Protective effects of NACA and markers of neurodegenerative disease in hypoxia. An experimental study in newborn piglets.

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2018
Protective effects of NACA and markers of neurodegenerative disease in hypoxia. An experimental study in newborn piglets
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Acknowledgements

The present work was carried out between 2012 and 2016 at the Institute of Surgical Research and the Department of Pediatric Research at Oslo University Hospital Rikshospitalet.

The work was funded by Helse-Sør Øst (South and Eastern Norway Regional Health Authority; Source number: 6051. Project number: 39570). Further, we received a grant from “Renée og Bredo Grimsgårds stiftelse" and Roche Diagnostics supported the study with ELISA kits for investigating S100B in CSF and blood.

I am very grateful to all collaborators, both domestic and international, who have contributed to my work and made the accomplishment of this thesis possible.

First of all, I would like to thank my principal supervisor Professor Ola D. Saugstad. I want to express my sincere gratitude for giving me the opportunity to work within research on neonatal hypoxia-ischemia and neuroprotection and I really appreciated his encouragement, ideas and scientific advices. I am also grateful for his great flexibility and support when my research, to some extent, took another way than initially planned, allowing me to search for possible associations between neonatal hypoxia and Alzheimer’s disease (AD).

I am very grateful to my co-supervisor Rønnaug Solberg, for all her support and feedback in the operation theatre and thereafter. She was the one who taught me how to work with the model. I am very impressed of her very high level of energy and her ability to guide and help us PhD-students in early
mornings as well as late evenings. She also gave me invaluable feedback during the writing process and throughout the whole project.

Further, I want to thank my second co-supervisor, Lars Oliver Baumbusch, for teaching me about molecular biology and his thorough and valuable advices, regarding different laboratory methods. It has always been a pleasure to discuss scientific problems with him.

Monica Atneosen, Grethe Dyrhaug and Camilla Schjalm have been invaluable when conducting the tests in the laboratory and it was always a pleasure to work with them.

I would like to thank several people in the Institute for Surgical Research. Professor Ansgar Aasen allowing me to work in his institute. Sera T. Sebastian, Aurora Pamplona and Vivi Stubberud for many hours and days assisting in the operation theatre. Every day working with you was a pleasure and the world would have been a better place if everybody had been as helpful and kind as you.

Geir Florholmen and Shakil Ahmed have both given me great help and scientific advices when discussing and analyzing my brain samples. I am really pleased about our cooperation. It was invaluable to discuss different scientific issues with Professor Magnar Bjørås from the Department of Microbiology, Oslo University Hospital.

I have had excellent cooperation with great researchers in different European countries, from whom I learned a lot. During the time of this thesis I got the opportunity to stay for a week at the excellent Center for Neurobiology of Cellular Interactions and Neurophysiopathology in Marseille, France, where the efficient and friendly Michel Khrestchatisky is in charge. Santiago Rivera and Aliane Charrat were very kind and patient when teaching us how to run In situ zymography on cerebellum. Santiago has ever been very accommodating when discussing various issues within this subject. In the course of my project I also went for a short visit to Valencia, Spain. I would like to thank Clara Alfaro-Cervello and Javier Escobar for a fruitful cooperation. I am very happy for their contribution.

From our neighbor country in the east I would like to thank Svante Nordgren for providing us with NACA and all his ideas and feedback during the writing process. Ewa Henckel, also located in Stockholm has been a great discussion
partner and she was always in a good mood when debating various scientific questions.
Anders Skinningsrud and Nils Bolstad, without them there would have been no article about a possible association between neonatal asphyxia and Alzheimer’s disease. I am very grateful for all the help you provided me when analyzing the samples of the Cerebrospinal fluid. The Professor of Statistics, Leiv Sandvik has been the best of sparring partners when discussing different statistical problems. It has always been very enjoyable to visit him in the office and we have had many laughs together, when he told about life in the 60´ies at a remote, windblown island west of Bergen.
I am very grateful to Sophia Manueldas for a fruitful cooperation regarding the cultivation and assessment of the cell line. Without her tremendous contribution there would have been no article about a cell culture in this thesis.
My good friend and co-worker Leonid Pankratov, together we have spent so many hours in the operation theatre. I really enjoyed working with him and we had great discussions about all kinds of topics, from pathophysiology by asphyxia to the travel of Leika, the first dog in space. It has been a pleasure to share the office with my friend Håvard Garberg. We shared ups and downs when it came to acceptance and rejections of our articles. Together we had many interesting arguments about many subjects, but mainly science and politics. Further, I would like to thank my dear brother Bjørn Petter Benterud for assisting me many hours in the operation theatre and for always being so patient. My parents Trond and Else Marie, have always supported me to all times during the study. I am very happy to have such wise and kind parents.
Finally, I want to thank my dear wife Anna for always listening to me and discussing different issues regarding the study. I really appreciate her love and encouragement and for reminding me that there are other things than research that matters in life. Last, but not least I owe thanks to my children Sara and Georg for their enthusiasm and encouragement.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>-OH</td>
<td>hydroxyl radicals</td>
</tr>
<tr>
<td>8-oxoG</td>
<td>8-oxoguanine</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid Beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AE</td>
<td>Adverse effects</td>
</tr>
<tr>
<td>aEEG</td>
<td>Amplitude integrated electroencephalography</td>
</tr>
<tr>
<td>Al</td>
<td>Aluminium</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BE</td>
<td>Base Excess</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>Cl</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>Cl-</td>
<td>Chloride</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CP</td>
<td>Cerebral palsy</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DHA</td>
<td>Docohexaenic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECK</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Labeled ImmunoSorbent Assay</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma amino-butyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Glu/NAA</td>
<td>Glutamate/n-acetylaspartate</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized Glutathione</td>
</tr>
<tr>
<td>H+MRS</td>
<td>Proton magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogenperoxide</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>Hg</td>
<td>Mercury</td>
</tr>
<tr>
<td>HIE</td>
<td>Hypoxic Ischemic Encephalopathy</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IQ</td>
<td>Intelligence quotient</td>
</tr>
<tr>
<td>kcal</td>
<td>Kilocalories</td>
</tr>
<tr>
<td>Lac/NAA</td>
<td>Lactate/n-acetylaspartate</td>
</tr>
<tr>
<td>LLA</td>
<td>Lower level of cerebral autoregulation</td>
</tr>
<tr>
<td>MABP</td>
<td>Mean arterial blood pressure</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix-metalloproteinases</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>Na+</td>
<td>Sodium</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
<tr>
<td>NACA</td>
<td>N-Acetylcysteine amide</td>
</tr>
<tr>
<td>NE</td>
<td>Neonatal encephalopathy</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-B</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-Aspartate</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron specific enolase</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O2-</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ONOO-</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PCr</td>
<td>Phosphocreatine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson´s disease</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic orthophosphate</td>
</tr>
<tr>
<td>PLIC</td>
<td>Posterior limb of the internal capsule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RCT</td>
<td>Randomized control trial</td>
</tr>
<tr>
<td>RMC</td>
<td>Random Mutation Capture</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UCH-L1</td>
<td>Ubiquitin carboxy-terminal hydrolase L1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Publications included in the thesis

Perinatal Asphyxia May Influence the Level of Beta-Amyloid (1-42) in Cerebrospinal Fluid: An Experimental Study on Newborn Pigs

N-Acetylcysteine Amide Exerts Possible Neuroprotective Effects in Newborn Pigs after Perinatal Asphyxia

III. Benterud T, Maanueldas S, Norgren S, Solberg R, Saugstad OD, Baumbusch LO
N-Acetylcysteine Amide (NACA) Reduces Cell Death after Oxidative Stress in a Porcine Embryonic Kidney Cell Line
Journal: Journal of Biomedical Science and Engineering 2016, 10(2), 31-36
**Introduction**

1.1 **INCIDENCE OF PERINATAL ASPHYXIA**

The World Health Organisation estimated in 2015 that out of 5.9 million children dying before the age of 5 years, 2.7 millions did not survive the neonatal period. Worldwide the three leading causes of death within the first 5 years of life are prematurity, 1.06 million (18%), pneumonia (0.92 million (16%) and perinatal asphyxia 0.69 million (12)% [1]. The incidence of severe perinatal asphyxia (causing death or severe neurologic impairment) in developed countries is calculated to 1/1000 in contrast to resource-poor countries, where it is suggested to be between 5 and 10/1000 for those born in hospitals [2]. However, these numbers must be interpreted with caution, because only a quarter of the world’s population lives in countries where more than 90% of births and deaths are registered [3].

1.2 **Definition of perinatal asphyxia**

The term asphyxia originates from Greek and means “pulseless”. Perinatal asphyxia is characterized by compromised placental or pulmonary gas exchange. If the exchange of oxygen (O₂) and carbon dioxide (CO₂) is deprived for a prolonged time the organism is in a situation of hypoxia, hypercarbia accompanied by anaerobic glycolysis, lactacidosis and metabolic acidosis [4]. A commonly used classification on severe asphyxia is defined by the American College of Obstetrics and Gynecology and includes the following criteria: a) Profound metabolic acidosis (pH<7.0 and Base excess...
(BE) ≤ -12 mmol/L in umbilical artery blood, b) Apgar score ≤ 3 for more than 5 minutes
c) Signs of Hypoxic Ischemic Encephalopathy (HIE), and d) Multi-organ
dysfunction [5].

1.2.1 Diagnosis
Perinatal asphyxia causes disturbance of several biochemical and clinical features. Since 1969, all children in Norway have been assessed with Apgar scores at 1, 5 and 10 minutes after birth. Apgar score characteristics are a combination of heart rate, respiration, muscular tone, respiration and reflexes of the newborn child. However, Apgar score alone is a poor predictor of outcome and should always be accompanied by other laboratory tests and clinical assessments.
Perinatal asphyxia influences various organs of the organism, but HIE is the most studied and related to the most severe sequelae. In 2002, a few years before the use of therapeutic hypothermia was established in term neonates exposed to asphyxia, Hankins et al. reported that out of 46 patients suffering from clinical central nervous injury, a damage of the liver was present in 80%, in the heart 78% and in the kidneys 72% [6]. Pulmonary dysfunction, coagulopathy, and impairment of the gastrointestinal system are also frequent complications after perinatal asphyxia [7].

1.2.2 Hypoxic Ischemic Encephalopathy
Encephalopathy of the newborn is a clinical entity of abnormal neurological function. It is characterized by irregular levels of consciousness, feeding difficulties, abnormal tone and reflexes, apnea and sometimes seizures [8]. Neonatal encephalopathy (NE) could be caused by different types of brain injury or conditions resulting in dysfunction of the Central Nervous System (CNS), including infections, hypoglycemia, HIE, focal infarction and inborn errors of metabolism [9]. The idiom HIE is used when NE is caused by a hypoxic-ischemic injury. The most common way to grade the severity of HIE was first formulated by Sarnat and Sarnat in 1976 [10], table 1.
Table 1
Extract from the Sarnat grading scale of HIE.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Mild, Grade I</th>
<th>Moderate, Grade II</th>
<th>Severe, Grade III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of consciousness</td>
<td>Hyperalert</td>
<td>Lethargy, obtundent</td>
<td>Stuporous</td>
</tr>
<tr>
<td>Neuromuscular control</td>
<td>Over reactive</td>
<td>Reduced spontaneous movements</td>
<td>Reduced or absent movements</td>
</tr>
<tr>
<td>Muscular tone</td>
<td>Normal</td>
<td>Hypotonic</td>
<td>Flaccid tone of the extremities and trunk (floppy)</td>
</tr>
<tr>
<td>Posture</td>
<td>Mild distal flexions</td>
<td>Distal flexions</td>
<td>Stretch spasms</td>
</tr>
<tr>
<td>Moro reflex</td>
<td>Strong</td>
<td>Weak</td>
<td>Absent</td>
</tr>
<tr>
<td>Autonomic functions</td>
<td>Sympathetic</td>
<td>Parasympathetic</td>
<td>Both suppressed</td>
</tr>
<tr>
<td>Pupils</td>
<td>Dilated</td>
<td>Constricted</td>
<td>Often unequal sized</td>
</tr>
<tr>
<td>Respiration</td>
<td>Spontaneous</td>
<td>Variable apneas</td>
<td>Frequent apneas</td>
</tr>
<tr>
<td>Heart rate</td>
<td>Tachykarida (&gt;160)</td>
<td>Bradykardia (&lt;100)</td>
<td>Variable</td>
</tr>
<tr>
<td>Intestinal motility</td>
<td>Normal-reduced</td>
<td>Diarrhoea</td>
<td>Variable</td>
</tr>
<tr>
<td>Convulsions</td>
<td>None</td>
<td>Common, focal or multifocal</td>
<td>Uncommon (excluding decerebration)</td>
</tr>
<tr>
<td>EEG</td>
<td>Normal</td>
<td>Early low voltage continuous delta and theta, later periodic seizures focal, 1-1.5 Hz spike-wave</td>
<td>Isopotential EEG or burst suppression</td>
</tr>
<tr>
<td>Duration</td>
<td>&lt;24 hours</td>
<td>2-14 days</td>
<td>Hours to weeks</td>
</tr>
</tbody>
</table>
1.2.3 Aetiology and risk factors

Asphyxia causing HIE might happen before, during or after the delivery. In many cases it is difficult to conclude if antepartum, intrapartum or postpartum events induced the HIE.

The Western Australian case control study enrolled 164 term neonates with moderate-to-severe neonatal encephalopathy (NE) born at term [11]. They were investigated, addressing possible risk factor leading to neonatal encephalopathy. Preconceptual and antepartum risk factors were identified in 69% of the cases; 24% of infants had a combination of antepartum and intrapartum risk factors, while barely 5% of infants had only intrapartum risk factors. No identifiable risk factors were found in 2% of the investigated population.

Both maternal and placental irregularities may cause antepartum asphyxia. Asphyxia may be subsequent to preeclampsia, maternal hypotension, placental abruption, umbilical cord suppression or other causes. There are many various fetal causes of asphyxia such as hypoplasia of the lungs, hemolytic anemia due to incompatibility of the Rhesus-system between the mother and her offspring [12]. Impaired cerebral blood flow of the fetus is a consequence of the reduced oxygenation and perfusion in all pathophysiological disorders mentioned above. Postnatal causes of asphyxia include neonatal sepsis, cardiopulmonary abnormalities or obstruction of the airways [9]. Last, but not least, a postnatal injury may follow after insufficient resuscitation or neonatal support.

1.2.4 Prognosis

According to Roberton’s textbook of Neonatology children suffering from HIE have the following risks of death or severe handicap: For mild HIE: 1.6%, for moderate HIE 24% and for severe HIE 78% [13]. Individuals suffering from moderate HIE build a heterogenous group in respect to long-term consequences. It is important to be aware that the neurological consequences of HIE are often indistinguishable from the complications observed after NE induced by other causes, such as infections or inborn errors of metabolism.
The assumption that cerebral palsy (CP) could be subsequent to complicated labor and diminished blood flow to the fetal brain at birth was proposed in 1862 by Little, when he first described the disorder[14]. A century ago, the medical society believed that severe perinatal asphyxia was the main cause of CP [15]. Little by little, researchers and clinicians agreed that most cases of CP were due to abnormal development of the brain, premature birth or perinatal infections. In 1986 Nelson et al. published an article where they revealed that of children without congenital malformations or other factors contributing to an adverse outcome, only 9% of children with CP had been exposed to birth asphyxia [16]. This number is comparable with the number described in review article from 2016, where the authors claim that less than 12% of children diagnosed with CP had been exposed to perinatal asphyxia [17].

A Californian case-control study including 231 582 singletons born after end of the 35’th gestational week, revealed that chorioamnionitis during the pregnancy was associated with a four-fold increased risk of developing CP, and the population attributable fraction of chorioamnionitis for CP was 11%. [18].

Van Handel et al. demonstrated that 7 to 9 years old children exposed to moderate perinatal asphyxia had lower IQ in comparison to their peers, but within normal range. They frequently scored below average in the domains reading, spelling and arithmetic/mathematics [19]. The same research group published a study in 10 years old children subjected to mild or moderate HIE, where they revealed that those exposed to HIE exhibited a significantly higher rate of anxiety, attention regulation and social problems than their age-matched peers. Children exposed to mild HIE had also more problems than expected regarding social functioning, even though the problems were milder than in the moderate HIE group [20].

10 years old children exerted impaired working memory, verbal and visuo-spatial long-term memory and learning capacity, which was associated with the degree of NE. The augmented memory problems were generally discovered in children without CP, but were more pronounced for children suffering from CP [21]. In the TOBY trial, a randomized control trial (RCT) including 326 newborns subjected to HIE, the authors showed that 52% of
the survivors exposed to hypothermia along with standard care and 39% of those just receiving standard care had an IQ of more than 85 when 7 years old [22]. Even though they observed a very positive effect of hypothermia, the IQ in both groups is much lower than in the regular population, where 84% have an IQ of 85 or more. These reports indicate that mild and moderate HIE, even without development of motor deficit, may have a subtle impact on intellectual capacity, lasting at least throughout the first 10 years of life. These differences between the groups will probably remain into adulthood.

1.2.4.1: HIE and adult neurodegenerative diseases

To the best of our knowledge, publications connecting HIE to adult neurodegenerative diseases, such as Parkinson’s disease and Alzheimer’s Disease (AD), are scarce. Borenstein et al. reviewed two decades of research and proposed that there are several risk factors in early life for developing AD. These risk factors include birth weight and early life brain development (head growth), nutrition and growth, both prenatally and in childhood, lack of environmental enrichment as well as head injury [23]. Amyloid Beta (Aβ), peptides of 36–43 amino acids, fundamentally involved in the pathogenesis of AD as the main component of the amyloid plaques present in the brains of Alzheimer patients, accumulated in swollen axons in the brains in 9 out of 12 patients exposed to a single lethal head injury [24]. Further, Mendez et al. reviewing more than 20 years of literature regarding head injury, discovered that increasing severity of a single moderate to severe Traumatic Brain Injury augments the risk of subtle AD significantly [25]. Mortimer et al. reanalyzed data from 11 case-control studies and found that a significant higher risk (RR=1.82) of developing AD was present when the person had suffered from a head trauma with loss of consciousness [26]. In another publication, studying the change of the AD biomarkers Tau and Aβ1-42, the authors revealed that increased Tau-levels and decreased Aβ1-42 levels in brain extracellular space followed after severe head trauma [27]. These changes are similar to those seen in Alzheimer patients and could indicate that severe head trauma may have long-term implications.
Finally, a controversial topic is when we could expect to find signs of neuro-pathologic changes in AD? A large epidemiologic study from 1997, included 61 autopsies from patients 26-30 years of age. The authors revealed that 20% of the cases were in Braak neurofibrillary stage 1, the mildest AD pathologic stage [28].

1.2.5 Mechanisms of brain injury in HIE

Various biochemical and cellular pathways are involved in the mechanisms eventually leading to the brain injury following perinatal asphyxia [4,9,29]. A simplified figure of the different mechanisms involved is illustrated in figure 1.

**Figure 1**
The figure is a simplified illustration of the different stages following perinatal asphyxia.
The process can be divided into several phases, characterized by the energy state, as originally described by Lorek et al. through calculation of mean cerebral phosphocreatine concentration [PCr]/inorganic orthophosphate concentration [Pi] [30].

The biochemical cascade evolving after perinatal asphyxia can be divided into the primary phase with energy failure and reduced microcirculation of the brain, a latent phase and the second phase of secondary energy failure. In the initial phase the mitochondrial red-ox chain will generate reactive oxygen species (ROS) and it comes to a deficiency of energy along with depolarization and influx of Ca\(^{2+}\), Na\(^{+}\) and Cl\(^{-}\) ions, generating cytotoxic edema and excitotoxicity, mainly due to an overwhelming release of Glutamate, which may lead to necrosis of the neurons. The excess of free cytosolic calcium activates lipases, proteases and other enzymes, degrading proteins, phospholipids and DNA and it induces generation of the free radical nitric oxide which diffuses into the adjacent cells prone to nitric-oxide toxicity [31, 32]. Due to lack of available substrate to the energy dependent reuptake mechanisms of glutamate, glutamate accumulates and increases to excitotoxic concentrations, overactivating N-methyl-D-Aspartate (NMDA) receptors leading to influx of Ca\(^{2+}\) and Na\(^{+}\) [31, 33]. The mixture of acidosis, energy failure, excessive intracellular calcium, glutamate release, generation of ROS and lipid peroxidation orchestrate the cell death of the neurons.

A third phase where harmful factors induce further injury has recently been proposed. This third phase is suggested to include release of cytokines and other factors causing chronic inflammation which in turn leads to epigenetic changes and impairment of neurogenesis along with impairment of axonal growth and alteration of synaptogenesis [34].

Briefly, in phase 1 it comes to a failure of energy substrates due to lack of generated ATP because of hypoxia, which may lead to necrosis of the neurons. Then, subsequent to the reoxygenation, a “latent” phase, with recovering of aerobic metabolism, repolarization, and restoring energy and many cellular functions will ensue [4]. In phase 2, starting 6-48 hours after the hypoxic challenge, excessive free radicals are produced and the levels of antioxidants will diminish. In addition to necrosis and apoptosis, inflammatory
reactions with release of cytokines may play an important role. The secondary
energy failure evolves with mitochondrial failure heading towards delayed cell
death [35]. The third phase which may evolve slowly after days or weeks may
gradually induce neurodegenerative changes.

1.2.5.1: Oxidative stress

The term oxidative stress reflects a disturbed balance between the amount of
ROS and the capability of the organism to detoxify these reactive
intermediates through antioxidants. A reduction or emptying of endogenous
antioxidants is a consequence of oxidative stress. ROS are chemical reactive
oxygen species containing oxygen, including hydrogen peroxide (H$_2$O$_2$),
superoxide (O$_2^-$), hydroxyl radicals (•OH) and singlet oxygen. ROS are free
radicals, which mean that they are very reactive atoms containing one or
more unpaired electrons. The main source of ROS in mammals is the
mitochondrial respiratory chain. ROS is also produced in the hypoxanthine-
xanthine oxidase system, by activated macrophages and oxidation of
catecholamines and arachidonic acid [36].

Even though ROS play crucial roles in various processes such as intracellular
signaling and cell growth, the overload of free radicals may lead to lipid
peroxidation, disturbances of the cell membranes and damages to proteins
and DNA [37-39].

Until a few years ago, the use of 100% oxygen was recommended in neonatal
resuscitation [40]. This practice was questioned by Saugstad and co-workers
in a report from 1980, after demonstrating that hypoxanthine, an ATP-
breakdown metabolite, had increased concentrations after birth asphyxia [41].
Due to the fact that hypoxanthine is also a potential generator of oxygen
radicals they became concerned that high oxygen concentration could be
harmful in resuscitation. The following decades, the use of different rates of
oxygen in various resuscitation models was subjected to extensive research.
However, first three decades later in 2010, the use of room-air was
implemented in the international guidelines for neonatal resuscitation by the
International Liaison Committee on Resuscitation [42].
Nevertheless, several authors have demonstrated that also reoxygenation with room-air will cause oxidative stress, although to a significant lesser extent than when reoxygenated with 100% O$_2$ [39, 43].

Oxidative stress plays a role in numerous diseases and is suggested to play a role in various disorders of aging, such as atherosclerosis, Parkinson’s disease and AD [44].

1.2.5.2: Inflammation

The inflammatory response evolving during and after asphyxia is a key player in the hypoxic-ischemic injury [9]. Oxidative stress can induce the activation of the inflammatory markers IL18 and L-1β through the NLRP3 inflammasome pathway [45] and activate inflammatory transcription factors, including NF-kβ [46]. Inflammation of the brain tissue, which is characterized by migration of leukocytes, release of cytokines, such as IL-1β, activation of astrocytes, microglia and endothelial cells, may cause cell death of the neurons [47].

Rognlien et al. described that hypoxia-reoxygenation augmented expression of several inflammatory genes, including NFkB1 in the brains of neonatal mice [48] and using a similar model, Wollen et al. demonstrated an accumulation of microglia in the striatum and hippocampus of the rodents [49] three days after the hypoxic challenge.

Even though the levels of cytokines are not used for prognostic purposes, a prospective study conducted on 24 term infants subjected to HIE, revealed that the level of IL-1β, measured within 24 hours after the asphyctic event, correlated with the severity and clinical outcome at 12 months of age [50]. Furthermore, Nelson et al. showed that children suffering from perinatal asphyxia and diagnosed with spastic CP displayed significantly higher levels of IL-1β, IL6 and TNFα at birth than children experiencing asphyxia without developing CP [51]. The importance of IL-1β, regarding cerebral inflammation and neuronal damage have also been shown by Girard et al. demonstrating that Anakinra, an (IL)-1-receptor antagonist, preserved motor function when administrated after a hypoxic-ischemic insult [52].

1.2.6 Distribution of injury
The vulnerability of the immature brain to hypoxia depends on the gestational age of the infant. For early preterms (26th – 34th gestational week) the white matter surrounding the ventricles is very susceptible to variations in oxygen tension and circulation. In the late preterm infant (34th – 36th gestational week) hypoxic insults mainly affect the grey matter, but the brain stem is more commonly involved than in term infants. In contrast to the term neonate, the cortex around the central sulci is less frequently affected [53].

In term neonates, basal ganglia, the posterior limb of the internal capsule (PLIC), cerebral cortex, thalamus, hippocampus, and cerebellum are cerebral regions often injured after perinatal asphyxia [54-56]. Abnormal signal intensity in the PLIC in MRI is a good predictor for abnormal neurodevelopmental outcome in term neonates exposed to HIE [57].

Two main patterns regarding affected areas after HIE are distinguished:
1) Basal ganglia–thalamus pattern primarily affecting bilaterally the central grey nuclei (ventrolateral thalami and posterior putamina) and perirolandic cortex. This pattern of damage is most commonly observed after an acute sentinel event, e.g. a placental abruption or a prolapsed cord, and can be described as a pattern following “acute near total asphyxia”. 
2) Watershed predominant pattern of injury, also referred to as a pattern observed after “prolonged partial asphyxia”, for instance after hypotension or infection. The vascular watershed zones (anterior–middle cerebral artery and posterior–middle cerebral artery) are affected, involving white matter and in more severely cases also the overlying cortex [58].

The areas of the brain affected will have an important bearing of the experienced symptoms and a large Australian population-based case-control study revealed that if CP was present at 6 years after neonatal encephalopathy in term infants, the type of CP was more likely to be of the dyskinetic or spastic types [59].

1.2.7 Mitochondria and Mitochondrial DNA (mtDNA)
Mitochondria, which are double-membrane organelles found in abundance in the cytosol of the eukaryotic cells, carry out a crucial role for the cellular energy metabolism through oxidative phosphorylation in the Electron
Transport Chain (ETC) for the production of ATP [60]. Like the nuclear genome, the mitochondrial genome has double-stranded DNA encoding for genes. Each mitochondrion contains 2-10 copies of the mitochondrial genome. In humans, the mtDNA consists of 16 569 base pairs and encodes for 13 subunits of the electron transport complex as well as 2 rRNAs and 22 tRNAs [61]. The mtDNA is more susceptible to mutations than the nuclear genome [62, 63]. ROS, which are produced by the ETC during and after perinatal asphyxia, may damage the mtDNA and thus lead to mitochondrial dysfunction of the neurons [64]. In our study mtDNA was isolated from cerebellum and an embryonic kidney cell line, respectively.

1.2.8 Biomarkers

A biomarker is a measurable indicator of the severity or presence of some medical conditions and may indicate what kind of treatment should be administered to the patient.

Currently, the diagnosis and prognosis of neonatal HIE are based on nervous system clinical manifestations, imaging and EEG. However, the evaluation of the neurological examination will vary between different investigators. Furthermore, EEG seems to be of suboptimal value when used within the first 6 hours of therapeutic hypothermia [65]. Therefore, it is a need for biomarkers reflecting the severity and anticipating the outcome after HIE when the clinicians decide whether neuroprotective therapies, with possible adverse effects, should be initiated or not.

The biomarker should fulfill the following criteria such as 1) It should be well studied in the pediatric population and reference ranges for term as well as preterm babies should exist. 2) The biomarker must be measurable and reproducible by commercial kits.

Potential biomarkers which may be capable to anticipate the severity of a possible brain damage following perinatal asphyxia, are different molecules belonging to various families of proteins, such as Neuron specific enolase (NSE), S100B, GFAP, UCH-L1, and Tau protein [66, 67].
NSE, a glycolytic enzyme, abundant in the neurons, has displayed promising results as in one report the authors describe that a distinct cut-off value of the serum-level can distinguish between infants with good and poor outcome [68]. However, some other studies have found a variation between infants suffering from different severity of HIE, so further research is needed before using NSE routinely after perinatal asphyxia.

There are several heterogenous factors, which can influence the level of a specific biomarker. Small amounts of the above-mentioned proteins are present in extra-cerebral cells, e.g. NSE in lymphocytes, S100B in adipocytes. Therefore, at least two biomarkers should be applied to establish an optimized scheme and Lv et al. suggested that the biomarkers GFAP and UCH-1 have the greatest potential of predicting long-term neurological handicaps and combined they are good supplements to brain imaging methods [67].

Tau-proteins are proteins stabilizing the microtubules in the neurons of the CNS and they have been shown to play a role as biomarkers in AD. Increased levels of Tau in CSF have been found after repeated head traumas [69], as well as after cerebral ischemia in humans and in bilirubin encephalopathy of the neonate [70].

Last, but not least, Tau is augmented in CSF in early phases of AD.

To the best of our knowledge, no previous studies have conducted research on Tau using a neonatal HIE-model.

Further, Masaro et al. showed that increased plasma levels of the protein S100B, and NSE measured several times during therapeutic hypothermia were associated with the severity of the outcome at 15 months [71].

1.2.8.1: Biomarkers of oxidative stress:

The reactive intermediates of oxidative stress are very difficult to measure due to their extremely short half-life. The half-life of O$_2^-$, H$_2$O$_2$ and OH$^-$ are $10^{-6}$, $10^{-5}$ and $10^{-9}$ seconds respectively [72]. Therefore, more stable end products proved to be affected by oxidative stress, such as lipid peroxidation, protein carbonylation, different inflammation factors or the level of 8-oxoguanine are employed for quantifying the degree of oxidative stress exposed to the organism.
1.3 Alzheimer`s Disease (AD)

AD is an adult neurodegenerative disease defined clinically by a gradual reduction in memory and other cognitive functions along with neuropathological changes such as gross atrophy of the brain and the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles [73-75]. It is a clinically heterogenous disease with a strong genetic component. In addition, there exist several risk factors for developing AD such as repeated head injuries and insulin resistance, increasing the susceptibility to the disorder [23]. It is estimated that in the US alone, approximately 5.4 million individuals suffer from AD [76].

Currently, there is no cure for AD; however, a Cochrane review concluded that Rivastigmine, an inhibitor of cholinesterase, had a small effect concerning the decline of cognitive functions in patients with mild to moderate AD [77]. It is still lack of knowledge about the etiology and pathophysiology of the disorder, but it is obvious that β-Amyloid plays an important role in development of AD. According to the Amyloid hypothesis β-Amyloid (Aβ) aggregation and deposition leads to hyperphosphorylation of Tau (a microtubule stabilizing protein) inducing formation of neurofibrillary tangles and subsequently a chronic neuroinflammation, neuronal injury and synapse loss along with neuronal death will evolve [78, 79], figure 2. Aβ has a tendency to oligomerize and making it less soluble and this may explain the reduced levels of Aβ found in CSF during early phases of AD.

Figure 2
Oxidative stress may play a key role in development of AD and numerous oxidative modified proteins were recognized in a recent report [80]. Siegel et al. found that 4-Hydroxynonenal, a product of lipid peroxidation modifies Aβ and triggers its aggregation [81]. These aggregations will eventually lead to neuronal death. Furthermore, a reduction or loss of function of antioxidants has been reported in patients with AD [82]. Whether massive exposure to oxidative stress in childhood will enhance the susceptibility to AD in late adulthood is still an open question.

Some other observations supporting the theory that the brain of AD patients are under increased oxidative stress are listed below:

1. Increased concentrations of brain Fe^{2+}, Al^{3+}, and Hg^{2+} in AD, capable of stimulating free radical generation;

2. Increased lipid peroxidation and decreased poly-unsaturated fatty acids in the AD brain, and increased 4-hydroxynonenal, an aldehyde product of lipid peroxidation in AD ventricular fluid;

3. Increased levels of protein and DNA oxidation in the AD brain;

Supporting indirect evidence comes from a number of in vitro studies demonstrating that free radicals are involved in neuron degeneration and death in the AD brain [83]. Although there has been extensive research on preventing the decline in cognitive function in AD, several Cochrane reviews
state that the existing medications have a limited influence on the progression of the disease [77, 84].

1.4 Therapy and Intervention Strategies in perinatal asphyxia
To reduce the complications after perinatal asphyxia it is important to identify neonates at risk for evolving organ injuries and to recognize who may benefit from therapeutic interventions, which may ameliorate the development of a possible brain injury [85]. Furthermore, supportive care aiming at a stable organ perfusion is crucial to maintain energy metabolism, including facilitating important nutrients to the brain. In many cases, the secondary injury failure starts about 6 hours after the hypoxic challenge. Therefore, it is probably essential to start the therapeutic intervention as early as possible and at least within this time window [32]. It is utterly important to perform risk stratification before the onset of the secondary energy failure to choose the patients eligible for potential treatment. Recognizing these neonates remains a challenge and further research on possible biomarkers is needed to identify these infants within the time-window of 6 hours. In Norway, a risk stratification of children eligible to hypothermia consists of an evaluation of Apgar score, BE and pH, amplitude EEG as well as a neurological examination where the degree of the encephalopathy is evaluated.

1.4.1 Hypothermia
Today, the only therapeutic intervention used extensively worldwide is therapeutic hypothermia. A Cochrane meta-analysis from 2013 including 11 RCTs and 1505 term and late preterm infants, revealed that the use of hypothermia has reduced the burden of disease remarkably [86]. There was a significant reduction in death or major neurodisability in survivors at 18 months of age, Risk Ratio (RR) 0.75 (95% CI 0.68 to 0.83) number needed to treat (NNT) 7. In regard of neuromuscular disability, results from the Cool Cap trial, a multicenter RCT including 256 patients, suggest that hypothermia is of more benefit in children suffering from moderate hypoxia, than in neonates exposed to severe hypoxia [87]. In spite of the promising data from several trials a large number of children are still suffering from severe long-lasting
consequences of perinatal asphyxia [86, 87]. It is noteworthy that an endogenous hypothermic response will evolve during HIE and Reinboth et al demonstrated in a mice model that this thermoregulatory response induces neuroprotective effects [88].

1.5 Possible neuroprotective substances

Several possible neuroprotective substances, supposed to influence different pathways playing a role in neonatal asphyxia, have been studied in order to reduce complications after perinatal asphyxia [89], however, none of them are used regularly in the clinics today. The administration of potential neuroprotective compounds such as Melatonin, Allopurinol, Erythropoetin and stem cells have exerted promising effects in various animal and/or pilot clinical studies [89, 90].

Of many different possible therapeutics investigated, I have concentrated on drugs, which have been or are under examination as neuroprotectors after perinatal asphyxia at our department. In all trials, the pig model was applied. These medications include Hydrogen gas, Nicotine, Docohexaenic acid (DHA), Cannabinoid and N-Acetylcysteine amide. In addition, N-Acetylcysteine is described due to the fact that its potential pulmonary protective abilities have been examined within the same model, and its proximity to NACA.

1.5.1 Hydrogen

Hydrogen is a colorless and odorless gas which is used in different chemical industries, including manufacturing of fertilizers and methanol [91]. Working with Hydrogen is potential hazardous, due to its low molecular weight it may easily leak out, it is highly flammable and will burn in air at concentrations between 4 and 75% [92]. However, several reports have described that Hydrogen may have positive effects in medical science. Already in 1975, Dole et al. showed a significant regression of squamous cell carcinoma in mice treated with hyperbaric Hydrogen [93]. Since then, many reports have revealed positive effects of Hydrogen in various disease models in several organs such as brain and liver [94]. Hydrogen has antioxidant properties, is a
free radical scavenger, and diminishes the levels of hydroxyl radicals (·OH), and peroxynitrite (ONOO⁻) [95]. Both of these latter substances are oxidants reacting with lipids, nucleic acids, proteins, which may lead to lipid peroxidation, protein inactivation and fragmentation of the DNA. Furthermore, Hydrogen increases the levels of various antioxidant enzymes such as superoxide dismutase, catalase and heme oxygenase [96, 97]. Domoki et al. showed that 2% Hydrogen-gas improved neuropathological scores in various cerebral regions, including cortex, hippocampus, basal ganglia, cerebellum and the brainstem and in neonatal pigs exposed to hypoxia [98]. Moreover, Li et al. reported in an Alzheimer rodent model, that rats receiving daily intraperitoneal injections of hydrogen-rich saline for 2 weeks improved their cognitive and memory functions by preventing neuro-inflammation and oxidative stress [99]. Many different reports have been published describing possible beneficial effects in animal studies in various brain diseases such as Parkinson’s disease, newborn asphyxia, brain infarction and AD [94].

1.5.2 Nicotine

Nicotine binds to nicotinic cholinergic receptors, increasing the release of neurotransmitters such as dopamine, glutamate, and gamma amino-butyric acid (GABA). These substances play a key role in induction of nicotine dependency [100]. In the brain it increases the release of glutamate and dopamine, explaining its ability to induce dependency. Throughout the last 50 years, the negative effects of nicotine have been in the center of attention. It is well known to the public that the use of nicotine as smoked tobacco may have negative consequences to the respiratory system and other organs. A review from 2001 concluded that there is a dose-dependent association between maternal smoking and low birth weight and the authors describe a possible association between maternal smoking and impaired neurodevelopment and even an increased susceptibility to psychiatric problems [101]. Nevertheless, there are some epidemiological studies which may indicate that nicotine could have some positive effects, e.g. it may decrease the susceptibility to Parkinson’s disease [102]. Previous studies conducted at our department showed that short-term low-dose infusion of nicotine increases the levels of mRNA of Brain Derived Neurotrophic Factor after neonatal hypoxia in a
neonatal pig model [103]. Furthermore, a reduction in the levels of glutamate in striatum in the nicotine group compared with the placebo group was found [104]. These results may indicate that nicotine could have neuroprotective properties after perinatal asphyxia.

1.5.3 Docosahexaenoic acid (DHA)

DHA is an omega-3-fatty acid and a component of different organs, such as brain and retina. It can be synthesized from alpha-linoleic acid or obtained from various kinds of food, such as breast milk or fish oil. Through inhibition of NF-κB, DHA suppresses microglial activation and reduces the inflammatory response [105]. In a neonatal rat model, Berman et al. noticed that DHA augmented hypothermic neuroprotection after hypoxia, evaluated through functional improvement and reduced brain injury [106]. In 2017, our group published an article describing significantly reduced levels of isoprostanes, a marker of damage to myelin, in cortical and hippocampal tissue in pigs receiving DHA after neonatal hypoxia [107]. When measuring the levels of neuroprostanes, indicative of considerable neuronal injury, a decrease of more than 50% in cerebral cortex was found. In another study issued in 2017, our group demonstrated that neuroprostanes measured in urine is significantly lower in pigs exposed to hypothermia plus DHA compared with pigs exposed to only hypothermia 570 minutes after hypoxia [108].

The exact mechanisms of DHA are not fully understood; however, there are compelling results indicating that DHA have both anti-apoptotic and anti-necrotic properties after hypoxia-ischemia [109]. It reduces lipid peroxidation and a mice model of Alzheimer’s disease revealed that the animals receiving DHA had a significantly slower impairment of learning ability, than their counterparts [110]. In spite of these promising results, the supplementation of DHA does not seem to reduce the impairment in cognitive function in patients with established AD [111]. Further research is needed before DHA could be considered investigated in a clinical neonatal setting.

1.5.4 Cannabidiol (CBD)

CBD, the main non-psychotrophic substance in cannabis, is one of more than 60 active cannabinoids found in the plant cannabis [112]. It is believed to have
a therapeutic potential in several diseases, due to clinical reports showing limited side effects and it has minimal interference with psychological functions.

CBD has displayed promising neuroprotective properties in rodent models of stroke and AD [113, 114]. A recently published review article conclude that In vivo, CBD do probably not exert its effects in neurological diseases through the endocannabinoid system, but rather through other molecular pathways like the 5-HT$_{1a}$ (serotonin) or pathways linked to intracellular calcium levels [115]. Few studies have investigated the effects of CBD after hypoxia-ischemia in neonatal piglet models. Three of these papers, published between 2008 and 2013 describe that infusion of CBD intravenously (iv) may exhibit neuroprotective effects [116-118]. CBD improved short-term cerebral hemodynamics and brain metabolic activity along with diminished severity of brain edema and seizures following hypoxia-ischemia. Further, CBD reduced the levels of NSE and S100B in CSF 6 h after HI, as well as improved neurobehavioral score at 72 h [117]. Last but not least, the infusion of CBD alleviated the increase in the proton magnetic resonance spectroscopy (H+MRS) biomarkers lactate/n-acetylaspartate (Lac/NAA) and glutamate/n-acetylaspartate (Glu/NAA) ratios [118]. The latter (Glu/NAA) is supposedly the best MRI biomarker for predicting the neurological outcome after NE [119, 120]. Decreased neuronal damage in histopathological investigation in piglets that received CBD was found in all three studies. In contrast to the above mentioned reports, Garberg et al. did not discover any significant neuroprotective effects of CBD, following hypoxia-ischemia in their trial of 2016 [121]. Due to these divergent results, caution is warranted when considering investigating CBD in clinical trials.

1.5.5 N-Acetylcysteine (NAC)

NAC is a precursor of Glutathione, the major endogen antioxidant in the cells. NAC neutralizes ROS generated after hypoxia-reoxygenation and has a weak, but detectable ability to scavenge ROS prior to conversion to reduced Glutathione (GSH) [122].

Moreover, it reduces the toxicity of lead poisoning [123] and it is considered safe during the pregnancy [124]. In rodent studies high doses did not induce
any teratogenic effects or changes in reproductive capacity [125]. Since the 1960s, NAC has been applied to reduce the viscosity of the mucus in various lung diseases, such as cystic fibrosis and chronic obstructive pulmonary disease, since the 1960s [126]. Researchers discovered that thiols could have mucolytic abilities and that one of the most efficient agents was the amino acid L-cysteine. When used as a mucolyticum, NAC breaks disulfide bonds in mucus polymers [127]. However, L-cysteine is very susceptible to oxidation and it is rapidly transformed to the inactive disulfide cystine. Acetylation of the N-terminus of Cysteine was found to sufficiently stabilize the molecule, did not precipitate upon oxidants and was therefore superior regarding mucolytic effects. The Acetyl-group increases the water solubility, accelerates the absorption and distribution of orally ingested NAC and reduces the reactivity of the thiol (R-SH) to oxidation, diminishing the potential toxicity of NAC compared to cysteine. There are also indications that NAC could reduce pulmonary inflammation after perinatal asphyxia In a newborn hypoxia-reoxygenation piglet study, where the animals were reoxygenated with 100% oxygen [128]. In that study, Østerholt et al. revealed that NAC was associated with reduced levels of the oxidant peroxynitrite, less cytokine expression and decreased accumulation of inflammatory cells in the lungs 150 minutes after hypoxia.

Nowadays, NAC is probably best known for its abilities as an antidote against acetaminophen (Paracetamol) poisoning. The tremendous effect of NAC after accidental ingestion of Paracet is due to the fact that NAC is a precursor of GSH [124]. Intracellular hepatic GSH detoxifies the reactive metabolite N-acetyl-p-benzo-quinone-imine [129].

After cleavage of the acetyl-group, reduced cysteine will be available for incorporation into the intracellular antioxidant Glutathione. Glutathione is a tripeptide and consists of the three amino acids, γ-glutamyl, cysteine and glycine.

The synthesis of GSH proceeds in two steps, starting with the combination of glutamic acid & cysteine and finishing with the addition of glycine. Because there is an abundance of glycine and glutamic acid in the cells, the
accessibility of cysteine will limit the amount of Glutathione. Reduced Glutathione (GSH) is an important scavenger of ROS, *figure 3*. 

**Figure 3.**

![Cellular GSH status diagram](image)

Cellular GSH status is maintained in the reduced state by the GSH peroxidase and GSSG reductase system, known as the GSH redox cycle, which is coupled to the oxidized and reduced nicotinamide dinucleotide phosphate (NADP⁺/NADPH) redox couple.

Patients with HIV have often abnormal low levels of Glutathione and a review article from 2010 described several RCTs where supplementation of NAC had a positive outcome, such as reduced mortality and higher levels of Natural killer cells [130]. NAC also seems to be efficient in protecting the kidneys against contrast-agent induced injuries [131] and it is found to be effective for the prevention of cardiotoxicity by doxorubicin and haemorrhagic cystitis from oxaza-phosphorines [124].

Throughout the past decades, a multitude of clinical reports addressing advantageous effects of NAC in neurological and psychiatric disorders including schizophrenia, autism, traumatic brain injury and AD have been documented. Based on the level of evidence for a number of different trials Deepmala et al. published a systematic review article in 2014, where they suggest a grade of recommendation for the use of NAC for the various disorders [132].

Further, one double-blind controlled placebo study including 81 US soldiers serving in Iraq, displayed a significant improvement in symptoms of mild traumatic brain injury and outcomes on neuropsychological testing seven days after the head injury [133]. A neonatal rat study using a modified Levine model of hypoxic-ischemic injury with one-sided ligation of the right common
carotid artery and 2 hours exposure to 8% O$_2$, revealed that the administration of NAC in addition to hypothermia significantly reduced the activation of the pro-apoptotic factor Caspase 3 and iNOS expression [134]. In addition, hypothermic female rats treated with NAC had a smaller infarct volume and a significantly better long-term neuromotor outcome than hypothermic, saline treated counterparts.

1.5.5.1: Adverse effects (AE):
An *in vitro* study investigating the effects of NAC employing a human mast cell line 1 (HMC-1) and human peripheral blood mononucleocytes exhibited that high levels of NAC lead to an enhanced secretion of Histamine in both cell types and that the release was alleviated when the cells were co-incubated with Paracetamol [135]. These results are in line with several observations reporting frequent clinical signs of histamine release, when NAC is administered after accidental ingestion of Paracetamol. There have been numerous reports of AE after intake of NAC; however, most RCT did not report major AE compared to placebo control group and just a few trials have been discontinued because of potential severe AEs after orally ingested NAC [132]. A retrospective study including 250 patients reported that frequent adverse effects after iv administration of NAC are headache, abdominal pain, rash, dry mouth, bronchospasm and hypotension [136], and probably some of these effects could be associated with an increased secretion of Histamine. Furthermore, a few reports have described pro-oxidant properties of NAC when administered in high doses [137, 138].

Even though NAC displayed beneficiary effects in experimental animals and clinical trials, systematic reviews and meta-analysis studies question its efficacy in the above-mentioned pathologies.

1.6 NACA
1.6.1 Chemistry
The failure of NAC to provide significant effect could be explained by its low bioavailability and hydrophobicity. At physiological pH, NAC acquires negative charge trough the loss of a proton from the carboxyl-group and thus its penetration through the Blood Brain Barrier is hindered [139].
Therefore, a group from Israel invented a thiol compound, supposed to have far better membrane permeability and bioavailability in order to improve the replenishment of GSH.

The chemical formula of N-Acetylcysteine amide (NACA) is quite similar to NAC, but it contains an amide group instead of a carboxyl group, increasing its lipophilicity, *figure 4*:

Due to the amide group (NH$_2$), the molecule improves its ability to cross biological membranes including the blood brain barrier and it has enhanced reducing capacity compared with NAC [139].

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**Figure 4**

![Chemical structure of N-Acetylcysteine (NAC) and N-Acetylcysteine amide (NACA)](Image)

R $\rightarrow$ S

Sulfhydryl group

**Thiol compound**

36
1.6.2 Antioxidant properties of NACA

Ates et al. investigated the antioxidant properties of NACA by employing various *in vitro* assays, such as reducing power, peroxidation-inhibiting activity, metal-chelating capacity and free radical scavenging capacities [139]. They compared NACA with NAC and three antioxidants (ascorbic acid, butylated hydroxytoluene and a-tocopherol). NACA revealed higher reducing power and radical scavenging ability than NAC. Comparative analyses of NACA versus NAC have shown that the former has a better ability to chelate lead than NAC.

Grinberg et al. applied a human red blood cell model to demonstrate the superior membrane permeability of NACA compared to NAC. Further, their results indicate that NACA was approximately five times more potent in inhibiting intracellular oxidation induced by tert-Butyl hydroperoxide [140] and the capability of NACA to restore the content of GSH, was significantly higher than for NAC, 91% vs 15% respectively. In an *in vitro* study on blood cells from β-thalassemic patients NACA significantly reduced oxidative stress and the cells exposed to NACA had higher amounts of GSH along with lower contents of ROS in red blood cells, thrombocytes and polymorphonuclear leukocytes [141]. In addition, NACA reduced the phagocytosis by macrophages in a higher degree than NAC.

NACA has the potential to reduce the ter-butyl hydroperoxide-induced oxidative stress in retinal epithelial cells [142]. The cells pre-incubated with NACA diminished the levels of malondialdehyde and augmented the GSH level and Glutathione Peroxidase (GPx) activity, thereby reducing oxidative stress in tBHP-treated cells. Further, it prevented the reduction in transepithelial resistance, which let the authors suggest that NACA efficiently maintained the cellular homeostasis and outer blood retinal integrity. In addition, NACA administered to mice intraperitoneally reduced the damaging effects of phototoxic doses of light to the retinal pigment epithelial cell layer and it preserved photoreceptor function and visual potential. The authors of this study concluded that NACA may be beneficial in hindering loss of vision.
associated with oxidative stress in age-related cataract and macular degeneration.

1.6.3 Anti-apoptotic properties

Gong et al. demonstrated in a cell culture of renal proximal tubular epithelial cells that both NACA and NAC protected against apoptosis induced by toxic doses of iohexol, one of the most extensively used contrast agents, however; the effect of NACA was more pronounced compared to NAC. Using the same type of cells, the authors showed that NACA was also able to reduce the degree of apoptosis for kidney cells exposed to toxic doses of Gentamycin. In both cases, cellular apoptosis was reduced through suppression of the p38 MAPK/iNOS-signaling pathway and the expression of the pro-apoptotic proteins Bcl2, Bax and NF-κB was reverted [143, 144].

1.6.4 Anti-inflammatory properties

Chavko et al. demonstrated in a lung contusion model that NACA reduced the increase in mRNA expression levels of several inflammatory cytokines [145]. The thiol NACA has exhibited anti-inflammatory abilities in mice suffering from diesel engine exhaust-induced inflammation. In that study mice were pretreated with 250 mg/kg NACA orally, before exposure to either diesel particles or filtered air. Histological evaluation revealed that rodents pretreated with NACA had lower levels of macrophages and less mucus plug formation in the lungs than the control group [146]. These findings may indicate that NACA may play a crucial role in reducing inflammation in different pulmonary disorders.

1.6.5 NACA as a neuroprotectant

A recent study demonstrated that NACA alleviated signs of neurodegeneration and apoptosis in the brain of rats exposed to focal penetrating head trauma [147].

Using cell lines and rodent models, the possible neuroprotective effects of NACA have been explored in various types of neurological disorders, such as Multiple sclerosis, Parkinson’s disease and AD [148]. Aβ is a crucial
component of amyloid plaques in the brains of patients developing AD. In a neuronal cell line NACA attenuated Aβ-induced oxidative stress and toxicity and pretreatment with NACA augmented the viability of neuronal cells exposed to Aβ by reducing oxidative stress accompanied by attenuation of MAPK phosphorylation cascade [149].

Taking all these above-mentioned reports into consideration, it is reasonable to believe that NACA may have important protective effects in a great variety of disorders associated with oxidative stress.

2. Objectives of the study

The hypothesis of this work is based on the theory that an excessive generation of ROS follows hypoxia-reoxygenation and that an antioxidant may have a crucial effect improving the outcome.

We wanted to investigate:

1) If the antioxidant NACA could reduce the mortality in a porcine epithelial-like embryonic EFN-R kidney cell line exposed to H₂O₂.

2) To analyze the neuroprotective effect of NACA after perinatal hypoxia-reoxygenation with emphasize on pro-inflammatory cytokines and the transcription factor NF-kB in the brain of neonatal pigs.

3) Whether NACA, alleviate signs of the neurological complications after neonatal asphyxia?

4) If there is a correlation between the levels of CSF T-tau, p-Tau, Aβ42 (biomarkers of adult neurodegenerative disorders), S100B, NSE and hypoxia-reoxygenation in the newborn piglet and establish a possible link between perinatal hypoxia-reoxygenation and any of these biomarkers.

3. Materials and Methods

3.1 The pig model

Even though there are several good cell models and computerized models, there is a need for animal experiments imitating medical conditions.
When conducting animal experiments, we have an obligation to consider the “3 R’s”, which are abbreviations for Replacement, Reduction and Refinement [150].

- Could similar information be achieved when replacing the animal experiments with *in vitro* models, such as cell cultures?
- Reduce the number of sacrificed animals as much as possible, while maintaining the statistic significance as strong as required. This was not possible for this thesis.
- Refine the models to increase the quality of information from each conducted experiments.

In addition, it is important to relieve the animals from any distress through appropriate anesthesia and generally cautious handling throughout each experiment.

The Norwegian Council for Animal Research approved the experimental protocol for our studies. The animals were cared for and handled in accordance with the European Guidelines for the use of experimental animals by researchers who have been certified by the Federation of European Laboratory Animals Science Association (FELASA). We have studied between 12 and 36 hours old Noroc pigs, delivered early in the morning on the day of the experiment. During the transport they were kept in the thermo-neutral zone, avoiding excessive use of energy. Until the experiments were initiated they were kept in a warm incubator to avoid dehydration. Usually, 2 pigs were delivered each morning. The pigs would be excluded from the experiments if the hemoglobin level was less than 5 g/dL or in a reduced general condition. The weight span of the included animals was from 1650 grams to 2270 grams, with an average weight of 1919 grams.

No animal model is ideal reflecting the complexity of the brain of the human child. However, the physiology, biochemical values and anatomy between pigs and humans are comparable [151]. The porcine model is regarded as a good model for investigating organ injuries due to neonatal hypoxia-ischemia and potential neuroprotection [152-154]. The normal duration of the pregnancy of a pig is 115 days. When born at term the brain maturation, myelinisation, growth and distribution of grey versus white matter are comparable with a human baby born in gestational week 37-38 [155, 156].
Further, the cerebral structures of humans and pigs are going through a comparable growth spurt around birth [155]. Moreover, the newborn pig exhibits a similar type of organ damage and cardio-vascular response to global hypoxia-ischemia. The response of cerebral blood flow [157] and vulnerability of the different regions of the brain are comparable[158] and there is an analogous pattern along with the time course of secondary energy failure [30]. Previous studies demonstrated that the lower level of cerebral autoregulation (LLA) is supposed to be between 35 and 40 mmHg for neonatal pigs [159, 160] which is comparable to the level for homo sapiens born at term [161].

Various models of inflicting asphyxia/ hypoxia-ischemia are frequently used in the neonatal pig. In the Vanucci model the carotid arteries are clamped bilaterally combined with inhalation of low concentrations of oxygen to induce global hypoxia and cerebral ischemia [162, 163]. In the global hypoxia model, which has been applied for several years in our department [154, 164], the animals are exposed to hypoxia. The hypoxia will influence the circulation of the animal and after a while the MABP will start to drop. When MABP declines to levels below the LLA ischemia occurs.

Several groups are monitoring the pigs with aEEG as an indicator of brain activity and they use it to regulate the concentration of FiO$_2$ [158, 165]. In our model, we apply a constant FiO$_2$, when inflicting hypoxemia to the pigs, and we decide the length of the hypoxic challenge through monitoring MABP and BE. In our study, the pigs were followed for 9.5 hours after hypoxia.

There are some weaknesses of the applied model. A relatively small number of animals were investigated so the statistics would have been improved if more pigs had been included. The age of the pigs were 12 - 36 hours, thus, to some extent, the pigs were adapted to the extra-uterine life, when investigated. An area of uncertainty concerns the variability in vulnerability between different species, although the vulnerability between individuals within one species may be larger than that between different species. Due to the genetic diversity of the pigs, we suggest that our pig model is more realistic than many rodent models, where often a high number of the animals are of the same litter. Last, but not least, the follow-up was only 9.5 hours, so no long-term follow-up was conducted.
3.2 Anesthesia

When conducting pig experiments it is important to reduce pain and distress of the animals as much as possible. In the present work, Pentobarbital was used to induce the anesthesia and thereafter midazolam and the analgesic fentanyl were administered during the whole trial to maintain the anesthesia and all efforts were made to minimize suffering.

The use of anesthetics during the trials should always be considered when interpreting the results from animal experiments. In animals, all currently available sedatives and anesthetics that have been examined, including midazolam, diazepam, clonazepam, ketamine, pentobarbital, propofol [166], chloral hydrate, halothane, sevoflurane and isoflurane, have been demonstrated to trigger neurodegeneration in the immature brain [167]. Further, the anesthetic drugs applied during the experiments may have long-lasting effects on the neural system [168-170]. The experiments in the present studies were accomplished in general anesthesia; however, we tried to optimize the dosage of the anesthetics to minimize their possible side effects.

When interpreting the analysis from animal studies it is important to be aware of the anesthesia and it potential influence on the results. Because the sham group was subjected to the same anesthetic and surgical protocol as the intervention groups, these procedures will influence all the pigs in a similar way.

3.3 Study design

Thirty newborn pigs, in good general condition were included in the study. Following the introduction of a cannula into an ear vein, fentanyl 50 microg/kg, midazolam 1 mg/kg and pentobarbitone 15-20mg/kg were administered as bolus injections for induction of anesthesia before intubation. Thereafter they were placed in the supine position and washed for sterile procedures.

Anesthesia was maintained by a continuous infusion of fentanyl (50 microg/kg/h) and midazolam (0.25 mg/kg/h; IVAC P2000 infusion pump). If required, a bolus of fentanyl (10 microg/kg) and midazolam (1 mg/kg) were administered (need for medication being defined as increased blood pressure
or/and heart rate, shivering and augmented muscular tone evaluated by passive movements of the limbs.

Pentobarbitone (5 mg/kg) was a few times added if there was increased muscular tone that did not respond to repeated administration of fentanyl or midazolam. A continuous iv. Infusion of salidex, consisting of saline 0.3% and glucose 3.5%, 10 ml/kg/h was administrated until start of hypoxia. From 15 min after start of hypoxia the infusion was reduced and continued at 5 ml/kg/h. The pigs were ventilated with a pressure-controlled ventilator (Babylog 8000+; Drägerwerk, Lübeck, Germany) IMV mode, humidification by Fisher and Paykel MR730, 39°C. Normoventilation (arterial carbon dioxide tension (PaCO2) 4.5–6.0 kPa) was achieved by adjusting the ventilatory rate or the peak inspiratory pressure.

In addition to monitoring the heart frequency and MAP we regularly pinched the animals between the toes to check their response to painful stimuli.

3.3.1 Anesthetics used in the study:

3.3.1.1: Pentobarbitone

An intermediate acting barbiturate was administered for induction of the anaesthesia and for terminating the pigs at the end of the study. The drug has been extensively used in animal research. The predominant effect after induction with barbiturates is venodilatation followed by peripheral pooling of the blood. Furthermore, it may depress the myocardial contractility [171], have strong anti-convulsive properties, may reduce the cerebral blood flow along with the cerebral metabolic rate of oxygen. However, a recent Cochrane review showed that the administration of prophylactic Pentobarbitone after perinatal asphyxia, did not lead to any reduction in neither morbidity nor mortality for infants suffering from the consequences of perinatal asphyxia [172].

3.3.1.2: Midazolam

Midazolam is a short-acting benzodiazepine used widely in human and veterinary medicine. It is an effective sedative with minimal influence on the cardiovascular system [173]. Ahmad et al. showed that in a piglet model, continuously infusion of midazolam 0.1 mg/kg/hr augmented the cerebral
functional oxygen extraction along with a reduction of the sagittal sinus vein blood pressure, but it did not affect systemic hemodynamics or blood gases [174].

3.3.1.3: Fentanyl
The analgetic opioid fentanyl has a fast onset after administration. Rey-Santano et al. showed that moderate doses of fentanyl (3 μg/kg/hr), are infused continuously in newborn pigs, systemic cardiovascular parameters remain unmodified [175]. However, the cerebral oxygenation, which was used as a surrogate of cerebral blood flow, was reduced in pigs subjected to fentanyl along with a decline of the EEG background activity. Rigidity of the chest wall is described as a possible side effect of fentanyl in neonates [176]. To our knowledge, there is no other papers describing this phenomenon in a porcine model, but during our trials we observed this effect and therefore fentanyl was administered slowly when needed.

3.4 Surgical preparation
The left jugular vein was cannulated using an arterial cannula with FloSwitch (20G/1,10mm x 45mm. B.D. Faraday Road, Swindon, UK) and the right carotic artery was cannulated using avenflon (BD Venflon Pro, 22GA, 0,9mm x 25mm. Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden). Both procedures were accomplished under sterile conditions and the cannulas were sutured to the skin. Thereafter, the pigs were placed in a prone position. Rectal temperature was kept between 38.5 and 39.5°C with a heating blanket and a radiant heating lamp. MABP was measured continuously in the right carotic artery using BioPac systems MP150-CE.

3.5 Experimental protocol
Twelve pigs were included in each experimental group and six pigs were in the control group (sham-operated group). One of the pigs in the sham operated group was excluded, due to significant abnormalities in several clinical as well as biochemical parameters. This pig was excluded in all calculations because we considered it to be in a bad shape previous to the experiments. After about one hour of stabilization the animals in the
intervention groups went through global hypoxia and reoxygenation with air. The pigs in the first experimental group, described in article 1 (figure 5a), were subjected to global hypoxia (8% O₂ in Nitrogen) until Base BE reached -15 mmol/l (moderate hypoxia). The animals in the second experimental group (severe hypoxia) were exposed to 8% O₂ until BE reached -20 mmol/l and/or MAP fell below 20 mmHg. During hypoxia, CO₂ was added, aiming at a PaCO₂ 8.0–9.5 kPa, to imitate perinatal asphyxia. After the hypoxic challenge, the pigs were reoxygenated with air and observed for 9.5 hours, before terminated with an overdose of Pentobarbitone. The pigs in the sham group were not exposed to hypoxia.

Figure 5a
Invasive blood pressure and ECG were measured continuously. The experimental protocol used in article II and IV was slightly different from the above described protocol, *figure 5b*. Both intervention groups, placebo controls and NACA group were exposed to 8% $\text{O}_2$ until BE reached -20 mmol/L and/or MAP fell below 20 mmHg. Immediately after end of hypoxia the pigs would receive 300 mg/kg (150 mg/ml) of NACA. Additionally, NACA, in the same dose, was administered 270 minutes later. The placebo controls received the same amount of ml as saline. The sham group was not subjected to hypoxia, but underwent the same anesthetic and surgical procedures as the piglets in the intervention groups.

*Figure 5b*
Arterial blood gases were collected at several time points during the experiment. Further, various blood samples for measuring different biochemical markers expected to be influenced of the hypoxia were taken throughout the study. Between 0.5 and 1.0 ml of CSF was collected via lumbar puncture with a 22G spinal needle from each animal at the end of the study and frozen at -70°C for further investigations. Urine was collected twice, 270 and 570 minutes after end of hypoxia. All collected body fluids were immediately snap frozen at -70°C and stored for further investigations. Immediately after termination the brains were removed and the left hemisphere was immersion-fixed in formalin.

From the right hemisphere, small parts of hippocampus, cerebellum, prefrontal cortex and striatum were sampled for further analysis.

3.6 EFN-R embryonic kidney cells
Porcine epithelial-like embryonic EFN-R kidney cells were provided by the Friedrich-Löffler Institute, Federal Research, Institute for Animal Health, Greifswald-Insel Riems, Germany. The cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) (Sigma-lifescience, USA), supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin (Sigma-lifescience, USA) in a humidified chamber at 37°C with 5% CO2. Cells were cultured until they were approximately 80% confluent. About 150,000 cells were seeded in each well of a 12-wells plate. The cells were incubated in serum-free medium for 24 hours and the confluent cells were divided into five groups and treated
with 100 μM of hydrogen peroxide, H₂O₂ and/or 750 μM NACA (PharmaZell GmbH, Germany), as described in table 1. The cell viability was measured by MTT assay[177].

3.7 Analyses

3.7.1 MTT assay.
The MTT assay is a simple, reliable and established method for measuring the viability of cells [177] and was applied in the in vitro study. The principle of the assay is based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert MTT (3,4,5 Dimethylthiazol-2,5 diphenyl tetrazolium) to a purple formazan precipitate, which was measured spectrophotometrically. The amount of the dehydrogenases may reflect the number of viable cells in the experiment. Almaas et al. demonstrated a high correlation between the cleavage of MTT and the release of lactate dehydrogenase (LDH) in a cell line [178].

3.7.2 Pathology
A number of types of cell death exists, such as apoptosis, autophagy, necrosis and necroptosis and several mechanisms of cell death are activated by hypoxia-ischemia [179-181]. For simplification, the two main forms of cell death are necrosis and apoptosis.
Cell death occurring passively, in an uncontrolled and unregulated way is called necrosis. The process develops when injuries to the cell are too severe to overcome and the organism is not able to trigger the energy-dependent apoptotic pathway. Necrosis is characterized by swelling of the organelles and the cell following increased membrane permeability. This leads to release of enzymes from the lysosomes into the cellular matrix. Inflammation of the neighbouring cells is often a consequence of various intracellular materials which are thrown into the extracellular matrix due to disruption of the cell membrane.
In our experiments, Hematoxylin & Eosin staining (HE) was applied to assess the different grades of necrosis in brain tissue.
The HE stained sections from prefrontal cortex were divided into five different categories when evaluated. Areas of necrosis were defined by the presence of vacuolated neuropil and shrunken neurons with pyknotic nuclei. The histopathologic evaluation of the HE stained sections were accomplished by an experienced neuropathologist who was blinded to the treatment of the pigs.

Apoptosis is the other main death process of the cell. It develops in a controlled manner with blebbing of the cellular membranes, shrinkage of the cells, condensation of chromatin leading to disruption of the DNA and in due course formation of apoptotic bodies. These bodies are removed by phagocytes to prevent inflammation. Through apoptosis unwanted, misplaced or damaged cells are eliminated and therefore it plays important roles in different physiological processes, such as embryologic development, proliferation and tissue homeostasis. However, apoptosis may also be initiated by different exogenic factors, including UV-radiation and hypoxia. In our study we used TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), a well-established method, to evaluate the grade of apoptosis in cortex of the pigs.

3.7.2.1: Quantification of apoptosis with TUNEL assay

Nuclei positive for (TUNEL) were identified with the In-Situ Cell Death Detection Kit, AP (Roche Applied Science, Penzberg, Germany) in paraffin-embedded sections. One blinded investigator counted the TUNEL+ cells in the upper cortical layers of the deeper parts of the sulci of the prefrontal cortex.

We excluded TUNEL+ cells within or adjacent to blood vessels to avoid counting TUNEL+ white blood cells.

3.8 Analysis of gene expression.

Polymerase chain reaction (PCR) is used to diagnose diseases, identify viruses and bacteria, match criminals to crime scenes, and in many other ways. The reverse transcription combined with the polymerase chain reaction (RT-PCR) is an important method to quantify gene expression. A polymerase catalyzes the regeneration of a specific gene with a chain reaction, which is
repeated again and again to exponentially amplify the target gene. Fluorescence technology is used for monitoring the amplification process. The fluorescent signal augments proportionally with the amount of PCR product during each cycle in real time. To correct for variability between different samples, normalization of gene expression was performed against ribosomal RNA from the housekeeping gene P0. Relative expression was quantified using the comparative CT method of relative quantification (RQ), calculated with the formula $2^{-\Delta \Delta CT}$, where $\Delta CT$ is the normalized signal level in a sample. ($\Delta CT = CT$ of target gene $– CT$ of endogenous control gene) [182].

### 3.9 Protein analysis

#### 3.9.1 ELISA

Enzyme Labeled ImmunoSorbent Assay (ELISA) is an important method, which uses antibodies and color change for quantifying a specific substance. For detection, the antibody is either labeled with an enzyme or a fluorescent, which is linked with a chromogenic substrate to develop colour. In article I and II ELISA was used for measuring the levels of various proteins.

#### 3.9.2 Western blot.

Western blot is a method used for detecting proteins of interest, based on antibodies ability to bind specific antigens. Initially, the proteins are separated based on their size (by SDS-PAGE). Thereafter, they are transferred to a membrane by electrotransferring. Following the second step, the unspecific binding sites are blocked before they are labeled with primary antibodies. A secondary antibody which identifies the Fc region of the primary antibody is added. The secondary antibody usually possesses a conjugated reporter enzyme for example horse radish peroxidase (HRP). A specific substrate for this enzyme is then added to generate a detectable signal. HRP and peroxide catalyzed oxidation of the Lumigen PS-3 Acridan substrate will produce acridinium ester intermediates. This intermediate reacts with peroxide under slight alkaline conditions, and produces a chemiluminescence, which may be quantified by densitometric
analysis of the immunoreactive bands using Image J software or other similar programs.

3.10 Analysis of 8-oxoguanine, HPLC

The DNA contains four bases, the Purines Cytosine (C) and Guanine (G) and the Pyrimidines Thymine (T) and Adenosine (A). Guanine has a low oxidation potential and is readily oxidized to 8-oxoG, one of the major DNA lesion formed from ROS. This base modification is very mutagenic and may induce GC to TA transversions if not identified and repaired [183]. Our group has previously described that a graded inspiratory fraction of oxygen leads to increased oxidative stress and caused a dose-dependent production of 8-oxoG [39]. Sejersted et al demonstrated that the amount of 8-oxoG was increased in rodents resuscitated with 100% oxygen compared to animals resuscitated with air after neonatal hypoxia [184].

3.11 In situ zymography

In situ zymography enables the localisation of the activity of matrix-metalloproteinases (MMPs) in histological sections. The laboratory technique was applied for measuring the activity of MMPs in cerebellum. Frozen sections are placed on glass slides coated with fluorescently labelled matrix proteins. Following incubation the activity can be viewed as black holes in the fluorescent background because of proteolysis of the matrix protein [185]. Alternatively, frozen tissue sections can be incubated with matrix proteins conjugated to quenched fluorescein. Proteolysis of the substrate by MMPs leads to the release of fluorescence. The latter technique was used when evaluating the activity of MMPs in the Purkinje cells in cerebellum, described in article IV.
Figure 6: Fluorescence photomicrograph of a cerebellar section demonstrating *in situ* zymography in one of the pigs exposed to hypoxia. The fluorescence signal represents the proteolytic activity and a stronger signal represents an increased activity. In our study, we compared the intra-nuclear fluorescence signal in the Purkinje cells (white arrow) between the different groups.

### 3.12 Random Mutation Capture (RMC)

In our study, we applied the Random Mutation Capture (RMC) method to test a possible protective feature of NACA for mtDNA exposed to oxidative stress. The method is based on a PCR-reaction in combination with restriction enzyme digestion [186], *figure 7*. 
Figure 7

Taq digest

Restriction digestion with TaqI enzyme in mitochondrial 12S gene. TaqI restriction enzyme recognizes T^CGA and cuts at this site.

A restriction enzyme digestion of the DNA-template prior to the PCR reaction will cut the wild-type template preventing a PCR product, but a mutation in the target site will lead to a PCR-reaction product. The ratio between the amount of wild-type (WT) (cut) and mutation (uncut) product can be estimated, if the total amount of template is determined by another PCR-reaction product with primers covering a close-by sequence excluding the restriction enzyme cutting site for the respective restriction enzyme. The product of a WT and the mutant template will result in different ct-value signals in a qRT-PCR reaction, the mutant (uncutted) product will display a higher ct-value than the WT (cutted). The mutation rate is calculated with the $2^{-\Delta\Delta CT}$ method.

Due to the findings of Lunnon et al., who recently exhibited that in blood, alterations in genes coding the oxidative phosphorylation subunits are present at very early stages of AD [187] and a review article from 2015 where the authors describe an association between mutations of mtDNA and AD [188], we suggest that the RMC method could be useful in AD research, as well.
4. Statistics

When calculating the variables with normal distributions, an independent samples t test was performed when the distribution was normal. Levene’s test for equality of variance was conducted before executing the t-test. If Levene’s test displayed a significant difference between the compared groups, a t-test assuming different variances was conducted. Otherwise, a t-test assuming equal variances was carried out. For variables with non-normal distributions Mann-Whitney U test and Kruskal-Wallis test were applied. The obtained results for the transcription factor NF-kB were markedly skewed, therefore, we winzorized the data before using the t-test.

The biomarkers S100B and NSE were log-transformed to obtain a normal distribution.

Pearson’s correlation was used for calculation of the correlation between the log-values of NSE and S100B and between Lactate and AB42. The statistical analyses were performed by SPSS Statistics 19.0 (SPSS Inc., Chicago, IL, USA).

Chi-square test without Yates’ correction was applied when comparing dichotomous variables.

Main results of the study

Paper 1

Perinatal Asphyxia May Influence the Level of Beta-Amyloid (1-42) in Cerebrospinal Fluid: An Experimental Study on Newborn Pigs

The purpose of the study was to investigate if biomarkers used in adult neurodegenerative diseases, primarily AD, may play a role in risk stratification after perinatal asphyxia in term neonates. The level of Aβ42 in CSF drops in the early stages of AD.

We explored that the concentration of Aβ42 in CSF was significantly lower for neonatal pigs exposed to severe hypoxia compared with the control group. Moreover, the CSF-levels of Aβ42 and S100B were moderately negative correlated along with a negative correlation between Aβ42 in CSF and Lactate in blood at the end of hypoxia.
As anticipated, the concentration of S100B in serum and the log-value of S100B in CSF were significantly higher in the severe hypoxia than in the control group.

The significant decrease of Aβ42 in CSF after severe hypoxia may indicate that there is an association between perinatal asphyxia and neurodegenerative diseases as AD in late adulthood. Could the reduced concentration of Aβ42 in CSF be a sign of aggregation of Aβ42, which in turn damages the neurons, thus making the neurons more prone to oxidative stress in late adulthood when the production of ROS increases [189, 190]? However, further experimental and epidemiological studies must be conducted in order to strengthen this hypothesis.

Paper 2

N-Acetylcysteine Amide Exerts Possible Neuroprotective Effects in Newborn Pigs after Perinatal Asphyxia

The objective of the study was to investigate the possible neuroprotective properties of the antioxidant N-acetylcysteine amide (NACA) in neonatal pigs exposed to severe global hypoxia. Each of the two intervention groups included 12 pigs along with 5 pigs in sham operated group. The animals in the intervention groups were exposed to 8% oxygen until Base excess dropped to -20 mmol/l or the mean arterial blood pressure fell below 20 mmHg. When reaching this value, the pigs were reoxygenated with air and exposed to either 300 mg/kg of NACA (NACA-pigs) or saline (Placebo controls) iv. The sham operated pigs were subjected to similar surgical and anaesthetic procedures as the pigs in the intervention groups, but they were not exposed to hypoxia. Following hypoxia the pigs were observed for 9.5 hours, before termination and collection of the prefrontal cortex. During the study period blood, urine and CSF were collected at different time points.

In cortex the levels of IL-1β and the transcription factor NF-κB were lower for the pigs treated with NACA compared with saline. Furthermore, the first half hour following hypoxia, the NACA-pigs had a more profound decline in the levels of TNFα than the placebo controls.
The reduction in inflammatory markers measured in prefrontal cortex in pigs treated with NACA, may indicate that NACA alleviates cerebral inflammation after perinatal asphyxia and therefore it may display neuroprotective effects.

**Paper 3**

**N-Acetylcysteine Amide (NACA) Reduces Cell Death after Oxidative Stress in a Porcine Embryonic Kidney Cell Line**

In contrast to the previous articles, this paper deals with investigations on a cell culture. A porcine embryonic EFN-R-kidney cell line was used as a model for examinations of the protective effects on cells exposed to high levels of oxidative stress induced by H$_2$O$_2$.

The cells were exposed to different concentrations of H$_2$O$_2$, seeking the optimal dose-response relation for the trials. Based on the results of these experiments, the cells were subjected to either 100 μMol of H$_2$O$_2$ and/or 750 μM of NACA for 24 hours.

The evaluation of the cells revealed that the EFN-R cells subjected to NACA one hour before exposure to H$_2$O$_2$ displayed a significantly higher viability than those not pre-treated with NACA, p< 0.001. Further, EFN-R-cells receiving NACA one hour after treatment with H$_2$O$_2$ also showed increased viability compared with the control group not subjected to NACA, p< 0.01.

Our results may indicate that NACA plays an important role in diminishing the injury potential of oxidative stress in embryonic cell population and therefore have protective effects in neonates exposed to perinatal asphyxia.

**Paper 4**

**Cerebellum susceptibility to neonatal asphyxia: Possible protective effects of N-Acetylcysteine Amide (NACA)**

Improved neuroimaging modalities have pointed out that cerebellum is more affected after severe perinatal asphyxia than previously suggested. In addition to being a well-known coordinator of motoric functions, it plays a role in higher cognitive functions.

In this paper we evaluated whether NACA could reduce signs of neuroinflammation using the same group of pigs, which was used in paper 2. *In situ* Zymography of the Purkinje cells in cerebellum was used for evaluating
the possible anti-inflammatory effect of NACA. The sham-operated animals displayed significantly less proteolytic activity in the nucleus of the Purkinje cells than the pigs in the intervention groups, (p<0.05). There was a tendency to less proteolytic activity for pigs exposed to NACA after hypoxia (NACA-pigs), compared with pigs receiving saline following hypoxia (placebo controls), p= 0.08. Further, our examinations revealed that the cerebellum of the NACA-pigs showed less mutations of the mitochondrial DNA (mtDNA) than placebo controls (p<0.05).

**General Discussion**

Perinatal asphyxia remains one of the major contributors to perinatal morbidity and mortality worldwide. Today, the only extensively used therapy is moderate hypothermia, and the American Heart Association Guidelines for Cardiopulmonary Resuscitation from 2015 states that hypothermia should be induced to neonates exposed to hypoxia, born at more than 36 weeks of gestation with evolving moderate to severe hypoxic-ischemic encephalopathy \[191\]. Even though induced hypothermia reduces the neurological consequences along with the mortality for a large number of asphyctic infants \[86\], there is still a need for other compounds to improve the outcome after asphyxia.

As mentioned earlier, there are several agents with possible neuroprotective properties being extensively studied of various groups throughout the world. The solution could be a mixture of various agents with synergistic effects, as it has already been displayed of Huun et al. that the combination of DHA and hypothermia may have neuroprotective effects \[108\]. NACA could be one of the agents with positive effects.

**6.1 The role of oxygen and antioxidants**

Oxygen has been used for decades in resuscitation of the newborn. On the one hand oxygen is inevitable, but on the other hand too much of it may cause detrimental injuries to the brain, especially after the child has been exposed to hypoxia \[36, 38\]. An extensive biochemical cascade evolves during and after perinatal hypoxia-reoxygenation, including release of ROS
and triggering of inflammation [9]. Due to the rapid increase of ROS a
increase of the concentration of oxidized Glutathione (GSSG) will follow [192].
The brain has a low level of antioxidants and a high abundance of easily oxidotive lipids, making it particularly vulnerable to free radical damage [193] and scavenging of reactive oxygen species [141] may inhibit injuries inflicted after perinatal asphyxia.

In animal models, it has been shown that administering an antioxidant immediately after perinatal asphyxia may alleviate the evolving neuronal damage [134, 194]. However, selecting the optimal dose in this setting is a difficult task. We must take into consideration that the inflicted hypothermia will influence the metabolism of the administered drug and the fact that some antioxidants, may have severe side effects [124, 136, 195].

6.2 NACA as an interventional strategy
The aim of this thesis was to explore if the antioxidant NACA could play a role in reducing cerebral injuries in newborns exposed to oxidative stress in an established newborn hypoxia-reoxygenation pig model [128].
The molecule NACA has antioxidant properties by acting directly with ROS, but above all, its antioxidant effect is due to the properties of its metabolite Glutathione.
As stated in the introduction and described by Sunitha et al. in a review article, NACA has certain antioxidant, anti-apoptotic and anti-inflammatory effects in cell cultures and animal studies [148]. The reduced levels of IL-1β and NF-κB in the cerebral tissue along with TNF-α in plasma of pigs exposed to NACA after hypoxia, described in article I, are in line with another study, suggesting that NACA may alleviate the inflammatory response by regulating activation of NF-κB [196]. The use of different models have revealed that NACA may have protective effects in many kinds of tissues, exposed to toxic doses of a diversity of compounds, including lungs, liver, neural tissue and kidneys [147, 197-199].
In paper III, we describe a significantly increased viability in a cell culture of embryonic kidney cells and these results correspond well with the findings of
other studies showing advantageous properties of NACA in cell cultures exposed to oxidative stress [198, 200]

Moreover, NACA has displayed promising results in various disease models, such as cataract, beta-thalassaemia, asthma, lung contusion, Multiple Sclerosis, Parkinson’s disease, Tardive dyskinesia, HIV-1 associated dementia, contrast-induced nephropathy [197] AD and macular degeneration [200].

Due to the chemical similarity between NAC and NACA, it is likely that NACA also alters the cerebral glutamatergic transmission visible in drug withdrawal. A reduction of cocaine-seeking behaviour was demonstrated by Jastrzębska et al. in a rat model where cocaine was withdrawn from drug-dependant animals and therefore the authors concluded that NACA has a potential clinical role in cocaine use disorder [201].

The alleviation of apoptosis [143, 144] and inflammation [145, 196] along with scavenging of reactive oxygen species [141] may inhibit injuries inflicted after perinatal asphyxia.

### 6.3 What is new in our research on NACA

As far as we know, our study is the first study carried out on neuroprotective effects of NACA in neonatal pigs. We suggest that our findings of less expression of the inflammatory proteins IL-1β and phosphorylated NF-kB (p65) in the brain as well as TNF-α in plasma, may indicate that NACA could play a role, alleviating the inflammatory process evolving after perinatal hypoxia-reoxygenation.

Further, the decreased levels of mtDNA in cerebellum in pigs subjected to NACA after the hypoxic challenge, along with the tendency to less proteolytic activity in the Purkinje cells, illustrated in article IV, could strengthen the theory that NACA has anti-inflammatory and neuroprotective properties after perinatal asphyxia.

Moreover, the promising results described in article III, dealing with the viability of embryonic kidney cells exposed to oxidative stress, point out that NACA may have protective effects in other organs in addition to cerebrum, when the neonate is exposed to oxidative stress. These articles add important
information to our knowledge of NACA, as we are the first to describe its effects in a neonatal pig model as well as in an embryonic cell line. It is worth mentioning that in a cell line of primary neurons, NACA was able to partly reverse the toxic effects of $\alpha\beta 42$ on the neurons, therefore NACA may reduce the degree of neurotoxicity induced by $\alpha\beta 42$ in the brains of AD patients suffering from AD [149] and we speculate that NACA could alleviate the development of AD in a number of patients.

6.4 Is there a link between perinatal asphyxia and AD

During the German occupation in the winter 1944-1945 the population of the western parts of the Netherland suffered from famine. The average intake of calories for pregnant women was only 400-800 kcal/day, about a quarter of the recommended amount of calories [202], and therefore many foetuses received less nutrients than recommended. This birth cohort, born in winter, spring or summer 1945, has later on been extensively investigated. Studies on the Dutch Famine Birth Cohort, which consists of 2414 men and women from Amsterdam born around the time of the dutch famine, have revealed a link between prenatal conditions and typical diseases of aging [203, 204].

Even though several genes have been identified which contribute to AD, the most important being APP, Presenilin 1 and 2 and Apo$\varepsilon 4$ [205], environmental factors seems to have an impact on AD. The Dutch Famine Birth cohort has a higher prevalence of some disorders associated with ageing. Individuals, who were in their early gestation during the famine, exhibited an increased risk of cardio-vascular and metabolic diseases more than 60 years later. Compared with persons who were born before the famine, they had significantly lower scores on cognitive tests [206]. These results are in line with other reports describing that intrauterine exposure to famine may lead to increased risk of diabetes, obesity, cardiovascular disease, schizophrenia and cognitive aging [203].

Therefore, it is not surprising if neonates exposed to severe hypoxia at birth could be more prone to adult neurodegenerative diseases such as AD.
6.5 What is new in our research on β-Amyloid

To our knowledge, we are the first who have investigated the biomarkers Tau and Aβ42 in a neonatal population, and the reduction of Aβ42 in CSF after perinatal asphyxia has not been previously described.

The function of Aβ and its precursor APP are not fully elucidated. B-APP knockout mice display severe cognitive defects, indicating that Aβ may have important functions in the nervous system [207]. However, the protein Aβ is able to misfold and aggregate into oligomers and these oligomers are thought to have a toxic influence on the neurons in AD [208]. Lambert et al. showed in a rodent model that small diffusible Aβ oligomers could be fatal to mature neurons in hippocampus [209]. Further, Pillot et al. were able to demonstrate that small non-fibrillar complexes of Aβ could disturb the plasma membrane of neurons and induce toxicity in primary culture from cortical neurons via an apoptotic pathway [210, 211]. The theory that humans subjected to perinatal asphyxia are more susceptible to AD in late adulthood is further strengthened by the findings of Bernert et al. who demonstrated that neurodegeneration and neurotransmitter changes were present in adult guinea pig 3 months after exposure to perinatal asphyxia [212].

Several papers have described a possible association between traumatic head injury and AD [23] and Magnoni et al. showed that the level of Aβ in the extracellular space was increased after head trauma [27]. Furthermore, a leakage of S100B from the astrocytes and an elevation of S100B in CSF and plasma evolve after traumatic brain damage [213, 214]. The above mentioned findings, regarding high levels of S100B in CSF and plasma after head injury and decreased levels of Aβ in the extracellular space after trauma to the head are comparable with our study, where we demonstrated a decrease of Aβ42 combined with an increase of CSF- and plasma-S100B. It is tempting to speculate that the lower levels of Aβ42 in the pigs exposed to hypoxia could be a sign of aggregation of Aβ42, which in turn attacks the neurons, triggering a long-lasting process, which makes them more susceptible to AD in late adulthood, when the organism is more exposed to ROS. The absent changes of the biomarkers T-tau and p-Tau could be due
to the limited time of our experiments, 9.5 hours, and we suggest that this time frame is too short to develop tau changes. Individuals who are in their 60’ies and 70’ies today, the age when the first signs of AD are usually presented, were born at a time when no systematic assessment for asphyxia, such as Apgar or blood gases, were performed. From 1969 onwards, Apgar score has been measured in all Norwegian children. Therefore, it would be highly interesting to conduct a register-based study from 2030 onwards, to compare if neonates scored with a low Apgar score, show more signs of AD than their peers.

7. Considerations

Quantifying the expression and activation of genes and proteins at one time-point does not reflect the whole pathophysiological process after perinatal asphyxia. Further, one may argue that more pigs should be included to increase the statistical power, however; in our research a higher number of animals were investigated than in many comparable studies. Finding the right dose of an antioxidant may be a time-consuming and difficult task as several antioxidants may be chemically modified to pro-oxidants when administered in higher doses [137].

Estrella et al. showed three decades ago that Acetylcysteine may have pro-oxidant properties and the degree of pro- versus antioxidant effects was dependant on the administered dose [138]. Therefore, we cannot exclude the possibility that even NACA may have pro-oxidant abilities when given in high doses.

7.1 Implications for further research

Our work may implicate that NACA have more effects regarding neuroprotection than previously known. However, more studies, including different concentrations of NACA, must be conducted before NACA should be considered used in a clinical trial. One important question which should be answered before initiating such a trial is if NACA, like N-Acetylcysteine, may initiate release of Histamine [135] and cause side effects such as anaphylaxis and hypotension [215]. In future studies, assessing the effects of NACA after
perinatal asphyxia, hypothermia should be inflicted to the animals, because hypothermia is now a well established prescribed procedure started after hypoxia-reoxygenation in the newborn [216, 217].

8. Summary

In our paper addressing the question whether NACA may reduce the mortality in a porcine epithelial-like embryonic EFN-R kidney cell line exposed to H₂O₂, we demonstrate that NACA may reduce cell death after exposure to oxidative stress. Further, we present that the levels of IL-1β and NF-kB in cortex were lower in pigs exposed to NACA after hypoxia compared to pigs exposed to saline, which may indicate that NACA may reduce signs of cerebral inflammation. However, of the cytokines investigated, only a few were influenced by the administration of NACA.

In cerebellum we found that NACA may reduce signs of proteolytic activity and mutations of mtDNA which may indicate that NACA may exhibit some neuroprotective effects.

Due to the findings of the reduced concentration of Aβ42 combined with the increased levels of S100B in the CSF of the NACA-pigs, we suggest that biomarkers of adult neurodegenerative diseases may play a role in the evaluation of HIE.
9. Reference list


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Perinatal Asphyxia May Influence the Level of Beta-Amyloid (1-42) in Cerebrospinal Fluid: An Experimental Study on Newborn Pigs

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Abstract

Objective

Total tau (T-tau), phosphorylated tau (p-Tau) and Beta-Amyloid 1–42 (AB42) in Cerebrospinal Fluid (CSF) are useful biomarkers in neurodegenerative diseases. The aim of the study was to investigate the role of these and other CSF biomarkers (T-tau, p-Tau, AB42, S100B and NSE), during hypoxia-reoxygenation in a newborn pig model.

Design

Thirty newborn pigs were included in a study of moderate or severe hypoxia. The moderate hypoxia group (n = 12) was exposed to global hypoxia (8% O2) until Base excess (BE) reached -15 mmol/l. The pigs in the group exposed to severe hypoxia (n = 12) received 8% O2 until BE reached -20 mmol/l or mean Blood Pressure fell below 20 mm Hg, The control group (n = 6) was kept at room air. For all treatments, the CSF was collected at 9.5 hours after the intervention.

Results

The level of AB42 in CSF was significantly lower in the pigs exposed to severe hypoxia compared with the control group, 922(SD +/-445)pg/ml versus. 1290(SD +/-143) pg/ml (p<0.05), respectively. Further, a non-significant reduction of AB42 was observed in the group exposed to moderate hypoxia T-tau and p-Tau revealed no significant differences between the intervention groups and the control group, however a significantly higher level of S100B was seen in the CSF of pigs receiving hypoxia in comparison to the level in the control group. Further on, there was a moderate negative correlation between the levels of AB42 and S100B in CSF, as well as a moderate negative correlation between Lactate in blood at end of hypoxia and AB42 in CSF.
Interpretation
This is the first study to our knowledge that demonstrated a significant drop in AB42 in CSF after neonatal hypoxia. Whether or not this has an etiological basis for adult neurodegenerative disorders needs to be studied with additional experiments and epidemiological studies. AB42 and S100B are significantly changed in neonatal pigs subjected to hypoxia compared to controls and thus may be valuable biomarkers of perinatal asphyxia.

Introduction
Intrapartum events are among the most common causes of neonatal death with more than 800,000 annual cases worldwide [1].

Even though the majority of the children exposed to severe perinatal asphyxia will survive, many of them will suffer from long-term sequelae, such as cerebral palsy and cognitive deficits. In severe cases, perinatal asphyxia may lead to Hypoxic-Ischemic Encephalopathy (HIE), which may cause permanent neurological damage.

Proteins, such as S100B and Neuron specific Enolase (NSE), released into the cerebrospinal fluid (CSF) during neuronal injury, might be useful as biomarkers in reflecting disease severity and predicting the clinical outcome after perinatal asphyxia [2].

We addressed the question whether total-tau (T-tau), phospho-Tau (p-Tau) and Beta-Amyloid 1–42 (AB42), biomarkers of adult neurodegenerative disorders, could serve as markers of perinatal asphyxia [3,4,5].

In pediatric populations altered T-tau levels in CSF have been found in patients with brain tumors [6] and West syndrome (Infantile spasms) [7]. Magnoni et al.(2012) found that T-tau in the brain extracellular space was increased and negatively correlated with Beta-Amyloid levels in the extracellular space after traumatic brain injury and that T-tau may be helpful when predicting the clinical outcome [8].

In two retrospective studies Rondell et al. and Zetterberg et al. showed increased serum levels of Tau-protein and AB42, respectively, after hypoxia due to Cardiac arrest [9, 10]. Few, if any, experiments have been conducted to investigate if there is an association between asphyxia in the neonates and the levels of these markers.

In addition to T-tau, p-Tau and AB42, we also addressed the question of how NSE and S100B were affected in our model of neonatal hypoxia-reoxygenation.

Objective
The objective was to determine a possible correlation between the levels of CSF T-tau, p-Tau, AB42, S100B and NSE after hypoxia-reoxygenation in the newborn pig and establish a possible association between perinatal hypoxia-reoxygenation and any of these markers.

Materials and Methods
Study design
Thirty newborn pigs, age 12–36 hours, Hb > 5g/dl and in good general condition were included in the study.

The pigs were given fentanyl 25microg/kg, midazolam 1.0mg/kg and pentobarbitone 20mg/kg intravenously as bolus injections for induction of anaesthesia before they were intubated and placed on their backs and washed for sterile procedures. Anaesthesia was maintained by a
continuous infusion of fentanyl (50 microg/kg/h) and midazolam (0.25 mg/kg/h; IVAC P2000 infusion pump). When necessary, a bolus of fentanyl (10 microg/kg) and midazolam (1 mg/kg) were administered (need for medication being defined as shivering, increase in blood pressure and/or pulse and increased tone assessed by passive movements of the limbs). Pentobarbitone (2.5 mg/kg) was a few times added if there was increased muscular tone that did not respond to fentanyl or midazolam. A continuous iv. infusion, Salidex: saline 0.3% and glucose 3.5%, 10 ml/kg/h was given until start of hypoxia. From 15 min after end of hypoxia the infusion was continued at 5 ml/kg/h.

The pigs were ventilated with a pressure-controlled ventilator (Babylog 8000+; Drägerwerk, Lübeck, Germany) IMV mode, humidification by Fisher and Paykel MR730, 39°C. Normoventilation (arterial carbon dioxide tension (PaCO2) 4.5–6.0 kPa) was achieved by adjusting the peak inspiratory pressure or ventilatory rate.

Surgical preparation
The left jugular vein was cannulated with an arterial canula with FloSwitch (20G/1.10mm x 45mm. B.D. Faraday Road, Swindon, UK), and the right carotic artery was cannulated using a venflon (BD Venflon Pro, 22GA, 0.9mm x 25mm. Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden). Both procedures were conducted under sterile conditions, and the canulas were sutured to the skin. The animals were thereafter placed in a prone position for the rest of the experiment. Rectal temperature was maintained between 38.5 and 39.5°C with a heating blanket and a radiant heating lamp. Mean arterial blood pressure (MABP) was measured continuously in the right carotic artery using BioPac systems MP150-CE.

Experimental protocol
Twelve pigs were included in each experimental group and six pigs were in the control group. After 1 hour of stabilization the pigs in the intervention groups went through global hypoxia and reoxygenation with air.

The pigs in the first experimental group were exposed to global hypoxia (8% O2 in Nitrogen) until Base Excess (BE) reached -15 mmol/l (moderate hypoxia). The animals in the second experimental group (severe hypoxia) were exposed to 8% O2 until BE reached -20 mmol/l and/or mean blood pressure fell below 20 mmHg. During hypoxia, CO2 was added, aiming at a PaCO2 8.0–9.5 kPa, to imitate perinatal asphyxia. After the hypoxic challenge the pigs were reoxygenated with air and observed for 9.5 hours. The pigs in the control group were not exposed to hypoxia. Invasive blood pressure, EEG and ECG were measured continuously. The experiments were performed under Midazolam and Fentanyl anaesthesia, and all efforts were made to minimize suffering. Between 0.5 and 1.0 ml of CSF was collected via lumbar puncture with a 22G spinal needle from each pig at the end of the study and frozen at -70°C for further analysis (Fig 1). Blood for measuring Hb and S100B was collected before hypoxia and at the end of the study and frozen at -70°C. In addition arterial blood gases were collected at several time points throughout the experiment.

Method of sacrifice
After 9.5 hours of reoxygenation the pigs were subjected to euthanasia with an overdose of Pentobarbitone (150mg/kg).
Approval

The Norwegian Council for Animal Research approved the experimental protocol (approval number 4630). The animals were cared for and handled in accordance with the European Guidelines for the use of experimental animals by researchers who have been certified by the Federation of European Laboratory Animals Science Association (FELASA).

Sampling

CSF was sampled from 30 pigs via lumbar puncture. The quality of the CSF of three pigs was insufficient; therefore these were not included in the study. The samples were stored at -70°C until further analyzes. Then they were thawed, mixed and diluted and analyzed according to the manufacturers’ instructions. For T-tau, p-Tau and AB42 ELISA kits were applied. (Innotest hTau Ag, Innotest Phospho-Tau (181P) and Innotest Beta-Amyloid; all Innogenetics, Gent, Belgium). S100B was measured using an electrochemiluminescent immunometric assay (ECLIA) on Cobas e 601 immunoassay platform (Roche Diagnostics, Mannheim, Germany). S100B cannot be assayed in EDTA-plasma, and thus serum was derived from the originally collected EDTA-plasma by adding 20 microL 2M CaCl₂ to 1 mL sample. The samples were left to clot for 1 hour and centrifuged at 2500g for 15 minutes prior to assay of S100B. Neuron-specific Enolase was measured using the Kryptor NSE assay (Thermo Fischer Scientific B.R.A.H.M.S, Asnières, France), an automated homogenous immunometric assay based on the Time Resolved Amplified Cryptate Emission (TRACE) technology [11].

Brain tissue from hippocampus was homogenized in 5M guanidine HCl buffer with protease inhibitor and PMSF using Omnitip (Omn International USA). Homogenate was diluted with sample dilution buffer to 1:50 and 1:200. Final guanidine HCl concentrations were below 0.1 m. Sample duplicates were run on AB42 specific sandwich colorimetric ELISAs following the protocol of the manufacturer (BioSource, Camarillo, CA, USA). Optical densities at 450 nm of each well were read on a Multiscan Ascent (Thermo Scientific, MA, USA) and sample Aβ42 concentrations were determined by comparison with the AB42 standard curves.

Immunohistochemistry

Four microm thick sections, which had been stored in 4% formaldehyde, were deparaffinized and rehydrated. The sections kept in citrate buffer, were heated in microwave for 15 min. The
sections were then incubated with Formic acid 80% for 10 minutes, prior to blocking in a Peroxidase Block, 3% H2O2 for 10 minutes.

Following brief washes with Blocking buffer (5% goat serum, 5% BSA) for 30 minutes, the sections were incubated with Primary Antibody 6E10 (1:1000), a mouse monoclonal antibody, APP (Biolegend, MA, USA), and incubated over night at -4°C. The next day the slices were incubated with ImmPRESS (anti-mouse IgG, Vector Laboratories, CA, USA) for 30 minutes. For visualization DAB plus Peroxidase Substrate (Vector Laboratories, CA, USA) Kit were used.

Expression analysis of Amyloid Protein Precursor (APP) from Hippocampus

Total RNA was extracted from tissue samples using the EZNA Total RNA Kit II (Omega BioTek, Inc, Norcross GA, USA) according to manufacturers’ instructions.

Purified RNA was quantified using NanoDrop ND-1000 (NanoDrop Technologies, Delaware, USA) and 2 microg were reverse transcribed into cDNA with the High capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Tech, CA, USA).

Primers were designed using Primer Express 3.0 Software (Applied Biosystems, USA).

Primer sequence: Forward: 5-´ CAGATCCGATCCCAGGTTATGA-3´
Primer sequence: Reverse: 5-´ AGCAGGAACGTTGTAGAGCAGG-3´.

A tenfold dilution of each primer showed efficiency of between 90% and 110%.

Amplification was performed for both target genes and reference gene P0 in a ViiA 7 Real Time PCR System, universal settings (Applied Biosystems, Life Tech, CA, USA).

An amount of 50 ng of each sample was run in duplicate with 400 nmol/l primers and Power SYBR Green Master Mix (Applied Biosystems, Warrington, UK).

Data were analyzed by the comparative Ct method (Delta-delta Ct method).

Statistics

The data were statistically analyzed using the Kruskal-Wallis test and Mann-Whitney U test for variables with non-normal distributions, and independent samples t test when the distribution was normal. Levene’s test for equality of variance was performed before performing the t-test. If Levene’s test documented a significant variance difference between the compared groups, a t-test assuming different variances was performed. Otherwise, a t-test assuming equal variance was performed.

The biomarkers S100B and NSE were log-transformed to obtain a normal distribution.

Pearson’s correlation was used for calculation of the correlation between the log-values of NSE and S100B and between Lactate and AB42. The statistical analyses were performed by SPSS Statistics 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Comparing the two treatment groups of either moderate or severe hypoxia and the control group, there were no significant differences in weight, hemoglobin level, age, BE, PaCO2 or glucose level at start (Table 1). Table 2 describes the arterial blood gases, lactate and glucose taken at 5 different time points during the experiment. For one of the pigs in the group exposed to moderate hypoxia the BP fell below 20mmHg before reaching the predestined value of BE = -15mmol/l, (BE = -11mmol/l).

At the end of hypoxia and 30 minutes after end of hypoxia there were significant differences between both intervention groups and the control group regarding the levels of BE, pH, lactate and glucose (p<0.05). Until 90 minutes after end of hypoxia there were still significant
differences in all 4 parameters between the group exposed to severe hypoxia and the control group and BE and lactate remained significantly different until 120 minutes after end of hypoxia (p < 0.05). Blood gases taken at later time points of recovery showed no significant differences between the groups.

Between the different groups of the study cohort, there were no significant differences in weight, Hb level, age, BE, PaCO2 or glucose at start. Values are presented as mean (+/- SD) BE: base excess.

Table 1. Physiological parameters in the animals of the different groups at start.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>SEVERE HYPOXIA N = 12</th>
<th>MODERATE HYPOXIA N = 12</th>
<th>CONTROL N = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>1924 (124)</td>
<td>1982 (140)</td>
<td>1923 (76)</td>
</tr>
<tr>
<td>Haemoglobin g/dl</td>
<td>7.0 (1.0)</td>
<td>7.8(0.9)</td>
<td>7.7(1.8)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>6/6</td>
<td>6/6</td>
<td>3/3</td>
</tr>
<tr>
<td>Duration of Hypoxia (min)</td>
<td>33(12)</td>
<td>32 (9)</td>
<td>0</td>
</tr>
<tr>
<td>Age (h)</td>
<td>28.6 (3.5)</td>
<td>26.3 (4.6)</td>
<td>22.5 (1.5)</td>
</tr>
<tr>
<td>BE (mmol/l) Start</td>
<td>-0.3 (3.6)</td>
<td>1.6 (5.5)</td>
<td>4.3 (2.9)</td>
</tr>
<tr>
<td>Lactate (mmol/l)Start</td>
<td>2.8 (1.0)</td>
<td>1.8 (0.5)</td>
<td>2.3 (1.1)</td>
</tr>
<tr>
<td>Arterial pH Start</td>
<td>7.45 (0.04)</td>
<td>7.45 (0.07)</td>
<td>7.44 (0.12)</td>
</tr>
<tr>
<td>PaCO2 (kPa) Start</td>
<td>5.0 (1.1)</td>
<td>5.7 (1.4)</td>
<td>5.0 (1.1)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.7 (2.3)</td>
<td>6.0 (0.9)</td>
<td>5.0 (1,2)</td>
</tr>
</tbody>
</table>

Between the different groups of the study cohort, there were no significant differences in weight, Hb level, age, BE, PaCO2 or glucose at start. Values are presented as mean (+/- SD) BE: base excess.

doi:10.1371/journal.pone.0140966.t001

Table 2. BE, pH, PaCO2, Lactate and Glucose at 5 different time points after hypoxia.

<table>
<thead>
<tr>
<th>BE, mmol/L</th>
<th>End hypoxia</th>
<th>30 min reox</th>
<th>90 min reox</th>
<th>270 min reox</th>
<th>570 min reox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sev. Hypoxia</td>
<td>-19.0 (3.9)</td>
<td>-15.1 (3.5)</td>
<td>-5.7 (4.6)</td>
<td>-1.4 (5.0)</td>
<td>-3.7 (5.4)</td>
</tr>
<tr>
<td>Mod. Hypoxia</td>
<td>-15.8 (1.9)</td>
<td>-10.4 (2.8)</td>
<td>-1.3 (6.2)</td>
<td>-0.8 (5.7)</td>
<td>-4.0 (5.6)</td>
</tr>
<tr>
<td>Control</td>
<td>4.1 (3.8)</td>
<td>4.4 (1.9)</td>
<td>4.2 (2.3)</td>
<td>2.9 (4.5)</td>
<td>0.5 (3.4)</td>
</tr>
<tr>
<td>Arterial pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sev. Hypoxia</td>
<td>6.92 (0.11)</td>
<td>7.18 (0.07)</td>
<td>7.35 (0.08)</td>
<td>7.39 (0.10)</td>
<td>7.39 (0.09)</td>
</tr>
<tr>
<td>Mod. Hypoxia</td>
<td>6.99 (0.05)</td>
<td>7.24 (0.06)</td>
<td>7.39 (0.10)</td>
<td>7.38 (0.06)</td>
<td>7.32 (0.10)</td>
</tr>
<tr>
<td>Control</td>
<td>7.43 (0.05)</td>
<td>7.48 (0.04)</td>
<td>7.45 (0.06)</td>
<td>7.42 (0.12)</td>
<td>7.38 (0.06)</td>
</tr>
<tr>
<td>PaCO2, kPa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sev. Hypoxia</td>
<td>7.7 (1.1)</td>
<td>4.4 (0.6)</td>
<td>4.6 (0.7)</td>
<td>5.2 (0.5)</td>
<td>4.5 (0.6)</td>
</tr>
<tr>
<td>Mod. Hypoxia</td>
<td>8.0 (1.7)</td>
<td>5.1 (0.7)</td>
<td>5.1 (0.8)</td>
<td>5.4 (0.5)</td>
<td>5.6 (1.3)</td>
</tr>
<tr>
<td>Control</td>
<td>5.7 (0.3)</td>
<td>5.4 (0.6)</td>
<td>5.5 (0.7)</td>
<td>5.5 (0.6)</td>
<td>5.9 (0.8)</td>
</tr>
<tr>
<td>Lactate, mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sev. Hypoxia</td>
<td>13.2 (3.1)</td>
<td>11.2 (0.6)</td>
<td>6.3 (2.1)</td>
<td>2.3 (2.2)</td>
<td>2.1 (2.0)</td>
</tr>
<tr>
<td>Mod. Hypoxia</td>
<td>11.9 (3.0)</td>
<td>9.4 (2.7)</td>
<td>3.9 (1.8)</td>
<td>1.7 (1.0)</td>
<td>2.3 (2.1)</td>
</tr>
<tr>
<td>Control</td>
<td>2.4 (2.1)</td>
<td>1.8 (1.1)</td>
<td>1.5 (0.6)</td>
<td>1.3 (0.4)</td>
<td>1.7 (1.0)</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sev. Hypoxia</td>
<td>9.1 (3.7)</td>
<td>7.5 (3.6)</td>
<td>6.7 (2.3)</td>
<td>4.9 (0.9)</td>
<td>4.7 (1.6)</td>
</tr>
<tr>
<td>Mod. Hypoxia</td>
<td>8.9 (3.4)</td>
<td>7.0 (3.1)</td>
<td>5.2 (1.6)</td>
<td>4.4 (1.2)</td>
<td>4.8 (1.4)</td>
</tr>
<tr>
<td>Control</td>
<td>5.0 (1.5)</td>
<td>4.7 (0.8)</td>
<td>4.9 (0.6)</td>
<td>4.3 (0.6)</td>
<td>4.3 (1.5)</td>
</tr>
</tbody>
</table>

Mean and (SD) for arterial blood gases (BE, pH, PaCO2, Lactate and Glucose) at end of hypoxia, 30, 90, 270 and 570 minutes after end of hypoxia. For the Control group the arterial blood gases stayed stable throughout the experiment. For the control group the described time points are corresponding time points.

doi:10.1371/journal.pone.0140966.t002
Cerebrospinal fluid

Because of insufficient quality of CSF from 3 pigs, there were 5 pigs in the control group, 10 pigs in the group exposed to moderate hypoxia and 12 pigs in the group exposed to severe hypoxia. Fig 2 describes the level of AB42 in CSF for each group.

There was a slight correlation between AB42 and LogS100B in CSF as well as AB42 in CSF and arterial Lactate (Fig 3A and 3B).

We measured the levels of S100B in CSF and serum and the concentration of NSE in CSF (Fig 4A and 4B).

In spite of no significant difference of NSE in CSF between the hypoxia and control groups, p = 0.11, we found a strong correlation between the levels of NSE and S100B in CSF, (Fig 5).

(S1 and S2 Files are captions for supporting information files "correlations_LogNSE--Logs100b and Lactate-S100b.pzfx")

There were no differences between the intervention groups and the control group for T-tau and p-Tau in CSF.

Brain tissue

AB42 could not be detected by ELISA in brain tissue homogenates and aggregated AB42 were not observed in any part of the brains by immunohistochemistry or in any of the slices and any of the animals examined.

The groups revealed no differences the gene expression of Amyloidal Precursor Protein in Hippocampus or Cortex.
Fig 3. (A): Correlation between Log S100B and AB42 in CSF. There was a moderate negative correlation between Log S100B and AB42 in CSF, R = -0.418, p < 0.05. (B): Correlation between Arterial Lactate and AB42 in CSF. Arterial Lactate at end of Hypoxia had a moderate negative correlation with AB42, R = -0.419, p = 0.03.

doi:10.1371/journal.pone.0140966.g003

Fig 4. S100B in CSF and serum. (A) shows the Log-values of S100B in CSF for both intervention groups and the Control group. Log-S100B was significantly higher in the group exposed to hypoxia than in the control group, 1.0 (SD +/-0.3) pg/ml vs. 1.4 (SD +/-0.4) pg/ml, p < 0.05. Mean difference was 0.4 (95% CI: 0.2–0.9). (B) depicts the Delta-Value from End of Hypoxia to End of experiment, p = 0.05. There was no difference between the group exposed to moderate vs. severe hypoxia regarding the levels of S100B, neither in CSF nor in blood, therefore we decided to combine both hypoxia groups into one intervention group.

doi:10.1371/journal.pone.0140966.g004
The newborn pigs exposed to severe hypoxia revealed significantly lower levels of AB42 in CSF compared to the control group. To our knowledge the present study is the first to report a significant reduction in the level of AB42 in CSF and hypoxia-reoxygenation in a neonatal model. A similar tendency was observed, although not significant, for those exposed to moderate hypoxia.

In accordance with the amyloid hypothesis decreased AB42 in CSF is supposed to be the first biomarker change to occur in AD [12,13]. A possible explanation for the absent T-tau and p-Tau changes in our experiment might be that the available limited time of 9.5 hours in our model is too short to develop tau changes. With longer observation time one might expect increased T-tau, an unspecific marker of neuronal injury.

AB42 and its protein precursor, Amyloid Beta protein precursor (B-APP) have played a central role in research on AD. However, the function of B-APP and AB in the nervous system is controversial.

B-APP-knockout mice show severe behavioral deficits, possibly indicating that B-APP has important physiological functions in the nervous system.

**Discussion**

The newborn pigs exposed to severe hypoxia revealed significantly lower levels of AB42 in CSF compared to the control group. To our knowledge the present study is the first to report a significant reduction in the level of AB42 in CSF and hypoxia-reoxygenation in a neonatal model.

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B-APP-knockout mice show severe behavioral deficits, possibly indicating that B-APP has important physiological functions in the nervous system.
In the prodromal and preclinical stages of AD the level of AB42 in CSF is reduced [14] and we found a similar pattern in the present neonatal hypoxia-reoxygenation model. In AD, the level of soluble AB42 correlates with synaptic changes and disease severity [15], indicating an imbalance between production and clearance of AB42.

B, resulting in accumulation of toxic AB aggregates, neuroinflammation and neuronal cell death [16].

AB are known to self-assemble into oligomers, which are thought to be an important source of toxicity by damaging the neurons [17].

Lambert et al.(1998) presented in a mouse model that neurotoxins comprising oligomers of AB42 could kill neurons in hippocampus [18]. AB induces liposome fusion in vitro, which may suggest that AB in a non-fibrillar form may play a role in the progression of AD by directly disturbing the plasma membrane of neurons and altering its property [19]. Pillot. et al (1999) were able to show that in a primary culture from cortical neurons, AB could induce neurotoxicity via an apoptotic pathway [20].

In accordance to other studies we found that S100B in CSF was significantly higher for those exposed to hypoxia [21, 22]. However, a significant moderate negative correlation between AB42 and S100B in CSF was also observed.

We did not detect any signs of AB42 in the brain homogenate with ELISA, possibly due to a low concentration of AB42 in the brain of the newborn pigs.

Neither did we detect any signs of aggregation of AB42 in the brain tissue with the optical microscope, however a possible presence of small aggregates of AB42, below the detection limit of an optical microscope, cannot be excluded.

Taking these points into consideration, it is tempting to speculate that the reduction of AB42 in CSF and the negative correlation with S100B after neonatal hypoxia-reoxygenation, could be a sign of aggregation of AB42, which in turn attacks the neurons, triggering a long-lasting process.

In addition, it is an interesting observation that the same cognitive skills which are very often reduced for relatively well-functioning children after perinatal asphyxia are similar to the skills which are influenced in the earliest phases of AD, such as attention and visuospatial skills [23–25].

The moderate negative correlation between AB42 and lactate at end of hypoxia could strengthen the speculation that perinatal asphyxia might inflict neurodegenerative changes, as a study on newborn lambs from 2014 showed a high correlation between lactate 4 hours after asphyxia and histological degeneration of hippocampus 72 hours after asphyxia [26].

Could the neurons injured after neonatal hypoxia-reoxygenation be more prone to increased oxidative stress in late adulthood than their peers and could this make them more prone to neurodegenerative disorders such as AD?

It would be worthwhile to study whether AB42 and S100B in CSF could represent useful biomarkers at an early stage of brain damage after perinatal hypoxia-reoxygenation.

Limitations of the study

We are aware that a relatively small number of animals were investigated. The pigs were sacrificed 9.5 hours after end of hypoxia, thus there were no long term follow-up.

It would have been highly interesting examining the brain of the pigs with electron microscope and search for possible changes in the neurons. However, to find the regions of interest, when we could not discover any changes in the optical microscope would have been very difficult.

As this is an animal study caution should be taken interpreting it on humans.
Conclusion
To our knowledge, this study is the first to show an association between AB42 in CSF and perinatal hypoxia. Whether or not the reduction of AB42 in CSF after perinatal hypoxia has an etiological basis for adult neurodegenerative disorders needs to be studied with additional experiments and epidemiological studies.

AB42 and S100b are significantly changed in neonatal pigs subjected to severe hypoxia compared to controls, thus they may be valuable biomarkers of perinatal asphyxia.

Supporting Information
S1 File. Graphpad-File for the values of LogNSE and LogS100B for each pig. (PZFX)
S2 File. Graphpad-File for the values of Lactate and S100B for each pig. (PZFX)

Acknowledgments
The authors wish to thank Vivi Stubberud, Aurora Pamplona, Sera Sebastian, Grethe Dyrhaug, Monica Atneosen-Åsegg, Santiago Rivera and Eliane Charrat. Roche Diagnostics supported the study with ELISA kits for investigating S100B in CSF and blood.

The study was funded by Helse-SørØst (South and Eastern Norway Regional Health Authority; Source number: 6051. Project number: 39570.

Author Contributions
Conceived and designed the experiments: TB RS ODS. Performed the experiments: TB LP RS. Analyzed the data: TB NB AS LS. Contributed reagents/materials/analysis tools: NB AS. Wrote the paper: TB LP NB AS RS LB LS ODS.

References


N-Acetylcysteine Amide (NACA) Reduces Cell Death after Oxidative Stress in a Porcine Embryonic Kidney Cell Line

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https://doi.org/10.4236/jbise.2017.102004

Received: December 28, 2016
Accepted: January 21, 2017
Published: January 24, 2017

Abstract

Introduction: Oxidative stress may have detrimental effects on different structures of the cells, such as the DNA. Recently, we have published a study demonstrating that N-Acetylcysteine amide (NACA) displayed anti-inflammatory properties on the brain after exposure to oxidative stress in an established neonatal piglet model, imitating perinatal asphyxia. As different clinical studies have shown an association between the severity of hypoxic-ischemic encephalopathy and damage of the kidneys, we investigated a possible protective effect of NACA against H₂O₂-induced oxidative stress using a porcine epithelial-like embryonic kidney cell line (EFN-R). Objective: To investigate a potential protective effect of NACA on cells of a porcine embryonic kidney cell line exposed to H₂O₂. Methods: We subjected the cells to different concentrations of H₂O₂ for variable time periods, seeking the optimal dose-response for the experiments. Based on the results of these investigations, we exposed the cells to 100 μMol of H₂O₂ and/or 750 μM of NACA for 24 hours. Some of the cells would receive NACA either one hour before or one hour after exposure to H₂O₂. Results: The viability of the investigated EFN-R cells revealed that both, the group treated with NACA before exposure to H₂O₂ and the group treated with NACA after exposure to H₂O₂, exhibited significantly higher cell viability compared to the H₂O₂ group (p < 0.001 and p < 0.01, respectively). Discussion: The increased viability of the cells may indicate that NACA could play an important role in reducing oxidative stress. Taking the results from our previous study into consideration, our findings may streng-
then the theory that NACA may have organ protective properties for neonates exposed to oxidative stress.

**Keywords**

N-Acetylcysteine Amide (NACA), Cell Lines, Oxidative Stress

1. **Introduction**

Reactive oxygen species (ROS) are important in different processes of the organism, including cell-signaling [1]. However, during oxidative stress, too much ROS is produced, which may have detrimental effects on different structures of the cells. ROS play a role as a mediator of apoptosis and may induce damage to the DNA. One important member of ROS is \( \text{H}_2\text{O}_2 \), which may be harmful to the DNA and leads to cell injury.

Recently, we have published a study demonstrating that NACA displayed anti-inflammatory properties after exposure to oxidative stress in an established neonatal piglet model, imitating perinatal asphyxia [2]. Our piglet model has been established for many years and several authors have shown a significant increase in markers of oxidative stress after the inflicted asphyxia [3]. As different clinical studies have shown an association between the severity of hypoxic-ischemic encephalopathy and damage of the kidneys [4] [5], we decided to investigate possible protective effects of NACA using the porcine epithelial-like embryonic kidney cell line EFN-R exposed to \( \text{H}_2\text{O}_2 \). Previous studies have shown that NACA may reduce the injury in epithelial kidney cells exposed to toxic doses of the antibiotic Gentamycin and the contrast agent Iohexol [6] [7]. Prior to the experiments with NACA, dose-response investigations of \( \text{H}_2\text{O}_2 \) were conducted to estimate the appropriate dose for the treatment of cells of a cell line.

2. **Objective**

To investigate a potential protective effect of NACA on cells of an embryonic kidney cell line exposed to \( \text{H}_2\text{O}_2 \).

3. **Methods**

*Cell culture:* The porcine epithelial-like embryonic kidney cell line EFN-R (catalogue number CCLV-RIE 86) was generated and provided by courtesy of the Friedrich-Loeffler Institute, Federal Research, Institute for Animal Health, Greifswald-Insel Riems, Germany.

EFN-R cells were grown using Dulbecco’s modified eagle’s medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA), 10% fetal bovine serum (FBS) and 1% streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA). The cells were incubated in a cell chamber at 36.8°C and 5% CO\(_2\). Cells were cultured when they were approximately 80% confluent. The confluence describes the percentage of
cells in growth. For subculturing, the cells were washed with PBS, trypsinated and incubated in the cell chamber for 3 minutes. Cells were then centrifuged at 3000 rpm for 3 minutes, and the pellet was dissolved in fresh cell medium.

A Bürker-chamber was used to calculate cell numbers. 100 μl of cell suspension together with 900 μl tryptan blue solutions (Life Technology, UK) was added onto a Bürker-chamber glass plate and covered with a slide and placed under a microscope. The amount of cells was counted in minimum of five squares, and the average value of number of cells per square was calculated. The cells were counted only on the top and the left edge of each square, to avoid cells be counted twice. The desired amount of cells was adjusted and transferred to plates for further experiments.

**MTT:** For measuring the number of viable cells the MTT-test was conducted. The MTT-test is a reliable, simple, and established method to measure cytotoxicity, proliferation, and activation in cell lines [8]. The MTT (3,4,5 dimethylthiazol-2,5 diphenyl tetrazolium, Sigma-Aldrich, St. Louis, Missouri, USA) viability assay is based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert MTT to a purple formazan precipitate. These mitochondrial succinate dehydrogenases may reflect the number of viable cells present. The resulting crystals are subsequently dissolved using dimethyl sulfoxide (DMSO) and the optical density of each well is measured using a multiscan acsent plate reader (Thermo Electron Corporation, Waltham, Massachusetts, USA). Cells were exposed to H₂O₂ for 1 hour. After incubation, the medium was removed and 200 μl MTT was added and incubated for 1 hour. MTT was removed and 100 μl of DMSO was added. The cells in the control group received DMEM.

**Cell line experiments:** The EFN-R cells were used as model system for stress experiments. 150,000 cells were seeded in each well in a 12-wells plate. The cells were starved for 24 hours and the confluent cells were treated with H₂O₂ for various incubation time and concentrations. In the dose-response investigation of H₂O₂ we sought the preferable dose of H₂O₂ to be used in the main experiment. The cell plate was divided into four groups with triplicates, and each group was treated with different concentrations of H₂O₂ (Figure 1).

**Statistics:** The analyses were performed using SPSS software v21 (SPSS Inc. Chicago, Illinois, USA). The data were analyzed using the Kruskal-Wallis test and Mann-Whitney U test for variables with non-normal distributions. For normal distributions, Student t-test and ANOVA were performed.

**4. Results**

EFN-R cells, exposed to H₂O₂ at various concentrations and for different time periods, revealed a decline in cell viability with increasing concentrations and exposure time.

Based on the results of the experiment described above, we decided to subject the EFN-R cells to 100 μMol of H₂O₂ and/or 750 μM of NACA for 24 hours.

Our second experiment revealed that cells exposed to NACA 1 hour after treatment with H₂O₂ had a lower mortality than cells subjected to H₂O₂ alone,
p < 0.01. Also the group subjected to NACA 1 hour before exposure to H$_2$O$_2$ displayed significantly higher viability 24 hours later (p < 0.001) (Figure 2).

5. Discussion

In this study, our aim was to investigate a possible protective effect of NACA to a porcine embryonic kidney cell line exposed to oxidative stress by H$_2$O$_2$ treatment.

The dose of 750 μM of NACA was decided because of previous reports demonstrated that NACA was protective to neural cells exposed to oxidative stress induced by Glutamate [9].

Figure 1. Effect of different concentration of H$_2$O$_2$ at different time points, measured by the MTT-assay. Cells were treated with different concentrations (50, 100, and 200 μMol) of H$_2$O$_2$ and incubated either for 1 (n = 8) or 24 (n = 9) hours. Viable cells were assessed by MTT-assay, presented in percentage compared to control for the representative time point. Values represent means ± standard deviation (SD). Statistically different values of *p < 0.05, **p < 0.01 were calculated with t-test and compared to cells without H$_2$O$_2$ treatment (control).

Figure 2. Cells exposed to H$_2$O$_2$ had a significantly higher mortality rate compared to the control group not exposed to H$_2$O$_2$ (p < 0.001). The NACA group was exposed to NACA, but not H$_2$O$_2$. Evaluation of the viability of the EFN-R cells revealed that both the group treated with NACA before exposure to H$_2$O$_2$ (Pre) and the group treated with NACA after exposure to H$_2$O$_2$ (Post), exhibited significantly higher cell viability compared with the H$_2$O$_2$ group (p < 0.001 and p < 0.01, respectively).
Even though several studies have shown advantageous outcomes of NACA in different cell cultures subjected to oxidative stress \[10\] \[11\], we are, to our knowledge, the first who confirm the positive effects using a porcine epithelial-like embryonic kidney cell line.

6. Conclusions

The augmented viability of the EFN-R cells may indicate that NACA could play a crucial role when reducing oxidative stress.

Taking the results from our previous study into consideration, we suggest that NACA may have organ protective properties for neonates exposed to perinatal oxidative stress.

We underscore that our results are based on experiments on isolated cells and more studies should be accomplished before considering transferring them into a clinical trial.

Conflict of Interests

There are no conflicts of interests to declare.

References


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Research Article

Cerebellum Susceptibility to Neonatal Asphyxia: Possible Protective Effects of N-Acetylcyesteine Amide

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Received 1 May 2017; Revised 7 September 2017; Accepted 7 December 2017; Published 30 January 2018

Academic Editor: Hubertus Himmerich

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Background. After perinatal asphyxia, the cerebellum presents more damage than previously suggested. Objectives. To explore if the antioxidant N-acetylcysteine amide (NACA) could reduce cerebellar injury after hypoxia-reoxygenation in a neonatal pig model. Methods. Twenty-four newborn pigs in two intervention groups were exposed to 8% oxygen and hypercapnia, until base excess fell to $-20$ mmol/l or the mean arterial blood pressure declined to <20 mmHg. After hypoxia, they received either NACA (NACA group, n = 12) or saline (vehicle-treated group, n = 12). One sham-operated group (n = 5) served as a control and was not subjected to hypoxia. Observation time after the end of hypoxia was 9.5 hours. Results. The intranuclear proteolytic activity in Purkinje cells of asphyxiated vehicle-treated pigs was significantly higher than that in sham controls ($p = 0.03$). Treatment with NACA was associated with a trend to decreased intranuclear proteolytic activity ($p = 0.08$). There were significantly less mutations in the mtDNA of the NACA group compared with the vehicle-treated group, $2.0 \times 10^{-4}$ ($\pm 2.0 \times 10^{-4}$) versus $4.8 \times 10^{-5}$ ($\pm 3.6 \times 10^{-5}$, $p < 0.05$). Conclusion. We found a trend to lower proteolytic activity in the core of Purkinje cells and significantly reduced mutation rate of mtDNA in the NACA group, which may indicate a positive effect of NACA after neonatal hypoxia. Measuring the proteolytic activity in the nucleus of Purkinje cells could be used to assess the effect of different neuroprotective substances after perinatal asphyxia.

1. Introduction

Globally, approximately 45% of the cases of child death within the first five years of life occur during the neonatal period [1]. Despite the numbers of fatal cases due to the complications of perinatal asphyxia have been remarkably reduced over the last 15 years, there are still many children suffering from extensive neurological consequences after perinatal asphyxia.

It is widely recognized that in neonatal basal ganglia, the cerebral cortex, thalamus, and hippocampus are the most vulnerable brain areas after perinatal hypoxia [2]. However, improved neuroimaging modalities have shown that the cerebellum is more damaged after perinatal
asphyxia than previously suggested [3]. In addition to being a coordinator of motor function, the cerebellum plays a role in higher cognitive functions and several authors argue that the abnormalities in the cerebellum may play a pivotal role in different mental disorders, such as attention deficit and hyperactivity disorder (ADHD) and schizophrenia [4, 5].

Our group has recently described anti-inflammatory and possible neuroprotective effects of the antioxidant N-acetylcysteine amide (NACA) after neonatal hypoxia-reoxygenation in a neonatal pig model. Further, NACA reduced the levels of the proinflammatory cytokine IL-1β and the transcription factor NF-κB in the prefrontal cortex of the brain after neonatal hypoxia-reoxygenation [6]. The substance NACA has many similarities with N-acetylcysteine, which has been used as an antioxidant precursor to glutathione in the treatment of paracetamol overdose for more than 30 years [7]. However, due to the amide group which increases its lipophilicity, NACA has an augmented ability to penetrate the blood-brain barrier and the cellular membranes [8, 9]. Moreover, we showed in a pig epithelial-like embryonic EFN-R kidney cell line that NACA had a protective effect on cells exposed to H₂O₂-induced oxidative stress [10].

In the present study, we wanted to explore if NACA treatment after hypoxia-reoxygenation reduces cerebellar injury, using the same group of pigs. The model is well established, and it has been used for years in our department to induce oxidative stress [11].

Matrix metalloproteinases (MMPs) display proinflammatory activity and exert deleterious actions in numerous neuropathological settings, including hypoxia and ischemia [12, 13]. Moreover, some MMPs have been located in the nucleus of neural cells [14, 15] and are associated with neuronal DNA degradation upon oxygen-glucose deprivation [16]. Therefore, we used in situ zymography, which reflects the net metalloproteinase activity in the tissue, to measure proteolytic activity in the cerebellum upon hypoxia-reoxygenation.

Furthermore, reactive oxygen species (ROS) produced during and after perinatal asphyxia may induce lesions of mitochondrial DNA (mtDNA) and subsequently lead to impaired function of neural cells [17]. In this study, we investigated mtDNA in the cerebellum after hypoxia-reoxygenation and if the pigs subjected to NACA after hypoxia (NACA group) would be less susceptible to mutations of mtDNA. The objective of the present study was to evaluate the damage-reduction potential of NACA on cerebellar injury after hypoxia-reoxygenation in neonatal pigs.

2. Methods

2.1. Study Design. A total of 29 newborn pigs, age 12–36 hours, hemoglobin >5 g/dl, and in good general condition, were included in this study (Figure 1). The pigs were anesthetized, ventilated, and surgically prepared, including insertions of central venous and arterial lines, as previously described by Benterud et al. [18].

The experimental protocol has been thoroughly described in our previous article [6]. Briefly summarized, twenty-four pigs were randomized into two intervention groups. Both groups were subjected to 8% oxygen until base excess (BE) values declined to ~20 mmol/l or mean arterial blood pressure (MABP) fell below 20 mmHg. During hypoxia, CO₂ was added, to achieve a PaCO₂ of 8.0–9.5 kPa, in order to imitate perinatal asphyxia. At the end of hypoxia, 12 of the pigs were treated with NACA 300 mg/kg diluted in saline 0.9%, while the other 12 received normal saline (vehicle-treated group). An additional dose of NACA or saline was administered 270 minutes after the hypoxic challenge. The pigs were reoxygenated with air for 9.5 hours until they were terminated with an overdose of pentobarbital 150 mg/kg.

The five pigs in the sham-operated group underwent the same procedures as described in our previous article and were not exposed to hypoxia.

Due to the fact that there might be some subtle gender differences between neonatal pigs [19], the same number of male and female animals were included in each group.

3. Laboratory Methods

3.1. In Situ Zymography. We focused our investigations of the cerebellum on Purkinje cells, due to their high vulnerability to hypoxia [20, 21]. Furthermore, the nucleus of Purkinje cells presented the highest level of fluorescence and seemed to be particularly affected by hypoxia-reoxygenation. In situ zymography is commonly used as an index of net metalloproteinase activity resulting from the balance between gelatinases (principally MMP-9 and MMP-2) and the tissue inhibitors of MMPs (TIMPs) that are present in the tissue. In situ zymography was performed to localize net gelatinolytic activity in cerebellar brain sections, with minor modifications compared to the method previously described for brain tissue [22]. Sections of fresh frozen brain tissue (20 μm thick) from the cerebellum were generated using a cryostat (Leica CM3050S, Nussloch, Germany). Nonfixed brain sections were incubated for 2 hours at 37°C in a humid dark chamber in a reaction buffer that contained 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM CaCl₂, 2 mM sodium azide (pH 7.6), and 80 μg/ml of intramolecularly quenched FITC-labeled DQ-gelatin (EnzCheck collagenase assay kit; Thermo Fisher Scientific, Waltham, Massachusetts, USA). After the incubation, the tissue was fixed in 4% paraformaldehyde Antigenfix solution (Diapath, MM France, Brignais, France), incubated for 5 minutes with 0.5 μg/ml Hoechst 33258 (Thermo Fisher Scientific), and mounted in Prolong Gold Antifading reagent (Thermo Fisher Scientific). The sections were incubated with 1 mM phenanthroline (Thermo Fisher Scientific), a broad-spectrum metalloproteinase inhibitor. Samples were observed with a confocal microscope (LSM 700 Zeiss, Jena, Germany), and images were analyzed using the Zen (Zeiss) and ImageJ softwares (NIH, Bethesda, MD, USA). Gelatin-FITC cleavage by tissue gelatinases releases quenched fluorescence representative of net proteolytic activity. Sections incubated without DQ-gelatin were not fluorescent. We used 8 piglets per experimental group and 5 from the control...
group, and we analyzed three slices per animal. The 8 pigs in each experimental group were randomly selected.

3.2. Histopathology. After removal of the brain, one hemisphere was immersion fixed in formalin. Tissue blocks (0.5 cm thick) from the cerebellum were embedded in paraffin, sliced in 4 μm thick sections, and stained with hematoxylin and eosin (H&E). An experienced neuropathologist evaluated the slices. Because of suboptimal conservation of the cerebellum, a simplified assessment was conducted. Cerebellar damage was categorized into two variables: (1) generalized damage and (2) localized/no damage.

The term generalized was used if the injuries were observed in all parts of the tissue section, in comparison to localized, where only small and limited parts of the tissue section were involved. Due to the limited amount of tissue, 23 pigs were evaluated, 5 in the sham group and 18 in the intervention groups. Of the pigs in the intervention groups, 8 were in the NACA group and 10 in the vehicle-treated group.

3.3. DNA Extraction from Cerebellum. Total DNA from the cerebellum was isolated using DNA blood and tissue kit (Qiagen, Hildesheim, Germany). 10–25 mg of tissue from each pig was lysed and dissolved according to manufacturer’s protocol with slight modifications (For a more detailed description, please read the Supplementary Materials section).

3.4. Mutation Rate of Mitochondrial DNA. Random mutation capture (RMC) was performed to assess the rate of mutations of mtDNA in the cerebellum. The method is thoroughly described in the Supplementary Materials section.

3.5. Gene Expression. Real-time quantitative PCR (RT-qPCR) was performed to investigate the expression levels of genes involved in the NLRP3 inflammatory pathway, including IL-1β, IL18, and NLRP3. RT-qPCRs were performed using the RT-RNA PCR kit following the instruction of the producer (Applied Biosystems, now Life 21 Technologies, Carlsbad, CA, USA). The final reaction volume was 25 μl, including 12.5 μl of universal master mix (Life 21 Technologies), 200 nmol of forward and reverse primers, and 5 μl of the diluted cDNA product (1:12.5 dilutions). The 96-well plate reactions were carried out with an initial cycle at 50°C for 2 minutes, a heating stop at 95°C for 10 minutes, followed by 45 cycles of 30 seconds at 95°C, and 60 seconds at 60°C. All reactions were performed on a Viia7 Sequence Detection System (Life 21 Technologies). Experiments were performed in triplets, and all transcript quantification data were normalized to the endogenous reference gene P0.

The primer sequences 5‘–3‘ were as follows: NLRP3 (forward primer) AAAAAACGTGAGTTGACCATTGTCTG and (reverse primer) CACCATCTTTATACACACCAGATGTGCTC; IL-1β (forward primer) GTATGGCAAGTGGCA GTCT and (reverse primer) GTGGCCAGCCAGCACTAC; and IL-18 (forward primer) GCCCTCAGTAGGTCTGGC AGTA and (reverse primer) GGACTCATTTCCCTAA AGGAAAGAGTT.

3.6. ELISA. To determine the protein concentrations of IL-1β, enzyme immunoassays kit was used as instructed by the manufacturer (R&D Systems, Oxford, UK).

4. Statistical Analysis

The analyses were performed using SPSS software v21 (SPSS Inc., Chicago, IL, USA). The data were analyzed using the Kruskal-Wallis test, Mann–Whitney U test, or Student t-test documented a significant variance difference between the compared groups, a t-test assuming different variances was performed. Otherwise, a t-test assuming equal variance was performed.

All the differences were considered significant if p < 0.05. When calculating the results of the histopathological evaluation, chi-square test without Yates’ correction was performed.

5. Results

We did not find any significant difference between the genders, and therefore, the data for both genders are merged.

5.1. Physiological Parameters. At baseline, there were no differences in weight, hemoglobin, pH, BE, lactate, pCO2, or
Table 1: Background and physiological parameters throughout the experiment.

<table>
<thead>
<tr>
<th></th>
<th>Control n = 5</th>
<th>Hypoxia + NACA n = 12</th>
<th>Hypoxia + saline n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight (g)</strong></td>
<td>1923 (±76)</td>
<td>1874 (±184)</td>
<td>1924 (±124)</td>
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<tr>
<td><strong>Hypoxia time (min)</strong></td>
<td>33 (±12)</td>
<td>40 (±13)</td>
<td>33 (±12)</td>
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<tr>
<td><strong>Hb g/100 ml start</strong></td>
<td>7.7 (±1.8)</td>
<td>8.0 (±1.2)</td>
<td>7.9 (±1.0)</td>
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<tr>
<td><strong>Hb g/100 ml end</strong></td>
<td>5.9 (±1.2)</td>
<td>6.3 (±0.8)</td>
<td>7.1 (±2.7)</td>
</tr>
<tr>
<td><strong>Gender (male/female)</strong></td>
<td>3/2</td>
<td>6/6</td>
<td>6/6</td>
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<tr>
<td><strong>pH</strong></td>
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<td></td>
</tr>
<tr>
<td>Start</td>
<td>7.44 (±0.04)</td>
<td>7.42 (±0.04)</td>
<td>7.45 (±0.04)</td>
</tr>
<tr>
<td>End hypoxia</td>
<td>7.43 (±0.05)</td>
<td>8.07 (±0.08)</td>
<td>6.92 (±0.11)</td>
</tr>
<tr>
<td>30 min reox</td>
<td>7.46 (±0.04)</td>
<td>7.18 (±0.09)</td>
<td>7.18 (±0.07)</td>
</tr>
<tr>
<td>90 min reox</td>
<td>7.45 (±0.06)</td>
<td>7.45 (±0.04)</td>
<td>7.35 (±0.08)</td>
</tr>
<tr>
<td>270 min reox</td>
<td>7.42 (±0.12)</td>
<td>7.45 (±0.04)</td>
<td>7.39 (±0.10)</td>
</tr>
<tr>
<td>570 min reox</td>
<td>7.38 (±0.06)</td>
<td>7.42 (±0.04)</td>
<td>7.39 (±0.09)</td>
</tr>
<tr>
<td><strong>BE (mmol/l)</strong></td>
<td></td>
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</tr>
<tr>
<td>Start</td>
<td>4.3 (±2.9)</td>
<td>4.1 (±3.6)</td>
<td>4.4 (±1.9)</td>
</tr>
<tr>
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<td>18.9 (±2.2)</td>
<td>13.3 (±4.7)</td>
</tr>
<tr>
<td>30 min reox</td>
<td>4.4 (±1.9)</td>
<td>13.3 (±4.7)</td>
<td>15.1 (±3.5)</td>
</tr>
<tr>
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<td>4.2 (±2.3)</td>
<td>3.9 (±5.0)</td>
<td>5.7 (±4.6)</td>
</tr>
<tr>
<td>270 min reox</td>
<td>2.9 (±4.5)</td>
<td>2.2 (±4.4)</td>
<td>1.4 (±5.0)</td>
</tr>
<tr>
<td>570 min reox</td>
<td>0.5 (±3.4)</td>
<td>6.5 (±6.6)</td>
<td>3.7 (±5.4)</td>
</tr>
<tr>
<td><strong>Lactate (mmol/l)</strong></td>
<td></td>
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<tr>
<td>Start</td>
<td>2.3 (±1.1)</td>
<td>2.3 (±1.1)</td>
<td>2.8 (±1.0)</td>
</tr>
<tr>
<td>End hypoxia</td>
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<td>14.3 (±2.7)</td>
<td>13.2 (±3.1)</td>
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<td>6.3 (±2.1)</td>
</tr>
<tr>
<td>270 min reox</td>
<td>1.3 (±0.4)</td>
<td>1.7 (±0.8)</td>
<td>2.3 (±2.2)</td>
</tr>
<tr>
<td>570 min reox</td>
<td>1.7 (±1.0)</td>
<td>1.4 (±1.0)</td>
<td>2.1 (±2.0)</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
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<td></td>
</tr>
<tr>
<td>Start</td>
<td>5.0 (±1.2)</td>
<td>6.4 (±2.1)</td>
<td>6.7 (±2.3)</td>
</tr>
<tr>
<td>End hypoxia</td>
<td>5.0 (±1.5)</td>
<td>9.6 (±3.4)</td>
<td>9.1 (±3.7)</td>
</tr>
<tr>
<td>30 min reox</td>
<td>4.7 (±0.8)</td>
<td>8.0 (±3.5)</td>
<td>7.5 (±3.6)</td>
</tr>
<tr>
<td>90 min reox</td>
<td>4.9 (±0.6)</td>
<td>6.5 (±2.3)</td>
<td>6.7 (±2.3)</td>
</tr>
<tr>
<td><strong>pCO₂ (kPa)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>5.0 (±1.1)</td>
<td>5.2 (±0.9)</td>
<td>5.0 (±1.1)</td>
</tr>
<tr>
<td>End hypoxia</td>
<td>5.7 (±0.3)</td>
<td>8.4 (±1.4)</td>
<td>7.7 (±1.1)</td>
</tr>
<tr>
<td>30 min reox</td>
<td>5.4 (±0.6)</td>
<td>5.1 (±0.8)</td>
<td>4.4 (±0.6)</td>
</tr>
</tbody>
</table>

5.2. In Situ Zymography. Compared to sham animals, a significant increased proteolytic activity was found in animals exposed to hypoxia alone (vehicle group, p = 0.03), by contrast to NACA animals where a significant difference was not found (p = 0.08). Representative images obtained from five (sham) and eight (NACA and vehicle-treated groups) animals in each group are shown in Figure 2.

5.3. Histopathology. Significantly, more pigs in the intervention groups had generalized damage than in the control group (p < 0.05) (Table 2).

There was no difference between the two intervention groups (p = 0.67).

Figure 3 shows an example of the cerebellum of a pig with a localized damage in different magnifications.

5.4. mtDNA Mutation. There were significantly fewer mutations in the NACA group than in the vehicle-treated group (2.0 × 10⁻⁴, SD ± 2.0 × 10⁻⁴ versus 4.9 × 10⁻⁴, SD ± 3.6 × 10⁻⁴) (p < 0.05) (Figure 4).

There was no significant difference when comparing the mtDNA mutation rate between the sham (2.2 × 10⁻⁴, glucose level between the groups. Arterial blood gases were taken at 6 different time points during the experiment. There were no significant differences between the 2 intervention groups in any of these variables. The physiological parameters and their change during the experiments are thoroughly described in Table 1.
and vehicle-treated groups ($p = 0.11$); however, the sham group consists of only five pigs.

5.5. Quantitative Real-Time PCR (qRT-PCR). Between the groups, there were no significant differences in gene expression of NLRP3, IL-18, and IL-1β.

5.6. Protein Concentrations of IL-1β in the Cerebellum. The concentrations of IL-1β did not differ between the groups. In the NACA group, the concentration was $15.9 \pm 9.6$ versus $16.9 \pm 8.2$ in the vehicle-treated group ($p = 0.82$). A figure of the concentrations of IL-1β is included in the Supplementary Materials.

6. Discussion

To our knowledge, the present study is the first to use in situ zymography of the cores of Purkinje cells as a marker of hypoxic damage. Measuring the net gelatinolytic activity may be a relevant method to assess the grade of inflammation in cerebellar tissue. Net gelatinolytic activity reflects the proteolytic activity of gelatinases in a specific tissue [22]. The proteolytic activity of MMPs is tightly associated with the activity of TIMPs. Altered balance between MMPs and TIMPs results in a less-controlled equilibrium and may lead to an abrupt increase of proteolysis and pathological processes, including inflammation [12, 13]. This assumption has been strengthened by numerous observations relating to increases in gelatinase activity with glial reactivity and neuronal demise and has been demonstrated in rodents after global cerebral ischemia [22, 23] and excitotoxic seizures induced by kainate [24]. In the latter model, gelatinolysis increased in neurons as early as eight hours after excitotoxic insult and remained high for several days in blood vessels and reactive glial cells of vulnerable areas, in relation with neuroinflammation.

Moreover, Chen et al. showed that exposing newborn rats to a broad-spectrum inhibitor of MMPs after hypoxia-ischemia provided a long-term protection in both neuronal morphology and neurological function in the immature
point to eosinophilic Purkinje cells, representing neurons with hypoxic injury. The black arrows point to normal Purkinje cells.

Our group has previously investigated the association between net gelatinolytic activity and gene expression of MMP-2 and MMP-9 in various tissues, such as the liver, lungs, and striatum [27, 28]. The gene expression of MMP-2 and MMP-9, as well as the protein levels of active MMP-2 and MMP-9, was associated with increased activity in the liver and lungs, whereas no such association was found in the striatum. Due to the important role of Purkinje cells in the developing brain [29], we speculate that the analysis of gelatinolytic activity in these cells could serve as an important tool in evaluating the various effects of neuroprotective substances after hypoxia in different models. The observation that pigs treated with NACA had a tendency to lower levels of gelatinolytic activity, compared with the vehicle-treated group may indicate that NACA reduces inflammation in the cerebellum.

Our results are in line with previous investigations of our group by Solberg et al. on the striatum of neonatal piglets, which observed an increased gelatinolytic activity in the nuclear compartment as well as in the cytoplasm of the neurons 9.5 hours after hypoxia. At that time point, the differences between the groups were not visible on HE stainings [30]. Furthermore, Hill detected an increased intranuclear gelatinolytic activity immediately after reoxygenation in a primary culture of cortical neurons after oxygen and glucose deprivation [16]. In the same study, they treated rats with an inhibitor of MMPs before they were subjected to occlusion of the middle cerebral artery. The rats exposed to the MMP inhibitor displayed significantly less apoptosis than the control group. These observations may indicate that the increased intranuclear gelatinolytic activity in neurons, such as Purkinje cells, could be an early marker of future neuronal degeneration.

Regarding the gene expression of NLRP3, IL-1β, and IL18, there were no significant changes between the groups. These results could be in line with previous findings by our group exhibiting that for rats exposed to hypoxia and sham-operated rats, the mRNA expression of these components were similar in some cerebral subregions, including the cortex and the subventricular zone, at 24 hours after hypoxia [31]. Therefore, it is not surprising that no variability between the groups was revealed at one specific time point in our study. Further studies should be conducted on the time dependency of the compounds of the NLRP3 inflammasome pathway.

In addition, investigations did not reveal any significant changes between the groups in the levels of IL-1β as early as 9.5 hours after hypoxia, which stands in contrast with another report, showing that cerebellar IL-1β concentrations were significantly changed for all time points between 3 hours and 7 days in neonatal rats subjected to hypoxia [32]. The differences between these studies could be due to different methodology or simply reflect specific reactions to hypoxic injury across animal species.
An increased production of ROS may cause mutations in the mtDNA leading to a critical effect on the activity of the mitochondrial electron transport chain, which subsequently may lead to mitochondrial dysfunction, apoptosis/necrosis, and diseases [33]. Wang et al. showed that damage to mtDNA may lead to diminished mitochondrial bioenergetics and hamper the maturation of neural stem cells [34]. We speculate that our results consisting of a significant reduced mutation rate of mtDNA in pigs subjected to NACA after hypoxia may be associated with a better neurological outcome, which stands in line with the findings of Patel et al. who demonstrated that NACA preserved mitochondrial bioenergetics and improved functional recovery after inflicted spinal trauma [35]. The lack of significant difference between the sham-operated and vehicle-treated groups could be due to the limited number of pigs included in the sham group (n = 5).

Our findings suggest that NACA could reduce the mitochondrial damage and thereby have a positive influence on the energy metabolism of neural cells. Histopathological analyses of the cerebellum revealed no significant differences between the intervention groups but a significant difference between the sham and the intervention groups. The limited signs of cell death observed in some of the sham pigs may be due to possible harmful effects of anesthesia on the brains of newborns, as described in other publications [36–38]. Moreover, all pigs underwent surgical procedures which could be potentially harmful. On the other hand, some anesthetics may have neuroprotective features and a recent study by Liu et al. demonstrated that midazolam may protect against neuroapoptosis induced by physiological and oxidative stress [39]. The anesthetic regime was similar for each pig, and therefore, these effects should be consistent across all animals.

After severe perinatal hypoxia, a certain degree of cell death will occur, during and immediately after the hypoxic challenge. Between 6 and 24 hours later, a phase of secondary energy failure may evolve with declines in phosphocreatinine and ATP, impaired mitochondrial function, and further neuronal cell death. Many animals in our study have probably not reached the phase of secondary energy failure, and there are few signs of cell death visible on histological sections 9.5 hours after hypoxia. At this time point, however, the mechanistic measures of injury may differ between animals exposed to NACA and those who were vehicle treated. We suggest that if we had run the trials for an extended period of time, we would have seen a difference in histopathology between the two intervention groups.

7. Conclusion

Generally, pigs exposed to NACA after hypoxia revealed a tendency to reduced gelatinolytic activity in cerebellar Purkinje cells, measured with in situ zymography and a significant reduction of the mutation rate of mtDNA in the cerebellum. We therefore speculate that NACA may have neuroprotective capabilities after perinatal asphyxia. Our observations are in line with our previous study using the same group of animals, where we described possible anti-inflammatory effects of NACA in the cortex of neonatal pigs [6]. However, more studies are needed before NACA could be considered useful in a neonatal clinical setting.

Finally, our results indicate that using in situ zymography in the investigation of Purkinje cells could be a valuable biomarker to compare different neuroprotective substances in hypoxia-reoxygenation models.

8. Limitations of the Study

The mutations of mtDNA were only measured at one time point. Gel zymography of MMPs was not conducted; however, we assume that evaluating the net gelatinolytic activity reflects the actual proteolytic activity better than measuring the activity of one specific MMP. Although animal experiments have largely contributed to our understanding of human pathophysiology, we should be cautious when translating the results from animals to humans. We postulate that the differences between the groups could have been larger if the animals had been observed over a longer time period. Also, we are aware that the inclusion of both genders in a small study could possibly attenuate observable differences.

Other points of concern are that the number of animals in each group was relatively small and the study time was limited to 9.5 hours, so there was no long-term follow-up. Furthermore, electrophysiological surveillance of the pigs with EEG while anesthetized could have provided us with valuable information; however, this was not performed in this trial.

Prior to this investigation, the interindividual distribution of the proteolytic activity and rate of mutations in mitochondrial DNA following asphyxia were unknown. Thus, a proper power calculation to determine the adequate sample size could not be performed. In retrospect, the small sample size is troublesome and limits the statistical analysis.

Ethical Approval

The Norwegian Council for Animal Research approved the experimental protocol (approval number 4630). The animals were cared for and handled in accordance with the European Guidelines for the Use of Experimental Animals by researchers who have been certified by the Federation of European Laboratory Animals Science Association (FELASA).

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The NACA used was a kind gift of Dr. Glenn Goldstein, New York, NY, USA. The authors thank Ms. Eliane Charrat for her technical support and Professor Sandvik for the advice on statistical analysis. The authors are also grateful for the assistance from Vivi Stubberud, Aurora Pamlona, Sera Sebastian, Leonid Pankratov, Bjørn Petter Benterud, Grethe

Disease Markers
Dyrhaug, Monica Atneosen-Åsegg, and Ashley Kim. The study was funded by Helse-Sør Øst (South and Eastern Norway Regional Health Authority; source number 6051; Project no. 39570).

Supplementary Materials

Cerebellum susceptibility to neonatal asphyxia: possible protective effects of N-acetylcysteine amide (NACA).

References


Supplementary Materials: Cerebellum susceptibility to neonatal asphyxia: Possible protective effects of N-Acetylcysteine Amide (NACA)

Extraction of mtDNA from cerebellum
All extractions were performed using the Qiagen kit (DNeasy® Blood and Tissue Kit, Hilden, Germany), following the instruction by the producers. Briefly, lysis of the samples was performed by the addition of 180 μl buffer ATL and 20 μl proteinase K and incubation over night at 56°C. Thereafter, 200 μl 100% ethanol was added, the mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube, and centrifuged at 8000 rpm for 1 minute. The spin column was placed into a new 2 ml collection-tube and processed in two separate wash steps with buffers AW1 and AW2. Finally the DNA was eluted by adding 200 μl buffer AE to the center of the spin column membrane and centrifuged for 1 minute at 8000 rpm. Concentration and purity of DNA was assessed by NanoDrop ND 100 (Life Science, USA) and diluted to a final concentration of 6 ng/μl and 15 ng/μl DNA.

The Random Mutation Capture (RMC) method to estimate mtDNA damage
The RMC method is a technique to estimate the mutation rate of mtDNA in a sample, based on a PCR-reaction combined with restriction enzyme digestion [1]. Basically, primers for a PCR-reaction cover a sequence containing a restriction enzyme target site, e.g. TCGA for TaqI. A restriction enzyme digestion of the DNA-template prior to the PCR reaction will cut the wild-type template preventing a PCR product, but a mutation in the target site will lead to a PCR-reaction product. The ratio between the amount of wild-type (WT) (cut) and mutation (uncut) product can be estimated, if the total amount of template is determined by another PCR-reaction product with primers covering a close-by sequence excluding the restriction enzyme cutting site for the respective restriction enzyme (figure 1). The product of a wild-type (WT) and the mutant template will result in different ct-value signals in a qRT-PCR reaction, the mutant (uncutted) product will display a higher ct-value than the WT (cutted). The mutation rate is calculated with ΔΔct method.
**mtDNA Restriction Enzyme test**

Primers were designed using the primer 3 plus program and the efficiency tested by a 10-fold qRT-PCR dilution reaction using the pet101 plasmid (Invitrogen, Paisley, UK) and cerebellum samples. The correct fragment size was confirmed and the mtDNA samples were digested using 1U restriction enzyme *TaqI* (Invitrogen, Paisley, UK) with smart cut buffer and nuclease free water in a total volume of 30 μl. The restriction digestion was performed at 65°C for 15 minutes followed by inactivation of the enzyme for 10 minutes at 95°C.

The primer sequences for mtDNA 12S were: Forward primer (5’-3’): CGCAACTGCTAAACTCAA, Reverse primer (5’-3’): TAGCCCATTTCTTTCCAACC

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**Analyses of mtDNA damage**

The mastermix for one reaction contained 6x SYBR green, 0.5 μM forward- and reverse primer and nuclease free water to a final volume of 20 μl. To mastermix with target 12S gene, 1U *TaqI* enzyme was added. The digestion step was performed at 15 minutes at 65°C for enzyme digest and 10 minutes at 95°C for inactivation.
The product was analyzed with qRT-PCR. DNA mutation frequency was calculated using $\Delta CT$ method following, $2^{\exp(- (c_{\text{Targ}} - c_{\text{ctrl}}))}$, where $c_{\text{Targ}}$ and $c_{\text{ctrl}}$ represent CT values of enzyme-treated and non-treated DNA.

**Figure 2**

The figure display the concentrations of IL-1β in each group

Reference: