

From DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH
Karolinska Institutet, Stockholm, Sweden

**ON NEONATAL ASPHYXIA:
Clinical and Animal Studies Including Development
of a Simple, Safe Method for Therapeutic Hypothermia
With Global Applicability**

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Institutet**

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Cover: The world we live in. The Iceberg or “PCM” symbolizing our limited knowledge about what hypothermia does to the human body, the smart piglets of my experiments, and the polar bear (me) swimming towards a safer rock of knowledge.

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*This book is dedicated to:
all children suffering from brain disabilities,
all entrepreneurs with great ideas, helping the world,
but most of all to my friends and loved ones.*

ABSTRACT

Recent randomized clinical trials show that hypothermia can decrease brain dysfunction in newborn infants at risk for hypoxic-ischemic encephalopathy. One goal of the present study was to develop an alternative to current relatively complex and expensive cooling methods dependent on electricity and continuous water supply. An effective and cheap cooling method for global implementation both during transportation and in hospitals based on Phase Changing Material (PCM) was developed. It was found that a specific Glauber salt composition fulfilled safety, cooling and easy of handling criteria and the material was tested in piglets and newborn babies with results comparable to those with conventional cooling. A second goal was to evaluate near red infrared spectroscopy (NIRS) for non-invasive in vivo monitoring of cortical vascular haemodynamic responses to sensory stimuli. NIRS revealed that infants respond more strongly to their mothers' faces than to that of strangers. Preliminary results suggest NIRS may become a useful method for monitoring effects of hypoxic ischemia and its treatment by cooling. When newborn infants at risk are born outside a hospital with cooling facilities, cooling during transport may be beneficial. We found that passive induction of hypothermia during transport is possible, although temperatures of the infants will vary depending on climate and other circumstances, and that such passive measures can lead to unintended excessive cooling necessitating careful monitoring of body temperature. The PCM cooling material was tested as an alternative to water bottle cooling in a piglet hypoxic ischemia model and found to be effective and possibly leading to a more stable target temperature. To better understand how hypoxic ischemia affects different brain areas, brains from piglets subjected to standardized hypoxic ischemia and treatment protocols consisting of cooling, xenon or a combination thereof were analysed with respect to transcriptional activity of key genes, using quantitative in situ hybridization. Analysing mRNA species coding for BDNF, MANF, HSP70, GFAP, NgR, MAP2, LDH-A and LDH-B revealed marked effects of the hypoxic ischemic insult, partial counteraction of mRNA alterations by the treatments and differences between brain areas, as well as possibly between core and mantle regions. In a separate set of animals, different cooling temperatures were compared with respect to the activity of the same set of genes. Cooling to 33°C appeared to be advantageous, while cooling to a rectal temperature of 30°C appeared to be associated with some unwanted effects. It is concluded that cooling can be better controlled and at the same time more easily be made globally available using PCM material, and that cooling partially counteracts some, but not all changes of a selected set of brain mRNA species observed 2-3 days after hypoxic ischemia in a piglet model.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. Carlsson J, Lagercrantz H, Olson L, Printz G, Bartocci M. Activation of the right fronto-temporal cortex during maternal facial recognition in young infants. *Acta Paediatrica* 97, 1221-1225, 2008
- II. Hallberg B, Olson L, Bartocci M, Edqvist I, Blennow M. Passive induction of hypothermia during transport of asphyxiated infants: a risk of excessive cooling. *Acta Paediatrica* 98, 942-946, 2009
- III. Olson L, Lothian L, Åden U, Lagercrantz H, Martin V, Settervall F. Phase change Glauber salt solutions for medical applications. *Applied Energy* (in revision)
- IV. Iwata S, Iwata O, Olson L, Kapetanakis A, Kato T, Evans S, Araki Y, Kakuma T, Matsuishi T, Setterwall F, Lagercrantz H, Robertson NJ. Therapeutic hypothermia can be induced and maintained using either commercial water bottles or a “phase changing material” mattress in a newborn piglet model. *Arch. Dis. Child.* 94, 387-391, 2009
- V. Olson L, Faulkner S, Lundströmer K, Chandrasekaran M, Kato T, Ådén U, Settervall F, Raivich G, Lagercrantz H, Olson L, Robertson NJ, Galter D. Hypoxic ischemia-induced alterations of eight key genes in regions of the newborn piglet brain; partial normalization by hypothermia and/or xenon (manuscript)
- VI. Olson L, Faulkner S, Lundströmer K, Kerenyi A, Kelen D, Chandrasekaran M, Ådén U, Olson L, Golay X, Lagercrantz H, Robertson NJ, Galter D. Comparisons of three hypothermic target temperatures for the treatment of hypoxic ischemia: mRNA level responses of eight genes in the piglet brain (manuscript)

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LIST OF ABBREVIATIONS

aEEG	amplitude-integrated electro-encephalogram
ARC	activity-regulated cytoskeletal-associated protein
BDNF	brain derived neurotrophic factor
Bp	base pairs
ctx	cortex cerebri
CBF	cerebral blood flow
CBV	cerebral blood volume
CMC	cethyl methyl cellulose
Cnx43	connexin 43
E	embryonic day
FiO ₂	fraction of inspired oxygen
F _{TOE}	fractional tissue oxygen extraction
Gap43	growth-associated protein 43
GFAP	glial fibrillary acidic protein
HbO ₂	oxygenated haemoglobin
H_{fs}	heat of the fusion
HHb	deoxygenated haemoglobin
HI	hypoxic ischemia
HIF	hypoxia-induced factor
HSP 70	heat shock protein 70
KI	Karolinska Institutet
LDHA, B	lactate dehydrogenase A, B
LWP	large white pig
MAP2	microtubule-associated protein 2
MANF	mesencephalic astrocyte-derived neurotrophic factor
MR	magnetic resonance
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
Na ₂ SO ₄	sodium sulfate
NICU	neonatal intensive care unit
NIDCAP	newborn individualized development care protocol
NIRS	near infrared spectroscopy
NMDA	<i>n</i> -metyl- <i>d</i> -asparate
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NgR	nogo-66 receptor
NOGO	neurite outgrowth protein A
NSE	neuron specific enolase
P	postnatal day
PCM	phase change material
<i>PCr/Pi</i>	ratio of phosphocreatine to inorganic ortophosphate
Q_{lat}	sensible and latent heat stored
ROP	retinopathy of prematurity
rSO ₂	regional oxygen saturation
SaO ₂	saturation of oxygen

TH	tyrosine hydroxylase
T_c	charging temperature given by the heat source
T_{ls}	phase change temperature
TOBY	total body hypothermia
TOI	tissue oxygenation index
T_{rectal}	rectal temperature
TrkB	tropomyosin related kinase B
UCL	University College of London (In most cases this refers to our collaborators in the Robertson research group)
VAchT	vesicular acetylcholine transporter
∇T	reachable temperature difference
Xe	xenon

1 EXTENDED SUMMARY

Being born is a stressful event (Lagercrantz & Slotkin 1986, Taeusch et al 2005). While such stress is natural and regarded as important for transition to extra-uterine life and initiation of breathing, a major risk of being born is oxygen deprivation, which may cause brain damage leading to early death or life-long functional impairments. Researchers therefore seek ways to identify babies at risk for hypoxic ischemia and strategies to minimize the consequences thereof.

In Sweden, an estimated 200 children are born each year with hypoxic ischemic asphyxia or oxygen deprivation during delivery of a severity necessitating treatment, to reduce future handicap. In the developing countries the problem is even more common. Not only the brain, but also other organs, such as the heart, liver or kidney can be damaged by an episode of hypoxic ischemia.

Early experimental support for the hypothesis (Westin et al 1959) that hypothermia would be beneficial in hypoxic ischemia was provided among others by Thoresen et al (Laptook & Corbett 2002, Laptook et al 1999, Thoresen 1999). Results from current randomized clinical trials (Azzopardi et al 2008, Edwards & Azzopardi 2006, Gluckman et al 2005, Jacobs et al 2007b, Jacobs et al 2011, Shankaran et al 2005) suggest that treatment with mild or moderate hypothermia of newborn infants born with hypoxic-ischemic encephalopathy is helpful. The patients appear less handicapped at 18 months of age. Adverse effects of cooling do not appear to be serious if only a mild therapeutic cooling is applied and temperature kept stable.

In adults stroke is the most common cause of acute brain damage. Stroke is the third most common cause of death and the most common cause of handicap in adults in the western world, with 30.000 new cases/year in Sweden (Wahlgren 2011). The time between stroke and hospital care is extremely important, with every minute counting. Recent research suggests that cooling may have beneficial effects also in this condition when initiated during the pre-treatment/transportation phase to the hospital (Baldwin et al 2010, Den Hertog et al 2009, Uren et al 2009).

The rationale of therapeutic cooling of the brain, is that lower temperature will allow time to metabolize potentially detrimental compounds without triggering apoptotic events or other harmful processes and thus reach homeostasis before returning to normal body temperature. To achieve cooling, current practice is based on methods requiring electricity and clean circulating water. These methods are also relatively expensive. There is therefore a need for a method providing effective and safe cooling to a defined temperature, which is inexpensive and easy to use also in remote areas.

One focus of this thesis has been to develop and test a phase change material (PCM) composition suitable for the induction and maintenance of therapeutic hypothermic treatment of neonatal asphyxia and possibly other conditions threatening brain tissue integrity. This work benefitted from access to current clinical protocols and techniques and the author's participation in clinical trials of hypothermia as treatment for neonatal ischemic hypoxia. Collaboration with Dr. Nicola Robertson's group at University College

in London, has been the basis of a second focus, the elucidation of gene activity alterations in the brains of newborn piglets subjected to transient hypoxic ischemia, and the degree to which such alterations can be modified by cooling to different temperatures, xenon or a combination of cooling and xenon.

PCM offers an alternative to conventional cooling without the need for electricity or water while providing several additional benefits. PCM's are chemical mixtures able to take up large amounts of energy before changing temperature and phase and therefore suitable for cooling anything that is put next to it. An added benefit would be that no temperature undershoot can occur while cooling with a PCM material. One candidate PCM material for medical applications is Glauber salt.

A specific Glauber salt composition was found to fulfil safety, cooling and ease of handling criteria and the material was tested in piglets and newborn babies with results comparable to those with conventional cooling.

Close collaborations with experts in Sweden in the fields of material physics, a leading experimental lab in England using a piglet model, as well as the neonatal and stroke wards in Stockholm, has allowed work to develop and evaluate PCM-based cooling mattresses both physically, experimentally and clinically. A specific advantage of a PCM solution is the possibility for global implementation of a safe and simple, reusable, environmentally safe cooling method.

Near infrared spectroscopy (NIRS) was evaluated as a non-invasive in vivo method to monitor cortical vascular haemodynamic responses to sensory stimuli. The method could demonstrate that infants respond more strongly to their mothers' faces than to that of strangers. Preliminary results suggest NIRS may also become a useful method for monitoring effects of hypoxic ischemia and its treatment by cooling as described later.

When newborn infants at risk are born outside a hospital with cooling facilities, cooling during transport may be beneficial. We found that passive induction of hypothermia during transport is possible, although temperatures of the infants will vary depending on climate and other circumstances, and that such passive measures therefore can lead to unintended excessive cooling, necessitating careful monitoring of body temperature.

The PCM cooling material was tested as an alternative to water bottle cooling in a piglet hypoxic ischemia model and found to be effective and possibly leading to a more stable target temperature.

To better understand how hypoxic ischemia affects different brain areas, brains from piglets subjected to standardized hypoxic ischemia and treatment protocols consisting of cooling, xenon or a combination thereof were analysed with respect to transcriptional activity of key genes, using quantitative in situ hybridization. Analysing mRNA species coding for BDNF, MANF, HSP70, GFAP, NgR, MAP2, LDH-A and LDH-B revealed marked effects of the hypoxic ischemic insult, partial counteraction of mRNA alterations

by the treatments and differences between brain areas, as well as possibly between core and mantle regions.

In a separate set of animals, different cooling temperatures were compared with respect to the activity of the same set of genes. Cooling to 33°C appeared to be advantageous, while cooling to a rectal temperature of 30°C appeared to be associated with some unwanted effects.

It was concluded that cooling can be better controlled and at the same time more easily be made globally available using PCM material, and that cooling partially counteracts some, but not all changes of brain mRNA observed 2-3 days after hypoxic ischemia in a piglet model.

2 INTRODUCTION

2.1 GENERAL BACKGROUND

Neonatal hypoxic ischemic encephalopathy in term infants constitutes a serious health problem, not the least due to its often life-long consequences. An estimated 3-5 of every 1000 live term births are affected, a quarter of which with severe symptoms; 10-30% of the affected children do not survive, 30% suffer life-long disabilities (Badr Zahr & Purdy 2006, Edwards 2009, Ellis et al 2000, Johnston et al 2011b, Kumar et al 2009, Pfister & Soll 2010, Savman & Brown 2010, Thayyil et al 2009, Wachtel & Hendricks-Munoz 2011). The incidence may be 10-fold higher in the developing world. In Sweden, an estimated 200 children are born each year with hypoxic ischemic asphyxia or oxygen deprivation during delivery of a severity necessitating treatment, in order to reduce future handicap. Not only the brain, but also other organs, such as the heart, liver or kidney can be damaged by an episode of hypoxic ischemia.

Hypothermia as treatment to prevent damage caused by hypoxic ischemia is in a transition phase from preclinical to clinical use (Hayden 2010), with an increasing number of hospitals adopting cooling protocols for treatment of HIE. To optimize treatment rapid identification of newborns in need of treatment is necessary. Screening of newborns with fNIRS (Kusaka et al 2004a, Wilcox et al 2005) and aEEG (Hellstrom-Westas 1992, Hellstrom-Westas 2005, Hellstrom-Westas et al 1995) could detect small hidden seizures by monitoring brain activity with simple tools, and thus minimize the risk of missing children that should undergo treatment.

Current independent randomized clinical trials provide evidence that mild hypothermic treatment of newborn infants at risk for hypoxic-ischemic encephalopathy is clinically valuable (Azzopardi et al 2008, Edwards et al 2010, Gluckman et al 2005, Jacobs et al 2007b, Perlman 2006b, Pfister & Soll 2010, Shankaran et al 2005). To the extent that these and other on-going trials are based on electricity and/or continuous water supply, facilities that are not globally available, there is a need for alternative techniques, which could be implemented both during transportation and in hospital care, irrespective of electricity and water facilities.

In this thesis, the development of a novel cooling method using Phase Changing Material (PCM) is described. PCM requires no power or specific maintenance to be effective. Our studies have led to a PCM-based cooling mattress, that we have tested as a cooling aid in a piglet brain ischemia model to generate proof-of-concept (Paper III). Based on the results we have obtained, and complementary studies by other research groups, we feel there is enough evidence for a future clinical trial of cooling during transport and as a cooling method for newborn infants in the developing world, where safe and simple methods for mild cooling of neonates are needed.

Organisation of the group is shown below:

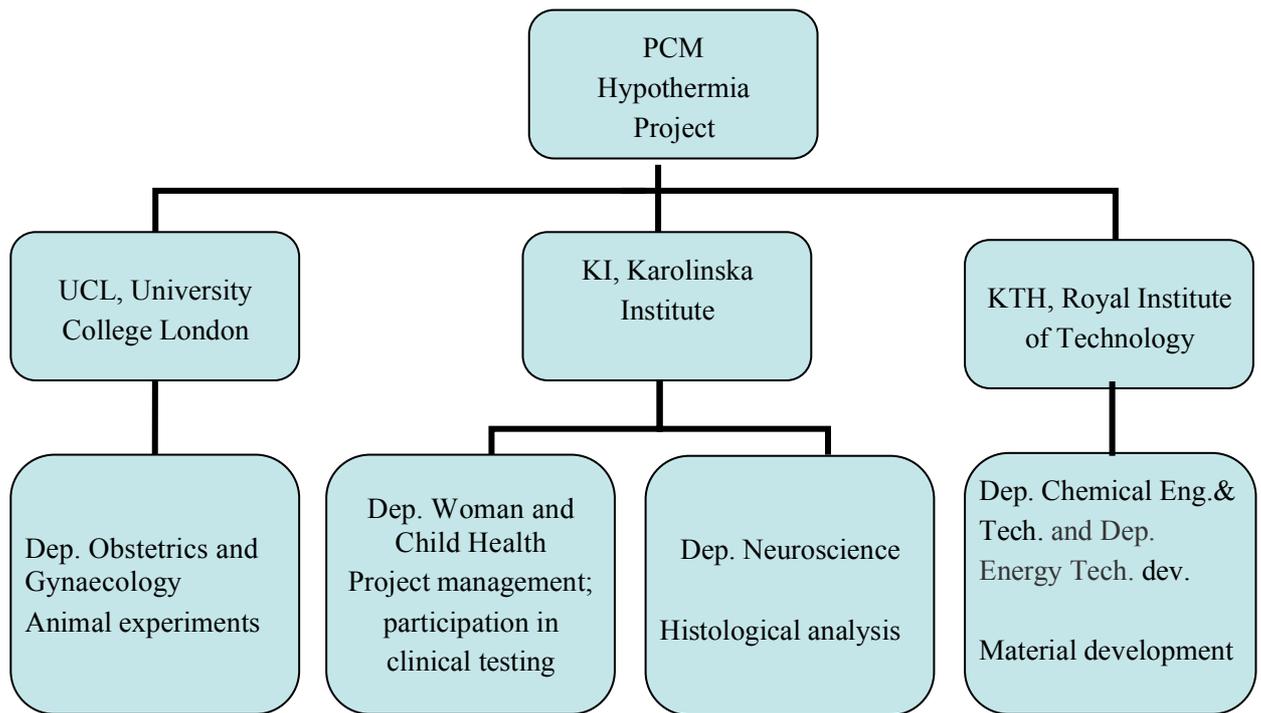


Figure 1: The project has been structured in different modules to facilitate development and testing of PCM material and to learn more about how the transcriptional activity of several key brain genes are influenced by HI and its treatment in a well controlled piglet model.

2.2 PERINATAL ASPHYXIA

The medical condition Perinatal or Neonatal asphyxia is the result of deprivation of oxygen to a newborn infant for a time long enough for the brain to become affected in a physically harmful way. The condition can be caused in several different ways. During delivery, blood flow to the infant's brain can become compromised, e.g. if the umbilical cord is too tight around the infant's neck, if there is a decrease in maternal blood pressure, or when in transfer from the anoxic environment of the womb to an environment where breathing is necessary. While hypoxia can damage most of the infant's organs (heart, lungs, liver, gut, kidneys), the focus of this thesis is brain damage.

In most cases, there is no or only a small amount of brain damage, but in severe cases, infants who survive suffer physical and/or mental symptoms such as developmental delay, intellectual disability, spasticity and other physical impairments. In most forms of cerebral palsy, asphyxiation during the birth process is a major causative factor. As mentioned above, about 200 children born at term every year in Sweden, need treatment, to reduce future handicap. In Sweden, the Levene classification system (Levene et al 1986) is used for grading level of HIE as mild (I), moderate (II) or severe (III), based on the scoring proposed by Sarnat and Sarnat (Sarnat & Sarnat 1976). For our hypothermia treatments we first targeted level I and II as described by Hallberg (Hallberg 2010).

Despite major advances in monitoring technology and knowledge of foetal and neonatal pathologies, perinatal asphyxia, causing hypoxic-ischemic encephalopathy (HIE), remains a serious condition associated with significant mortality and long-term morbidity.

2.2.1 A global view

Statistics from UN and different countries show that the incidence of HIE is high in countries with limited resources, although exact figures are hard to come by, since most births out of hospitals tend not to enter official statistics. A qualified guess is that birth asphyxia is the cause of some 23% of all neonatal deaths worldwide. This makes HIE one of the top causes of death and disability factors in people worldwide. Birth asphyxia is estimated to account for nearly two million neonatal deaths or stillbirths every year. Another million infants survive birth but will suffer from cerebral palsy, mental retardation, learning difficulties, and other disabilities. Incidence and causes may vary also within regions (Badr Zahr & Purdy 2006). Nepal was noted for more than average risk factors (Ellis et al 2000) and in the sixties there were similar problems in northern Europe pointed out then by Gandy et al (Gandy et al 1964a, Gandy et al 1964b) and portable equipment was suggested.

A specific problem in developing countries and rural areas is the lack of, or limited access to electricity, water and medical equipment. As pointed out elsewhere, we aimed to develop and test novel simple and safe cooling methods, based on PCM that could also be implemented under such conditions.

2.2.2 Characteristics of perinatal asphyxia

Prediction is essential for children affected by perinatal asphyxia for several reasons. Information about the timing of the injury, its estimated location and the extent of damage are needed to predict outcome and inform the parents. Such information is also needed to inform about available treatment options and prognosis with respect to infant health after such treatment. Timing of the hypoxic ischemic event is also vital for the planning of when and how hypothermic and other neuroprotective treatments should be initiated (Cowan et al 2003), or if damage is so severe that treatment should not be initiated. Coupled to the difficult decision of withdrawal of treatment, are a number of additional factors included in the global perspective. The typical course of untreated perinatal asphyxia or HIE includes an initial phase of intra-cerebral energy depletion followed by a phase of secondary energy failure about six to fifteen hours later, when ATP is depleted and lactate alterations are found (Azzopardi et al 1989a, Azzopardi et al 1989b). This secondary energy failure is thought to play a major role for the adverse outcome and ensuing permanent brain injury. At the cellular level, the biochemical disturbances of brain homeostasis include increased amounts of free radicals and nitric oxide which may cause oxidative damage, severe mitochondrial dysfunction, which, in addition to severe disturbance of energy homeostasis may initiate apoptosis, high intracellular calcium levels, inflammatory responses, cell swelling and oedema (Edwards et al 1997, Edwards et al 1995, Hagberg et al 1987, Kjellmer et al 1989, Mehmet et al 1994b, Mehmet et al

1994c, Miller 1991, Moore et al 2011, Ross et al 2010). Animal models based on sheep and piglets, with brains more similar to the human brain than those of rodents, have been instrumental for the understanding of the complex alterations of brain tissue caused by HI (Gunn et al 1997, Gunn et al 1998, Lorek et al 1994, Taylor et al 1999, Thiringer et al 1987, Thoresen et al 1995, Thoresen et al 1997).

Most infants suffering from perinatal asphyxia or hypoxia have different degrees of cyanosis (visible as a bluish colour of the lips), poor skin colour, affected muscle tone, impaired blood circulation, low responsiveness to sensory stimuli, a low Apgar score (Apgar 1953) at 5 min and respiratory distress. If the infant suffers very severe or prolonged asphyxia, cardiac arrest and death may follow unless resuscitation is started.

2.2.3 Selected HIE facts and complications

Facts

Age	By definition the newborn period of term infants. Preterm infants can also suffer from HIE, but the pathology and clinical manifestations are different.
Ethnicity	No predilection has been observed.
Gender	No marked predilection is observed. Morbidity slightly higher in boys.
Morbidity	Dependent on severeness, but as much as 25-50% in severely affected groups within the first week, due to multiple organ failures.

Complications*

Mental development index (MDI)	Functional impairments of mental abilities are common.
Psychomotor development index (PDI)	In more severe HIE common; in minor HIE less common.
Disabling cerebral palsy	Some reports suggest about every third case.
Epilepsy	From our clinical experience, about every sixth have seizures.
Blindness	Due to ROP and similar problems, partial or complete blindness may occur.
Severe hearing impairment	From our clinical experience \approx one out of 20.

*The incidence of long-term complications depends on the severity of hypoxic-ischemic encephalopathy.

2.2.4 Secondary energy failure

A HI insult leads to a series of harmful events. During the initial stage nerve cells and other cells are affected by energy depletion. A high consumption of glucose and anaerobic metabolism leads to loss of energy, accumulation of metabolites, depolarization and Ca^{2+} influx, release of glutamate and other excitatory amino acids and cytotoxic oedema. The production of lactate in the early phase together with depletions of energy storage causes metabolic acidosis. Timely resuscitation leads to a period when cells may recover such that metabolism and nerve cell function is restored. More severe HI will also cause the secondary energy failure, including increased production of free radicals (Kjellmer et al 1989), increasing calcium levels (Miller 1991, Siesjo et al 1995), the release of nitric oxide (Dawson et al 1993), mitochondrial dysfunction (Blumberg et al 1996, Robertson et al 2009, Siesjo et al 1999), activation of apoptotic pathways (Edwards et al 1997, Mehmet et al 1994a) and inflammatory responses (Bona et al 1998). This stage starts about 2-6 hours after the first phase and can continue for days. Seizures are often found after 8-24 hours. Common to all interventions aiming to protect the brain is to stop or dampen this secondary energy failure phase. Detailed discussions of these issues have been published (Hallberg 2010, Lorek et al 1994, Thoresen et al 1995).

2.2.5 Size of injury

The anatomical location and size of injury caused by perinatal asphyxia varies between individuals, although many features are common to those affected (Berger & Garnier 1999, Berger & Garnier 2000). Animal studies suggest that size of subject and injury is of importance in order to decide which form of cooling to be used (Iwata et al 2006). The individual injury and the type of injury as well as size can be studied by MRI (Rutherford et al 2006). MRI scans can be obtained during the first hours after birth, but if the person examining such scans is not a trained paediatric radiologist, mistakes may occur not the least because the neuronal injury in hypoxic-ischemic encephalopathy is an evolving process, while the brain is also undergoing major programmed developmental changes. The magnitude of the final outcome/damage depends on duration and severity of the initial insult as well as the degree of reperfusion injury, including the triggering of apoptotic events. Biochemically, HIE is the result of a cascade of events. After a therapeutic hypothermia treatment period, as well as 10 days later, the Swedish hypothermia treatment protocol, like that of many other countries, calls for MRI exams to verify injury and as a prognostic aid. The protocol further suggests that a follow up at 18 months should also include a MRI study. Teenagers, who suffered HIE as newborns before the introduction of therapeutic hypothermia have also been studied (Nagy et al 2005). While cerebral palsy cannot be predicted by imaging alone (de Vries et al 2011), it can be useful to verify injury and its treatment by hypothermia (Hagmann et al 2011).

MRI can also be used to deduce local brain temperatures (Kozak et al 2010). If this technology can be further developed, it might help in attempts to achieve regionally differentiated cooling of different brain areas. Since neonatal HI is a risk factor for cerebral palsy and early neonatal death, individuals should be followed up at 18 months

to examine white matter, central grey matter and the brain stem. Infants with only cortical or white matter injury or only grey matter injury must not be excluded, since these different forms of injury can all lead to dysfunction that cannot be compensated for despite the neonatal brain's remarkable plasticity and ability to compensate for lost functions.

A general problem connected to magnitude of damage is severity of cognitive impairments. Depending on size and position of injury, symptoms can vary from mental retardation to deficits in attention, motor control and perception disorders. This can affect children that do not develop overt cerebral palsy, but have problems such as learning deficits, short-term and working memory deficits as well as motor control and social problems similar to those of ADHD (Klingberg et al 2005, Lindstrom et al 2006, Nagy et al 2005).

Other common attributes of HIE are focal cerebral infarction (Cowan et al 2003, Cowan et al 1994), still births (Edwards et al 1997), and several other forms of cerebral injuries of different degrees, see "The newborn brain"(Lagercrantz et al 2010). In this book, prominent researchers describe issues from how the brain is built to the difficult question of when and to which degree consciousness is present in the newborn as well as how the brain is effected when problems occur. Consciousness is also described by Lagercrantz and Changeux (Lagercrantz & Changeux 2009).

2.2.6 Amplitude-integrated EEG and neurophysiology

One way to diagnose perinatal asphyxia is aEEG. Examination of brain activity by this form of 2-4 channel EEG is a promising way to monitor activity during the first few days of life (Hellstrom-Westas et al 1995, Spitzmiller et al 2007, Tao & Mathur 2010, Toet et al 1999). The simplified recordings that the method provides can be readily understood by clinicians in the neonatal units and if the results call for a more complete EEG data set, a full EEG scan (Walsh et al 2011) can be carried out. In two of the clinical hypothermia trials (Azzopardi et al 2009, Gluckman et al 2005), aEEG was included as a criterion for starting hypothermia and to monitor such treatment with respect to brain cortical activity and the occurrence of seizures. Cortical activity is of course influenced by a number of different factors such as body temperature, ventilator volume and speed, pharmaceuticals etc. (Hellstrom-Westas 1992, Hellstrom-Westas et al 1995, Hellstrom-Westas et al 1992, Levy et al 2003).

2.3 PCM

A material with phase change properties can be a chemical element, a solution or a substance with high melting energy (Lázaro et al 2005, Mehling et al 2008). It melts/solidifies at a precise temperature and can store considerable amounts of energy (heat) before changing from one phase to another. We have used elements or solutions that change between solid and fluid phases within a narrow temperature interval. The most common use of PCM today, is for energy storage by changing between solid and fluid

phases. Phase changes that include other PCMs, high temperatures and/or gas phases are less useful in medical applications due to the need of either large volumes in a low pressure setting or smaller amounts in a high pressure setting, increasing the risk for mistakes or secondary injury of medical staff or patients.

PCMs can be divided into three groups: Organic (waxes, vegetabilic solutions, oils and extracts, sugar alcohols, polyethylene glycols), inorganic (salt solutions such as Glauber salt solutions (Marliacy et al 2000)), or eutectic ones (mixtures of inorganic, organic, or inorganic and organic compounds with a minimal melting point compared to concentration, often lower than 0°C) (Günther & al. 2009, Yinping & Yi 1999, Zalba et al 2004).

Comparing organic and inorganic PCMs, one notes that organic materials tends to expand when undergoing phase changes, be more fire instable, have a lower enthalpy (organic 100 – 240 kJ/kg; inorganic 150 – 400 kJ/kg), and also have a lower melting point. In terms of chemical stability, all-organic PCMs are to prefer, because they can be recycled between phases better and, most importantly, they have no undershoot while going between phases, which may happen with inorganic PCM if nothing is done about this matter (Mehling et al 2008).

Salt based inorganic PCM products such as hydrates, nitrates and carbonates have melting temperatures of 90-120°C, while in solutions together with water the temperatures can be modified across a wide range. A disadvantage with these salt solutions is often that they are corrosive and hygroscopic, leading to the need of being securely encapsulated. They also quite often display an undershoot at melting temperature which causes them to store/give away energy less effectively than organic PCMs that do not completely solidify at a given melting temperature (Cabeza & Mehling 2007, Eicker 2010, El-Sebaili et al 2009, G. Belton 1973, Kravvaritis et al 2010, Lázaro et al 2005, Medrano et al 2009, Sharma et al 2009).

2.3.1 PCM for additional medical applications

In addition to the specific PCM application discussed in this thesis, there are many other ways in which PCM can be used in the medical field. PCM blocks can be placed in containers for medicines, other drugs, or specimens from humans or animals that need to be transported within a defined temperature interval. The length of time that stable temperature conditions can be maintained depends on many factors, including amount and type of PCM, as well as ambient temperature. Air transports using PCM technology are in operation (WWW.aircontainer.com). PCM can also be placed into a specific liquid to cool it down or to restore temperature of e.g. blood. The optimal body temperature is 36.3-37.6°C. Our metabolic heat production is dependent upon physical activity and ambient temperature and about 1000W during "normal" working situations, 2000W for top athletes, and 100W when we are resting (Çengel 1998, Gagge & Hardy 1967). The body strives to cope with these variable heat production situations by regulating breathing, sweat production, shivering and by skin exposure to ambient air (evaporation, convection, radiation, conduction).

To help control body temperature, PCM can be microencapsulated into fibres woven into various fabrics (Mondal 2008, Shin et al 2005a, Shin et al 2005b, Sorrentino et al 2008). These materials can then be used for different textiles such as jackets or coats, or the top of a PCM mattress to maintain a comfortable temperature zone by dampening short-term ambient temperature fluctuations. For longer temperature variations a vest with macroencapsulated PCM in pouches may be used, e.g. to keep a surgeon or fire fighter in the comfort zone, enabling them to make better decisions.

PCM heating bags for hand warming purposes are probably the most well known. Such bags, however, needs triggering by breaking a plastic divider or snapping a piece of metal inside the bag allowing materials to mix and crystallize. Other suggested uses of PCM in medicine (not yet scientifically proven) include: to encapsulate PCM into shoe inlets, to incorporate PCM in a head bandana as a treatment of headaches, as well as cooling the back for other conditions. It remains to be seen if these applications of PCM are better than placebo.

Lately, there have been tests using PCM slurries for different medical purposes in order to obtain faster and better controlled cooling to a minimum temperature while avoiding temperature undershoot (Bang & Suslick 2010, Goehring et al 2006, Lampe & Becker 2007, Laven et al 2007). Another biological application is to use PCM as an interface between a laptop and a persons lap, to avoid recently reported skin problems like irritation and rashes caused by heat generated by laptops (Karlsson & Linde 2010). (<http://www.laptopcooler.se/>). Secondary advantages using PCM in this case is that the PCM will match the form of the knee while melting and also be completely silent, not disturbing the surroundings, compared to other cooling methods.

2.4 NEAR INFRARED SPECTROSCOPY

Near infrared spectroscopy is increasingly used to monitor the condition of the human brain, particularly in infants. Functional Near Infrared Spectroscopy (fNIRS) is based on neurovascular coupling in the brain and almost non-invasively monitors the difference between HbO₂ and HHb in real-time. The technique can be used to monitor blood flow in the brain both in cortical areas and in deeper brain structures. A light source (optod) transmits near infrared light into the tissue of the brain where the light becomes scattered in different ways and absorbed by oxyhaemoglobin, and deoxyhemoglobin. A different optode measures reflected light from a given brain area. Since the pioneering experiments of Meek and co-workers, fNIRS has been used for functional studies of visual function in infants (Kusaka et al 2004a, Kusaka et al 2004b, Meek et al 1998, Otsuka et al 2007, Taga et al 2003), and can be used to assess micro-vascular haemodynamic changes coupled to cortical activation in response to sensory stimulation (Bartocci 2006, Bartocci et al 2006, Obrig & Villringer 2003a, Obrig & Villringer 2003b, Obrig et al 2000, Zaramella et al 2001).

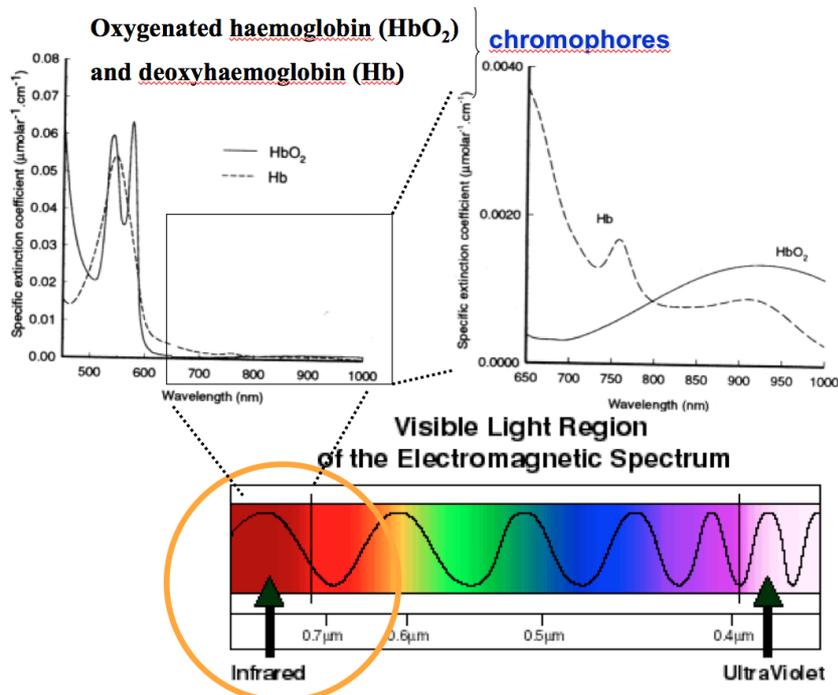


Figure 2: General principle of NIRS: The infrared light spectrum is located between 700 and 900 nm. Deoxy- and oxy- haemoglobin have different absorption modalities in this interval, which is used to monitor HbO₂ and Hb as well as cytochrome aa3. Composed from power point presentation by Marco Bartocci

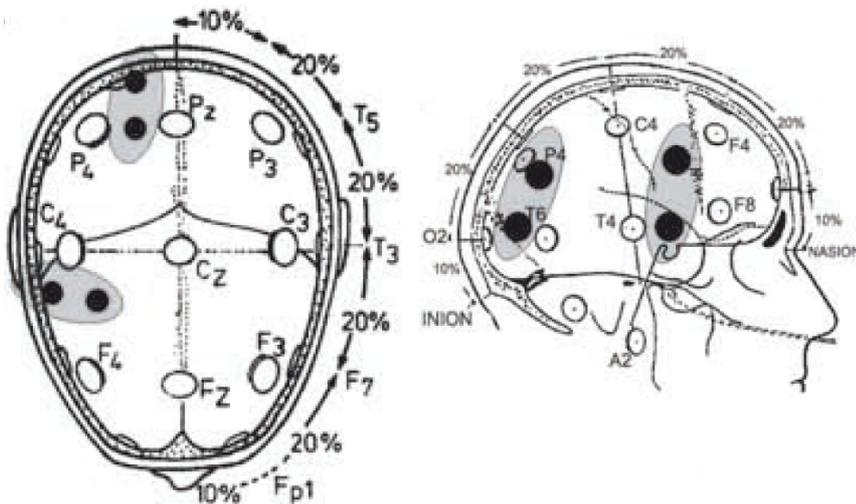


Figure 3: Schematic illustration of typical coordinates used for fNIRS recordings. Each recording site is based on an emitter of near infrared light and a detector which records reflected light. Courtesy of Dr. Marco Bartocci.

2.4.1 NIRS technique

We used a dual-channel NIRS device (NIRO 300, Hamamatsu Photonics, Hamamatsu, Japan) to record alterations of oxygenated [HbO₂] and deoxygenated haemoglobin [HHb]. The device has two channels, each consisting of a light transmitter and a receiver. The emitted light has a wavelength between 700 and 1000 nm. Sampling at 2 Hz was used to collect data via an RS-232 interface to a computer.

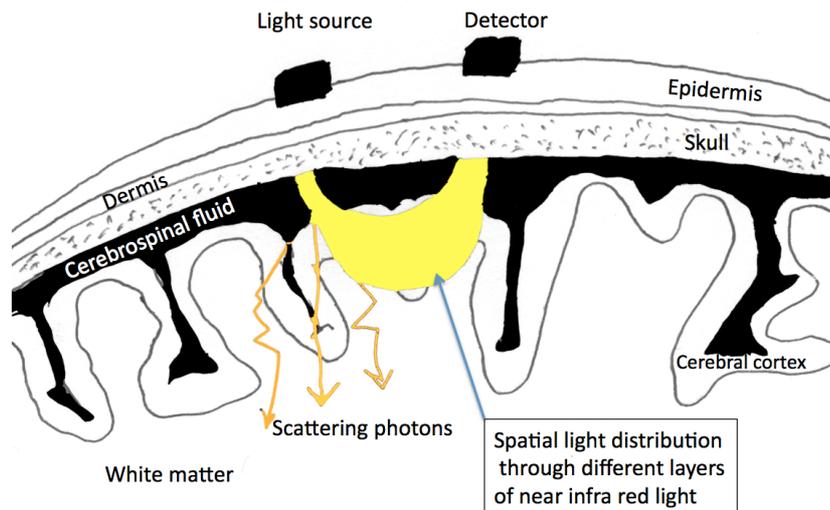


Figure 4: NIRS. A more detailed view, where emission to detection in tissue is described.

Key areas for face recognition are located in the right temporal and prefrontal cortex, which are target areas of the ventral stream. These areas should be functionally developed at 6-8 months of age in full-term born infants.

Previous studies of term and preterm children (Cady & Azzopardi 1989, Wyatt et al 1989), have shown that NIRS can be used to monitor activity in children with low versus full activity (Bartocci 2006). It was found that preterm infants run a considerable risk of developing cognitive impairments, including difficulties recognizing facial expressions or familiar faces. We therefore used NIRS in an attempt to quantify such problems as caused by HIE in term infants, because of the ease of operation. The infant could be in one parent's lap during the test, minimizing stress, and, unlike the case with certain other imaging modalities, NIRS allowed the child to move slightly during recording sessions. We found that term infants recognized their own mother while children born preterm had bigger problems differentiating between their own mother and other women.

Preliminary data have also been obtained with regard to the usefulness of NIRS for studies of children undergoing hypothermia treatment, see results section.

Already in the early sixties, Adamsons found that blood pH and temperature could have a connection, and that levels could be put into a diagram (Adamsons et al 1964). These and other results made it interesting to test NIRS as a method for examining the haemoglobin

in the hypothermic infant as a biomarker for of how well the hypothermic treatment is working (Abdul-Khaliq et al 2001, Gucuyener et al 2011). NIRS reveals two different patterns for asphyxia and hypoxic injuries, allowing us to analyse the effects of oxygenation and type of injury by studying the concentration of cerebral Hbtot, HHb and HbO₂ in term and preterm infants and the shifts in the time line curves for these concentrations. It was found that while hypoxia caused increased carotid-blood flow and cerebral blood volume and a fall in carotid vascular resistance, asphyxia did not cause a significant rise in CBV, a fall in carotid blood flow and a rise in carotid vascular resistance (Bennet et al 1998, Faris et al 1991, Livera et al 1991).

Limitations and assumptions when using NIRS.

When using NIRS, one assumes that tissues are optically homogeneous, that there are only three chromophores present at all times (HHb HbO and Cyt aa3), that the spatial distribution of all chromospheres is constant at all times, that the transmitting and receiving optodes are fixed in the most optimal way, that the extracerebral haemoglobin is stable (haemoglobin levels in the epidermis, dermis and skull, normally very stable in an infant) and that the extra light scattered in the tissue has constant properties, not inflicting with the measurements. For most studies saturation should also be recorded as close to the patient's brain as possible (often on the right arm). Although these assumptions cannot be expected to be maintained in a precise manner, NIRS results from groups of individuals and comparisons between groups can still be both reliable and valid.

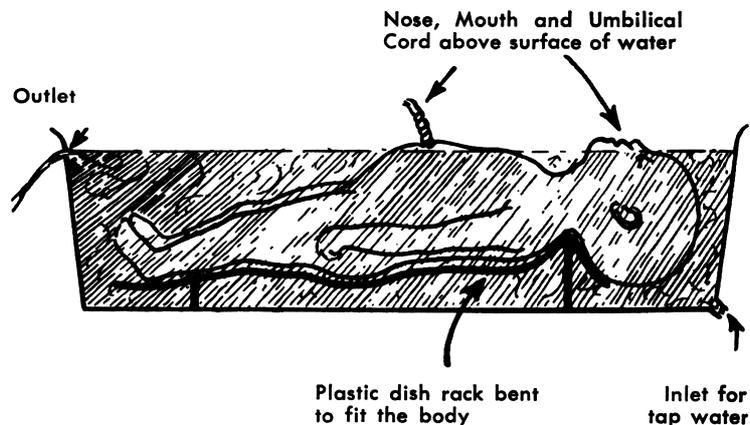
2.5 QUANTITATIVE RADIOACTIVE IN SITU HYBRIDIZATION

Specific messenger RNA species can be localized and levels quantified in tissue sections by quantitative in situ hybridization (Dagerlind et al 1992, Olson et al 2011). The sensitivity, unsurpassed spatial resolution and quantitative nature of in situ hybridization is not always appreciated in comparisons to Northern blots and other ways to measure RNA amounts in tissues. However, it is a precise technique that allows direct quantitative determinations of mRNA amounts in specific defined brain areas, and, when needed, in individual cells, without the need for reverse transcriptase or PCR steps. An example of detection of mRNA in individual neurons of cortex cerebri (in this case even without the aid of microscopical enlargement of signals) is shown in the results section.

Because there is typically a positive coupling between mRNA amount and gene activity, mRNA determinations, and, particularly alterations of mRNA levels by specific, controlled experimental treatments, as determined by in situ hybridization, reflect transcriptional activity of the corresponding genes. We therefore used in situ hybridization with radioactive oligo-DNA probes as described previously (Dagerlind et al 1992) to address gene regulation changes caused by HI in piglets, and how such changes might be affected by the cooling and xenon treatment protocols.

2.6 DEVELOPMENT OF HYPOTHERMIA AND CLINICAL EVIDENCE

Therapeutic cooling of infants and adults is not a new idea even though we now have better ways not only to control temperatures, and ventilation, but also to monitor effects in treated infants. Methods such as NIRS and MR can be used to confirm damage to the brain implicated by biomarkers in blood and urine. In 1954, Enhörning and Westin observed prolonged asphyxia in fetuses (Enhörning & Westin 1954). In 1957, Brodie and colleagues showed how the heat production was influenced by hypoxic conditions (Brodie et al 1957). The following year Burnard and Cross described rectal temperature differences in infants with birth asphyxia (Burnard & Cross 1958), and Silverman and colleagues (Silverman et al 1958) showed how survival was influenced by temperature. Thorn and Heimann (Thorn & Heimann 1958) demonstrated how anoxia, ischemia and asphyxia together with hypothermia affected ammonia levels in different organs including brain regions. In 1959, Westin and coworkers presented a simple device for the induction of hypothermia in newborns (Westin et al 1959)(right).



In the sixties, several studies were published

suggesting positive effects of hypothermia and emphasizing the importance of maintaining correct levels of oxygen both in animal and in humans (Adamsons et al 1964, Auld et al 1962, Dunn & Miller 1969, Ehrstrom et al 1969, Gandy et al 1964a, Gandy et al 1964b, Little 1966, Miller et al 1964). During the seventies and eighties there was increasing concern about therapeutic hypothermia expressed by medical societies both in Europe and the USA, based on alarming reports of negative effects of uncontrolled hypothermia for asphyxic infants (Dudgeon et al 1980, Michenfelder & Milde 1977, Oates & Harvey 1976, Pulsinelli et al 1982). In Russia, hypothermic treatment was further developed, although publications in Russian journals did not reach western scientists and clinicians. Nevertheless, a garment to control the temperature of the human body was presented in 1972 (Webb et al 1972). There have also been early animal studies of hypothermic treatment (Busto & Globus 1989) before as well as after an ischemic event (Boris-Moller et al 1989) in a second era, guiding the planning of clinical trials of hypothermia (Boris-Moller et al 1989). Deep hypothermia ($\approx 30\text{-}32^{\circ}\text{C}$) has also been proposed and effects reported (Compagnoni et al 2008). However, until recently, and despite previous trials, no therapeutic intervention following delivery has been shown to robustly improve outcome in perinatal HI, a concern that made the clinical neonatal field careful in planning new trials (Edwards & Azzopardi 1998). Thus it is not until the last several years that clinical practise has been transformed by the results of several large randomised clinical trials of treatment of neonatal encephalopathy by mild hypothermia (Azzopardi et al 2008, Edwards & Azzopardi 2006, Gluckman et al 2005, Hobbs et al 2008, Jacobs et al 2007b, Jacobs et al 2011, Robertson et al 2008, Shankaran 2009, Shankaran et al 2005, Thayyil et al 2009, Whitelaw & Thoresen 2001, Whitelaw &

Thoresen 2002). The first study used a cool cap to selectively cool the head such that a rectal temperature of 34.5°C was obtained. The second study used total body cooling to an oesophageal temperature of 33.5°C, while the third study cooled to a rectal temperature of 33.5°C. The decreased temperature was maintained for 72 hours in all three studies. No significant side effects were noted with this degree of cooling. Together, the data 1,5 years - (until school age) after treatment indicate that hypothermia reduces the incidence of death and/or severe disability. The most significant effects were seen in cases with modest encephalopathy and those without EEG signs of seizures (Edwards et al 2009, Edwards et al 2010, Hobbs et al 2008, Janata et al 2008, Perlman 2006a, Pierrat et al 2005, Strohm & Azzopardi 2010).

Most of the data above suggest that hypothermia is positive (Polderman & Girbes 2006) but there is always the risk of believing to much in an idea, as well explained in the article by Bertolizio et al (Bertolizio et al 2011), suggesting that before all centres cool brains, additional clinical evidence is needed. This also reflects on the staff, that they have the correct training and sufficient amounts of hypothermia cases to keep up the knowledge.

The current standard hypothermic therapy protocol in clinics and experimental work with large animals, is based on work by Gluckman, Gunn and collaborators on sheep (Gunn et al 1997), (for rodents the standard protocol at Karolinska Institutet is based on Vannucci's studies (Vannucci 1990, Vannucci & Perlman 1997)). The temperature was set to 34°C and the cooling time for humans to 72 hours, based partly also on rodent work (Sirimanne et al 1996), and hypothermia was to be initiated no later than 6 hours after birth as described by Thoresen (Thoresen et al 1995), to avoid secondary energy failure. Temperature studies have shown that that rectal temperature reflects core brain temperature (Battin et al 2003, Burnard & Cross 1958, Clarke et al 1997, Gandy et al 1964b, Iwata et al 2006, Okken A 1995).

Different aspects of the protocol have been discussed, such as the issue of whole-body or head cooling, and whether or not children should be sedated during hypothermia. A study of piglets by Thoresen and colleagues finds no effects of either anaesthesia alone (36 hour) or cooling (24 hours) alone on a number of histological and transcriptional markers for cell death and brain maturation (Gressens et al 2008). Indeed, the same group found that cooling was not effective without anaesthesia, probably due to stress, as evidenced by a marked increase of cortisol levels (Gressens et al 2008). Similar results have been noted by others (Liu et al 2011a, Liu et al 2011b).

In addition to the choice of hypothermia temperature, several other factors may influence outcome, such as the interval between insult and the induction of hypothermia, the length of cooling (Colbourne & Corbett 1994, Colbourne & Corbett 1995, Taylor et al 2002, Wagner et al 2002) as well as the speed of rewarming (Thoresen & Whitelaw 2000, Thoresen & Whitelaw 2005). Too short cooling periods such as 1 hour, have been reported to lack effects (Laptook et al 1999). The cooling protocol used in the present piglet experiments is documented elsewhere (Faulkner et al 2011); the Swedish clinical protocol (In Swedish) www.sfog.se/ARG.../natupplaga/ARG57inkl%20errata%20sid%2018.pdf guided us with respect to interval to treatment.

2.7 COMBINING HYPOTHERMIA WITH OTHER TREATMENTS

To complement hypothermia, several different neuroprotection strategies have been proposed, such as (1) NMDA-receptor blockage with xenon, or magnesium sulphate, (Buchan & Pulsinelli 1990, Hobbs et al 2008), (2) Ca²⁺ blockers, (3) melatonin, (4) scavengers of reactive oxygen species (Chaudhari & McGuire 2008, Chen et al 2009), and (5) ventilation of the infant with from 100% oxygen to room air strategies (Saugstad 2010). For all these strategies the ambient air temperature must also be taken into account when planning the hypothermia treatment (Gunn & Bennet 2001), as well as the newborn's individualized direct contacts with the environment, where strategies like NIDCAP can become useful (Kleberg et al 2000, Westrup 2007, Westrup et al 2004). Due to failure of additive results of Ca²⁺ in animal experiments (Levene et al 1990) such treatment is being abandoned. Similarly, magnesium sulphate is being abandoned, even though there are some seemingly positive results (Levene et al 1995, Spandou et al 2007). Erythropoietin seems to be a drug that can work together with hypothermia even though it is too early to be conclusive (Scalia et al 1998, Zhu et al 2009). In the present work we have focused on hypothermia and xenon as a complement to treat experimental hypoxic ischemia in newborn piglets. Work by Chakkarapani et al suggest that xenon could be an effective add-on (Chakkarapani et al 2010, Chakkarapani et al 2009).

Care must be taken when interpreting results from piglet experiments (our own mRNA data and other histological data) which may show small or no evidence that xenon would improve the hypothermic effect with animals studied for 48 h compared to 72h treatments incorporated into new improved clinical protocols (Gunn & Bennet 2010).

2.8 THE NEED FOR A SIMPLIFIED SAFE COOLING PROTOCOL

In order to start a new cooling centre, there are many factors to take into consideration, both locally and globally, including the availability of doctors and nurses 24 hours a day, as well as the number of cots. There are also several of the technical aspects, which must be dealt with, including the risk of too much cooling (Barks 2008, Worner & Oddo 2010).

A “low tech” method to safely cool infants with perinatal hypoxia-ischaemia is needed for transports and in developing countries, with more advanced models with servo control and rigorous monitoring systems for the developed world (Horn et al 2009, Horn et al 2010, Horn et al 2006, Robertson & Iwata 2007). However, too simple solutions might risk overcooling, unless rigorous monitoring of the neonate is practised at all times. Work presented in this thesis suggests that PCM mattresses containing PCM material as presented here can overcome these problems (Papers III and IV).

The standard method of cooling in the on-going international whole body cooling study in the developed world (TOBY trial; <http://www.npeu.ox.ac.uk/TOBY/>) is a mattress (Tecotherm), which needs a power supply, consumables (a regular supply of coolant) and carries a substantial cost (£13,000). Given high birth rates and limited hospital facilities in

many developing countries, the need of a “low tech” method for low-cost safe cooling of infants with perinatal hypoxia-ischaemia is strong and immediate.

We are proposing to pilot such a low-tech method that can effectively cool and maintain the core temperature of an infant at a constant level. PCM appears suitable for this purpose. PCMs do not require electricity; they are biologically safe for humans, cheap, can be reused and are likely to provide a more stable cooling temperature than other low tech methods such as ice packs. PCMs are already used for containers, which require precise temperature regulation during transport, and for the insulation of buildings.

3 AIMS

The research aims at developing methodology and equipment to monitor and counteract hypoxic injury taking into account both transport and global applicability issues, and to study in a large animal model how hypoxic ischemia and its treatment affects the transcriptional activity of genes encoding neural and glial proteins.

3.1 Paper I

- To test and evaluate fNIRS as a method to monitor face recognition in children
- To compare face recognition abilities between preterm and term infants.
- To learn how to implement NIRS as commonly used equipment in a neonatal intensive care ward.

3.2 Paper II

- Analyze how newborn children become passively cooled during transport and monitor their temperature regulatory mechanisms. Find optimal pre-transport strategies in preparing for transport from the delivery room.
- Compare temperature during transport to outcome.

3.3 Paper III

- Develop a thermo-compatible material, based on PCM properties, suitable for medical cooling purposes and modeling.
- Use the material to develop a mattress for the induction of therapeutic hypothermia, suitable for use also in developing countries (limited data presented due to choice of technical, rather than medical journal).

3.4 Paper IV

- Study if controlled hypothermia can be achieved in newborn piglets subjected to HI, by simple methods such as soft water-filled containers or PCM.

3.5 Paper V

- Analyze alterations of neural and glial gene activity patterns as reflected by alterations of mRNA levels at the cellular level in the piglet brain in response to hypoxic ischemia and its treatment by cooling, xenon or the combination thereof.

3.6 Paper VI

- Analyze neural and glial gene activity patterns at the cellular level in piglet brain tissue as above. Test different temperatures of hypothermia to verify if the standard cooling temperature used clinically is optimal.

4 CHOICE OF MATERIALS AND METHODS; ETHICAL CONSIDERATIONS

4.1 DEMANDS ON A PCM COOLING MATERIAL FOR MEDICAL USES

By changing different constituents of PCMs (usually a salt hydride, fatty acid and ester or paraffin, such as octadecane) the melting point can be altered. For medical applications, PCMs should ideally meet a number of criteria: 1. Have a controlled set temperature. 2. Be non-toxic. 3. Have a cooling effect that is rapid but with no overshoot (the PCM should not be able to cool below the set temperature of the specific chemical PCM composition). 4. Be stable for at least 72 hours. 5. Be of low cost so the final product becomes cost-effective. 6. Have no interbatch variability. 7. Be possible to cycle through at least 100 cooling phase changes. 8. Have a reversed phase change no longer than 8 hours. 9. Be effective without the need for an outside energy source. 10. Have limited expansion and not in any other way be potentially harmful for the aimed target. 11. Be magnetic resonance camera (MR) compatible, to allow continuous cooling for as long as needed.

A group of materials that meets many, if not all of the above criteria is Glauber salt-based products. However, the materials used as PCM do not cover the 24-40°C interval completely. Typically a given material covers a narrow temperature interval, and knowledge about how mixtures of different compounds or the different compounds themselves behave with respect to PCM characteristics has been scant. One of the temperature gaps where more work is needed is 32 to 35°C, another is just outside the interval mentioned above, just above 42°C. A problem we soon discovered was that the specifications for different materials differed between batches and that materials that would otherwise fit our needs were toxic. The salt solutions we have chosen as a base for our experiments have either been Glauber salt-based products (Climsel, Climator Sweden AB, <http://www.climator.com>) or other salt solutions (Rubitherm GmbH, <http://www.rubitherm.de/>). Since there will always be a small air pocket (as well as packaging material around the PCM) between the material and the skin of the patient one needs a PCM material that continually cools to a lower temperature than the aimed skin temperature. In our first pilot studies we performed phantom experiments, which included containers filled with water heated to 37,5°C. We found that the set temperature for the glauber salt should be 28°C if we aimed to achieve a rectal temperature of 32 to 34°C. These results and our first animal pilot studies have made us focus on finding novel PCMs that can come closer to the aimed target of 33,5°C. A variety of methods to find novel material mixtures with the desired properties have been used.

4.2 PCM VERIFICATION METHODS

A variety of methods have been used to find novel material mixtures with the desired properties, including:

1. DSC, Differential scanning calorimetry
2. T-method, T-history method
3. HCM, the Heated/Cooled Microscopy method.
4. AC, Adiabatic calorimeter
5. EB, the Energy Balance method
6. EB-Chinese version

We needed a well-defined method to verify melting as well as freezing points of the materials of interest and the mixtures between them as well as of the individual chemical constituents. Hence we chose a simplified version of method 1 above and a modified method 2.

4.2.1 DSC (Differential Scanning Calorimetry)

Phase equilibrium studies. With this technique one can decide the amount of energy that is needed for temperature equilibrium between a substance and a reference. The kinetic energy is monitored and the area under the integral is measured to reflect heat flow, and δ -enthalpy. This is explained in Anderson Materials science for engineers (Anderson 2003), in the web page of Anderson Materials Evaluation, Inc. (<http://www.andersonmaterials.com/dsc.html>) and elsewhere (Borreguero et al 2011, Marin & et al. 2003). For a complete description of DSC, see Speyer, Hemminger Hühne, Cammenga and Epple (Cammenga et al 1993, Epple & Cammenga 1993, Hohne et al 1990, Speyer 1994).

By selecting PCMs with phase change temperatures at either side of the desired 33.5°C, and then mix such PCMs, the result should be a material with the desired phase change temperature. The concept can be exemplified as follows: a compound A, with a melting point of 20°C, is mixed with a compound B, with a melting point of 57°C to form a mixture of A and B with a melting point that makes it possible to reach the target 33.5°C. Stable mixtures with desired properties can only be found by careful studies of such phase equilibria between two compounds. Assessment of the phase change temperatures (melting and freezing) of mixtures with varying compositions can be used to generate a so-called phase diagram for candidate systems. This will help compose the best mixture with regard to the desired target temperature, as well as obtaining knowledge about mixture stability and the possible formation of undesirable irreversible phases. The T-history method described below was used to acquire the necessary data.

4.2.2 T-history method

The method was first proposed as a way to validate small samples of PCM by Zhang et al (Zhang & Jiang 1999) being an economic and simple method to determine the heat stored compared to the temperature function. The temperatures of three entities are simultaneously recorded: (1) the control substance (normally sterile water), (2) the reference ambient median temperature and (3) the temperature of the substance being tested. Control studies are carried out of the reference and the tested substances during heating and cooling phases. In our T-history like set up, one test tube contains the sample

to be tested, one exactly the same volume of control substance. The test tubes were then lowered into a water bath. PT100 thermometer test probes in the tubes measured the temperature and sent a signal to a computer, recording temperatures in the surrounding encapsulated environment as bath temperature is raised and lowered. From such recordings a diagram is generated from which melting and freezing points of the tested substances can be deduced.

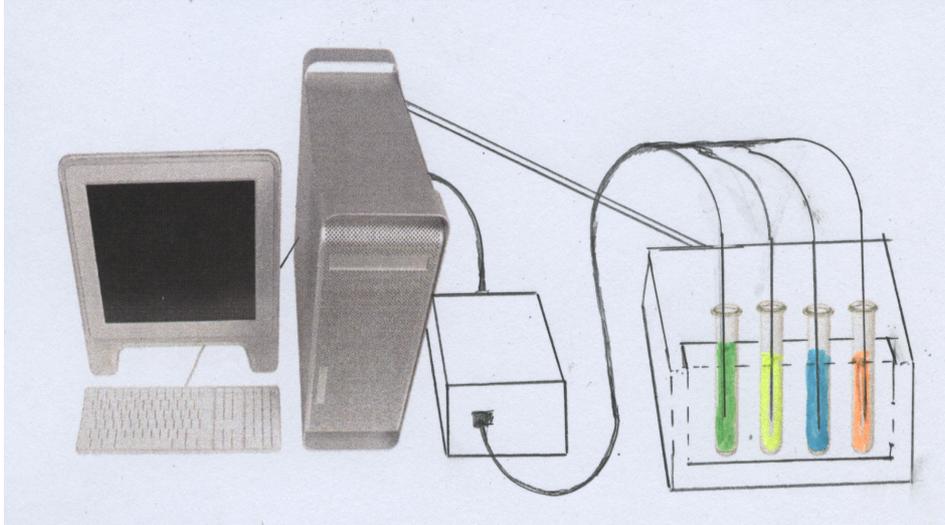


Figure 6: PCM: Computer with connections to screen, analog/ digital converter for temperature measurements, water bath (here shown cut of) with test tubes in the water; Green: control substance, Yellow: our PCM mixture, Blue: gelified water and Orange: regular water.

The T-history method generates values of good quality, although not as exact as other methods listed above. An advantage of the T-history method is that one can observe the complete procedure without damaging any material. With measuring tubes positioned vertically, volume expansion due to temperature increases will not be a disturbing component in the heat-absorption parts of the calculations (Hong et al 2004, Lázaro et al 2006, Mehling et al 2008, Mondieig et al 2003, Wang et al 2007).

4.3 THE CHOICE OF ANIMAL MODEL

The piglet model is suitable because the degree of maturation at birth is similar to that of the human newborn infant. There is also similarity of size and brain anatomy between piglets and neonatal humans. Cardiovascular, respiratory and metabolic homeostasis can be maintained for periods in excess of 48 hours. The numbers of piglets are kept at the minimum required to obtain meaningful information about group differences.

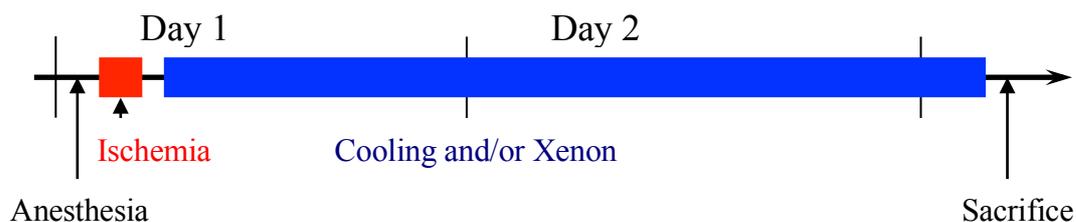


Figure 7: Time course of a piglet hypoxic ischemia experiment.

4.3.1 Hypoxic ischemia and treatment experiments

Piglet experiments were carried out at the University College of London as directed by Dr. Nicola Robertson. Under carefully controlled circumstances including MR spectroscopy, anaesthetized piglets were subjected to a period of hypoxic ischemia and then kept anaesthetized and receiving no treatment, hypothermia, xenon, or a combination of hypothermia and xenon. While still anaesthetized piglets were then brought to normal body temperature and sacrificed and one half of each brain used to analyze several histological parameters. The first results of these experiments, including a detailed description of methods has recently been published (Faulkner et al 2011). The other half of the brains from piglets used for these experiments were brought to the Department of Neuroscience at Karolinska Institutet for in situ hybridization analyses.

Neonatal, large white, piglets were born at farms in a controlled way in England. Within 24h of birth they were taken to UCL where a veterinarian examined them and blood samples were taken to assure health. The animals were then surgically prepared under general anaesthesia (isoflurane, ~3% during surgery and 1.5-2.5% thereafter). During this time the piglets received intensive life support including continuous physiological monitoring of arterial oxygen as measurement (O'Brien et al 2006). A tracheostomy tube was used to maintain normal PCO₂ and PO₂ (temperature adjusted) and animals were mechanically ventilated throughout the experiment. Maintenance fluids, continuous morphine infusion and antibiotics (benzyl penicillin and gentamicin) were given using an umbilical venous catheter, and by an umbilical arterial catheter we could monitor heart rate, blood pressure, and blood gases. Placing vascular occluders (OC2A, In Vivo Metric, Healdsburg, CA, U.S.A.) around the carotid arteries allowed us to interrupt this blood flow later. To simulate the proceedings of the clinical set up as much as possible, aEEG (BRM2 Brain Monitor, Brainz Instrument, Manukau, New Zealand) recordings, were also carried out throughout the experiment.

Following surgery, piglets were positioned in a prone position in an open incubator covered with a polytrianfoam mattress. Rectal temperatures were recorded (Arbo N44-91, Kendall, Powell, TN, U.S.A.). Initially the temperature was kept normal (38.5 ± 0.5°C) by covering the piglet with blankets. The piglets were next exposed to transient HI. The protocol we used called for the inspired oxygen fraction (FiO₂) to be reduced to 12% while closing of the carotid artery occluders. A fixed protocol of induction (10 minutes with FiO₂ 12%) and maintenance (15 minutes with FiO₂ 16%) was followed. This caused a 70% NTP deprivation and moderate cerebral injury with 30-40 % cell death in cortex, based on previous experiments using ³¹P-MRS. After this time period, occluders were opened and resuscitation initiated. The FiO₂ increased and normal saturation was found. The injury outcome was established by the aEEG and MRS.

Animals were randomized to treatment groups. Temperatures were recorded during cooling induction, maintenance and rewarming. Physiological data were continuously monitored. Two hours following transient HI and resuscitation, the piglet was placed on cooling devices if randomized for this (one way of cooling was accomplished using circulating water as used in humans (Azzopardi et al 2008)), aiming at a temperature of 33.5± 0.5°C as well as other temperatures for paper VI. If T_{rectal} deviated from the target

temperature adjustments were done. After the experimental period, rewarming by 0.5°C/h was commenced. Intravenous phenobarbital (20 mg/kg as the primary dose and 10 mg/kg thereafter up to 40 mg/kg in total) was used when seizures were clinically detected. After rewarming to normothermia, animal was sacrificed (intravenous pentobarbitone overdose). Brain and other organs were removed and either perfused or deep-frozen for further studies. Brains sent to Sweden were perfused with 4% formalin and then placed in a sucrose solution before shipment for in situ hybridization and immunohistochemistry studies.

4.3.2 Sectioning of the newborn porcine brain

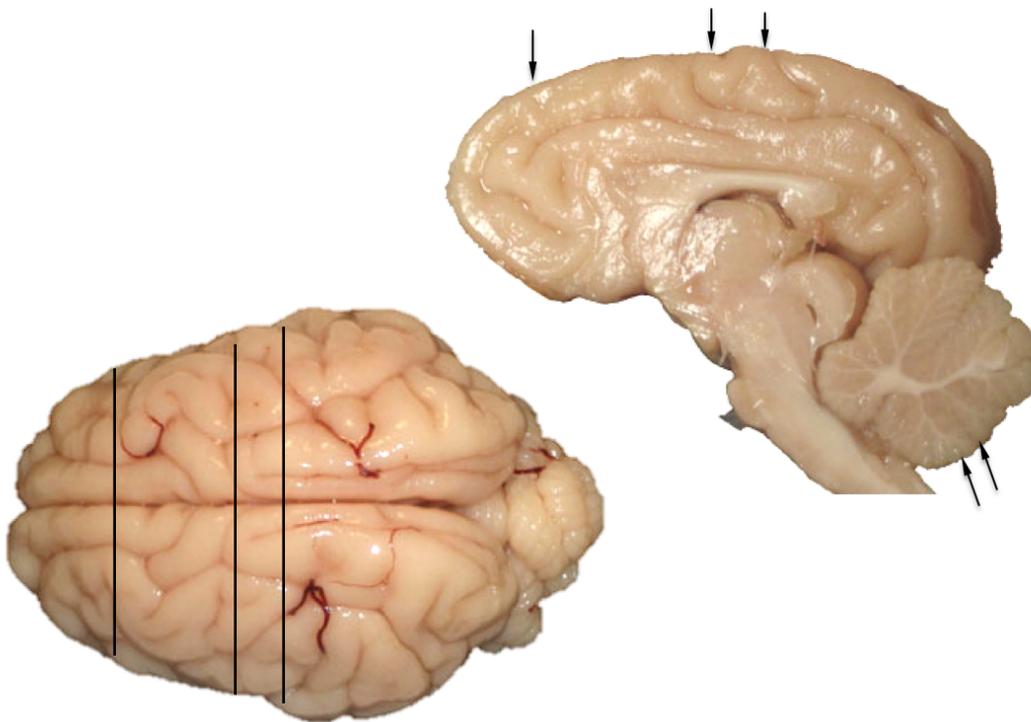


Figure 8: Photographs of newborn piglet brains used in papers V and IV. Levels for coronal sections of the brains used to obtain defined areas of prefrontal cortex, cortex cerebri, striatum, hippocampus and thalamus are shown in a dorsal view and indicated by arrows in a mid-sagittal view.

Tissues were collected as follows: Anterior level: Prefrontal cortex; middle level: A slice at this level was divided into triangle-shaped halves carrying cortical and striatal tissue, respectively; parietal cortex and striatum; posterior level: A slice at this level was trimmed and divided by a 30-45° angled cut into two pieces containing thalamus and hippocampus, respectively. Cerebellar tissue was collected from the area indicated in the sagittal view by double arrows.

All brain halves and remaining pieces that could be of interest were kept in sucrose until final decisions. Areas of interest were then frozen to keep the brain as fresh as possible until further studies can be performed. Areas that have been saved this way include cortical areas above hippocampus, thalamus, the primary visual cortex and associated areas.

4.4 IN SITU HYBRIDIZATION

To analyse transcriptional activity of different genes, as reflected by mRNA levels in situ, we used radioactive oligomeric complementary DNA probes and the protocol developed by Dagerlind et al (Dagerlind et al 1992) for rodents, slightly modified for piglets for papers V and VI.

Brains are either fresh frozen or perfusion-fixed prior to freezing and cryostat sectioning. In our case, the large piglet brain was first perfused with fixative and one hemisphere used for the in situ hybridization studies. Following the crucial step of rinsing in sucrose solutions, and as guided by the available literature on pig brain anatomy (Felix et al 1999), different brain areas were next dissected and frozen, according to a strict protocol. We then generated serial 14 µm coronal cryostat (Microm, HM500M) sections from different brain areas of interest. Sections were thawed onto coated slides (Super frost, Menzel-Gläser, Braunschweig, Germany) and stored at -20°C until use. Oligonucleotide probes specific to the mRNA species of interest were 3' end-labelled with [³³P] dATP (Perkin-Elmer, Massachusetts, USA) by terminal deoxynucleotidyl transferase (Fermenta, Helsingborg, Sweden) and purified (G50 Microcolumns, GE Healthcare, Stockholm, Sweden). Sections were incubated overnight (42°C) with hybridization cocktail (Dagerlind et al 1992).

To detect radioactivity in the sections, three methods were employed:

(1) For fast screening, selection of probes and estimation of exposure times for film autoradiography, we used phosphoimaging (Fujix, Bas3000, Plates: Fujix, Bas 3000 IP-Bas UR, Softwares: Fujix, Bas 3000 reader 3.14, Aida Array Compare 3.27 and Aida Image Analyzer 3).

(2) To quantify mRNA amounts in defined regions we used film autoradiography, apposing hybridized sections to photographic X-ray film (Biomax, Kodak, Rochester, N.Y., USA), in the dark. Typically, exposure is needed for 2-4 weeks, given that ³³P has a halftime of 24 days, and dependent on the abundance of the mRNA species.

The film autoradiographs were digitized by high resolution scanning (Epson Perfection V750 Pro). By using trans-illumination rather than recording reflected light, we obtained a much better grey scale resolution. In several cases this system allowed the visualization of individual labelled nerve cells in different layers. A standard is needed to measure the densities; we used a ¹⁴C step standard (Amersham Autoradiographic Microscale, Amersham, USA) that was placed next to the slides for each experiment. Digitized images could next be analysed using appropriate software (ImageJ), and densities compared to the ¹⁴C step standards. Statistical analyses were made using appropriate software (GraphPad Prism versions 5).

(3) For high-resolution autoradiographic analysis of mRNA species, slides (counterstained by cresyl violet) were dipped in photographic emulsion (NTB emulsion diluted ≈1:3, Kodak, VWR Sweden) and kept in the dark for ≈ 2-6 weeks, followed by development.

Silver grains in the emulsion overlying the section were then detected by light or dark field microscopy, allowing resolution at cellular and partly subcellular levels, such as presence of mRNA in dendrites.

We tested two to four probes for each gene of interest, and selected probes with best signal to noise ratio for autoradiographic quantification of mRNA species. In a first selection process, radioactivity patterns from hybridized sections were mapped using phosphoimaging to determine usefulness of individual probes and estimate suitable times for future exposure on X-ray film.

Based on phosphoimaging and preliminary film autoradiography data, the following probe sequences were selected for analysis of our genes of interest in the entire material: LDHA: NM_001172363.1, nt:1412-1363; LDHB: NM_001113287.1 nt: 353-505 GFAP, GI:29335682, nt: 89-138; HSP70, NM_001123127, nt 1676-1627; MAP2, XM_001926244 nt: 265-217; MANF, GI:52351138, nt: 223-272; BDNF, NM_214259 nt: 561-512; NgR, XM_001927797.1 nt:876-827.

4.5 ETHICAL CONSIDERATIONS

All studies in this thesis are based and were carefully planned with ethical considerations being an important factor. Paper I, where we studied face recognition using NIRS equipment, is based on human ethical approvals Dnr 2006/1:10 and 2006/1093-31. Paper II, where we study children transported before and during passive cooling is based on human ethical approval Dnr AD088-05 T659-05. Paper III is a physico-chemical study based on common knowledge as well as clinical and technical considerations for which ethical approval is not needed. Papers IV -VI are based on ethical approvals to Dr. Nicola Roberts at University College of London, England, UK Home Office Project License, ethical board. For bringing tissue samples between sites we have a document approved by both the authorities in Sweden, Jordbruksverket, and England, UK Home Office. To verify that our new equipment may provide the same or better results as compared to traditional cooling equipment, and to carry out additional histological analysis, the brain tissue samples were brought to the Karolinska Institute. This follows Swedish regulations for bringing dead tissues into Sweden. Sharing data and tissues between London and Stockholm minimizes the number of experimental animals needed. The PCM compositions and temperature intervals are not considered harmful to animals or newborns.

The following are considerations were also taken into account:

- The development of PCM could lead to new medical products coming from Sweden. PCM is compatible with magnetic resonance analysis, yet inexpensive and easy to handle. All PCM bars used in the study have been tested for toxicity, by sending a batch sample to a lab in Lund. The specific temperature interval is of no harm to piglets or future human beings. All PCM should be reusable.
- All PCM we use shall be possible to recycle after end usage.

We started our project with the 3R's Reduce, Refine, Replace in mind. Therefore, we have used dummy models whenever possible. The first evaluations of how PCMs work were carried out with dummies.

We have strived to technically refine our dummy model to replace animal and human studies as far as possible. To reduce or minimize the number animals used in our study we decided to use a well established model in the research field in use in at University of Collage London.

The piglet model is suitable because the degree of maturation is similar to that of the human newborn infant and the similarity of the piglet size and anatomy allows the application of neonatal intensive care techniques with which we have wide experience. Cardiovascular, respiratory and metabolic homeostasis can be maintained for periods in excess of 48 hours. The laboratory at UCL where conducting experiments on these animals and there was surplus brain tissue available for a collaborative effort with respect to PCM mattress efficacy and in situ hybridization studies. Numbers of piglets could thus be reduced while obtaining more information/piglet than otherwise possible.

Being a member of the Clinical research group for cooling in Sweden using traditional methods, the author will obtain the latest knowledge about traditional cooling available. This will help reduce variations in our experimental model. The author will also have the opportunity to refine the cooling model whenever needed, according to clinical and ethical practice.

5 RESULTS AND DISCUSSION

This thesis attempts to address the problem of how to treat neonatal hypoxic ischemia at several levels, focussing on therapeutic hypothermia. It spans from ways to monitor functions in the newborn baby brain (Paper I) and passive cooling of newborn infants during transport (Paper II), to the development of a PCM based cooling technique (Paper III), and via tests of such cooling in a phantom and then in piglets (Paper IV), to the effects of oxygen deprivation on the newborn brain and the possible beneficial effects of cooling and xenon (Paper V) and the choice of different cooling temperatures (Paper VI), studied at the level of transcriptional activity of selected genes in the brain as reflected by regional alterations of mRNA levels. While the focus of this work is the newborn brain, cooling may also be beneficial in protecting the adult brain from secondary damage and/or reperfusion damage, e.g. in stroke or cardiac arrest (Castren et al 2010).

A key issue of this thesis has been to develop and test a novel cooling method, for which a number of criteria should be met, such as being effective, safe, reusable, inexpensive, harmless to users and patients, and independent of access to water and electricity. This is why we focused on Glauber salt PCM, the composition of which was studied in Paper III. In order for a Glauber salt PCM to change phase from solid to liquid, it needs to take up a large amount of energy from its surroundings, in our case from the animal/patient. During this energy transfer the PCM will remain at its melting temperature, and the energy transfer will cool the animal/patient.

5.1 MONITORING THE NEWBORN BRAIN WITH NIRS (PAPER I)

Near infrared spectroscopy (NIRS) has been shown to be useful to monitor the infant brain, not the least during cooling. We have tested if NIRS might also become helpful to identify HI conditions and monitor effects of treatment of such conditions. Paper I describes the face recognition results. Preliminary data (see below) suggest usefulness of NIRS also to follow hypoxic ischemic damage and treatment effects.

In the face recognition study the infants were shown a grey screen followed by either an image of their mother or an unknown individual, a grey screen and a second face unknown, or mother, respectively. Out of 27 infants fulfilling inclusion criteria, data were obtained from 19, while 8 cases had to be excluded for various technical reasons. Regardless of whether the mother's image came first or last, it elicited a significant increase of $[HbO_2]$ in the fronto-temporal area on the right side, while no such increase was noted in the occipital area. We also found that if the mother's image was shown first, the $[HbO_2]$ response declined when the grey screen was shown again. Notably, $[HbO_2]$ decreased significantly in the occipital area in response to seeing the unknown face. $[HHb]$ did not change significantly in response to any of the three different images. Seeing the mother's face was often also accompanied by signs of increased attention and pleasure.

Studies of cerebral blood volume and HbO₂ by Van Bel et al and by Ilves et al (Ilves et al 2004, van Bel et al 1993) show how a decrease the first 12 h of life correlate to neurological abnormalities in asphyxiated neonates. Using NIRS we can determine if the HIE is mild, moderate or severe, and conclude that a post-hypoxic ischemic reperfusion injury of the brain during the first hours of life is crucial information with regard to pathogenesis and neurological outcome and sequelae in neonates with moderate/ severe birth asphyxia.

Tsuji and colleagues (Tsuji et al 1998) concluded from Doppler and NIRS measurements that monitoring global cerebral haemoglobin oxygen saturation provided by NIRS could serve as a therapeutic guide, since signs of cerebral ischemia during blood pressure fluctuations appear rapidly.

When studying the regional oxygen saturation ($rSO_2 = (HbO_2)/(HbO_2 + HbH)$) or the tissue oxygenation index (TOI) with NIRS (dependant on type of NIRS equipment) Toet et al (Toet et al 2006) and our own preliminary data show that rSO_2 seems to reflect the reperfusion period and to be strongly correlated to outcome in neonates with birth asphyxia. Particularly the TOI and the FTOE (fractional tissue oxygen extraction ($(SaO_2 - rSO_2)/ SaO_2$)) seem to be good prognostic tools to study infants during hypothermia to detect prognostic improvements during a 72 h period.

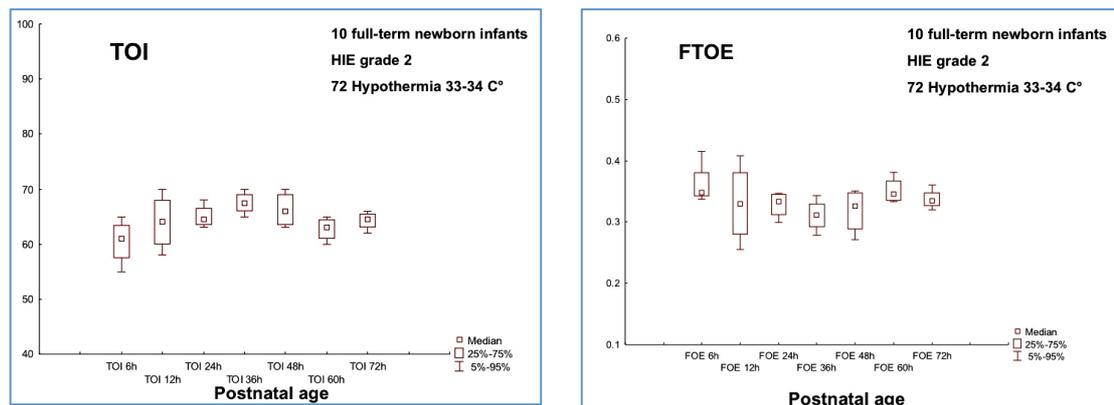


Figure 9: Preliminary results from NIRS recordings of 10 full term infants with grade 2 HIE treated by hypothermia. TOI appears to rise during the first 36 hrs. and then decrease somewhat while staying above the 6 hrs. entrance value. FTOE shows a somewhat reciprocal development during the first 36 hrs, followed by recovery. The largest variations between subjects of both measures are noted at 12 hrs. These results correlate well with Toet et al (Toet et al 2006) and appears to provide good prediction for the children. The x axis shows postnatal age in hours. From unpublished studies by Bartocci (with permission) and the Karolinska Neocool group.

To conclude, NIRS can be used to differentiate between asphyctic/ischemic and hypoxic injury, which can guide treatment strategies. NIRS can be used to monitor different haemoglobin changes, which is informative with regard to CBV, CBF and FTOE responses to HIE injury. One can also study seizures with NIRS (not described above) to get predictive values that might indicate future problems or how pharmaceuticals should be set in. A therapeutic window of opportunity often described in the literature can be confirmed by NIRS. Hypothermia may influence cerebral tissue oxygen extraction during

the first 72 h and we can follow this with NIRS and take measures to counteract pathological levels. Continuous monitoring with NIRS may help to prognosticize and could also be used before and after transport especially in combinations with aEEG monitoring.

5.2 PASSIVE COOLING AND TRANSPORT (PAPER II)

Severely asphyxiated outborn children were studied with regard to rectal temperature before, during and after transport to the two hospitals with cooling facilities. We were interested in following the temperature after rectal temperatures have been recorded, and active heating procedures stopped, as the children were transported to one of the two hypothermia treatment centers.

The greater Stockholm area has five obstetric and four neonatal clinics in five hospitals. Two of these neonatal clinics served as regional centers for hypothermic treatment of infants during the study period of paper II, and thus transportation to these two centers was an important issue in terms of times and care during transport. Even if the longest distance between centres in Stockholm is only 32 km, traffic and weather situations prolongs transport time.

The same protocol with the same inclusion criteria was followed as in the TOBY trial. Inborn infants selected for therapy were placed on a cooling mattress (Tecoterm[®], TeoCom, Germany) with a temperature of 33-34°C within 6 hrs. after birth.

Infants included in Paper II were cooled for 72 hrs, after which normal body temperature was reinstated by increasing the temperature by no more than 0.5°C/hr. Data were entered into the national perinatal database PNQ, where hypothermia treated individuals can also be entered and that collects inclusion criteria, transportation data, treatments, complications, follow up data, and more. A total of 37 infants fulfilled treatment criteria, which corresponds to an incidence of about 90/100 000 live births. For different reasons 3 of the 37 infants did not receive hypothermia treatment. About 53%, or 18 of the included newborn babies had to be transported after birth. We compared the baseline parameters of these babies to those of inborn babies included in the hypothermia study, and found no differences between the two groups. Almost all transported infants EEG recordings were initiated prior to transport.

Rectal temperatures ranged from 33 to 36.4°C at the start of transport. Within this range were 8 normothermic infants and 4 with a temperature in the intended hypothermic therapy range. There were no infants with rectal temperatures below the intended hypothermic temperature or above normal body temperature. Logistic difficulties precluded recordings of rectal temperatures at the start of transport in 4 infants.

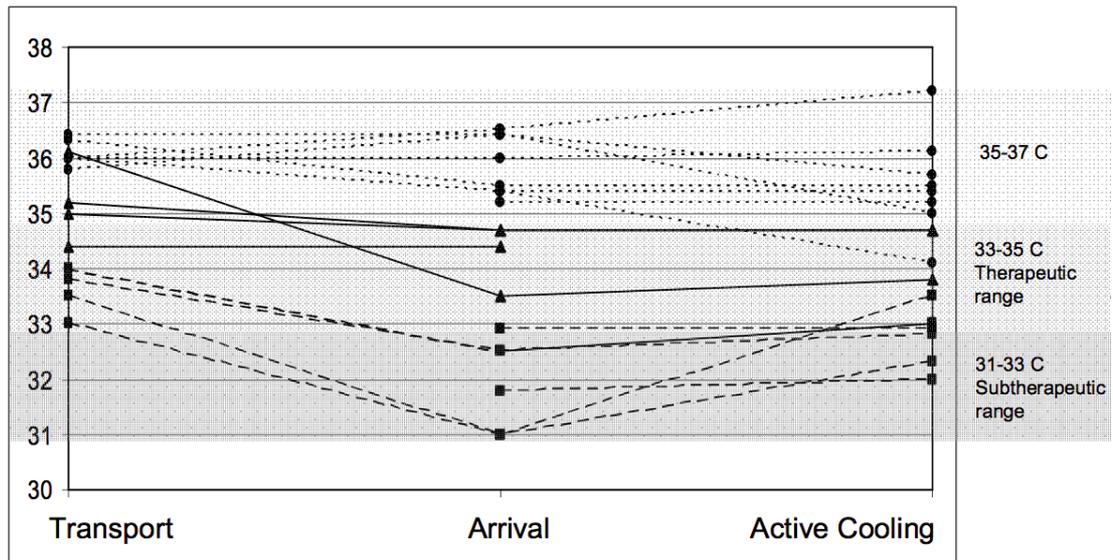


Figure 10: Temperature of a group of the studied newborn infants subjected to passive induction of hypothermia by stopping active warming before transport, on arrival at the hypothermia centre and at start of active cooling.

Upon arrival to a hypothermia therapy center, the range of temperatures of transported infants had widened to 31.0 - 36.5°C, with no less than 6 infants having sub-therapeutic rectal temperatures. Two infants displayed small increases of body temperature, a total of nine arrived with lower temperatures than when the transport started. In one case body temperature had decreased 2.6°C. We found that individuals arriving with subtherapeutic temperatures had instable temperatures when transferred to the mattresses, a situation that necessitated frequent changes of mattress temperature. We also found that the temperatures recorded when infants arrived to the hypothermia treatment unit were strongly correlated to the postnatal age when all active warming was turned off prior to transport. Perhaps not surprising, the lowest temperatures found at arrival were more often noted in infants with low birth-weights. There was no clear correlation between temperatures and treatment with anticonvulsants. Likewise, there was no significant correlation between mechanical ventilation (n=6) and temperature, although perhaps a tendency of ventilated infants to arrive with a lower than average temperature. Finally, four of the 34 infants died, three of those that died had been transported, and two of these arrived with temperatures that were sub-therapeutic.

We conclude that passive cooling before and during transport to a hypothermia treatment center leads to a rather wide range of body temperatures. Several infants arrive with subtherapeutic temperatures, and appears more difficult to rewarm to and then maintain at the planned therapeutic temperature. While the cohort is too small for statistical analysis based on different stratifications of the material, it also appears as if being passively cooled to a subtherapeutic temperature could be a disadvantage.

The instability of infant temperatures shown in figure 2 in paper II related to unstable passive cooling during transport might have been avoided if we could have had a more stable cooling method during transport. In paper IV, we found PCM to have the needed

cooling characteristics in model animals. Presumably, infants in transit would benefit from less temperature variations and avoid the risk of subtherapeutic temperatures.

5.3 PCM DEVELOPMENT (PAPER III) AND PHANTOM STUDIES

Early work by Brodie et al and Gagge et al (Brodie et al 1957, Gagge & Hardy 1967) described the production of heat by newborn infants and the effects of hypoxia on such heat production, and how heat exchange through radiation from humans can be studied by calorimetric methods, respectively. In this study we set up a phantom system to test the possible usefulness of PCM material. In these pilot phantom experiments, which are not included in Paper III, a container filled with water was heated by a heater coil to 37,5°C, to serve as a dummy for an infant with normal temperature. We then measured the PCM temperature as well as the temperature inside and outside of the cylinder.

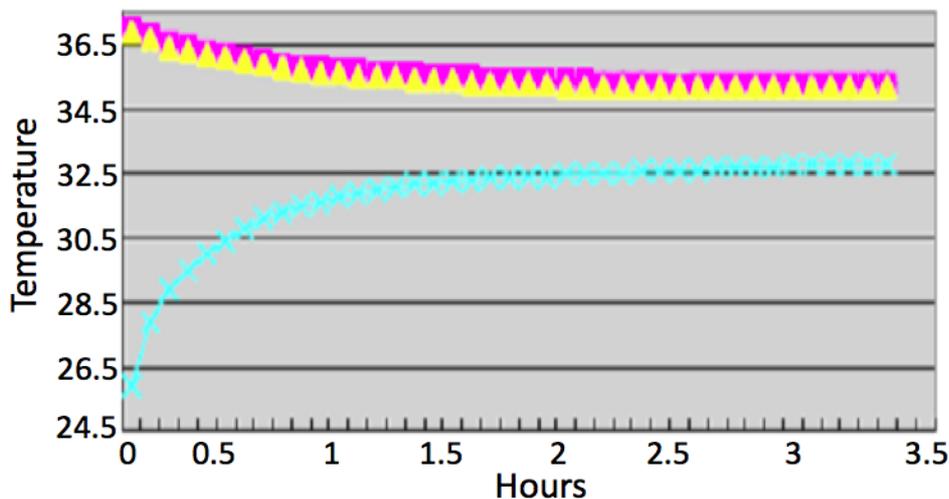


Figure 11: Pilot phantom study. Cooling of a water-filled and continuously heated (37.5°C) phantom by PCM 32 with a melting point of 32°C. The experiment was carried out at an ambient temperature (T_2 , not shown) of 20°C. Purple: T_3 , “Rectal” probe recording temperature inside one end of the phantom; Yellow: T_5 , “Skin” probe, recording temperature at the outer surface of the phantom; Blue: T_4 recording temperature of the PCM.

We postulated that the set temperature for the Glauber salt should be 28°C when we aimed to achieve a rectal temperature of 32 to 34°C. These results and our first animal pilot studies made us focus on finding novel PCM’s that can come closer to the aimed target of 33.5°C as studied in Paper III described below.

In paper III, we studied the behaviour of different mixtures between NaCl and Na₂SO₄, in water solutions. By adding different amounts of NaCl into the PCM solution, modifications of the melting temperature could be obtained. From the situation with

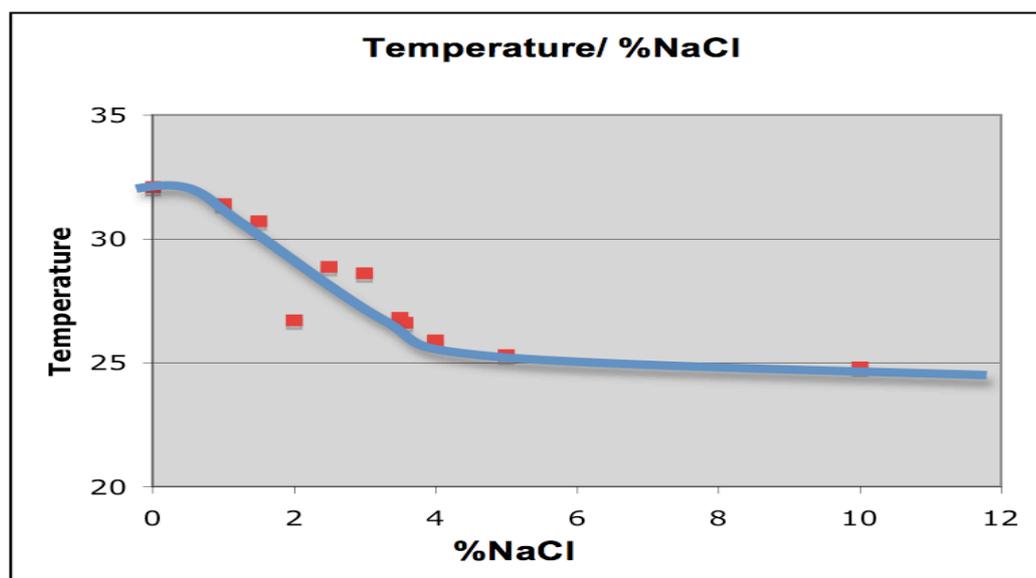


Figure 12: Melting points of PCM material containing different amounts of NaCl.

In the absence of NaCl, the melting point (red) is 32°C. Increasing NaCl decreases the melting point to about 25°C when 5% NaCl is present. A further increase of NaCl did not markedly change the melting point. It should be pointed out that the blue approximated curve does not exclude that there may exist additional complexity of the melting point temperatures in the 1.5 - 3.0 % range that were not resolved due to technical limitations of the experimental setup.

absence of NaCl and a melting point of 32°C we could decrease the melting point down to 25°C by adding up to 5 % NaCl. Additions of more NaCl had little further effect on the melting point.

The salt solutions studied have temperature change curves that differ from the water curve when warmed up to about 42°C and that flattens out briefly around 30-32°C, depending on the mixture. It is in this temperature interval that the salt solution mixtures function as PCM as intended.

The different materials in our experimental set ups were less diverse during cooling than when being heated, but even during cooling small variations of the slopes of the curves could be noted. When the amount of added Na_2SO_4 was low, crystallization with 7 rather than 10 H_2O molecules often occurred. Decreasing bath temperature to 5°C, caused a rise of PCM temperature to 10 or 12 °C because the salt acted in a non-regulated way until re-heating the solution.

When 0-10% NaCl and 30% Na_2SO_4 was dissolved in water and 0.833g of CMC was added as a gelifier, our system confirmed published phase diagrams for temperature vs. system percentage. We allowed 0.2-0.3 degrees of tolerance in between measures, based on reliability of the water bath servo control variability such as forms of crystallization. When a particular salt solution adopts a 7 instead of 10-crystal water configuration, the next cycle will be effected.

Viscosity of the gel could be controlled by the amount of CMC (3 to 8%). Keeping the proportions of salt the same, such viscosity changes did not alter the phase change temperature curves much. Thus the system was robust to viscosity changes as needed for different PCM applications. However, in mixtures containing low amounts of NaCl, changing CMC amounts too much was associated with risk of gel compartmentalization and inhomogeneous behavior.

Taken together, the results from Paper III suggest that PCM can be formulated and packaged to serve as a reliable low-tech, low-cost, reusable cooling device that does not carry the risk of overcooling its target. As will be shown in paper IV, PCM compares favorably to soft containers of tepid water as a coolant for newborn babies during transport.

5.4 PCM IMPLEMENTATION IN AN ANIMAL MODEL (PAPER IV)

A next step in order to move PCM towards clinical use was to test its cooling ability in a large animal model, for which newborn piglets (large white strain) were chosen. The piglets were anaesthetized and subjected to controlled hypoxic ischemia as described in papers IV, V and VI and by Faulkner et al (Faulkner et al 2011). Piglets were kept anaesthetized and divided into two groups receiving therapeutic hypothermia by either PCM (n=6) or water bottles with tepid (25°C) water (n=5).

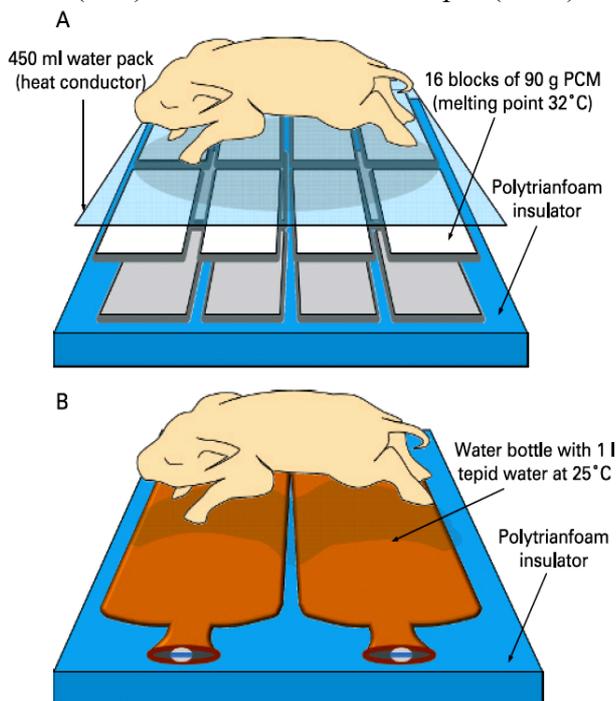


Figure 13: Diagrams demonstrating the construction of and the position of piglets on the PCM cooling mattress and the water bottles. (A) PCM mattress: 16 PCM packs with a specific melting point of 32°C were built into two layers of 4x2 PCM sheets. The upper surface of the PCM mattress was covered by a plastic water bag to improve thermal conductance. (B) Commercial water bottles: two water bottles were filled with 1 litre each of tepid water at 25°C. These cooling devices were placed underneath the piglet's head, trunk and limbs. An insulator mattress was placed underneath the cooling

devices so as to allow heat exchange only with the ambient air (25°C) and not with the incubator surface. From paper IV, Iwata et al 2009.

PCM and water bottle cooling were similar in terms of time to reach a target therapeutic rectal temperature of 33-34°C in the piglets. PCM caused significantly more stable cooling and led to a longer period at the target temperature. Water bottles had to be exchanged twice for most piglets.

Physiological parameters (HR, blood pressure...see table 1) before, during and after hypoxic ischaemia did not differ significantly between the two cooling methods. Likewise, numbers of piglets requiring pentobarbital due to seizures was similar between groups. There were no group differences noted during rewarming of the piglets.

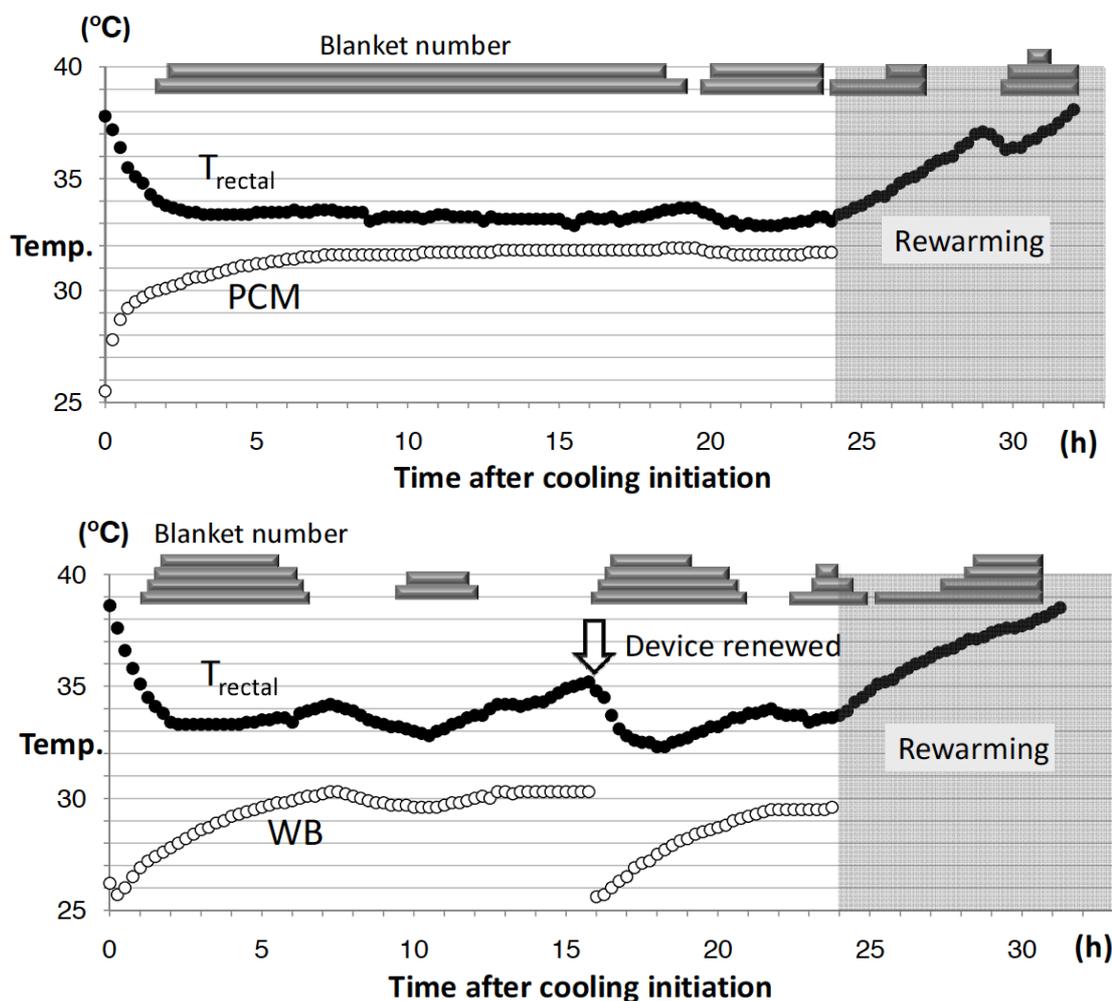


Figure 14: Temperature of the cooling agent, PCM (top) or water containers (bottom), and of piglets subjected to HI, during initiation of cooling, after having reached the target temperature, and during rewarming. Numbers and periods of blankets used to control piglet temperature are also indicated in the figure.. PCM (top) causes effective cooling to the target temperature, which is kept relatively stable until rewarming. Using containers of tepid water the piglet temperature was more variable despite more frequent alterations of blanket arrangements in order to dampen temperature variations. Shown is also that the water containers had to be changed, and the effects on piglet temperature thereof.

Table 1 Physiological variables

	Baseline*	Nadir of HI*	2 h*	14 h†	26 h†	End of experiment†
HR (min ⁻¹)						
PCM	165 (29)	208 (24)	160 (16)	121 (18)	117 (29)	170 (8)
WB	156 (19)	224 (23)	168 (31)	128 (22)	129 (18)	171 (20)
MABP (mm Hg)						
PCM	51.4 (15.5)	46.6 (20.0)	42.2 (5.1)	36.0 (4.5)	40.0 (5.8)	42.3 (7.9)
WB	41.6 (5.9)	37.4 (9.6)	40.4 (4.3)	36.4 (4.7)	45.4 (6.8)	47.2 (9.9)
Arterial blood gas analysis						
pH						
PCM	7.47 (0.06)	7.47 (0.08)	7.55 (0.11)	7.49 (0.09)	7.53 (0.07)	7.44 (0.10)
WB	7.39 (0.05)	7.37 (0.08)	7.43 (0.06)	7.40 (0.13)	7.37 (0.17)	7.36 (0.10)
Pco ₂ (kPa)						
PCM	5.46 (0.96)	5.04 (0.82)	4.39 (1.05)	5.13 (1.22)	4.09 (0.67)	5.83 (1.49)
WB	6.62 (0.92)	6.13 (1.56)	5.45 (0.49)	6.65 (1.51)	6.92 (1.67)	6.99 (0.76)
Po ₂ (kPa)						
PCM	11.20 (6.70)	2.61 (0.73)	11.68 (5.34)	9.20 (5.22)	8.22 (5.94)	10.42 (3.53)
WB	12.24 (1.50)	3.08 (0.86)	12.10 (1.58)	12.26 (4.88)	13.85 (2.49)	11.67 (5.29)
BE (mmol/l)						
PCM	5.3 (2.7)	3.6 (3.0)	5.9 (2.8)	5.6 (2.7)	2.8 (1.8)	4.6 (1.8)
WB	4.4 (6.5)	0.7 (5.9)	2.9 (5.9)	5.7 (6.8)	3.8 (7.1)	3.0 (6.7)
Lactate (mmol/l)						
PCM	2.67 (1.71)	3.68 (1.60)	2.17 (1.01)	1.35 (0.64)	1.46 (0.61)	1.21 (0.43)
WB	2.66 (0.84)	6.45 (1.71)	2.44 (1.64)	0.97 (0.32)	1.04 (0.33)	1.23 (0.12)

BE, base excess; FiO₂, fraction of inspired oxygen; HI, hypoxia-ischaemia; HR, heart rate; MABP, mean arterial blood pressure; PCM, phase changing material; WB, water bottle. Data are shown as mean (SD).

*6 PCM and 5 WB subjects included; †5 PCM and 5 WB subjects included.

Taken together, experiments reported in paper IV demonstrates that simple low-tech cooling devices such as water bottles with tepid 25°C water or PCM with a melting point of 32°C can be used to induce and maintain therapeutic hypothermia in piglets for 24 hrs. Although fine-tuning of target temperature using blankets was needed with both cooling methods, PCM caused a more stable therapeutic temperature of a longer lasting nature in our study. The rewarming phase was here achieved by a gradual rewarming by reducing the surface area of the piglet skin exposed to the cooling device. No adverse effects were apparent at the completion of rewarming.

5.5 HI AND COOLING EFFECTS ON BRAIN TRANSCRIPTS (PAPER V)

5.5.1 Tissue quality tests for immunohistochemistry and in situ hybridization

We first set out to evaluate the usefulness of formalin-fixed piglet brain tissue, taking into account that some of the material had been stored for considerable times (up to ≈ 6 months) after formalin fixation, and that this was neonatal porcine tissue, rather than rodent material. Our immunohistochemistry protocols almost invariably involves fixation by formalin perfusion, preferably including picric acid, while our in situ hybridization protocols are based on fresh frozen tissue. For immunohistochemistry, another issue is the scarcity of well-documented antibodies suitable to detect larger porcine molecules, although antibodies against small molecules, such as 5HT, should work across species.

Using standard immunohistochemistry protocols, we found that the piglet brain tissue lent itself well to immunohistochemistry using several different markers. Thus, catecholaminergic, serotonergic and cholinergic systems in the newborn piglet brain could be visualized by antibodies against tyrosine hydroxylase, serotonin, and vesicular

acetylcholine transporter, respectively. Likewise a GFAP antibody was identified with good signal-to-noise ratio for detection of the sparse amounts of GFAP-immuno-reactive astrocytes of the piglet brain. A neurofilament antibody also provided useful signals, while available antibodies against NSE and NF- κ B did not generate satisfactory signals. Taken together, these pilot tests (data not shown), suggest that the current protocol for collecting piglet brain tissue is suitable also for tests at the protein level of findings made in the present work at the mRNA level, provided that appropriate antibodies can be obtained for gene products of interest.

Like for immunohistochemistry, one issue was possible sequence differences between porcine and rodent or human gene sequences. We solved this by choosing preserved areas of the genes and by generating several complementary probes for each gene of interest and then test for probes with good signal-to-noise ratio and distributions compatible with the known distribution of transcriptional activity of the selected genes in different species.



Figure 15: HSP70 mRNA: Using a transillumination scanning technique allows detailed observations of the distribution of neurons containing HSP70 mRNA in different cortical layers. The arrows point to the surface of the brain with pia mater barely visible. Below the surface, the outermost molecular layer of cortex cerebri is devoid of HSP70 mRNA signals. Scale bar: 1 mm.

We found that in spite of formalin perfusion, neonatal piglet brain tissue lent itself well to in situ hybridization and, indeed that such fixation appeared to increase resolution of emulsion-dipped material. An example of HSP70 mRNA induction in neurons is shown in Fig. 15. Moreover, exposure to autoradiography film allowed reproducible quantitative data of mRNA amounts. Film autoradiography of 3 examined mRNA species and an example of effects of treatment are shown in Fig. 16.

A control piglet subjected to 48 hrs. of anaesthesia only, expressed the selected genes to approximately the same extent as naive piglets, suggesting that anaesthesia *per se*, did not cause major alterations of the activity of the chosen genes.

The objective of the present study was to compare mRNA levels of the chosen genes in different brain areas resulting from 48 hours of cooling the hypoxic ischemic anesthetized piglets to decrease body temperature by 3.5, 5 or 8°C to help determine if there is a preferable cooling level. Hypothermia to 33.5 °C was also combined with Xenon and compared to Xenon alone. Messenger RNA levels were determined by quantitative in situ hybridization (Dagerlind et al 1992, Olson et al 2011) a technique that allows direct quantitative determinations of mRNA amounts in precisely defined brain areas and, when needed its localization to individual cells, without the need for any PCR step.

5.5.2 A rationale for selection of mRNA species to analyse in papers V and VI

The extremely complex gene expression patterns, regulations and interactions of the brain are humbling. To begin understanding some of the possible effects of neonatal hypoxic ischemia and its treatment on gene regulatory events, we have chosen a set of genes reflecting important functions of neurons and astrocytes as well as energy metabolism, and for which there is background knowledge.

LDH-A and LDH-B mRNA. Our collaborators in London have used 1H-MRS to obtain lactate/NAA peak area ratios in the piglets from which the current brain tissue was obtained. They demonstrated that the lactate/NAA ratio was increased by the hypoxic ischemic episode and that the treatments, particularly the combination of cooling and xenon, appeared to counteract this increase (Faulkner et al 2011). This supports previous findings that lactate levels can serve as a biomarker of perinatal asphyxia and/or hypoxic ischemic encephalopathy (Karlsson et al 2010, Kruger et al 1999). LDH enzymes are widely distributed in different body tissues (Champe & Harvey 1994, Harvey & Ferrier 2010).

Brain lactate levels also serve as a biomarker of the aging brain, not the least the prematurely aging brain of mice with a genetically increased load of mitochondrial DNA mutations (Ross et al 2010).

HSP70. HSP70 is an immediate early gene that is rapidly up-regulated in neurons by a plethora of stress factors, including temperature changes (Giffard et al 2008). HSP70 also has a role in control of brown fat activity (Argyropoulos & Harper 2002, Cannon & Nedergaard 1985), which has a crucial role in temperature regulation in infants. The

functions of HSP70 in the brain are not fully understood even though it is abundantly expressed by many neurons in response to stressful events.

MAP2. MAP2 has been used to monitor the extent of brain injury after ischemia (Ishii et al 1998). It is a neuron-specific protein, known to be down-regulated by ischemia in cortex cerebri (Dehmelt & Halpain 2005, Lingwood et al 2008a, Lingwood et al 2008b, Schwab et al 1998, Svensson & Aldskogius 1992).

GFAP. GFAP is the classical marker of astrocytes, and more or less unique to this class of brain cells. Astrocytes are key elements in brain energy metabolism and contribute to the integrity of the blood brain barrier. Various stressors, including disturbances of metabolism or the blood brain barrier cause astrocytes to become reactive, a condition characterized by up-regulation of GFAP (Shen et al 2010, Shin et al 2008).

BDNF. BDNF (Lewin & Barde 1996) is by far the most abundantly expressed member of the NGF family of neurotrophic factors in the brain and expressed by neurons in pigs and rodents alike (Wetmore et al 1990). BDNF is typically up-regulated by neuronal activity, including stress, and is of key importance for synaptic plasticity (Greenberg et al 2009, Isackson et al 1991, Karlen et al 2009, Wetmore et al 1994).

MANF. MANF is a more recently discovered trophic protein, which is widely expressed in the brain. This protein has been reported to reduce endoplasmic reticulum stress, be trophic for embryonic dopamine neurons, and to increase after cerebral cortex brain ischemia (in rodents, increase is reported to decrease injury) (Airavaara et al 2009, Apostolou et al 2008, Lindholm et al 2008). MANF also plays a role in other organs, notably as a cardiomyokine the heart (Glembotski 2010).

NgR1. The newborn child's brain is able to learn by structural plasticity of its developing synaptic networks. NgR1 mRNA is neuron-specific and rapidly down-regulated by neuronal activity (Josephson et al 2003) at the same time as BDNF mRNA is up-regulated. There is now genetic evidence that such activity-driven down-regulation of NgR1 is needed in order to form lasting memories (Karlen et al 2009). In addition to BDNF mRNA as a marker of brain plasticity, we therefore chose to analyse NgR1 mRNA. For a review of Nogo receptor, see Schwab (Schwab 2004, Schwab 2010).

5.5.3 Effects of HI and the treatment regimes

LDH-A and LDH-B mRNA. The recent study of the same material as used here (Faulkner et al 2011) found that the lactate/NAA peak increased during 48 hrs. after HI and that cooling and, particularly, cooling combined with xenon counteracted these increases. We found a decrease of LDH-A and an increase of LDH-B in cortex cerebri decreasing the LDH-A/B ratio markedly in this area, while this was not the case in striatum. The decreased ratio in cortex was maintained in the treated groups. The decreased ratio seen in cortical areas is compatible with the increased lactate levels seen in the HI group in the parallel study (Faulkner et al 2011), if viewed as an increased efficiency in converting excess lactate to pyruvate. The difference in LDH-A/B ratios between cortex and striatum of HI piglets, may explain why cortex seems to be better

protected than striatum. This may be of clinical relevance explaining why many children with cerebral palsy seem to have intact cortex with relatively normal cognitive development.

BDNF mRNA. In the piglet brain, BDNF mRNA was expressed by neurons at relatively low levels. Hypoxic ischemia increased BDNF mRNA levels in parietal cortex as would be expected for a stressful event. However, despite the various treatments BDNF mRNA levels remained markedly increased as measured after rewarming, although there was a modest tendency to counteract the expression of BDNF mRNA by the cooling in parietal and prefrontal cortex. The overall effect of the hypoxic ischemic insult was also observed in the hippocampal formation, more marked in CA1 but visible also in the dentate gyrus. In the CA1 pyramidal layer, cooling counteracted the HI-induced BDNF mRNA increase, while this effect was not seen when cooling was combined with xenon. The HI effect of increasing BDNF mRNA levels was also noticeable in striatum, although less pronounced, and in thalamus there was hardly any effect of either HI or its treatment.

Experience from mice suggest that the most marked activity/stress-driven increases of BDNF mRNA are to be expected in cortex and hippocampus and that effects would be less in areas such as striatum or thalamus, which is fully compatible with the expression alterations found in the current piglet study.

Taken together, out of the 18 analysed treatment situations (3 treatments x 6 brain areas) then mean BDNF mRNA levels were lower than in the corresponding mean BDNF mRNA levels in HI treated brain areas in 16 of the 18 cases, suggesting an overall dampening effect of treatment at the investigated time after treatment. Of the treatments, it appears that the most pronounced effect was that of cooling in CA1.

MANF mRNA. We found MANF mRNA hybridization to occur ubiquitously in grey and white matter at relatively high levels. For instance, we calculated MANF mRNA levels in naive cortex to be \approx 15-fold higher than BDNF mRNA levels. In stark contrast to the effects of HI on BDNF mRNA levels, MANF mRNA levels were down-regulated by HI in all investigated areas. Also unlike the situation for BDNF mRNA, but in support of normalizing effects of treatment, all three treatments led to higher levels of MANF mRNA in parietal cortex than seen in the non-treated HI-group. In prefrontal cortex MANF mRNA levels were lower than in parietal cortex, although we calculated they were still \approx 9-fold higher than the BDNF mRNA levels in this area. HI strongly decreased MANF mRNA levels in prefrontal cortex, and none of the treatments appeared to counteract this decrease as seen after 2 days of treatment and rewarming (when relevant). The HI-induced MANF mRNA decrease was marked also in hippocampus, the dentate gyrus, striatum and thalamus (although lesser so in this region) and there did not appear to be any clear effects of any of the three treatments.

HSP70 mRNA. HSP70 mRNA levels were low in the entire naive piglet brain. HI caused a general increase of HSP70 mRNA levels was found in all investigated areas, with the hybridization signal restricted to neurons. Effects of HI in parietal cortex were so dramatic that we were able to detect hybridization signals from single individual neurons using film autoradiography, thus without the aid of a microscope. The HI induced increase of HSP70

mRNA was further augmented by hypothermia and xenon. This pattern of response was thus different from that of both BDNF mRNA and MANF mRNA.

In parietal cortex cerebri the HI-induced HSP70 mRNA increase in neurons was \approx 10-fold. The augmentation of this increase by treatment was particularly marked in parietal cortex and CA1 of hippocampus, and strongest when xenon and hypothermia were combined.

HSP mRNA levels were not measurable in the dentate gyrus in any of the groups, suggesting that HSP70 does not play a role in this area, at least at the time point chosen for our analysis. Levels were low in prefrontal cortex and thalamus, increased by HI but not further increased by the different treatments. In striatum, there was also a significant increase by HI and a tendency for this increase to be lowered by treatments.

Taken together the HSP70 gene is robustly activated by HI in all areas except the dentate gyrus, although the most marked effects are clearly seen in parietal cortex and CA1 of hippocampus. To the extent that such increases are beneficial in a situation with HI-induced metabolic stress, it is possibly good that the increase is augmented in cortical and hippocampal areas by the treatments. However, we cannot exclude that the treatment-induced increases constitute responses to continued secondary degeneration events. Alternatively, given the role of HSP70 in uncoupling needed to generate heat (Argyropoulos & Harper 2002) the HSP70 mRNA response could be related to heat generation from brown fat in the newborn piglets. In hippocampus, the two groups receiving cooling (as single treatment or combined with xenon, respectively) both have higher HSP70 mRNA levels than the two groups not subjected to hypothermia treatment (HI and HI + xenon groups). Our piglet data are compatible with recent findings with respect to the effects of different hypothermia temperatures on members of the HSP70 family in rats (Xiao-Yan & Yi-Xin 2011).

GFAP mRNA. There is generally much less GFAP immunoreactivity and GFAP mRNA in newborn versus adult brains. This was clearly the case for the newborn piglet brains with low levels of GFAP mRNA in naive brain tissue in parietal and prefrontal cortex and striatum, relatively low amounts in thalamus and no detectable amounts in hippocampus or the dentate gyrus. In parietal cortex, HI induced an increase of GFAP mRNA that was largely upheld also in the three treatment groups. Thus none of the treatments was able to counteract the astrocytic reaction to HI, as measured 2-3 days after HI. In prefrontal cortex there was a tendency of increase of GFAP mRNA by HI and in animals treated with xenon or xenon+hypothermia, while the modest HI-induced increase was not seen in the hypothermia group.

Evaluated across areas, HI with or without treatment was often associated with modest increases of GFAP mRNA, while there was no clear pattern of effects of the treatments.

MAP2 mRNA. Map2 (Dawson & Hallenbeck 1996, Dehmelt & Halpain 2005, Dinsmore & Solomon 1991) was found in low to moderate amounts in all investigated areas of naive newborn piglets. HI caused a robust pan-regional decrease of MAP2 mRNA levels, most marked in parietal cortex, hippocampus, gyrus dentatus and thalamus. In parietal cortex,

where HI levels were 40% of naive levels, the different treatments all appeared to partially counteract this decrease. In prefrontal cortex, presumably in a more immature state than parietal cortex at birth, MAP2 mRNA levels were lower in naive piglets, although there was a tendency for levels to be decreased by HI also in this region and no indication of any effect of any of the treatments. HI induced decreases of MAP2 mRNA were dramatic in hippocampus and gyrus dentatus and treatments seemed largely ineffective. In striatum and thalamus HI-induced decreases were less severe and there were no clear effects of treatments.

Summarizing effects on MAP2 mRNA one finds a general, and in some regions severe decrease of the transcriptional activity of this gene. With the exception of parietal cortex, where treatments may counteract such decreases partly, none of the three treatments appeared able to counteract the MAP2 mRNA losses present at sacrifice. Because MAP2 mRNA loss is a marker of neurodegenerative events these observations strongly suggest that none of the treatments is able to fully protect the newborn piglet brain, subjected to a severe hypoxic ischemic event, from degenerative events.

NgR mRNA. As expected for a developing brain, fully capable of structural plasticity, we found very low levels of NgR mRNA in the different areas of the newborn piglet brain. In parietal cortex cerebri, HI nevertheless caused a significant decrease of NgR mRNA. This decrease may be viewed as an activity/stress-induced down-regulation of NgR mRNA, to allow needed compensatory structural plasticity, in line with similar regulatory responses to increased neuronal activity in adult mice and rats (Josephson et al 2003, Karlen et al 2009, Kilic et al 2010). In this area all three treatments led to mean NgR mRNA levels that were somewhat higher than in the HI group, suggesting a degree of protection, best seen in piglets treated by hypothermia.

Somewhat unexpectedly, HI tended to cause an increase of NgR mRNA levels in prefrontal cortex and hippocampus, although there was no clear pattern of effects of treatments except perhaps a normalization of this increase by cooling in hippocampus. NgR mRNA levels in the dentate gyrus, striatum and thalamus were extremely low and there were no clear effects of HI or its treatment.

Due to the low amounts of NgR mRNA in the newborn piglet brain, down-regulation of this gene by HI, as presumably would have been expected in an adult brain, was difficult to detect, although we did detect such a down-regulation in parietal cortex. Likewise, any possible effects of treatment were difficult to detect, although normalization by cooling of the HI-induced decrease in parietal cortex and the HI-induced increase in hippocampus cannot be excluded.

Conclusions from Paper V

In Paper V, we have studied gene regulatory events caused by HI, and how such events are influenced by two different treatment principles, hypothermia, xenon and a combination of them. We conclude from examination of mRNA encoding 8 different genes in 6 different areas of the piglet brain that alterations are strikingly gene specific,

with some genes being down-regulated and others up-regulated by the HI insult. We also find several situations in which the treatments normalize mRNA levels regardless of whether HI caused levels to decrease or increase. However, there are also situations in which an HI-induced alteration of mRNA levels is enhanced by treatment. Finally some mRNA species are much less affected by HI than others. In terms of the different treatments, we failed to find convincing evidence of xenon-specific effects. If anything, the presence of xenon, either alone or together with hypothermia tended to counteract HI effects less well than hypothermia alone. Together, our observations demonstrate that moderate hypothermia does not lead to a generalized down-regulation of gene activities, at least not as seen immediately after rewarming. Instead, gene regulatory events are different for different genes, suggesting that many of the gene regulatory mechanisms are operative. Typically, transcriptional activity, as reflected by mRNA levels is most marked in parietal cortex. It is also in this superficial region that effects of treatments are best seen. However, none of the HI-induced alterations of mRNA levels, including the probably detrimental decrease of MAP2 mRNA could be fully and robustly counteracted by treatment. This can be one explanation why in the clinical situation not all patients recover fully. It should be noted that, the degree of injury in HIE infants varies more than in the controlled piglet experiments studied here, and could thus be both less and more severe than in our animal model.

5.6 EFFECTS OF TEMPERATURE ON SOME HIE MARKERS (PAPER VI)

5.6.1 Background, hypothermia therapy parameters and rationale

Therapeutic hypothermia for the treatment of neonatal HI is becoming increasingly clinically accepted. However, there are few systematic studies aiming to optimize several different key hypothermic therapy parameters. Thus the rate of cooling, the target temperature, the length of the cooling period and the rate of rewarming are at least partly unexplored issues. The most important of these parameters is arguably the possible differences between different target temperatures.

The cooling device, mode of cooling, such as cooling the head versus the body, and the methods of estimating brain temperature are additional important hypothermia-related parameters to consider. There are reports to suggest that outcome may vary due to type of cooling device (Agnew et al 2003, Christensson et al 1993, Iwata et al 2009, Jacobs et al 2007a). Rectal temperature can be regarded as a reasonable reflexion of inner brain temperature in newborns of different species (Battin et al 2001, Battin et al 2003, Burnard & Cross 1958, Clarke et al 1997, Gandy et al 1964b, Okken A 1995, Pierrat et al 2005). However, a given cooling device may lead to different degrees of lowering the temperature of core and mantle regions of the brain. In order to understand if different degrees of damage between core and mantle regions is due to intrinsic differences in HI susceptibility between different brain regions or are the result of different degrees of cooling, possible temperature differences between different brain areas during therapeutic hypothermia needs to be evaluated.

Paper VI builds on the information obtained in Paper V about the effects of HI and cooling to 33.5°C on expression of 8 key genes in different areas of the brain, as determined by quantitative in situ hybridization. The results from Paper V, summarized in a paragraph above, demonstrate that practically no mRNA alterations caused by HI are fully compensated for by the treatments, when analysed immediately after rewarming to normal body temperature. This set of observations is only one of many to suggest that better protocols are needed if we are to improve treatment results for neonatal HI.

Like paper V, paper VI is collaboration with Dr. Nicola Robertson and her colleagues in London. The same carefully controlled newborn piglet brain HI model (Faulkner et al 2011) was used to evaluate the effects of a higher target temperature, 35°C, and a lower target temperature, 30.5°C, as compared to the temperature used in Paper V, 33.5°C, on HI-induced alterations of the same set of genes as studied in Paper V. Paper VI thus compares the effects of decreasing piglet rectal temperatures by 3.5, 5.0 or 8.0°C during (to 35, 33.5 or 30°C) 48 hrs. after the HI insult. In situ hybridization methods and the rationale for selection of genes to analyse are the same as for Paper V.

LDH-A and B and the LDH-A/B ratio. The mean LDH-A/LDH-B mRNA ratio was decreased in parietal cortex in all treated groups and significantly so in the 33.5°C group compared to the normothermic HI group. This result is similar to that seen in Paper V, although less pronounced. In line with Paper V, we also found that the LDH-A/B ratio was not altered in striatum, while significant decreases were seen in thalamus (35 and 33.5°C) and hippocampus (35°C) compared to the normothermic HI group. Thus, there was a tendency for 35 and 33.5°C to lead to significant compensatory decrease of the LDH-A/B ratio while no significant effects were found in the lowest temperature group.

BDNF mRNA. Levels of BDNF mRNA were low in all areas of the naive newborn piglet brain. There were robust increases of BDNF mRNA levels after HI in all five investigated areas. No clear effects of any of the three cooling temperatures were noted in this study, although the mean levels were marginally lower when cooling to 33.5°C than at the other two cooling temperatures. The HI-induced increase is similar to that seen in Paper 5, while the cooling effects, if any were considerably smaller. Subtle differences between the experiments, including the length of the rewarming process may explain these differences.

MANF mRNA. Like in Paper V, MANF mRNA was found in relatively high levels in grey and white matter of naive piglets and the HI insult caused levels to decrease in all examined areas. In all areas analysed, Cooling to 33.5°C appeared to modestly counteract the effects of HI in all analysed areas (likelihood of being a chance observation \approx 3%), although not significantly so in any individual area. In three areas (cortex, striatum and thalamus), cooling to 30°C was associated with lower MANF mRNA levels than in any of the other four tested conditions, while cooling to 35°C did not seem to affect MANF mRNA levels much. It should be noted that none of the effects of cooling reached significance.

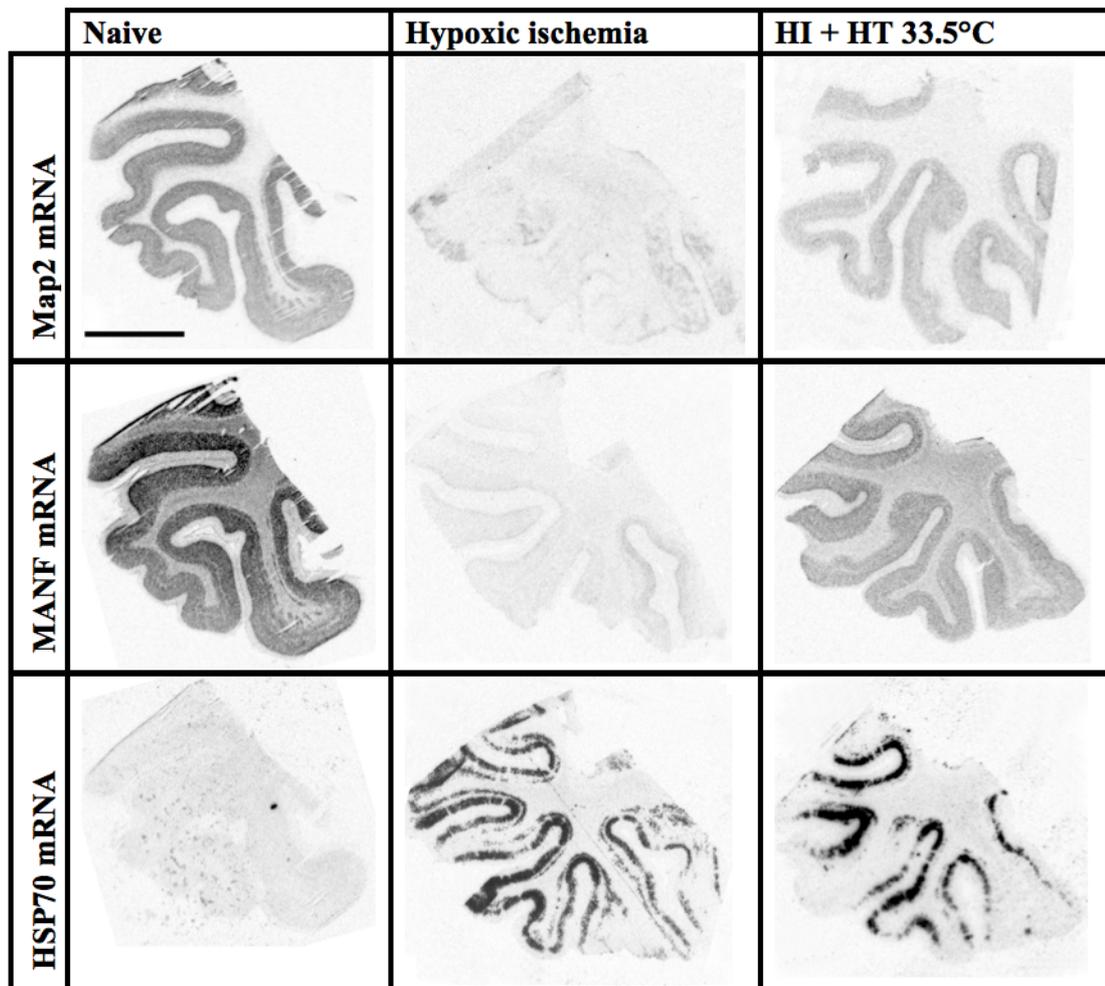


Figure 16: Representative in situ hybridization results from sections of parietal cerebral cortex obtained from naive piglets, animals subjected to transient hypoxic ischemia and animals subjected to the same hypoxic ischemia followed by 33.5° hypothermia treatment (HI+HT33.5°C). The autoradiographic images of radioactive probes for MAP2, MANF and HSP70 mRNA are shown. Note that the hypoxic ischemic insults cause decreases of MAP2 and MANF mRNA labelling but increases of HSP70 labelling and that the opposing effects of HI seen for MAP2 and MANF are partly counteracted by the hypothermia treatment while the effect of HI on HSP70 mRNA is potentiated by cooling. Calibration bar 5 mm. From Paper VI.

HSP70 mRNA. We found HSP70 mRNA levels to be low in the naive piglet brain, and like in Paper V, that HI caused levels to increase, and that cooling tended to cause a further increase of HSP70 mRNA levels in cortex and hippocampus. There was a non-significant tendency for cooling to 33.5°C to cause the largest increase of HSP70 mRNA in cortex and hippocampus. All three cooling temperatures were associated with higher mean HSP70 mRNA levels in cortex and hippocampus than was seen in the normal temperature group (1.5% chance of being a random observation). We also note that the finding in Paper V that cooling to a rectal temperature of 33.5°C caused slightly decreased (rather than increased) HSP70 mRNA levels in striatum was in line with findings in Paper VI.

GFAP mRNA. GFAP mRNA levels were very low in the newborn naive piglet brain. These low levels made the detection of robust differences following HI and treatments difficult. However, there was one rather robust pan-regional effect, cooling to a rectal temperature of 30°C caused GFAP mRNA levels to be very low. This effect was significant both in relation to the levels in the 35 and the 33.5°C groups.

MAP2 mRNA. As expected HI led to a severe decrease of MAP2 mRNA in cortex cerebri, hippocampus, the dentate gyrus and thalamus. A similar effect was not seen in striatum, possibly due to too large variability in the naive group. There was a very weak tendency for cooling to 35 and 33.5°C, but not 30°C to counteract the HI-induced decrease of MAP2 mRNA amounts.

NgR mRNA. Very low amounts of NgR1 mRNA made comparisons difficult. The overall picture suggests that there might be were small decreases of NgR mRNA levels in all groups subjected to HI in all areas except striatum.

By and large, the results in Paper VI support those of Paper 5, although partly less clearly so. Taken together the data can be seen as supporting cooling to 33.5°C as the best compromise, with cooling to only 35°C being less effective and cooling to as low a rectal temperature as 30°C being associated with some negative effects, while providing less protection. However, like the data from Paper V, the data from paper VI show that none of the changes of mRNA levels caused by HI could be effectively counteracted by any of the three cooling temperatures as tested immediately after rewarming and sacrifice.

5.6.2 Comments on preliminary studies of additional mRNA species

In addition to the mRNA species encoded for by the eight genes reported in papers V and IV, several other relevant mRNA species have been considered, based on importance during development, neuronal activity or stress, or other roles in neurons and glia. Suggestions have also been made to explore candidates closely related to what other parts of the neonatal group have in their future plans. We have not yet carried out systematic studies of these genes, but a few observations are worth mentioning:

Nestin. Nestin is an intermediate filament and early marker of the neuronal and astroglial lineage. Important as a cytoskeletal component for axonal growth (Dahlstrand et al 1992, Lothian & Lendahl 1997, Michalczyk & Ziman 2005). We found nestin mRNA to be expressed at low levels with no marked differences between treatments, which could be viewed as compatible with a cytoskeletal role.

HIF. Hypoxia-inducible factor 1 (HIF-1) is a transcriptional regulator for genes regulating mammalian oxygen homeostasis. HIF-1 has been proposed to affect the reactive oxygen species pathway, and therefore of interest to us (Hwang & Lee 2011, Page et al 2002). HIF-1 also appears to be a mediator of angiogenesis in HIE (Huang et al 2004).

Our first preliminary results indicated only small variations and it seemed that intra-group variations were larger than inter-group variations. The cause of these variations remains to be understood.

KCC2. K⁺- Cl⁻ cotransporter 2 is a neuron specific chloride cotransporter involved in controlling the intracellular Cl⁻ concentration in neurons. Animal studies have shown that lack of KCC2 disturbs respiratory rhythmogenesis, leading to premature intrauterine death. KCC2 has a significant role in central nervous system development and function, see theses by Zachi Horn <http://publications.ki.se/jspui/handle/10616/40384> and Hong Li <http://urn.fi/URN:ISBN:978-952-10-4493-9>. Very preliminary data suggest low expression levels in prefrontal cortex of newborn piglets, but further tests of probes and exposure times are needed before group effects could be evaluated.

Connexin 43. Connexin 43 is a gap junction protein found only in vertebrates. The protein forms gap junctions between astrocytes, functionally connecting large groups of astrocytes. Our preliminary data suggest a modest decrease of connexin 43 mRNA by hypothermia, with no clear differences between cooling temperatures.

Arc. Activity-regulated cytoskeleton-associated protein is normally localized to be in a position to activate different NMDA receptors in an activity dependent way. The protein is believed to be part of the molecular processes of learning and memory (Steward & Worley 2001, Wallace et al 1998). Arc mRNA studies may thus serve to complement our BDNF mRNA studies. Sections from the same piglets as analysed in Paper V suggest that Arc mRNA is increased by HI in prefrontal and parietal cortex cerebri, and that such increases are counteracted by cooling, particularly in parietal cortex. Sections from piglets used in Paper VI indicated similar results. Figure 17 depicts preliminary data on Arc mRNA levels.

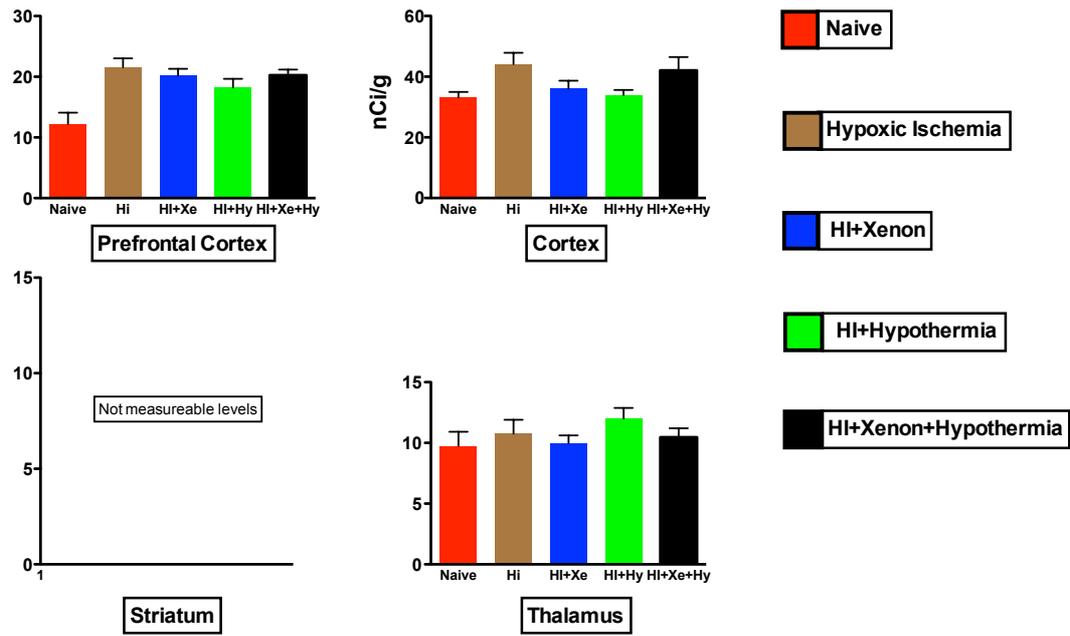


Figure 17: Arc mRNA levels in sections from 4 brain regions of the same piglets as used for Paper V.

6 CONCLUSIONS

In paper I, we used NIRS to monitor brain function, and demonstrated its usefulness to test face blindness, a possible side effect of HIE. We also established NIRS as a way to monitor haemoglobin oxygen binding changes, which is informative with regard to CBV, CBF, TOI and FTOE responses.

In paper II, we conclude that there is a major risk for patients to become too cold using passive cooling. It also becomes more difficult to stabilize a target hypothermic temperature, which lowers the odds for successful treatment.

In paper III we found that by alternating the composition of Glauber salt, a range of materials could be developed for inducing and maintaining hypothermia and that PCM was ready for clinical trials. A composition of the salt defined by the amount of added NaCl and gelifier to assure even distribution of the salts, was shown to operate well at the optimal cooling temperature of 28°C.

In paper IV, material developed in paper III was tested carefully for effectiveness in piglets, and found to work as a reliable, stable and simple cooling source. The composition of Glauber salt-based PCM, as obtained in paper III, may fulfil the need for a safe, low-cost, low-tech cooling material useful also in remote settings of the developing world.

In papers V and VI, analysis of transcriptional activity of selected genes in defined brain areas was presented. Transcriptional activity, as reflected by levels of mRNA, demonstrated that HI causes gene-specific alterations of mRNA levels, and that such changes are often area-specific, tending to be more pronounced in cortical and hippocampal areas. We found that none of the examined gene expression patterns (BDNF, MANF, HSP70, MAP2, GFAP, NgR, LDH-A, LDH-B) had been fully normalized by treatment at the time of examination. For instance, MAP2 mRNA decreases are indicative of neurodegenerative events. A LDH-A/LDH-B ratio shift suggests disturbed energy homeostasis. We noted that cooling does not uniformly decrease gene activity. Our study identified three different patterns for different genes, (1) HI induced decrease, counteracted by cooling, (MANF and MAP2 mRNA), (2) HI induced increase, counteracted by cooling (BDNF and GDNF mRNA), and (3) HI induced increase, further potentiated by cooling (HSP70 mRNA). Overall, we found that cooling to 33.5°C is better than less or more cooling, with cooling to 35°C having some protective effects and cooling to 30°C being associated with certain negative effects. We suggest studies of dynamic changes of defined mRNA species may become particularly helpful in order to evaluate effects of pharmaceuticals e.g. to counteract apoptosis, as a complement to cooling.

To summarize, we have addressed HIE issues by evaluating NIRS as a possible diagnostic method, by identifying unsatisfactory results of mere passive cooling of infants in transport, by the identification and testing of a Glauber salt with phase change properties, in phantom experiments and as a coolant in a well established piglet HI model.

We have also searched for additional biomarkers of HI and its treatment using quantitative in situ hybridization to monitor transcriptional activity of selected genes in different brain regions in piglets undergoing HI and therapeutic hypothermia.

7 FUTURE DIRECTIONS

As alluded to previously, there is room for improvements at all levels in our attempts to improve quality of life for children at risk for HIE. Faster and more precise diagnostic tools should guide better decisions about suitable treatments. It is anticipated that different forms of non-invasive functional in vivo brain imaging/on line recording technologies will continue to develop, including magnetoencephalography (MEG), MRI, MRS, EEG and NIRS in addition to novel forms of CT scans to help monitor the functional status and energetics of the newborn brain. Several of these techniques may allow bedside evaluations.

In terms of treatment, there is room for improvements with respect not only to cooling methods, but also to cooling protocols, and ways to monitor local temperatures and effects of cooling during on going hypothermia. It is anticipated that several other complimentary or possibly stand-alone methods will be developed. Although there are differences, there are also principle similarities between the catastrophic local loss of blood circulation in stroke and neonatal HIE. Therefore, the development of pharmacological means to dampen the effects of strokes, may find use in the treatment of neonatal HI and vice versa. However, despite enormous efforts, and despite positive effects in small animal models, neuroprotective drugs that also work in large animal models and human stroke have been very hard to find. One explanation may be that most drugs rescue the penumbra zone, which is similar in thickness across brain sizes, and therefore a much larger percentage of a stroke volume in a small brain than in a large brain. A recent report that imatinib normalizes the blood-brain barrier in a rodent model of stroke and thereby causing better recovery (Su et al 2008) is promising, and may potentially have effects that go deeper than the penumbra zone. However, the smaller volume of the newborn brain would also suggest that drugs that “only” protects the penumbra might be more valuable in newborns than in adults.

In the future, combination treatments for perinatal hypoxic ischemic events may become common practise. In addition to drugs mentioned above, hypothermia could be combined with pharmaceuticals to increase plasticity, or to target IL-1b and inflammatory processes as shown by Clausen et al in mice (Clausen et al 2011). There are several NMDA blockers (including xenon) that work in small animals and should be explored in larger animal models. There are also models that involve drugs that will lower body temperature for short periods of times. It has been suggested that low doses of such drugs might be a way to control the initial instability as well as the rewarming stages after cooling.

One area of particular future interest is HI-induced disturbances that cannot presently be detected at a time when protective treatments might have helped. Various forms of diffuse damage, alterations of neuronal migration patterns or timing, irregularities of normal developmental apoptosis, temporary disturbances of the developing blood-brain barrier, possibly with invasions of blood-borne cells causing low level immune and inflammatory reactions, lasting effects in the form of abnormal epigenetic alterations, disturbances of astroglial function or myelination, may all escape current early diagnosis tools. Symptoms

of various types, may nevertheless emerge years after birth. Thus there is a need of improved early diagnostic tools.

It is unlikely that there will ever be satisfactory treatments for all neonatal HI injuries. Therefore, an important future need is to also improve later treatment and training programs for children and adults who suffered from neonatal HIE. In recent years, the plasticity of the brain has come into focus. Learned skills and lasting memories are stored in the form of structural alterations of synaptic circuitry. The molecular events underlying such reorganisations are currently being revealed. BDNF is invariably increased and NgR appears to be invariably decreased (Josephson et al 2003) by increased neural activity, allowing synaptic changes to occur and to carry new abilities and memories. Recently, it was shown in a genetic mouse model that inability to down-regulate NgR leads to inability to form lasting memories (Karlén et al 2009). These mechanisms are operational also in adults. Thus in stroke, as well as in other conditions with brain damage, systematic, intense and focussed training may lead to better recovery than previously appreciated.

It has been a rewarding experience to participate in the Lagercrantz group with associated researchers and clinicians addressing neonatal development and treatments of disturbances thereof. From this horizon one finds that stem cells, e.g. from the umbilical cord, may be engineered and used to improve recovery as studied by Lothian and colleagues (Frisen et al 1998, Johansson et al 2002, Lothian et al 1999). Herlenius and colleagues have recently provided interesting data to suggest that grafted neural stem cells may provide neuroprotective effects through gap junction connections with host cells, formed by Connexin 43, Gap43, and that the potassium-chloride transporter, KCC2, needs to be taken into account when designing stem cells for combination treatments (see Theses by Jäderstad J. (<http://publications.ki.se/jspui/handle/10616/40283>) and Horn Z. (<http://publications.ki.se/jspui/handle/10616/40384>)).

A better understanding of the proinflammatory TNF- α pathway may also lead to novel ways of dampening neurodegenerative events caused by HI (Aden et al 2010).

Resting state fMRI is rapidly developing (Bruhn et al 2001, Fransson et al 2011, Fransson et al 2009, Fransson et al 2007), and may help focus on areas that may be important for memory and brain areas active later in life. Results from our colleagues at UCL in London, and the neonatal clinics units in Stockholm provide a hopeful outlook in terms of large animal modelling and clinical applications of improved HI diagnosis and treatment protocols.

Continuous work to find good biomarkers for HIE (Bennet et al 2010) including regulation at the transcriptional and posttranscriptional level of proteins by HI insults and the treatment of such insults is ongoing. Parts of the present thesis work is an attempt to contribute to the biomarker search and to understand how important genes are influenced by HI and its treatment, including genes of importance for brain plasticity and repair. Findings concerning HIE injuries in prefrontal cortex and hippocampus combined with the understanding of how prefrontal cortex inflicts memory tasks (Wendelken & Bunge 2010) may lead to new improved treatments based on individualized tasks and working memory training, as shown by e.g. Klingberg et al (Klingberg et al 2005).

There are several ways to obtain mild hypothermia. In addition to whole body cooling and head cooling, an interesting approach developed for heart patients is intranasal cooling (Castren et al 2010, Janata et al 2008) as also shown in animal studies with ice slurries (Janata et al 2008). In both these cases PCM could be used in different ways, and adopting the clinical hypothermia protocols to be used in future treatments for neonatal asphyxia. While brain cooling would be faster, fast heating and cooling may increase risk of blood vessel rupture as has been shown previously for fast heating. This may actually cause more damage than conventional hypothermia treatments. The relation between brain and tympanic temperature in humans has been described (Mariak et al 1994). The correlation allows reasonable estimates of brain temperature from tympanic temperature. Slurries of other compositions of PCM than used in the present studies have beneficial thermal properties, although they are not yet non-toxic (Augood et al 2001, Zhang et al 2010, Zhang & Niu 2010). In the future, these could be used in the form of microencapsulated material that will be under constant heat flux (Alvarado et al 2007) while circulating through a bag inserted into the patient's nasal cavity.

Implementation of new techniques such as hypothermia with or without PCM in low resource settings is challenging for a number of additional socioeconomic and medical reasons. Children in some areas are badly resuscitated due to extreme situations, weather and other health factors, including costs that cannot be met by the parents/caretakers. The need for modern equipment (Wyatt 2008), overcrowded delivery rooms, overcrowded rooms for the first days of care, and parents that cannot be away from work to take care of their newborn, are all factors that must be taken into consideration (Mullany 2010, Pasha et al 2010, Trevisanuto et al 2011, Wall et al 2009). Successful implementation of cooling methods in low resource settings must include the delivery of sustainable knowledge that can be handled by the local hospital and its staff.

The PCM possibilities in medical practice are numerous. One of the more challenging parts of the field is stroke prevention and stroke patient transport to regional hospitals. One interesting field to explore is fever convulsions in children. All children who develop high fever need to be taken care of and sometimes lowering the fever with an external heat sink rather than by pharmaceuticals might be preferred. This also can come in handy in discussions about multi-resistant bacteria, now becoming a big issue, when traditional cooling with antibiotics may be futile.

One aspect of cooling is simplicity and stability, because the results of cooling in terms of body and brain temperatures in piglets can vary with device and environmental factors (Christensson et al 1993, Jacobs et al 2007b). PCM may be designed to offer stable cooling to a set temperature (Mehling et al 2008) also in situations where there is a lack of water and/or electricity such as during transport or in small remote hospitals (Kumar et al 2009, Robertson et al 2008, Thayyil et al 2009). The current work has provided evidence that a composition of Glauber salt-based PCM may fulfil the need for a safe, low-cost, low-tech cooling material allowing the implementation of neonatal cooling in remote settings of the developing world.

From a research standpoint the results we have presented in paper V and VI, open up a lot of possibilities such as what happens at the protein level rather than the mRNA level. We

can also explore other important genes or areas in the brain, such as other cortical areas, cerebellum and other areas or regions of interest, to provide information in response to other groups who need answers from large animal studies. Not only is it then of translational importance that the studies were carried out in an almost human-sized mammal, the size of the brains also allows for a large number of different genes to be studied in each brain, which increases the statistical power of gene comparisons while limiting the number of animals needed.

7.1.1 Implementing PCM at hospitals and during transport

The results from our research and that of our collaborators both when it comes to hypothermia and transports (Hallberg et al 2009, Robertson et al 2010) suggest that we should start clinical trials using the mattress that we have produced. Trials should start not only in the Western world, where we, together with colleagues in Uppsala have tried it for helicopter transports (preliminary results show that it works for this type of transport even though the PCM Glauber salt solution was regarded as somewhat rigid) and at UCL, where we have just started transport studies, together with Medical Cooling Sweden AB, but also in a global perspective in countries like India, Moldavia, Uganda, Ecuador or Vietnam. We suggest to run these clinical trials in a similar way to the ones carried out by other groups (Jacobs et al 2011, O'Reilly et al 2011, Thoresen et al 2009, Uren et al 2009), but using PCM instead of ice or cold water gloves. In less fortunate rural areas, we suggest to also look into use of the mattress as the method of choice for the entire hypothermia protocol period. In the industrial world, there are situations where servo-controlled transport equipment (Johnston et al 2011a) can not be used, such as emergency or unplanned transports between hospitals or in small private clinics, to start the hypothermia procedures before the 6 h time limit and before transport to the regional cooling centre.

To get the best out of the PCMs we should keep searching/developing new materials as a collaboration between academia and commercial entities specialized in the field (e.g. Medical Cooling Sweden AB, Rubitherm GmbH, TST AB, Climator AB) and others in the field of engineering and energy transport. It is essential to further follow up these fields (Chen et al 2008, Chen et al 2006, Goldstein et al 2010, Mehling & Cabeza 2008) and collaborations with other groups are important to understand energy transport in PCM and combinations of PCMs. Our mattress must also work in different climate zones, and therefore might need to contain different amounts and compositions of PCM and be of different designs to be as efficient as needed in different climates. We also suggest to widen transport applications to other medical needs such as stroke patients and other medical subfields. Work of others (Baldwin et al 2010, Castren et al 2010, Gunn & Bennet 2010) also suggests there are unmet medical cooling needs for the future. The development then will have to deal with compartmentalization and maybe larger areas of PCM that are not covered by the "patient", in order to last for longer times, and/or to be as soft and flexible as possible while still optimized for the hypothermia in need.. New durable textiles (Shin et al 2005a, Shin et al 2005b, Sorrentino et al 2008) compatible with PCM inside that minimizes the loss of energy transport between the main PCM block and the patient during transport should be used.

8 SWEDISH SUMMARY

Denna doktorsavhandling sammanfattas som forskning kring vad som sker när nyfödda drabbas av allvarlig syrebrist samt 1. Hur man implementerar högteknologisk diagnosutrustning i sjukvården (Artikel I), 2. Vad som sker under transport med temperaturen hos barn med syrebrist (Artikel II), 3. Om vi med fasförändringsmaterial (PCM, engelska för Phase Change Material) på ett enkelt men effektivt sätt kan finna en kylbehandling som kan fungera både under transport samt globalt för hela behandlingen, i områden där risken för syrebristskador hos nyfödda är betydligt större (Manuskript III), 4. Implementera den PCM-baserade kylmetoden i djurmodell (Artikel IV) 5. Samt analysera några av de gener som påverkas vid hypotermibehandling vid olika temperaturer samt med kombinationsbehandling med xenon (Manuskript V och VI).

Allvarlig syrgasbrist vid födelsen, Nyföddhetsasfyxi, drabbar ca 1-2 barn/1000 födslar och ungefär hälften utvecklar en medelsvår eller allvarlig hypoxisk-ischemisk encephalopati (HIE). Internationell konsensus enligt Cochrane efter de multicenter studier som gjorts, är att de barn som haft medelsvår neonatal encephalopati (NE) fått en förbättrad prognos (d.v.s. kombinerat utfall död och CP) efter tidig start (inom 6h) av nedkylning.

Frågeställning: Går det att med en alternativ kylmetod, PCM nedkylning, (PCM är en billig och säker metod med stora fördelar jämfört med vattenbaserade metoder då den är tekniskt mindre krävande och med lätthet kan användas i utvecklingsländer där elektricitet, vatten och tekniskt kunnande är bristvaror), få likande resultat som dagens vattenmadrassbaserade lösning?

Arbetsplan och metoder: Vi har först testat en högteknologisk metod för att mäta hemoglobinnivåer i hjärna hos barn med problem med ansiktigenkänning. Jag har varit med för att lära mig metoden då den i viss mån kan användas på de syrebristskadade barnen samt som teknisk expert. Vidare har studierna gått ut på att medverka vid ett antal djurexperiment med en djurmodell för syrebristskador och behandling av sådana skador i England, och där hjärnor genomgått vidare histologisk analys i Sverige med avseende på ett antal nyckelgener. Djuren har behandlats med kylning till olika temperaturer eller i en kombination med gasen xenon, (som påverkar de så kallade NMDA-receptorerna). Parallellt med detta har utveckling av fasförändringsmateriel skett i Sverige. Vi har sedan implementerat de slutsatser vi kommit fram till från våra PCM-saltstudier, på ett antal försöksdjur och därmed försökt påvisa att hypotermi med PCM är lika effektiv som de nuvarande vattenbaserade metoder som används för att minska hjärnskadeutbredning hos nyfödda barn med encephalopati.

I en analytisk fas har vi sedan: 1 undersökt aktiviteten hos nyckelgener i hjärnan på RNA, med hjälp av in situ hybridisering och immunohistokemi. Undersökta gener är Nogoreceptor, som påverkar långtidsminnet och plasticiteten, BDNF, hjärnans viktigaste neurotrofiska faktor, MANF en nyligen identifierad trofisk faktor, HSP70 en viktig s.k. "immediate early gene", MAP2, viktig för nervträdars integritet, och GFAP, ett strukturprotein i astrocyter. 2: försökt fortsätta utveckling av PCM-produkten för att finna rätt smälttemperatur, hårdhet på materialet etc. I en framtida fas planeras att barn som

drabbats av syrebrist kyls ned med PCM-madrasser under transport (PCM är oberoende av el och vatten) till centra där fortsatt nedkylning kan ske med den vattenbaserade metoden då sådan finns att tillgå inom ramen för BLFs neonatalsektions riktlinjer. I annat fall kan barn nedkylas under hela behandlingsperioden d.v.s. 72 timmar med enbart PCM material. Vår forskning har försökt bidra med ökad kunskap om PCMs egenskaper i intervallet 20- 45°C. Detta kan utnyttjas i en rad ytterligare olika medicinska och tekniska tillämpningar, såsom kylning av brännskador, i dialysmaskiner eller för upprätthållande av kroppstemperatur hos barn och vuxna med störd temperaturreglering.

Betydelse: Vi hoppas öka förståelsen av vad som sker i hjärnan hos det skadade barnet vid kylningsprocessen och med en tekniskt enklare metod såsom PCM metoden, minska skadeutbredningen hos barn utsatta för syrebrist. Vidare kan det vara så att genom att utnyttja vår framtagna produkt kan fler användningsområden med kylning som behandlingsmetod testas med material som ligger i en temperatur-zon som tidigare inte haft vetenskapligt stöd.

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