LEGAL PROTECTION FOR CHILDREN

PAEDIATRIC FORENSIC MEDICINE

PROGRAMME

THURSDAY MAY 19TH

Death scene investigation in sudden death in infants and small children

Welcome. Torleiv Ole Rognum

10.00: Why death scene investigation? *Director Geir Stene-Larsen, Norwegian Institute of Public Health*

10.10 Death scene investigation. Lesson from the UK. *Peter Fleming*

10.50: Death scene investigation as a voluntary health service. *Torleiv Ole Rognum*

11.10: Death scene investigation from the parents' point of view. *Trond Mathiesen*

11.25: Coffee break

11.40: Australian practice in sudden infant death. Police investigation or health care? *Roger W Byard*

11.55: Death scene investigation. Interplay between the police and the health professionals, accuse or comfort? *Unni Heltne*

12.15: Research on sudden unexpected deaths ▢ A new case control study in New Zealand. *Ed Mitchell*

12.40: The role of professionals involved in SIDS cases. *Jan Sperhake*

12.50: Summing up: Death scene investigation in SUDI. Moderator: *Frøydis Heyerdahl at The Ombudsman for Children*

13.00: Lunch

Child protection

14.00: Should doctors keep professional secrecy when child abuse is suspected? *Jens Grøgaard*

14.20: Police investigation in child abuse: health professional - partners or opponents? *Anne Alræk Solem*

14.40: The roles of the professionals in suspected child abuse. *Kristine Kiær*

15.00: Plenary discussion. Moderator: *Director Lars E Hanssen, Norwegian Board of Health Supervision.*

15.30: Coffee break

Case discussion

15.45-17.00: Battered or a domestic accident? In any case left in helpless condition! Arne *Stray-Pedersen*

A method to disclose inflicted suffocation? *Sara Moberg, TO Rognum*

Why and how do babies die? *Mike Wailoo*

The cry and die syndrome. *Roger W Byard*

Case. *Thomas Bajanowski*

Shaking? or Neglect? *Jens Grøgaard*

17.05: UNICEF: Caring for your baby at night leaflet. Is there a sensible way forward? *Peter Blair*

17.20-18.00: Discussion

FRIDAY MAY 20 TH

Brain stem research and SIDS

09.00: 25 years of brain stem research in SIDS. Where do we stand? Where do we go? *Hannah Kinney*

09.45: Is there any correlation between brain stem pathology and risk factors for SIDS? *David Paterson*

10.15: Aquaporins and their function in the brain. *Mahmood Amiry-Moghaddam*

10.45: Aquaporin 4 and SIDS. *Siri Hauge Opdal*

11.10 Coffee break

Shaken baby syndrome – does it exist?

11.30: Current status on shaken baby syndrome. Henry F. Krous

12.15: Case: von Willebrand and other coagulation disorders – possible contributory factors in shaken baby syndrome?
Arne Stray-Pedersen

12.25: The devil's advocate: The models for investigating shaken baby syndrome, do they fit? *Roger W Byard*

13.00: Lunch

Domestic violence and effect on early brain development

14.00: Post traumatic stress in infants, does it exist? *Jens Grøgaard*

14.20: Domestic violence: Impact on brain development. *Magne Raundalen*

15.00: Coffee break

15.10: A paediatric view on long time effects of child abuse and neglect. *Peter Sidebotham*

15.30: Abused children get traumatized parents. *Ada Sofie Austegard*

15.45: Effects of neglect on neurocognitive development. *Annika Melinder*

16.05: Plenary discussion

16.45-17.05: GWAS-studies in SIDS □ possibilities and challenges. Mechtild Vennemann.

18.00: ISPID Epidemiology working group

SATURDAY MAY 21ST

SIDS – predispositions and death mechanisms

09.00: SIDS: Fatal interplay between microbiological agens, environmental risk factors and genetic make-up?
Caroline Blackwell

09.30: SIDS: Fatal reaction to "harmless" stimuli? *Linda Ferrante*

09.50: Newborn hearing screening and prediction of increased risk for SIDS? *Peter Fleming*

10.00: Brain stem auditory pathways – significance for SIDS? *Hannah Kinney*

10.10: The inner ear: role in CO_2 detection & can the newborn hearing rest detect SIDS? *Daniel Rubens* and *Travis Allen*

10.25: Disturbed brainstem mechanisms and their implications for SIDS. *Jan-Marino Ramirez, Alfredo J. Garcia III, Henner Koch, Frank Elsen, Aquan Wei, Atsushi Doi*

10.40: Discussion and coffee

11.00: The Universal Age Distribution of the Sudden Infant Death Syndrome. *David T. Mage*

11.15: A proposed model that explains the gestalt of SIDS. *David T. Mage*

11.30: Coffee break

Pathological findings in SIDS

11.40: Return to pathology; Why do majority of SIDS cases reveal consistent findings?
Evidence of a single pathogenetic process? Genetics, preterm and postnatal insults, innate pathogen recognition, infection, and testosterone … Coinciding contributors to SIDS. *Paul N. Goldwater*

11.55: Sudden deaths in the first 7 days of life occuring within the birth clinics. *Jan Sperhake*

12.10: What are the effects on bereaved parents of investigation by professionals following the death of their child?
Joanna Garstang

12.25: Sudden Unexpected Death in Infancy (SUDI) Toolkit. *Allan Lynsay*

12.40: Discussion

13.00: Lunch

SIDS - predispositions and death mechanisms

The inner ear: role in CO2 detection & can the newborn hearing test detect SIDS?

Daniel Rubens, Travis Allen,
Center for Integrative Brain Research,
Seattle Children's Research Institute

We originally identified a hearing difference in 31 SIDS cases versus controls by their newborn hearing screen tests (Figure2). We then hypothesized that the inner ear plays an unrecognized integral role in respiratory control. We therefore investigated whether mice with induced inner ear injury demonstrated an alteration in their respiratory response to inhaled CO_2. Intra-tympanic gentamicin injections were administered to induce inner ear damage. Hearing loss and vestibular

an average of 50% over their baseline values for both parts of the experiment. In contrast, the ear-damaged group values increased by only 2-11%. Inner ear damage significantly reduces the respiratory response to CO2 inhalation. In addition to the established role of the inner ear organ in hearing and balance, this alludes to an unidentified function of the inner ear and its interconnecting neuronal pathways in respiratory regulation. This finding may offer valuable new clues for SIDS. Histological ex-
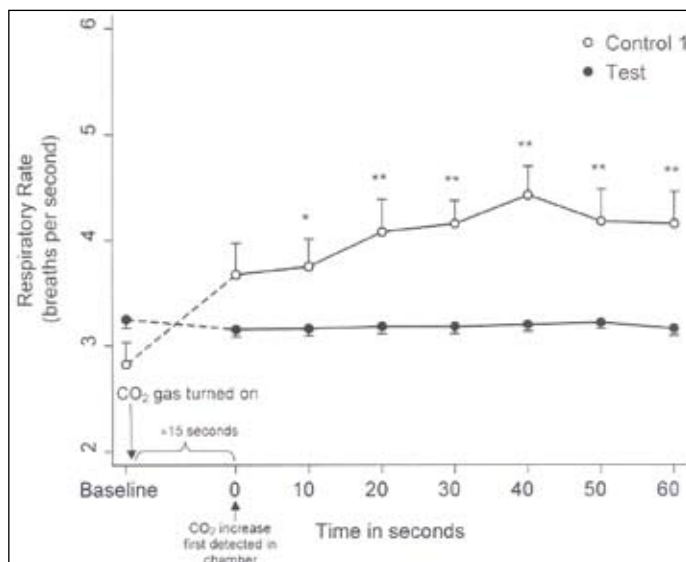

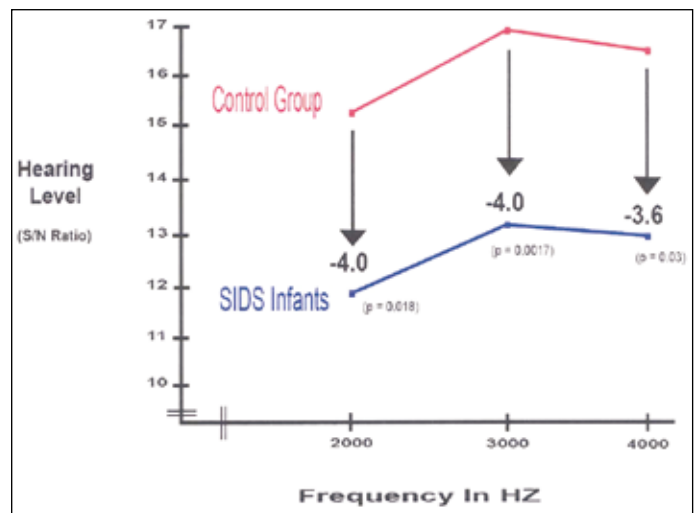Figure 1: (Study 1) Respiratory Response to CO2 Over Time

Figure 2: Graph demonstrating the right sided signal to noise ratiodifferences in thirty one SIDS infants versus matched controls

dysfunction were tested one week after injections to confirm presence of inner ear insult, following which the animal's respiratory response to inhalation of 8% CO_2 was examined. Mice with inner ear injury (n = 60) displayed a significantly diminished hypercapnic ventilatory response (HCVR) (See Figure 1). This contrasted with the normal HCVR seen in control mice that had not undergone tympanic injections (n = 30), controls that received tympanic injections with saline (n = 5), and controls that had gentamicin administered systemically (n = 5). In response to inspired CO_2, the mean respiratory frequency of control mice increased by

amination supported inner ear damage following intra-tympanic gentamicin injections without gross morphological insult to the respiratory centers of the brainstem. Our data supports an integral role of the inner ear and its interconnecting pathways in respiratory control that has not been previously studied. Further investigation of this mechanism is underway. In addition, we are planning a follow up hearing study in the UK to include a population of approx 15 million infants over a 2 year period. We will collect neonatal hearing screening data from all SUDI (approx 200 cases), with a control group of approx 600 infants.

Disturbed brainstem mechanisms and their implications for SIDS

Jan-Marino Ramirez, Alfredo J. Garcia III, Henner Koch, Frank Elsen, Aguan Wei and Atsushi Doi.

Center for Integrative Brain Research, Seattle Children's Research Institute, and the Departments of Neurological Surgery and Pediatrics, University of Washington School of Medicine, Seattle

Sudden Infant Death Syndrome (SIDS) has been associated with disturbances in the generation of sighs and gasps. These respiratory activities are activated under hypoxic conditions and serve as important autoresuscitation mechanisms. SIDS is also associated with disturbances in aminergic neuromodulators (in particular serotonin and norepinephrine), but it remains unknown how these different disturbances relate to each other and lead to SIDS. Here we propose that the pre-Bötzinger complex, an area located within the ventrolateral medulla plays a critical role in the events leading to SIDS. This area is critical for the generation of breathing, sighs and gasps. Under normoxic conditions multiple cellular, neuromodulatory and synaptic network mechanisms contribute to the generation of breathing and are capable of continuously compensating for each other in case any of these mechanisms is disturbed. This can be demonstrated in whole animals in which neuromodulators can be selectively lesioned. It can also be demonstrated in the isolated respiratory network in which different cellular and synaptic mechanisms can be abolished without eliminating respiratory rhythm generation. When challenged in hypoxia, however, the pre-Bötzinger complex reconfigures and becomes dependent on the persistent sodium current as well as serotonergic and noradrenergic mechanisms that are critical for the generation of gasping. Disturbances in any of these mechanisms lead to the failure to gasp and autoresuscitate. This increased vulnerability can be demonstrated by either blocking serotonergic, noradrenergic or persistent sodium-dependent mechanisms, and disturbing any one of these mechanisms is sufficient to lead to the failure to gasp. Prior exposures to hypoxia, oxidative stress or other stressors and risk factors will affect the response of this network and will increase its vulnerability. These stressors act directly on the cellular mechanisms that underlie autoresuscitation. Thus, we hypothesize that victims that succumb to SIDS have no apparent respiratory deficits under normal conditions because the respiratory network is capable of compensating for multiple genetic, metabolic or environmental disturbances. However, when challenged under severe hypoxic conditions evoked for example by a failure to respond to hypercapnic conditions, the reconfigured brainstem network will reveal these disturbances and will fail to autoresuscitate.

A Proposed Model that Explains the Gestalt* of SIDS

David Mage, Ph.D and Maria Donner, Ph.D

ABSTRACT "I varje utredning, hävdade Borkmann, finns det en punkt bortanför vilken vi egentligen inte behöver mer information. När vi nått fram till den vet vi redan tillräckligt för att lösa fallet med hjälp av enbart tankearbete." [Håkan Nesser, Borkmanns punkt, 1994]. We propose that the mystery of SIDS is now at Borkmann's Point where there is sufficient published information for it to be understood and completely explained. Based on what we know, SIDS is characterized by the following 14 clues that together constitute its gestalt and must be linked by any proposed model for the phenomenon of an infant dying suddenly and unexpectedly for no apparent reason:

1) Characteristic and unique 4-parameter lognormal age distribution;
2) Increased risk of prone sleep position;
3) Prone and supine SIDS have same age distribution;
4) Male and female SIDS have same age distribution;
5) Sudden unexpected respiratory deaths 'at home' have same age and gender distribution as SIDS;
6) No evidence of cause of death at autopsy or death scene;
7) SIDS spares infants at birth;
8) Seasonality: winter maximum, summer minimum;
9) Increasing SIDS rate with parity (live birth order);
10) Consistent male excess of ~50%: 0.62 White male fraction, 0.57 Black male fraction;
11) Low increased risk of SIDS in subsequent siblings of SIDS;
12) Parental smoking is a risk factor for SIDS;
13) ALTE is not a risk factor for SIDS.

Each of these 14 clues will be addressed and woven into a modified version of the late John Emery's model of SIDS that we propose to use now to solve its mystery.

*German: Gestalt = 'essence or shape of an entity's complete form.'

Twenty-five Years of Brainstem Research: Where do We Stand? Where do We Go?

HC Kinney,

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The brainstem is essential for mediating protective responses to homeostatic stresses that can complicate infant sleep, such as hypoxia, hypercarbia, or cardiovascular imbalances in the prone (face-down) position, or hyperthermia due to overbundling. Thus, SIDS, or a subset of SIDS, may be due to brainstem abnormalities that impair the the infant's ability to respond to homeostatic stresses during sleep, thereby leading to sleep-related sudden death. Charged by the inviting committee of this meeting to address the challenging questions in the above title, I will respond by reviewing: 1) the rationale for the brainstem hypothesis in SIDS; 2) the historical origins of SIDS brainstem research; 3) current brainstem findings in SIDS cases, with an emphasis upon those from our laboratory; 4) potential mechanistic consequences of SIDS brainstem pathology that are based upon illustrative studies in animal models; and 5) suggested directions for research to come.

Selected References:
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Pathological findings in SIDS

Return to pathology; why do a majority of SIDS cases reveal consistent findings? Evidence of a single pathogenetic process? Genetics, preterm and postnatal insults, innate pathogen recognition, infections, and testosterone . . . coinciding contributors to SIDS

Paul N. Goldwater

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Many paediatric pathologists expert in SUDI maintain that deaths from asphyxia are indistinguishable on gross pathology from SIDS without providing supportive evidence. While to the contrary, there is, however, evidence that pathological findings in SIDS are salient and consistent. They consist of 1) Intrathoracic petechial haemorrhages; 2) thymomegaly, 3) encephalomegaly, 4) evidence of microcardia, 5) liquid unclotted blood in the chambers of the heart, 6) kidney growth-restriction and 7) an empty bladder and rectum. Given the fact that most SIDS cases share this common pathological picture, it would in all probability, be reasonable to suppose these pathological features are the result of a single cause or a related underlying pathophysiology. While not congruent with mainstream consensus, it would seem improbable that such a regular spectrum of pathologies found in a majority of SUDI cases would be the result of a myriad or even a few possible causes. In the minority of cases of SUDI where the pathology is not typical of SIDS then it would be logical to consider pathogenetic mechanisms of a different nature in this subgroup.

My paper will draw the abovementioned pathologies (including brainstem and cardiac changes) into context with genetics (including gender), antenatal insults contributing to a vulnerable host (e.g. smoke exposure), bacterial and viral infection, evidence of defective innate pathogen recognition, postnatal insults (e.g. smoke exposure), testosterone and immunosuppression, the testosterone peak of infancy, and waning transplacentally-acquired immunity. The effect of proneness on brainstem-cardiac control will also be discussed. Supportive evidence for plausible links between prone sleep position and infection have been proposed based on bacterial colonization and toxin induction with raised nasopharyngeal temperatures prone, and bacterial contamination of the sleeping surface promoting colonization of the infant's nasopharynx and gut. Many aspects of this paper coincide with Caroline Blackwell's "Fatal interplay between microbiological agents, environmental risk factors and genetic make-up?"

Sudden Unexpected Death in Infancy (SUDI) Toolkit

Lynsay Allan, SUDI project lead.

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Organisation:
Healthccare Improvement Scotland

Aim: To improve the multiagency response to SUDI in Scotland by providing an easily accessible resource for all professionals involved.

Methods:
An overarching multi-agency Steering Group, sub-groups and short life working groups have progressed the following:
- Development of a resource which provides advice on good practice in the event of a SUDI for Crown Office and Procurators Fiscal service, community midwives, community health visitors, Emergency Department staff, general practioners, neonatologists, pathologists, paediatricians, public partners, the Scottish ambulance service and social workers.
- Inclusion of information about the SUDI review process for coordination of timeous SUDI review multidisciplinary case discussion meetings.
- Identification of a national dataset to: promote information sharing between agencies, inform professionals involved in the SUDI review and to evaluate the data collected and the review process.
- Engagement with bereaved families has provided feedback from those affected by SUDI to inform the SUDI Steering Group, sub groups and component parts of the project.

Results:
1. A toolkit for professionals was launched by the Scottish Government on January 18th 2011 and is available on www.sudiscotland.org.uk

2. The SUDI Scotland toolkit informs multi-agency professionals involved in SUDI to respond appropriately. Users may readily access the resources required and are signposted to current national guidance for their profession. The toolkit contains interactive timelines and flow charts, demonstrating the involvement of all professionals at various stages and their likely interactions. The toolkit provides information leaflets for staff and bereaved parents and details of support organisations and personnel available for both families and professionals.

3. The national data collection forms are available in the toolkit. National data collection will report the : incidence of SUDI, identify risk factors (which may inform future practice) and evaluate SUDI reviews from a professional and parental perspective.

The 'History and Examination' data collection form has been designed to promote information sharing thus avoiding the need for each agency to request the same information from parents, so reducing unnecessary distress at an extremely difficult time.

Conclusion:
NHS QIS has produced a multi agency professional resource for SUDI, unique for Scotland. The toolkit contains professional feedback forms. Each web page accessed and any resource downloaded, printed or emailed is tagged to allow evaluation of user engagement by Google Analytics reporting. The project will report on the evaluation of the toolkit, including its usefulness, ease of access, relevance of content, impact on response, impact on holding a timeous SUDI review case discussion meeting and provision of support resources for parents and professionals.

Soria Moria Abstract May 2011
Dr Joanna Garstang

What are the effects on bereaved parents of investigations by professionals following the death of their child?

Abstract: Since April 2008, all unexpected child deaths in England are subject to a multi-agency investigation by police, health and social services. The investigation includes a full medical history, a complete autopsy, death scene analysis and social services enquiries. The process may be highly invasive of the privacy of the grieving family in the days immediately after their child's death. At present there are limited data on the effectiveness of the multi-agency approach and no data on the experience of this approach on the bereaved parents. A large scale project is underway, in the West Midlands, to evaluate the multi agency approach and to learn of both the bereaved parents and professionals experiences.

In the initial stages of the project a mixed studies systematic review of the literature is being undertaken. It is focusing on the effects on the bereaved parents of interactions by professionals. I will present the aims and methodology for this study, along with findings of the literature review and reflections on how we can effectively study professional interaction with parents following a sudden unexpected infant death.

Intraalveolar macrophages – the sign of suffocation?

Sara Moberg, Torleiv Ole Rognum

Institute of Forensic Medicine,
University of Oslo

It has been proposed that intraalveolar haemorrhage and iron containing macrophages may help the diagnose of suffocation in cases of sudden unexpected death in infants (1,2). Half of the infants that die from suffocation have intraalveolar haemorrhage (1,3). In cases of repeated episodes of suffocation iron containing macrophages has been described in lung tissue (2). Some studies also show that SIDS victims may have interstitial iron containing macrophages (2), but that intraalveolar macrophages is more often seen in victims of suffocation (2).

The aim of the study was to:
1. Develop a reproducible semiquantitative scoring system for estmation of amount of intraalveolar haemorrhage (IAH), interstitial iron-containing macrophages (IICM) and intraalveolar iron-containing macrophages (IACM) in the lungs.
2. To investigate a possible relationship between the amount of IAH, IIMC and IACM in cases of suffocation and controls (SIDS).

Subjects. Thirtysix infants and small children (mean age 21 months, range 0 – 102 months) from Southeast Norway, diagnosed with suffocation or suspicious suffocation at the Institute of Forensic Medicine, Oslo between 1989 and 2010, were investigated. As controls 34 aged matched SIDS cases and cases of sudden death in todlers were used (mean age 15 months, range 0 – 111 months).

Tissue sections. One peripheral and one central section from each lung lobe (all together 10 sections) were investigated from each case. The sections were stained with Perls and HAS (Hematoxyline-Azophloxine-Safran).

Evaluation. The sections were scored for amount of intraalveolar haemorrhage (IAH) according to a semi-quantitative scoring system propsed by Krous et al (4), and for amount of interstitial iron-containing macrophages (IICM) and intraalveolar iron-containing macrophages (IACM) according to a semiquantitative scoring system modified after Hanzlick et al (5), 0 representing virtually no haemorrhage or positve staining and +4 extensive haemorrhage or extensive distribution of IICM and IACM. The scoring system for IICM and IACM was validated by morphometric technique counting IICM and IACM in 12 cases by means of ocular grids. At least 15 grids had to be counted to obtain stable means. The same 12 cases were also scored blindly by means of the semi quantitative scoring system. When comparing the results of the scoring and those of the morphometric counting by nonparametric correlation method, a good correlation was found (tau=0.7, p<0.01). Two observers (SM and TOR) scored the material in parallel; SM repeated the scoring blindly after 6 weeks. Inter individual and intra individual reproducibility for IAH were good (kappa= 0,7), and fair (kappa= 0,5), respectively. The inter- individual reproducibiliy for IICM and IACM were good (kappa= 0.65 and 0.62, respectively).

The mean score for intraalveolar haemorrhage, intraalveolar iron containing macrophages and interstitial iron containing macrophages was calculated in each case.

Results. The score for intra-alveolar iron containing macrophages was significantly higher in victims of suffocation than in the controls (p=0.041, X2 test, Monte Carlo). Score >2 was only observed in the cases of suffocation. There was no difference between the amount of intra-alveolar haemorrhage or the content of interstitial iron containing macrophages between the victims of suffocation and the controls (p>0.05, X2 test).

Conclusion. The number of cases with a clear-cut history of suffocation is relatively limited in this study. Nevertheless, the result indicate that a high score for intraalveolar iron containing macrophages (>2) support the diagnose of suffocation if no other explanation are available.

The failure to relate IAH to suffocation is in accordance with the finding of Krous et al (4).

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Risk factors in Sudden infant death syndrome (SIDS)

Marianne Cathrine Rohde, Lisbeth Lund Jensen, Jytte Banner

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ABSTRACT: The recommendation against prone sleeping was introduced in December 1991 in Denmark, and was followed by a marked reduction in SIDS cases. In the period from 1992 to 2008, 82 of 233 autopsied infant deaths were classified as SIDS at our institution, which serves 2.2 million citizens. The number of SIDS infants was reduced from 13 per year in the two first years after the introduction of the 'Back to sleep campaign' (1992 and 1993) to 3.7 per year in the following 15 years (1994-2008). Risk factors in two time periods were compared. We found an expected decrease in the number of SIDS infants placed and found prone from the first to the second time period and that the mothers placing their infant prone were older than the ones placing them supine. None of the epidemiologic parameters such as age at death, male ratio, prematurity, birth weight, month of death, and maternal age had changed significantly between the two time periods. While looking at signs of infection, a significant increase in the proportion of cases with positive bacterial culture was seen, in spite of an unchanged post-mortem interval. At the same time, a decrease in the proportion of cases diagnosed with beginning/possible interstitial pneumonia on histology was seen. To conclude it seemed that stress related risk factors were changed to some extent in the "post epidemic phase" parallel to the decrease in SIDS incidence, and that the few remaining infant deaths that we can still not explain may be less related to prone sleeping.

INTRODUCTION

Sudden death in infancy is a much debated and studied area in forensic and pathological research. In Denmark we experienced a significant rise in the numbers of Sudden Infant Death Syndrome (SIDS) cases in the late seventies, and this lead to the foundation of a Nordic collaborative study group of pathologist, epidemiologist and pediatricians, to unite forces [1-3]. After the campaign against prone sleeping and the introduction of cool sleeping environment recommendations in December 1991, an almost visually vertical decline in the SIDS contribution to death rates were seen in all the Nordic Countries [4]. Today only 1-3 infant deaths per year are categorized as SIDS in our institution if any, perhaps both due to a decline in the incidence and change in diagnostic capabilities and routines. As a consequence of the decline in SIDS numbers in Denmark only few papers have been published on SIDS with Danish data from 1996 and later [5-7].

In spite of the years of research in SIDS the pathophysiological mechanisms behind SIDS are still unknown and SIDS remains a diagnosis of exclusion [8;9]. The widely accepted triple risk hypothesis states that SIDS is caused by a combination of three factors a) predisposition b) vulnerable period in the infant's life and an c) exogenous stressor [10]. Prone sleeping, overheating and signs of infection are known risk factors for SIDS and have been suggested as this above-mentioned exogenous stressor [11-14]. The same factors are known to lead to heat stress and the induction of cellular stress responses such as increased synthesis of the inducible heat shock proteins [15;16]. This response is a part of a defense mechanism in the cells to protect against irreparable damage that may be the result of stressors like elevated temperature [15]. Risk factors affecting cellular stress response are of the outmost interest in SIDS research, and we have studied heat shock responses in SIDS cases and found that they may have a different response after stress exposure in selected genes of the stress response compared to control cases [17].

The aim of this study is to display differences in factors inducing cellular stress in SIDS infants between two periods in the post-epidemic phase. We focused on differences in the frequency of stress potentiating factors between the period straight after the campaign against prone sleeping was introduced in Denmark where the campaign was under adaption (1992-1993), and the following 15 years hereafter (1994-2008) where the number of SIDS declined dramatically and stabilized at a low number of approximately 0.2 per 1000 live born infant.

MATERIAL AND METHODS

Case files from Department of Forensic Medicine at Aarhus University, Denmark were examined for infant cases from a 17 year period from 1992 to 2008. The Department of Forensic Medicine at Aarhus University covers the majority of Western Denmark and was in 2007 serving around 2.2 million of Denmark's around 5.5 million citizens. A total of 233 infant cases were autopsied from 1992-2008 and 82 of these were classified as SIDS.

Two study periods were defined in the present study. A national study showed that after 1993 prone sleeping was reduced to 5% [18], compared to 50 % percent before the campaign, therefore 1993 was used as cut point to illustrate differences before and after the new recommendations. Twenty-six of the 82 infants died in the first study period (1992-93), and 56 died in the second period (1994-2008). At the Department of Forensic Medicine at Aarhus University it is procedure that the senior pathologist assigned to the case is coding the case using a system based on the SNOMED codes. The code 'Unexplained infant death' is equivalent to SIDS as defined in the Nordic SIDS criteria. At the end of each year all cases are again reviewed by a senior pathologist. In the study period 1992-2008 cases were reviewed by the same senior pathologist to ensure consistency in the way the cases were coded, and the Nordic SIDS criteria have been applied for research purposes [19]. All the investigated cas-

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es had undergone full autopsies. The extent of ancillary tests in the SIDS cases varied depending on the year of the autopsy. Details of the cases were reviewed and relevant data including epidemiological data, circumstance of death, available medical history of the infants, manners of the death, and any significant autopsy findings, histology result and results of ancillary tests together with coded cause of death were entered in a database using EpiData 3.1 software. Information on epidemiological factors (age, sex, season of death, birth weight, mothers age), environmental factors (prone sleeping, co-sleeping, head coverage when found) and available information on potential risk factors related to heat stress such as possible signs of infections and hyperthermia (positive growth of bacteria in culture, reactive changes on lung histology, signs of infection on lung histology, symptoms on infection prior to death) were selected and extracted from the database. As the material primarily included cases autopsied prior to the implementation of viral diagnostics as a part of the autopsy protocol we chose to focus on bacterial microbiology. Right and left lung tissue, blood extracted from the heart and cerebrospinal spinal fluid is routinely investigated in all cases of infant death with no apparent cause of death, and the results of the microbiological tests are interpreted with regard to location of the sample, usual flora of the site, pathogenic potential, histology and reported symptoms. Non-routinely additional relevant tissues, bodily fluids or samples are tested (e.g. middle ear and tracheal secretion, urine or stool samples), according to the findings and the history.

SIDS infants dying in the first two years after the campaign against prone sleeping (1992-1993) and infants who had died the following fifteen years (1994-2008) were then compared on the selected variables. For statistical analyses STATA software version 10.1 was used.

RESULTS

A reduction in the number of SIDS deaths from 13 per year to 3.7 per year was found (fig. 1). In the latest seven years (2002 to 2008) only eight SIDS
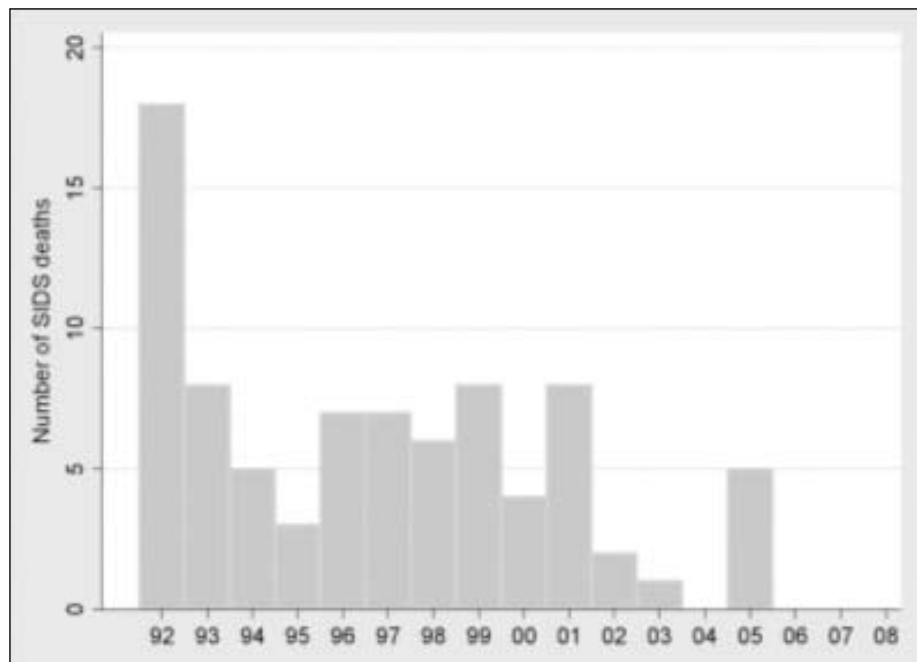

Figure 1: Distribution of SIDS death from 1992 to 2008 in Department of Forensic Medicine Aarhus University. X-axis shows years, and Y- axis absolute numbers of infants categorized as SIDS.

deaths equaling 1.1 SIDS deaths per year were registered.

Epidemiologic data
In the first two years after introduction of the campaign against prone sleeping, there was a seasonal variation in the number of SIDS deaths with a higher incidence in the winter months (fig 2). This seasonal change

was less pronounced in the following 15 years, but the difference between the two periods was not significant.

There seemed to be a slightly increased proportion of premature infants in first period (17% versus 11%), however this difference was not significant either (table 1). Neither age, sex, birth weight, prematurity nor maternal age showed any significant
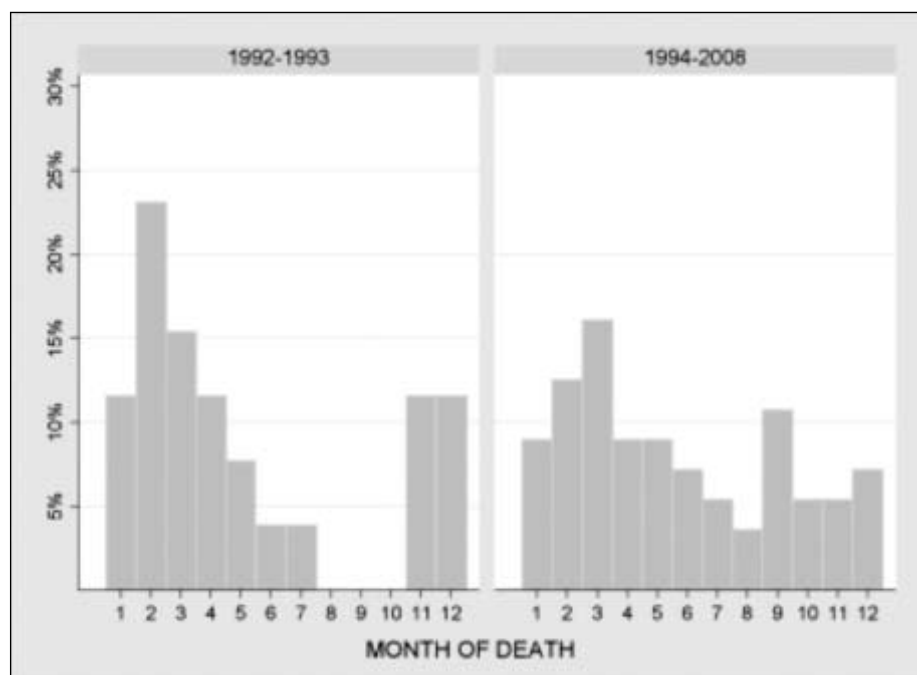
There Figure 2: Seasonal change in SIDS death between first period and second period shown in percent of total number of SIDS deaths in the periods respectively.

Table 1: Distribution of epidemiologic variables over the two time periods.

VARIABLES	1992-1993 (n=26)	1994-2008 (n=56)	Total (n=82)	P-value
Age, weeks				NS
Mean (min-max)	12.2 (2.7-21.4)	13.8 (0.4-45.6)	13.3 (0.4-45.6)	
Sex				NS
Male ratio	69%	59%	61%	
Female ratio	31%	43%	39%	
Prematurity				NS
Number of cases with data	18	36	54	
Premature (%)	11% (2/18)	17% (6/36)	15% (8/54)	
Birth weight, g				NS
Number of cases with data	18	35	53	
Mean (min-max)	3151 (2130-4000)	2955 (1010-4090)	3021 (1010-4090)	
Peak season of year	February	March	February=March	NS
Maternal age				NS
Number of cases with data	13	39	52	
Mean (min-max)	27 (21-44)	27 (18-37)	27 (18-44)	

differences between the two periods of time (table 1).

Environmental stress factors

Infants dying in the first period were placed prone in 69% of the cases while infants during the second period were reported to have been placed prone in 5 (19%) of the cases, whereof two were in 1994, one in 1999 and two in 2000. The difference was significant (Fisher's exact, p=0.003) though information on how the infant was placed were missing in (n=39) 48% of the cases. The proportion of infants found prone was significantly (Fisher's exact, p=0.007) higher in the first period compared to the second period (77% versus 38%) (table 2). The age of the mothers appeared to have influence on whether the infants were placed prone or supine with a significant tendency for mothers placing their infants prone to be older than the mothers who placed their infants supine with a of mean age 29.9 y versus 25.7 y respectively (Mann-Whitney, p=0.0266). There were no significant differences in the age of the mothers between the two periods of time when not considering how the infants were placed. When comparing the infants placed prone versus supine in first period and second period respectively there were no significant differences as well. The proportion of SIDS infants co-sleeping with their parents seemed to have become more common in the second period increasing from 17% in the first period to 36%, but this rise was not statistically significant. This proportional increase

Table 2: Distribution of environmental stress factors in the two periods. Number of cases with data reflects the number of the total 82 SIDS infants were we had information on this specific issue. The percentages presented are of the total number of cases with data in the period.

VARIABLES	1992-1993 (n=26)	1994-2008 (n=56)	Total (n=82)	P-value
Placed in prone position				
Number of cases with data	16	27	43	
%	69% (11/16)	19% (5/27)	37% (16/43)	P=0.003
Found dead in prone position				
Number of cases with data	22	39	61	
%	77% (17/22)	38% (15/39)	52% (32/61)	p=0.007
Face downward or head covered				
Number of cases with data	18	23	41	
%	61% (11/18)	61% (14/23)	61% (25/41)	NS
Co-sleeping with one or more adults				
Number of cases with data	23	55	78	
%	17% (4/23)	36% (20/55)	31% (24/78)	NS

was primarily due to a fall in SIDS infants not co-sleeping as the absolute number of co-sleepers were 4 and 15 in the two time periods respectively corresponding to 2 per year in the first period and 1.3 per year in the second period. In 10 of the 12 cases of co-sleeping where information on sleeping position was present, the infants were found supine. One infant from the first period was found prone and one from the second period found on its side. Face covering did not prove any significant differences between the periods of time.

Heat stress factors – signs of infection and/or hyperthermia

A review of the microbiology showed a significantly (Fisher's exact, p=0.003) larger percentage of cases with bacterial growth believed to be either 'normal flora/contamination' or 'unknown whether contributing to death' in the second period than the first (89% versus 58%) (table3). This was due to a higher amount of cases with 'normal flora/contamination' as there was almost no difference in the frequency of cases where microbiology was described as unknown whether contributing to death. The most common bacteria detected in the samples described as 'normal flora/contamination' was non-hemolytic streptococci (33% of the samples), Staph. aureus (17%), coagulase negative staphylococci (9%), E. coli (5%), K. oxytoca (4%), S. pneumoniae (4%), and Staph. albus (4%). Routinely samples were taken for bacterial microbiology from four places (lungs, blood and spinal fluid), and then non-routinely supplemented with extra samples from areas of interest in the specific case. The amount of sites sampled had increased from the first to the second period from 4.1 sites per case to 4.4 sites per cases. There were no significant differences in the post mortem interval (time from death/ found death to autopsy) between the two time periods.

While 36% of the SIDS cases from the first period were mentioned having slight reactive changes (beginning/possible interstitial pneumonia included) only 20% of the cases from the second period showed this kind of change, however this difference was not significant. When focusing strictly

Table 3: Distribution of signs of inflammation that may be present in SIDS and indications of exposure to hyperthermia over the two periods

VARIABLES	1992-1993 (n=26)	1994-2008 (n=56)	Total (n=82)	P-value
Bacterial growth in culture (normal bacterial flora and/or contamination/unknown if contributing)				
Number of cases with data	26	56	82	
%	58% (15/26)	89% (50/56)	79% (65/82)	p=0.003
Normal bacterial flora/contamination				
Number of cases with data	26	56	82	
%	46% (12/26)	82% (46/56)	70% (58/82)	p=0.002
Unknown if contributing				
Number of cases with data	26	56	82	
%	8% (2/26)	7% (4/56)	9% (6/82)	NS
Minor interstitial inflammation (lymfocytic)				
Number of cases with data	25	56	81	
%	16% (4/25)	0%	5% (4/81)	p=0.008
All reactive changes in lung tissue (interstitial and alveolar change)				
Number of cases with data	25	56	81	
%	36% (9/25)	20% (11/56)	25% (20/81)	NS
Symptoms of infection prior to death				
Number of cases with data	20	40	60	
%	35% (7/20)	53% (21/40)	47% (28/60)	NS
Temperature above 38°C in the days prior to death				
Number of cases with data	5	9	14	
%	0%	33% (3/9)	21% (3/14)	NS
Temperature above 37°C measured during resuscitation				
Number of cases with data	10	18	28	
%	10% (1/10)	28% (5/18)	21% (6/28)	NS
Was the infant moist when found				
Number of cases with data	5	18	23	
%	40% (2/5)	39% (7/18)	39% (9/23)	NS

on the cases where beginning/possible interstitial pneumonia was described in the autopsy report the difference between the two time periods was significant (Fisher's exact, p=0.008) with 16% of the cases from first period with this diagnosis compared to none in the second period. In three of the four variables concerning the history of the child (symptoms of infection prior to death, fever with temperature above 38°C in the days prior to death, and rectal temperature above 37°C measured during resuscitation), the proportion of positive cases were highest in the second period. The difference between the two periods ranged from 18 to 33 percentage points (table 3), but were however not significant. The variable 'was the infant moist when found' was almost evenly distributed over the two time periods and showed no significant differences.

DISCUSSION

Epidemiologic data
The peak number of infants dying of SIDS was found in 1992 where recommendations on supine sleeping had only recently been introduced, and therefore was not followed by all. The success of the campaign is evident in the following years, as the reduction in the number of deaths is already seen in 1993. We decided to divide data in two periods, to reflect a period where prone could be considered the most important risk factor, and the second period, where prone sleeping should be reduced, and therefore become less important as a risk factor. Worldwide campaigns against prone sleeping have markedly reduced the number of SIDS deaths and as a side-effect have led to changes in the epidemiologic characteristics of SIDS [20-23]. Data from the UK have shown an increase in the proportion of pre-term infants over the past 20 years and a less pronounced seasonal variability which is consistent with the trends of our findings although our result were not sta-

tistically significant [20;24]. Neither age of the infant, maternal age, male ratio nor birth weight had significantly changed from the period right after the introduction of the campaign against prone sleeping in Denmark and the following fifteen years which also is in keeping with the literature[20;24].

Environmental stress factors
As expected the proportion of infants placed prone and found prone was significantly reduced from first period to the second period where parents apparently adapted recommendations from the health care system. In some materials there has been a discrepancy between the carers information on how the child was found and the livor mortis, suggesting that the child had been found prone in some of the cases where it had been reported as being found supine [25]. In our dataset the lividity pattern correlated with the information reported by the carers in all cases with available information (data not shown). When correlating the information on how the infants were placed with the age of the mother we found that mothers placing their infants prone in general were older than the ones placing their infants supine. The older age of the mothers placing their infants prone could suggest that fewer of them were first time mothers and they trusted their own good experiences with placing their first children prone and that this fact made them less susceptible to information against prone sleeping. Unfortunately we lacked information on how many of the mothers who had more than one child, but other studies have shown a tendency for mothers with more than one child to be more likely to place their infants prone in the period right after the "Back to Sleep" campaign [26].

While prone sleeping is a universally accepted risk factor, co-sleeping as a risk factor for SIDS per se is a considerably more controversial subject [27;28]. It is nevertheless relatively widely agreed that co-sleeping can increase the risk of SIDS under certain circumstances such as co-sleeping on sofas, parental smoking and alcohol consumption and in the first few months of the infants life [27;29-31]. In our study we found an increasing but not significant proportion of

infants co-sleeping with their parents in the second period compared to the first period and this tendency have been recorded in other studies as well [20;23]. Blair et al. found that the increase was owing to the fact that the absolute number of co-sleeping SIDS infants had decreased less than the non-co-sleeping and that the fact that fewer co-sleeping infant had been placed prone compared to non-co-sleeping previously and hence the "Back to Sleep" campaign would have greater impact on the non-co-sleeping infants[20]. However as the absolute number of co-sleeping SIDS infants in our material was relatively stable and the vast majority found supine in both time periods this does not explain the increase in our material.

Airway/head covering has been reported a risk factor for SIDS and was present in 61% of our cases respectively in our material but with identical distribution over the two time periods suggesting that this still may be a major modifiable risk factor in Danish SIDS infants [32].

Heat stress factors

As mentioned above a part of the triple risk hypothesis is the presence of an exogenous stressor[10]. Incipient or weak infections have been suggested as possible exogenous stressors and hypotheses based on bacterial toxins and cytokines have been forwarded [33-37]. Interpreting bacterial growth in tissue and body fluid samples is notoriously difficult as some bacteria such as E. coli and Staph. aureus can be part of the normal flora (depending on the location), be an indication of contamination, but also have pathogenic propensity. Furthermore post mortem sampling and bacterial outgrowth often results in a low sensitivity, as some of the relevant pathogenic bacteria will not survive the cooling of the body, or are outgrown by microorganisms not involved in the causality of death [38]. The interpretation of a possible growth has to take the location of the sample, usual flora of the site, and pathogenic potential of the microorganism in to account together with the histology and the reported symptoms of the infant [39]. We found a significantly increased proportion of cases with bacterial growth of some sort in the second pe-

riod primarily due to a significantly increased amount of cases interpreted as normal flora and/or contamination. The cause of this increase is nevertheless uncertain as the post mortem interval has not increased significantly between the two time periods. Possible explanations could be changing procedures for collecting samples or improved methods for culturing and the addition of more specific and sensitive methods in general e.g. PCR techniques. The possibility that some of the bacteria interpreted as contamination in blood or spinal fluid with e.g. S. aureus or E.coli may be subtle or early infections cannot entirely be ruled out. They were considered influential only if there was growth in several samples and/or pathological signs are present in the tissue samples. The number of cases where it was uncertain whether bacterial growth had contributed was low and unchanged; therefore, it seems reasonable to conclude that the possible significance for death was unchanged. By definition, of course cases where bacterial culture is believed to be of any significance are excluded from this study a priori. Even though there seemed to be an increase (although not statistically significant) of cases with symptoms of infections and/or overheating in the second period this is contrasted by a significant decrease in cases with signs of histological confirmed beginning/possible interstitial pneumonia. So data give no support for the belief that subtle infections have increased in SIDS. We did not review all cases histological, so therefore cannot be certain that the criteria for lung histology were followed. A possible explanation for the decreasing number of cases with beginning/possible interstitial pneumonia could be a diagnostic shift in the evaluation of infant cases with reactive change on lung histology as this has been known to cause classification problems with SIDS [40]. Even though uniform diagnostic criteria were used in both periods, these are still subject to individual interpretation, and therefore also possibly affected by trends in the community and diagnostic habits for the forensic pathologists, as the data spread over 17 years. Therefore it cannot be out ruled that the almost disappearing of SIDS in the latest years

of the second period (fig. 1) perhaps also partly were affected by this. To further investigate this the same cases as presented here have since been reclassified according to the San Diego criteria [9] the results of which will presented in a separate paper.

Possible limitations

Because our data collection has been limited to archival autopsy files with considerable inconsistencies among the type of data collected by the investigating police officers, we have been forced to leave out some important parameters; maternal smoking habits, number of siblings, ambient temperature and socioeconomic status being the most important, because the data only was present in a very small number of cases. To avoid a lack of this vital information in future studies uniform guideline for police officers and forensic pathologists working with infant cases will need to be implemented in our institution.

CONCLUSION

By comparing risk factors the first two years (1992-1993) after the introduction of the campaign against prone sleeping with the following fifteen years (1994-2008) we found an expected decrease in the proportion of infants placed and found prone, while neither of the epidemiologic parameters such as age at death, male ratio, prematurity, birth weight, month of death and maternal age changed significantly between the two time periods. While looking at possible indicators for infection such as symptoms of infection, positive lung histology and positive bacterial growth we found no indication of increased growth of bacteria that may have influenced on death. But the increase in total positive growth in culture may indicate that methods and techniques have improved, and this in turn will have the side effect that more cases that would earlier have been SIDS are now categorized as explained. A decrease in the proportion of cases diagnosed with beginning/possible interstitial pneumonia was seen. We speculate that the latter may be caused by a change in the evaluation of lung histology over the years and that these

diagnostic changes may partly explain why the numbers of SIDS deaths have almost disappeared since 2001.

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Cytokine gene polymorphisms in sudden infant death

Ferrante L

SUMMERY OF THESIS

Three of the papers included in the thesis "Cytokine gene polymorphism in sudden infant death" are the results of genetic studies of functional polymorphisms in cytokine genes and their possible associations with characteristic features found in SIDS victims and cases of infectious death[1]. The studies also relates the different genetic patterns to known environmental risk factors for SIDS. The main aim of the thesis is to search for predisposing factors according to the hypothesis of the fatal triangle for SIDS (1-3).

During the last decade SIDS research has been increasingly focused on the study of genetic predisposing factors, and so far genes involved in the regulation of the immune system, cardiac function, the serotonergic network and brain function and development have emerged as the most important with regard to SIDS (4). Various mutations and polymorphisms in these genes have been found associated with SIDS, but so far only mutations in genes involved in long QT syndrome (LQTS) may be viewed as independent cause of death. Concerning the other possible genetic predisposing factors to SIDS, they are most likely part of a polygenic inheritance pattern, contributing to triggering a death mechanism when combined with environmental risk factors, such as maternal smoking, prone sleeping position or infection.

A large study of 201 Norwegian SIDS cases demonstrated that 9.5% of cases diagnosed as SIDS carried functionally significant genetic variants in the LQT genes, indicating that sudden arrhythmic death is an important contributor to the group of sudden unexpected death in infants (5). The most commonly observed mutation was in the SCN5A gene, which is of particular interest since mutation in this gene is known to cause arrhythmias during sleep, when most deaths in infants occur.

Stoltenberg et al have reported immune stimulation in both the upper airways and intestines, showing that SIDS cases had higher number of IgM immunocytes in the tracheal wall than controls, but significantly lower numbers of IgA and IgM immunocytes than cases of infectious death (6). In the duodenal mucosa the number of IgA immunocytes was higher in SIDS cases than in controls (6).

These findings indicate that the mucosal immune system is activated in a large proportion of SIDS cases. It is also shown that SIDS babies have higher IgG and IgA-immunocyte density in the palatin tonsillar compartments than controls (7). Furthermore, salivary glands have a higher number of CD45+ stromal leukocytes, as well as intensified epithelial expression of HLA-DR and secretory component, and increased endothelial expression of HLA class I and II (8). These observations confirm that the immune system is activated in SIDS, probably with release of certain cytokines that are known to up-regulate epithelial expression of HLA-DR and secretory component (8).

A real breakthrough for the immunological overreaction theory was the demonstration by Vege et al (9), who showed that SIDS victims who have had signs of slight infection prior to death had both increased number of IgA-immunocytes and increased HLA-DR expression in their laryngeal mucosa, as well as increased levels of IL-6 in their cerebrospinal fluid (CSF) (*Figure 1*). In fact, half of the SIDS victims had CSF IL-6 concentrations in the same range as victims of meningitis and septicemia (10).

A further support for the infection theory is a study performed on registry data from Norway and Sweden which suggests that there is a co-variation between epidemics of whooping cough and SIDS (11). The association was stronger in Sweden than in Norway, which may reflect that Swedish infants not are vaccinated against Bordetella pertussis while the Norwegian infants are (11).

Recently, Stray-Pedersen et al showed that SIDS victims with positive Helicobacter pylori Stool Antigen (HpSA) immunoassay had elevated IL-6 levels in the cerebrospinal fluid
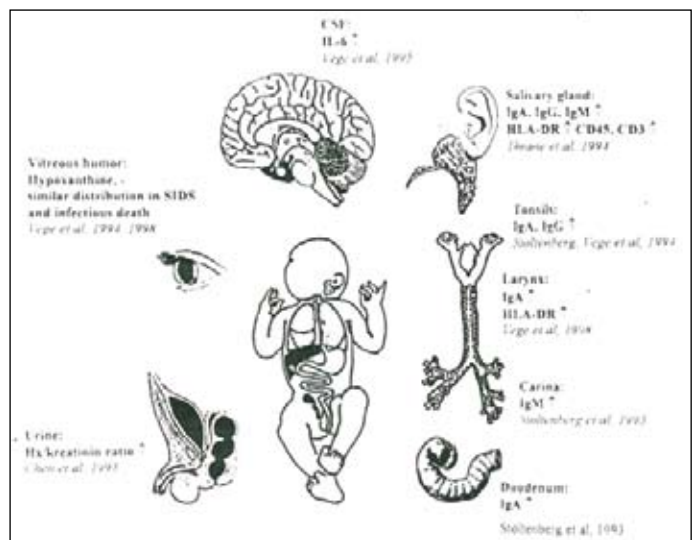

Figure 1: The salivary glands, tonsils, laryngeal and duodenal mucosa show increased number of immune producing cells compared to controls (6-8). The number of IgA-immunocytes and HLA-DR expression are increased in laryngeal mucosa, and the CSF show elevated IL-6 concentration (9). Vitreous humour and urine contain increased amount of hypoxanthine due to ante mortem hypoxia (16-18)

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[1]A fourth paper included in the thesis is submitted for publication and thus not mentioned here.

compared to SIDS victims with negative HpSA test (12). Furthermore, detection of helicobacter pylori antigen in stool was found associated with SIDS and death due to infection, indicating that this bacteria may represent a contributing factor to sudden death during the first months of life (12).

Surfactant protein A (SP-A) is a protein produced in the lungs, with a major purpose to reduce the surface tension at the alveolar air-liquid interface. Furthermore, it takes part in regulation of the inflammatory process. Interestingly, with regard to SIDS there is a drop in alveolar SP-A expression in the first months after birth (13), corresponding to the classical age peak of SIDS. Thus it may be hypothesized that this transient low expression of SP-A may be a part of the increased vulnerability for SIDS at that age (13).

The level of IgA imunocytes in larynx are found to be higher in SIDS victims than in controls (9, 14). Furthermore, the relationship between laryngeal immune stimulation, clinical signs of slight infection prior to death and high levels of IL-6 in CSF (9) may indicate an interaction between the immune system and the central nervous system (CNS). The assumption of such a relationship is strengthened by the recently reported increased IL-6 receptor expression on serotonergic cells in brain stem nuclei involved in respiratory regulation in SIDS cases compared to controls (15). The fact that a large proportion of SIDS victims whose brain stems had been investigated had had a common cold prior to death, strengthens the hypothesis that there may be a connection between activation of the mucosal immune system and a dysfunction of the seretonergic network regulating respiration and heart function in SIDS.

In summary, these studies indicate that a significant proportion of SIDS victims have an activated immune system that seems to cause autonomic dysregulation inducing down-regulation of respiration, irreversible hypoxia and death (2, 17, 18). The purpose of the thesis was to search for genetic factors that may explain the fatal disturbances of this immunological homeostasis. Most important for such a mechanism seems to be the genes encoding the interleukins.

SIDS and genes

Cytokines are specialized peptides, proteins, and glycoproteins that are produced by different immune cells, for instance when exposed to a pathogen. Important functions of the cytokines are to supervise cell communication and regulate the cells activity during the inflammatory process, making the cytokines important intercellular mediators. The pro-inflammatory cytokines are immunoregulators that favor inflammation while anti-inflammatory cytokines are immunoregulatory cytokines that control the pro-inflammatory cytokines by reducing their production, and thus counteract their biological effect.

The candidate genes most likely to explain the vulnerability to infection in SIDS are the cytokine genes. Several studies have investigated these genes in an attempt to uncover associations between SIDS and different genetic variants (19-24). IL-10 is an important immune regulatory cytokine, and the SNPs in the promoter region in position -1082, -819 and -592, as well as the microsatellite IL-10R and IL-10G, are collectively responsible for the production of the protein (25). Both SIDS cases and cases of infectious death have been reported associated with the ATA haplotype and the ATA/ATA genotype of this gene (19, 20). In addition, a higher percentage of the genotypes G21/G22 and G21/G23 in cases of infectious death compared to SIDS, and a higher percentage of G21/G22 in the SIDS cases compared to controls have been reported (19). Based on these findings it may be speculated that in some situations an infant with an unfavorable IL-10 genotype may exhibit aberrant IL-10 production, which in turn leads to a disturbed immunological homeostasis.

The polymorphisms IL-1ß-511C/T and +2018T/C, located in the IL-1Ra gene, have a significant effect on the IL-1ß levels, but no association to SIDS has so far been established (21). Another study investigated the 89bp VNTR in the IL-1Ra gene in Australian SIDS cases, and found that carriage of the 2/2 genotype increased the risk for SIDS compared with the predominant 1/1 genotype (26). Homozygous carriers of allele 2 show a more severe and also prolonged proinflammatory

immune response compared to other IL-1Ra genotypes (27), which may contribute to the vulnerability to infection seen in SIDS.

A British study including common polymorphisms in the genes encoding IL-4, IL-6, IFNγ, TGF, and VEGF found significant differences for the genes encoding IL-6 and VEGF: the genotypes -174GG, and -1154AA were more frequent in SIDS cases than in controls (22). Even though only a small number of SIDS cases was included, the authors suggest that the causation of SIDS is related to both fetal lung development and an infant's innate ability to mount an inflammatory response to infection (22). The findings regarding the IL-6 gene has been confirmed in a study of Australian SIDS cases (23), but not in a Norwegian study (24).

Surfactant protein A (SP-A) and surfactant protein D (SP-D) are humoral molecules involved in the innate host defense against various bacterial and viral pathogens. Ten SNPs that might influence expression of the genes encoding these two surfactants have been investigated in SIDS cases and controls (28). No difference in genotype distribution was found, even though there was a tendency for the most common SP-A haplotype, 6A2/1A0, to be overrepresented in cases with low immunohistochemical SP-A expression (28). The SP-D expression was not influenced by any of the investigated SNPs.

Subjects and methods

Subjects

The subjects included in the studies of this thesis were all examined at the Institute of Forensic Medicine, University of Oslo, during the period 1988-2006. The autopsies were performed as soon as possible after death and for most cases within 36 hours. Tissue samples and body fluids were collected in all cases based on a well established protocol (29-32), and the post mortem examinations of every infant was performed by the same forensic pathologists (TOR, ÅV, MA, ASP). All individuals were Caucasians from the south-eastern part of Norway.

The subjects included 148 SIDS cases, 56 borderline SIDS cases, 41 cases of infectious death, and 131 adult diseased controls (*table 1*). The SIDS

Table 1: Survey of the patients

Group	Numbers of case	Diagnosis	Gender (F/M)	Age at time of death, median (range)
SIDS	148	SIDS	57/91	3.8 mo (0.1-24 mo)
	56	Borderline SIDS	20/36	3.5 mo (0.5-39mo)
Infectious death	41	19 pneumonia	13/28	7.6 mo (0.8-39mo)
		16 septicaemia		
		1 meningitis		
		2 CMV infection		
		1 encephalitis		
		1 myocarditis		
		1 peritonitis		
Controls	131	52 disease	43/88	49 y (11-86yrs)
		23 intoxication		
		56 violent death		

and borderline SIDS cases were diagnosed according to the Nordic criteria (31), were it is demanded that both clinical history, autopsy findings and investigation of the circumstances of death provides no explanation for death. The cases undergo a full body X-ray prior to autopsy, and before a diagnosis of SIDS or borderline SIDS can be established, toxicological screening has to be negative, and mutations and functional polymorphisms in the LQTS genes, as well as the A985G mutation in the MCAD gene, have to be excluded. Afterwards, an informed consent questionnaire was send to the SIDS parents to obtain knowledge of the history of the child's health in the days before death, parental smoking, position when found dead and sleeping environment. All subjects included in the group of infectious death died suddenly and unexpectedly, without a history of severe illness prior to death. The causes of death in the control group were acute non-infectious disease, trauma, and intoxication.

Genotyping methods

Sequenom
The SNPs included in table 2 and figure 2 were genotyped using MassARRAY™ on a SEQUENOM® platform, in cooperation with CIGENE, Norwegian University of Life science, Ås. As core facility under the Norwegian Functional Genomics program (FUGE), CIGENE is a national platform for SNP analysis. The total process is automated using highly specialized instruments such as Beckman Biomek Fx pipetting robot (Beckman Coulter), a Beckman Multimek96 pipetting robot (Beckman Coulter), a nano-dispenser chip-printing robot, a mass spectrometer, and a Laboratory Information Management System (LIMS system). The mass difference were detected using a matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) and genotypes were called using type 3.1 software (SEQUENOM®).

TaqMan
One of the SNPs in the TNF gene, -1031C/T, was analyzed using TaqMan SNP genotyping assay (Applied Biosystems) and Mx3000p real-time PCR machine (Stratagene, a Jolla, CA).

VNTR
Two VNTRs were analyzed in the papers included in this thesis, a VNTR in intron 2 of the IL-1ra gene and a VNTR in intron 6 of the IL-1α gene. The polymerase chain reaction was performed using standard protocols for normal amplification. Primers for both VNTRs were designed using the program Primer3 (http://frodo.wi.mit.edu). Amplified products were detected by gel electrophoresis on 2% agarose gels in the presence of ethidium bromide, and the DNA bands were visualized using ultra violet (UV) light.
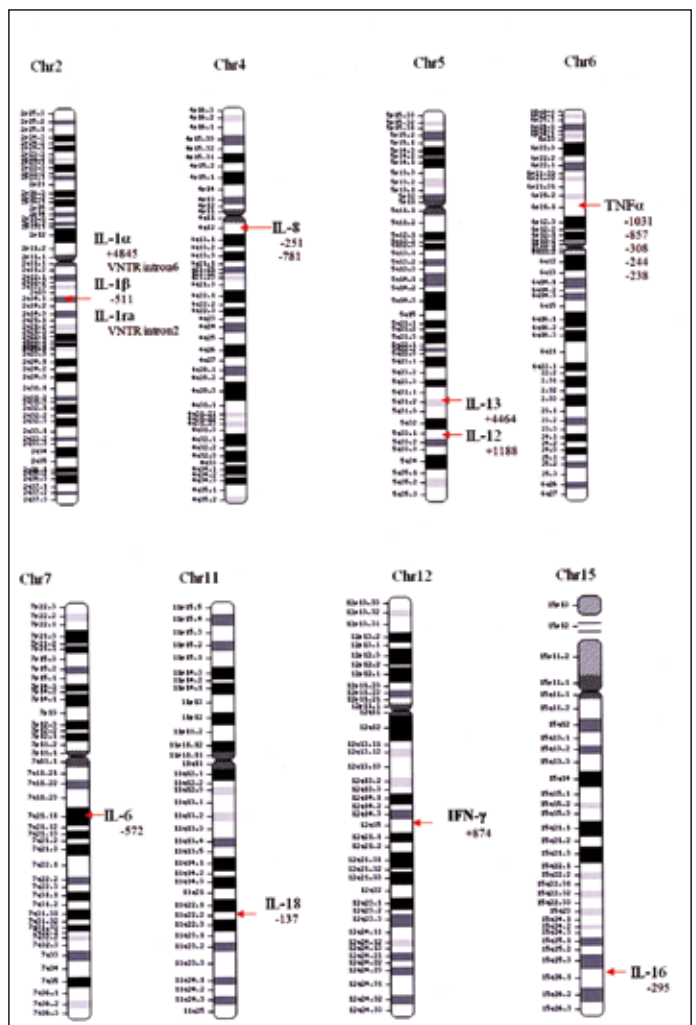
Table 2: Functional SNPs investigated using MassArray

Gene	Polymorphism	rs number
IL-1α	+4845G/T	rs17561
IL-1ß	-511C/T	rs16944
IL-6	-572G/C	rs1800796
IL-8	-781C/T	rs2227306
	-251A/T	rs4073
IL-12	+1188A/C	rs3212227
IL-13	+4464 A/G	rs20541
IL-16	-295T/C	rs4778889
IL-18	-137G/C	rs187238
TNF-α	-1031C/T	rs1799964
	-857 T/C	rs2507961
	-244A/G,	rs673
	-238 A/G	rs361525
IFN-γ	+874A/T	rs2430561

Fiogure 2: Chromosomal localization of the polymorphisms studied in the present thesis.

Summary of papers

Paper I

The aim of paper I "TNF-α promoter polymorphisms in sudden infant death" Ferrante L, et al. (33) was to investigate functional polymorphisms in the promoter region of the TNFα gene in order to investigate any possible associations between TNFα genotype and SIDS. SNPs at the position -1031T/C, -857C/T, -308G/A, -244G/A, and -238G/A were included. Associations between -238GG and SIDS (p=0.022) and between -308GA and borderline SIDS (p=0.005) were found. Furthermore, two SNP profiles -1031CT, -238GG, -857CC, -308GG and -1031TT, -238GG, -857CC, -308AA were found significantly more often in SIDS than in controls, and may thus be unfavorable. The findings of the study add evidence to the theory that an unfavorable genetic profile in the TNFα gene may be involved in SIDS. Both high levels and prolonged exposure to TNFα, combined with reduced release of anti- inflammatory cytokines, might cause a deleterious immunological imbalance in an infant at vulnerable developmental stage of the central nervous system and the mucosal immune system, and thus contribute to SIDS.

Paper II

In paper II "Cytokine gene polymorphisms and sudden infant death syndrome", Ferrante L, et al (34) the aim was to uncover any possible unfavorable genetic predisposition in genes encoding, IL-6, IL-8, IL-12, IL-13, IL-16, IL-18 and IFNγ. The genotypes IL-8 -251AA/AT and IL-8 -781CT/TT were significantly more frequent in SIDS cases found dead in a prone sleeping position, compared with SIDS cases found dead in other sleeping positions. In addition, there was an association between fever prior to death and the genotype IL-13 +4464GG in the cases of infectious death. In conclusion the results from this study support the hypotheses that specific cytokine genotypes are a part of genetic make-up that make infants sleeping prone at risk for SIDS. The study also supports the view that SIDS is multifactorial, including both environmental risk factors and a predisposing inheritance pattern.

Paper III

In the paper entitled "IL-1 gene cluster polymorphisms and sudden infant death syndrome" Ferrante L, et al (35) we wanted to investigate two polymorphisms in the IL-1α gene; a variable number of tandem repeat (VNTR) in intron 6 and a single nucleotide polymorphism in +4845G/T, as well as the -511C/T polymorphism in the gene encoding IL-1β, and a VNTR in intron 2 of the competitive antagonist IL-1Ra. An association between the gene combination IL-1α VNTR A1A1/IL-1ß +4845TT and SIDS was disclosed (p < 0.01). In the SIDS group it was also found that the genotypes IL-1ß -511CC/CT were significantly more frequent in the SIDS victims found dead in a prone sleeping position, compared with SIDS victims found dead in other sleeping positions (p = 0.004). The findings indicate that specific interleukin gene variants may be a predisposing factor for sudden unexpected infant death, and one may speculate that this genotype combination may cause an elevated expression of IL-1 that might be unfavorable.

Conclusion

The thesis indicates that there exists a predisposition for SIDS due to genetic variation in the cytokine network. Combined with environmental risk factors and a vulnerable developmental stage of the infant, this may be fatal. This means that a genetic predisposition by no means implies that SIDS will ensue. Most multifactorial diseases and conditions are not caused by just one unfortunate gene variation. Therefore, the development of multiplex chip analysis is becoming more and more important in research into genetic diseases. Although new knowledge has been obtained through the present studies, they also point to challenges of how to proceed in mapping genetic risk factors for SIDS. Last, but not least, we need to learn how to transform the knowledge into prevention of SIDS deaths.

To sum up the conclusions for the thesis:

- An unfavorable genetic profile in the TNFα gene may be involved in SIDS, by exposing the infant to both a high level of and a prolonged exposure to TNFα.
- Genetic variation in the genes encoding IL-8 and IL-1ß are a part of a genetic make-up that make infants sleeping prone at risk for SIDS.
- Genetic variation in the IL-1 gene cluster may also contribute to SIDS, by giving a changed expression of both IL-1α and IL-1ß and thus a disturbance in the immunological response.
- SIDS cases with HLA-DR activation in laryngeal mucosa more often have rare genotypes of IL-1α, further strengthening the finding of an involvement of IL-1 in SIDS.
- Taken together, the findings in the thesis indicate that specific interleukin gene variants may be predisposing factors for sudden unexpected infant death and support the theory of the fatal triangle (figure 3).
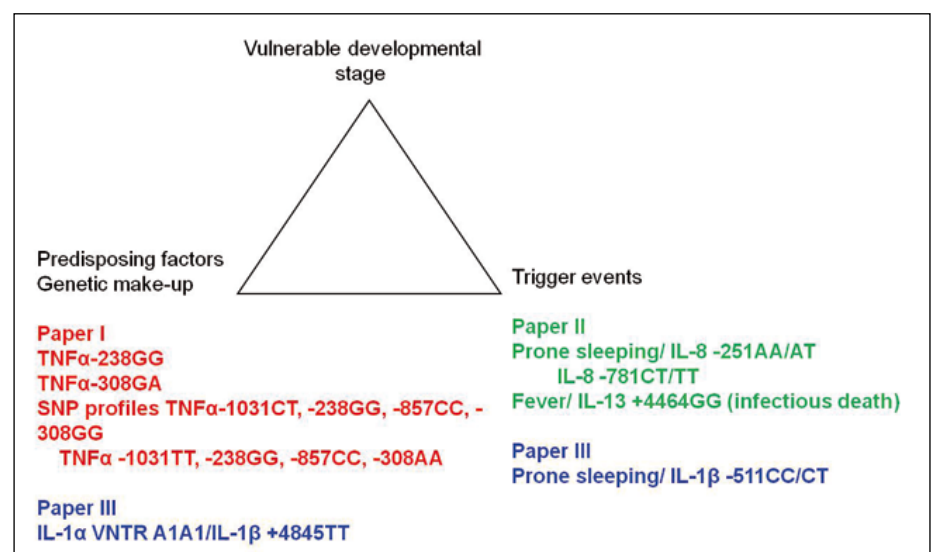

Figure 3: The fatal triangle, modified after Rognum and Saugstad (2).

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AQP4, brain edema and SIDS

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SUMMERY Aquaporins (AQP) provide a way of rapid transport of water across the plasma membrane. So far at least 13 different aquaporines have been described. AQP4 is the most abundant water channel in the brain and spinal cord, and it is shown that AQP4 plays a significant role in brain edema. A link between AQP4 and infection has also been indicated. It is shown that APQ4 is strongly upregulated in meningitis, and that both IL-1ß and TNFα have the ability to induce AQP4 expression. The gene encoding AQP4 is located on chromosome 18, and mutations giving reduced water permeability as well as gain-of-function mutations have been reported. Several studies report findings of increased brain weight and edema in SIDS, and one might speculate that the enlarged brain seen in some SIDS victims is due to a genetically determined disturbance of the water homeostasis in the brain. Based on this hypothesis, four tag SNPs in the gene encoding AQP4 have been investigated in Norwegian SIDS cases and controls. An association between the genotypes CT/TT in of rs2075575 and SIDS (p<0.01) was found, and also an association between brain/body weight ratio and rs2075575 genotype in SIDS cases aged 0.3-12 weeks (p=0.014). This study indicates that rs2075575 may be of significance as a predisposing factor for SIDS.

KEYWORDS Aquaporin-4 - Brain edema - SIDS

INTRODUCTION

The most important proteins with regard to water transport are the aquaporins (AQP), which provide a way of rapid transport of water across the plasma membrane. Aquaporins have been found in a variety of human tissues, including kidney, lung, liver, colon, brain, and different parts of the eye, and so far at least 13 different aquaporines have been described. Mammalian AQPs are divided into two groups, those that transport only water and those that also transport glycerol and other small polar molecules. The latter are called aquaglyceroporins, and include AQP3, AQP7 and AQP9 [1].

AQP4 is the most abundant water channel in the brain and spinal cord, but also AQP1 and AQP9 have in the central nervous system [2, 3]. AQP4 is expressed in astrocyte end-feet at the fluid-tissue barriers (blood-brain and brain-cerebrospinal fluid) [4]. The AQP4 protein is expressed as two major polypeptides of 323 and 301 aminoiacids, which arise from two different translating-initiating methionines, M23 and M1 [5]. In the human brain, the M23 and M1 isoforms represents around 75% and 25% of the total AQP4, respectively [6]. M23 forms higher order assemblies within the plasma cell membrane, termed orthogonal arrays of particles (OAPs), while M1 exists as individual tetramers [7].

The membrane localization of AQP4 requires a dystrophin-associated protein complex involving α-syntrophin, and it is shown that mice homozygous for disruption of the gene encoding α-syntrophin (α-Syn$^{-/-}$) lack AQP4 in perivascular astrocyte endfeet membranes [8, 9]. In addition, the K$^+$ channel Kir4.1 is also co-expressed with AQP4, and together they compose a multifunctional unit responsible for clearance of K$^+$ and/or H$_2$O following neural activity [10].

AQP4 AND BRAIN EDEMA

Several studies suggest a key role for AQP4 in brain edema. AQP4 becomes upregulated in conditions that cause brain edema, such as malignant astrocytomas, bacterial meningitis, and subarachnoid hemorrhage [11-14]. When exposed to acute water intoxication and ischemic stroke APQ4 knock-out mice show less brain tissue water content, better neurological outcome and also improved survival compared to wild-type mice [15]. Also studies of α-syntrofin null mice (α-Syn$^{-/-}$), which due to a subsequentAQP4 expression may be regarded as alternative models for the AQP4-null genotype, show the same; a reduced development of postischemic brain edema [9]. However, the converse is also true: the efficiency of intravenously administered hypertonic saline in extracting water from the brain by osmosis is impaired in α-Syn$^{-/-}$ mice, and mice overexpressing AQP4 develop more brain edema after water intoxication than wild type mice [16, 17].

The finding of an improved survival in AQP4 knock-out mice may indicate that lack of AQP4 is protective against brain edema, and thus beneficial [15]. The opposite could however easily be true, since elimination of brain edema is essential in the treatment of patients suffering from head injuries, and it is shown that AQP4 deletion in mice increases vasogenic brain edema caused by brain tumors [18]. AQP4 permits bidirectional water transport, and consequently, AQP4 may not only provide the major pathway for water entry into the brain but also for water exit [9]. AQP4 inhibitors are thus predicted to reduce brain swelling in cytotoxic edema, which is accumulation of intracellular fluid that occurs without disruption of the blood-brain barrier. AQP4 activators, on the other hand, are predicted to reduce brain swelling in vasogenic edema, which is accumulation of fluid through a leaky blood-brain barrier [19].

AQP4 AND INFECTION

It is shown that APQ4 is strongly upregulated in meningitis, resulting in a ~5 fold higher water permeability

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across the blood-brain barrier in infected compared with non-infected wild type mice [13]. Furthermore, meningitis produced a marked astrocyte foot process swelling in wild type but not in AQP4 null mice, and the AQP4 null mice had lower intracranial pressure, lower brain water accumulation, and a reduced mortality, indicating that lack of AQP4 is beneficial [13]. However, it has also been reported a protective effect of AQP4 on brain swelling in brain abscess-associated edema induced by injecting live *Staphylococcus aureus* into the stratum [20]. In this model, the AQP4 null mice showed both a higher intracranial pressure and brain water content, compared to wild-type mice, suggesting that AQP4 induction may reduce vasogenic edema associated wtih cerebral infection [20].

Interleukin-1ß (IL-1ß) is together with TNFα important pro-inflammatory interleukins in the brain that are known to play a role in the formation of brain edema after various types of injury. Of special interest is that these two interleukins also are shown to induce AQP4 expression, indicating a link between infection, interleukin response, and AQP4 expression [21, 22].

GENE STUDIES

The gene encoding AQP4 is located on chromosome 18, and mutations giving reduced water permeability as well as gain-of-function mutations have been reported [23, 24]. These gene variants are potentially clinically relevant. Just as AQP4 null mice showed improved survival after cytotoxic edema could even partial knock-down SNPs in humans have similar protective effects [15, 24].

The first study to investigate the AQP4 gene in relation to disease was a Japanese study of schizophrenia [25]. Scan studies for schizophrenia have suggested a linkage to 18q, and based on this Muratake et al investigated four SNPs in the AQP4 gene in 261 schizophrenia subjects and 278 controls, but the study did not reveal any association between these SNPs and disease [25]. Also the genetic role of AQP4 gene variation in migraine, as well as in Menière's disease, have been investigated, but without any significant findings [26, 27].

Kleffner et al investigated 10 SNPs in the AQP4 gene in patients with middle cerebral artery occlusion [28]. The study disclosed an association between the SNP rs9951307 and severe brain edema, and the authors speculate that genetic variation is of importance with regard to brain edema formation [28].

The most comprehensive study regarding AQP4 gene variation and disease is by Heuser et al, who investigated the AQP4 gene in 218 patients with temporal lobe epilepsy (TLE), and in addition included SNPs in the gene encoding the potassium channel Kir4.1[29]. They detected several SNPs in the AQP4 gene, and a multivariate analysis showed that a combination of three SNPs in the AQP4 gene (ss119336753, ss119336754 and rs1053074) together with SNPs in the genes encoding the potassium channels Kir4.1 (KCNJ10) and Kir3.3 (KCNJ9) was associated with TLE. This study supports the suggestion that perturbations of water and K⁺ transport are involved in the etiopathogenesis of TLE [29].

AQP4 AND SIDS

Several studies report findings of increased brain weight and edema in SIDS [30-32]. A study of 208 sudden infant death syndrome (SIDS) cases suggested that the enlarged brains were present from birth rather than a result of disproportional postnatal growth [30]. This is confirmed by Kadhim et al, who speculates that the excessive brain weight might reflect abnormal cerebral development and could be detrimental to vital neural control [31]. In addition is brain edema a common feature of non-accidental head injury in infancy, such as shaken baby syndrome [33].

One might speculate that the enlarged brain seen in some SIDS victims are due to a genetically determined disturbance of the development of the water homeostasis in the brain. Based on this, we have performed an investigation of four tag SNPs in the gene encoding AQP4, including rs2075575, rs4800773, rs162004 and rs3763043, in 141 SIDS cases and 179 controls [34]. It was found an association between the genotypes CT/TT in of rs2075575 and SIDS; 84.4% of

the SIDS cases had these genotypes, compared to 74.3% of the controls (p<0.01). In addition, an association was observed between brain/body weight ratio and rs2075575 genotype in SIDS cases aged 0.3-12 weeks (median ratio CC 10.6, median ratio CT/TT 12.1, p=0.014).

It was also found that the CT/TT genotypes tended to be associated with maternal smoking, 65.7% of the 67 SIDS cases exposed to nicotine had these genotypes, compared to 36.4% of the 11 SIDS victims not exposed to nicotine (p=0.09). It is shown that nicotine induces edema [35], and one may speculate that nicotine, in combination with the rs2075575 CT/TT genotypes, induces a less effective AQP4 protein synthesis and thus a disturbed water homeostasis. No associations were found between AQP4 genotype and sleeping position or infection prior to death.

The findings in this study fit with the hypothesis of a fatal triangle in SIDS [36], indicating that rs2075575 may be of significance as a predisposing factor to SIDS [34]. One might speculate that rs2075575 is part of a genetic pattern that is important for the expression of AQP4 in the brain and thereby also for the development of water homeostasis. One may speculate that the T allele, or another allele or genotype in disequilibrium with the T allele, induces a delayed maturation of the processes underlying water and ion homeostasis. Such delay may predispose for an increased water flow into the brain tissue, and thus an abnormal brain/body weight ratio, which may be particularly unfavorable in the first months of life.

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