Water Transport Through Perinatal Skin

Barrier Function and Aquaporin Water Channels

BY

JOHAN ÅGREN
List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


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Abbreviations

TEWL Transepidermal water loss
TEWL_{50} Transepidermal water loss corrected to 50% relative humidity
AQP Aquaporin
ANS Antenatal corticosteroid treatment
ER Evaporation rate
RH Relative humidity
SGA Small for gestational age
E Embryonic
P Postnatal
IWL_{s} Insensible water loss through the skin
BSA Body surface area
pF picoFarad equivalents
W/D Wet/dry weight ratio
RT-PCR Reverse transcriptase-polymerase chain reaction
Introduction

Improvements in maternal and neonatal care have led to increased survival of preterm infants during the last decade (1, 2), especially of infants born extremely preterm. These latter infants constitute a significant group of patients with specific needs related to the immaturity of several important organ functions. The function of the skin as a barrier, limiting water loss to the environment, has a unique relevance to infants born extremely preterm. While providing an appropriate interface with the aqueous environment in utero, the skin of preterm infants offers a poor barrier against the gaseous environment after birth, leading to large losses of weight (3) due to excessive loss of water through the skin (4, 5). This high transepidermal water loss (TEWL) can lead to hypothermia (6, 7), dehydration, and hyperosmolarity (8, 9), all of which have negative consequences for the outcome and health of these infants. The mechanisms that influence water transport through the immature skin are not fully understood, and at present the barrier to water loss is considered to depend solely on the physical properties of the stratum corneum (10-14). Since this skin layer clearly is not fully developed in the immature fetus and preterm infant (15), factors influencing the water content of the skin can determine the amount of water accessible for evaporation and thus indirectly influence TEWL.

In the present project we addressed the question whether there might be factors in the skin that could influence the rate and direction of water transport through perinatal skin. One such factor could be aquaporin (AQP), a fairly recently described (16, 17) integral membrane protein which acts as a selective water channel, mediating transcellular water transport in many tissues (18, 19). This investigation has focused on aspects of water transport through developing skin, first by determining TEWL in a group of extremely preterm infants. Further, in an experimental rat model we analyzed the expression and distribution of AQP in the perinatal skin in relation to biophysical parameters of the skin barrier such as TEWL, skin surface hydration, and the water content of the skin. The investigation included an analysis of developmental aspects as well as the effects of a clinically relevant form of pharmacological treatment, namely antenatal corticosteroids (ANS) and of a pathophysiological intervention (restriction of fluid and nutrient intake).
Background

Structural aspects of cutaneous development

The skin consists of the epidermis (an epithelium), the dermis (a connective tissue) and the cutaneous appendages (hair follicles, sebaceous glands, and sweat glands). The skin also harbors capillaries and specialized cells providing tactile sensation and host-defense functions.

The epidermis

In the embryo, the single layered epidermis is covered by a layer of peridermal cells providing a barrier to the amniotic fluid (20). The periderm begins to regress and is shed at the time when keratinization of the epidermis begins, at about 18 weeks of gestation (20). The epidermis gradually increases in thickness as the keratinocytes proliferate, differentiate, migrate outwards and then progressively involute. During terminal differentiation the cells extrude their lipid content into the extracellular space and become incorporated in sheets of lipids like bricks in mortar, forming the stratum corneum (20). At about 22 weeks of gestation the epidermis is two to three cell layers thick and a thin stratum corneum has formed (15). As gestation proceeds, there is a steady increase in the number of cell layers of the epidermis and at about 34 weeks there is a structurally mature epidermis with a well-defined stratum corneum (15).

Increasing knowledge is being gained about the process of skin organogenesis. It has been demonstrated that embryonic skin development is patterned (21) and that nuclear receptors might be involved in the process of maturation (22, 23). During skin development, several changes in the gene expression (24, 25) and in the molecular composition of the epidermis (10, 26, 27) take place, and it has been proposed that these changes represent signals in the maturational process. As gestation proceeds, ion concentration gradients appear within the epidermis (28-30), events which might be related to the expression of ion transporters and receptors (31, 32). Water-
immobilizing extracellular matrix molecules such as hyaluronan, and the expression of its receptors, are also related to skin development (33-35).

The vernix caseosa is a unique feature of human fetal skin biology (36). Vernix is a lipid-rich material which also contains desquamated corneocytes and gradually covers the fetal skin as the maturing epidermis forms the stratum corneum (37). Vernix has a high water content and yet is highly viscous, as the water is retained within the cells (38). This property presumably accounts for the contribution of vernix to the fetal skin barrier (39).

The dermis
Like the epidermis, the dermis is thin in the early stage of gestation (20, 33). It is rich in proteoglycans such as hyaluronan and chondroitin sulfate, a fact which accounts for the high water content and gel-like appearance of this tissue (33, 34). Gradual accumulation of collagenous and elastic connective tissue, and the appearance of fibroblasts, take place as dermal maturation proceeds, and by about 22-23 weeks of gestation both the dermal vasculature and the sensory apparatus have developed (33, 40, 41). Sebaceous glands are also present at this time and produce part of the lipids constituting the vernix (42). Sweat glands are present early in gestation but are not functionally developed until about 36 weeks of gestation (43-45).

Postnatal skin development in the preterm infant
In the newborn preterm infant, postnatal life has a stimulating effect on epidermal development so that after a few weeks postnatally the histological structure of the epidermis resembles that of a term infant (15). In parallel, experimental studies have demonstrated that exposure to a dry environment promotes functional maturation of the fetal skin (46, 47), but it is not known whether this also applies to the human immature neonate.

Postnatally, very preterm infants commonly accumulate a significant nutrient deficit in the first weeks after birth (48), which can result in retardation of body growth. The question whether the postnatal intake of fluids and/or nutrients can influence the epidermal barrier in newborn infants has not been studied previously.

Comparative aspects of the skin - human versus animal
In this study, as in several others (27, 28, 37, 49-52), the possible mechanisms involved in developmental skin biology have been explored with use of an animal model. In most cases the rat has been used in these
studies. The skin of an adult rat is very thin and covered with fur, and obviously does not look much like that of a human. However, the skin of the perinatal rat shares several features with human skin. In contrast to the adult rat, the perinatal rat has a thick interfollicular epidermis structurally resembling that of human skin. Moreover, in spite of a tremendous difference in the duration of gestation, the development of the structure of rat skin and its barrier function display similarities to that of human skin (10, 27). Nevertheless, caution has to be observed when applying results obtained in experimental studies to human biology.

Functional properties of the developing skin

TEWL and the skin barrier
Evaporation of water from the skin surface takes place at the water/air interface, i.e., where there is a most marked drop in water vapor pressure. Since the skin surface is readily accessible for biophysical measurements, most studies on skin water transport rely on such measurements, and TEWL (53) and skin surface hydration (54) are often used as an estimate of skin barrier function. Little is known about the way in which water transport to or from deeper structures of the skin regulates the water content of the skin.

The function of the skin as a barrier serving to limit water loss is considered to reside in the stratum corneum, and skin barrier function has been found to be related to the thickness of the epidermis as well as to the synthesis, deposition and organization of the stratum corneum lipids (10, 55, 56). Several aspects of epidermal development and integrity correlate with gestational age (27) and are affected by perturbation of the skin, for example by adhesive tape removal (57). The transepidermal water transport in itself seems to be a signal in the establishment and recovery of the barrier. Thus, if skin with a poorly developed or damaged barrier is exposed to factors limiting water loss, such as when it is bathed in fluid or occluded by an impermeable covering, the formation of an effective stratum corneum is impaired (28, 58). Transepidermal water transport may influence barrier formation indirectly by its effect on intraepidermal ion concentration gradients (29). It is not clear how such gradients are established in fetal skin, which is continuously exposed to amniotic fluid, but vernix may play a role in this context (39). While the sebaceous lipids of the vernix provide a hydrophobic barrier protecting the fetal stratum corneum, desquamation of well-hydrated vernix corneocytes might enable net transepidermal water transport and promote the formation of epidermal ion concentration
gradients during fetal life. Accordingly, other factors with an impact on epidermal water transport or hydration, or water-binding capacity might indirectly influence processes involved in the formation of the skin barrier.

**TEWL: relation to age and intrauterine growth**

Evaporation of water from the skin surface commences immediately after birth. After the initial evaporation of amniotic fluid, TEWL is low in term infants and comparable to that of adults (59, 60). The rate of evaporation from the skin of term infants changes little with increasing postnatal age, but is elevated as a result of sweating in a warm environment and during increased activity (44, 61).

In the preterm newborn infant, however, TEWL is high and inversely related to gestational age at birth (4, 62, 63). Importantly, the exponential relationship between TEWL and gestational age has a very steep course at gestational ages ≤ 28 weeks (4, 5, 63). The high evaporative water loss in the most preterm infants rapidly declines during the first week after birth and then decreases further with increasing postnatal age (62). However, TEWL in infants born very preterm (GA 25-27 weeks) is significantly higher at a postnatal age of 4 weeks than in term infants (62, 63), indicating that skin barrier function does not fully develop in parallel with the accelerated structural maturation observed after birth in these infants (15). In preterm infants born small for gestational age (SGA), TEWL is lower than in appropriately grown infants of the same gestational age (63), indicating a less permeable or less hydrated skin in SGA infants.

**Environmental influence on TEWL**

The by far most important environmental factor influencing TEWL is the ambient vapor pressure (or relative humidity; RH) (59). TEWL is inversely related to ambient RH, so that if preterm infants are nursed at a high RH, TEWL is substantially reduced (59). TEWL is also influenced by ambient temperature and body temperature, but to a lesser extent (61). Thus, the ambient conditions can have a significant impact on the fluid balance of newborn infants, and in studies on factors influencing TEWL, detailed knowledge is required about the environmental humidity and temperature.
Clinical consequences of an inadequate skin barrier

Fluid balance

The skin is the major route for fluid loss in preterm infants early after birth. It is estimated that infants born at a gestational age of 25-27 weeks lose water through the skin at a rate corresponding to approximately 130 ml/kg body weight during the first day after birth if they are nursed at an ambient humidity of 50% (60). As a consequence, the TEWL in these infants can lead to hypothermia, dehydration, and hyperosmolarity, of which the latter two conditions are considered to increase the risk of cerebral hemorrhage (9, 64, 65).

As discussed above, TEWL is substantially reduced by nursing the infants in a highly humidified environment (66). In addition, to balance these losses, active enteral and parenteral administration of fluids is required (67, 68), but with avoidance of excessive fluid administration, which according to different reports may increase the incidence of persisting ductus arteriosus (69), necrotizing enterocolitis (70, 71), and chronic lung disease (72). From a clinical perspective, the ambient conditions in which the infant is nursed have to be taken into account when the supply of fluids and nutrients is planned.

Cutaneous absorption and invasion by microorganisms

Another consequence of a poor epidermal barrier is the risk of penetration of harmful substances through the skin. Several reports describe the hazards of various topically applied agents (73, 74). Clearly, many potentially harmful substances can penetrate the skin and thus contribute to short- or long-term morbidity. Moreover, such effects might be difficult to recognize during the care of a critically ill immature neonate. Thus, avoidance of use of topically applied agents or ointments appears to be the safest principle in neonatal skin care practice (73).

Percutaneous respiration is significant in the preterm infant, the skin allowing transfer of both oxygen and carbon dioxide, representing a potential benefit of the highly permeable skin of these infants (75).

Another possible consequence of a poor barrier is the potential risk of entry by microorganisms. The vernix has been shown to have innate antimicrobial properties (76, 77), but it is presently not known whether the differences in skin structure and function between preterm and term infants imply differences in the microbial flora or the innate immunity of the skin. It is possible that repeated abrasive stress to the thin and vulnerable epidermis
will add to the generally increased risk of microbial infection in these infants.

**Current concepts of managing high TEWL**

In neonatal intensive care, an adequate supply of fluid and nutrients is of vital importance to the extremely preterm infant. To be able to provide appropriate amounts of fluid and nutrients, information is needed on infant maturity, weight change, circulatory status and general condition, but also about the environmental conditions in which the infant is nursed. Nursing preterm infants in highly humidified incubators is generally an appropriate measure for lowering their otherwise high TEWL (4, 60, 62, 63, 66, 78).

Alternative approaches to reduce TEWL are to apply a plastic dressing (79) or an ointment (80, 81) to the skin. Such measures are effective in reducing TEWL, but might be associated with serious disadvantages such as increased skin trauma and a risk of cutaneous absorption of harmful substances and nosocomial infections (73, 82, 83) as discussed above.

Antenatal administration of corticosteroids (ANS) has become a standard treatment when there is a possibility of preterm delivery, to induce fetal lung maturation (84), thereby reducing the risk of respiratory distress syndrome in preterm infants (85). As with the pulmonary epithelium, the immature epidermis is highly permeable to water, even after exposure to air, as reflected by the high postnatal transepidermal water loss. It might therefore be expected that after ANS, changes may also occur in the epithelium covering the body surface after ANS. Experimental studies in the rat have demonstrated that ANS promotes development of a thicker and less water-permeable stratum corneum compared to that in age-matched controls (86), but studies in infants have so far failed to show such an effect (87), although indirect measures of insensible water loss such as levels of serum sodium are lower in infants exposed to ANS than in unexposed infants (88).

**Aquaporin (AQP) water channels**

The **AQP**

The high water permeability displayed by certain epithelia has long been observed (89, 90). In contrast to membranes displaying low permeability to water, where water flux takes place by diffusion through the hydrophobic lipid bilayer, these “leaky” epithelial membranes were first considered to harbor some kind of “water shuttle” (91). However, about two decades ago
the concept of water pores began to receive increasing attention (92, 93). The isolation and identification of a membrane protein from red cells eventually led to the discovery of a new family of integral membrane proteins exhibiting high and selective permeability to water (94) The proteins were recognized as the long sought for water channels, and were named aquaporins and numbered in order of discovery. The basic channel-like structure of AQP1 is depicted in Figure 1. (95). The AQP monomer consists of membrane-spanning subunits that fold together to form an aqueous pathway through the cell membrane, and the monomers form tetramers in the membrane (95).

Figure 1. The molecular structure of AQP1 (adapted from Jung et al 1994, republished with permission by The Journal of Biological Chemistry)
The channels are inhibited by mercurial compounds (96), and in some cases a pH-dependent permeability has been demonstrated (97, 98). The family of aquaporins consists of two groups. The aquaporins of one group are permeable only to water and are named orthodox aquaporins. Those of the other group also exhibit permeability to small solutes such as glycerol and urea, in addition to water, and are called aquaglyceroporins.

**AQP function, distribution and regulation, and implications for disease**

Transport of water through AQP channels is selective but passive, the flux being driven by external forces such as those created by osmotic effects of macromolecules or ions. Accordingly, water transport through AQPs occurs exclusively downstream any given gradient. The AQPs are distributed throughout several organs and display tissue-specific expression (99). So far, ten mammalian aquaporins have been identified and numbered in order of identification, and some interesting localizations may be noted: AQP0, previously called the major intrinsic protein of the lens, is expressed in the eye lens fiber cells, and exhibits low permeability to water; mice lacking this AQP suffer from congenital cataract (99). AQP1 is expressed in small blood vessels such as the peribronchial capillaries, where it may participate in the absorption of lung fluid at birth (100). Interestingly, the expression of AQP1 in the lung is induced by glucocorticoids (101). AQP1 is also found in the apical and basolateral plasma membranes of renal proximal tubule cells (102), accounting for part of the renal water reabsorption (99). AQP2 is expressed in renal collecting duct cells in response to vasopressin; thus it mediates the vasopressin response and is involved in the pathogenesis of diabetes insipidus (103). AQP2 mutations have been shown to account for a few of the genetic defects in patients with nephrogenic diabetes insipidus, a disease more commonly attributed to hereditary mutations of the vasopressin receptor. AQP3 is present in the kidney (99), in the airways (100, 104), and in the skin (105, 106). AQP4 is expressed in astroglial cells of the central nervous system and it has been proposed that it may be involved in the pathogenesis of brain edema (107, 108). AQP5 has been isolated from the salivary gland (109) and from alveolar pneumocytes (110). AQP6 is an odd member of the AQP family, being located intracellularly in renal collecting ducts, and it might possibly be involved in renal acid excretion (97, 111). AQP7, AQP8 and AQP9 have been identified, but their expression and functional significance have not yet been characterized (99).

Some of the AQPs have been shown to be developmentally and physiologically regulated in several tissues. For instance, AQP1 exhibits a complex developmental pattern with transient expression either prior to or
after birth in some tissues, and long-term constitutive expression in others (99, 101, 102). In the rat the development of AQP2 expression in the kidney is related to the development of the urinary concentrating capacity (112). In the lung and in the airways, also, the expression of AQP displays a developmental pattern (101).

AQP in the skin

AQP1 and AQP3 have been identified in adult rat skin (105), but the physiological role of AQPs in the skin is largely unknown. AQP1 is a member of the orthodox aquaporin group and AQP3 is one of the aquaglyceroporins permeable to glycerol in addition to water. Matsuzaki et al (106) state that since AQP3 is present in many epithelial tissues, this AQP might provide epithelial cells with water to protect them from dehydration. Preliminary results from our group have shown expression of AQP1 and AQP3 in perinatal skin and we have speculated that these AQPs might be involved in the large losses of water from the skin of infants born extremely preterm (113). In mice lacking AQP3, Verkman and co-workers have found impairment of stratum corneum hydration and a reduced epidermal glycerol content compared to wild-type mice, suggesting that transport of glycerol through AQP3 might be of importance for the epidermal barrier (114, 115). Recently, functional aspects of AQP3 expression have been studied in adult human skin (116).

The present investigation has for the first time delineated developmental aspects of skin AQP expression and its relation to characteristics of the skin barrier in the perinatal period.
Present investigation

Aims
In order to gain further knowledge of the mechanisms that contribute to the high transepidermal water loss that may cause serum hyperosmolarity in very preterm infants early after birth, studies were conducted

• to determine the magnitude and postnatal course of transepidermal water loss in extremely preterm newborn infants;

• to elucidate the developmental pattern of transepidermal water loss in the experimental rat model;

• to map the expression and distribution of aquaporin in perinatal rat skin;

• to determine the effects of antenatal administration of corticosteroids and of postnatal restriction of fluid and nutrient intake on the skin barrier and aquaporin expression in the perinatal rat.

Methodologically, we chose firstly to study a group of extremely preterm infants in a clinical setting, and secondly to use an experimental approach. In an experimental rat model, instrumental biophysical measurements of skin barrier parameters were combined with analysis of aquaporin expression, using molecular biotechnology.

Subjects (I)
In 13 consecutively delivered newborn infants with a gestational age equal to or less than 25 weeks (24 w: n=3; and 25 w: n=10), measurements of TEWL were performed on the first day of life at a mean postnatal age of 13 (range 5-22) hours and then at postnatal ages of 1, 3, 7, and 28 days. All measurements were performed during routine neonatal intensive care and
monitoring, and only when the infants were judged by the attending neonatologist to be in a clinically stable condition.

The infants were nursed in incubators with controlled ambient temperature and RH and measurements were performed with the infants lying naked on their side, uncovered by bedding material. To avoid condensation of vapor on the Evaporimeter sensors, the RH inside the incubator was lowered from the 85% routinely used during the first postnatal days, to a value of approximately 65%. To compensate for the resulting increase in evaporative heat loss, the ambient air and ventilator gas temperatures were increased. Measurements were then performed 45 minutes after lowering the RH and after at least 10 minutes of stable environmental conditions. The study was approved by the Uppsala University Ethics Committee. Detailed data on the infants are given in paper I.

Experimental animals (II, III and IV)

Experimental studies were performed on timed-gestation Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) of different embryonic (E) and postnatal (P) ages (days). The normal length of gestation in these rats is 21 days and day E0 was defined as the day of observation of a vaginal plug. P0 was defined as the day of birth. In the studies of rat pups delivered preterm (II and III), the dams were anesthetized with an intraperitoneal injection of thiobutabarbital (8 mg/100 g body weight), cesarean section was performed, and TEWL and skin surface hydration were measured (see below). In study II, whole-thickness skin samples were obtained from newborn rat pups delivered at E19 and E20, and from rats at a postnatal age of 40 days (adult). Skin samples from adult rats were used as immunohistochemical preabsorption controls. In study III, experiments were performed at E17-E18 and the effect of ANS was studied by administering betamethasone (60 µg/100 g body weight) intraperitoneally to the dams (n=2) at E17, 24 and 18 hours prior to delivery at E18, when measurements and skin sampling were performed. NaCl-injected animals (n=3) of the same gestational age were used as controls. In study IV, P1 and P7 rat pups were subjected to short-term restriction of fluid and nutrient intake. After spontaneous birth at term, which occurred at night, the P1 pups were kept with their dams (n=4) until the morning, when after random selection half (n=19) of the offspring were separated from their dams and kept in incubators at a temperature of 26 °C, and deprived of intake of fluids and nutrients for 18 hours. The other rat pups (n=18) served as controls and were left with their dam suckling ad libitum during the 18 h period. Measurements of TEWL and skin surface
hydration, and skin sampling, were then undertaken (see below). P7 pups that were also born at term (dams; n=3) were fasted in a similar manner for 18 hours beginning at a postnatal age of 6 days, and measurements of TEWL, skin surface hydration, and skin sampling, were performed at a postnatal age of 7 days (n=13). Suckling P7 pups were used as controls (n=13). The control rat pups were allowed to adapt to the same environmental conditions as the fasted pups by keeping them in the incubator for a 1 h period prior to the measurements. Each rat pup was individually weighed before and at the end of the 18 h period to determine any change in body weight.

All skin samples were taken from the back of the rats. Skin samples for analysis of AQP mRNA expression (II, III and IV) were immediately frozen on dry ice after removal. The dams and adult study rats were fed a standard rat diet (Bantin & Kingman Ltd, UK) and received tap water ad libitum. The studies were approved by the Uppsala Animal Ethics Committee.

Methods

TEWL and insensible water loss from the skin in infants (I)

The rate of evaporation (ER; g m$^{-2}$ h$^{-1}$) was measured with an Evaporimeter (Servo Med, Stockholm, Sweden) as described previously (53). This instrument, which also provides data on RH and vapor pressure, calculates ER from the RH measured with two sensors at two distances from the skin surface. The sensors are located in a probe that is held lightly against the skin during the measurement. Since ER depends on the ambient vapor pressure (59, 117), the instrument was also used to determine RH and vapor pressure in the surrounding air. To allow determination of total TEWL (g m$^{-2}$ h$^{-1}$) in the infant study, ER was measured from the skin surface on the chest, an interscapular skin area, and a buttock. TEWL was then estimated as previously (59), according to the equation:

$$\text{TEWL} = 0.92 \times \text{ER}(A, B, C) + 1.37$$

where ER(A, B, C) is the mean of the ER values obtained from the three sites of measurement. Since the same ambient RH could not be achieved during all the measurements, the values obtained were corrected to an RH of 50% (TEWL$_{50}$), using the two-point equation for ER over RH at each measurement as described previously (118).
The insensible water loss through the skin (IWL; g kg\(^{-1}\) 24 h\(^{-1}\)) at 50% RH was calculated as previously (63), according to the equation:

\[
\text{IWL}_s = \text{TEWL}_{50} \times \text{BSA} \times 24
\]

where BSA is the body surface area (m\(^2\)), and 24 is the correction factor for time (24 h). BSA was calculated from the du Bois (119) length-weight formula, with the proportionality constant changed as proposed by van Graan (120):

\[
\text{BSA} = 0.2157 \times W^{0.425} \times H^{0.725}
\]

where W is the body weight (kg) and H is the height (m) of the infant.

The ambient temperature, incubator inner wall temperatures, esophageal temperature, and skin temperatures were measured with a tele-thermometer and probes (YSI, Yellow Springs, OH, USA).

**TEWL in rat pups (II, III and IV)**

In the experimental studies the evaporation rate measured with the Evaporimeter from the back of the rat pups was used as an estimate of TEWL (g/m\(^2\) h). In study II, TEWL was measured in E18 (n=8), E20 (n=4) and P4 (n=10) rats. In study III, TEWL was measured in E18 rat pups exposed to betamethasone antenatally (n=10) and in control rat pups (n=15) of the same gestational age.

After delivery, the fetus was cautiously removed from the amniotic sac with its placental connection intact. The fetus was handled carefully with a sterile compress and placed in the prone position without wiping the skin surface area. TEWL measurements were then made from the upper back of all rat pups every two minutes until a stable level was recorded, which occurred 8-12 minutes after delivery. In postnatal (P4) rats, which were born at term, TEWL was measured every two minutes during a ten-minute period. During that period the TEWL values obtained remained stable and the mean of the last two values was calculated.

In study IV, TEWL was measured in P1 (n=37) and P7 (n=26) rat pups at the end of an 18-hour fasting period and in suckling littermate control pups. The evaporimeter probe was also used to measure RH and PH\(_2\)O in the air 10 cm above the animal where the air temperature was also measured, ensuring that all measurements were made under similar environmental conditions.

Air temperature and skin temperature on the back of the rat pups, were measured with a tele-thermometer and probe (YSI, Yellow Springs, OH,
USA). The TEWL measurements were made from a slightly smaller skin surface area than that used in measurements in infants, by reducing the area of skin from which evaporation could take place, and the readings were recalculated so as to be valid for the skin area exposed without affecting the accuracy of the method (Öberg PÅ, personal communication).

Skin surface hydration in rat pups (II, III and IV)
The hydration of the skin was assessed by determining the surface electrical capacitance of the skin, using the NOVA dermal phase meter DPM 9003 (NOVA Research Corp, Gloucester, MA, USA) with a 3 mm probe (121). This instrument consists of a probe with two concentric brass rings separated by nonconducting material, and operates between 90 for a low, and 999 for a high reading, expressed as an arbitrary value of picoFarad equivalents (pF). The probe was held lightly against the skin, and the value recorded shortly (5 seconds) after its application to the skin was considered to reflect baseline surface hydration of the skin (54, 121).

In studies II and III skin surface hydration was measured intermittently as described for the TEWL measurements, on the skin of the lower back of the rat pups, caudal to the area where TEWL was measured. When a stable level was reached, the mean of the last two values was calculated. In study IV two measurements were performed with an interval of 2 minutes and a mean was calculated.

Skin water content in rat pups (III and IV)
Whole-skin samples were analyzed for their tissue water content by determining the tissue wet/dry weight ratio (W/D) as previously described (122). After removal, the samples were placed in pre-weighed tubes, individually weighed and frozen and then freeze-dried for 72 hours. The samples were then re-weighed and the skin water content was calculated as the ratio of the weight before freeze drying to the weight after drying.

AQP immunohistochemistry (II)
After fixation, immunohistochemical analysis was performed using thin cryosections (10-12 µm) or thin paraffin sections (2 µm) incubated with the primary antibodies against the different AQP (800 ng IgG/ml). The labeling was visualized as previously described (123), using horseradish peroxidase-conjugated secondary antibodies (P0448, DAKO, Denmark; diluted 1:100). Controls in which the primary antibodies were preabsorbed with the immunizing peptides displayed no labeling.

AQP gene expression (II, III and IV)
The expression of skin AQP1 and AQP3 mRNA was analyzed by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) in studies II-IV. The method allows quantification of specific mRNA and it is semiquantitative in the sense that the expression of specific mRNA is determined in relation to the expression of an internal standard. To avoid the use of genes that might be up- or downregulated during maturation or by the action of our interventions, we chose the 18S rRNA as internal standard (124).

Skin samples from 6 rat pups in each group were used for extraction of total RNA using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The yield of total RNA was measured with a spectrophotometer (Beckman DU 640, Fullerton, CA, USA). Reverse transcription was carried out in a 20 µl reaction volume containing 3 µg total RNA, standard M-MLV RT-buffer (Promega, Madison, WI, USA), 40 ng oligo-dT15 (Promega), 1 mM dNTP (Amersham Pharmacia Biotech, Little Chalfont, UK), 60 U rRNasin (Promega) and 300 U M-MLV RT (reverse transcriptase) (Promega), and incubated for 60 minutes at 42º C. The RT reactions were terminated by inactivation at 65º C for 10 minutes followed by chilling to 4º C. Five microliters of RT reaction solution was then converted to a 50 µl polymerase chain reaction (PCR) mixture containing standard PCR buffer (Promega), 2.5 mM MgCl2, 0.2 mM each dNTP (Amersham Pharmacia Biotech) and 0.6 µM AQP1 primers (or 0.8 µM AQP3 primers), 0.4 µM Classic II Internal Standards (Ambion, Texas, TX, USA), and 10 U AmpliTaq Gold (Perkin Elmer, Foster City, CA, USA). The PCR mixtures were divided into three reactions of 15 µl each and subsequently amplified in 26, 28, and 30 cycles (94º C for 30 sec, 65º C for 1 min), starting at 95º C for 8 minutes and finishing at 65º C for 5 minutes. The PCR products were run on 1.5% agarose gel with 1×TAE buffer, containing 0.5 µg/ml GelStar stain (BioWhittaker Molecular Applications, Rockland, ME, USA). GeneRuler 100 bp DNA ladder (Fermentas, Vilnius, Lithuania) was used for sizing of PCR fragments. Digital images were acquired with use of a Fluor-S MultiImager and analyzed with the original software (Quantity One, version
4.2.1. Bio-Rad Laboratories, Hercules, CA, USA) after subtraction of matched backgrounds. All contents (except RNA and cDNA, rRNasin, M-MLV RT) in semiquantitative RT PCR were premixed to eliminate errors during pipetting. All RNA samples were analyzed in triplicate. The level of 18S rRNA expression was measured in all samples and used to normalize the AQP expression, thus correcting for any sample-to-sample differences in RNA concentration, RNA quality, and efficiency of the RT and PCR reactions. The values for AQP expression are presented as the ratio of AQP to 18S signals.

The primers for AQP1 and AQP3 were designed on the basis of their reported sequences and obtained from CyberGene, Huddinge, Sweden. The AQP primers were selected from different exons to avoid amplification of genomic DNA. The Classic II 18S Internal Standards (Ambion) primer set was used as internal control, in accordance with the manufacturer’s protocol.

Blood analyses (IV)
After measurement of TEWL and skin temperature, the rat pups were decapitated and blood samples were collected (P1: n=19; P7: n=8) for determination of serum cortisol (Spectria coated tube radioimmunoassay, Orion Diagnostica, Espoo, Finland) and acid-base status, S-Na and S-K (ABL 770, Radiometer, Copenhagen, Denmark).

Treatment of data
Values are presented as means ± SD. ANOVA on repeated measurements (I) and Student’s t-test on unpaired observations (II, III and IV) were used to test for statistical significance and p < 0.05 was considered significant.

Results

TEWL in extremely preterm infants (I)
In a group of extremely preterm infants (n=13, GA 24-25 w), TEWL corrected to 50% RH (TEWL_{50}) was 58.4 ± 14.8 g m^{-2} h^{-1} on the first day after birth. The TEWL_{50} remained at the same high level at a postnatal age of 1 day (59.3 ± 17.6 g m^{-2} h^{-1}), and then gradually decreased with increasing postnatal age. At a postnatal age of 28 days TEWL_{50} was 24.2 ± 7.7 g m^{-2} h^{-1}. From the data on TEWL_{50} and from the infants’ body weight and length, a calculation of insensible water loss through the skin (IWL_{s}) was made. It
was estimated that the high initial evaporation observed in these infants correlated to an IWL of $164 \pm 46$ g kg$^{-1}$ 24 h$^{-1}$. With increasing postnatal age and in parallel with the decrease in TEWL, the infants were able to maintain a normal body temperature at lower ambient temperatures. Data on TEWL$\text{50}$ in relation to postnatal age are displayed in Figure 2.

![Figure 2](image_url)

Figure 2. Transepidermal water loss, corrected to 50% relative humidity (TEWL$\text{50}$), in relation to postnatal age in 13 infants born at 24-25 completed weeks of gestation (adapted from paper I, republished with permission from Acta Paediatrica)

AQP1, AQP3, and TEWL in perinatal rat skin (II)

Immunohistochemical analysis of E20 and adult rat skin revealed that AQP1 and AQP3 were abundantly expressed on day 20 prenatally and in adult skin. Representative immunohistochemical photographs of the AQP3 expression are displayed in Figure 3.
Figure 3. Immunocytochemical localization of AQP3 in rat skin at E20 (top) and adult rat skin (bottom). AQP3 is present in basal cell layers of the epidermis (adapted from paper II, republished with permission from Pediatric Research).

AQP1 staining was observed exclusively in dermal capillaries in both E20 and adult rat skin. The staining of AQP1 was denser at E20 than in adult skin because of the relatively higher abundance of capillaries in the immature skin. AQP3 was expressed in the epidermis. In E20 skin the expression was more abundant and localized to the apical-lateral plasma membrane of the two to four basal cell layers of the epidermis (Fig. 3). In adult rat skin AQP3 expression was less abundant, and in the epidermis it was limited to the most basal cell layer (Fig. 3). AQP3 was also found in sebaceous glands and hair follicles. No staining was observed in the most superficial layers of the epidermis. The expression of AQP1 and AQP3 mRNA was several times higher in embryonic than in adult skin (p<0.001). At E19 the AQP to 18S
ratio (see methods) for AQP1 was 1.5 ± 0.4 and for AQP3 it was 2.5 ± 0.3. The corresponding values in adult skin were 0.2 ± 0.1 and 0.6 ± 0.2 respectively.

TEWL and skin surface hydration were measured from the skin of E18, E20 and P4 rats. TEWL was highest at E18 (133 ± 18 g/m²h) and then decreased markedly, reaching a value of 25 ± 1 g/m²h at E20 and 9 ± 2 g/m²h at P4. Skin surface hydration was also related to maturity, being 794 ± 15 pF at E18, 109 ± 11 pF at E20 and 0 ± 0 pF at P4.

Antenatal corticosteroids, TEWL and AQP3 in preterm rats (III)
In study III, preterm rat pups delivered at E18, after exposure to betamethasone 24 and 17 hours prior to delivery, had TEWL values approximately 30% lower (134±12 g/m² h) than those of unexposed control pups (183±14 g/m² h) (p<0.001). In parallel with the findings concerning TEWL, exposure to ANS resulted in lower skin surface hydration as reflected by a lower capacitance of 783±20 pF compared to 822±20 pF in the controls (p<0.001). There were no differences in ambient RH or air temperature, or in skin temperature, between the groups.

To provide an estimate of total skin water content, whole-thickness skin samples were analyzed for their wet/dry weight ratio. W/D was lower in betamethasone-exposed rat pups (8.8±0.7) than in unexposed control rat pups (10.7±0.8) (p<0.001).

Skin AQP mRNA expression was determined by use of semiquantitative RT-PCR. The epidermal AQP3 mRNA expression was twice as high in skin from rat pups exposed to betamethasone as in skin from control rat pups (191 ± 21 % vs. 100 ± 33 %; p<0.01). Analysis of the dermal AQP1 mRNA expression revealed no significant differences between the groups.

TEWL after restriction of fluid and nutrient intake in newborn rats (IV)
TEWL was determined in rat pups subjected to restriction of fluid and nutrient intake for a period of 18 hours at a postnatal age of 1 and 7 days and in littermate control rat pups that were suckled by their dam. At P1, TEWL was twice as high in the fasted rat pups (5.0 ± 0.9 g/m² h) as in the controls (2.6 ± 0.6 g/m² h, p<0.001). At P7, however, the 18-hour fasting period resulted in a slightly lower TEWL of 5.9 ± 1.4 g/m² h compared to a TEWL of 8.1 ± 2.2 g/m² h in the controls (p<0.05). There were no significant differences in ambient conditions or skin temperature between the fasted group and the control group at P1, but at P7 the skin temperature was lower
(p<0.05) in the fasted (30.0 ± 1.2 °C) than in the control rat pups (31.2 ± 1.4 °C).

W/D was higher (p<0.001) in the skin of P1 rat pups than in that of P7 pups. Furthermore, at P1 the period of fasting resulted in an increased (p<0.001) skin water content as reflected by a higher W/D in the skin of fasted rat pups (7.9 ± 0.8) than in the controls (6.5 ± 0.3). Also at P7, W/D was slightly higher after fasting, being 3.6 ± 0.6 in the fasted rat pups and 3.1 ± 0.4 in the controls (p<0.05).

The serum cortisol value was found to be lower (p<0.001) in the P1 rat pups than at P7, and the S-cortisol value was twice as high (p<0.001) in fasted as in control rat pups in both age groups studied.

Analysis of the mRNA expression of the dermal AQP1 and of the epidermal AQP3 revealed no significant differences between the rat pup groups.
Discussion

Excessive losses of water from the skin of very preterm infants may have clinically important consequences, such as hypothermia, dehydration, and hyperosmolarity. This thesis concerns the water transport through perinatal skin and explores the potential role of aquaporin water channels in the regulation of skin hydration and transepidermal water transport.

An assessment of transepidermal water loss in a group of infants born at 24 and 25 weeks of gestation (I) showed that their TEWL was very high after birth and then decreased at a slower rate than has previously been reported for infants born at a gestational age of 25-27 weeks. These data, which are clinically useful, also formed the basis for the experimental part of the investigation. Using a rat model, we investigated the distribution and gene expression of AQP1 and AQP3 in perinatal skin, in relation to biophysical measurements of TEWL and skin surface hydration (II). In rats, AQP1 and AQP3 were abundantly expressed prenatally, AQP1 in dermal capillaries and AQP3 in basal cell layers of the epidermis. The mRNA expression of both AQPs was higher prenatally than in adult rat skin. TEWL and skin surface hydration were inversely related to maturity, i.e., they showed the same developmental pattern as in infants. Antenatal corticosteroid treatment improved the skin barrier in preterm rat pups, resulting in lower TEWL, lower skin surface hydration and a lower water content of the skin than in unexposed control pups (III). Antenatal steroids also increased epidermal AQP3 expression (III). In addition, in newborn term rat pups short-term restriction of fluid and nutrient intake caused an increase in TEWL and in the skin water content early after birth (IV). The localization and expression of AQP in the perinatal skin are such that they might be involved in the hydration and transport of water through the skin of the fetus and/or newborn infant.

In study I it was found that in infants born at 24-25 weeks of gestation TEWL remained at a higher level 4 weeks after birth than has previously been reported for a group of slightly more mature infants (62). On the first day after birth, the TEWL values were of the same magnitude as has previously been observed in infants born at a gestational age of 25-27 weeks (62). From TEWL, an estimation of IWLs (63) was made, based on calculations of body surface area and on the hypothetical situation where the
entire skin of the infant is exposed to a homogeneous environment with a relative humidity of 50%. This is not the case in the clinical situation, since areas of the infant’s skin may be in contact with other skin areas, clothing, bedding material, and monitoring devices, all of which may create a microclimate close to the skin that will presumably influence TEWL. Nevertheless, the magnitude of IWL, illustrates the importance of carefully controlling the environment in which the infant is nursed. As TEWL is inversely related to the vapor pressure (or relative humidity) of the environment, nursing extremely preterm infants in highly humidified incubators is an effective and convenient way to reduce their losses of fluid through the skin (4, 60, 62, 66, 78, 125). The high TEWL in the most preterm infants often leads to a transient hyperosmolarity during the first days after birth, even when they are nursed in incubators with a high humidity (126). It is not clear whether this finding can be attributed solely to the poor physical properties of the skin barrier, or whether other mechanisms, such as the expression of AQP, might influence skin hydration and transepidermal water transport.

The present investigation clearly demonstrates a similarity in evaporation of water from human and rat skin during maturation (II). As in infants (4, 62), the amount of water lost from the skin of rat pups was inversely related to gestational and postnatal age (II). Thus, we consider that our experimental model can be used for studying mechanisms involved in the transport of water through the perinatal skin. The results obtained are in accordance with previously published experimental data on the acquisition of a competent skin barrier, obtained with different techniques. Specifically, the following previously reported observations may be noted: 1) an improvement in rat skin barrier function with advancing maturity (10, 27), which is in conformity with the findings in study II, demonstrating that the development of an epidermis capable of limiting water loss to the environment is a gradual process; 2) a maturational effect of antenatal steroids on barrier formation in the fetal rat (52, 86), which is supported by the finding in study III that TEWL was lowered by antenatal corticosteroid treatment. In addition, it has been found that exposing the skin to a dry environment accelerates maturation of the epidermal structure and barrier function (46, 47). In several of the previously published studies (10, 46, 86) the skin barrier was analyzed by the closed chamber technique, with the skin occluded by a measuring chamber, which thereby created a microenvironment close to the skin to which water was lost and could be estimated. In newborn preterm infants the skin is exposed to air after birth. To allow comparisons with available data on the development of epidermal permeability in infants, we measured TEWL by evaporimetry, a method used extensively in infant studies (127), as it allows accurate determinations.
of both high and low evaporation rates during free evaporation from the skin surface (53, 117). Capacitance measurements of the skin barrier have also been performed previously in infant (54) and experimental studies (128), not only as a measure of baseline hydration but also as an estimate of TEWL by analyzing the rate of water accumulation beneath the measurement probe (54). The instrument allows a measurement of surface hydration with a detection depth approximately corresponding to the thickness of the whole mature stratum corneum or part of it (121). In the present investigation (II and III), skin surface hydration was determined in preterm rat pups with a very thin stratum corneum, which means that this method might have measured hydration of deeper structures (e.g., epidermis). Readings obtained immediately after application of the probe are considered to reflect the baseline surface hydration, while those obtained after a longer period of occlusion are influenced by TEWL through the accumulation of water under the probe (128). Our estimate of skin hydration was based on the reading 5 seconds after application of the probe. Since TEWL was very high in the most immature rats, it may have partly contributed to the high capacitance readings obtained in studies II and III.

Although the skin provides an adequate interface with the aqueous environment in utero, its ability to limit water loss from the body surface after preterm birth is poor (4, 62, 129). In the newborn preterm infant, postnatal life has been reported to stimulate the structural development of the epidermis so that its histological structure a few weeks after birth resembles that of a term infant (15). However, our data (I) demonstrate that infants born extremely preterm have four times higher TEWL at a postnatal age of 4 weeks than term infants. The reason for this continued compromise of the skin barrier after preterm birth is not known, but it might be due to the environment to which these infants are exposed, or to the metabolic changes that take place at and after birth. The skin of a preterm infant, as compared to that of a fetus, is exposed to factors that might influence the formation of an effective barrier against the environment. The most striking difference between the prenatal and postnatal environment is the exposure to air after birth. The effect of dry air, so-called xeric stress, has been proven experimentally to influence both the structure and function of the skin barrier (46, 47), but the influence of different levels of xeric stress on the development of the barrier function in preterm infants has not been investigated. A further factor, nutrition, also has the potential to influence growth of the skin, and fetal growth has been found to influence skin development in the rat. Rat pups with intrauterine growth retardation display a reduced epidermal thickness and an altered content of the stratum corneum (130), but this does not seem to be related to any loss of barrier function (131). Studies have shown, rather, that preterm SGA infants have lower
TEWL than appropriately grown infants of the same gestational age (132), which might imply that SGA infants have a more mature epidermal barrier.

In study IV, newborn rat pups subjected to a period of restricted fluid and nutrient intake early after birth showed higher TEWL and a higher water content of the skin than suckling control pups, while this response was not observed when the same the intervention was carried out at an age of 6 days.

In rat pups fasted at a postnatal age of 6 days, the water content of the skin was higher but TEWL slightly lower at the end of this period than in controls, indicating that developmental changes of the skin had occurred and that increased skin hydration does not always result in higher TEWL. To speculate, the increased skin hydration observed in rat pups after fasting might be due to a mechanism that helps to conserve water in the skin in response to dehydration. Since our assessment of the skin water content was based on determinations of wet/dry weight ratios, the changes observed might simply represent an altered content of “dry” elements of the skin in response to corticosteroids (III) or to restriction of fluid and nutrient intake (IV). The results of study IV might be of clinical relevance. At present, it is not known whether routines for administration of fluids and nutrients can influence the insensible losses of fluids in newborn infants.

The data obtained in rats (II) on the distribution of AQP1 in the dermis and AQP3 in the epidermis suggest that AQP has a physiological role in the skin. Furthermore, we found the gene expression of the AQP to be clearly higher in the fetal than in the adult rat skin (II) indicating a difference in function in relation to maturity or birth. Preterm rat pups that had been exposed to corticosteroids antenatally (III) had a lower TEWL, skin hydration and water content than unexposed controls, indicating a maturational effect. The mRNA expression of the epidermal AQP3 was clearly higher in rat pups exposed to ANS than in control rat pups, while there was no change in the expression of the dermal AQP1 related to ANS. This finding might suggest that the effect of the administered corticosteroid represents a regulatory response in the skin rather than an unspecific gene induction.

What then is the significance of the AQP in the developing skin? Apparently, these channels should not simply be regarded as membrane surface pores leaking water through the skin, with TEWL directly related to the number of water channel proteins expressed. AQP3 might serve the role of hydrating basal layers of the mature skin exposed to a dry environment (106). This concept is supported by the finding that AQP3-null mice, as compared to the wild-type, show an impairment of stratum corneum hydration (115). However, in the absence of a competent stratum corneum limiting water loss, the AQP could influence the rate of water transport through the skin by facilitating movement of water across the basal cell
layers into the epidermis, resulting in a high water content in the skin and a high rate of water evaporation from the skin surface. Most of the amniotic fluid formed early in gestation is considered to leak through the skin (133). This transfer might at least partly take place through AQP3, representing another possible physiological function.

How should the effects of antenatal steroids observed in study III (improved barrier function and increased AQP3 expression) be interpreted? One possible explanation could be that the AQP might be involved in the transfer of water from superficial to deeper layers of the skin. If, depending on existing gradients, AQP3 promotes transport of water inwards from the surface, this might represent a mechanism whereby epidermal hydration is reduced as an effect of ANS. Support for the suggestion of such water transfer is provided by the previous finding of a negative TEWL in some preterm SGA infants (63, 132), and the demonstrated up-regulation of AQP3 by hypertonicity (134) might imply a role of AQP3 in this context. Alternatively, the role of AQP in the skin might partly concern processes other than those purely involved in water transport. Since ion concentration gradients in the epidermis have been shown to be an important regulator of epidermal differentiation (28, 29), AQP-mediated changes in the epidermal water content might also have the potential, indirectly, to influence processes involved in the maturation of the epidermal barrier. Another possibility, as suggested in a recent paper by Hara et al (114), is that transport of glycerol through AQP3 is important to barrier formation and function. If so, the increased AQP3 expression observed after ANS might have provided the maturing skin with components necessary for the improvement of the improved skin barrier.

In study IV we did not find any significant changes in AQP gene expression in response to fasting. This finding indicates that although AQP expression might be a prerequisite for appropriate basal skin hydration, the fasting-induced changes in TEWL and skin water content in the term newborn rat were not regulated directly by alterations in AQP expression.
Conclusions

The following conclusions can be drawn from the findings in the present investigation:

• In extremely preterm infants, transepidermal water loss is very high early after birth. It then decreases with increasing postnatal age, but at an age of 4 weeks it still remains 4 times as high as in infants born at term. The amount of water lost from the skin of extremely preterm infants has important clinical consequences and measures should be taken to reduce and/or compensate for these losses.

• The developmental pattern of transepidermal water loss in the perinatal period in the rat resembles that of infants.

• The aquaporin water channels AQP1 and AQP3 are distributed in locations where they can influence hydration and water transport in the skin. Their expression is higher in fetal skin than in mature skin.

• Antenatal corticosteroid treatment induces functional changes in the skin of preterm rat pups, resulting in a lower TEWL, lower skin surface hydration, and a lower water content of the skin. These changes occur in parallel with an upregulation of AQP3 in the epidermis.

• Short-term restriction of fluids and nutrients has an impact on the skin water content and TEWL in the newborn rat pup.
Acknowledgements

I wish to express my sincere gratitude to:

**Gunnar Sedin**, my main supervisor, for sharing with me his vast knowledge of neonatal physiology, and for solid guidance, concern and friendship.

**Barbro Kjällström** and **Sabina Albinsson**, for excellent laboratory and technical assistance, genuine friendship and support.

**Gunnar Flemström**, my supervisor at the Department of Physiology, for valuable discussions on membrane physiology.

**Anita Aperia**, my supervisor at Karolinska Institute, for kindly providing excellent working conditions at the Astrid Lindgren Children’s Hospital and for teaching me to appreciate the unexpected.

**Gunnar Sjörs**, friend and co-author, for stimulating collaboration and for focusing our scientific discussions on clinical issues.

**Sergey Zelenin**, co-author, for extraordinary expertise in molecular biology, guidance through the mysteries of RT-PCR, and friendship.

**Ann-Christin Eklöf**, co-author, for always making me feel welcome at the Astrid Lindgren Children’s Hospital and for sharing her knowledge on the handling of laboratory animals.

**Soren Nielsen** and **Lene N Nejsum**, co-authors, for constructive collaboration and outstanding performance in immunohistochemistry, and for fruitful discussions during my visit in Denmark.

**Uwe Ewald**, my main clinical mentor, for posing thought-provoking questions and generous friendship.

**Torgny, Hans, Anders, Richard, Bo, Barbro, Erik** and **Katarina**, my fellow neonatologists, for generous friendship, support and clinical training.
All staff at the neonatal care unit 95F/H, for helping out with the clinical studies and for being such good friends and teachers of clinical skills.

Maud Marsden, for excellent linguistic revision of my manuscripts.

My wife Ulrika and our children Simon, Klara and Lisa, for everything that really matters to me.

This investigation was supported by external grants from the Swedish Research Council, HRH Crown Princess Lovisa’s Association for Child Medical Care, and the Gillberg Foundation.
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