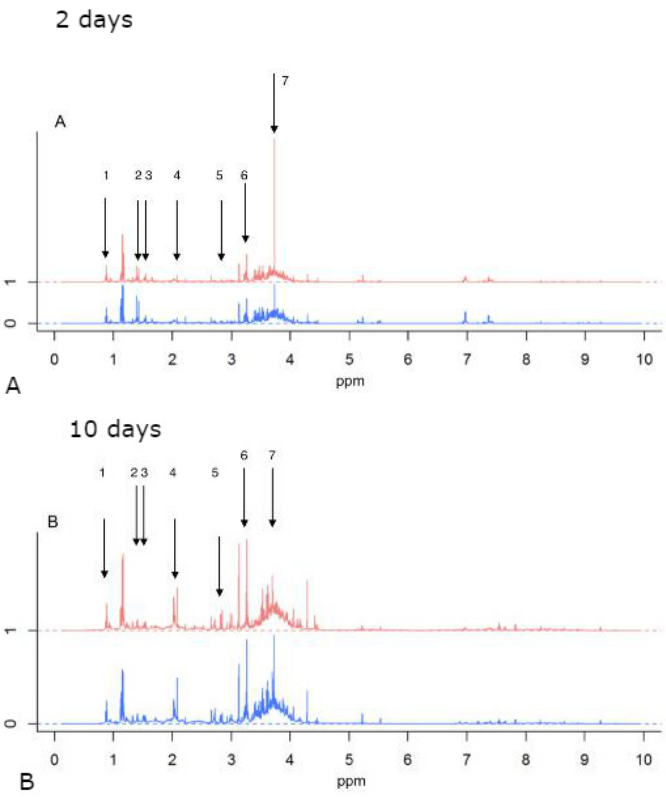
Metabolites	2 d				10 d			
	WM score		cGM score		WM score		cGM score	
	normal mild N=20	moderate severe N=10	normal mild N=18	moderate severe N=12	normal mild N=20	moderate severe N=10	normal mild N=18	moderate severe N=12
leucine	o	ooo			o	o	o	o
3-OHbut							ooo	oo
3-NH2isobut			oo	o				
3-OHisoval	o	o			oo	oo	o	o
threonine			o	ooo	o	o	oo	oo
lactate	oo	oooo	o	oo				
alanine	o		oooo	oooo	oooo	oooo	oooooo	oo
N-acetylated * at 2.02 ppm					oooooo	oooooo	ooooooooooooo	oooooooo
at 2.05 ppm					oo	oo	oooooo	oo
acetate	o	o				oo	o	o
acetone	oo	oo	o	o				
pyruvate							o	o
succinate	o	o	oo	oo	oo	oo	oo	oo
dimethylamine			o	o	oooo	oooo		
dimethylglycine	oooo	oo			o	o	o	o
citrate	oooo	oooo	oo	oo	ooooooooo	ooooooooooo	ooooooo	ooooooo
creatine	o	o			o	o	o	o
creatinine	oooo	oooo	oooo	oooo	ooooooooo	ooooooooo	ooooooo	ooooooo
carnitine	ooo	ooo	ooo	ooo	ooo	ooo	oo	oooo
betaine	oooooo	oooooo	ooooo	ooooo	oooooo	oooooo	oooooo	oooooo
myoinositol	oo	oo	oooo	ooo	ooooooo	ooooooo	oo	oo
glycine	ooooo	ooooo	o	ooo	oooo	oooo	ooo	oooo
glucose	ooooo	ooooo	oooooo	oooooo	oooo	oooo	ooooooo	ooooooo
fumarate							o	o
hippurate					ooooooooo	ooooooooo	oo	oo
formiate	oo	oo	ooooo	ooo	ooo	ooo	o	o
NMNA			oo	oo	o	o	o	o

The circles indicate the score of increasing metabolites compared to the average spectra (from low (O) to very high >10 O), in red: significant metabolites. Abbreviations: 3-OHbut, 3-hydroxybutyrate; 3-NH2 isobut, 3-amino-isobutyrate; 3-OHisoval, 3-hydroxyisovalerate; NMNA, N-methylnicotinamide; * N-acetylated, N-acetylated compounds (metabolites like N-acetylalanine, -glycine,-glutamine and many others with very similar chemical shifts).

Citrate increased at 10 days in all groups. A complete overview of the metabolites detected in the spectra is shown in table 3. An example of spectra for moderate/severe abnormal cGM score compared to the normal/mildly abnormal group of infants is shown in Figure 1.

Using the ^{1}H-NMR spectra, ROC curves were obtained. The ROC curves for prediction of moderate-severe WM abnormalities showed the following values: [2 d: AUC 0.71, specificity (SP) 80%, sensitivity (SE) 72% and 10 d: AUC 0.69, SP 64%, SE 89%] (Figure 2).

Figure 1 | Mean spectra of metabolites in patients with and without cGM moderate to severely abnormal MRI score (total cGM score ≥2) at 2 days (A) and 10 days (B) after birth.



Mean spectra of metabolites in patients with and without moderately/severely abnormal cGM MRI score (total cGM score ≥2) at 2 days (A) and 10 days (B) after birth. Horizontal axis shows ppm. Chemical shifts of relevant signals in ppm: Leucine 0.95 and 0.97; threonine 1.33; lactate 1.41; acetate 2.08; citrate 2.89; carnitine 3.22 and glycine 3.72. 0 = normal or mildly abnormal GM score, 1 = moderate-severely abnormal cGM score.

Figure 2 | ROC curves showing the predictive value of the panel of metabolites showed in table 3 for moderate/severely abnormal WM MRI score (total WM score ≥5).

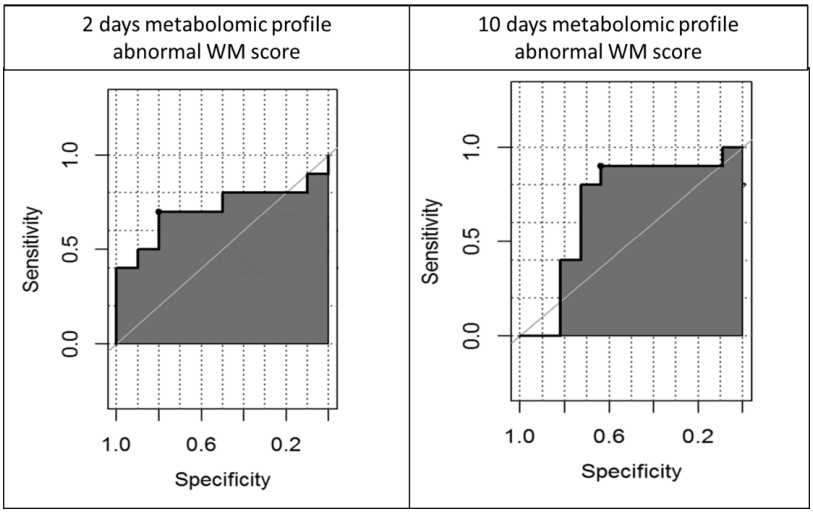
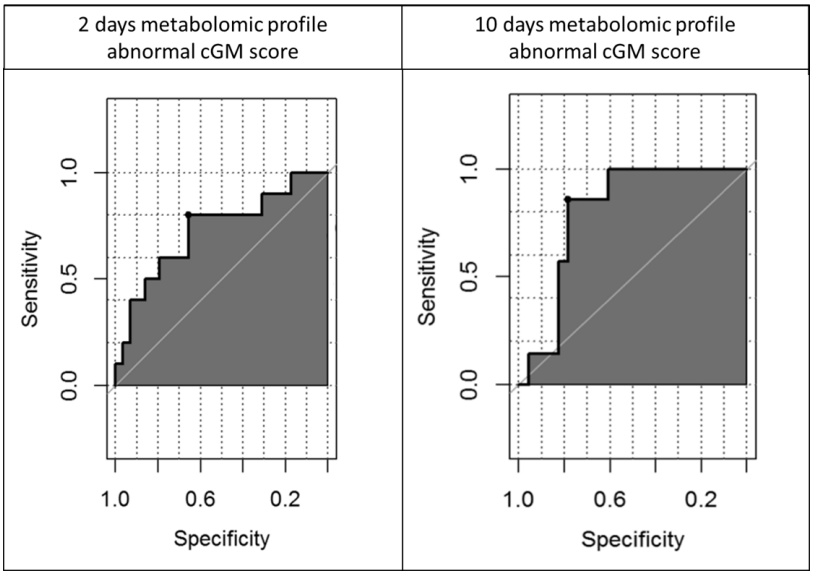


Figure 3 | ROC curves showing the predictive value of the panel of metabolites showed in table 3 for moderate/severely abnormal cGM MRI score (total cGM score ≥2).



The ROC curves distinguished neonates at both 2 and 10 days after birth who later developed markedly less mature cGM score from the others (with normal or mild abnormalities) [2 d: AUC 0.72, specificity (SP) 65%, sensitivity (SE) 75% and 10 d: AUC 0.80, SP 78%, SE 80%] (Figure 3).
Neither moderate/severe abnormal dGM score nor cerebellum score showed a significant association or could be predicted by the urinary metabolomics profile. Moreover we could not find any association/prediction between urinary metabolic profile and moderately to severely abnormal global MRI score.

Discussion

Specific urinary metabolic spectra of extremely preterm infants at 2 and 10 days after birth were followed by moderately to severely abnormal cGM and WM scores at TEA. This relation was stronger for cGM score compared to WM score with a higher specificity and sensitivity. Thus, the present study suggests, for the first time, that metabolomics can provide a valid metabolite signature potentially usable as prediction model in preterm infants. However, the PCA could not find a significant clustering of metabolic spectra for infants with moderately to severely abnormal MRI scores in all the evaluated regions (WM, cGM, dGM and cerebellum), since probably, the extent of change could depend on many factors and comorbidities such as prematurity related diseases (ROP, IVH, PDA, length of mechanical ventilation, sepsis).

Our results suggest that urine is a promising source of biomarkers for brain maturation and that, using metabolomics techniques in urine in the early postnatal period, is useful to identify infants at higher risk of brain disease. Sample collection is easy to do and free from clinical risk. Furthermore, different from blood, urine is always available in preterm newborns.
In our study, several significantly altered metabolites were identified, with different trends over time in infants with normal or mildly abnormal and moderately to severely abnormal MRI scores. These results demonstrate that probably the whole profile is more important for the prediction of MRI score than the single metabolites.

Analyzing differences in spectra between normal and mildly vs moderately to severely abnormal MRI score, we found that altered metabolites are mainly implicated in the important processes of energy metabolism, mithocondrial function and mielin production, all impaired in preterm infants.[18-21] In particular, we hypothesize that the increase in lactate and acetate in infants with WM abnormalities is due to activation of anaerobic glycolysis due to mitochondrial dysfunction and the degeneration of fatty acids, probably due to impaired cerebral blood autoregulation in preterm infants.[22] Developmental immaturity of the preterm brain circulation derives from both altered vasoreactivity and immaturity of vasoactive signaling and leads to impaired cerebral blood blow and consequently to intraventricular hemorrhage and ischemic injury to the white matter.[23] Lactate is generally increased after hypoxia, due to

the shift from aerobic to anaerobic metabolism.[24,25] Preterm infants are particularly exposed to cerebral hypoxia, especially in the first days after birth. The main reason is the hemodynamic instability secondary to the transition phase of the respiratory and circulatory systems.[26] Hypoxia may result in impaired neurodevelopmental outcome in preterm newborns.[25]

The white matter, is also particularly vulnerable to hypoxic-ischemic and oxidative injury, being sensitive to decreased blood supply.[25] Glycine and threonine increases have been implicated in hypoxia response, oxidative stress and inflammation, well known phenomena in preterm infants.[24] Both are glucogenic amino acids, which may be converted to pyruvate during protein metabolism. Increased levels of plasma glycine may be caused by reduced amino acid oxidation or reduced gluconeogenesis as a strategy to conserve amino acids.[27] In most mammals, the pre-postnatal transition is accompanied by important adaptations in carbohydrate metabolism due to the abrupt change from the placental supply of nutrients to a cyclic supply via breast milk, however, preterm infants experience enteral feeding difficulties in the neonatal period linked to the impaired gut function.[27] Furthermore glycine is a necessary substrate for the biosynthesis of reduced glutathione, the main antioxidant molecule in the brain.[28]

Although we believe that urine metabolomics represent a promising non-invasive approach to study brain diseases in the neonatal population, data are too limited to draw definite conclusions regarding the use of metabolomics profile in clinical practice. Potential confounders should be analyzed in detail and will benefit from studies on a larger number of patients to identify the effect of environmental factors and comorbidities on the metabolomics spectra. A validation of our results in a new and larger cohort of extremely preterm infants is also necessary to check reproducibility of spectra. Furthermore the effects of nutrition might have played an essential role, although all infants received the same parenteral nutrition solution and the increase in oral feeding was performed using the same steps, following the local protocol of the UMC Utrecht. Finally the lack of standards in both analytical methods as well as “normality ranges” for urine metabolites in newborns is a big limitation to this promising technique.[29]

Conclusions

Urine spectra of extremely preterm infants at 2 and 10 d discriminate patients with moderately to severely abnormal cGM score and WM score at TEA. The main metabolic changes could be connected to 2 different metabolic pathways: energy metabolism and protein metabolism. Thus, urine ^{1}H-NMR appears to be a promising tool for early identification of neonates at high risk of subnormal brain development and to better understand the multifactorial pathogenesis of neonatal brain injury. However, studies on a larger number of patients are needed to confirm our findings.

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part three

DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

8

Discussion and future directions

The general aim of this thesis was to describe the use of early clinical and biochemical biomarkers, or the combination of biomarkers, and its interpretation in a clinical setting for understanding and prediction of brain development and brain injury in preterm infants. To achieve this goal, we firstly evaluated the use of early biomarkers in clinical practice and the possible effect of confounders, such as medications, as described in **Part 1**. In **Part 2** the relation between single and combined clinical and biochemical biomarkers and their association with brain development are discussed. In **Part 3** the summary of the findings and future directions are reported.

Part 1 The use of biomarkers – clinical interpretation

Chapter 2 | The effect of morphine on aEEG/EEG

EEG and aEEG are increasingly used for neuromonitoring in preterm infants and many studies support the hypothesis that the quality of early brain activity, together with the quantitative assessment of aEEG/EEG metrics, are important for shaping neuronal connectivity during the last trimester of gestation.[1] SATs are a primary feature of the preterm EEG, they are represented by bursts of activity capable to guide the development of brain connections.[1,2] SATs correlate with faster growth of total brain volume and deep gray matter volume.[3] The presence of prolonged periods of brain inactivity (inter SAT (bursts) intervals (ISI)) is associated with poor outcome at 2 years of age.[4] In this context, the use of sedatives in the NICUs, such as morphine, known to depress aEEG/EEG activity, can potentially alter later brain development.[4-6] In a recent paper, the depressive effect was mainly seen on the total maturity score and the cycling subscore.[6]

Thus, we aimed to quantify the specific effect of morphine and its cumulative dose on quantitative aEEG/EEG measures in 174 extremely preterm infants born in three different European centers. Infants were monitored during 72 hours after birth with aEEG and epochs were selected from the recordings at specific time-points. Traces were authomatically analyzed to obtain quantitative aEEG/EEG measures. The results show that morphine administration and especially higher cumulative doses, have a significant effect on quantitative aEEG/EEG measures, causing depression of early brain activity in all epochs. Both morphine administration per se and an increase in the total amount, significantly decreased the number of SATs and increased periods of brain inactivity.

The results from **Chapter 2** suggest that morphine administration is likely to induce disturbances in brain activity which are also dose-dependent.[10] From a neurophysiological perspective, morphine administration may cause a sort of functional thalamo-cortical disconnection with

a suppressed EEG/aEEG activity.[6,7] Studies support the hypothesis that opioids administration can reduce neuronal density and dendritic length, as well as cause apoptosis in rodents and *in vivo* models of human microglia and neurons.[8,9] Moreover, sicker infants, who need mechanical ventilation for a longer period, receive higher doses of morphine to reduce stress and pain and are generally at higher risk for brain injury and long term developmental delay. However, there are not enough data to establish the role of morphine on short and long term neurological outcome of preterm infants. Thus, the advice is to perform an adequate and constant pain assessment in order to minimize the use and dose of sedative drugs in this high risk population.[10] Furthermore, our results suggest that sedative administration should always be considered when interpreting aEEG/EEG, since medication can affect its predictive value through the adverse effect on background activity.

To further explore the role of morphine and other sedatives on the preterm brain, future research should focus on the effects of sedatives on brain activity and development together with both short and long term neurodevelopmental outcome.

Chapter 3 | Oxidative stress

Extremely preterm newborns spend the last trimester of gestation in neonatal intensive care units, where they continue their development. During the last trimester of gestation, nervous system grows more than in any other life period.[11,12] In addition, development is susceptible to the "Free Radicals Disease" (FRD) that includes bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), necrotizing enterocolitis (NEC), and intraventricular haemorrhage (IVH).[12-14] Oxygen, while essential for life, may also generate toxic free radicals species that have the potential to cause cellular and organ damage.[15] The exact starting fraction of inspired oxygen (FiO_2) for delivery room resuscitation in preterm infants has become a matter of great debate over the last few years, especially because those infants usually need more oxygen due to their pulmonary immaturity.[16,17] The use of room air for neonatal resuscitation, for more than two or three minutes, leads to hypoxia. Thus, nowadays 21%-30% O_2 is suggested to start neonatal resuscitation in preterm infants.[18] However, in developing and low-resources countries many clinicians continue to use 100% oxygen, because they are still uncertain about the consequences of using less oxygen but also because of the high cost to provide blenders and oximeters in delivery rooms.[19]

In **Chapter 3** we provide the preliminary results of the TO2RPIDO (targeted Oxygen for the Resuscitation of Preterm infants and their Developmental Outcomes) study, enrolling preterm infants <32 weeks of completed gestation who are randomised at birth to receive either 100% oxygen or room air (www.anzctr.org.au). FiO_2 is targeted to pre-ductal SpO_2. Oxidative stress biomarkers were measured at birth, two and 12-hours after birth. Significantly higher levels of AOPP and isoprostanes concentrations were found in the 100% oxygen group.

Results form **Chapter 3** should alert clinicians that the use of 100% oxygen in the delivery room, even in a targeted approach, may cause lipid as well as protein damage. Pure oxygen had been freely used in delivery room resuscitation of newborn infants for more than two centuries,[20,21] but there is evidence, primarily derived from studies on asphyxiated full-term infants, suggesting that using less oxygen, is a feasible alternative and that this practice might even decrease oxidative stress and improve short and long-term outcome.[22,23] International resuscitation guidelines were changed from 2005 to recommend the use of less oxygen for the resuscitation of all newborn infants.[24,25] Significantly more information regarding the impact of these relatively new recommendations on a high-risk and already vulnerable population are therefore urgently required. In this study, we mainly focused on oxidative stress markers. Whether these changes are reflective of significant clinical outcomes could not be elucidated in the present report, since the recruitment of patients of the TO2RPIDO trial was still on-going when the laboratory analysis was conducted. Thus, well-designed studies on the specific relation between different FiO_2 during resuscitation in preterm infants and outcomes are urgently required since this has the potential to affect more than 15 million newborn infants each year.[26]

Chapter 4 | Cord blood biomarkers of placental disease

Histological chorioamnionitis (HCA) is responsible for approximately 60-70% of pregnancies complicated by preterm prelabor rupture of membranes (pPROM) and consequently preterm birth.[27] Histological analysis of placenta is a useful tool to detect the etiology and recurrent risk of pregnancy disorders. There is a paucity of information regarding the relationship between the presence of HCA or placental vascular underperfusion (VU) lesions, the most common risk factors for pPROM and preterm birth, and OS markers in the umbilical cord. In **Chapter 4** we investigated whether placental lesions, indicating HCA or VU, were associated with increased levels of OS biomarkers in cord blood. Thus, 120 neonates born below 30 weeks of gestational age were enrolled and their placenta was classified based on histological anaysis, while cord blood concentrations OS biomarkers were measured. OS biomarkers were significantly increased in HCA group compared to controls. Significant associations were also found between VU and oxidative injury to proteins, low GA and the presence of fetal growth restriction. The findings in **Chapter 4** strongly support the hypothesis that the increase in OS during pregnancy, in response to various maternal stimuli, is the final end-point of multiple mechanisms, such as hypoxia and infections. Fetal exposure to enhanced OS during pregnancies may contribute to altered placental function, impaired fetal growth and preterm delivery.[27] Thus, OS may be the general underlying mechanism linking adverse intrauterine environment with fetal programming.

Clinicians should be aware that hypoxia and inflammation during pregnancy may regulate placental development/function through OS, with plausible effects on fetal programming, and that those infants would probably benefit from antioxidant protection during pregnancy and immediately after birth.

Part 2 Combining biomarkers in clinical practice

Chapter 5 | Combining EEG and NIRS in the early neonatal period

The simultaneous assessment of NIRS and (a)EEG couples monitoring of oxygen supply and delivery to cerebral activity and could therefore yield new insights into brain metabolism and detect potentially vulnerable situations. Previous studies on the combination of NIRS with aEEG/EEG monitoring, showed that a higher cFTOE was associated with a narrower aEEG bandwidth, suggesting higher oxygen utilization to meet higher metabolic demand in case of a more mature aEEG/EEG.[28] In Chapter 4, the relationship between NIRS and quantitative EEG parameters such as SAT rate or ISI is investigated in the first few hours after birth in forty-four hemodynamically stable babies with a GA < 28 weeks. Both parameters of oxygen utilization and delivery were associated with quantitative EEG metrics. These results clearly underline that oxygen delivery and utilization are directly related to functional brain activity during the first hours after birth, with an increase in oxygen extraction in preterm infants with more active early electro-cerebral activity. Increased brain metabolism is accompanied by an increase in cerebral oxygen consumption and consequently by an increase in CBF as part of the so called neurovascular coupling.[29] Moreover, a significant association between superior vena cava flow and aEEG at 12 h after birth was reported.[30] Thus, hemodynamic changes and especially changes in CBF that occur immediately after birth, may affect cerebral circulation and also neuronal activity as shown in our results. Higher $rScO_2$ and lower cFTOE values were observed before brain injury became apparent, and these changes were highly indicative for subsequent development of a severe GMH-IVH.[31,32] When looking at aEEG/EEG, background activity is depressed during the first days after birth In the presence of a GMH-IVH and the extent of depression correlates with the degree of GMH-IVH.[33,34] Thus, the combined monitoring of cerebral oxygen delivery/utilization and brain activity can be useful for early identification of infants at risk of developing GMH-IVH; and these changes in continuous monitoring are seen before the injury became visible on ultrasound examination.[31,35] In this perspective, the results shown in **Chapter 5** suggest that the simultaneous monitoring of NIRS and aEEG and consequently of $rScO_2$/cFTOE and electrocerebral activity may be a noninvasive useful biomarker of brain function in high-risk, hemodynamically stable preterm infants.

Chapter 6 | Brain activity and brain development

The last trimester of gestation is characterized by huge fetal brain growth and development, where brain undergoes a 5-fold increase in volume and the majority of sulcal and gyral formation takes place.[31,36,37] A recent study by our group showed that increased electrical brain activity in preterm infants, in the first postnatal days, correlated with faster volumetric growth of total brain volume and deep gray matter up to term equivalent age.[3] The hypothesized correlation of brain functional and structural maturation creates an early potential interesting predictor of altered brain development. However, the effect of early brain activity on microstructural brain development was never investigated. The aim of **Chapter 6** was to evaluate the changes in brain morphology and microstructure, detected using serial MRI, in relation to early brain activity, monitored with aEEG/EEG in extremely preterm infants. Thus, a quantitative analysis of EEG metrics was performed in 33 preterm neonates who were continuously monitored with electroencephalography (EEG) during the first 48h of life. Infants underwent brain MRI around 30 and 40 weeks of postmenstrual age.

Results showed that increased early brain activity is associated with cerebellar and cortical growth, two structures with rapid development during preterm life and with higher microstrusctural growth in the CC. An approximately three-times increase in cerebellar volume from 28 to 40 weeks of gestation has been documented, making the cerebellum, more than the cortical gray matter, the brain structure showing the largest relative growth in this period.[26] This suggests that cerebellar and cortical growth are the most vulnerable to disturbances (in brain activity) in this early period of life. Both cerebellar and cortical alterations are associated with neurodevelopmental sequelae in the domains of cognition and motor development.[38-40] This study, suggesting developmental dependency upon early brain activity of both structures, puts forward that cortical alterations might already find their origin in the first period of life in extremely preterm neonates. Furthermore, increased brain activity was related to FA microstructural changes in the CC, a region responsible for the majority of inter-hemispheric connections. These results underline the importance of early life neuro-monitoring in extremely preterm neonates, in order to identify those at risk of altered brain development and should widen the standard use of aEEG/EEG in the neonatal period.

Chapter 7 | Urinary metabolomics to predict brain injury

Metabolomics has recently received a lot of attention due to its high sensitivity in detecting thousand of metabolites serving as biomarkers of many diseases.[41-43] Many studies support the hypothesis that urinary metabolomics, reflecting the state of the body, can give informative biomarkers for brain diseases such as Alzheimer's and Parkinson's disease, multiple sclerosis and stroke.[44-46] Thus, metabolomics in urine can be a potential, non-invasive source

of biomarkers of brain disease.[7, 8] Sample collection is easy to do and free from clinical risk. Furthermore, when looking at preterm infants, differently from blood, urine is always available in large amounts. In **Chapter 7**, we tested the hypothesis that urinary metabolic profile at 2 and 10 days after birth in preterm neonates could be predictive of abnormal MRI score at TEA, thus, if urine could be a source of biomarkers of brain disease.

ROC curves about urinary metabolic profile of thirty preterm infants could distinguish already at two and ten days after birth, neonates who later developed a markedly abnormal cGM and WM score from the others. Results from **Chapter 7** suggest that urine proton MR is a promising tool for early identification of neonates at high risk of brain damage and for a better understanding of the multifactorial pathogenesis of abnormal neonatal brain development. However, potential confounders should be analyzed in detail and later studies are needed to evaluate the effect of environmental factors and comorbidities on the metabolomics spectra.

Part 3 Conclusions and future directions

Conclusions

The results of the present thesis describe the use and potentialities of biomarkers, their combination and possible use in a clinical setting. Furthermore, they support the conclusion that the use of single or combined clinical and biochemical biomarkers can help clinicians to have more insight into brain development and injury and, thus, to predict outcome in extremely preterm infants.

Future directions

Despite the improvement in the surival rate of extremely preterm infants over the last decades, the incidence of abnormal neurodevelopmental outcome remains high and is a cause of important concern. MRI is currently the gold standard for the assessment of neonatal brain structure and development and is predictive for later neurodevelopmental outcome. However, MRI cannot be used bedside, is expensive and transport and sedation are difficult in critically ill infants. Therefore, cheap and bedside biomarkers allowing continous monitoring of brain development and maturation are urgently needed. Prediction of outcome and finding the best biomarker, or the best combination of biomarkers, able to predict brain development and injury in extremely preterm infants, are the most challenging and important aims for neonatologists and neonatal neurologists in the last decades.[47-49] Prediction of brain

injury and development is fundamental in order to warrant suriveillance and early intervention to this high risk group of patients. In this context, many attempts were made in the field of neonatology with the aim to identify those biomarkers.

Among all neuromonitoring tools, EEG is easily accessible and, especially aEEG, relatively easy to interpret, allowing for continuous cerebral function monitoring in neonates. This technique can be considered complementary to ultrasound and MRI, however, it is currently used as standard of care only in term infants with asphyxia. Nevertheless, in preterm infants there is increasing evidence that early aEEG/EEG are predictive of long-term neurological outcome,[50] but this does not yet influence decision making in this group of patients. For the future, we aim that cerebral function monitoring will become a standard tool in NICUs also for preterm infants, since we demonstrated that it can be an informative biomarker for later brain development.[51] In this perspective, it will be important to explore the relation between advanced MRI techniques, such as resting-state functional MRI (rsfMRI), used for the assessment of functional brain networks, and EEG detected brain function. Moreover, the combination of aEEG/EEG with NIRS could give clinicians valuable information about brain metabolism and could early detect potentially vulnerable conditions. Thus, we aim that further studies will be done on this correlation and on the prediction of long term clinical outcomes.

Besides neuromonitoring tools, plasma OS biomarkers have the potential to early identify newborns at high risk for brain damage. Nevertheless, there are few publications that validated the role of OS biomarkers of brain injury with more accurate brain damage assessment, such as brain MRI. The pathogenesis of perinatal brain damage in preterm infants is complex with multiple contributory pathways and mechanisms of injury. Thus, the use of combined, instead of single biomarkers will probably give more successful results. Our aim for the future is to combine multiple neuromonitoring tools, such as NIRS and aEEG/EEG, together with biochemical biomarkers of OS, in an common algorithm. This will give the chance to authomatically quantify brain acitvity, detect oxygen delivery and utilization, assess FRs injury to brain macromolecules and will thus increase the predictive power for the development of brain injury.

Finally, the present thesis showed some new potentials of the metabolomics as an incredible source of biomarkers possibly in relation to severe MRI brain injury. Metabolomics is becoming increasingly popular in the field of neonatology due to its unique potentialities which pave the way for future personalized neonatal care. This technique has the potential to photograph the interaction between the genome and the environment and thus, explore the metabolic status of an organism in different conditions determined by multiple factors such as drug treatments, environment, nutrition, gene expression. This can be important to generate specific biomarkers useful to guide diagnosis, monitor postnatal metabolic changes and probably identify new therapeutic targets for a future personalized therapy.[52,53]

Our study showed that metabolomics can be informative about the risk of abnormal brain development and to investigate on the pathogenesis of neonatal brain injury. Further studies are needed on the possibility of metabolomics to eventually predict long term outcome in preterm newborns, and possibily to design a tailored nutrition and individual preventive strategies based on the specific genetic profile of each newborn.

The discovery of a panel of early clinical and biochemical biomarkers, arising from a combination of different techniques, which can predict brain injury and development in preterm infants, will pave way for future research and hopefully therapeutic opportunities, such as antioxidants, for the developing brain.

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9

Summary

Nederlandse samenvatting

Prematuriteit is een belangrijke oorzaak van neonatale sterfte. Ongeveer 6% van de kinderen wordt extreem vroeg geboren (≤28 weken zwangerschapsduur), en zij lopen een risico van 25-50% op een veranderde hersenontwikkeling die zorgt voor een afwijkende neurologische ontwikkeling. Om die reden is er dringend behoefte aan vroege, non-invasieve en veilige biomarkers voor hersenontwikkeling om een voorspelling te kunnen doen over ontwikkeling, en om neuroprotectieve therapieen te beginnen. Het algemene doel van dit proefschift is om het gebruik van vroege klinische en biochemische biomarkers, of een combinatie hiervan, te beschrijven. Door de interpretatie van biomarkers kunnen we hersenontwikkeling en hersenschade in te vroeg geboren kinderen begrijpen en voorspellen. Om dit doel te bereiken, evalueerden we het gebruik van vroege biomarkers en de mogelijke effecten van confounders, zoals medicatie, beschreven in deel 1. Deel 2 bediscusseert de relatie tussen een enkele en een combinatie van klinische en biochemische biomarkers en hun associatie met hersenontwikkeling. Deel 3 bevat een samenvatting van de bevindingen en geeft aanbevelingen voor de toekomst.

Deel 1 Het gebruik van biomarkers – klinische interpretatie

Hoofdstuk 2 | Het effect van morfine op het aEEG/EEG

Het gebruik van EEG en aEEG is enorm toegenomen in de neuromonitoring van premature kinderen. Veel studies ondersteunen de hypothese dat de kwaliteit van vroege hersenactiviteit, samen met de kwantitatieve beoordeling van aEEG/EEG maten, belangrijk zijn voor de vorming van neuronale connectiviteit tijdens het laatste trimester van de zwangerschap. SATs zijn een primair kenmerk van het preterme EEG. SATs worden voorgesteld als pieken (bursts) van activiteit die de ontwikkeling van hersenverbindingen leiden. SATs hangen samen met snellere groei van het totale hersenvolume en het volume van de diepe grijze stof. De aanwezigheid van langere perioden van herseninactiviteit (inter SAT [bursts] intervals [ISI]) hangt juist samen met slechte neurologische uitkomst op 2-jarige leeftijd. Hiermee verband houdend is het gebruik van sedativa op de NICU, zoals morfine, die er om bekend staan om aEEG/EEG activiteit te verlagen, en mogelijk daardoor toekomstige hersenontwikkeling veranderen. Daarom hadden wij als doel om het precieze effect van morfine en toenemende doseringen van morfine, op kwantitatieve aEEG/EEG maten te meten in 174 extreem prematuur geboren kinderen uit drie verschillende Europese centra. De resultaten laten zien dat morfine toediening en vooral hogere doseringen van morfine een significant effect hebben

op kwantiatieve aEEG/EEG maten, leidend tot een verlaging van vroege hersenactiviteit. Vanuit een neurofysiologisch perspectief kan morfine toediening (door een verlaagde aEEG/EEG activiteit) mogelijk zorgen voor een soort functionele disconnectie tussen thalamus en cortex / thalamo-corticale disconnectie. Verschillende studies ondersteunen de hypothese dat opioiden zorgen voor een afname van neuronale dichtheid en lengte van de dendrieten. Daarbij is uit knaagdierstudies en humane *in vivo* modellen gebleken dat opioden zorgen voor apoptose van microglia en neuronen. Bovendien krijgen ziekere kinderen, die een langere periode beademingsbehoeftig zijn, hogere doses morfine om stress en pijn te verminderen. Deze kinderen hebben ook een hoger risico op hersenschade en een ontwikkelingsachtertand op de lange termijn. Het advies is om adequaat en continu pijn te beoordelen in deze hoogrisico populatie zodat het gebruik en de dosering van sedativa tot een minimum kan worden beperkt. Daarnaast wijzen onze resultaten erop dat de toediening van sedativa altijd in overweging moet worden genomen bij de interpretatie van het aEEG/EEG, omdat medicatie de voorspellende waarde kan beinvloeden door een ongunstig effect op het achtergrondpatroon. Toekomstig onderzoek zou zich moeten richten op de effecten van sedativa op hersenactiviteit en ontwikkeling, evenals neurologische ontwikkeling op de korte en lange termijn.

Hoofdstuk 3 | Oxidatieve stress

Zuurstof is essentieel voor het leven, maar kan ook de vorming van toxische vrije radicalen tot gevolg hebben. In prematuur geboren kinderen zouden deze kunnen leiden tot “Vrije Radicalen Ziekte” dat verschillende aandoeningen omvat waaronder bronchopulmonaire dysplasie (BPD), prematuren retinopathie (ROP), necrotiserende enterocolitis (NEC) en intraventriculaire bloeding (IVH). Toch hebben extreem vroeggeboren neonaten zuurstof nodig tijdens de opvang vanwege immaturiteit van de longen. Tegenwoordig wordt aanbevolen om te starten met 30% zuurstof tijdens de neonatale opvang van te vroeg geboren neonaten. In ontwikkelingslanden gebruiken veel clinici echter nog steeds 100% zuurstof, onder andere vanwege de hoge kosten van blenders en saturatiemeters in de verloskamers. In hoofdstuk 3 geven we de prelimiaire/voorlopige resultaten van de TO2RPIDO (Targeted Oxygen for Resuscitation of Preterm Infants and their Developmental Outcomes) studie, waarin premature neonaten <32 weken zwangerschapsduur werden gerandomiseerd bij geboorte voor toediening van 100% zuurstof of kamerlucht. In de 100% zuurstof groep werden significant hogere bloed concentraties advanced oxidative protein products (AOPP) en isoprostanen gevonden. De resultaten van hoofdstuk 3 moeten artsen er op attent maken dat het gebruik van 100% zuurstof in de verloskamer, zelfs bij een gerichte aanpak, kan zorgen voor lipide en eiwit schade. In de afgelopen twee eeuwen werd puur zuurstof vrijelijk gebruikt in de verloskamers bij de opvang van pasgeborenen, maar aanwijzingen uit studies met voldragen pasgeborenen met perinatale asfyxie suggereren dat het gebruik van minder zuurstof een haalbaar alternatief is.

Deze toepassing kan mogelijk ook oxidatieve stress verminderen en de korte en lange termijn uitkomst van te vroeg geboren neonaten verbeteren. Er is dringend meer informatie nodig over de impact van deze relatief nieuwe aanbevelingen voor een hoog-risico en reeds kwetsbare populatie. We hebben ons in deze studie met name gericht op oxidatieve stress markers. Of deze veranderingen een goede reflectie zijn van klinisch significante uitkomstmaten kon in het huidige artikel niet worden opgehelderd omdat het werven van patienten voor de TOR-2RPIDO studie nog niet was afgerond ten tijde van de laboratorium analyses. Er is dringend behoefte aan goed opgezette studies die de relatie tussen verschillen in zuurstof concentratie tijdens de opvang van te vroeg geboren neonaten en neurologische ontwikkeling onderzoeken, want dit zal ieder jaar meer dan 15 miljoen pasgeborenen betreffen.

Hoofdstuk 4 | Navelstreng biomarkers voor placentaire aandoeningen

Voor zwangerschappen met voortijdig gebroken vliezen (preterm premature rupture of membranes [=pPROM]) die leiden tot vroeggeboorte/prematuriteit, blijkt histologische chorioamnionitis (HCA) in 60-70% verantwoordelijk. Histologische analyse van de placenta is een bruikbaar hulpmiddel om de etiologie en het risico op herhaling van zwangerschapscomplicaties op te sporen. HCA en vasculaire ondervulling (vascular underperfusion [=VU]) zijn de meest voorkomende risicofactoren voor pPROM en vroeggeboorte. Er is echter een gesprek aan informatie over de relatie tussen het voorkomen van HCA of VU in de placenta en oxidative stress (OS) biomarkers in de navelstreng. In hoofdstuk 4 hebben we in 120 neonaten geboren onder een zwangerschapsduur van 30 weken onderzocht of placentaire schade, wijzend op HCA of VU, geassocieerd is met verhoogde OS biomarkers in het navelstrengbloed. OS biomarkers waren significant verhoogd in de HCA groep vergeleken met controles. Daarnaast vonden we significante relaties/associaties tussen VU en oxidatieve schade door eiwitten, lage zwangerschapsduur en de aanwezigheid van foetale groeiretardatie. De bevindingen van hoofdstuk 4 ondersteunen sterk de hypothese dat de toename van OS tijdens de zwangerschap, als gevolg van verschillende maternale stimuli, het definitieve eindpunt is van meerdere mechanismen, zoals hypoxie en infecties. De blootstelling van toegenomen OS aan de foetus tijdens de zwangerschap kan bijdragen aan veranderde placenta functie, vertraagde foetale groei en vroeggeboorte. Clinici moeten zich ervan bewust zijn dat hypoxie en inflammatie tijdens de zwangerschap de functie van de placenta kunnen beinvloeden via OS. Het is aannemelijk dat dit gevolgen heeft voor foetale programmering en neonaten met inflammatie en oxidatieve stress zullen daarom waarschijnlijk baat hebben bij antioxidant bescherming.

Deel 2 Het combineren van biomarkers in de praktijk

Hoofdstuk 5 | Combineren van EEG en NIRS in de vroege neonatale periode

Het gelijk toepassen van NIRS en (a)EEG monitoring van zuurstoftoediening en -afgifte aan cerebrale activiteit kan leiden tot nieuwe inzichten in het metabolisme in de hersenen en het opsporen van mogelijke kwetsbare situaties. Eerdere studies naar de combinatie van NIRS met aEEG/EEG monitoring lieten zien dat een hogere cFTOE was geassocieerd met een smallere aEEG bandbreedte, wat suggereerde dat meer zuurstufgebruik nodig was voor de hogere metabolische vraag in het geval van een meer ontwikkelde aEEG/EEG. In hoofdstuk 5 is de relatie tussen NIRS en kwantitatieve EEG parameters zoals SAT rate of ISI onderzocht in de eerste uren na geboorte in 44 baby's met een GA < 28 weken. Beide parameters van zuurstofgebruik en-afgifte waren geassocieerd met kwantitatieve EEG resultaten. Deze resultaten laten duidelijk zien dat zuurstofafgifte en-gebruik direct gerelateerd zijn aan functionele hersenactiviteit gedurende de eerste uren na de geboorte, met een verhoogde zuurstofextractie in te vroeg geboren baby's met meer actieve vroege elektro cerebrale activiteit. Verhoogd metabolisme in de hersenen gaat gepaard met een verhoogde cerebrale zuurstofconsumptie en ook door een verhoogde van de cerebral blood flow (CBF) als deel van zo genoemde neurovasculaire verbinding. Bovendien werd een significante associatie tussen de superior vena cava flow en aEEG op 12 uur na de geboorte gezien. Daarom kunnen hemodynamische veranderingen en met name veranderingen in CBF die direct na de geboorte plaatsvinden de cerebrale circulatie beïnvloeden. Hogere $rScO_2$ en lagere cFTOE waarden werden gezien voor hersenschade duidelijk werd en deze veranderingen waren hoog indicatief voor daaropvolgende ontwikkeling van een ernstig GMH-IVH. Wanneer we naar aEEG/EEG kijken, wordt de achtergrond activiteit onderdrukt gedurende de eerste dagen na de geboorte in aanwezigheid van een GMH-IVH en de mate van depressie correleert aan de maate van GMH-IVH. Deze veranderingen in continue monitoring werden gezien voordat schade zichtbaar werd op ultransound examinatie. In dit perspectief suggereren de resultaten in hoofdstuk 5 dat de simultane monitoring van NIRS en aEEG en daaropvolgend van $rScO_2$/cFTOE en elektrocerebrale activiteit noninvasieve bruikbare biomarkers zijn voor hersenfunctie in te vroeg geboren baby's met hoog risico.

Hoofdstuk 6 | Hersenactiviteit en hersenontwikkeling

Het laatste trimester van de zwangerschap wordt gekarakteriseerd door hoge fetale hersengroei en-ontwikkeling, waar de hersenen een vijfvoudige toename in volume en het grootste deel van de sulcale en gyrale formatie plaatsvindt. Een recente studie door onze onderzoeksgroep toonde aan dat verhoogde elektrische hersenactiviteit in te vroeg geboren baby's in de eerste postnatale dagen correleerde aan snellere volumetrische groei van het totale hersenvolume en diepe grijze massa tot aan de leeftijd equivalent aan de geboorteleeftijd. De verwachte correlatie van functionele en structurele hersenvolgroeiing creëert een vroege interessante voorspeller van veranderde hersenontwikkeling. Het doel van hoofdstuk 6 was om te evalueren of veranderingen in hersen morfologie en microstructuur, gedetecteerd door seriële MRI te gebruiken, in relatie tot vroege hersenactiviteit, gemonitord kan worden met aEEG/EEG in extreem vroeg geboren kinderen. Daarom werd kwantitatieve analyse met continue monitoring via EEG gedaan in 33 neonaten gedurende de eerste 48 uur na de geboorte. Daarnaast ondergingen ze seriële MRI. De resultaten gaven aan dat verhoogde vroege hersensactiviteit geassocieerd werd met groei van het cerebellum en de cortex. Dit zijn twee hersendelen met snelle ontwikkeling voor de geboorte en met hogere micro structurele groei in het CC. Een ongeveer drievoudig hogere toename van het volume van het cerebellum van 28 tot 40 weken werd gedetecteerd. Dit maakt het cerebellum, meer dan de corticale grijze massa, de hersenstructuur met de grootste relatieve groei in deze periode. Dit suggereert dat toename van het cerebellum en de cortex de meest kwetsbare hersendelen zijn voor verstoringen in deze vroege periode van leven. Veranderingen in het cerebellum en de cortex zijn geassocieerd met neurodevelopmentale restverschijnselen in de domeinen van cognitie en motorische ontwikkeling. Deze studie suggereert dat afhankelijkheid van de ontwikkeling van bij vroege hersenactiviteit van beide hersendelen, corticale veranderingen naar voren brengt die mogelijk al de oorsprong vinden in de eerste periode van het leven van extreem vroeg geboren neonaten. Bovendien was verhoogde hersenactiviteit gerelateerd aan FA micro structurele veranderingen in het CC, een regio verantwoordelijk voor het grootste deel van de interhemispherische connecties. Deze resultaten onderstrepen het belang van hersenmonitoring op vroege leeftijd in extreem vroeggeboren neonaten om het risico van veranderde hersenactiviteit in kaart te brengen en zouden het standaard gebruik van aEEG/EEG moeten laten toenemen in de neonatale periode.

Hoofdstuk 7 | Urine metabolomics om hersenschade te voorspellen

Metabolomics heeft kortgeleden veel aandacht gekregen door de hoge sensitiviteit in het detecteren van duizenden metabolieten, die biomarkers kunnen zijn voor vele ziekten. Veel studies ondersteunen de hypothese dat urine metabolomics, kunnen dienen als informatieve biomarkers door de staat van het lichaam weer te geven voor hersenziekten zoals Alzheimer en Parkinson, MS en beroertes. Metabolomics kan dus een mogelijke, non-invasieve biomarker zijn voor hersenziekten; verzameling van monsters is gemakkelijk en vrij van klinisch risico. In hoofdstuk 7 onderzochten we de hypothese dat het metabole urineprofiel van 30 te vroeg geboren baby's op 2 en 10 dagen na de geboorte voorspellend kan zijn voor abnormale MRI scores op TEA, dus of de urine een bron van biomarkers kan zijn bij hersenziekten. ROC curves van het metabole urineprofiel konden op dag twee en tien neonaten die later een abnormale cGM en WM score ontwikkelden opsporen. Resultaten van hoofdstuk 7 suggereren dat urine metabolomics een veelbelovende tool is voor vroege identificatie van neonaten met hoog risico op hersenschade en voor een beter begrip van de multifactoriële pathogenese van abnormaal neonatale hersenontwikkeling. Potentiële confounders moeten nog geanalyseerd worden tot in detail en grotere onderzoeken zijn nodig voor het effect van omgevingsfactoren en comorbiditeiten in metabolomics spectra.

Conclusie

De resultaten van de huidige thesis beschrijven het gebruik en de mogelijkheden van biomarkers, de combinatie en het mogelijk gebruik in de klinische setting. Bovendien ondersteunen ze de conclusie dat gebruik van een of gecombineerde klinische en biochemische biomarkers artsen kan helpen om meer inzicht in de hersenontwikkeling en -schade te krijgen en dus helpen bij het voorspellen van uitkomsten in extreem vroeggeboren baby's.

Riassunto in Italiano

La prematurità è la prima causa di morte nel periodo neonatale. Circa il 6% delle neonati nasce estremamente pretermine (≤ 28 settimane di gestazione); di questi, il 25-50% é a rischio di alterato sviluppo cerebrale e danno neuroevolutivo a lungo termine. Pertanto, l'uso e la validazione di biomarkers precoci, non invasivi e privi di rischio, in grado di predire lo sviluppo e la maturazione cerebrale sono fortemente necessari al fine di avviare strategie neuroprotettive precoci. L'obiettivo generale di questa tesi è stato quello di descrivere l'uso precoce di biomarkers clinici e biochimici, in combinazione tra loro o singolarmente e la loro interpretazione clinica per comprendere e predire lo sviluppo e il danno cerebrale nei neonati estremamente pretermine. Per raggiungere questo obiettivo, abbiamo valutato in primo luogo l'utilizzo di biomarkers precoci nella pratica clinica e il possibile effetto di eventuali fattori confondenti, come ad esempio i farmaci, come descritto nella PARTE 1. Nella PARTE 2 é invece stata discussa la relazione tra biomarkers clinici e biochimici utilizzati singolarmente o in combinazione tra loro e la loro associazione con lo sviluppo cerebrale. Nella PARTE 3 sono stati discussi I risultati ottenuti e valutate le potenziali implicazioni future.

Parte 1 L'uso di biomarcatori – interpretazione clinica

Capitolo 2 | L'effetto della morfina sull'aEEG/EEG

L'aEEG e l'EEG sono sempre più utilizzati per il monitoraggio neurologico nei neonati prematuri e molti studi sostengono che la qualità dell'attività cerebrale, nelle prime ore di vita, e la sua valutazione quantitativa, siano importanti per la lo sviluppo delle connessioni neuronali durante l'ultimo trimestre della gestazione. Le SATs (spontaneous activity transients) rappresentano la caratteristica primaria dell'EEG dei neonati pretermine. Le SATs consistono in burst di attivitá elettrica, in grado di guidare lo sviluppo delle connessioni cerebrali. Il numero di SATs correla inoltre, con una crescita maggiore del volume cerebrale totale e del volume della sostanza grigia profonda. La presenza di periodi prolungati di inattività del cervello (intervalli inter-SAT (bursts) (ISI)) è invece associata ad outcome neurologico avverso a 2 anni di età. In questo contesto, l'uso di farmaci sedativi, come la morfina, nelle NICU, determinando depressione dell'attività cerebrale, puó potenzialmente alterare lo sviluppo del cerebrale nelle etá successive. Pertanto, abbiamo cercato di quantificare l'effetto specifico della morfina e della sua dose cumulativa sull'analisi quantitativa dell'aEEG/EEG in 174 neonati estremamente pretermine nati in tre diversi centri europei. I risultati mostrano che la somministrazione di morfina, e soprattutto in dosi più elevate, ha un effetto significativo sull'aEEG/EEG, causando

la depressione dell'attività cerebrale. Da una punto di vista neurofisiologico, la somministrazione di morfina può causare una sorta di disconnessione funzionale talalamo-corticale con una depression dell'attivitá all'EEG. Alcuni studi sostengono che la somministrazione di oppioidi possa ridurre la densità neuronale e la lunghezza dendritica, nonché causare apoptosi neuronale nei roditori e in modelli di microglia e neuroni umani. Inoltre, i neonati che necessitano di ventilazione meccanica per un periodo prolungato, ricevono dosi elevate di morfina per ridurre lo stress e il dolore e sono generalmente a maggior rischio di sviluppare lesioni cerebrali e ritardo di sviluppo neuromotorio a lungo termine. Il consiglio è quello di eseguire una valutazione del dolore adeguata e costante al fine di ridurre al minimo l'uso e la dose di farmaci sedativi in questa popolazione ad alto rischio. Inoltre, i nostri risultati suggeriscono che la somministrazioone di tali farmaci dovrebbe essere sempre tenuta in considerazione quando si interpreta l'aEEG/EEG poiché puó influenzare il valore predittivo di tali strumenti attraverso l'effetto depressivo sull'attività di fondo. In futuro, la ricerca scientifica dovrebbe concentrarsi sugli effetti a breve e lungo termine dei farmaci sedativi sull'attività e sullo sviluppo cerebrale dei neonati pretermine.

Capitolo 3 | Lo stress ossidativo

L'ossigeno, pur essendo essenziale per la vita, può anche generare specie tossiche di radicali liberi e, nei neonati prematuri, ha la potenzialità di causare la "malattia libera dei radicali" (FRD) che comprende la displasia broncopolmonare (BPD), la retinopatia della prematurità (ROP), l'enterocolite necrotizzante (NEC) e l'emorragia intraventricolare (IVH). Tuttavia, i neonati estremamente pretermine hanno bisogno di ossigeno durante la rianimazione a causa dell'immaturità polmonare. Attualmente, la percentuale di ossigeno suggerita per la rianimazione dei neonati pretermine é del 30%. Tuttavia, nei paesi in via di sviluppo, molti medici continuano ad utilizzare l'ossigeno al 100% anche a causa dei costi elevati per l'acquisto di blender e pulsiossimetri nelle sale parto. Nel capitolo 3 sono mostrati i risultati preliminari dello studio TO2RPIDO, che ha arruolato neonati prematuri <32 settimane di gestazione, randomizzati alla nascita per ricevere ossigeno al 100% o aria ambiente in sala parto. Livelli significativamente più alti di AOPP e isoprostani sono state osservati nel gruppo al 100% di ossigeno. I risultati del Capitolo 3 suggeriscono che l'uso di ossigeno al 100% in sala parto, anche in un approccio "targeted", può causare danni significativi a lipidi e proteine. Il 100% di ossigeno é stato liberamente utilizzato nella rianimazione dei neonati per più di due secoli, ma ci sono prove concrete, prevalentemente su neonati a termine con asfissia, che l'uso di una percentuale inferiore si ossigeno sia un'alternativa sicura e che questa pratica potrebbe anche ridurre lo stress ossidativo e migliorare l'outcome a breve e lungo termine. Sarebbe importante valutare l'impatto di tali raccomandazioni su una popolazione ad alto rischio e già vulnerabile come i neonatoi estremamente pretermine. In questo studio, ci siamo con-

centrati principalmente su biomarkers di stress ossidativo. Se questi cambiamenti riflettano significativi risultati clinici non é possbile chiarirlo mediante il presente studio, anche perché l'arruolamento dei pazienti per lo studio TO2RPIDO era ancora in corso quando è stata condotta l'analisi di laboratorio. Quindi, sono necessari studi sulla relazione specifica tra diverse FiO2 durante la rianimazione nei neonati prematuri e l'outcome a breve e lungo termine, tali risultati avrebbero il potere di influire sul trattamento di più di 15 milioni di neonati ogni anno.

Capitolo 4 | Biomarkers plasmatici di patologia placentare

La corioamnionite istologica (HCA) è responsabile di circa il 60-70% di rottura prematura delle membrane (pPROM) e conseguentemente di parto pretermine. L'analisi istologica della placenta è uno strumento utile per valutare l'eziologia e il rischio ricorrente di tali complicanze. Attualmente esiste una scarsa quantità di informazioni riguardanti la relazione tra la presenza di lesioni di HCA o ipoperfusione vascolare placentare (VU), i fattori di rischio più comuni per la pPROM, e la nascita pretermine, e i biomarkers di stress ossidativo nel cordone ombelicale. Nel capitolo 4 viene valutato se le lesioni placentari, indicanti HCA o VU, siano associate a livelli più elevati di biomarkers di stress ossidativo nel sangue cordonale, in 120 neonati nati con etá gestazionale inferiore alle 30 settimane. I biomarkers di stress ossidativo sono risultati significativamente aumentati nel gruppo HCA rispetto ai controlli. Sono state inoltre osservate anche associazioni significative tra VU e danno ossidativo proteico, bassa etá gestazionale e presenza di ritardo di crescita intrauterina. I risultati del Capitolo 4 sostengono fortemente l'ipotesi che l'aumento dello stress ossidativo, durante la gravidanza, in risposta a vari stimoli materni, sia lo step finale di una catena di insulti multipli, come ipossia e infezioni. L'esposizione fetale ad elevato stress ossidativo durante la gravidanza può contribuire ad alterare la funzione placentare e a ridurre la crescita fetale. I clinici dovrebbero essere consapevoli che l'ipossia e l'infiammazione durante la gravidanza possono regolare lo sviluppo/funzionalità placentare attraverso lo stess ossidativo e la produzione di radicali liberi in eccesso, con effetti sul programming fetale. Questi neonati potrebbero probabilmente beneficiare di evebtuali terapie neuroprotettive antiossidanti.

Parte 2 L'utilizzo combinato dei biomarkers nella pratica clinica

Capitolo 5 | L'uso combinato di EEG e NIRS in epoca neonatale precoce

La valutazione simultanea di NIRS ed aEEG/EEG permettono il monitoraggio dell'apporto e dell'utilizzo di ossigeno da parte del sistema nervoso centrale e potrebbero quindi fornire importanti informazioni circa il metabolismo cerebrale nonché individuare situazioni potenzialmente a rischio. Studi precedenti sull'utilizzo combinato di NIRS ed aEEG/EEG hanno mostrato che, in presenza di una cFTOE elevata, si osserva un a tracciato CFM con ampiezza inferiore. Ció suggerrebbe un maggiore utilizzo dell'ossigeno per soddisfare una domanda metabolica superiore in caso di un aEEG/EEG maggiormente maturo. Nel capitolo 4 é stata valitata la relazione tra NIRS e parametri quantitativi di EEG come il numero SAT per minuto o l'ISI in secondi, nelle prime ore dopo la nascita in quarantacinque bambini con etá gestazionale <28 settimane. Entrambe le variabli di apporto ed utilizzo di ossigeno, valutati con la NIRS, sono risultati essere associati alle variabili quantitative di EEG. Questi risultati sottolineano chiaramente come l'apporto e l'utilizzo di ossigeno siano direttamente correlati all'attività cerebrale durante le prime ore dopo la nascita, con un aumento dell'estrazione di ossigeno nei neonati prematuri con attività elettrica cerebrale maggiore. L'aumento del metabolismo cerebrale è accompagnato da un aumento del consumo di ossigeno cerebrale e quindi da un aumento del flusso ematico, come parte del cosiddetto accoppiamento neurovascolare. Inoltre, è stata riportata una significativa associazione tra il flusso ematico in vena cava superiore e l'aEEG 12 h dopo la nascita. In tal modo, i cambiamenti emodinamici che si verificano immediatamente dopo la nascita possono influenzare la circolazione cerebrale ed anche l'attività neuronale, come mostrato nei nostri risultati. Valori piú elevati di $rScO_2$ e inferiori di cFTOE sono stati osservati in pazienti che avrebbero in seguito sviluppato emorraggia intraventricolare di grado severo. Quando si esamina l'aEEG/EEG, l'attività di base è depressa durante i primi giorni dopo la nascita in presenza di GMH-IVH ed il grado di depressione dell'attivitá di fondo, correla con il grado di GMH-IVH. Inoltre, queste modifiche sono visibili mediante neuromonitoring, prima che l'emorraggia venga evidenziata con esame ultrasonografico. I risultati mostrati nel Capitolo 5 suggeriscono che il monitoraggio simultaneo utilizzando NIRS e aEEG e di conseguenza mediante utilizzo di $rScO_2$/cFTOE e dell'attività elettrocerebrale può essere un valido biomarker non invasivo di fuonzionalitá cerebrale in neonati prematuri ad alto rischio.

Capitolo 6 | Attività e sviluppo cerebrale

L'ultimo trimestre della gestazione è caratterizzato da un'enorme crescita e sviluppo del sistema nervoso centrale del feto, che si traduce in un aumento di volume di 5 volte con la formazione di solchi e giri. Un recente studio ha dimostrato come l'aumento dell'attività elettrica corticale nei neonati prematuri, nei primi giorni dopo la nascita, correli con una maggiore crescita volumetrica cerebrale e della sostanza grigia profonda al raggiungemnto del termine. La correlazione ipotizzata tra la maturazione funzionale e strutturale del cervello crea un potenziale biomarker precoce di alterato sviluppo cerebrale. Lo scopo del Capitolo 6 é stato quello di valutare i cambiamenti morfologici e microstrutturali cerebrali, rilevati utilizzando la risonanze magnetiche seriate, in relazione all'attività cerebrale precoce, monitorata mediante aEEG/EEG in neonati estremamente pretermine. L'analisi quantitativa dell'EEG è stata eseguita in 33 neonati pretermine che sono stati continuamente monitorati con EEG durante le prime 48h di vita e sottoposti a MRI seriate. I risultati hanno mostrato che la presenza precoce di maggiore attività cerebrale era associata a maggiore crescita cerebellare e corticale nonché a migliore sviluppo microstrutturale del CC. Il cervelletto e la corteccia cerebrale sono due strutture in rapido sviluppo durante la vita prenatale ed in particolare nell'ultimo trimestre di gestazione. È stato documentato un aumento di circa tre volte del volume cerebellare tra le 28 e le 40 settimane di gestazione, ció rende il cervelletto, ancor più che la sostanza grigia corticale, la struttura con maggiore crescita relativa in questo periodo. Si ritiene pertanto che queste due strutture, proprio a causa del lotro massivo sviluppo in questa fase evolutiva, siano anche maggiormente vulnerabili ai danni della prematuritá. Infine, alterazioni a carico di cervelletto e corteccia, sono associate a sequele neuroevloutive di natura sia cognitiva che motoria. Questo studio, suggerendo l'esistenza di una relazione tra attività cerebrale precoce e sviluppo di queste strutture, mette in evidenza come le alterazioni corticali potrebbero già trovare la loro origine nel primo periodo di vita nei neonati estremamente pretermine. Inoltre, l'aumento dell'attività corticale é associato ai cambiamenti microstrutturali di FA nel CC, una regione responsabile della maggior parte delle connessioni inter-emisferiche. Questi risultati sottolineano l'importanza del neuromonitoraggio precoce anche nei neonati estremamente pretermine, al fine di identificare quelli a rischio di alterato sviluppo cerebrale e suggeriscono pertanto l'ampliamento dell'utilizzo standard dell'aEEG/EEG nel periodo neonatale.

Capitolo 7 | L'uso della metabolomica per predire il danno cerebrale

La metabolomica ha recentemente ricevuto molta attenzione dalla comunitá scientifica a causa della sua elevata sensibilità nel rilevare migliaia di metaboliti utili come biomarkers di numerose patologie. Molti studi sostengono l'ipotesi che lo studio metabolomico nelle urine, riflettendo le la situazione metabolica dell'organismo, possa servire da biomarker anche per numerose malattie cerebrali come la malattia di Alzheimer e di Parkinson, la sclerosi multipla e l'ictus. La metabolomica urinaria fornirebbe dunque potenziali biomarkers non invasivi di malattia cerebrale; anche in quanto la raccolta dei campioni è facile e priva di rischi clinici. Nel capitolo 7 abbiamo testato l'ipotesi che il profilo metabolico urinario a 2 e 10 giorni dopo la nascita nei neonati pretermine possa essere predittivo di alterato score di risonanza magnetica cerebrale al raggiungimento del termine. Le curve ROC sono state in grado di distinguere già a due e dieci giorni dopo la nascita, i neonati che successivamente avrebbero sviluppato un punteggio di cGM e WM gravemente alterato rispetto dagli altri. Lo studio é stato effettuato sulle urine di trenta neonati pretermine. I risultati del Capitolo 7 suggeriscono che la metabolomica urinaria sia uno strumento promettente per la precoce identificazione dei neonati ad alto rischio di danno cerebrale e per una migliore comprensione della patogenesi multifattoriale di alterato sviluppo cerebrale. Tuttavia, i potenziali fattori confondenti dovrebbero essere analizzati in dettaglio e sono pertanto necessari studi su un maggior numero di campioni, al fine di valutare l'effetto dei fattori ambientali e delle comorbidità sugli spettri metabolomici.

Conclusioni

I risultati della presente tesi descrivono l'uso e le potenzialità dei biomarkers ed il loro possibile utilizzo singolo o combinato nella pratica clinica. Inoltre, l'utilizzo di biomarkers clinici e biochimici singoli o in associazione, può essere di aiuto ai clinici al fine di comprendere a fondo la patogenesi multifattoriale dell'alterato sviluppo cerebrale e di poter quindi eventualmente predire l'outcome neuroevolutivo nei neonati estremamente pretermine.

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List of abbreviations

1H-NMR	Proton magnetic resonance spectroscopy
% of time <5µV	Percentage of time spent <5 µV
aEEG	Amplitude-integrated EEG
AOPP	Advanced Oxidative Protein Products
BE	Base Excess
BPD	Bronchopulmonary dysplasia
BW	Birth weight
CBF	Cerebral blood flow
CC	Corpus callosum
cFTOE	Cerebral fractional tissue oxygen extraction
cGM	Cortical grey matter
CI	Confidence interval
CPAP	Continuous positive airway pressure
CR	Corona radiate
cTCD	Coronal transverse-cerebellar diameter
dGM	Deep grey matter
EEG	Electroencephalography
FA	Fractional anisotropy
FiO2,	Fractional inspired oxygen
FRs	Free radicals
GA	Gestational age
GI	Gyrification index
GMH-IVH	Germinal matrix-intraventricular hemorrhage
Hb	Hemoglobin
HCA	Histological chorioamnionitis
HCO_3^-	Bicarbonate
HFO	High frequency oscillatory ventilation
HIE	Hypoxic-ischemic encephalopathy
HPLC	High performance liquid cromatography
IHD	Inter-hemispheric distance
IR	Interquartile range
ISI	InterSAT interval
IsoPs	F2-Isoprostanes

IVH	Intraventricular hemorrhage
min aEEG	Minimum amplitude of aEEG
MS	Mass spectrometry
MRI	Magnetic resonance imaging
NAA	N-acetylaspartate
NEC	Necrotizing enterocolitis
NEOBRAIN	Neonatal estimation of brain damage risk and identification of neuroprotectants
NICU	Neonatal intensive care unit
NIRS	Near-InfraRed spectroscopy
NLEO	Nonlinear energy operator
NPBI	Non-Protein Bound Iron
OS	Oxidative stress
$PaCO_2$	Partial pressure of carbon dioxide
PCA	Principal Components Analysis
PDA	Patent ductus arteriosus
PHVD	Post-hemorrhagic ventricular dilatation
PLIC	Posterior limb of the internal capsule
PLSDA	Partial least square discriminant analysis
PMA	Post-menstrual age
PNA	Postnatal age
pPROM	Preterm prelabor rupture of membranes
RA	Room air
ROC	Receiver-operating characteristic
ROP	Retinopathy of prematurity
$rScO_2$	Regional cerebral oxygen saturation
SAT	Spontaneous activity transients
SATrate	SAT/min
SD	Standard deviation
SGA	Small for gestational age
SIMV	Synchronized intermittent mandatory ventilation
SPO_2	Peripheral oxygen saturation
TEA	Term equivalent age
VU	Vascular underperfusion
WM	White matter
WMI	White matter injury

Curriculum Vitae

Maria Luisa Tataranno was born on March 9, 1985 in Policoro, Italy. She grew up in Bernalda, a small village in the South of Italy, with her parents and two brothers. After gradutating scientific high school in 2003 with a score of 100/100, she moved to Siena to start medical school at the University of Siena. During her medical training, she discovered her love for traveling and exploring different cultures, thus she performed two clinical internships abroad: the first one in pediatrics at the *Pequeno Anjo* University Horpital in Itajaì, S. Catarina, Brasil, under the supervision of prof. Jose Alfonso Monestel Montoya and the second one in neonatology at the Civil Hospital *Fray Antonio Alcalde* in Guadalajara, Mexico, under the supervision of prof. Teresita De Jesus Peregrina Sandoval. She graduated medical school magna cum laude and in 2009 and she started her residency program in Pediatrics in 2010 at the University of Siena. Because of her passion for research, she decided to move to The Netherlands, at the department of neonatology of the Wilhelmina Children's Hospital in 2013, for a research fellowship of 15 months, under the supervision on prof. Manon Benders. During this research period, she started her PhD on biomarkers of brain development at UMC Utrecht under the supervision of prof. Manon Benders, prof. Linda de Vries, dr. Floris Groenendaal and dr. Jeroen Dudink. She did a clinical fellowship of 2 months at the Santobono-Pausilipon Children's Hospital in Naples. In May 2015, Maria Luisa graduated cum laude in Pediatrics and Neonatology at the University of Siena. The same year she started to work as a neonatologist at the neonatal intensive care unit of the S. Salvatore Hospital in L'Aquila and she always continued to work on her PhD project. In July 2016, she was offered a position as a fellow on neonatal neurology at the Wilhelmina Children's Hospital in Utrecht and moved again to The Netherlands, where she is currently working. In October 2016, she became the secretary of the Italian study group of clinical biochemistry.

Acknowledgements

"I am out with lanterns, looking for myself"

Emily Dickinson

Looking at the white page, how can I summarize these last 4 years in a few words? How can I be able to tell what this period meant to me and how important it was? When I look back what I see is a different me. The PhD years made me grow up together with my project. I had difficult moments and moments that I loved my project, with all my strength. There were moments that I thought about quitting with everything. But then, imagining my life without research, made me feel so empty that I decided to start working again, with a new spirit, a new energy, a new belief. And everytime I could not find a reason to go on, I found it in the encouragement of a colleague, in that patient for whom I hoped I could do more, in a coffee break with nice people, in the words of a good friend, in the enthusiasm of my supervisors, and in the support of my family who strongly believed that I could make it. Those last 4 years were so intense, so full of feelings and happenings inside and outside myself that I can easily say that this was the most difficult, exciting, frightening, inspiring and challenging period of my career.

"The mind is not a vessel to be filled, but a fire to be kindled"

Plutarch

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"If you want to go fast, go alone.
If you want to go far, go together"

African proverb

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"Happiness is only real when it is shared"

Cristopher McCandless

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"Having somewhere to go is home.
Having someone to love is family.
Having both is a blessing"

Unknown

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"If you love a flower that lives on a star,
it is sweet to look at the sky at night.
All the stars are a-bloom with flowers"

Antoine de Saint-Exupéry

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Notes

Notes

“Non lasciarti tentare dai campioni dell’infelicità, della mutria cretina, della serietà ignorante. Sii allegro. [...] T’insegneranno a non splendere. E tu splendi, invece.”

Pier Paolo Pasolini
Lettere Luterane