FACULTY OF HEALTH AND MEDICAL SCIENCES
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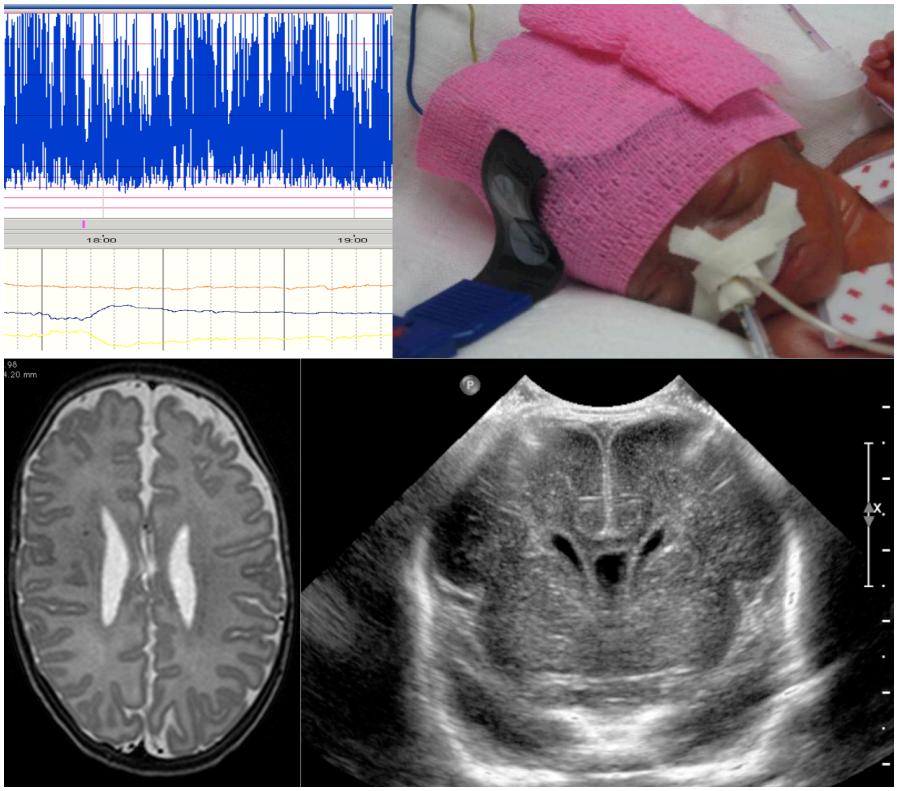
MEASURING THE EFFECT OF EARLY CEREBRAL HYPO- AND HYPEROXIA IN EXTREMELY PRETERM INFANTS



PhD thesis by Anne Mette Plomgaard

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Titel og evt. undertitel:	Måling af effekten af tidlig cerebral hypo- og hyperoxi hos ekstremt præmature børn
Title / Subtitle:	Measuring the effect of early cerebral hypo- and hyperoxia in extremely preterm infants
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Submitted:	January 30, 2016

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Acknowledgments

This thesis was funded by a grant from The Danish Strategic Research Council.

I would like to thank *Gorm Greisen* for giving me the opportunity to be part of the European working group conducting the SafeBoosC II Trial. I have learned a lot from him professionally as well as personally. I thank him for teaching me not only skills in the field of research but also for trying to teach me about the art of diplomacy.

Thanks to *Carsten Thomsen* and the "crew" at the Department of Diagnostic Radiology for helping me setting up the MRI-part of the SafeBoosC II study, helping me out with animal experiments, and for carefully commenting on the very first draft of this thesis.

Thanks to *Manon Benders* for inviting me to her home in Utrecht and giving me the opportunity to practice the clinical skills of cranial ultrasound and afterwards for spending many hours during early mornings and late evenings scoring all cranial ultrasound scans, and for being part of and housing the MRI-scoring-group. The ultrasound scans were scored in collaboration with *Cornelia Hagmann*, who was part of the MRI-scoring group, she deserves great thanks for her the work with the images and for always listening. Thanks to *Monica Fumagalli* for joining the MRI-scoring-group.

Thanks to *Tue Hvass Petersen*, who Gorm set me up with, when I was about to give up on the technical challenges of the EEG analysis and to *Per Winkel* for statistical assistance.

I would also like to thank the rest of the *SafeBoosC II study group* – it has been interesting, challenging, and mostly fun working with everybody[*].

Thanks to all the *parents and the infants* who joined the SafeBoosC II trial. Without them, this thesis would not have been possible.

I was lucky being seated together with skilled and humours *Ph.D. fellow students* who would always try to cheer me up – or calm me down – when the workload, the statistics, or the computer was against me.

Last, but definitely not least, I wish to thank my family. My boyfriend *Mikkel Kragelund,* who actually managed to read the entire thesis, for always standing beside me and cheering at me through the ups and downs of the Ph.D. study. My children *Smilla* and *Sirius* for reminding me about the most important things in life such as eating pancakes on Saturday mornings, watching TV in the sofa, not being too healthy, and fighting over nothing…

[*] Gerhard Pichler, Martin Wolf, Axel Frantz, Christian Gluud, Adelina Pellicer, Olivier Claris, Gene Dempsey, Frank van Bel, Wim Van Oeveren, Topun Austin, Petra Lemmer, Stefan Kleiser, Simon Hyttel-Sørensen, Joan Riera, Thomas Alderliesten

Contents

Included manuscripts

This thesis is based on the following three manuscripts, which are referred to in the text by their roman numerals.

I. Brain injury in the international multicentre randomized SafeBoosC phase II feasibility trial: cranial ultrasound and magnetic resonance imaging assessments
AM Plomgaard, C Hagmann, T Alderliesten, T Austin, F van Bel, O Claris, E Dempsey, A Franz, M Fumagalli, C Gluud, G Greisen, S Hyttel-Sorensen, P Lemmers, A Pellicer, G Pichler, and M Benders.
Pediatr Res. 2015 Nov 16. doi: 10.1038/pr.2015.239. [Epub ahead of print].

II. The SafeboosC II randomised trial: treatment guided by near-infrared spectroscopy reduces cerebral hypoxia without changing early biomarkers of brain injury
AM Plomgaard, W van Oeveren, TH Petersen, T Alderliesten, T Austin, F van Bel, M Benders, O Claris, E Dempsey, A Franz, M Fumagalli, C Gluud, C Hagmann, S Hyttel-Sorensen, P Lemmers, G Pichler, A Pellicer, P Winkel, and G Greisen.
Pediatr Res. 2015 Dec 17. doi: 10.1038/pr.2015.266. [Epub ahead of print].

III. Early biomarkers of brain injury and cerebral hypo- and hyperoxia in the SafeBoosC II trial
AM Plomgaard, T Alderliesten, T Austin, F van Bel, M Benders, O Claris, E Dempsey, M Fumagalli, C Gluud, C Hagmann, S Hyttel-Sorensen, P Lemmers, G Pichler, W van Oeveren, A Pellicer, TH Petersen, P Winkel, and G Greisen.
Submitted, PLoS ONE, January 30, 2016.

Related publications

Cerebral near infrared spectroscopy oximetry in extremely preterm infants: phase II randomised clinical trial. S Hyttel-Sørensen, A Pellicer, T Alderliesten, T Austin, F van Bel, M Benders, O Claris, E Dempsey, A Franz, M Fumagalli, C Gluud, B Grevstad, C Hagmann, P Lemmers, W van Oeveren, G Pichler, **AM Plomgaard**, J Riera, L Sanchez, P Winkel, M Wolf, and G Greisen. *BMJ (Clinical Research Ed.), 350, g7635, 2015.*

The SafeBoosC phase II clinical trial: an analysis of the interventions related with the oximeter readings. J Riera, S Hyttel-Sorensen, M Carmen Bravo, F Cabañas, P López-Ortego, L Sanchez, M Ybarra, E Dempsey, G Greisen, T Austin, O Claris, M Fumagalli, C Gluud, P Lemmers, G Pichler, **AM Plomgaard**, F van Bel, M Wolf, and A Pellicer. *Arch Dis Child Fetal Neonatal Ed 2015;0:F1–F6*

Summary

Background

Extremely preterm infants (infants born more than 12 weeks preterm) carry a high risk of death or long-term cerebral impairment. The mortality is 25% and 25% of the surviving infants live with some degree of handicap spanning from cerebral palsy to low intelligence quotient, cognitive disabilities, and behavioural and learning disabilities. The brain injuries are partly consequences of the immature circulatory- and respiratory systems causing clinical instability and episodes of cerebral hypo- and hyperoxia in the developing brain, which may initiate a "chain of causation" starting with an acute cerebral reaction potentially leading to later to structural brain damage and eventually impaired cognitive or psychomotor outcome.

The SafeBoosC II Trial was a multicentre, randomised feasibility trial. The primary objective of the trial was to determine the possibility of stabilising the cerebral oxygenation in extremely preterm infants monitored by cerebral near infrared spectroscopy (NIRS) oximetry and thereby reduce the burden of cerebral hypo- and hyperoxia, with the purpose of ultimately reducing the risk of brain injury.

Patients and methods. A total of 166 extremely preterm infants were included in the SafeBoosC II trial before 3 hours of age: 86 infants were randomised to the experimental group and 80 to the control group. The intervention period was 72 hours.

- Experimental group: cerebral NIRS monitoring combined with an evidence-based intervention guideline for NRIS values out of range (55-85%).
- Control group: blinded collection of NRIS data combined with treatment as usual.

The primary outcome of the SafeBoosC II trial was the time spent outside the target range of 55-85% multiplied by the mean absolute deviation during the first 72 hours after birth (the burden of cerebral hypo- and hyperoxia) expressed in percentage hours (%hours). One hour with an oxygenation of 50% gives 5%hours of hypoxia. The burden of cerebral hypo- and hyperoxia was significantly reduced by 58% (p=0.001) in the experimental group. This was mainly due to decreased hypoxia as the hyperoxia was unaffected by the intervention.

The secondary and explorative outcomes of the SafeBoosC II trial. To investigate the potential consequences of reducing the cerebral burden of hypo- and hyperoxia, the number of deaths and the cerebral effects were monitored in various ways form birth till term equivalent age (TEA).

At 6 and 64 hours of age blood samples were collected and analysed for three biomarkers of brain

injury: S100beta, brain fatty acid binding protein (BFABP), and neuroketal. Standardised cranial ultrasound (cUS) was performed at day 1, 4, 7, 14, 35 and at TEA. The images were analysed by two experts, who categorised the images as: no injury, mild/moderate injury, or severe injury. Electroencephalography (EEG) was performed at 64 hours of age and analysed for burst rate and spectral edge frequency 95%. Magnetic resonance imaging (MRI) was conducted at TEA. The images were analysed by three experts and categorised as: no injury, mild/moderate injury, or severe injury. All cerebral outcome measurements were analysed centrally without knowledge of allocation and medical history of the infant.

The aims of this thesis were

i) To determine whether the intervention in the SafeBoosC II trial reduced the risk of adverse cerebral outcomes and death
ii) To investigate whether the burden of cerebral hypoxia regardless of allocation in the SafeBoosC II trial was associated with an increased risk of adverse cerebral outcomes and death
iii) To discuss the relevance of the cerebral outcomes in a large scale randomised clinical trial

Results

Serial cUS was evaluated in 157 infants. The number of severe brain injury as detected by cUS did not differ significantly between the groups, i.e. 10/80 in the experimental group vs. 18/77 in the control group ($p = 0.10$). MRI at TEA were evaluated in 84/134 infants and the numbers of severe brain injury were similar in the experimental (5/46) and the control group (3/38), ($p = 0.72$). Fewer infants in the experimental (12/86) than in the control group (20/80) died before TEA ($p = 0.08$). The composite outcome of death or severe brain injury on cUS was significantly reduced in the experimental group (19/82 vs. 30/78, $p = 0.041$).

EEGs from 133 infants were evaluated. The two groups did not differ regarding burst rates (experimental 7.2 vs. control 7.7 burst/min.) or spectral edge frequency 95% (experimental 18.1 vs. control 18.0 Hertz). Neither did the two groups differ regarding the blood levels of S100beta, BFABP, and neuroketal concentrations at 6 or 64 hours ($n = 123$ participants).

Post hoc analysis of the data showed that significantly more infants with a cerebral burden of hypoxia within the highest quartile versus infants within the three lower quartiles were diagnosed with severe intracranial haemorrhage (11/39 vs. 11/117, $p = 0.003$), had low burst rate on EEG (12/28 vs. 21/103, $p = 0.015$), or died (14/41 vs. 18/123, $p = 0.006$), whereas none of these events

were associated with cerebral hyperoxia. The three blood biomarkers were not associated with the burden of cerebral hypo- or hyperoxia. After multiple logistic regression the following variables remained significantly associated with the composite outcome of severe brain injury on cUS or death; intervention ($p = 0.003$), gestational age below 26 weeks ($p = 0.007$), use of vasopressors ($p = 0.014$), and blood transfusion ($p = 0.016$).

Conclusions

i) There were fewer deaths and severe brain injuries as detected by cUS in the experimental group and the composite outcome of severe brain injury and death was significantly reduced. However, it must be kept in mind that the SafeBoosC II was a feasibility trial and underpowered to determine if a reduction in the cerebral burden of hypo- or hyperoxia would significantly reduce the risk of adverse cerebral outcomes or death.

ii) The risk of severe brain injury as assessed by cUS, EEG-burst rate and death was highest in the infants with the highest burden of cerebral hypoxia. Statistical analyses found co-linearity between burden of cerebral hypoxia and the intervention, and after multiple logistic regression analysis, the risk of death or severe brain injury on cUS was significantly associated with intervention, gestational age below 26 weeks, use of vasopressors, and blood transfusion and not to the burden of cerebral hypoxia.

iii) In future large randomised trials investigating the neuro-protective effects of cerebral NIRS it could be suggested using a composite outcome of death or severe brain injury on cUS, as the primary outcome of the intervention. MRI might be conducted in a sub-group of infants admitted to centres with MRI-dedicated neuro-imaging groups, thereby making it possible to investigate if subtle differences are present between groups. As there was no evidence that EEG burst rate or spectral edge frequency 95% was affected by the reduced burden of cerebral hypoxia in the experimental group, these outcome measures may not be useful in future studies of the accumulated burden of cerebral hypoxia during the first 72 hours of life. The blood biomarkers of brain injury was exploratory and the finding of no association to the burden of cerebral hypo- or hyperoxia, suggests that these blood biomarkers might not be useful in future studies on the accumulated burden of cerebral hypo- and hyperoxia measured by NIRS.

Abbreviations

aEEG:	amplitude-integrated electroencephalogram
BFABP:	brain fatty acid binding protein
cUS:	cranial ultrasound
DTI:	diffusion tensor imaging
DWI:	diffusion weighted imaging
EEG:	electroencephalogram
ICC:	inter cluster correlation
IVH:	intraventricular haemorrhage
IQ:	Intelligence quotient
MRI:	magnetic resonance imaging
NIRS:	near infrared spectroscopy
OR:	Odds ratio
PVHI:	periventricular haemorrhagic infraction
$rStO_2$:	regional tissue oxygen saturation
S100β:	S100beta
SEF95:	spectral edge frequency 95%
TEA:	term equivalent age

Background

Extremely preterm infants (infants born more than 12 weeks preterm) carry a high risk of death or long-term cerebral impairment. The mortality is approximately 25% and 25% of the surviving infants live with some degree of handicap spanning from cerebral palsy to low intelligence quotient, cognitive disabilities and behavioural and learning disabilities[1-3]. The number of infants hospitalized after extremely preterm birth is increasing and while the mortality rate has been declining over the past decades, the morbidity rate has not been reduced to the same degree[4]. In Europe alone about 25,000 extremely preterm infants are born every year. The survival of these infants depends on highly specialised intensive care and in the long run many of these infants may rely on specialised medical care, rehabilitation, and special education.

The vulnerable preterm brain

The vital organs of the extremely preterm infant have functional limitations. The respiratory and circulatory systems are immature and the cerebral autoregulation is impaired, especially during the first days of life[5,6]. In addition the oxygen transportation may be impaired by suboptimal or low haemoglobin levels[7]. This makes the developing premature brain susceptible to fluctuations in the cerebral blood flow [8] and the haemodynamic instability may cause episodes of cerebral hypo- and hyperoxia, which in turn through complex interactions between developing, repairing and destructive mechanisms may lead to brain injury[9]. Thus, an episode of cerebral hypo- or hyperoxia may cause an acute cerebral reaction to the brain tissue with microglia activation, astrogliosis and arrested oligodendrocyt maturation or death resulting in decreased myelination[10]. Later the damage to the brain tissue may result in structural brain damage and eventually an impaired cognitive or psychomotor outcome. This cascade of events is illustrated as “the chain of causation” from clinical instability to permanent cognitive and psychomotor impairment **(Figure 1)**.

Figure 1. The chain of causation: From clinical instability to cognitive and psychomotor outcome in extremely preterm infants – the role of hypo- and hyperoxia.

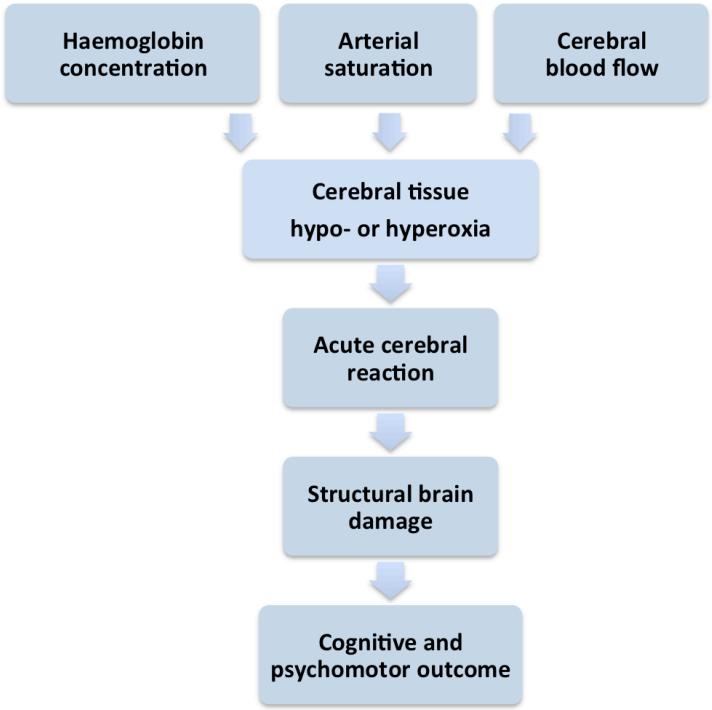


Figure 1. The chain of causation is an illustration of the cascade of events from the first cerebral exposure to a potentially harmful mechanism – in this case cerebral hypo- or hyperoxia – resulting in an acute cerebral reaction, which may lead to structural brain damage and eventually to cognitive or psychomotor impairment.

Near infrared spectroscopy (NIRS)

NIRS, a measurement of cerebral tissue oxygenation

Near infrared spectroscopy (NIRS) oximetry is a non-invasive method allowing the estimation of regional tissue oxygenation (rStO2) in a depth of 2-3 centimetres. The thin skull of preterm infants makes cerebral NIRS measurements well suited for this population. A sensor is placed over the region of interest and light within the infrared spectrum of 700-1000nm is applied to the tissue. In biologic tissue haemoglobin is the main chromofore absorbing light in the near infrared spectrum. The absorption dependents on the binding of oxygen, which differs between oxy-haemoglobin and deoxy-haemoglobin[11]. $rStO_2$ is expressed in percentage as *oxy-haemoglobin / (oxy-haemoglobin + deoxy-haemoglobin),* and reflects the saturation in veins, capillaries and arteries[12]. Continuous cerebral NIRS monitoring may be used as a measure of fluctuations in the cerebral blood flow[13]. It has therefore been suggested that NIRS oximetry

could give additional information in the early monitoring of the extremely preterm infants[12].

Target range for cerebral NIRS measurements

Several piglet experiments have investigated the outcome of low cerebral NIRS values. In term piglets NIRS values of 55% during 30 minutes allows the maintenance of a normal energy-metabolism in almost all animals[14]. Cerebral oxygenation must be kept below 30% before severe abnormalities occur in the electroencephalogram (EEG)[15,16] and below 35% for hours to induce significant histological changes in the brain tissue[17]. The latter study suggests that NIRS values returning to baseline levels after hypoxic episodes, are less likely to induce brain injury than if cerebral hypoxia is replaced by hyperoxia. Low levels of cerebral oxygenation and low cerebral blood flow in preterm infants have been associated with intraventricular haemorrhage (IVH), reduced brain activity, and lower developmental score at 2 years of age[18-22], whereas another study found cerebral hyperoxia prior to severe intracranial haemorrhage[23]. Recently, reference values for cerebral NIRS in extremely preterm infants during the first 72 hours of age were published and suggest that the 10^{th} centile is approximately 55%[24]. The reference values are based on 350 infants, and show correlations to gestational age, sex, intra uterine growth restriction and haemodynamically significant patent ductus arteriosus. Unfortunately these reference values are unable to define the optimal NIRS level.

Cerebral NIRS monitoring is currently being implemented in some neonatal intensive care units as part of the standard care of extremely preterm infants. Yet it remains to be determined whether monitoring the cerebral oxygenation actually prevents cerebral injury, improves neurological outcome, and/or increases the survival rate of the extremely preterm infants[25].

Measuring the cerebral effects of neuro-protective interventions in randomised clinical trials

When and how should the potential effect of a neuro-protective intervention be measured?

In neonatal randomised trials on neuro-protective interventions most studies report the primary outcome as a measure of psychomotor developmental impairment at 18-24 months corrected age either as a single measure or as a composite outcome also including death[26-29]. Extremely preterm infants are exposed to a variety of events (sepsis, necrotising enterocolitis, mechanical ventilation, persistent ductus arteriosus, hypotension), which may contribute to death or neurodevelopmental impairment independent of the actual neuro-protective intervention. It has therefore recently been questioned whether the 2-year outcome should remain the gold standard

outcome for neuro-protective interventions conducted in the early period after extremely preterm birth[29]. The correlation between Bayley III at 2 years and intelligence quotient (IQ) in adults is poor, whereas the IQ at 8 years is almost perfectly correlated to the adult IQ[30]. It may therefore be argued that the children should be followed at least into early school age. As neurodevelopment and IQ are influenced by many other factors than the neuro-protective intervention, it could also be argued that the primary result of a neuro-protective intervention should be measured at a time-point reflecting the direct linkage between the intervention and the outcome measure[29]. The chain of causation allows measurements of the potential effects of an early neuro-protective intervention at different time-points **Figure 2.**

Figure 2. Measurements of potential brain injury biomarkers at different time-points in the chain of causation – from clinical instability to cognitive and psychomotor outcome in extremely preterm infants, the role of hypo- and hyperoxia.

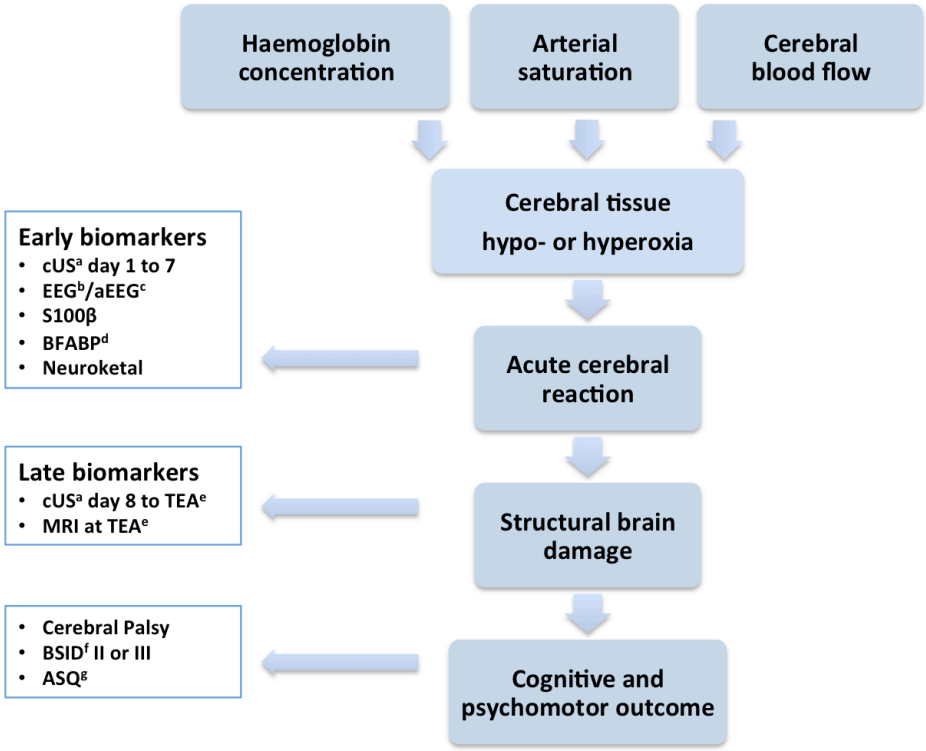


Figure 2. Illustration of time points for measuring the potential effects of a neuro-protective intervention. The early and late biomarkers are either correlated to later neurodevelopmental outcomes (cUS, MRI and EEG or aEEG) or possible markers of acute reactions in the brain, not routinely measured in preterm infants (blood biomarkers S100β, brain fatty acid binding protein (BFABP), and neuroketal).
[a] Cranial ultrasound, [b] Electroencephalography, [c] amplitude integrated electroencephalography, [d] Brain fatty acid binding protein, [e] Term equivalent age, [f] Bayley Scale of Infant Development edition II or III, [g] Ages and Stages Questionnaire

The biomarkers listed in **Figure 2** are either well-established biomarkers (cranial ultrasound (cUS), magnetic resonance imaging (MRI) and EEG or amplitude integrated electroencephalogram (aEEG)) known to correlate with later neurodevelopmental outcomes, or experimental outcome measures (blood biomarkers; S100beta (S100β), brain fatty acid binding protein (BFABP), and neuroketal) of the acute cerebral, which are not routinely measured in preterm infants.

Cranial ultrasound

cUS is the most widely used neuroimaging tool for assessing brain injury in preterm infants. The imaging modality has many advantages. It can be conducted bedside in the sick and instable preterm infant, be repeated as often as indicated, caries no risks, and is relatively inexpensive. The major disadvantage of cUS is that the examination is dynamic and user dependent and in clinical trials often only selected still images are available. Different types of brain injuries occur at different time-points from birth till term equivalent age (TEA), therefore serial cUS is recommended during the this period[31]. The most common acute brain injury developing during the transition from intra- to extra-uterine life, detectable by cUS is intracranial haemorrhage, which is present in 30 to 35% of all infants born before 32 weeks of gestation[32,33]. The haemorrhages are categorised according to severity[34]; IVH grade I including germinal layer haemorrhage, IVH grade II, IVH grade III, and periventricular haemorrhagic infarction (PVHI). Severe brain injuries detected by early cUS are mainly represented by IVH grade III and PVHI which in most cases develop during the first days of life[35,36]. Also large cerebellar haemorrhages are detected by cUS[37] and later cystic periventricular leukomalacia may be present – even though the number of infants suffering from this this diagnosis have declined dramatically over the past decades[38]. Diffuse damages to the white matter may be visualised on later cUS, but are more easily identified on MRI[39]. Severe findings on cUS are correlated with later neurodevelopmental outcomes and especially if conducted sequentially, cUS can be used in the prediction of later neurodevelopmental outcome[33,35,36,40,41]. Post-test probabilities for adverse outcomes have been estimated using the EPIPAGE study[42] of 1954 very preterm infants as reference material reporting a pre-test risk of cerebral palsy of 9%[43]. They report a 5% post-test probability of cerebral palsy in infants with normal cUS and a post-test probability of cerebral palsy of 26 and 53% for IVH III and PVHI respectively, and 74% in infants with cystic periventricular leukomalacia. In infants with normal cUS findings approximately 5% have severe cognitive impairments at 2 years of age, where as this number is 11 to 23% in infants with severe findings on cUS[44].

Electroencephalography and amplitude-integrated electroencephalography

Even before structural brain damage is visible, there are other ways of detecting adverse cerebral reactions. EEG and aEEG are commonly used for monitoring the spontaneous background cortical brain activity in term asphyxiated infants, and are known as predicting variables for neurodevelopmental outcome and death[45,46]. The method is easy to apply and can be conducted continuously for hours or even days with only minimal disturbance of the infant. The EEG in

extremely preterm infants has a characteristic maturation pattern starting with large bursts of activity dominated by low frequency waves. As the infant grows older, the EEG undergoes a specific maturation pattern – the number of bursts increases, the inter-burst interval decreases, and the frequency-pattern shifts towards faster rhythms[47]. The maturational changes are also present in the aEEG, where the proportion of continuity and cyclicality increase in combination with decreasing upper margin and bandwidth narrowing[48]. Several cohort studies in preterm infants have demonstrated correlations between early EEG and aEEG parameters and later neurodevelopmental outcomes[49-51].

Blood biomarkers

S100beta (S100β) is a small calcium binding protein present in high concentration in Schwann cells, maturing oligodendrocytes and astrocytes[52]. After severe cerebral damage it leaks from the tissue into the systemic circulation. S100β has been associated with hypoxic ischemia, severity of IVH and low NIRS-values[53-55] and is routinely used in the screening of patients with mild to moderate head trauma[56].

Brain fatty acid binding protein (BFABP), a brain-specific marker, is a small molecule (15kD), which is rapidly released from astrocytes as a response to ischemia, mechanical, and oxidative brain damage[57]. It is known to be elevated in adult patients with acute ischaemic stroke[57] and is increased in patients with various neurodegenerative diseases[58].

Neuroketal are compounds produced by free radical induced peroxidation of docosahexenoic acid, which is solely present in the brain and especially vulnerable to oxidative stress[59]. Therefor neuroketal is a potential marker of cerebral hyperoxia, which have been associated with adverse cerebral outcomes in animal[17] and human studies[23,60]. In addition elevated levels of neuroketal in cerebrospinal fluid have been reported in preterm infants with cerebral white matter damage on MRI[61].

Magnetic Resonance Imaging

MRI at TEA is increasingly being used in the imaging assessment of extremely preterm infants. This method has several advantages. It is not as user dependent as cUS, the images are acquired with high resolution, quantitative measurements are possible and functional images can be acquired. MRI also has disadvantages. The child cannot be assessed bedside and must lie still during the procedure, which may require sedation. In addition the method is relatively time consuming and expensive.

Conventional T1 and T2 weighted images show additional details of the brain injuries detected by cUS – IVH grade I to III and PVHI as well as PVL grade I to III, and small cerebellar haemorrhages which are not always visualised on cUS[37,62]. These findings have been associated with later learning disabilities, cognitive impairment and behavioural dysfunction in preterm infants[63]. In addition, the more subtle white matter injuries such as punctuate white matter lesions and diffuse white matter injury, may not be detected by cUS but are known to be associated with mild cognitive impairment and behavioural problems[39]. Conventional MRI visualises small injuries to the white and grey matter and scoring systems combining these injuries with biometric measurements thereby making a more comprehensive and objective evaluation of the abnormalities have been developed, [64,65]. MRI has also proven superior to cUS in predicting neurodevelopmental outcome, especially cerebral palsy at 2 years of age[66,67]. A recent study found 2% of the infants with normal MRI at TEA had cerebral palsy, where as this number was 61% in infants with severe white matter abnormalities[44]. For severe cognitive impairments these numbers were 4 and 22%, respectively.

In addition to conventional MRI it is possible to conduct more sophisticated imaging acquisitions for quantitative analyses such as diffusion weighted imaging (DWI) and diffusion tensor imaging (DTI). It has been suggested that these MRI procedures could be used in the evaluation of neuro-protective treatments as they are able to detect even subtle differences in brain development as opposed to cUS and conventional MRI[68-72]. Thus MRI may reduce the number of infants needed in future randomised neuro-protective trials.

The SafeBoosC II trial

- *Safeguarding the Brain of our smallest Children II*[73]

Primary objective

The primary objective of the SafeBoosC II trial was to determine the possibility of stabilising the cerebral oxygenation in extremely preterm infants monitored by cerebral NIRS oximetry and thereby reduce the burden of cerebral hypo- and hyperoxia with the purpose of ultimately reducing the risk of brain injury **(Figure 3)**.

Figure 3. The SafeBoosC II trial and the chain of causation: From clinical instability to cognitive and psychomotor outcome in extremely preterm infants.

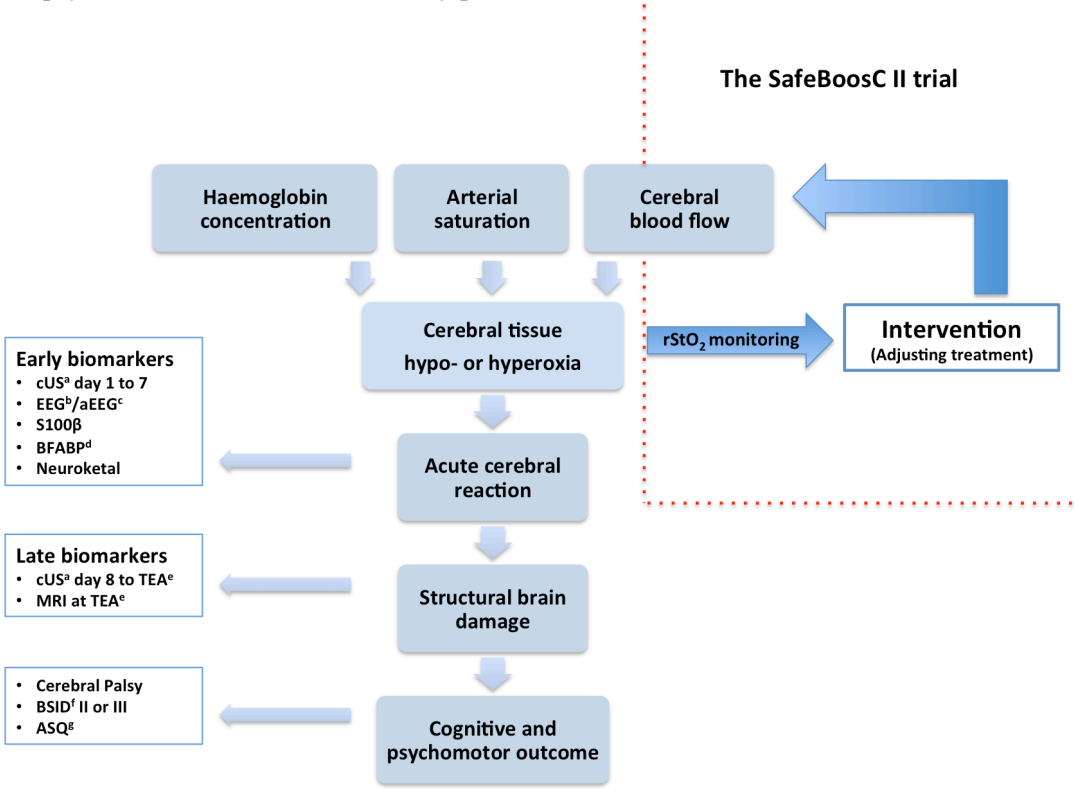


Figure 3 The theory of the SafeBoosC II trial was that monitoring the cerebral tissue oxygenation ($rStO_2$) with NIRS in it would be possible to adjust the physiological parameters (haemoglobin, arterial saturation and cerebral blood flow) causing the cerebral hypo- or hyperoxia, and there by reduce the time spent out of the target range of 55-85% $rStO_2$. Possible treatment interventions of the given parameters were listed in an evidence-based treatment guideline[74]. The secondary and exploratory outcome measures of the potential cerebral effects monitored in the SafeBoosC II trial are listed at different time-points in the chain of causations.
[a] Cranial ultrasound, [b] Electroencephalography, [c] amplitude integrated electroencephalography, [d] Brain fatty acid binding protein, [e] Term equivalent age, [f] Bayley Scale of Infant Development edition II or III, [g] Ages and Stages Questionnaire

Study design

A phase II multicentre randomised single blinded, parallel clinical feasibility trial.

Sample size estimation

The sample size estimation was based on unpublished data from 23 extremely preterm infants with a mean burden of 76.0 (SD 83.2) %hours (1.64 (SD 0.50) after log transformation). Based on a sample size estimation allowing a detection of a 50% reduction in the burden of hypoxia and hyperoxia with a type I error (α) of 5%, a power of 95%, a twin proportion of 30%, and an intra cluster correlation coefficient (ICC) of 0.33[75] this required 166 infants.

Subjects

A total of 166 extremely preterm infants from 8 European Countries were included.

The inclusion criteria were:

- Gestational below 28 weeks
- Decision to conduct full life support
- Possibility to place the cerebral NIRS-sensor within 3 hours after birth
- Obtained parental informed consent

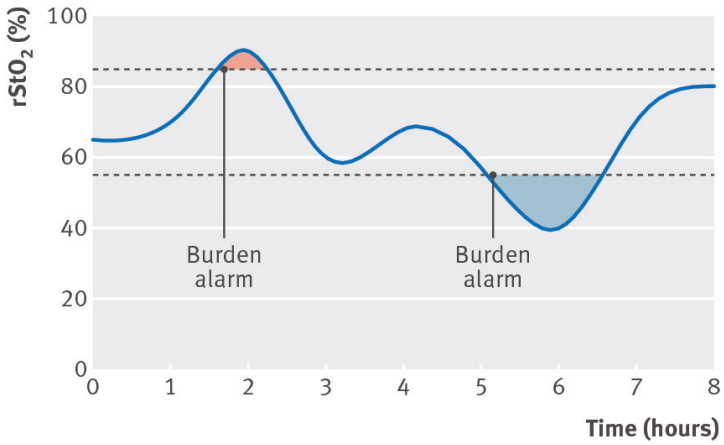
Randomisation, intervention, and primary outcome

Randomisation was performed within 3 hours after birth. The allocation sequence was 1:1 with block sizes of 4 or 6 in random order concealed for the investigators. The allocation was stratified by gestational age (above or below 26 weeks). Singletons were randomised individually and twins were allocated to the same treatment group. The infants were randomised into experimental group or control group. The intervention period was 72 hours.

- Experimental group: Monitoring of cerebral oxygenation using NIRS in combination with an evidence-based treatment guideline[74], recommending possible interventions if the cerebral oxygenation level was out of the predefined target range (55-85%).
- Control group: Monitoring with blinded collection of NIRS-data combined with standard care.

The pre-specified primary outcome was the time spent outside the target range of 55-85% multiplied by the mean absolute deviation during the first 72 hours after birth (the burden of hypoxia and hyperoxia), expressed in percentage hours (%hours), illustrated in **Figure 4**.

Figure 4. Schematic illustration of concept of burden of hypo- and hyperoxia in the SafeBoosC II trial.



Hyttel-Sørensen et al, BMJ (Clinical Research Ed.) [76]
$rStO_2$ = regional tissue haemoglobin oxygen saturation

Results

In total 86 infants were randomised to the experimental group and 80 to the control group. Baseline characteristics did not differ between groups. The median burden of hypo- and hyperoxia was 36.1%hours (interquartile range 9.2-79.5%hours) in the experimental group versus 81.3 (38.5-181.3) %hours in the control group, a reduction of 58% (95% confidence interval 35% to 73%, P < 0.001). In the experimental group the median burden of hypoxia was 16.6%hours (interquartile range 5.4-68.1) versus 53.6%hours (17.4-171.3) in the control group (P = 0.0012). The median burden of hyperoxia was small and similar between the groups: 1.2%hours (interquartile range 0.3-9.6) in the experimental group versus 1.1%hours (0.1-23.4) in the control group (P = 0.98)[76].

Conclusions: The burden of cerebral hypo- and hyperoxia was significantly reduced in the experimental group. Whereas the burden of cerebral hyperoxia was small and similar in the experimental and the control group, the cerebral burden of hypoxia was significantly reduced in the experimental group.

The secondary and explorative cerebral outcomes of the SafeBoosC II trial – the aims of this thesis

This thesis is based on the secondary (cUS, EEG and death) and explorative (MRI and blood biomarkers of brain injury; S100β, BFABP and neuroketal) outcomes in the SafeBoosC II trial.

The aims were

i) To determine whether the intervention in the SafeBoosC II trial reduced the risk of adverse cerebral outcomes and death

ii) To investigate whether the burden of cerebral hypoxia regardless of allocation in the SafeBoosC II trial was associated with an increased risk of adverse cerebral outcomes and death

iii) To discuss the relevance of the cerebral outcomes in a large scale randomised clinical trial

Methods in the assessment of cerebral outcomes in the SafeBoosC II trial

For all assessments of the cerebral outcomes the investigators were blinded to allocation and medical history of the infant. The methods are described in detail in manuscript I – III.

Cranial Ultrasound

Standardised serial cUS was performed on day 1, 4, 7, 14, 35, and at TEA. Two experts and one inexperienced cUS reader (Manon Benders, Cornelia Hagmann and Anne Mette Plomgaard) performed the central assessment of the cUS images according to predefined diagnosis **(Table 1)**. Each cUS was categorized on a three-level-scale: no brain injury, mild/moderate brain injury, or severe brain injury **(Table 1**). For each infant the scores of the serial cUSs were combined into an over-all cUS-score reflecting the worst scan.

Table 1. Predefined diagnosis for cranial ultrasound scans.

No brain injury
• None of the findings below
Mild/moderate brain injury
• Intra ventricular haemorrhage grade I-II (including germinal layer haemorrhage)
• Isolated ventriculomegaly with ventricular index <p97[a]
• Inhomogeneous flaring persisting after day 7
• Global thinning of corpus callosum at TEA
Severe brain injury
• Intra ventricular haemorrhage grade III (ventricular index >p97 during the acute phase)[a]
• Post haemorrhagic ventricular dilatation
• Parenchymal/periventricular haemorrhagic infarction
• Unilateral porencephalic cysts
• Cystic periventricular leukomalacia (bilateral)
• Cerebellar haemorrhage
• Cerebral atrophy at term age[b]
• Stroke

[a]Reference values according to Brouwer et al. Radiology, 2012. [77]
[b]Brain atrophy was defined as the combination of enlarged subarachnoid spaces, widened interhemispheric fissure, and reduction in complex gyral folding with or without concomitant ventriculomegaly, according to Horsch et al. Acta Paediatr., 2005.[78]

Magnetic Resonance Imaging

At TEA (corrected age 40-44 weeks) the infants were offered a cerebral MRI. An additional informed consent was required, if MRI at TEA was not routine local clinical practice. T1 and T2 weighted images were used for brain injury scoring. In addition DWI and DTI were planned. Three experts and an inexperienced reader scored the MRIs in consensus. The MRIs were scored on a

three-level-scale: No brain injury, mild/moderate brain injury, or severe brain injury as described by Kidokoro[65].

Electroencephalography and amplitude-integrated electroencephalography

At postnatal age of 64 hours 120 minutes of EEG with aEEG tracing was recorded, and the use of opioids or sedative medications was documented.

The raw EEG was converted into range-EEG (rEEG)[79]. Artefacts in the rEEG were visually identified independent by two authors. The analyses were afterwards conducted on the artefact-free data. The rEEGs were classified into four categories i) severe burst suppression, ii) burst suppression, iii) discontinuous, and iv) continuous.

Inter burst intervals were measured as the time between bursts of nested (high frequency) oscillations within large slow-wave depolarisations using an extraction algorithm based on the co-occurrence of a slow (0.5– 2 Hz) wave and higher (8–22 Hz) frequency oscillation[80].

For spectral analysis the EEG data were segmented into epochs of 2 seconds with an overlap of 50%. After Fast Fourier Transformation, the spectrum was subdivided into frequency bands: Delta (0.5 – 4 Hz), theta (4.5 – 8 Hz), alpha (8.5 – 13 Hz), and beta (13.5 – 30 Hz). The spectral distribution was calculated as the square root of the power in each band and expressed as percentages adding up to 100%. Spectral edge frequency 95% (SEF95) for each infant was, defined as the frequency between 0.5 and 30 Hz, below which 95% of the power was present.

Blood biomarkers

At the age of 6 and 64 hours 1ml of blood was collected. After inclusion of the last patient, the samples were analysed at a central laboratory (HaemoScan, Groningen, The Netherlands). *S100β* (50µl) and *BFABP (5*0µl) were assessed by enzyme-linked immunosorbent assay. *Neuroketal* (60µl) determination was performed by competitive enzyme immunoassay.

Adverse cerebral outcomes

In study III adverse cerebral outcomes were defined as: death, severe brain injury on cUS, EEG burst rate (burst/minute) within the lowest quartile, SEF95 within the highest quartile and S100β, BFABP, and neuroketal with an increase from 6 to 64 hours within the highest quartile.

Statistics

The statistics are described in detail in manuscript I-III.

The analysis performed in study I and II were planned per protocol. The distribution of the cUS and MRI scores of no brain injury, mild/moderate brain injury and severe brain injury between groups were analysed by Chi-Square tests and odds ratios (OR) for severe versus no severe brain were calculated. In addition the OR of the composite outcome of severe brain injury or death was calculated. EEG outcomes were analysed using general univariate regression models of each EEG quantity on gestational age (≤ 26 weeks) and each of the indicators of opioid treatment, sedative treatment, EEG-filter, EEG-sampling rate, and type of EEG device, was conducted. Indicators with significant effect on the EEG outcomes were included in multiple regression models. Thereafter the estimated mean (SE) was calculated and comparisons between the two groups were conducted. For blood biomarkers a linear mixed model analysis using time, intervention (experimental or control), and the interaction between time and intervention was conducted. An unstructured covariance matrix was used. The analyses were adjusted by gestational age (above or below 26 weeks) and centre. If the data did not follow a normal distribution the Mann-Whitney test was used.

The outcomes of EEG (inter burst intervals and SEF95) and the blood biomarker concentrations were tested for ICC. A mixed model analysis was carried out for each of the EEG- and biomarker outcomes: using birth (twin pair) as a random intercept. If ICC was less than 0.15 then all infants were included in the analysis for that outcome. If the ICC was higher than 0.15, one twin from each cluster was removed at random to avoid bias assessment regression[81] and the between-groups analyses were conducted on the remaining infants.

Kappa statistics were used to calculate the agreement of the local and the central cUS readers and the agreement of the two EEG artefact readers.

Study III. The between twins ICC for the burden of hypo-and hyperoxia was low (0.02)[76]. Therefore all infants were included in the explorative analysis. The median and inter quartile range (IQR) were determined for the burden of cerebral hypo- and hyperoxia. The infants were divided in groups according to a burden within or below the 4^{th} quartile of the burden of cerebral hypo- and hyperoxia, respectively. The infant characteristics were compared between the groups using the chi-square test or independent t-test as appropriate. ORs were determined for adverse outcomes for infants within the 4^{th} quartile of the burden of hypo- or hyperoxia versus infants in the three lower quartiles. Thereafter univariate correlation analysis was conducted to determine the patient characteristics associated with the composite outcome of severe brain injury or death. Multiple logistic regression was used with the composite outcome as dependent variable, centre, gestational age ≤26 weeks, and intervention as forced entry independent variables, and the patient

characteristics with significant correlation to the composite outcome as independent variables in a backward stepwise elimination procedure (P-out 0.1).

The statistics was performed using the software IBM SPSS Statistics for Windows Version 20.0.0 (Armonk, NY) and SAS version 9.3, (SAS Institute Inc., Cary, NC). For all analyses the two-sided P-value with a threshold of 0.05 was considered significant.

Results

Baseline characteristics for the 166 infants included in the SafeBoosC II trial did not differ between the two groups (**Table 2).**

Table 2 Baseline characteristics for the infants in the SafeBoosC II trial. Values are numbers (percentages) unless stated otherwise.

Characteristics	NIRS (n=86)	Blinded NIRS (control) (n=80)
Median (range) birth weight (g)	806 (410-1286)	880 (490-1330)
Median (interquartile range) gestational age at birth (weeks)	26.6 (25.7-27.4)	26.8 (25.5-27.6))
Gestational age <26 weeks	28 (33)	25 (31)
Male sex	44 (51)	34 (43)
Twins	20 (23)	14 (18)
Prenatal steroids (complete course)	58 (67)	56 (71)
Prolonged rupture of membranes	26 (31)	32 (40)
Maternal clinical chorioamnionitis	6 (7)	7 (9)
Apgar score ≤5 at 5 minutes	15 (18)	14 (18)
Mean (SD) umbilical arterial pH	7.33 (0.088)	7.31 (0.096)

NIRS=near infrared spectroscopy oximetry.

Hyttel-Sørensen et al, BMJ (Clinical Research Ed.)[76]
NIRS = experimental group, Blinded NIRS = control group

Study I

Cranial ultrasound. 157/166 (95%) of the infants included in the SafeboosC II trial acquired a central overall cUS-score. The number of uploaded images for the infants with central scorings declined from 96% on day 7 to 72% at TEA. Fifty-five percentage of the infants in the experimental and 65% in the control group received all serial cUS from day 1 till death or TEA.

The distributions of brain injury on day 1 and at TEA differed significantly between the experimental and the control group whereas the secondary outcome of the SafeBoosC II trial the overall cUS-score did not (p = 0.053) **(Table 3)**. The OR of severe brain injury versus no severe brain injury was insignificant at all time-points and for the overall cUS-score. The number of severe intracranial haemorrhages (IVH grade III and PVHI) was lower in the experimental (8/80 (10%)) than in the control group (14/77 (18.2%)) but this was not significant (OR 0.50 (95% confidence interval 0.20 to 1.27)).

Table 3. Central readings for each sequential cranial ultrasound scan and for the overall cUS-score. Values are given as number of infants.

	Day 1	Day 4	Day 7	Day 14	Day 35	TEA	Overall cUS-score
Experimental (n=80)							
Death	0	2	3	6	10	10	-
No injury	54	42	44	30	32	10	21
Mild/moderate injury	19	22	22	27	29	31	49
Severe injury	1	7	7	8	6	5	10
Control (n=77)							
Death	0	3	9	13	15	19	-
No injury	55	41	36	26	23	23	26
Mild/moderate injury	10	18	18	24	19	17	33
Severe injury	6	12	11	10	10	6	18
Chi-square test, p-value[a]	0.043	0.42	0.44	0.78	0.26	0.01	0.053
Severe vs. no severe brain injury							
Chi-square test, p-value[b]	0.06	0.33	0.21	0.46	0.11	0.76	0.10
Odds ratio[c]	0.15	0.54	0.52	0.66	0.41	0.81	0.47
(95% confidence interval)	(0.02-1.27)	(0.20-1.46)	(0.19-1.44)	(0.26-1.92)	(0.14-1.22)	(0.23-2.88)	(0.20-1.09)

[a] Chi square analysis of the between group distribution of brain injuries in the three categories; No injury, mild/moderate injury or severe injury
[b] Chi square analysis of the between group distribution of brain injuries in the three categories; severe injury versus no severe brain injury
[c] Odds Ratio for severe versus no severe

Magnetic resonance imaging. 87/134 (65%) of the infants alive at TEA had an MRI scan. Of these scans 57 (66%) were conducted according to the pre-specified time-point of 40 to 44 weeks of gestational age. Nine MRIs were excluded due to movement artefacts (n = 4) or slice thickness above 2 mm unless severe injury was clearly seen (n = 5), though 24 infants in each of the groups were included in the analysis of MRI according to the pre-specified time-point.

The distributions of brain injury as detected by conventional MRI at TEA did not differ between groups (p = 0.89), neither did the analysis of MRIs conducted at any time-point and regardless of slice thickness (OR = 1.4 (0.3 – 6.4) (**Table 4).**

Table 4. MRI brain injury severity between experimental and control group. Values are numbers (percentage) unless stated otherwise.

MRI at corrected age 40-44 weeks	Experimental n = 24	Control n = 24	P-value[a]	
Corrected age (week), median (range)	41.4 (40.7-43.6)	41.2 (40.3-44.0)		
No injury	3 (13)	2 (8)		
Mild/moderate injury	18 (74)	19 (79)	0.89	
Severe injury	3 (13)	3 (13)		
MRI at any time-point	**n = 46[b]**	**n = 38**		**OR(95% CI)**
Corrected age (week), median (range)	41.4 (36.7-52.7)	41.4 (39.8-53.9)		
No severe brain injury	41 (89)	35 (89)		
Severe injury	5 (11)	3 (8)	0.72	1.4 (0.3-6.4)

[a]Chi-squared test for equal distribution of brain injures between the groups. [b]Three infants were excluded from the analysis as the quality of the MRI was of insufficient quality

The composite adverse outcome of the SafeBoosC II trial. In the experimental group 12/86 infants died before TEA, whereas this number was 20/80 in the control group (p = 0.08). The composite outcome of severe findings on cUS or death differed significantly between groups; 19/82 in the experimental versus 30/78 in the control group (p = 0.04, OR = 4.48 (95% CI = 0.24-0.96). (This result is not presented in manuscript I, as it was not planned per protocol)

Study II

Electroencephalography and amplitude-integrated electroencephalography. EEG was recorded in 150/158 (95%) of the infants alive at 64 hours of age. Eighty-five percentage (133/158) was available for EEG-analysis. There was no difference between the groups according to EEG-recording details, background activity, burst rate (bursts/min) or SEF95 **(Table 5).**

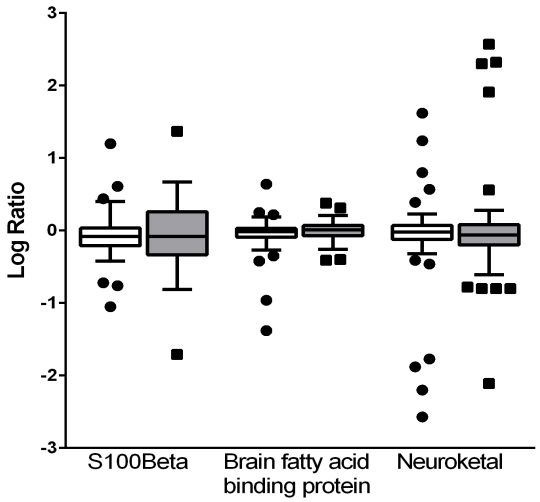
Table 5 EEG recording details and results.

EEG recording details	Experimental group n = 68	Control group n = 65	P-value
Age at EEG (hours), median (IQR)	64.8 (62.7 – 68.5)	65.8 (62.4-68.4)	NS
Sampling frequency 256 Hz, n (%)	54 (79)	50 (77)	NS[a]
Sampling frequency 200 Hz, n (%)	14 (21)	15 (23)	
Data filtered at 2 to 15 Hz, n (%)	13 (20)	10 (16)	NS
Opioid, n (%)	16 (24)	21 (32)	NS
Sedative, n (%)	9 (13)	5 (8)	NS
EEG results			
Severe burst suppression	3 (4)	2 (3)	
Burst suppression	11 (16)	7 (11)	0.75[b]
Discontinuous	41 (60)	41 (63)	
Continuous	13 (20)	15 (23)	
Burst rate (bursts/min) mean (SE)[c]	7.2 (0.72)	7.7 (0.73)	0.51
Spectral edge frequency 95%(Hz), mean (SE)[d]	18.1 (0.10)	18.0 (0.10)	0.51

IQR: inter quartile range, SE: standard error, NS: non significant.
[a] Chi-squared test of distribution of the sampling frequency between the experimental and the control group.
[b] Chi-squared test of distribution of the rEEG classification between the experimental and the control group.
[c] Mixed modelling with correction for EEG-filter at 2 to 15 Hz, opioid-treatment and after random exclusion of one twin form each twin-pair.
[d] Mixed modelling with correction for EEG sampling frequency and after random exclusion of one twin from each twin-pair.

Blood biomarkers. Blood samples were collected in 123 infants at 6 and 64 hours of age. The concentrations of the three biomarkers in the blood did not differ between the groups at 6 nor at 64 hours of age; neither did the main effect of the intervention (the increase in the blood biomarker concentration from 6 to 64 h of age) (**Figure 7**). The mixed model analysis or non-parametric analysis, in case of non-normal distribution of the data, confirmed the findings of no difference between the groups.

Figure 7. Intervention effect on the blood biomarkers illustrated as log ratio for the change in the concentration of the blood biomarkers from 6 to 64 hours of age. □ = Control ■ = Experimental



Study III

Cerebral oximetry data was missing in two of the 166 infants in the SafeBoosC II trial due to technical issues (n = 1) or consent withdrawn (n = 1).

The median (minimal - maximal) burden of cerebral hypoxia was 30.6 %hours (0.7 – 803.9) and the lower limit for the 4th quartile was 99.3 %hours whereas these numbers were 1.2 %hours (0.0 – 223.0) and 14.2 %hours for the burden of cerebral hyperoxia. Male gender was associated with less cerebral hypoxia and more cerebral hyperoxia. Furthermore, gestational age was positively associated with cerebral hyperoxia **(Table 6)**.

Table 6. Baseline characteristics and treatment during the first 72 hours of life according to burden of cerebral hypo- or hyperoxia split in the three lowest quartiles and the highest quartile. Values are numbers (percentages) unless stated otherwise.

	Burden of hypoxia			***Burden of hyperoxia***		
	Quartile 1 to 3 n = 123	**Quartile 4** n = 41	P-value	**Quartile 1 to 3** n = 123	**Quartile 4** n = 41	P-value[a]
Baseline characteristics						
Gestational age below 26 weeks	38 (31)	14 (34)	0.7	42 (34)	10 (24)	0.025*
Birth weight (gram), mean (SD)	847 (211)	875 (207)	0.47[b]	849 (208)	872 (216)	0.54[b]
Male sex	65 (53)	13 (32)	0.02*	53 (43)	25 (61)	0.047*
Twins	21 (17)	12 (29)	0.09	23 (19)	10 (24)	0.43
Antenatal steroids full course	82 (67)	31 (78)	0.2	88 (72)	25 (61)	0.18
Prolonged rupture of membranes	40 (33)	17 (36)	0.21	42 (34)	15 (38)	0.72
Maternal chorioamnionitis	6 (5)	5 (13)	0.1	9 (8)	2 (5)	0.59
APGAR-score <5 points at 5 minutes	21 (17)	8 (20)	0.69	20 (16)	9 (23)	0.38
Umbilical arterial pH, mean (SD)	7.32 (0.1)	7.31 (0.1)	0.62[b]	7.32 (0.1)	7.29 (0.1)	0.13[b]
Treatment during the first 72h of life						
Surfactant treatment	90 (73)	35 (85)	0.11	90 (73)	35 (85)	0.11
Mechanical ventilation	79 (64)	30 (73)	0.29	80 (65)	29 (70)	0.5
Patent ductus arteriosus treatment	17 (14)	4 (10)	0.52	15 (12)	6 (15)	0.71
Use of vasopressors/inotropes	22 (18)	16 (40)	0.004*	29 (24)	9 (22)	0.79
Any red blood cell transfusion	31 (26)	18 (45)	0.025*	41 (35)	8 (20)	0.07
Corticosteroids	4 (3)	4 (10)	0.1	6 (5)	2 (5)	0.99

[a] Chi square test. [b] Independent t-test. * Significant at 0.05 level.

One-hundred-and-fifty-five (155) infants were evaluated by central reading of cUS. Twenty-seven infants had severe brain injury (IVH grade III or PVHI, (n = 22), stroke (n = 2), cerebral atrophy (n = 2), or cerebellar hemorrhage (n = 1)). Severe brain injury, death and EEG burst rate were significantly associated with cerebral hypoxia, but unrelated to cerebral hyperoxia. The levels of the three blood biomarkers were not associated to either cerebral hypo- or hyperoxia (**Figures 8 and 9).**

Figure 8 Risk for adverse outcomes for infants with a ***burden of cerebral hypoxia*** within the highest quartile versus infants with a burden in the three lower quartiles. Odds ratio (OR) and 95% confidence interval.

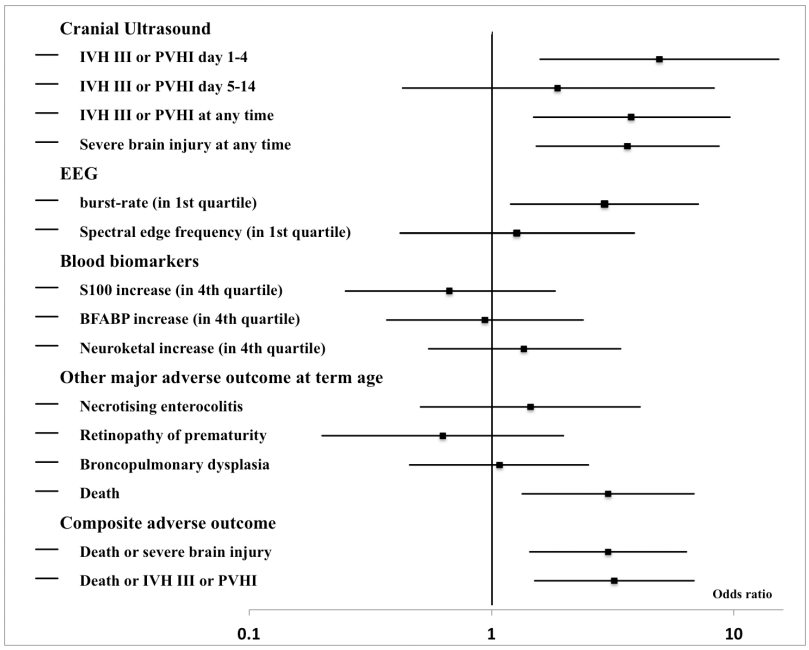
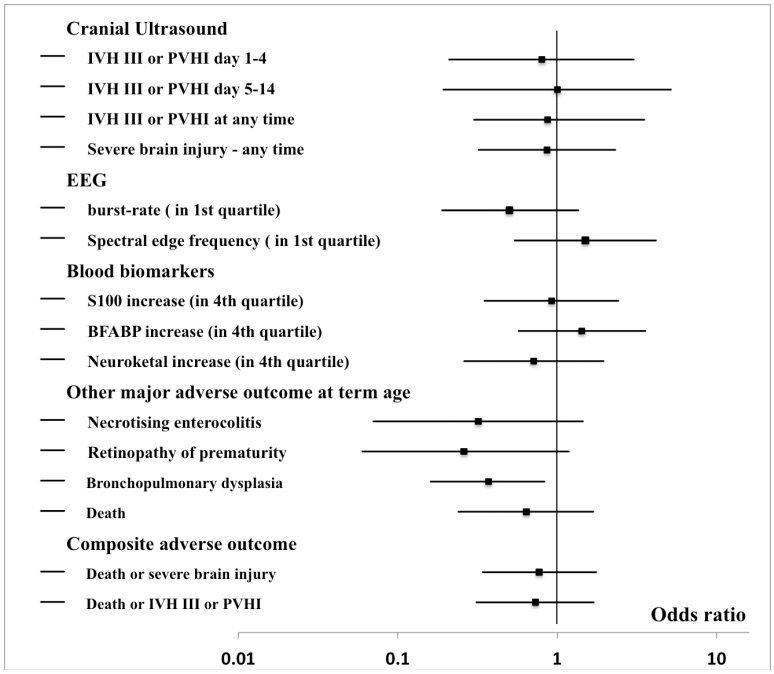


Figure 9 Risk for adverse outcomes for infants with a ***burden of cerebral hyperoxia*** within the highest quartile versus infants with a burden in the three lower quartiles. Odds ratio (OR) and 95% confidence interval.



Correlation analyses of the following variables showed a significant correlation ($p < 0.05$) between gestational age, birth weight, clinical chorioamnionitis, surfactant, mechanical ventilation, use of vasopressors, blood transfusions, and the burden of cerebral hypoxia within the 4th quartile on one hand and the composite outcome severe brain injury or death on the other. These variables were included in the multiple logistic regression model with backward stepwise elimination. Centre, gestational age below 26 weeks, and intervention were forced-entry variables in the model. The following variables remained statistically significant: intervention ($p = 0.003$, OR (95% CI) 0.29 (0.12 – 0.69)), gestational age below 26 weeks ($p = 0.007$, 3.33 (1.38 – 8.06)), use of vasopressors ($p = 0.014$, 3.26 (1.26 – 8.44)), and blood transfusion ($p = 0.016$, 2.97 (1.22 – 7.23)).

Discussion

Principal Findings

The significant reduction in the burden of cerebral hypo- and hyperoxia in the group receiving cerebral NIRS monitoring in combination with the evidence-based treatment guideline did not significantly reduce the risk of brain injury as assessed by the chosen biomarkers in the SafeBoosC-II trial.

The OR of severe brain injury versus no severe brain injury as assessed by blinded cUS-reading was insignificant at all time points and for the overall cUS-score, however the number of severe brain injuries as detected by cUS was lowest in the experimental group, mainly due to fewer cases of severe intracranial haemorrhages during the first week of life. Blinded evaluation of conventional MRI did not find any differences between the two groups. The EEG burst rates and SEF95 and the levels of the blood biomarkers, S100β, BFABP and neuroketal were similar in the experimental and the control group. The composite outcome of death or severe brain injury as detected by cUS was significantly reduced in the experimental group. However the SafeBoosC II trial was powered to detect a 50-percentage reduction in the burden of cerebral hypo- and hyperoxia, and not to conclude on the cerebral effects of such reduction.

Explorative analysis of the SafeBoosC II data showed that the early burden of cerebral hypoxia, but not hyperoxia, was associated with a reduction of brain electrical activity, severe brain injury as detected by cUS (especially early IVH III and PVHI), and death. There were no associations between the burden of cerebral hypo- or hyperoxia and the three blood biomarkers. Multiple logistic regressions found significant associations between intervention, gestational age below 26 weeks, use of vasopressors, and blood transfusion on one hand and the composite adverse outcome of severe brain injury detected by cUS or death on the other.

Methodological considerations

Strengths and limitations of each sub-study

The SafeBoosC II trial was powered to detect a 50-percentage reduction in the burden of cerebral hypo- and hyperoxia and not powered to demonstrate effects on clinically relevant outcomes of such reduction.

Study I

Cranial ultrasound. Local readers have the advantage of real time images whereas central readers only had access to selected still-images for assessment. The central readers were blinded to the allocation and medical history of the infant, which is important in a randomised clinical trial where blinding of the person performing the cUS is impossible as in the SafeBoosC II trial. cUS was performed from day 1 till TEA but not prior the inclusion. Therefore it is not possible to determine if the greater number of the severe brain injuries on day 1 in the control group was caused by an early effect of the intervention or if it was present before the enrolment in the trial. The percentage of uploaded cUS series decreased from day 7 onwards and at TEA only 72% of the infants received a cUS. Most infants were discharged or transferred to local hospitals at this age. As some lesions, such as cystic periventricular leukomalacia and cerebral atrophy, develop over time, we may therefore potentially have missed some cases[36,44].

Magnetic resonance imaging. The MRIs were analysed centrally by experts blinded to allocation and the medical history of the infants. MRI at TEA was optional and performed in 64% of the infants. This number was lower than expected; maybe because MRI at TEA was not standard clinical practice in most participating centres, and in these centres an additional informed consent prior to the MRI was required. MRI identified severe brain injury in two infants who were categorised with mild/moderate brain injury on cUS. Both infants had unrecognised cerebellar haemorrhages, this is one of the reasons that MRI is important in addition to cUS[31,37]. Subtle differences in MRI might be detected by sophisticated MRI techniques such as DTI and DWI[72,82]. Unfortunately in the SafeBoosC II trial we were unable to perform quantitative MRI analysis because of different scanner systems and poor quality of image acquisitions.

Study II

Electroencephalography. The EEGs were recorded with eight different devices, data were collected at two different sampling rates and with or without online filtration at 2-15Hz, therefore a direct comparison between the groups was impossible and a multiple regression model with corrections for the EEG-sampling-specifications (EEG-filter and EEG-sampling rate) was conducted. We did not measure the pCO_2 or seizure activity in the SafeBoosC II infants, but all other physiological variables were similar in the two intervention groups (fractional inspired oxygen, peripheral capillary oxygen saturation, mean airway pressure, heart rate, and arterial blood pressure)[76]. The burst rates were in line with previously published results in this group of infants[50,83,84], and the inter burst intervals were longer in preterm infants with IVH III or PVHI, which has also previously

been observed[85]. While it is feasible to use EEG as an outcome in a multicentre randomised clinical trial, it should be realised that working with different EEG devices may complicate the analyses by adding heterogeneity.

Blood biomarkers. The three blood biomarkers S100β, BFABP and neuroketal were explorative and unvalidated markers in the assessment of early brain injury in extremely preterm infants. Samples were scheduled for collection at 6 and 64 hours of age, but as a specific timestamp for each sample is missing the samples could potentially have been collected at other time-points than planned. However as the SafeBoosC II was a randomised clinical trial, we do not believe this would change the result of no difference between groups. The samples were analysed at a central laboratory and the technicians were blinded to allocation and medical history of the infants.

Study III

cUS was conducted at pre-specified days of life, but the exact timing of IVH grade III and PVHI and other severe brain damages is not available and therefore we cannot know if cerebral hypoxia preceded IVH or vice versa. Similarly, EEG was recorded only once. While the timing at 64 hours of age was expected to assess the potential effects of the accumulated burden over the intervention period, finer details of preceding or concurrent cerebral hypoxia could not be extracted, such as the relative significance of longer periods of moderate hypoxia versus peaks of severe hypoxia. Additionally data on continuous measurements of blood pressure, arterial oxygen saturation or cerebral blood flow, which may have contributed to both low cerebral NIRS values and severe brain damage[15,17,19] was not collected in the SafeBoosC II trial.

As the burden of cerebral hypoxia was reduced by 50% in the experimental group compared with the control group[76], the multiple logistic regression models were adjusted for not only the stratification variables (centre and gestational age below 26 weeks), but also the randomisation indicator (experimental vs. control group), which reduced the statistical significance of the burden of hypoxia. It must be kept in mind, that neither the composite outcome nor the particular statistical analyses were specified in the trial protocol; therefore this post-hoc finding must be interpreted conservatively.

The relevance of the chosen biomarkers in the SafeBoosC II trial

Cranial Ultrasound. Severe intracranial haemorrhages in the extremely preterm infants mainly occur during the first days of life[35,36]. Low cerebral NIRS-values and cerebral hypo-perfusion in preterm infants during the transition from intra- to extra- uterine life have been associated with

higher grades of IVH[19,22,86], but also cerebral hyper-perfusion just before the development of PIVH have also been reported[23]. We therefore believed that serial cUS would be a good marker of the potential neuro-protective effect of the intervention in the SafeBoosC II trial.

MRI is important in addition to cUS[31]. Furthermore, MRI at TEA is superior to cUS in predicting the risk of CP in preterm infants[67] and contributes with additional information to serial cUS when estimating risk of adverse neurodevelopmental outcome[44]. MRI at TEA therefore plays an important role in the risk stratification in these infants[67]. A recent review however, concludes that moderate to severe white matter abnormalities on MRI at TEA in very preterm infants predicts cerebral palsy with moderate specificity and sensitivity, and the predictive value of neurocognitive and behavioural impairment is limited[87]. In addition to conventional MRI it is possible to use the MRI techniques for quantitative analyses and it has been suggested as a future outcome in the evaluation of neuro-protective treatments[68-70,88]. MRI may detect subtle differences in brain injury and brain development [72], which are not identified by cUS. Therefore MRI could potentially reduce the number of infants needed for future randomised neuro-protective trials.

Electroencephalography. Low cerebral blood flow and low levels of cerebral NIRS have been associated with decreased EEG activity[20,89] which has also been shown in preterm infants with permissive hypercapnia[83,90]. In term new-born infants, severe hypoxic-ischaemic brain injury is associated with EEG burst-suppression, lasting hours to days and predicting later neurodevelopmental outcome and death[45,46]. Several studies in preterm infants have reported moderate to good predictive values of early EEG parameters and later neurodevelopmental outcomes [50,51]. Therefore, we hypothesised that EEG at the end of the intervention period would be the time with the highest likelihood of detecting an effect of a reduction in the burden of cerebral hypo- or hyperoxia.

Blood biomarkers. S100β. A small study in preterm infants found correlation between the levels of S100β in blood and the degree of IVH[53], a recent larger study did not confirm this finding[91]. Negative correlation between NIRS levels and S100β in critically ill children has previously been shown[92], we therefor considered S100β a potential marker of early cerebral hypoxia.
BFABP has not previously been investigated in extremely preterm infants, but in adults with acute ischaemic stroke BFABP is elevated 2-3 hours after the event and remains increased for at least 5 days[57], therefore we considered BFABP a potential marker of cerebral hypoxia. *Neuroketals* are

compounds produced by free radical induced peroxidation of docosahexenoic acid. Docosahexenoic acid is solely present in the brain and especially vulnerable to oxidative stress[59], neuroketals was therefore hypothesised to increase in the infants with high values of cerebral hyperoxia.

The effects of the reduced burden of hypo- and hyperoxia in the randomised SafeBoosC II trial

Our results of a tendency towards fewer deaths and less severe brain injuries on cUS in the experimental group and the significant reduction in the composite outcome of severe brain injury on cUS and death supported the hypothesis that the SafeBoosC II NIRS intervention would be effective in reducing brain injury. The effects in terms of MRI at TEA, early EEG outcomes and blood biomarkers did not support the hypothesis. Therefore it could be speculated that the reduction of the cerebral burden of hypoxia was not of clinical relevance – even if the burden was reduced by 58% in the experimental group[76]. Is it possible that the reduced burden of cerebral hypoxia was irrelevant for the brain? Although the 'burden of cerebral hypoxia' − the accumulated area under the curve expressed in %hours − amounted to more than 200 %hours in some infants. Or were the chosen biomarkers unable to measure the neuro-protective effects? In the SafeBoosC II trial only 25% of the alarms for cerebral oxygenation out of range was followed by a clinical intervention the remaining was not. This cloud suggest that the clinicians were content with the child status overall despite a cerebral oxygenation outside the monitoring range[93]. The threshold used as the alarm limit for intervention and for the calculation of the burden was 55%[73]. This limit corresponds to the recently published 10^{th} percentile of the cerebral NIRS reference values in extremely preterm infants during the first 72 hours of age[24], though it must be kept in mind that these reference values are unable to define the optimal NIRS level. Animal studies in term piglets have shown that cerebral oxygenation must be kept below 30% before severe abnormalities occur in the EEG [15,16]and below 35% for several hours to induce significant histological changes in the brain tissue[17], this might explain that the EEG burst rate and SEF95 in the SafeBoosC II trial were unaffected by the intervention. It cannot be excluded that more sophisticated MRI techniques or EEG analyses might have detected subtle differences between groups. S100β levels were similar in the intervention groups; this could be because the burden of cerebral hypoxia – even though the reduction in the experimental group was highly significant – was insufficient for the release of the intracellular S100β into the systemic circulation. There was no difference in the level of BFABP between the groups, which again might be explained by the insufficiency of a moderate global cerebral hypoxic exposure, measured in the SafeBoosC II trial – compared to stroke patients

experiencing an acute and severe local cerebral hypoxia[57]. As the burden of hyperoxia was unaffected by the intervention in the SafeBoosC II trial, it may not be surprising that the neuroketal levels were similar in the two groups.

2-year follow-up

The 2-year follow up of the SafeBoosC II participants is on-going and will further explore if cerebral hypoxia and/or the SafeBoosC II intervention is related to later neurodevelopmental outcomes such as psychomotor deficit. The patients are currently being evaluated by the Baylay Scales of Infant Development and a the parental Ages and Stages Questionnaire, which have previously been used in this population[94].

Risks associated with a high burden of cerebral hypoxia during the first 72 hours of life

The post hoc analysis found evidence that the early burden of cerebral hypoxia, but not hyperoxia, was associated with a reduction of brain electrical activity, severe brain injury (especially early IVH grade III and PVHI), and death, which is in line with previous findings[18-21]. Multiple logistic regressions showed significant associations between intervention, gestational age below 26 weeks, use of vasopressors, and blood transfusion on one hand and the composite adverse outcome of severe brain injury or death on the other. The association-analysis were based on the SafeBoosC II dataset, therefore the multiple logistic regression models were adjusted for the stratification variables (centre and gestational age below 26 weeks), as well as the randomisation indicator (experimental vs. control). As the burden of hypoxia was reduced by 50% in the experimental group compared with the control group[25], it was therefore not surprising that adjusting for the intervention indicator reduced the statistical significance of the burden of hypoxia. The statistical significance of the intervention indicator means that the risk of severe brain injury or death in the experimental group of the SafeBoosC-II trial was less in the experimental than in the control group when adjusted for a number of other factors. However, neither the composite outcome nor the particular statistical analyses were specified in the trial protocol. This post-hoc finding must therefore be interpreted conservatively.

Future considerations

SafeBoosC II was a feasibility trial and underpowered to determine if a reduction in the cerebral burden of hypo- or hyperoxia would reduce the risk of adverse cerebral outcomes or death, but as the composite outcome of severe brain injury detected by cUS and death was significantly reduced, there is reason to believe that monitoring extremely preterm infants with cerebral NIRS might reduce the risk of severe brain injury or death.

Cranial ultrasound. It was feasible to evaluate most of the infants by central reading of serial cUS and the risk of severe brain injury using this imaging modality revealed less severe brain injuries in the experimental than in the control group. It could be suggested that central reading of cUS is conducted in future trials, where blinding of the involved personnel is impossible. Further more it would be reasonable to perform cUS prior to inclusion in the trial – to insure that infants with cerebral congenital malformations or perinatal cerebral injury are excluded. Due to the nature of the randomised trial, these infants should be equally distributed between the experimental and the control group, but in the SafeBoosC II trial more severe brain injuries were present in the control group than in the experimental group at day 1, and unfortunately we were unable to determine if these injuries were present prior the initiation of the intervention.

Magnetic resonance. For future neuro-protective trials in extremely preterm infants it has been suggested that the number of infants needed to identify a potential neuro-protective effect might be reduced if sophisticated MRI techniques are used, as MRI could detect more subtle differences between groups than cUS[72,82]. This suggestion seems reasonably, but in the SafeBoosC II trial, the percentage of infants having an MRI was less than expected and the image quality, scanner systems, and image acquisitions differed between the including centres. In future large multicentre randomised controlled trials studying neuro-protective interventions; only MRI-dedicated neuro-imaging groups/centres might use MRI and the MRI protocol should be followed rigorously.

Electroencephalography and blood biomarkers. EEG and the blood biomarkers are not established biomarkers of brain injury in extremely preterm infants. However, we considered them prime candidates and therefore consider the absence of any difference between the groups as an important signal. Our results raise the possibility that cerebral hypoxia at the levels that are common in extremely preterm infants during the first days after birth are really not significant as an aetiology for brain (tissue) injury as detectable by EEG and the selected molecular biomarkers. However

combining continuous EEG monitoring in with cerebral NIRS might be useful in describing the acute adverse reactions related to low levels of cerebral oxygenation.

The composite outcome of severe brain injury and death. The composite outcome of severe brain injury as detected by central reading of cUS and death was significantly reduced in the experimental group, and as both outcomes showed a tendency towards less adverse events in the experimental group, it may be reasonable to use a composite outcome in future randomised trials investigating the effects of reducing the burden of cerebral hypoxia by monitoring cerebral NIRS in extremely preterm infants.

Conclusions

This thesis was based on the secondary (cUS, EEG and death) and explorative (MRI and blood biomarkers of brain injury) outcomes in the randomised clinical SafeBoosC II trial.

i) There were fewer deaths and less severe brain injuries as detected by cUS in the experimental group and the composite outcome of severe brain injury and death was significantly reduced. However, it must be kept in mind that the SafeBoosC II was a feasibility trial and underpowered to determine if a reduction in the cerebral burden of hypo- or hyperoxia would reduce the risk of adverse cerebral outcomes or death.

ii) The risk of severe brain injury as assessed by cUS, EEG-burst rate and death was highest in the infants with the highest burden of cerebral hypoxia. Statistical analyses found co-linearity between burden of cerebral hypoxia and the SafeBoosC II intervention, and after multiple logistic regression analysis, the risk of death or severe brain injury on cUS was significantly associated with intervention, gestational age below 26 weeks, use of vasopressors, and blood transfusion and not to the burden of cerebral hypoxia.

iii) In future large randomised trials investigating the neuro-protective effects of cerebral NIRS monitoring it could be suggested using a composite outcome of death or severe brain injury on cUS as the primary outcome of the intervention. MRI might be conducted in a sub-group of infants admitted to centres with MRI-dedicated neuro-imaging groups, thereby making it possible to investigate if subtle differences are present between groups. There was no evidence that EEG burst rate or SEF95 was affected by the reduced burden of cerebral hypoxia in the experimental group, therefore these outcome measures may not be useful in

future studies of the accumulated burden of cerebral hypoxia during the first 72 hours of life. The blood biomarkers of brain injury were exploratory and the finding of no associations to the burden of cerebral hypo- or hyperoxia, suggests that these blood biomarkers might not be useful in future studies on the accumulated burden of cerebral hypo- and hyperoxia measured by NIRS.

Dansk resumé (Summary in Danish)

Baggrund. Ekstremt præmature børn (børn født mere end 12 uger for tidligt) har stor risiko for død eller neurologiske senfølger. Dødeligheden er 25%, og 25% af de overlevende børn lever med ét eller flere handicap i form af cerebral parese, lav intelligenskvotient, nedsat kognitiv funktion eller indlæringsvanskeligheder. En af årsagerne til hjerneskade er det præmature barns umodne cirkulatoriske- og respiratoriske system, som forårsager klinisk ustabilitet og dermed perioder med for lavt eller for højt iltindhold i hjernevævet (cerebral hypo- eller hyperoxi). Hypo- og hyperoxi i den sårbare præmature hjerne kan føre til en aktivering af en "årsagskæde" begyndende med en akut reaktion i hjernevævet, som senere kan medføre strukturelle hjerneskader og i sidste ende nedsat kognitiv eller psykomotorisk funktion.

SafeBoosC II studiet var et randomiseret multicenter feasibility studie. Det primære formål med studiet var at undersøge muligheden for at stabilisere hjernens iltning hos ekstremt præmature børn ved hjælp af monitorering med nær infrarød spektroskopi (NIRS) og derved reducere byrden af cerebral hypo- og hyperoxi.
En population på 166 ekstremt præmature børn blev inkluderet, senest 3 timer efter fødslen. Børnene blev randomiseret til to grupper. Forsøgsgruppe (n=86): cerebral NIRS-overvågning i kombination med en evidensbaseret retningslinje med anbefalede interventioner ved NIRS-værdier udenfor referenceområdet (55-85%), eller kontrolgruppe (n=80): blindet opsamling af NIRS-data kombineret med vanlig behandling. Interventionsperioden var 72 timer.
Det primære resultat af SafeBoosC II studiet var reduktion af den tid, hjernens iltning ikke var indenfor referenceområdet udtrykt som byrden af cerebral hypo- og hyperoxi i %-timer, beregnet som tiden uden for referenceområdet på 55-85% multipliceret med den gennemsnitlige absolutte afvigelse i de første 72 timer efter fødslen. En time med 50% oxygenering giver således en byrde på 5%-timer. Byrden af cerebral hypo- og hyperoxi blev reduceret med 58% ($p = 0,001$) i forsøgsgruppen sammenlignet med kontrolgruppen. Det skyldtes først og fremmest en reduceret byrde af cerebral hypoxi, da byrden af hyperoxi ens i de to grupper.
For at undersøge de mulige konsekvenser af den reducerede byrde af cerebral hypo- og hyperoxi blev effekter på hjernen monitoreret på forskellige måder fra fødslen og frem til forventet termin, og antallet af dødsfald blev optalt.
Da børnene var 6 og 64 timer fik de taget blodprøver, som blev analyseret for tre molekylære biomarkører for hjerneskade: S100beta, brain fatty acid binding protein (BFABP) og neuroketal. Standardiseret ultralydsundersøgelse af hjernen (cUS) blev foretaget på dag 1, 4, 7, 14, 35 og til

termin. Billederne blev analyseret af to eksperter, der inddelte billederne i 3 kategorier: Ingen hjerneskade, mild / moderat hjerneskade eller alvorlig hjerneskade. Elektroencefalografi (EEG) blev udført da børnene var 64 timer. EEG'erne blev analyseret centralt for burst rate og spectral edge frequency 95%. Ved termin fik børnene foretaget magnetisk resonans skanning (MRI). Billederne blev analyseret af tre eksperter, der inddelte billederne i 3 kategorier: Ingen hjerneskade, mild / moderat hjerneskade eller alvorlig hjerneskade. Alle analyser af de cerebrale resultater blev foretaget centralt uden kendskab til allokering og barnets sygehistorie.

Formålene med denne afhandling var

i) at afgøre, om intervention i SafeBoosC II studiet reducerede risikoen for negative cerebrale udfald og død.
ii) at undersøge, om byrden af cerebral hypoxi, uanset allokering i SafeBoosC II studiet, var forbundet med en øget risiko for negative cerebrale udfald og død.
iii) at diskutere relevansen af de cerebrale udfald i fremtidige randomiserede studier med cerebral NIRS-monitorering.

Resultater. I alt 157 børn blev kategoriseret ud fra cUS. Antallet af alvorlige hjerneskader var ikke signifikant forskelligt mellem grupperne (forsøgsgruppe (10/80) vs. kontrolgruppe (18/77), (p = 0,10)). MRI til termin blev foretaget hos 84 børn. Antallet af alvorlige hjerneskader var ens i de to grupper (forsøgsgruppe (5/46) vs. kontrolgruppe (3/38), (p = 0,72)). Der var færre dødsfald i forsøgsgruppen (12/86) end i kontrolgruppen (20/80) (p = 0,08). Det kombinerede resultat af død eller alvorlig hjerneskade kategoriseret ved cUS var signifikant mindre i den eksperimentelle gruppe (19/82) end i kontrolgruppen (30/78), (p = 0,041).
EEG fra 133 børn blev analyseret. Burst rate (forsøgsgruppe 7,2 vs. 7,7 burst/min i kontrolgruppe) og spectral edge frequency 95% (forsøgsgruppe 18,1 vs. 18,0 hertz i kontrolgruppe) var ens i de to grupper. Blodprøver for 123 børn blev analyseret, og blodets indhold af S100beta, BFABP og neuroketal var ens i de to grupper.
Post-hoc analyse viste, at børnene med en byrde af cerebral hypoxi i den højeste kvartil havde signifikant højere risiko for svær hjerneblødning (11/39 vs. 11/117, p = 0,003), lav burst rate på EEG (12/28 vs. 21/103, p = 0,015) og død (14/41 vs. 18/123, p = 0,006) i forhold til børn med en byrde af cerebral hypoxi i de 3 nedre kvartiler. Ingen af de ovenstående hændelser var forbundet med cerebral hyperoxi. Efter multipel logistisk regression var følgende variable signifikant associeret med det kombinerede resultat af død eller alvorlig hjerneskade på cUS: intervention (p = 0.003), gestationsalder under 26 uger (p = 0.007), brug af vasopressorstoffer (p = 0.014) og

blodtransfusion (p = 0.016). Der var ingen sammenhæng mellem byrden af cerebral hypo- eller hyperoxia og S100beta, BFABP eller neuroketal

Konklusion

i) Der var færre dødsfald og færre alvorlige hjerneskader målt ved cUS i forsøgsgruppen, og det kombinerede resultat af alvorlig hjerneskade eller død var signifikant lavere i forsøgsgruppen. SafeBoosC II var et feasibility studie og styrken af studiet var beregnet i forhold til at reducere byrden af cerebral hypo- og hyperoxi og altså ikke til at afgøre, om der var klinisk målbare effekter af en sådan reduktion.

ii) Risikoen for alvorlig hjerneskade vurderet ved cUS, EEG-burst rate eller død var højest hos børnene med den største byrde af cerebral hypoxi. Statistiske analyser fandt co-linearitet mellem byrden af cerebral hypoxi og interventionen i forsøgsgruppen, og efter multipel logistisk regressionsanalyse var risikoen for død eller alvorlig hjerneskade på cUS signifikant associeret med interventionen, gestationsalder under 26 uger, brug af vasopressorer og blod transfusion, og ikke med byrden af cerebral hypoxi.

iii) I fremtidige randomiserede studier, som beskæftiger sig med neuroprotektive effekter af cerebral NIRS monitorering, bør det overvejes at kombinere død og alvorlig hjerneskade på cUS, som primært resultat af interventionen. MRI kan gennemføres i en undergruppe af børn fra centre med MRI-dedikerede forskningsgrupper. Det vil gøre det muligt at undersøge, om der er diskrete forskelle mellem grupperne. Da der ikke var beviser for, at EEG burst rate eller spectral edge frequency 95% var påvirket af den reducerede byrde af cerebral hypoxi i forsøgsgruppen, anses disse EEG-udfald at være irrelevante i fremtidige studier af den akkumulerede byrde af cerebral hypoxi i de første 72 timer af livet. De molekylære biomarkører for hjerneskade (S100beta, BFABP og neuroketal) var hverken relateret til graden af hypo- eller hyperoxi, hvilket antyder, at disse markører ikke vil være nyttige i fremtidige undersøgelser af den akkumulerede byrden af cerebral hypo- og hyperoxi målt ved NIRS.

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Brain injury in the international multicenter randomized SafeBoosC phase II feasibility trial: cranial ultrasound and magnetic resonance imaging assessments

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BACKGROUND: Abnormal cerebral perfusion during the first days of life in preterm infants is associated with higher grades of intraventricular hemorrhages and lower developmental score. In SafeBoosC II, we obtained a significant reduction of cerebral hypoxia by monitoring cerebral oxygenation in combination with a treatment guideline. Here, we describe (i) difference in brain injury between groups, (ii) feasibility of serial cranial ultrasound (cUS) and magnetic resonance imaging (MRI), (iii) local and central cUS assessment.

METHODS: Hundred and sixty-six extremely preterm infants were included. cUS was scheduled for day 1, 4, 7, 14, and 35 and at term-equivalent age (TEA). cUS was assessed locally (unblinded) and centrally (blinded). MRI at TEA was assessed centrally (blinded). Brain injury classification: no, mild/moderate, or severe.

RESULTS: Severe brain injury did not differ significantly between groups: cUS (experimental 10/80, control 18/77, $P = 0.32$) and MRI (5/46 vs. 3/38, $P = 0.72$). Kappa values for local and central readers were moderate-to-good for severe and poor-to-moderate for mild/moderate injuries. At TEA, cUS and MRI were assessed in 72 and 64%, respectively.

CONCLUSION: There was no difference in severe brain injury between groups. Acquiring cUS and MRI according the standard operating procedures must be improved for future trials. Whether monitoring cerebral oxygenation during the first 72 h of life prevents brain injury should be evaluated in larger multicenter trials.

The circulatory adaption to birth is problematic for the vulnerable preterm infant. The immature respiratory and circulatory systems may cause episodes of cerebral hypoxia, which may lead to brain injury. Near-infrared spectroscopy (NIRS) has been used to monitor tissue oxygenation, and abnormal cerebral perfusion has been associated with higher grades of intraventricular hemorrhages and lower neurodevelopmental scores at 2 y of age (1,2). The multicenter feasibility trial SafeBoosC II (3) demonstrated that cerebral NIRS in combination with a dedicated treatment guideline (4) was able to reduce the median burden of cerebral hypo- and hyperoxia by 58% (95% confidence interval: 35–73%; $P < 0.001$) compared with a control group with blinded collection of NIRS data and treatment as usual (5). Hypoxia was reduced, while hyperoxia was unaffected. Whether the experimental intervention prevents brain injury remains to be determined.

Cranial ultrasound (cUS) is the most widely used neuroimaging tool for assessing brain injury in preterm infants, and cUS findings are often reported as an outcome measure in randomized clinical trials. The advantages of cUS are that it causes minimal disturbance to the preterm infant, has no side effects, can be conducted at the bedside, is easy to repeat as often as indicated, and is relatively inexpensive. Furthermore, severe brain injury as assessed by cUS correlates well with later neurodevelopmental outcome; hence, cUS, especially sequentially, can be used to predict later neurodevelopmental outcome (6–9). Magnetic resonance imaging (MRI) at term-equivalent age (TEA) though is superior to cUS in predicting neurodevelopmental outcome and cerebral palsy at 2 y of age (10,11). More recently, it has been suggested that MRI can be used for the evaluation of neuroprotective treatments by quantitative applications (12–14) as subtle differences in brain development can be detected (15). Thus, MRI may reduce the number of infants needed for future randomized neuroprotective trials. Additionally, MRI has been suggested for routine use in clinical settings for high-risk extremely preterm infants (13).

In SafeBoosC II, brain injury assessed by serial cUS was a secondary outcome and MRI at TEA was an explorative outcome. The aims of the present study were (i) to assess the difference in brain injury between the two treatment groups of

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Received 12 May 2015; accepted 31 August 2015; advance online publication 13 January 2016. doi:10.1038/pr.2015.239

the SafeBoosC II trial, (ii) to describe the feasibility of neuroimaging (cUS and MRI) according to a standard operating procedure, and (iii) to explore the differences between interobserver agreement of central and local readings of serial cUS in this randomized multicenter feasibility trial.

RESULTS

Birth weight and gestational age did not differ between the experimental and the control groups (Table 1).

Table 1. Patient characteristics

	Experimental group	Control group
Infants included in SafeBoosC II	*n* = 86	*n* = 80
Gestational age (wk), median (range)	26.9 (23.9–27.9)	26.9 (23.4–27.9)
Birth weight (g), median (range)	806 (410–1,286)	880 (480–1,330)
Infants with central overall cUS score	*n* = 80	*n* = 77
Gestational age (wk), median (range)	26.6 (23.9–27.9)	27.0 (23.4–27.9)
Birth weight (g), median (range)	806 (410–1,286)	880 (490 - 1330)
Infants with MRI at any time	*n* = 49	*n* = 38
Gestational age (wk), median (range)	27.0 (23.9–27.9)	26.7 (24.0–27.9)
Birth weight (g), median (range)	806 (520–1,180)	900 (515–1,330)
Infants with MRI at corrected age 40–44 wk	*n* = 28	*n* = 29
Gestational age (wk), median (range)	26.7 (23.9–27.9)	27.0 (24.9–27.9)
Birth weight (g), median (range)	800 (520–1,180)	934 (700–1,330)

cUS, cranial ultrasound; MRI, magnetic resonance imaging.

Differences in Brain Injury Between Groups

Cranial ultrasound

Local reading. The distribution of brain injury severity did not differ at any time-point between the experimental and the control groups; neither did the overall cUS-score.

Central reading. The raw and unadjusted P values for the between-group analysis showed a significant difference in the distributions of brain injury on day 1 ($P = 0.043$) and at TEA ($P = 0.01$), whereas the secondary outcome of the SafeBoosC II trial, the overall cUS score, did not differ significantly between the groups ($P = 0.053$). The number of severe injuries was lower in the experimental than in the control group, whereas the number of mild injuries was higher in the experimental than in the control group (Table 2). The odds ratio of severe brain injury vs. no severe brain injury was insignificant at all time points and for the overall cUS score. There were fewer severe intracranial hemorrhages (IVH grade III and periventricular hemorrhagic infractions) in the experimental group (8/80 (10%)) than in the control group (14/77 (18.2%)), but this was not significant (odds ratio 0.50 (95% confidence interval: 0.20–1.27)).

In 25 of the 28 preterm infants with severe brain injury, this was detected within the first 7 d of life. In the remaining three infants, severe brain injury on cUS was diagnosed by day 35 (posthemorrhagic ventricular dilatation, day 14; local cystic lesion, day 35; and cerebral atrophy, day 35).

Magnetic resonance imaging

Twenty-four MRIs conducted within the predefined corrected gestational age of 40–44 wk in each group were assessed ($n = 48$; Table 3). MRIs done outside the predefined corrected

Table 2. Brain injury severity for each of the sequential and the overall cUS score as assessed by central reading

	Day 1	Day 4	Day 7	Day 14	Day 35	TEA	Overall cUS score
Experimental group (*n* = 80)							
No scan/central score (*n*)	6	7	4	9	4	24	—
Death (*n*)	0	2	3	6	10	10	—
No injury (*n*)	54	42	44	30	32	10	21
Mild/moderate injury (*n*)	19	22	22	27	29	31	49
Severe injury (*n*)	1	7	7	8	6	5	10
Control group (*n* = 77)							
No scan/central score (*n*)	6	3	3	4	10	12	—
Death (*n*)	0	3	9	13	15	19	—
No injury (*n*)	55	41	36	26	23	23	26
Mild/moderate injury (*n*)	10	18	18	24	19	17	33
Severe injury (*n*)	6	12	11	10	10	6	18
χ^2 test, *P* value[a]	0.043	0.42	0.44	0.78	0.26	0.01	0.053
Severe vs. no severe brain injury[b]							
Odds ratio (95% confidence interval)	0.15 (0.02–1.27)	0.54 (0.20–1.46)	0.52 (0.19–1.44)	0.70 (0.26–1.92)	0.41 (0.14–1.22)	0.81 (0.23–2.88)	0.47 (0.20–1.09)

cUS, cranial ultrasound; TEA, term-equivalent age.
[a]χ^2 analysis of the between-group distribution of brain injuries in the three categories: no injury, mild/moderate injury, and severe injury. [b]Odds ratio for severe vs. no severe (no/mild/moderate) brain injury.

Table 3. MRI brain injury severity between experimental and control group

MRI at corrected age 40–44 wk	Experimental group ($n = 24$)	Control group ($n = 24$)	*P* value[a]	
Corrected age (wk), median (range)	41.4 (40.7–43.6)	41.2 (40.3–44.0)		
No injury, *n* (%)	3 (13)	2 (8)		
Mild/moderate injury, *n* (%)	18 (74)	19 (79)	0.89	
Severe injury, *n* (%)	3 (13)	3 (13)		
MRI at any time point	$n = 46$[b]	$n = 38$	*P* value[a]	OR (95% CI)
Corrected age (wk), median (range)	41.4 (36.7–52.7)	41.4 (39.8–53.9)		
No severe brain injury, *n* (%)	41 (89)	35 (89)		
Severe injury, *n* (%)	5 (11)	3 (8)	0.72	1.4 (0.3–6.4)

CI, confidence interval; MRI, magnetic resonance imaging.
[a]χ^2 test for equal distribution of brain injuries between the groups. [b]Three infants were excluded from the analysis as the quality of the MRI was of insufficient quality.

gestational age of 40–44 wk (n = 30 there of three with artifacts) and MRIs with movement artifacts (n = 4) or slice thickness above 2 mm unless severe injury was clearly seen (n = 5) were excluded. MRI brain injury scores did not differ between the two groups. Analysis including MRI at any time point (46 infants in the experimental group and 38 infants in the control group) was also carried out, and there was no difference between the groups (**Table 3**).

Discrepancies between cUS and MRI findings

None of the cUSs or MRIs scored as no brain injury was scored as severe in the other imaging modality, but in five of the MRI conducted within the predefined gestational age of 40 to 44 wk, there was disagreement of mild/moderate in one modality compared to severe brain injury in the other. Two preterm infants with IVH grade 1 or 2 seen on cUS (mild/moderate brain injury) on day 1 and day 7, respectively, were classified as severe brain injury on MRI (Kidokoro score of 12) (16). Both had cerebellar hemorrhages and additionally increased subarachnoid space or cerebral occipital atrophy, which was not identified by cUS.

One preterm infant had IVH grade III (day 7) and subsequently developed posthemorrhagic ventricular dilatation on cUS. MRI at TEA confirmed posthemorrhagic ventricular dilatation; however, according to the MRI Kidokoro scoring system, this resulted in mild/moderate global injury score, since the ventricles had returned to almost normal sizes (16). Cerebellar hemorrhage was seen on cUS in one preterm infant resulting in a severe cUS injury score. As there were no additional lesions and the cerebellar hemorrhage was unilateral on MRI, this resulted in mild/moderate global MRI injury score (16). A lenticulo-striatal stroke was seen on cUS in one preterm infant, this was classified as severe cUS brain injury. On MRI at TEA, the atrophy of the stroke area could be seen; however, in the global brain injury score, the sum of all lesions yielded a mild/moderate injury score.

Twenty-seven MRIs performed at other time points than corrected gestational age 40 to 44 wk were assessed. Four of these had severe brain injury on cUS, which in two of the cases also was identified on MRI. In the other two infants with severe brain injury on cUS, one infant was diagnosed on day 1 with unilateral hemorrhagic infarction; however, the MRI at 49 wk showed mild/moderate injury. The other infant was diagnosed with a lenticulostriatal stroke on cUS day 4 and the MRI at 47 wk of corrected gestational age showed mild/moderate injury.

Feasibility

Cranial ultrasound

Eight-hundred and thirteen of the 887 planned cUS series (cUSs as described in the cUS-standard operating procedure from birth to time of death or TEA) were available for central reading, each series consisting of up to 12 images. In 32 series, the quality was too poor for central image analysis. Seven-hundred and eighty-one (88%) of the planned cUS series were thus scored centrally. The number of uploaded and centrally scored cUS series was highest on day 7 (experimental group 95%, control group 96%) and lowest at TEA (experimental group 66%, control group 79%; **Figure 1**). In total, only 55% of the infants in the experimental and 65% in the control group had all cUS from day 1 till TEA or death.

Local overall cUS score was calculated in 165 out of 166 infants; 1 infant died prior to the first cUS. One-hundred and fifty-seven (157/166) infants (95%) had an overall central cUS score. Reasons for no central overall cUS score were death prior to the first cUS (n = 1), too few uploaded image series per patient (less than 3 cUS; n = 1), poor quality of the images (n = 3), or a combination of both (n = 4). Three of the infants without an overall central cUS score died before TEA, two in the experimental and one in the control group.

Magnetic resonance imaging

Eighty-seven (65%) of the 134 infants alive at TEA had an MRI. Median (range) corrected age at the time of the MRI was 41.4 wk (range: 36.8–53.9). Fifty-seven (66%) of the MRIs were conducted within the predefined corrected gestational age of 40 to 44 wk (median (range)) 41.4 wk (40.0–44.0) in accordance with the MRI-standard operating procedure. The reasons for no MRI were: lack of parental consent (n = 29), the infant could not lie still (n = 3), or other reasons such as technical problems, child discharged to other department, or failure to turn up at the appointment (n = 15).

Central vs. local readings cUS

Kappa values between the local and central readers were highest for periventricular hemorrhagic infarction (κ = 0.79 at day 7 and 0.75 at day 14) and posthemorrhagic ventricular

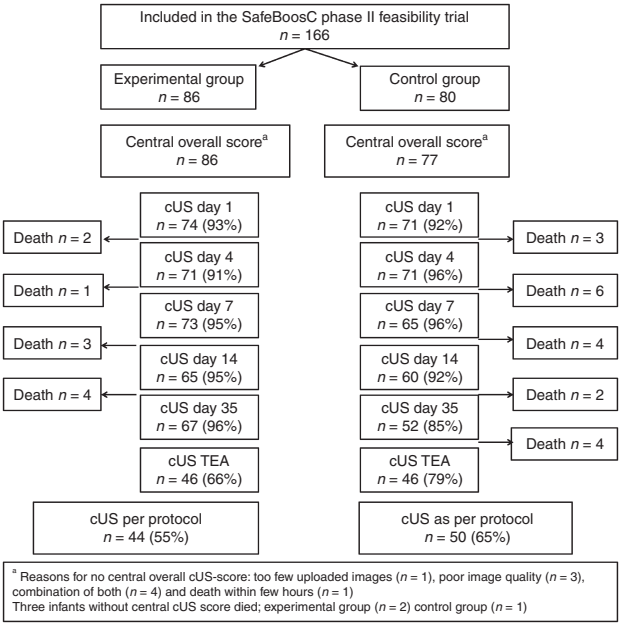

Figure 1. Flow chart from inclusion to term-equivalent age, number of uploaded serial cranial ultrasound scans in the two treatment groups.

dilatation ($\kappa = 1$ at day 7 and 0.72 at day 14), whereas the lowest κ values were found for intraventricular hemorrhage grade I and II ($\kappa = 0.32$ at day 1 and 0.34 at day 4) and persisting inhomogeneous flaring at day 14 ($\kappa = 0.04$). Kappa values (95% confidence interval) for the overall cUS score no, mild/moderate or severe brain injury was 0.61 (0.49–0.72) for the central readers and 0.40 (0.29–0.51) for local vs. central. Interobserver κ value for the overall cUS score for severe vs. no severe injury between the two central readers was 0.86 (0.75–0.96), whereas the agreement between the local and central reading was 0.57 (0.41–0.74). These κ values were similar for the images scored in DICOM- and in other formats.

DISCUSSION

Differences in Brain Injury Between Groups

The odds ratio of severe brain injury vs. no severe brain injury was insignificant at all time points and for the overall cUS score. In addition, conventional MRI did not suggest any differences between the two groups. However, cUS findings were only a secondary outcome and MRI an explorative outcome in the SafeBoosC II trial; therefore, the study was not powered to look at differences in brain injury between the two groups. More infants in the control group than in the experimental group had severe brain injuries at day 1, although the patient characteristics were similar. As the infants did not have cUS before inclusion of the trial, it is not possible to determine if these numbers reflect an early effect of the intervention or if it was present before the inclusion. Therefore, inclusions immediately after birth or neuroimaging before intervention are considerations for future studies.

The number of severe brain injury as assessed by blinded central cUS reading was lowest in the experimental group, mainly due to fewer cases of severe intracranial hemorrhages, as compared with the control group during the first week of life. However, there were more mild injuries on cUS in the infants in the experimental group compared with the control group. This could be a result of a shift from severe brain injury and death, which was highest in the control group, toward more mild injuries. This finding was not confirmed on MRI at TEA.

There is no gold standard for monitoring cerebral tissue oxygenation; therefore, the target values of cerebral oxygenation (55–85%) in the SafeBoosC II trial are based on the best possible evidence, which is the 95% confidence interval of cerebral NIRS values in 439 preterm infants born below 32 wk of gestation within the first 3 d of life (P. Lemmers and F. van Bel, unpublished data). In the SafeBoosC II trial, NIRS monitoring in combination with a dedicated treatment guideline for cerebral tissue oxygenation out of range was able to significantly reduce the burden of cerebral hypoxia during the first 72 h of life (5). It has previously been reported that low cerebral NIRS values in preterm infants during the first days of life are associated with higher grades of intraventricular hemorrhage and lower developmental quotients in very preterm infants (1,2,17), but in this study, we did not find a difference in the number of severe brain injuries between the experimental and the control groups neither in cUS nor in cerebral MRI. However, it must be kept in mind that the SafeBoosC II trial was not powered to look at the numbers of severe brain injuries between groups, as cUS and MRI were secondary and explorative outcomes, it could be speculated that the reduction of the cerebral burden of hypoxia was not of clinical relevance, but the burden was reduced by 58% in the experimental group (5). The cerebral oxygenation was only measured during the first 72 h of life, during the period where most severe intracranial hemorrhages are known to occur (7). We did find more severe intracranial hemorrhages in the control group than in the experimental group, but the difference was insignificant. Measuring NIRS during the first 72 h of life in extremely preterm infants may potentially reduce the number of severe brain injuries especially the early and intracranial hemorrhages, known to cause unfavorable neurodevelopmental outcomes, but the SafeBoosC II trial is not powered to make any final conclusions. Assessment of neurodevelopmental outcome at 2 y of age in this cohort is ongoing and will provide further information on the possible adverse effects of low cerebral oxygenation during the first 72 h of life in this population.

Feasibility of Neuroimaging in This Multicenter Trial

We showed that it is feasible though challenging to use central reading of cUS as an outcome measure in a randomized multicenter trial: most images could be centrally scored. Central reading of cUS in multicenter studies has previously proven possible (18). The percentage of uploaded cUS series decreased from day 7 onwards; at TEA, it was only 72%, and many infants were discharged or transferred to local hospitals at this age. As some lesions, such as cystic periventricular leukomalacia (19), cerebral atrophy (20), and posthemorrhagic ventricular dilatation develop over time, it has been argued that it is necessary

to perform serial cUS until TEA in order to capture the full extent of brain injury (21). However, in one study, only 4% of the severe brain injuries was detected after day 35 (19). None of our infants scanned at TEA shifted category from no severe brain injury to severe brain injury, but we may potentially have missed some cases.

MRI at TEA was optional and performed in only 64% of the infants. This number was lower than we had expected, maybe because MRI at TEA was not standard clinical care of preterm infants in most participating centers, and an additional informed consent for MRI was required separately from the consent to the randomized intervention. This is a limitation of our study. The infants in the SafeBoosC II trial were followed closely by serial cUS, and it is possible that the parents felt they were already well informed about the extent of the brain injury and the risk of later adverse neurodevelopmental outcomes and therefore did not want to take part in an additional MRI scan where the infant might be sedated and admitted to the hospital for this purpose alone. In this study, two infants who had mild/moderate brain injury on early cUS because of IVH grade I-II findings were categorized as having severe injuries on MRI because of cerebellar hemorrhages that were not recognized on serial cUS. Therefore, MRI is important in addition to cUS (21). Furthermore, MRI at TEA is superior to cUS in predicting the risk of CP in preterm infants (10) and contributes with additional information to serial cUS when estimating risk of adverse neurodevelopmental outcome (18)—MRI at TEA therefore plays an important role in the risk stratification in these infants (11). In our study, the number of severe and mild/moderate brain injuries on MRI was similar in the control and the experimental groups.

In future, large multicenter randomized controlled trials studying neuroprotective interventions; only MRI-dedicated neuroimaging groups/centers might use MRI. The MRI protocol should be followed rigorously, in order to make sure that the timing, slice thickness, and quality are comparable for all infants. With high resolution MRI, mild injury might be easier to pick up and fewer patients are needed to prove differences in quantitative measures that are related to long-term outcome (22). It is essential to obtain knowledge on the possible effects (benefits or harms) of the neuroprotective interventions in the vulnerable and developing preterm brain. In this study, it was not possible to perform quantitative MRI analysis because of different scanner systems and image acquisitions.

Central vs. Local Readings cUS

The overall κ values for the local and the central readers were moderate to good for severe injuries and poor to moderate for mild/moderate injuries. These findings are consistent with previously published interobserver cUS studies (23–25).

Local readers have the advantage of real-time images, whereas the central readers have only selected still images for assessment. However, central readers are blinded to the treatment groups, which are important in randomized clinical trials where blinding of the person performing the cUS is impossible, as in the SafeBoosC II trial. Central reading might be thus the preferred method of analyzing cUS images in randomized multicenter studies (24) without blinded intervention. The problems of poor image quality may be addressed by dedicated training in a future study.

Conclusions

The distribution of brain injury into no, mild/moderate, or severe injury did not differ among the groups neither for the overall cUS score nor for MRI. There was less severe brain injury in the experimental group as assessed by central reading of cerebral ultrasound, which is in line with the reduction of cerebral hypoxia in the experimental group of the SafeBoosC II trial, but MRI conducted at TEA in 64% of the infants did not support this finding. The problems in acquiring cUS and MRI data of good quality might be addressed by dedicated training. cUS as well as MRI images at TEA were often missing. Central, blinded readers of cUS achieved high interobserver agreement for severe brain injury. Large randomized clinical trials should be performed to evaluate the effect of the burden of cerebral hypoxia on brain injury in extremely preterm infants.

METHODS

Study Design and Population

One-hundred and sixty-six extremely preterm infants from eight European countries were included (1 June 2012 to 31 December 2013) (5). The infants were randomized to either the experimental group or the control group. Infants in the experimental group were monitored with NIRS, and continuous cerebral tissue oxygenation levels were available for the clinician together with a dedicated treatment guideline (4), listing possible interventions if the cerebral oxygenation level was out of range (55–85%). The infants in the control group were also monitored with cerebral NIRS, and the data were recorded but blinded to the clinicians. They were given standard care. In both groups, the cerebral NIRS monitoring was started within 3 h after birth and continued until 72 h of life (5).

The protocol of SafeBoosC II is published (3) and is available in full at www.safeboosc.eu. The study is registered at ClinicalTrial.gov, NCT01590316.

Cranial Ultrasound

On day 1 (any time during the first 24 h of life), 4 (±1), 7 (±1), 14 (±1), 35 (±1), and at TEA (week 38 to 44) standardized cUS (six coronal and five sagittal images through the anterior fontanel and 1 through the mastoid window) was performed. The cUS was performed unblinded and according to local standard, either by the clinical staff or by a suitable qualified sonographer. Local unblinded investigators reported the cUS findings in an electronic record form. The images were anonymized and uploaded as DICOM files for central reading. The local scorings from the electronic record form were converted into predefined diagnoses (**Table 4**), which correlate to later developmental outcomes (18,20,26,27).

Central reading was performed by two cUS readers (M.B. and C.H.) blinded to treatment group and medical history of the infants. Initial cUS images from two times 20 random infants were scored by the central readers to ensure common use of the scoring system. Then, the central readers scored all cUS series individually according to the predefined diagnoses (**Table 4**). Disagreements were resolved by consensus meetings. Each cUS was categorized on a three-level scale as no brain injury, mild/moderate brain injury, or severe brain injury (18,20,26,27). DICOM images were analyzed using the software program OsiriX version 6.0. (Pixmeo, Geneva, Switzerland). Images uploaded in other formats were viewed in Preview.

The scores of all cUS images for each infant were combined into an overall cUS score reflecting the worst scan in any of the serial cUSs. In case of death, the overall cUS score would reflect the worst scan in

Table 4. Predefined diagnosis for cranial ultrasound scans

No brain injury
• None of the findings below
Mild/moderate brain injury
• Grade 1–2 IVH (including germinal layer hemorrhage)
• Isolated ventriculomegaly with ventricular index <p97[a]
• Inhomogeneous flaring persisting after day 7
• Global thinning of corpus callosum at TEA
Severe brain injury
• IVH grade III (ventricular index >p97 during the acute phase)[a]
• Posthemorrhagic ventricular dilatation
• Parenchymal/periventricular hemorrhagic infarction
• Unilateral porencephalic cysts
• Cystic periventricular leukomalacia (bilateral)
• Cerebellar hemorrhage
• Cerebral atrophy at term age[b]
• Stroke

At each cUS the infant was diagnosed with one of the above findings.
cUS, cranial ultrasound; IVH, intraventricular hemorrhage; TEA, term-equivalent age.
[a]Reference values according to Brouwer *et al.* (21). [b]Brain atrophy was defined as the combination of enlarged subarachnoid spaces, widened interhemispheric fissure, and reduction in complex gyral folding with or without concomitant ventriculomegaly, according to Horsch *et al.* (20).

any of the cUSs performed before the time of death. Infants missing cUS examinations on more than two of the predefined time points were excluded from the analysis unless the brain injury was classified as severe in any of the existing images.

Magnetic Resonance Imaging

At TEA (corrected age 40–44 wk), the parents of the preterm infants were offered an MRI. The MRI procedure was performed according to local protocols; preferably on a 3T MRI system; if this was not available, images were acquired on a 1.5T system. T_1- and T_2-weighted images were used for brain injury scoring. The MRIs were anonymized and uploaded in DICOM format for central reading. A.M.P., C.H., M.F., and M.B. analyzed the MRIs blinded to treatment group, medical history of the infant, and cUS findings. MRIs were scored in consensus. The scoring was done according to a combination of previously published scoring systems (11,16,28). The scores were categorized into: normal brain (0–3 points), mild/moderate brain injury (4–11 points), and severe brain injury (12 points or more) as described by Kidokoro (16). All MRIs were evaluated in the software program OsiriX version 6.0.

Ethics

The SafeBoosC phase II feasibility multicenter trial was approved by each hospital's local research ethics committee (Hopital Femme Mere Enfants, Lyon, France; Rigshospitalet, Copenhagen, Denmark; La Paz University Hospital, Madrid, Spain; Cork University Maternity Hospital, Cork, Ireland; Wilhelmina Children's Hospital, Utrecht, The Netherlands; Medical University of Graz, Graz, Austria; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; and Rosie Hospital, Cambridge University Hospitals, UK) and where required (Austria, Denmark, and France) by the competent authority responsible for medical devices. Parental written informed consent was mandatory before inclusion in the trial. Additional informed parental consent was obtained for MRI at TEA if MRI was not clinically indicated or standard clinical care.

Statistics

Patient characteristics were summarized. The distribution of the brain injury severity on each day of the sequential cUS scans, the overall cUS score, and the MRI scores was tested between the groups by χ^2 test. The results were dichotomized into severe brain injury or no severe brain injury, and odds ratios were calculated. The percentage of the per-protocol images that were uploaded and scored was calculated for each day of the sequential cUS and for MRI. Kappa statistics was used to test the agreement between the two central readers and between the central and the local cUS readers. The statistics was performed using the software RStudio Version 0.98.501 (Boston, MA) and IBM SPSS Statistics for Windows Version 20.0.0 (Armonk, NY).

STATEMENT OF FINANCIAL SUPPORT

The Danish Council for Strategic Research financially supported this work through an unconditional and unrestricted grant of DKK 11,100,105. The funder had no role in the design, conduct, or analysis of the trial.

Disclosure: The authors have no financial relationships relevant to this article to disclose.

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The SafeBoosC II randomized trial: treatment guided by near-infrared spectroscopy reduces cerebral hypoxia without changing early biomarkers of brain injury

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BACKGROUND: The SafeBoosC phase II multicentre randomized clinical trial investigated the benefits and harms of monitoring cerebral oxygenation by near-infrared spectroscopy (NIRS) combined with an evidence-based treatment guideline vs. no NIRS data and treatment as usual in the control group during the first 72 h of life. The trial demonstrated a significant reduction in the burden of cerebral hypoxia in the experimental group. We now report the blindly assessed and analyzed treatment effects on electroencephalographic (EEG) outcomes (burst rate and spectral edge frequency 95% (SEF95)) and blood biomarkers of brain injury (S100β, brain fatty acid-binding protein, and neuroketal).

METHODS: One hundred and sixty-six extremely preterm infants were randomized to either experimental or control group. EEG was recorded at 64 h of age and blood samples were collected at 6 and 64 h of age.

RESULTS: One hundred and thirty-three EEGs were evaluated. The two groups did not differ regarding burst rates (experimental 7.2 vs. control 7.7 burst/min) or SEF95 (experimental 18.1 vs. control 18.0 Hz). The two groups did not differ regarding blood S100β, brain fatty acid-binding protein, and neuroketal concentrations at 6 and 64 h (n = 123 participants).

CONCLUSION: Treatment guided by NIRS reduced the cerebral burden of hypoxia without affecting EEG or the selected blood biomarkers.

The mortality in extremely preterm infants is approximately 25% and the prevalence of moderate and severe neurodevelopmental impairment in the surviving infants is as high as 25% (1). The etiology behind this neurodevelopmental impairment is multifactorial and partly related to the immaturity of the respiratory and circulatory systems leading to episodes of cerebral hypoxia. Cerebral hypoxia, in turn, through complex interactions between destructive and developmental disturbances may lead to micro- and macroscopic structural brain damage (2). The outcome of relevance to the individual child is neurodevelopmental impairment and possible ensuing disabilities, which cannot be determined before the child is fully grown. Therefore, there is a need for identifying early surrogate outcomes. Long before the age of reliable testing of neurological functions or intelligence, it is possible to visualize brain injuries such as intraventricular and parenchymal hemorrhage by early cranial ultrasound (3). Later cranial ultrasound may show ventricular dilatation as a sign of *exvacuo* dilatation caused by cerebral atrophy, or periventricular leukomalacia (2,4,5), whereas magnetic resonance imaging at term equivalent age may show minor degrees of white matter damage (3). Even before structural brain damage is visible, there are other means of detecting injury to the brain of the extremely preterm infant. Several cohort studies have demonstrated good correlations between early electroencephalography (EEG) and later developmental outcomes (6,7). A number of biomarkers show correlation with brain injury. Blood S100β is elevated in newborn infants with intraventricular hemorrhage (8) and hypoxic–ischemic encephalopathy (9). Blood brain fatty acid-binding protein (BFABP) is elevated in elderly patients with neurodegenerative diseases (10) and cerebrospinal fluid neuroketal is elevated in preterm infants with white matter damage on magnetic resonance imaging (11).

The SafeBoosC phase II randomized trial demonstrated that the combination of cerebral oxygenation monitoring by near-infrared spectroscopy (NIRS) combined with an evidence-based treatment guideline (12) vs. blinded collection of cerebral NIRS data and treatment as usual for 72 h significantly reduced the burden of cerebral hypoxia (13). To investigate the

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Received 17 June 2015; accepted 30 September 2015; advance online publication 20 January 2016. doi:10.1038/pr.2015.266

Table 1. Entry variables and 72-h outcomes of the included infants

	Infants with EEG		Infants with blood samples at 6 and 64 h	
	Experimental group ($n = 68$)	Control group ($n = 65$)	Experimental group ($n = 66$)	Control group ($n = 58$)
At entry variables				
Birth weight (g), median (range)	821 (410–1,286)	880 (490–1,330)	772 (410–1,286)	880 (515–1,330)
GA (wk), median (IQR)	26.6 (25.8–27.4)	27.0 (25.7–27.6)	26.6 (24.4–27.3)	27.0 (25.7–27.6)
Infants below 26 wk of gestation, *n* (%)	20 (29)	20 (31)	23 (35)	18 (31)
Twins, *n* (%)	15 (22)	13 (20)	14 (21)	10 (17)
Apgar below 5 at 5 min, *n* (%)	10 (15)	9 (14)	12 (18)	8 (14)
Antenatal steroids (complete course)	44 (65)	48 (74)	47 (71)	41 (71)
Outcomes at 72 h				
Burden of hyperoxia (%hours), median (IQR)	1.2 (0.4–11.8)	4 (0.2–29.5)	1.0 (0.3–6.2)	1.8 (0.2–21.3)
Burden of hypoxia (%hours), median (IQR)*	16.6 (5.2–65.4)	52.9 (17.2–152.9)	16.6 (6.1–63.5)	63.0 (21.2–174.8)

IQR, inter quartile range; EEG, electroencephalography.
* $P < 0.001$ between experimental and control groups (13).

possible benefits of the experimental intervention on putative surrogate outcomes (14–16), we recorded EEG with amplitude-integrated EEG tracing at the end of the intervention. We also analyzed the blood taken at the beginning and at the end of the intervention for the brain injury molecular biomarkers S100β, BFABP, and neuroketal. We hypothesized that the intervention would reduce the interburst interval (IBI) (6), which was a secondary outcome in the trial. Furthermore, an increased EEG spectral edge frequency 95% (SEF95) (17) and decreased levels of the brain injury molecular biomarkers S100β, BFABP, and neuroketal (exploratory outcomes) were expected.

RESULTS

Infant Characteristics

Patient characteristics at entry were similar across intervention groups but the burden of cerebral hypoxia was significantly lower at the end of intervention (72 h of age) in the experimental group vs. the control group (**Table 1**). There were no differences in GA and birth weight for infants with and without EEG or with or without molecular biomarkers at the two time points, although more infants without EEG had an Apgar score below five points at 5 min.

Electroencephalography

Eight infants died before 64 h of age (three in the experimental group and five in the control group) and another eight infants did not have any EEG recordings. Thus, one hundred and fifty EEGs were recorded. Seventeen of the recorded EEGs were not evaluable, which include impossible to read ($n = 7$), too short ($n = 2$), no artifact-free epochs ($n = 7$), or the infant was too old at the time of the EEG recording ($n = 1$). In total, we therefore analyzed EEGs from 133 infants. Data were collected using eight different EEG monitors: Micromed EEG system (Mogliano, Veneta, Italy) ($n = 29$); Nervus monitor (Cephalon, Norresundby, Denmark) ($n = 29$); Olympic cerebral function monitor (Natus, Pleasanton, CA) ($n = 29$); BRM2 and BRM3 (Natus, former Brainz monitor) ($n = 16$ and $n = 3$); NicoletOne video-EEG system (Carefusion, Madison, WI) ($n = 16$); g.recorder (g-tec, Graz, Austria) ($n = 7$); and Moberg CNS monitor (Moberg Research, Ambler, PA) ($n = 4$). The median age at the time of EEG recording, number of sedated infants, and the number of infants treated with opioid did not differ between the intervention groups (**Table 2**). The interobserver

Table 2. EEG recording details and results

EEG recording details	Experimental group ($n = 68$)	Control group ($n = 65$)	*P* value
Age at EEG (h), median (IQR)	64.8 (62.7–68.5)	65.8 (62.4–68.4)	NS
Epochs per infant, median (IQR)	12 (11–16)	12 (11–20)	NS
Sampling frequency 256 Hz, *n* (%)	54 (79)	50 (77)	NS[a]
Sampling frequency 200 Hz, *n* (%)	14 (21)	15 (23)	
Data filtered at 2–15 Hz, *n* (%)	13 (20)	10 (16)	NS
Opioid, *n* (%)	16 (24)	21 (32)	NS
Sedative, *n* (%)	9 (13)	5 (8)	NS
EEG results			
Severe burst suppression	3 (4)	2 (3)	
Burst suppression	11 (16)	7 (11)	0.75[b]
Discontinuous	41 (60)	41 (63)	
Continuous	13 (20)	15 (23)	
Burst rate (bursts/min) mean (SE)[c]	7.2 (0.72)	7.7 (0.73)	0.51
Spectral edge frequency 95% (Hz), mean (SE)[c]	18.1 (0.10)	18.0 (0.10)	0.51

IQR, inter quartile range; SE, standard error; NS, nonsignificant; EEG, electroencephalography.
[a]Chi-squared test of distribution of the sampling frequency between experimental and control. [b]Chi-squared test of distribution of the rEEG classification between experimental and control. [c]Result of mixed modelling after random exclusion of one twin from each twin pair.

agreement for the artifact rejection was 92% and the Kappa value was 0.61.

After inverse transformation, the IBIs were normally distributed and by multiplication by 60 s/min expressed as burst rate (bursts/min). Inter cluster correlations (ICCs) were above 0.15 for both burst rate and SEF95. Therefore, the between-groups analyses were conducted after randomly excluding one of each twin pair. Univariate analysis for burst rate and SEF95 was unaffected by GA ($P = 0.42$ and $P = 0.40$). The burst rate was significantly affected by EEG filter ($P < 0.0001$), type of EEG device ($P = 0.045$), and opioid treatment ($P = 0.004$). SEF95 was significantly affected by EEG sampling rate ($P < 0.0001$) and type of EEG device ($P<0.0001$). Type of EEG device and EEG filter and type of EEG device and EEG sampling rate were strongly correlated; therefore, only EEG filter and EEG sampling rate, opioid treatment, and GA were retained in the multiple regression models. In multiple regression, the mean (SE) burst rate did not differ between the two intervention groups: experimental 7.2 bursts/min (0.72) vs. control 7.7 bursts/min (0.73). The mean SEF95 (SE) did not differ between the intervention groups: experimental 18.1 Hz (0.10) vs. control 18.0 Hz (0.10) (**Table 2**). The delta-, theta-, alpha-, and beta-power bands did not differ between the intervention groups. The raw burst rates and SEF95 separated by EEG filter and EEG sampling rate are illustrated in **Figure 1a**,**b**. The unadjusted burst rate (SD) was reduced in infants with intraventricular hemorrhage grade III or periventricular hemorrhagic infraction on day 1–4 ($n = 10$): 5.6 bursts/min (3.6) compared to 9.8 bursts/min (5.0) in the remaining infants ($n = 120$).

Blood Biomarkers

One hundred and twenty-three infants (66 in the experimental group vs. 57 in the control group) had blood samples taken at both the time points (6 and 64 h of age). The concentrations of the three biomarkers in the blood did not differ between the intervention groups neither at 6 nor at 64 h of age (**Table 3**). The main effect of the intervention is illustrated in **Figure 2**.

The ICC between the twins was above 0.15 for all blood biomarkers. Therefore, all analyses were conducted after the exclusion of one random twin from each twin pair. The mixed-model analysis or nonparametric analysis, in case of non-normal distribution of the data, confirmed the findings above (**Table 3**). The blood biomarkers were similar in infants with and without intraventricular hemorrhage degree III or periventricular hemorrhagic infractions.

DISCUSSION

The reduction in the burden of cerebral hypoxia in the group receiving cerebral NIRS monitoring combined with an evidence-based treatment guideline was not reflected in any of the early biomarkers of brain injury that were assessed in the SafeBoosC II trial.

The Randomized Clinical Trial Design

The SafeBoosC phase II trial was a randomized clinical multicentre trial. We blindly evaluated and analyzed EEGs in 84% (133/158) and blood samples in 78% (123/158) of the infants alive at the time of the scheduled EEG recording and blood sampling. To avoid biased regression coefficients (18), we randomly excluded one of each twin pair, if the ICC was above 0.15.

The Challenges of EEG Recording

The EEGs were recorded with eight different devices. We therefore used a multiple regression model with corrections for EEG sampling specifications (EEG filter and EEG sampling rate). The difference in SEF95 given the different sampling rates of the EEG devices was not surprising since this was linked to the differences in frequency resolution of the Fast Fourier Transform given the different sampling rates. Some of the EEG

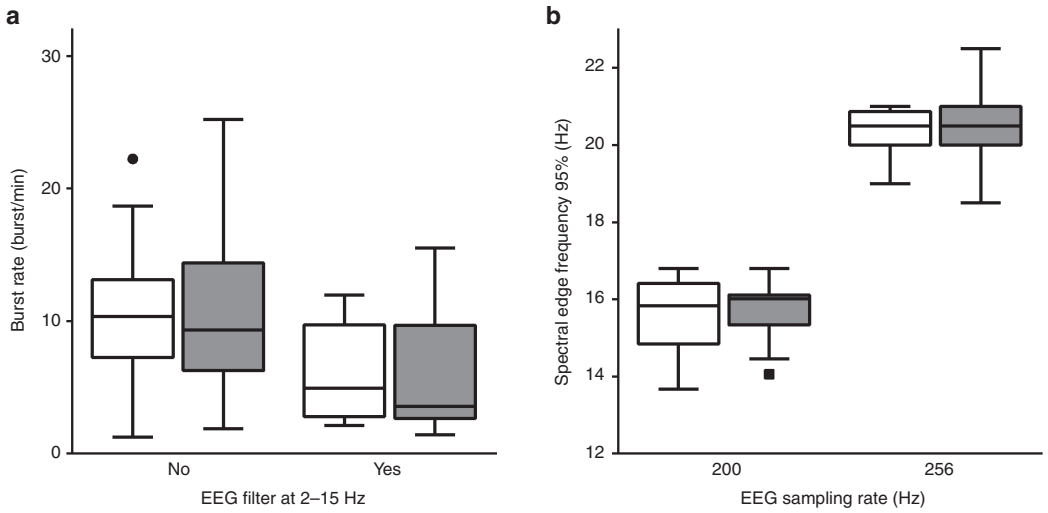

Figure 1. Intervention effects on EEG outcomes. (**a**) Intervention effect on burst rates, according to EEG filter of the device. (**b**) Intervention effect on spectral edge frequency 95%, according to the EEG sampling rate of the device. □ = control group, ■ = experimental group.

Table 3. Molecular brain injury biomarkers

Biomarker (time)	*n*	Median (pg/ml)	IQR (pg/ml)	*P* value	
				Unadjusted analysis	Adjusted analysis with exclusion of one random twin from each twin pair
S100Beta (6 h)	E = 75	696	341–983	0.26	0.50[a]
	C = 68	795	408–1,130		
S100Beta (64 h)	E = 70	602	267–1,024	0.51	
	C = 63	601	330–1,074		
BFABP (6 h)	E = 72	176	162–225	0.16	0.10[b]
	C = 67	193	168–246		
BFABP (64 h)	E = 69	181	155–227	0.47	
	C = 64	188	158–233		
Neuroketal (6 h)	E = 76	653	461–901	0.14	0.27[a]
	C = 68	589	329–798		
Neuroketal (64 h)	E = 69	576	280–856	0.61	
	C = 63	602	287–762		

The effect of the intervention at the 6 and 64-h time points in the middle column and the effect on the change over time to the right.
E, experimental group; C, control group; IQR, inter quartile range.
[a]Parametric statistics. [b]Nonparametric statistics.

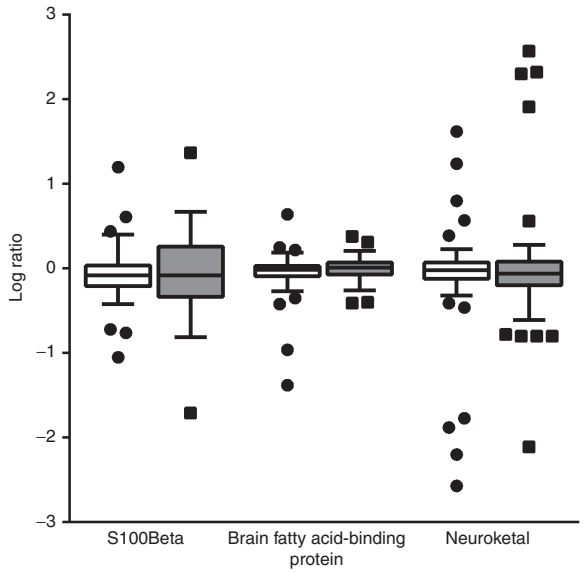

Figure 2. Intervention effect on the blood biomarkers illustrated as log ratio for the change in the concentration of the blood biomarkers from 6 to 64 h of age. □ = control group, ■ = experimental group.

amplifiers used were equipped with a band pass filter from 2 to 15 Hz. This clearly affected the detection of burst rates since the algorithm used for this was based on co-occurrence of slow- and high-frequency oscillations (19). Nevertheless, the burst rates in the SafeBoosC II trial were in line with previously published results in this group of infants (6,20,21) and the IBIs were longer in preterm infants with intraventricular hemorrhage grade III or periventricular hemorrhagic infractions, which has also previously been observed (22). However, we did not find a correlation between IBI and GA above or below 26 wk; this could be explained by the limited range of GA in the SafeBoosC trial. We therefore believe that the EEG data are robust and the results are reliable. While it is feasible to use EEG as an outcome in a multicentre randomized clinical trial, it should be realized that working with different EEG devices may complicate the analyses by adding heterogeneity.

The Relevance of the Chosen Biomarkers

EEG. In term newborn infants, severe hypoxic–ischemic brain injury is associated with EEG burst suppression, lasting hours to days and predicting later neurodevelopmental outcome and death (23,24). EEG burst rate is reduced in preterm infants when arterial P_{CO_2} and blood glucose concentrations are outside the normal range (20). Furthermore in preterm infants, permissive hypercapnia has been associated with increased power in the delta band of the EEG (25). Therefore, we hypothesized that EEG would be a sensitive surrogate marker of any clinically relevant brain effect of the reduction in cerebral hypoxia or hyperoxia that was aimed for in the SafeBoosC II trial. While the effect on cerebral hyperoxia was small and statistically insignificant, the reduction of cerebral hypoxia was highly statistically significant between the intervention groups, as well as potentially clinically relevant—amounting to more than a 50% reduction in cerebral hypoxia (13). We therefore expected the EEG to be different in the intervention groups. Furthermore, our present analyses are not able to determine if IBI or SEF95 is able to predict the long-term cerebral outcome of preterm infants. Several studies have investigated the predictive value of different EEG variables and later neurodevelopmental outcome, although the variables and the timing of the EEG recording that have been used differ (6,7,22), and therefore EEG should not yet be discarded

as a potential surrogate outcome of randomized clinical trials in preterm infants (14). A systematic review aiming to clarify the predictive value of EEG as a surrogate outcome for the developmental outcome in this group of infants is currently being conducted (26). We could not implement continuous EEG monitoring in this complex, multicentre SafeBoosC II trial and the age of 64 h was chosen as we hypothesized that the end of the intervention was the time with the highest likelihood of detecting an accumulated effect of a reduction of cerebral hypo- or hyperoxia in the experimental group—should such be found. This approach was chosen by extrapolation from term infants with severe hypoxic–ischemic brain injury in whom the EEG effects last some days (27).

We chose burst rate and SEF as the variables for the EEG analyses as these are the most commonly used in preterm infants and have been related to long-term outcomes (6,7,17). Certainly, other more sophisticated analytical methods could be used and may be more sensitive to subtle effects of moderate cerebral hypoxia (28).

Blood biomarkers. The three blood biomarkers of brain injury were explorative outcomes of the SafeBoosC II trial and the reduction in the burden of cerebral hypoxia was not reflected in any of them.

S100β is the best known of the three brain injury blood biomarkers and is routinely used in the screening of patients with mild to moderate head trauma (29). It is a small calcium-binding protein present in high concentration in Schwann cells and astrocytes (30) and after severe cerebral damage, it leaks from the tissue into the systemic circulation.

A small study in preterm infants found that the level of S100β in blood is correlated to the degree of intraventricular hemorrhage (31), whereas a recent larger study showed no correlation (32). The level of S100β is increased in term asphyxiated infants and the concentrations have been correlated to the degree of hypoxic–ischemic encephalopathy (9). Whereas hypoxic encephalopathy in term infants typically is the result of a terminal, short and severe hypoxia, the preterm infants in the SafeBoosC II trial were more likely exposed to various grades of cerebral hypoxia for shorter or longer periods during the first 72 h of life. There was no difference in S100β between the two intervention groups. This could be because the burden of cerebral hypoxia—even though the reduction in the experimental group was highly significant—was insufficient for the release of the intracellular S100β into the systemic circulation. Negative correlations between NIRS levels and S100β in critically ill children have been shown previously (33).

BFABP is a small molecule (15 kDa), which is rapidly excreted by the kidneys without cleavage. It is released from astrocytes as response to mechanical damage, ischemia, and oxidative brain damage (34). BFABP has not been investigated previously in extremely preterm infants, but is known to be elevated in patients with various neurodegenerative diseases (10). In adult patients with acute ischemic stroke, BFABP is elevated from 2 to 3 h after the event and remains increased for at least 5 d (34). There was no difference in the level of BFABP between the two intervention groups in the SafeBoosC II trial, which again could be explained by the insufficiency of a moderate global cerebral hypoxic exposure—compared to stroke patients experiencing an acute and severe local cerebral hypoxia.

Neuroketal concentrations in cerebrospinal fluid have been reported to be increased in preterm infants with cerebral white matter damage on magnetic resonance imaging (11). Neuroketals are compounds produced by free radical-induced peroxidation of docosahexenoic acid, which is solely present in the brain and especially vulnerable to oxidative stress (35). As the burden of hyperoxia was unaffected by the intervention in the SafeBoosC II trial, it may not be surprising that the neuroketal was the same in the two groups.

We did not observe any significant effects of cerebral oxygenation monitoring on blood levels of S100β, BFABP, or neuroketal. Our present analyses are not able to decide if these blood biomarkers are predictive of long-term cerebral outcome in this population. Accordingly, we do not yet know if these blood biomarkers are valid surrogate outcomes for cerebral damage in relation to NIRS (14–16).

Limitations of the Trial

We did not measure the P_{CO_2} in the SafeBoosC II infants, but all other physiological variables were comparable in the two intervention groups (fractional inspired oxygen, peripheral capillary oxygen saturation, mean airway pressure, heart rate, and arterial blood pressure) (13). The incidence of seizures in this population is high, however, the burden of the seizure is low; exceeding 90 s during 24 h of recording on day 3 in only 3% of the infants and thus only amounts to less than 0.2% of recording time (36). In this study, we did not investigate the seizure activity. The blood biomarker and EEG outcomes are unvalidated short-term surrogate outcomes of the intervention and assessment of neurodevelopmental outcome at 2 y of age in this cohort is ongoing. This may enable the validation of these surrogates (14–16).

Our results do not support the hypothesis that the SafeBoosC II NIRS intervention would be effective in reducing brain injury. First, the chosen surrogate outcomes may not be valid. EEG and the blood biomarkers are not established markers for brain injury in preterm infants. However, we examined them as prime candidates and therefore consider the absence of any difference between the groups as an important signal. Is it possible that the reduced burden of cerebral hypoxia was irrelevant for the brain? Although the "burden of cerebral hypoxia"—the accumulated area under the curve expressed in %hours—amounted to more than 200 %hours in some infants, the threshold used as the alarm limit for intervention and for the calculation of the burden was 55% (13). In piglet experiments at term, cerebral oxygenation has to be kept below 30% before severe abnormalities occur in the EEG (37,38) and below 35% for several hours to induce brain injury (39). Furthermore, preterm infants are normally exposed to arterial oxygen saturation of 70% or less in utero, and some neonatal units in the past (1990s) targeted arterial saturation

at low ranges and thereby reduced the risk of retinopathy of prematurity, without increasing the risk of cerebral palsy (40), though it has later been shown that low levels of arterial saturation increase mortality (41). Therefore, our results raise the possibility that cerebral hypoxia at the levels that are common in extremely preterm infants during the first days after birth is really not significant as an etiology for brain (tissue) injury as detectable by EEG and the selected molecular biomarkers at 64 h of age.

Conclusions

Whereas cerebral hypoxia was reduced to less than half in the experimental group, the result was neither reflected in the EEG, nor in the three assessed blood biomarkers.

METHODS

Study Design and Patient Characteristics

The SafeBoosC II randomized clinical trial is a multicentre, blinded, feasibility medical device trial (42). One hundred and sixty-six extremely preterm infants from eight European countries each represented by one NICU were included (1 June 2012–31 December 2013) (13). The infants were randomized to the experimental group (cerebral NIRS monitoring with visible cerebral oxygenation levels combined with an evidence-based treatment guideline (12) listing possible interventions if the cerebral oxygenation was out of range: 55–85%) vs. control group (blinded collection of cerebral oxygenation levels combined with treatment as usual). In both intervention groups, the NIRS monitoring was started within 3 h after birth and ended at 72 h of life. The trial is registered at ClinicalTrial.gov, NCT01590316; the protocol is available in full at http://www.safeboosc.eu.

The EEG Recording

Recording was specified in a standard operating procedure. At postnatal age of 64 h (±8) at least 120 min of good quality EEG with amplitude-integrated EEG tracing was recorded. Electrodes were placed at P3 and P4 positions according to the international 10–20 system. Needle, disc, or hydrogel electrodes could be used according to local practice and additional electrodes were allowed. The electrode impedance was less than 20 kΩ during the recording. If the child was treated with morphine, other opioids, or sedative medications this was noted in an electronic record form. The raw EEG data were anonymized and uploaded for central analysis via a file transfer protocol.

EEG Analysis

All EEG analysis was performed in Matlab version R2014b (MathWorks, Natick, MA).

Artifact rejection. The raw EEG was band pass filtered (0.5–30 Hz) using a zero-phase filter. Since the amplitude-integrated EEG trace was missing in some infants, the filtered EEG data were converted into range EEG (rEEG) (43) to enable a standardized artifact rejection. The rEEGs were split in 10-min epochs for visual inspection. Artifacts in the rEEG epochs were independently visually identified by two of the authors (GG and AMP) blinded to treatment allocation and the clinical history of the infant. Kappa statistics was used to calculate the level of agreement. For epochs with disagreements, the raw EEG was reviewed and the epoch rejected if the presence of artifacts was confirmed.

Classification of rEEG. The rEEGs were visually classified into four categories: (i) severe burst suppression; (ii) burst suppression (mainly flat lower margin); (iii) discontinuous (lower margin below 25 μV most of the time); or (iiii) continuous (lower margin above 25 μV).

IBIs were measured as the time (in seconds) between bursts of nested (high-frequency) oscillations within large slow-wave depolarizations using an extraction algorithm based on the co-occurrence of a slow (0.5–2 Hz) wave and higher (8–22 Hz) frequency oscillation. Consecutive events that occurred within 0.5 s of one another were counted as one and events of duration less than 4/22 of a second were discounted (19). IBIs were calculated for each of the artifact-free 10-min epochs and expressed as the median of all IBIs in all included epochs.

Spectral analysis. For each artifact-free 10-min epoch spectral analyses were conducted using Matlab routines (Neurospec 2.0, Neurospec.org). The EEG data were segmented into epochs of 2 s with an overlap of 50% (1s). After Fast Fourier Transformation, the spectrum was subdivided into frequency bands: delta (0.5–4 Hz), theta (4.5–8 Hz), alpha (8.5–13 Hz), and beta (13.5–30 Hz). The spectral distribution was calculated as the square root of the power in each band and expressed as percentages adding up to 100%. A grand mean of the spectral distribution for each infant was calculated as the mean of all epochs.

SEF95 for each infant was defined as the frequency between 0.5 and 30 Hz, below which 95% of the power was present. SEF95 is expressed as a mean of all epochs.

Brain Injury Biomarkers in Blood

Blood sampling, plasma storage and analyses were specified in a standard operating procedure. At the age of 6 h (±1) and 64 h (±1), 1 ml of blood was collected. A blood sample was only taken if the infant had an indwelling arterial or venous line. The blood was sampled in heparin tubes and centrifuged (1200×*g* for 12 min) before the plasma was transferred to a small screw-cap tube. The samples were kept at −40 to −20°C for a maximum of 1 wk, thereafter at −80°C (±10°C) for prolonged storage. After inclusion of the last patient, the samples were sent on dry ice to a central laboratory (HaemoScan, Groningen, The Netherlands).

Laboratory Analysis

The blood samples were analyzed for the three different brain injury biomarkers. The laboratory technician was unaware of the medical history and allocation of the infant. S100β (50μl) was assessed by ELISA. Clone 1B2 monoclonal antibody was used as capture antibody (Abnova, Taipei, Taiwan) and biotinylated clone 8B10 as detection antibody (Hytest, Turku, Finland). Intra-assay variance is 4.6% and the lower level of quantification (LLOQ) is 39 pg/ml. BFABP (50 μl) was determined by means of ELISA with BFABP polyclonal capture antibodies and monoclonal detection antibody (HaemoScan). Intra-assay variance is 6.4% and the lower level of quantification is 150 pg/ml. Neuroketal (60 μl) determination was performed by competitive enzyme immunoassay based on purified rabbit antibodies against KLH complexed neuroketal (Haemoscan). Intra-assay variance is 10% and the lower level of quantification is 4.1 pg/ml.

Ethics

The SafeBoosC phase II trial was approved by each hospital's research ethics committee (Hopital Femme Mere Enfants, Lyon, France; Rigshospitalet, Copenhagen, Denmark; La Paz University Hospital, Madrid, Spain; Cork University Maternity Hospital, Cork, Ireland; Wilhelmina Children's Hospital, Utrecht, The Netherlands; Medical University of Graz, Graz, Austria; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; and Rosie Hospital, Cambridge University Hospitals, United Kingdom), and where required (Austria, Denmark, and France) by the competent authority responsible for medical devices. Written informed parental consent was mandatory before inclusion in the trial.

STATISTICS

Between-Groups Analysis

EEG. The distribution of the IBIs and SEF95 were tested for normality, if not normal, the data were transformed. For the EEG outcomes, a general univariate regression model of each EEG quantity on GA (above or below 26 wk) and on each of the indicators of opioid treatment, of treatment with sedative, of EEG filter, of EEG sampling rate, and of type of EEG device was conducted to see which of these quantities had a significant ($P < 0.05$) effect on the EEG outcome. The variables with significant effect on the EEG outcomes were included in multiple regression models, thereafter the estimated mean (SE) was calculated and comparisons between the two groups were conducted.

Molecular biomarkers. If the molecular biomarkers were not normally distributed, logarithmic transformation was attempted. For data with normal distribution before or after transformation a linear mixed-model analysis using time, intervention (experimental or control), and the interaction between time and intervention was conducted. An unstructured covariance matrix was used. The analyses were adjusted by GA (above or below 26 wk) and centre. If the data did not follow a normal distribution, the Mann–Whitney test was used. For all analyses the two-sided *P* values with a threshold of 0.05 were used.

Inter Cluster Correlation

The outcomes of EEG (IBI and SEF95) and the biomarker concentrations were tested for ICC to determine the correlation between the infants in each pair of twins. A mixed-model analysis was carried out for each of the EEG and biomarker outcomes: using birth (twin pair) as a random intercept. If ICC was less than 0.15, then all infants were included in the analysis for that outcome. If the ICC was higher than 0.15, one twin from each cluster was removed at random (using a web-based randomization program (RANDOM.ORG, Dublin, Ireland)) and the between-groups analyses were conducted on the remaining infants.

Other Analyses

The interobserver agreement for the artifact rejection of the rEEGs was estimated as proportion and the Kappa value was estimated.

The analyses were conducted using IBM SPSS Statistics for Windows version 20.0.0, (IBM SPSS, Armonk, NY) and SAS version 9.3, (SAS Institute, Cary, NC).

ACKNOWLEDGMENT

The authors would like to acknowledge Caroline Hartley for willingly sharing the Matlab script for the inter-event intervals.

STATEMENT OF FINANCIAL SUPPORT

The Danish Council for Strategic Research financially supported this work through an unconditional and unrestricted grant of DKK 11,100,105. The funder had no role in the design, conduct, or analysis of the trial.

Disclosure: None of the authors have any conflicts of interest to disclose.

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Manuscript III

Early biomarkers of brain injury and cerebral hypo- and hyperoxia in the SafeBoosC II trial

Short title: Cerebral hypoxia and early markers of brain injury

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Abstract

Background. The randomized clinical trial, SafeBoosC II, collected data on cerebral oxygenation monitored by near-infrared spectroscopy in both the intervention and the control group. The primary outcome was the burden of cerebral hypo- and hyperoxia. Here we describe the associations between the burden of cerebral hypo- and hyperoxia and the secondary and explorative outcomes of this trial.

Methods. Cerebral oxygenation was continuously monitored during the first 72h of life in 166 extremely preterm infants. Cranial ultrasound was performed at day 1,4,7,14, and 35 and at term. Electroencephalogram (EEG) was recorded at 64h. Blood-samples taken at 6 and 64 hours were analysed for the brain injury biomarkers S100beta, brain-fatty-acid-binding-protein, and neuroketal.

Results. Significantly more infants with a cerebral burden of hypoxia within the highest quartile versus infants within the three lower quartiles were diagnosed with severe intracranial haemorrhage (11/39 versus 11/117, p=0.003), had low burst rate on EEG (12/28 versus 21/103, p=0.015), or died (14/41 versus 18/123, p=0.006), whereas none of these events were significantly associated with cerebral hyperoxia. The blood biomarkers were not significantly associated with the burden of cerebral hypo- or hyperoxia.

Conclusions. The explorative analysis showed that early burden of cerebral hypoxia but not hyperoxia was significantly associated with low brain electrical activity, severe intracranial haemorrhage, and death.

Introduction

Extremely preterm infants have an immature cardiorespiratory system and cerebral autoregulation can be impaired, especially during the first days of life[1,2]. This makes the developing brain of the preterm infant susceptible to fluctuations in the cerebral blood flow (CBF)[3] and may cause episodes of cerebral hypo- and hyperoxia. Near-infrared spectroscopy (NIRS) is a non-invasive method for estimating tissue oxygenation. Spatially resolved NIRS measures the ratio of the concentrations of oxygenated haemoglobin to total haemoglobin on an absolute scale with a range of 0% to 100%[4]. Changes in cerebral NIRS-values are correlated to CBF[5]. Severe intraventricular haemorrhage (IVH grade III) and periventricular haemorrhagic infarction (PVHI) mainly develop within the first 3 days of life[6], during the period of transition from intra- to extra uterine life when the brain is especially vulnerable. Low cerebral oxygenation, as estimated by NIRS, during this transition has been associated with higher grades of intraventricular haemorrhage and lower 2-year developmental quotients[7-9]. In addition high values of NIRS in animal studies are associated with brain injury[10], as confirmed in human asphyxiated term new-borns[11]. Cerebral NIRS monitoring is currently used in some neonatal intensive care units as part of the standard of care for extremely preterm infants and infants with hypoxic-ischemic encephalopathy Yet it remains to be determined if monitoring cerebral oxygenation actually prevents cerebral injury, improves neurological outcome, and/or increases the survival of the extremely preterm infants[12].

The phase II randomized clinical trial, SafeBoosC II, demonstrated that it is possible to reduce the burden of cerebral hypoxia during the first 72 hours of life[13]. The study was not powered to detect differences in clinical outcomes. In the present post hoc analysis, we use the SafeBoosC II data to explore the association between the burden of cerebral hypo- and hyperoxia regardless of

allocation, and the secondary and explorative outcomes of the trial: namely serial cranial ultrasound (cUS), electroencephalographic (EEG) measures, blood biomarkers of brain injury and death.

Patients and methods

Infant characteristics and study design

The SafeBoosC II is a multicentre randomised clinical feasibility trial[14]. A total of 166 extremely preterm infants were included in the SafeBoosC II trial before 3 hours of age: 86 infants were randomised to experimental group (cerebral NIRS monitoring in combination with an evidence based intervention guideline[15] for NIRS values out of range (55-85%)) and 80 infants to control group (blinded collection of NIRS values combined with treatment as usual). The intervention period was 72 hours. The infants were recruited from 8 European countries each represented by one neonatal intensive care unit (June 2012 to December 2013). The trial is registered at ClinicalTrial.gov, NCT01590316, the protocol is available in full at http://www.safeboosc.eu.

The burden of cerebral hypo- and hyperoxia

The primary outcome of the SafeBoosC II trial was the burden of hypo- and hyperoxia, calculated as time spent out the target range of cerebral oxygen saturation predefined as 55-85%. The burden of hypo- and hyperoxia was calculated as the time spent below or above the target limits multiplied by the mean deviation from the lower or the upper limit during the first 72 hours of life, expressed in percentage hours (%hours). The burden was computed from un-edited NIRS values and extrapolated to 72 hours, without knowledge of any other outcomes of the trial[13].

Cranial ultrasound

On day 1 (anytime during the first 24 hours of life), 4 (± 1), 7 (± 1), 14 (± 1), and 35 (± 1) and at term equivalent age (week 38 to 44) standardized cUS (6 coronal and 5 sagittal images through the anterior fontanel and one through the mastoid window) was performed. The images were anonymised and uploaded to a central server. The images were centrally analysed by two experts (CH and MB) using the software program OsiriX version 6.0 (Pixmeo, Geneva, Switzerland). The process of the central scoring is described in detail elsewhere[16]. IVH grade III, PVHI, post haemorrhagic ventricular dilatation, porencephalic cysts, cystic periventricular leukomalacia, cerebral atrophy at term, stroke and cerebellar haemorrhage at one or more of the scans were classified as severe brain injury, and thereby as severe adverse outcome.

Electroencephalogram

EEG was analysed in 133 infants, the median age at EEG-recording was 65 hours postnatal and the median time of recording was 2 hours[17]. Electrodes were placed at P3 and P4 position according to the international 10-20-system. Needle, disc or hydrogel electrodes were used according to local practice. The electrode impedance was less than 20kΩ during the recording. If the child was treated with morphine, other opioids, or sedative medications this was documented.

All EEG analysis was performed in Matlab version R2014b using custom build programmes (MathWorks, Natick, Massachusetts, USA), without knowledge of the medical history of the infant. The analysis of the EEGs is described in detail elsewhere[17].

The raw EEG was band pass filtered (0.5 – 30 Hz) using a zero phase filter and converted into range-EEG (rEEG)[18]. Artefacts in the rEEG were independently visually identified by two of the

121 authors (GG and AMP) blinded to the clinical history of the infant. The EEG analysis was
122 conducted on the remaining artefact-free data.
123
124 Burst rate was calculated as the number of bursts per minute. A burst was defined as nested (high
125 frequency) oscillations within large slow-wave depolarisations using an extraction algorithm based
126 on the co-occurrence of a slow (0.5– 2 Hz) wave and higher (8–22 Hz) frequency oscillation, as
127 described by Hartley et al.[19]. Consecutive events occurring within 0.5 seconds of one another
128 were counted as one and events of duration less than 4/22 of a second were discounted[19]. Burst
129 rates were significantly affected (decreased burst rate) by the use of morphine and EEG-recordings
130 with an online filtration at 2-15 Hz (decreased burst rate)[17]. Therefore the burst rates were
131 adjusted for these variables. Adjusted burst rate in the 1st quartile was considered an adverse
132 outcome.
133 Spectral-analyses were conducted using Matlab routines (Neurospec 2.0, Neurospec.org). The EEG
134 data was segmented into epochs of 2 seconds with an overlap of 50% (1 second). After fast Fourier
135 transformation the 95% spectral edge frequency (SEF95) for each infant was defined as the
136 frequency between 0.5 and 30 Hz, below which 95% of the power was present. SEF95 was
137 significantly affected by the EEG sampling frequency (high sampling frequency higher SEF95)[17].
138 Therefore the SEF95 was adjusted for this. Adjusted SEF95 in the 1st quartile was considered an
139 adverse outcome.
140

141 Blood biomarkers

142 At the age of 6 and 64 hours (±1 h) 1 ml of blood was drawn from 123 infants with an indwelling
143 arterial or venous line[17]. After inclusion of the last patient, the samples were shipped and
144 analysed centrally (HaemoScan, Groningen, The Netherlands) without knowledge of the medical

history of the infant. *S100beta* (50 µl) was assessed by ELISA: clone 1B2 monoclonal antibody (Abnova, Taipei, Taiwan) and biotinylated clone 8B10 (Hytest, Turku, Fi). Intra-assay variance is 4.6 % and the lower level of quantification (LLOQ) is 39 pg/ml. *Brain fatty acid binding protein (BFABP) (*50 µl) was determined by ELISA with BFABP polyclonal capture antibodies and monoclonal detection antibody (HaemoScan). Intra-assay variance is 6.4 % and the LLOQ is 150 pg/ml. *Neuroketal* (60 µl) was performed by competitive enzyme immunoassay (HaemoScan). Intra-assay variance is 10 % and the LLOQ is 4.1 pg/ml. The laboratory analyses are described in detail elsewhere[17]. An increase in the biomarker concentration from 6 to 64 hours was considered as a marker of potential cerebral injury during the intervention period, therefore and adverse outcome was defined as an increase in the absolute value of the biomarker concentration from 6 hours (baseline) to 64 hours within the 4th quartile.

Statistics

The between twins inter-cluster correlation for the burden of hypo-and hyperoxia was low (0.02)[13]. Therefore, there was no need to exclude one infant from each twin-cluster thus data from all infants were included in the analysis. The median and inter quartile range was determined for the burden of cerebral hypo- and hyperoxia. Thereafter, the infants were divided in groups according to a burden within or below the 4th quartile of the burden of cerebral hypo- and hyperoxia, respectively. The infant characteristics were compared between the burden-groups using the chi-square test or independent t-test as appropriate. Odds ratios with 95% confidence intervals were determined for adverse outcomes for infants within the 4th quartile of the burden of hypo- or hyperoxia versus infants in the three lower quartiles. Thereafter univariate correlation analysis was conducted to determine the patient characteristics associated with the composite outcome of severe brain injury or death. Finally, a multiple logistic regression was used with the composite outcome as

dependent variable, centre, gestational age above or below 26 weeks (the stratification variables used in the randomized SafeBoosC II trial), and intervention as forced entry independent variables, and the patient characteristics which had significant correlation to the composite outcome as independent variables in a backward stepwise elimination procedure (P-out 0.1). The statistics was performed using IBM SPSS Statistics for Windows Version 20.0 (Armonk, New York, USA).

Ethics

The SafeBoosC phase II trial was approved by each hospital's research ethics committee (Hopital Femme Mere Enfants, Lyon, France; Rigshospitalet, Copenhagen, Denmark; La Paz University Hospital, Madrid, Spain; Cork University Maternity Hospital, Cork, Ireland; Wilhelmina Children's Hospital, Utrecht, The Netherlands; Medical University of Graz, Graz, Austria; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; and Rosie Hospital, Cambridge University Hospitals, United Kingdom), and where required (Austria, Denmark, and France) by the competent authority responsible for medical devices. Written informed parental consent was mandatory before inclusion in the trial. The trial was conducted in compliance with the guidelines of the Declaration of Helsinki in its latest form and the International Conference on Harmonisation good clinical practice guidelines.

Results

One-hundred-and-sixty-six infants were included in the SafeBoosC II trial. Cerebral oximetry data was missing for two infants; technical issues (n = 1) and consent withdrawn (n = 1).

The median (min - max) burden of cerebral hypoxia was 30.6 %hours (0.7 – 803.9) and the lower limit for the 4th quartile was 99.3 %hours. The median (min - max) burden of cerebral hyperoxia was 1.2 %hours (0.0 – 223.0) and the lower limit for the 4th quartile was 14.2 %hours. Male sex was associated with less cerebral hypoxia and more cerebral hyperoxia**,** Table 1. Furthermore, gestational age was positively associated with cerebral hyperoxia whereas the other baseline characteristics were not associated with either cerebral hypo- or hyperoxia.

Table 1. Baseline characteristics and treatment during the first 72 hours of life according to burden of cerebral hypo- or hyperoxia split in the three lowest quartiles and the highest quartile.

	Burden of hypoxia			***Burden of hyperoxia***		
	Quartile 1 to 3	**Quartile 4**		**Quartile 1 to 3**	**Quartile 4**	
	n = 123	n = 41	P-value	n = 123	n = 41	P-value[a]
Baseline characteristics						
Gestational age (week), mean (SD)	26.4 (1.2)	26.5 (1.5)	0.78[b]	26.3 (1.3)	27.7 (1-0)	0.35[b]
Gestational age below 26 weeks	38 (31)	14 (34)	0.7	42 (34)	10 (24)	0.025*
Birth weight (gram), mean (SD)	847 (211)	875 (207)	0.47[b]	849 (208)	872 (216)	0.54[b]
Male sex	65 (53)	13 (32)	0.02*	53 (43)	25 (61)	0.047*
Twins	21 (17)	12 (29)	0.09	23 (19)	10 (24)	0.43
Antenatal steroids full course	82 (67)	31 (78)	0.2	88 (72)	25 (61)	0.18
Prolonged rupture of membranes	40 (33)	17 (36)	0.21	42 (34)	15 (38)	0.72
Maternal chorioamnionitis	6 (5)	5 (13)	0.1	9 (8)	2 (5)	0.59
APGAR-score <5 points at 5 minutes	21 (17)	8 (20)	0.69	20 (16)	9 (23)	0.38
Umbilical arterial pH, mean (SD)	7.32 (0.1)	7.31 (0.1)	0.62[b]	7.32 (0.1)	7.29 (0.1)	0.13[b]
Treatment during the first 72h of life						
Surfactant treatment	90 (73)	35 (85)	0.11	90 (73)	35 (85)	0.11
Mechanical ventilation	79 (64)	30 (73)	0.29	80 (65)	29 (70)	0.5
Patent ductus arteriosus treatment	17 (14)	4 (10)	0.52	15 (12)	6 (15)	0.71
Use of vasopressors/inotropes	22 (18)	16 (40)	0.004*	29 (24)	9 (22)	0.79
Any red blood cell transfusion	31 (26)	18 (45)	0.025*	41 (35)	8 (20)	0.07
Corticosteroids	4 (3)	4 (10)	0.1	6 (5)	2 (5)	0.99

Values are numbers (percentages) unless stated otherwise. P-values have not been corrected for multiple comparisons.

[a] Chi square, [b] independent t-test

Serial ultrasound scans of 155 infants were available for evaluation by central reading. Twenty-seven (27/155) had severe brain injury, of which 22 had IVH grade III or PVHI. The remaining five infants had stroke (n = 2), cerebral atrophy (n= 2), or cerebellar haemorrhage (n = 1). Cerebral hypoxia was significantly associated with severe brain injury and death, Table 2. There was no significant association between cerebral hyperoxia and severe brain injury and death, Table 3. Cerebral hypoxia was significantly associated with early occurrence (day 1 to 4) of severe intracranial haemorrhages whereas there was no association between cerebral hypoxia and later occurrence (day 5 to day 14) of severe intracranial haemorrhages, Table 2. Cerebral hypoxia was also significantly associated with low EEG burst rates at 64 hours of age whereas there was no association between cerebral hypoxia and SEF95 at 64 hours of age. Of the 14 infants with early severe IVH ten (10) infants had EEG measurement and of these five (5) infants had burst rates within the lowest quartile, for the infants without severe haemorrhages the number of recorded EEGs was 115 and hereof 26 had burst rates within the lowest quartile (chi-square analysis between groups p = 0.12). The three plasma-biomarker-levels were not associated with cerebral hypoxia, Table 2. Cerebral hyperoxia was not associated with EEG burst rate, SEF95 or plasma biomarkers, Table 3. The odds ratios (ORs) and 95% confidence interval for adverse outcomes are illustrated in Figs 1 and 2.

Table 2. Distributions of early and late adverse outcomes of cranial ultrasound, EEG variables, blood biomarkers, term diagnoses, and death according to the burden of cerebral hypoxia within or below the 4th quartile.

Burden of hypoxia	**Quartile 1, 2, and 3**	**Quartile 4**	
	n = 123	n = 41	P-value[a]
Cranial ultrasound			
IVH 3-4 day 1-4	6/117	8/38	0.003
IVH 3-4 day 5-14	5/117	3/39	0.40
IVH 3-4 at any time	11/117	11/39	0.003
Severe brain injury - any time	14/116	13/39	0.002
EEG variables time 64h			
Burst-rate in the 1st quartile	21/103	12/28	0.015
Spectral edge frequency in the 1st quartile	15/99	5/27	0.67
Plasma biomarkers difference between 6 and 64h			
S100beta increase in 4th quartile	25/92	6/30	0.43
BFABP increase in 4th quartile	24/89	8/31	0.90
Neuroketal increase in 4th quartile	22/92	9/30	0.51
Other major adverse outcome at term age			
Necrotising enterocolitis	13/123	6/41	0.48
Retinopathy of prematurity	18/123	4/41	0.43
Bronchopulmonary dysplasia	52/104	14/27	0.86
Death	18/123	14/41	0.006
Combined adverse outcome			
Death or severe brain injury	28/117	20/41	0.003
Death or IVH 3 or 4	25/118	19/41	0.002

Values are given as numbers of events / numbers of infants investigated for event. P-values have not been corrected for multiple comparisons.

[a] Chi square test

Table 3. Distributions of early and late adverse outcomes of cranial ultrasound, EEG variables, blood biomarkers, term diagnoses, and death according to the burden of cerebral hyperoxia within or below the 4th quartile.

Burden of hyperoxia	**Quartile 1, 2, and 3**	**Quartile 4**	
	n = 123	n = 41	P-value[a]
Cranial ultrasound			
IVH 3-4 day 1-4	11/116	3/39	0.74
IVH 3-4 day 5-14	6/117	2/39	>0.99
IVH 3-4 at any time	17/117	5/39	0.79
Severe brain injury - any time	21/117	6/38	0.76

EEG variables time 64h			
Burst-rate in the 1st quartile	27/95	3/36	0.17
Spectral edge frequency in the 1st quartile	13/91	7/35	0.43
Plasma biomarkers difference between 6 and 64h			
S100beta increase in 4th quartile	24/93	7/29	0.86
BFABP increase in 4th quartile	23/92	9/28	0.45
Neuroketal increase in 4th quartile	25/93	6/29	0.5
Other major adverse outcome at term age			
Necrotising enterocolitis	17/123	2/41	0.12
Retinopathy of prematurity	20/123	2/41	0.064
Bronchopulmonary dysplasia	55/97	11/34	0.015
Death	26/123	6/41	0.36
Combined adverse outcome			
Death or severe brain injury	38/120	10/38	0.53
Death or IVH 3 or 4	35/120	9/39	0.46

Values are given as numbers of events / numbers of infants investigated for event. P-values have not been corrected for multiple comparisons

[a] Chi squared test

Fig 1. Risk for adverse outcomes for infants with a *burden of cerebral hypoxia* within the highest quartile versus infants with a burden in the three lower quartiles.

Odds ratio (OR) and 95% confidence interval.

Fig 2. Risk for adverse outcomes for infants with a *burden of cerebral hyperoxia* within the highest quartile versus infants with a burden in the three lower quartiles.

Odds ratio (OR) and 95% confidence interval.

Correlation analyses of the following variables showed a significant correlation ($p<0.05$) between gestational age, birth weight, clinical chorioamnionitis, surfactant, mechanical ventilation, use of

vasopressors, blood transfusions, and the burden of cerebral hypoxia within the 4th quartile on one hand and the composite outcome of severe brain injury or death on the other. These variables were included in the multiple logistic regression model with backward stepwise elimination. As described in the methods, centre, gestational age below 26 weeks, and intervention were forced-entry variables in the model. The following variables remained statistically significant: intervention (p=0.003, OR (95% CI) 0.29 (0.12 – 0.69)), gestational age below 26 weeks (p=0.007, 3.33 (1.38 – 8.06)), use of vasopressors (p=0.014, 3.26 (1.26 – 8.44)), and blood transfusion (p=0.016, 2.97 (1.22 – 7.23)). There was no centre-effect.

Discussion

This post hoc analysis shows that early burden of cerebral hypoxia, but not hyperoxia, is associated with a reduction of brain electrical activity, severe brain injury (especially early IVH grade III and PVHI), and death. There were no significant associations between the burden of cerebral hypo- and hyperoxia and the three blood biomarkers. Multiple logistic regressions showed significant associations between intervention, gestational age below 26 weeks, use of vasopressors, and blood transfusion on one hand and the composite adverse outcome severe brain injury or death on the other.

Low cerebral oxygenation has previously been associated with IVH and lower developmental quotients at 2-year follow up[7-9]. In a study by Noori et al, involving 22 extremely preterm infants, lower levels of cerebral oxygenation, cardiac output, and cerebral hypoperfusion were found prior to the development of IVH II and PVHI[7]. In term piglet models, however, while low cerebral oxygenation when accompanied by low cerebral blood flow caused permanent brain damage, prolonged cerebral hypoxia alone seemed to be of less importance[20,21]. Piglets had to be

exposed to cerebral hypoxia as low as 30-35% for several hours before significant histological damage appeared[21].

In the present analysis the burden of cerebral hypoxia was associated with decreased EEG burst rates. This is in agreement with a previous demonstration of an association between low cerebral blood flow and suppressed EEG in preterm infants[22]. EEG burst rate is decreased in infants with severe IVH[23,24] and suppressed EEG is correlated to adverse developmental outcome[25,26]. In the present analysis we did not find significantly more infants with low burst rates and early severe IVH grade III or PVHI.

The three blood biomarkers measured in the SafeBoosC II trial were not significantly associated with either cerebral hypo- or hyperoxia. S100beta is an established marker of brain injury in adults[27]. It is a calcium binding protein present in high concentration in Schwann cells and astrocytes[28] and is released to the systemic circulation after cerebral damage. S100beta in blood and urine from term and preterm infants has previously been associated with the severity of hypoxic ischemia and IVH[29,30], and is reported elevated in infants with low cerebral oxygenation[31]. We did not find any significant associations between S100beta and the burden of cerebral hypoxia: this is surprising since an effect of cerebral hypoxia would also be increased by the confounding effect of severe intracranial haemorrhage.

BFABP, a brain-specific marker, is rapidly released from astrocytes as a response to ischemia, mechanical and oxidative brain damage[32], and is elevated in patients with various neurodegenerative diseases[33]. BFABP was chosen as a potential biomarker for brain injury in the SafeBoosC II trial, as it is known to rapidly increase in hypoxic-ischaemic stroke patients, and the high levels persist for several days after an event[32]. The present data shows no association

between BFABP and the burden of cerebral hypo- or hyperoxia – this could be because of the different nature of the acute and severe local hypoxic-ischemia occurring in stroke patients compared to the global relative cerebral hypoxia measured in the SafeBoosC II trial. Neuroketals are compounds produced by free radical induced peroxidation of docosahexenoic acid, have been associated with white matter damage on MRI in preterm infants[34] and are solely present in the brain[35]. Neuroketal is mainly released as a reaction to cerebral oxidative stress, we expected that the levels of neuroketal would have been increased in the infants with a high burden of cerebral hyperoxia, but that was not the case.

Limitations

The explorative post hoc analysis of the SafeBoosC II trial data presents the relationship between the early cerebral oxygenation and short-term adverse cerebral outcomes and death in extremely preterm infants. However, this study has some limitations. Most importantly, cranial ultrasound was conducted at pre-specified days of life, but the exact timing of IVH grade III and PVHI and other severe brain damages is not available and therefore we cannot know if cerebral hypoxia preceded IVH or vice versa. However, one small study in very preterm infants recently identified a significantly lower regional cerebral oxygenation during the early transition in infants who later developed IVH versus the infants who did not[36]. Similarly, EEG was only recorded once. While the timing at 64 hours of age was expected to assess the potential effects of the accumulated burden over the intervention period, finer details of preceding or concurrent cerebral hypoxia could not be extracted, such as the relative significance of longer periods of moderate hypoxia versus peaks of severe hypoxia. We did not collect data on continuous blood pressure, arterial oxygen saturation or CBF, which may have contributed to both low cerebral NIRS values and severe brain damage[7,20,21].

The analyses are based on a dataset from our randomised clinical trial; therefore, the multiple logistic regression models were adjusted for the stratification variables (centre and gestational age below 26 weeks), as well as the randomisation indicator (experimental vs. control group). As the burden of hypoxia was reduced by 50% in the experimental group compared with the control group[13], it was therefore not surprising that adjusting for the randomisation indicator did reduce the statistical significance of the burden of hypoxia. We do not think that this changes the main conclusion of the study. On the other hand, the statistical significance of the randomisation indicator means that the risk of severe brain injury or death in the experimental group of the SafeBoosC-II trial was less in the experimental group than in the control group when adjusted for a number of other factors. However, neither the composite outcome nor the particular statistical analyses were specified in the trial protocol. This post-hoc finding must therefore be interpreted conservatively. Finally, the 2-year follow up of the infants included in the SafeBoosC II trial will further explore if cerebral hypoxia and/or the intervention is related to patient-relevant outcomes such as psychomotor deficit. Larger randomised clinical trials investigating possible patient-relevant benefits of continuous NIRS monitoring in extremely preterm infants is needed before the method is implemented as standard care in this population.

Conclusions

Our analysis is currently the largest dataset published on cerebral oxygenation in extremely preterm infants providing data on short-term neurological outcomes. The results support the previous findings of associations between low cerebral oxygenation in extremely preterm infants during the first days of life and EEG suppression, severe intracranial haemorrhage, and death. However, our analyses are exploratory and we were unable to determine which came first: cerebral hypoxia or

severe intracranial haemorrhage. We did not find any evidence that cerebral hyperoxia is associated with either death or severe brain damage.

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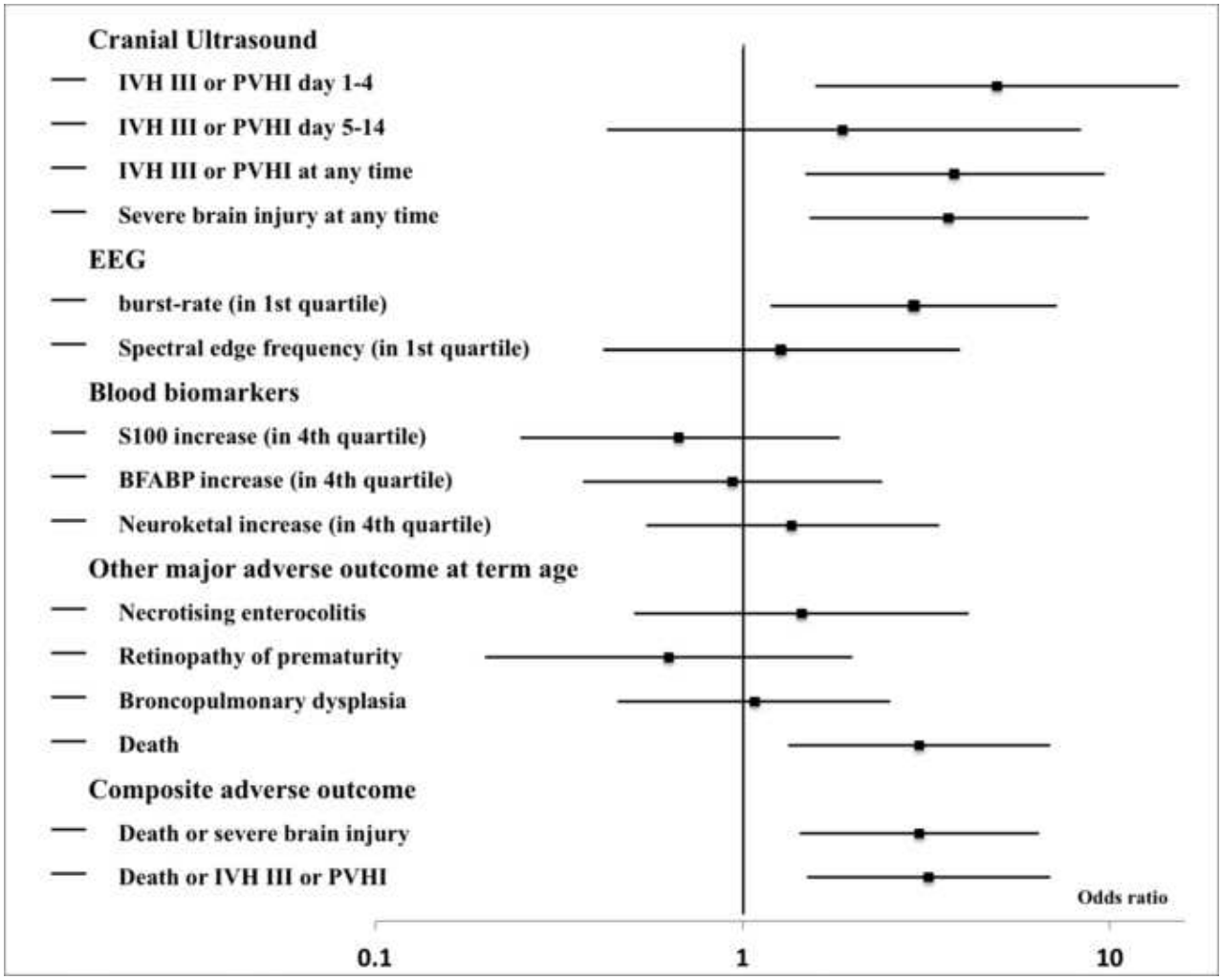

Figure 2

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