

The Pediatric Graduate School, Children's Hospital
Doctoral School in Health Sciences, Doctoral Programme in Clinical Research
Faculty of Medicine
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PERINATAL REGULATION OF LUNG LIQUID ABSORPTION IN PULMONARY ADAPTATION IN NEWBORN INFANTS

Cecilia Janér

ACADEMIC DISSERTATION

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Supervisors

Professor Sture Andersson
Department of Pediatrics
Division of Neonatology
Children's Hospital
Helsinki, Finland

Docent Olli Pitkänen
Department of Pediatrics
Division of Cardiology
Children's Hospital
Helsinki, Finland

Reviewers

Docent Ulla Sankilampi
Department of Pediatrics
University of Kuopio
Kuopio, Finland

Docent Hanna Soukka
Department of Pediatrics
University of Turku
Turku, Finland

Opponent

Professor Emeritus Hugo Lagercrantz
Division of Woman & Child Health
Karolinska Institute
Stockholm, Sweden

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*To my grandparents
Marita and Samuel*

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LIST OF ORIGINAL PUBLICATIONS

- I. Martelius L, Janér C, Süvari L, Helve O, Lauerma K, Andersson S. Delayed lung liquid absorption after cesarean section at term. *Neonatology*. 2013;24;104:133-136.
- II. Janér C, Pitkänen OM, Helve O, Andersson S. Airway expression of the epithelial sodium channel α -subunit correlates with cortisol in term newborns. *Pediatrics*. 2011;128: e414-421.
- III. Janér C, Pitkänen OM, Süvari L, Turpeinen U, Palojärvi A, Andersson S, Helve O. Cortisol, gestational age, mode of delivery, and molecular mechanisms of postnatal lung liquid clearance. *Neonatology*. 2015;107:27-33.
- IV. Janér C, Helve O, Pitkänen OM, Kari MA, Peltoniemi OM, Hallman M, Hovi P, Andersson S. Expression of airway epithelial sodium channel in the preterm infant is related to respiratory distress syndrome but unaffected by repeat antenatal beta-methasone. *Neonatology*. 2010;97:132-138.

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This thesis includes unpublished results.

ABBREVIATIONS

ATI	type I alveolar epithelial (cell)
ATII	type II alveolar epithelial (cell)
BE	base excess
cAMP	cyclic adenosine monophosphate
CK18	cytokeratin 18
CS	cesarean section
DBcAMP	dibutyl cyclic adenosine monophosphate
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel
FD	fetal day
FDLE	fetal distal lung epithelium
FT3	free triiodothyronine
mRNA	messenger RNA
GA	gestational age
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
Na-K-ATPase	sodium-potassium-adenosine-tri-phosphatase
Nedd4-2	neural precursor cell expressed developmentally downregulated protein 4-2
PD	potential difference
RDS	respiratory distress syndrome
RNA	ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
siRNA	small interfering RNA
SGK1	serum- and glucocorticoid-inducible kinase 1
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
TTN	transient tachypnea of the newborn
T3	triiodothyronine
VD	vaginal delivery

ABSTRACT

Respiratory distress is a major contributor to morbidity in newborn infants. Insufficient clearance of lung liquid at birth causes maladaptation (transient tachypnea of the newborn, TTN) primarily in late preterm (delivery at 34^{0/7} to 36^{6/7} gestational weeks) or term infants. In small preterm infants, lacking adequate surfactant levels, excess lung liquid contributes to respiratory distress syndrome (RDS). Major risk factors for respiratory morbidity include, among others, preterm delivery or delivery by elective cesarean section (CS). Importantly, the risk for respiratory morbidity remains elevated even in early term infants (i.e. delivery at 37^{0/7}- 38^{6/7} gestational weeks).

In both prematurity and CS, the hormonal milieu of the fetus, important in the adaptation to air-breathing, differs from that in term, vaginally delivered infants. In particular, glucocorticoids (GCs) play an important role in the maturation of the fetal lung. GCs are, administered to the mother in imminent preterm delivery, used in the prophylaxis of respiratory morbidity in preterm infants.

Although relevant to preventive and treatment strategies for respiratory morbidity, data on the role of airway ion and liquid transport and their hormonal regulation in human infants are limited. Therefore, the aim of this thesis was to acquire new data on lung liquid clearance, its molecular mechanisms and their perinatal hormonal regulation during pulmonary adaptation in newborn human infants.

We used ultrasound to study the amount of lung liquid in term newborn infants. The gene expression of the epithelial sodium channel (ENaC), Na-K-ATPase and serum- and glucocorticoid-inducible kinase 1 (SGK1) was measured in airway epithelial cells from nasal epithelium, which can be used as a surrogate for the lower airway epithelium. Gene expression was quantified with a real-time reverse-transcription polymerase chain reaction (RT-PCR). We determined the concentration of cortisol in umbilical cord blood and saliva with liquid-chromatography tandem-mass spectrometry or enzyme-linked immunosorbent assay (ELISA), and studied the associations of ENaC, Na-K-ATPase and SGK1 with cortisol in late preterm and term infants. In preterm infants, we studied the effect on airway ENaC expression of a repeated antenatal dose of the glucocorticoid betamethasone to the mother. The term infants and the majority of the preterm infants in the studies were born at the Women's Hospital, Helsinki University Central Hospital.

The lung liquid content of term, healthy infants at three hours postnatally was significantly higher in infants delivered by elective CS than in infants delivered vaginally (VD). Consistent with previous reports, the umbilical cord cortisol concentration was lower in CS than in VD infants. Also, CS was associated with lower SGK1 expression within hours after delivery. Within hours postnatally, the airway expression of ENaC, Na-K-ATPase, and SGK1 was lower in infants delivered late preterm and early term than in infants delivered ≥ 39 weeks. In late preterm

and term infants ENaC expression correlated positively with umbilical cord blood and salivary cortisol concentrations. However, a repeat antenatal dose of betamethasone given to the mother had no significant effect on ENaC expression in preterm infants.

The thesis confirms that the amount of lung liquid remains more abundant in CS than in VD infants, and that ultrasound is a useful tool for evaluating lung liquid content in newborns. Low SGK1 expression in CS delivery could contribute to the insufficient activation of transmembrane sodium transport and thus, of liquid. In infants delivered < 39 weeks of gestation airway expression of not only SGK1, but also of ENaC and Na-K-ATPase mRNAs was lower than in those delivered \geq 39 weeks. These physiological data are in line with the epidemiologic evidence of a higher risk for respiratory morbidity in infants delivered before 39 weeks of gestation, especially when delivered by CS. Thus, the data support the practice of postponing elective CS until 39 weeks. Our findings may provide a rationale for strategies aimed at preventing or treating respiratory distress in premature and term infants.

TIIVISTELMÄ

Hengitysvaikeudet aiheuttavat merkittävää sairastuvuutta vastasyntyneillä lapsilla. Täysiaikaisilla ja lievästi ennenaikaisilla lapsilla (syntymä raskausviikoilla 34^{0/7}-36^{6/7}) puutteellinen keuhkojen nesteenoisto voi johtaa keuhkoadaptatiohäiriöön. Pie-nillä keskosilla häiriintynyt nesteenoisto keuhkoista voi pahentaa puutteellisesta surfaktantin määrästä ja toiminnasta johtuvaa hengitysvajausta. Ennenaikaisuus ja syntymä keisarinleikkauksella ovat hengitysvaikeuksien merkittäviä riskitekijöitä. Hengitysvaikeuksien riski on kuitenkin lievästi suurentunut jopa täysiaikaiseksi katsottavilla vastasyntyneillä, jotka ovat syntyneet raskausviikoilla 37^{0/7}- 38^{6/7}.

Sikiön hormonaalinen ympäristö edistää sopeutumista ulkomaailmaan hen-gittämään ilmaa. Ennenaikaisesti tai keisarinleikkauksella syntyvien lasten hor-monaaliset vasteet poikkeavat täysiaikaisina ja alateitse syntyvistä. Glukokortikoi-di-hormonit ovat erityisen tärkeitä sikiön keuhkojen kypsymisen kannalta. Niitä käytetään lääkkeenä äideille annettuina ennen uhkaavaa ennenaikaista synnytystä ehkäisemään keskosten hengitysvaikeuksia. Vaikka hengitysteiden ioni- ja veden-kuljetus sekä niiden hormonaalinen säätely voivat olla merkityksellisiä hengitys-vaikeuksien ennaltaehkäisevien tai hoidollisten toimien kannalta, tunnetaan ne huonosti vastasyntyneillä lapsilla.

Tämän väitöskirjatyön tavoitteena oli tutkia vastasyntyneen lapsen keuhkojen nesteenoistoa, sen molekylaarisia mekanismeja ja hormonisäätelyä keuhkoadaptation aikana.

Tutkimme täysiaikaisten lasten keuhkonesteen määrää ultraäänellä. Määritimme hengitystie-epiteelin natriumkanavan (ENaC), Na-K-ATPaasin ja serum- and glucocorticoid-inducible kinase 1 (SGK1) geeni-ilmentymät sierainten epiteeli-soluissa RT-PCR:llä. Napaveren ja syljen kortisolipitoisuuksia määritimme nes-tekromatografia-tandemmassaspektrometrialla tai ELISA:lla. Keskosilla tutkimme äidille annetun ylimääräisen beetametasoni-glukokortikoidiannoksen vaikutusta hengitystie-epiteelin ENaC:in geeni-ilmentymään. Täysiaikaiset lapset ja valtaosa ennenaikaisista lapsista syntyivät Helsingin Yliopistollisen Keskussairaalan Nais-tenklinikalla.

Keisarinleikkauksella syntyneillä, terveillä täysiaikaisilla lapsilla keuhkojen nes-temäärä kolme tuntia syntymän jälkeen oli suurempi kuin alateitse syntyneillä lapsilla. Aikaisemmin kuvattujen tulosten mukaisesti napaveren kortisolipitoisuus oli matalampi keisarinleikkauksella kuin alateitse syntyneillä lapsilla. Lisäksi SGK1:n geeni-ilmentymä muutaman tunnin sisällä syntymästä oli matalampi keisarinleik-kauksen jälkeen. Hengitystie-epiteelin ENaC, Na-K-ATPase ja SGK1 geenien ilmen-tymät olivat matalammat lievästi ennenaikaisesti ja raskausviikoilla 37^{0/7}- 38^{6/7} kuin 39 raskausviikon jälkeen syntyneillä lapsilla. Lievästi ennenaikaisilla ja täysiaikaisilla lapsilla ENaC geenien ilmentymät korreloivat positiivisesti napaveren ja syljen kor-tisolipitoisuuksiin. Ylimääräisellä annoksella beetametasonia ei kuitenkaan ollut vaikutusta ennenaikaisesti syntyneiden lasten ENaC:in geeni-ilmentymään.

Tämä väitöskirja osoittaa, että vastasyntyneen lapsen keuhkojen nestemäärä on suurempi keisarinleikkauksen kuin alatiesynnytyksen jälkeen. Osoitimme myös, että keuhkojen ultraäänitutkimus on hyödyllinen työkalu vastasyntyneiden keuhkonesteen määrän arvioimisessa. Keisarinleikkauksella syntyneillä lapsilla matala SGK1-geenin ilmentymä saattaa vaikuttaa kielteisesti osmoottisesti aktiivisen natriumin siirtymiseen hengitystie-epiteelin läpi, jolloin nesteenpoistokin on puutteellista. SGK1 ilmentymän lisäksi ennen raskausviikkoa 39 syntyvillä lapsilla ENaC:in ja Na-K-ATPaasin ilmentymät olivat matalammat kuin sen jälkeen syntyneillä. Havaintomme ovat on yhteneväisiä epidemiologisten tutkimusten kanssa, joiden mukaan hengitysvaikeuksien riski on erityisen suuri keisarinleikkauksen jälkeen, jos lapsi syntyy ennen raskausviikkoa 39. Havaintomme puoltaa näkemystä, että elektiivinen keisarinleikkaus tulisi suorittaa viikolla 39 tai sen jälkeen. Löydöksemme antaa pohjan keskosten ja täysaikaisten lasten hengitysvaikeuksien ennaltaehkäiseville tai hoidollisille strategioille.

ABSTRAKT

En otillräcklig absorption av lungvätska kan leda till andningssvårigheter hos nyfödda barn. Hos små prematurer bidrar ett överskott av lungvätska till andningssvårigheter förorsakade av surfaktantbrist. Hos barn födda lindrigt prematura (gravitetsvecka 34^{0/7} till 36^{6/7}) och i synnerhet hos fullgångna barn (födda \geq 37 gravitetsveckor) födda med elektivt kejsarsnitt är ofullständig absorption av lungvätska vid födelsen den primära orsaken till pulmonell adaptationsstörning. Förutom prematuritet, ökar förlossning med kejsarsnitt risken för andningssvårigheter. Av särskild betydelse är att risken för andningssvårigheter är förhöjd även under tidig fullgången graviditet, dvs. under gravitetsvecka 37^{0/7}- 38^{6/7}.

Fostrets endokrina miljö är av stor betydelse för den pulmonella adaptationen. Vid såväl prematuritet som vid elektivt kejsarsnitt avviker fostrets hormonella respons från den vid normal, vaginell födelse efter fullgången graviditet. I synnerhet glukokortikoider är viktiga för mognaden av fostrets lungor och används, givna åt modern inför förlossningen, för att förebygga andningssvårigheter hos prematurer.

Trots att kunskapen om luftvägsepitelets jon- och vätsketransport samt deras hormonella reglering är av betydelse för interventioner som strävar till att förebygga eller behandla andningssvårigheter, är data från studier av nyfödda barn begränsad.

Målet för denna avhandling var därför att erhålla ny kunskap om absorptionen av lungvätska, dess molekylära mekanismer samt deras hormonella reglering vid den pulmonella adaptationen hos nyfödda barn.

Vi studerade mängden lungvätska hos fullgångna nyfödda barn med ultraljud. Genexpressionen av den epiteliala natriumkanalen ENaC, Na-K-ATPase och serum- and glucocorticoid-inducible kinase 1 (SGK1) i luftvägsepitel från näsans slemhinna, vilken kan användas som en modell för epitelet i de nedre luftvägarna och lungorna, kvaantificerades i lungorna, tegier somrrogat fåa andningssvårigheter hos prematurer och fullgångna barn. en rational för strategier sombestämdes med RT-PCR. Koncentrationen av kortisol i navelblod och saliv bestämdes med tandemmassaspektrometri eller ELISA. Hos barn födda lindrigt prematura eller efter fullgången graviditet studerade vi associationen mellan genexpressionen av ENaC, Na-K-ATPase samt SGK1 och kortisolkoncentrationerna. Vi studerade vi effekten av en upprepad dos av glukokortikoiden betametason, given åt modern före förlossningen, på luftvägsepitelets ENaC genexpression hos prematurer. Alla fullgångna barn i studierna samt majoriteten av prematurena föddes på Kvinnokliniken vid Helsingfors Universitets Centralsjukhus.

Friska, fullgångna barn födda med kejsarsnitt hade en större mängd lungvätska tre timmar efter födelsen än barn födda vaginellt. Koncentrationerna av kortisol i navelblod var lägre hos barn födda med elektivt kejsarsnitt än efter vaginell förlossning. Expressionen av SGK1 1.5 h efter förlossningen var också lägre efter kejsarsnitt. Barn som föddes lindrigt prematura eller efter tidig fullgången graviditet hade en lägre expression av ENaC, Na-K-ATPase och SGK1 i luftvägsepitel under

de första timmarna efter förlossningen jämfört med barn födda vid eller efter 39 graviditetsveckan. Expressionen av ENaC korrelerade positivt med kortisolkoncentrationen i navelblod och saliv. Däremot hade en upprepad dos av betametason åt modern ingen mätbar effekt på ENaC expressionen hos prematurer.

Denna avhandling bekräftar att mängden lungvätska är större hos barn födda med kejsarsnitt än efter vaginell förlossning. Den visar att ultraljud är en användbar metod för att estimerar mängden lungvätska hos nyfödda. Hos barn födda med kejsarsnitt, kan en låg expression av SGK1 bidra till otillräcklig aktivering av absorptionen av natrium, och därmed av vätska, över luftvägsepitelet. Förutom låg SGK1 expression, är genexpressionen av ENaC och Na-K-ATPase lägre hos barn födda före än efter graviditetsvecka 39. Dessa fysiologiska fynd överensstämmer med epidemiologiska data som visar en ökad risk för andningssvårigheter hos barn födda före vecka 39, särskilt då förlossningen sker via kejsarsnitt. Dessa resultat stöder principen att elektiva kejsarsnitt bör utföras efter graviditetsvecka 39. Resultaten i avhandlingen kan erbjuda en bas för strategier som strävar till att förebygga eller behandla andningssvårigheter hos såväl prematurer som fullgångna barn.

1 INTRODUCTION

At birth, the transition from having liquid-filled lungs to breathing air is a major challenge for newborn infants. When the clearance of lung liquid is inadequate, pulmonary adaptation may be compromised, resulting in respiratory distress. In late preterm or term infants, retained lung liquid manifests as transient tachypnea of the newborn (TTN), and in preterm infants, a high amount of lung liquid is encountered in respiratory distress syndrome (RDS) (O’Brodivich 1996). RDS requires intensive care and, although TTN is mild and self-limited in many cases, it may lead to severe respiratory failure, pulmonary hypertension, and the need for extracorporeal membrane oxygenation treatment (Keszler et al. 1992, Ramachandappa and Jain 2008). Thus, TTN and RDS are important contributors to morbidity in newborns.

The risk for respiratory morbidity rises if infants are born preterm, or even at early term, especially if delivered by elective cesarean section (CS) (Hansen et al. 2008, Melamed et al. 2009, Tita et al. 2009). Term, vaginal delivery (VD) is associated with maturational effects, including a rise in endogenous glucocorticoid (GC) concentrations and a surge in catecholamines due to labor (Murphy et al. 1982, Faxelius et al. 1983, Oh et al. 2006). These changes in the hormonal milieu of the fetus augment the absorption of sodium and liquid by the respiratory epithelium, thereby facilitating pulmonary adaptation.

A significant part of the data on lung liquid clearance, its molecular mechanisms and their regulation comes from animal experiments. This thesis examines the amount of lung liquid, the airway epithelial gene expression of molecules contributing to sodium and lung liquid clearance, and their hormonal regulation in human newborn infants. The studies were performed in the physiological situation of term, vaginal delivery as well as in clinical situations associated with a higher risk for respiratory morbidity (i.e. elective CS and premature delivery).

2 REVIEW OF THE LITERATURE

2.1 Lung development

In humans, fetal lung development is divided into four partially overlapping prenatal stages: the embryonic stage (0 to 5-6 weeks of gestation), the pseudoglandular stage (weeks 5-6 to 16-17 of gestation), the canalicular stage (16 to 24-26 weeks), and the saccular stage (week 24 to term) (Burri 1984, Bolt et al. 2001, Bizzarro et al. 2004, Smith et al. 2010). The final stage of lung development, the alveolar stage, begins at week 36 of gestation and continues after birth (Bolt et al. 2001 and Bizzarro et al. 2004).

During the first five to six weeks of gestation (i.e. the embryonic stage), the lung anlage appears as two ventral buds of the foregut, which later develop into the right and left lungs (Burri 1984, Schittny and Burri 2008). These lung buds first form the left and right main bronchi and subsequently the bronchial tree through repetitive growth and mainly dichotomous branching (Schittny and Burri 2008). The pseudoglandular stage includes continued growth and branching of the airways with the formation of the conducting airways and future terminal bronchioles (Bolt et al. 2001, Bizzarro et al. 2004, Smith et al. 2010). Towards the end of this stage, the development extends as far as the formation of alveolar ducts (Schittny and Burri 2008). The formation of respiratory bronchioles with the terminal bronchioles and alveolar ducts also takes place during the canalicular stage (Jobe 2011). The future gas exchange region of the lungs, originating from the last segment of the conducting airway, manifests as early acini (Schittny and Burri 2008). Importantly, during the canalicular stage, a thin blood-air barrier results from the fusion of the epithelial and vascular endothelial basement membranes (Smith et al. 2010, Jobe 2011). During the saccular stage, the peripheral airways form widened airspaces known as saccules or terminal sacs (Schittny and Burri 2008). Via septation of the saccules, alveolar ducts and, subsequently, alveolar sacs develop (Bolt et al. 2001, Smith et al. 2010). However, the majority of the alveoli form after birth, and alveolarization continues until the first 18 to 24 postnatal months (Bolt et al. 2001, Bizzarro et al. 2004, Schittny and Burri 2008). During the alveolar stage, the amount of interstitial tissue decreases, and the walls of the airspaces thin (Bolt et al. 2001, Bizzarro et al. 2004, Smith et al. 2010).

The structure of the airways and lungs develops in parallel with cellular differentiation, which begins during the pseudoglandular stage and proceeds centrifugally from the proximal airways, lined with high columnar epithelium, to the cuboidal epithelium of the distal airways (Smith et al. 2010, Schittny and Burri 2008). The canalicular stage includes the differentiation of cuboidal cells into type I and type II alveolar epithelial (ATI and ATII) cells with early type II cells representing the stem cells of type I and II pulmonary epithelial cells (Bolt et al. 2001, Bizzarro et al. 2004, Smith et al. 2010, Schittny and Burri 2008). The production of surfactant by ATII cells begins during the canalicular stage, with

small amounts present during weeks 22-24 (Schittny and Burri 2008). ATI cells contribute to the formation of the first thin air-blood barriers (Schittny and Burri 2008) and eventually constitute > 95% of the alveolar surface.

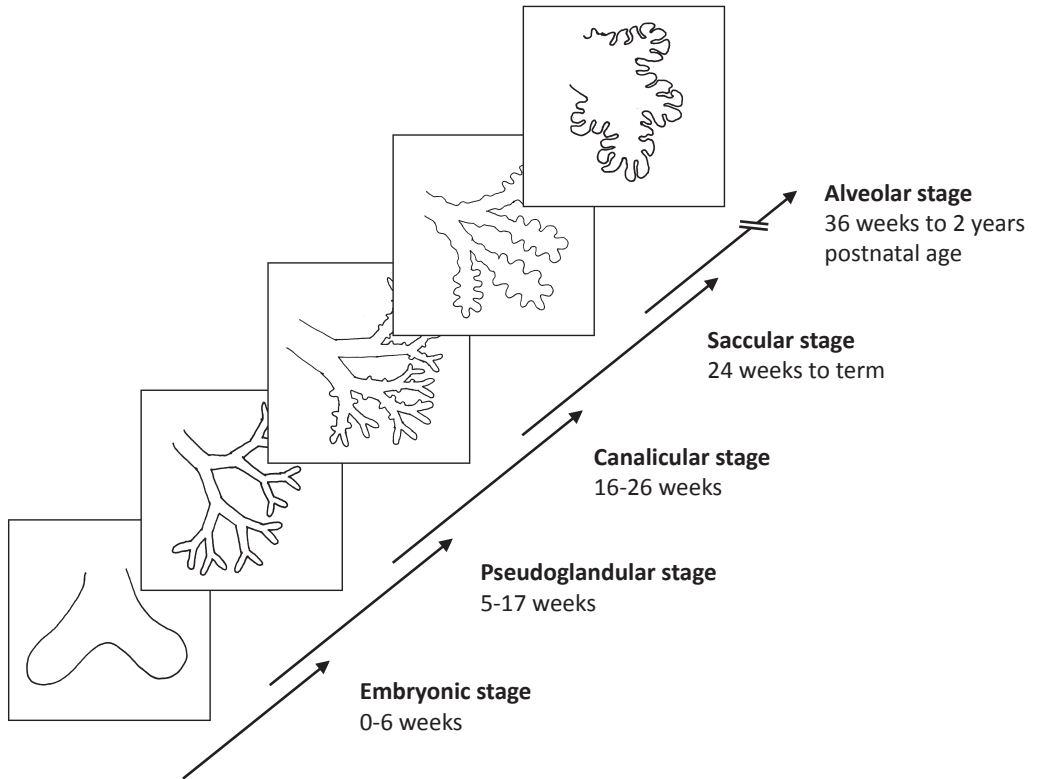


Figure 1. Stages of lung development. The stages overlap, and the timing of the different stages is approximate.

2.2 Lung liquid clearance at birth

In the fetus, the transport of water and solutes into the developing lumen distends the airways and allows for normal lung development (Alcorn et al. 1977), possibly via stretch-activated mechanisms (Nardo et al. 1998). The significance of intraluminal lung liquid is obvious in situations where it is reduced, as in oligohydramnios or congenital diaphragmatic hernia, where the lungs remain hypoplastic (Wigglesworth et al. 1988, Moessinger et al. 1983, Moessinger et al. 1986, Moessinger et al. 1987, Moessinger et al. 1990).

Lung liquid content already decreases during the last days of gestation (Kitterman et al. 1979, Dickson et al. 1986, Pfister et al. 2001), and labor further promotes the clearance of water from the lungs (Bland et al. 1979, Bland et al. 1982, Chapman et al. 1994, Berger et al. 1996). During labor, the activity of fetal

truncal muscles may result in the expulsion of some lung liquid via the trachea (Stockx et al. 2007). Prenatal reduction in the amount of lung liquid improves postnatal gas exchange with superior SaO_2 , PaO_2 and PaCO_2 values and results in less acidosis during the first hour after cesarean delivery than without liquid reduction before delivery (Berger et al. 1996). In animals delivered by cesarean section (CS), the first breaths promote liquid clearance and aeration of the lungs (Siew et al. 2009) but the absorption of lung liquid continues for several hours after birth (Bland et al. 1980, Bland et al. 1982). In adults, normal airway function and optimal gas exchange require the maintenance of an appropriate layer of liquid in the airways and the lung (Mac Sweeney et al. 2011).

2.2.1 Association of lung liquid clearance with hormonal stimuli

The rise in endogenous GC concentrations during late gestation allows for the subsequent activation of lung liquid absorption induced by high concentrations of catecholamines during labor. GCs increase the sensitivity and thereby the absorptive response of the lungs to catecholamines (Wallace et al. 1995, Wallace et al. 1996).

Catecholamines play an important role in regulating the transport of lung liquid. Their effects depend on the length of gestation: in lambs intravenous infusion of beta-agonists early in gestation inhibits or slows the secretion of liquid, but later (during the saccular stage of lung development) induces liquid absorption (Walters and Olver 1978, Brown et al. 1983, Barker et al. 1988). During labor, the endogenous rise in adrenaline concentrations results in slower lung liquid secretion followed by liquid absorption 50-150 minutes before delivery (Brown et al. 1983). Consequently, the absorption of lung liquid increases, lung water decreases, and animals delivered vaginally have less lung water postnatally than do animals delivered by cesarean section (Chapman et al. 1994, Bland et al. 1980, Bland et al. 1982, Bland et al. 1979). In humans, catecholamine concentrations in the umbilical cord blood correlate with lung compliance at two hours postnatally (Faxelius et al. 1983). In addition, maternal treatment with the beta-agonist terbutaline improves lung function in newborns after elective CS (Eisler et al. 1999).

Also, GCs, catecholamines and thyroid hormones have synergistic effects in promoting the maturation of the lungs and the absorptive capacity of the airway epithelium. In fetal lambs, cortisol infusion increases triiodothyronine (T3) concentrations, which may contribute to the absorptive response to catecholamines (Wallace et al. 1995). In thyroidectomized lamb fetuses, the absorption of lung liquid induced by adrenaline or its downstream mediator, cyclic adenosine monophosphate (cAMP), during the saccular stage of lung development is lacking (Barker et al. 1988). In contrast, when receiving a combination of T3 and hydrocortisone and upon adrenaline stimulation, thyroidectomized fetal lambs show absorption of lung liquid already during the canalicular stage (Barker et al. 1990). Thus, the presence of thyroid hormones may be necessary for the normal increase in fetal pulmonary sensitivity to adrenaline (Wallace et al. 1995).

2.2.2 The role of ion and water channels

Both the secretion of liquid into the respiratory tract and its absorption results from ion transport and the subsequent movement of water due to osmosis (Eaton et al. 2009). Thus, the transport of ions and water is an important feature of the epithelium that lines the airways. During fetal life, the secretion of chloride ions predominates (Olver and Strang 1973) and, in humans, is present at six weeks of gestation (McCray et al. 1992). Towards term, the secretion of chloride ions slows and the absorption of sodium ions through apical sodium channels begins (Wilson et al. 2007).

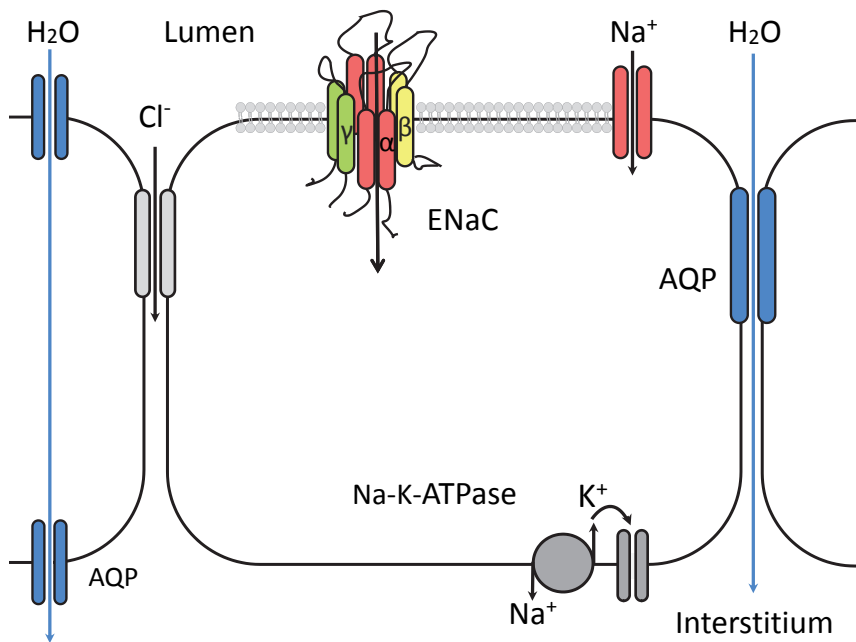


Figure 2. Sodium absorption through the apical cell membrane takes place via amiloride-sensitive (ENaC) and -insensitive channels. Basolateral Na-K-ATPase extrudes sodium ions to the interstitium to preserve electroneutrality. Water follows sodium osmotically, partially through water channels (aquaporins; AQP).

Apical sodium transport involves sodium channels that differ with regard to, for example, ion selectivity, conductance, and sensitivity to various blocking agents. The apical amiloride-sensitive epithelium sodium channel (ENaC), induced by catecholamines and GCs, plays a major role in lung liquid absorption during both labor and the early postnatal period (Hummler et al. 1996, Brown et al. 1983, Olver et al. 1986, O’Brodivich et al. 1990, Baines et al. 2000). ENaC is considered rate-limiting for perinatal lung liquid clearance, but other apical, amiloride-insensitive sodium channels (e.g. cyclic-nucleotide-gated channels) are also likely to play a role

(O’Brodivich et al. 2008, Eaton et al. 2009; Figure 2). Furthermore, to preserve electroneutrality, chloride is absorbed paracellularly and via, for example, the cystic fibrosis transmembrane conductor regulator (Eaton et al. 2009). Basolateral Na-K-ATPase extrudes sodium ions into the interstitial space, thus maintaining the electrochemical gradient for the net movement of sodium from the lumen of the airways and the alveoli into the interstitium (Hummler et al. 1996, Wilson et al. 2007; Figure 2). Water moves through the epithelium according to the osmotic gradient produced by ion fluxes. In part, this takes place via water channels known as aquaporins (Yasui et al. 1997, Ruddy et al. 1998, Zelenina et al. 2005; Figure 2).

2.3 ENaC

Animal experiments suggest that ENaC is critical in perinatal lung liquid clearance (Hummler et al. 1996, O’Brodivich et al. 1990, Li and Folkesson 2006). Also, evidence has emerged showing a role in both physiological and pathological lung liquid clearance in human newborns (Barker et al. 1997, Gowen et al. 1988, Helve et al. 2004, Helve et al. 2005, Helve et al. 2006, Helve et al. 2007). The airway epithelial ENaC is sensitive to amiloride and consists of three subunits, α -, β -, and γ , cloned in the 1990s (Canessa et al. 1993, Canessa et al. 1994, Voilley et al. 1994 and Voilley et al. 1995). The pore-forming α ENaC subunit alone can form non-selective sodium channels, but the formation of highly sodium-selective channels requires the presence of all three subunits (Jain et al. 2001, Johnson et al. 2006, Eaton et al. 2009). The co-expression of α -, β -, and γ ENaC potentiates the activity of ENaC manifold (Canessa et al. 1994, McDonald et al. 1995). Both highly selective and non-selective sodium channels are present in the apical cell membrane of ATI as well as in that of ATII cells (Johnson et al. 2006, Eaton et al. 2009). Although additional amiloride-sensitive sodium channels formed of the α -subunit, a δ -subunit, or both in combination with β - or γ ENaC subunits exist (McNicholas and Canessa 1997, Ji et al. 2006), these variations of ENaC are less studied.

2.3.1 ENaC in lung liquid clearance

Studies of fetal lambs show that sodium absorption occurs during late gestation (Wilson et al 2007). The inhibition of ENaC via the instillation of amiloride into the trachea of term, newborn guinea pigs causes respiratory distress and increases the amount of lung water (O’Brodivich et al. 1990). Research has identified the pivotal role of the α -subunit of ENaC by knocking out the α ENaC gene in mice. This results in flooding of the lungs of newborn mice pups, leading to their death from respiratory insufficiency within 40 hours of delivery (Hummler et al. 1996). A similar, organ-specific phenomenon has been demonstrated by using small interfering RNA (siRNA) to silence the α ENaC gene in newborn rat pups. In these experiments, trans-thoracal intrapulmonary injection of α ENaC-silencing siRNA

decreased whole-lung α ENaC mRNA and protein, and increased extravascular lung water content and mortality (Li et al. 2007). In adult rats, instillation of the siRNA into the lungs, besides decreasing α ENaC mRNA, protein and lung liquid absorption, had a negative impact, particularly on beta-agonist-stimulated absorption (Li and Folkesson 2006).

Efficient lung liquid clearance may also require adequate amounts of β ENaC. Although newborn mice lacking β ENaC show somewhat higher lung wet:dry weight, no respiratory distress is evident (McDonald et al. 1999). In adult mice homozygous for a mutant β -subunit with low amounts of β ENaC mRNA and no detectable protein, the alveolar liquid clearance, including the part inhibited by amiloride, decreases (Randrianarison et al. 2008). In this situation, stimulation by a beta-agonist failed to induce the clearance of alveolar liquid (Randrianarison et al. 2008). Furthermore, in mice, overexpression of the β ENaC subunit leads to the hyperabsorption of airway surface liquid (Mall et al. 2004).

Similarly to β ENaC knock-out mice, newborn mice lacking the γ ENaC gene do not die from respiratory distress (Barker et al. 1998). Nevertheless, they do experience a slower clearance of lung liquid 4-24 hours after birth and very little decrease in lung liquid content during the first 4 postnatal hours (Barker et al. 1998). In rat fetal distal lung epithelial (FDLE) cells, maximal epithelial liquid reabsorption in response to exposure to edema fluid requires the β - and γ ENaC subunits (Elias et al. 2007).

In humans, the ion transport over the proximal airway respiratory epithelium reflects that of lower parts of the airways (Barker et al. 1997) and correlates with lung function (Fajac et al. 1998, Helve et al. 2006). The potential difference (PD), reflecting primarily sodium transport, over the nasal epithelium increases with gestational age (GA) (Gaillard et al. 2005). In infants delivered by elective CS and in infants with TTN, the activity of ENaC in the nasal epithelium, corresponding to the amiloride-sensitive portion of PD, is lower than in vaginally delivered and healthy infants (Gowen et al. 1988). Also, during the first 24 h, both the maximal nasal transepithelial PD and the amiloride-sensitive part of PD are lower in preterm infants developing RDS than in those without RDS (Barker et al. 1997). In infants with RDS, the content of the β ENaC protein in tracheal aspirates is lower than in infants with TTN or in control infants (Li et al. 2009). We previously showed that ENaC activity, measured as the amiloride-sensitive nasal PD, at < 4 hours after delivery correlates with static lung compliance at 21-48 hours as well as with the change in lung compliance from < 4 to 21-48 hours (Helve et al. 2005). Adults prone to high-altitude pulmonary edema have not only lower nasal transepithelial PDs than do control subjects, but also a significantly smaller amount of amiloride-sensitive PD (Sartori et al. 2002). Furthermore, some have suggested that the functionality of transepithelial sodium transport could affect both the development and outcome of acute lung injury with associated pulmonary edema in adults (Mac Swezey et al. 2011).

2.3.2 Ontogeny of ENaC

α -, β -, and γ ENaC mRNAs have been detected in human lungs from 21-week-old and older fetuses (Venkatesh et al. 1997). We previously reported that the expression of the α -, β -, and γ ENaC subunits in the nasal epithelium of newborn infants correlates with GA (Helve et al. 2007). The expression of a 3.8-kB mRNA fragment corresponding to that of α ENaC is substantially higher in adult than in fetal lungs (Voilley et al. 1994).

Data on *in situ* ENaC expression in human distal airways are scarce (Smith et al. 2000, Gaillard et al. 2000). α ENaC mRNA is present from weeks 4-5 during the embryonic stage of lung development (Smith et al. 2000). During the embryonic stage, α ENaC is present in the epithelial cells lining the lung bud, predominantly at the basal and apical aspects of the cell. Both large and small airways express α ENaC mRNA during the pseudoglandular stage and during the canalicular stage α ENaC mRNA is ubiquitously present in the airway epithelium. In tissue from 28-32 weeks (saccular stage), α ENaC mRNA is present in most of the superficial airway epithelium, while in the distal lung, the expression is localized to the corner cells in an ATII cell pattern. During the alveolar stage, ATII cells express α ENaC and its mRNA is strongly present in the epithelium of the large airways but weaker in the small airways (Smith et al. 2000). The β - and γ ENaC proteins in human fetal lungs are present during the canalicular stage, but not during the pseudoglandular stage of lung development, and are localized in the apical domain of ciliated cells (Gaillard et al. 2000). The apical expression of β - and γ ENaC on ciliated cells in the superficial epithelium of large airways is higher after 24 weeks of gestation than during the canalicular period (Gaillard et al. 2000).

In various animal species, including the rat, guinea pig, rabbit and sheep, the mRNA and protein expression of the ENaC subunits increases with gestation (O'Brodivich et al. 1993, Tchepichev et al. 1995, Watanabe et al. 1998, Baines et al. 2000, Mustafa et al. 2004, Keller-Wood et al. 2005, Jesse et al. 2009). The mode of delivery may affect postnatal changes in expression: in the guinea pig, for example, a fall in α ENaC mRNA levels occurs after VD at term, whereas after CS both at as well as before term, α ENaC mRNA increases postnatally (Baines et al. 2000). In sheep, which have the longest gestation of the animals studied, α -, β -, and γ ENaC mRNAs decrease during the first 48 hours after delivery (Jesse et al. 2009). Likewise, the amount of α ENaC protein increases with gestation and then decreases postnatally (Keller-Wood et al. 2005, Jesse et al. 2009).

2.3.3 Regulation of ENaC

Glucocorticoids

Regulation of ENaC by GCs depends on exposure time, degree of maturation, target tissue and species, and is differential with regard to the subunits (Tchepichev et al. 1995, Venkatesh et al. 1997, Lazrak et al. 2000, Champigny et al. 1994, Nakamura et al. 2002). Since the regulation of ENaC and the expression of its subunits differ

depending on the target tissue, the following sections focus on studies performed on airway and lung tissue or cells.

GCs regulate transcriptional, translational and posttranslational mechanisms, and increase ENaC subunit mRNA, protein and currents in airway and lung-cell culture (Venkatesh et al. 1997, Itani et al. 2002a, Otulakowski et al. 2006, Lazrak et al. 2000, Champigny et al. 1994, Nakamura et al. 2002). A glucocorticoid response element (GRE) has been identified upstreams of the α ENaC promoter and is required to induce expression of the subunit (Sayegh et al. 1999, Otulakowski et al. 1999, Itani et al. 2002a, McTavish et al. 2009).

Data on the role of endogenous GCs (i.e. cortisol in guinea pigs, sheep, and humans or corticosterone in rats) are few. However, in both preterm and term guinea pigs delivered by CS, the postnatal increase in α ENaC mRNA parallels the rise in endogenous cortisol concentrations (Baines et al. 2000).

In a small population of ventilator-dependent infants born very preterm ($n = 4$, mean GA at birth 24.9 weeks), dexamethasone administered at 30-50 days postnatally, increased the airway expression of α - and β ENaC in all four infants, and of γ ENaC in three of the four infants 7-20 hours after the first dose (Helve et al. 2004). In human lung explants from 20- to 24-week-old fetuses treatment with dexamethasone increases α -, β -, and γ ENaC mRNAs beginning at eight hours, with maximal stimulation by 24 hours (Venkatesh et al. 1997).

A significant part of the data on the regulation of ENaC and sodium transport by GCs in human cells comes from cell lines from adult airway and lung epithelium. Human cell cultures have used cell lines from adenocarcinomas with characteristics of A549 cells (i.e. H441 and A549 cell lines). In H441 and A549 cells dexamethasone upregulates the transcription of α -, β -, and γ ENaC (Sayegh et al. 1999, Lazrak et al. 2000, Itani et al. 2002a, Rammingner et al. 2004, Watt et al. 2012). The timing of the increased transcription of the individual subunits differs, however: α ENaC mRNA increases significantly after two to four hours of GC exposure (Itani et al. 2002a). Increases in β - and γ ENaC mRNA may occur only after 24 hours of GC stimulation (Lazrak et al. 2000, Itani et al. 2002a), although γ ENaC mRNA has reportedly responded as early as 2 hours of GC exposure (Itani et al. 2002a). In 24 hours, dexamethasone also increases the protein amounts of α ENaC (Itani et al. 2002a) or β - and γ ENaC (Lazrak et al. 2000). However, the cell surface expression of α -, β -, and γ ENaC proteins increases already with brief (3 hours) dexamethasone stimulation (Watt et al. 2012). Furthermore, dexamethasone stimulates ENaC-dependent transmembrane currents (Sayegh et al. 1999, Lazrak et al. 2000, Itani et al. 2002, Rammingner et al. 2004). An initial increase in the transmembrane currents occurs at 4 hours, is evident at 6 hours, and continues for the following 18 hours (Itani et al. 2002a).

In cell cultures of lung epithelial cells from adult and fetal animals, GCs increase the amount of α -, β -, and γ ENaC mRNA and protein (Champigny et al. 1994, Nakamura et al. 2002, Otulakowski et al. 2006, Dagenais et al. 2001). The induction of α ENaC expression occurs earlier than does the increase in β - and γ ENaC mRNA (Nakamura

et al. 2002, Dagenais et al. 2001). Dexamethasone also increases amiloride-sensitive sodium currents (Champigny et al. 1994, Otulakowski et al. 2006).

The effects of GCs may also depend on environmental factors: in rat FDLE, the increases in α ENaC protein synthesis and amiloride-sensitive sodium transport by GCs require postnatal oxygen levels (Otulakowski et al. 2006). Furthermore, in adult rat ATII cell cultures, besides the presence of dexamethasone, air and permeable supports favor the formation of highly sodium-selective sodium channels (Jain et al. 2001). Culturing FDLE on an extracellular matrix derived from immature fetal rat lung reduces the portion of amiloride-sensitive sodium currents (Pitkänen et al. 1995).

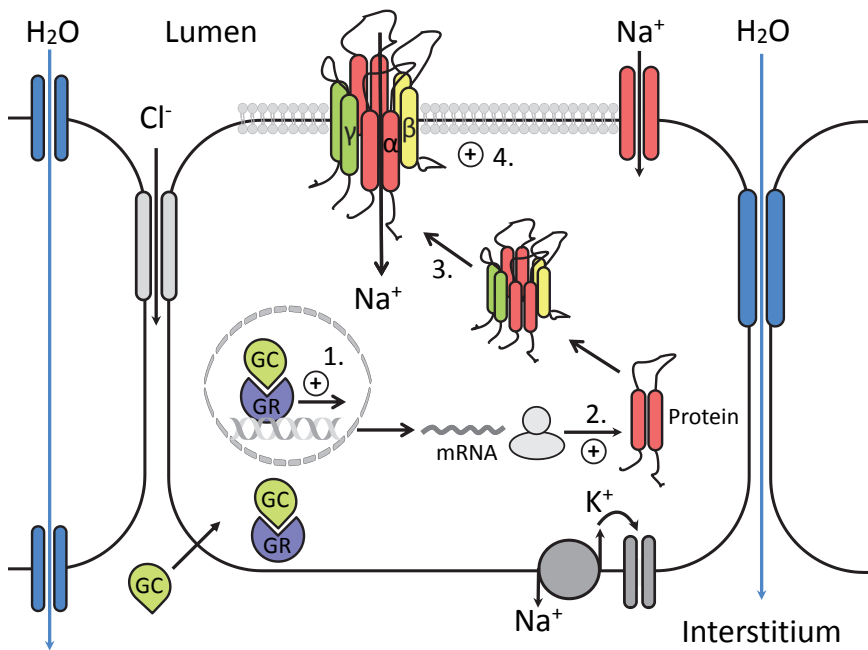


Figure 3. Effects of GCs include 1) transcription, 2) translation, 3) cell surface trafficking, and 4) ENaC activity.

Laboratory experiments in animals have often included the administration of exogenous GCs to the mother or fetus during pregnancy. These studies have shown that dexamethasone administered to the pregnant dam at 17-19 days of gestation, corresponding to the pseudoglandular to canalicular stage of lung development, induces the gene expression of α ENaC within eight hours, but not of β - or γ ENaC (Tchepichev et al. 1995). In fetal sheep, an implant that releases cortisol, which result in cortisol concentrations corresponding to those in late gestation (but before the final surge on the day of birth) or in a stressed preterm fetus, increases the amount of α ENaC mRNA in the lungs, but not of β - or γ ENaC

mRNA (Jesse et al. 2009). In thyroidectomized fetal sheep, the induction of lung liquid absorption by adrenaline infusion requires intravenously infusing T3 and hydrocortisone (Barker et al. 1991). Postnatally, in newborn, preterm rabbits, dexamethasone administered for 8 to 24 hours increases α ENaC mRNA and protein, but shows no effect on extravascular lung water content (Mustafa et al. 2004). In adult rats, the intraperitoneal administration of dexamethasone increases β ENaC mRNA and alveolar liquid clearance, predominantly related to the amiloride-sensitive (i.e. ENaC-mediated) component (Noda et al. 2003).

Thyroid hormones

In preterm lambs, thyroid hormones improve lung liquid absorption and lung function (Stein et al. 1994, Polk et al. 1995). Thyroid hormones may also potentiate the increase in ENaC transcription and activity induced by GCs (Champigny et al. 1994, Otulakowski et al. 1999). In fetal rat lung, the content of α ENaC mRNA at day 18 of gestation increases to a level corresponding to that at day 20, when the pregnant rat dams receive a combination of thyrotropin-releasing hormone (TRH) and dexamethasone (O'Brodivich et al. 1993). However, studies have reported contrasting results: according to Tchepichev and colleagues, rat fetuses of dams treated with dexamethasone on days 17 to 19 of gestation (pseudoglandular to canalicular stage) showed no additional effect of TRH on α ENaC gene expression (Tchepichev et al. 1995). Furthermore, adding T3 to human fetal lung explants treated with dexamethasone does not further increase ENaC mRNA (Venkatesh et al. 1997). However, T3 can positively affect the dexamethasone-induced conductance of ENaC without affecting α ENaC transcription (Richard et al. 2003). Accordingly, T3 potentiates the increased amiloride-sensitive sodium currents produced by dexamethasone (Champigny et al. 1994).

Catecholamines

In animals, during the later stages of gestation and labor, exogenous and endogenous beta-agonists induce the absorption of lung liquid (Walters and Olver 1978, Brown et al. 1983, Barker et al. 1988, Chapman et al. 1994, Bland et al. 1979, Bland et al. 1980, Bland et al. 1982). Amiloride can inhibit this effect, indicating a role for beta-agonist induction of ENaC (Olver et al. 1986). In human pulmonary tissue, the long-acting beta-agonist terbutaline increases liquid clearance in resected lung samples from cancer-surgery patients (Sakuma et al. 1994). Both amiloride and the beta-receptor antagonist propranolol inhibit this response (Sakuma et al. 1994).

Studies performed in cell cultures show that cAMP, a second messenger that mediates the effects of catecholamines and other beta-agonists, increases transepithelial sodium currents in both rat alveolar epithelial cells and human H441 cells (Dagenais et al. 2001, Thomas et al. 2004). Its rapid effect on sodium transport in human cells, observed within 5 min of cAMP stimulation, is likely to

be mediated via the protein kinase A and phosphoinositide-3-kinase pathways (Thomas et al. 2004). At least part of this increase in sodium currents is likely to result from the translocation of ENaC subunit proteins to the apical cell membrane (Thomas et al. 2004). The increase in sodium currents induced by cAMP is sustained > 24 hours and is associated with increased amounts of α ENaC mRNA and protein (Thomas et al. 2004). In rat alveolar cells, cAMP upregulates α -, β -, and γ ENaC mRNAs (Dagenais et al. 2001). The increase in transepithelial currents by cAMP stimulation parallels the increase in α - and γ ENaC mRNAs (Dagenais et al. 2001). The cAMP-induced increases in ENaC mRNA and protein are additive to those of GCs (Dagenais et al. 2001, Thomas et al. 2004).

Oxygen

Oxygen affects the transport of ions and water in the respiratory epithelium. In cultured rat FDLE, amiloride-sensitive and ouabain-sensitive currents (corresponding to ENaC and Na-K-ATPase activity, respectively) are greater under postnatal oxygen tension levels than under fetal ones (Pitkänen et al. 1996, Ramminger et al. 2002, Thome et al. 2003). Furthermore, the beta-agonist isoprenaline increases amiloride-sensitive currents in rat FDLE, cultured in the presence of dexamethasone and T3, more at postnatal than at prenatal oxygen tension levels (Ramminger et al. 2002). α -, β -, and γ ENaC mRNAs levels are higher after 48 hours at a pO₂ of 21%, as are their protein concentrations at the oxygen tension level of room air than at 5% (Pitkänen et al. 1996, Thome et al. 2003). However, during the transition from the fetal to a postnatal environment, some of the effects – especially the early effects – of the increase in oxygen tension levels on transepithelial lung liquid movement may be related to changes in interepithelial tight junctions (Pitkänen et al. 1996).

Hypoxia decreases amiloride-sensitive transepithelial sodium transport and associated alveolar liquid clearance in adult rat lungs and ATII cells (Pitkänen et al. 1996, Rafii et al. 1998, Vivona et al. 2001, Planès et al. 2002). The beta-agonist terbutaline reverses this decrease, an effect attributed to an increase in amiloride-sensitive sodium transport (Vivona et al. 2001, Planès et al. 2002). This increase in amiloride-sensitive sodium currents, in turn, may stem from the trafficking of ENaC subunits to the cell surface (Planès et al. 2002). The effect of hypoxia on ENaC subunit gene and protein expression in different studies varies. The effects on gene expression range from a decrease of α - and β ENaC mRNAs in the whole lungs of hypoxic rats (Wodopia et al. 2000) to no change in rat ATII cells cultured under hypoxic conditions (Planès et al. 2002) or even an increase in α ENaC mRNA in ATII cells from rats exposed to hypoxia (Vivona et al. 2001). In human A549 cells, α - and β ENaC protein amounts progressively decrease on exposure to hypoxia (3%) (Wodopia et al. 2000). Compared to normoxia or hypoxia in guinea pigs delivered preterm, hyperoxia (95%) increases postnatal α ENaC mRNA (Baines et al. 2000).

2.4 Na-K-ATPase

In human resected lung tissue blocking Na-K-ATPase inhibits nearly half of basal alveolar liquid clearance (Sakuma et al. 1994). In the lung, its major α - and β -subunits form a heterodimer with the final stoichiometry of 1:1. The genes for the subunits are located on different chromosomes, and their transcription may be independently regulated (Sznajder et al. 2002). The α -subunit has multiple transmembrane regions and forms a cationic pore (Chow et al. 1995, Sznajder et al. 2002). The glycosylated β -subunit increases the stability and trafficking of Na-K-ATPase to the cell membrane, as well as regulates its membrane-associated half-life (Chow et al. 1995, Sznajder et al. 2002). Both the α - and β -subunits have several isoforms. In adult rat ATI cells, α 1- and α 2-Na-K-ATPase mRNA and proteins are expressed, whereas ATII cells contain only the α 1-Na-K-ATPase subunit (Ridge et al. 2003, Johnson et al. 2002, Borok et al. 2002). Both ATI and ATII cells contain the β 1-subunit (Ridge et al. 2003, Johnson et al. 2002). However, in contrast to the adult rat lung, rat FDLE contain only the α 1- and β 1-subunits (O’Brodivich et al. 1993). In humans, the α 1-Na-K-ATPase protein occurs in tracheal aspirates from newborn infants (Li et al. 2009, Ringman Uggla et al. 2011).

2.4.1 Ontogeny of Na-K-ATPase

In rat peripheral lung tissue and FDLE, the gene expression of α 1-Na-K-ATPase increases from fetal day (FD) 17 to FD20 or postnatal day 1 (Ingbar et al. 1996, O’Brodivich et al. 1993). In whole rat lung tissue, α 1-Na-K-ATPase mRNA peaks perinatally (Yasui et al. 1997). β 1-subunit mRNA shows a similar increase up to FD20, but it is less abundant than α 1-Na-K-ATPase (O’Brodivich et al. 1993). In the fetal rat peripheral lung, changes in the amount of α 1-Na-K-ATPase protein in cell membranes parallels changes in the amount of mRNA (Ingbar et al. 1996). The activity of Na-K-ATPase in the fetal rat peripheral lung and FDLE increases during the last days of gestation, peaking at term (Ingbar et al. 1996, O’Brodivich et al. 1993).

In fetal sheep lung, α 1-Na-K-ATPase mRNA peaks at FD130 (term: 148-149 days), and the protein amounts of α 1-Na-K-ATPase in the cell membrane are significantly elevated at FD130-145 (Keller-Wood et al. 2009).

The data on the ontogeny of Na-K-ATPase in the human fetal lung are limited. In a small study of human infants who were intubated due to TTN, RDS or for various other reasons, the amount of α 1-Na-K-ATPase protein in tracheal aspirates increased with higher GA (Li et al. 2009).

2.4.2 Regulation of Na-K-ATPase

In the lung, GCs, insulin and aldosterone increase the gene transcription of Na-K-ATPase (Sznajder et al. 2002). The rat α 1-Na-K-ATPase subunit contains a GRE in the 5' flanking region of the major transcription site, as well as a cAMP response element (Sznajder et al. 2002). In the promoter region of the β 1-Na-K-ATPase gene, a common response element for GCs and mineralocorticoids is found in humans (Derfoul et al. 1998). The upregulation of β 1-subunit expression by GCs within six hours is also consistent with the activation of a GRE (Barquin et al. 1997, Chalaka et al. 1999).

The data concerning the effects of GCs on Na-K-ATPase in lung tissue and cells are somewhat contradictory. In one study, treatment of pregnant rat dams with dexamethasone, TRH or a combination of these failed to increase α 1- or β 1- Na-K-ATPase mRNA in the lungs of their fetuses (Tchepichev et al. 1995). In contrast, another study reported that maternal dexamethasone treatment increases both the mRNA and protein amounts of the β 1-Na-K-ATPase subunit (Ingbar et al. 1997). In rat FDLE, dexamethasone concentrations corresponding to physiological GC concentrations increases α 1- and β 1-subunit mRNAs (Chalaka et al. 1999). In contrast, in alveolar epithelial or ATII cells from adult rats, dexamethasone increases β 1-Na-K-ATPase mRNA with no significant increase in α 1-Na-K-ATPase mRNA (Dagenais et al. 2001, Barquin et al. 1997). Dexamethasone increases the protein amounts of both the α 1- and β 1-subunit as well as the activity of Na-K-ATPase in adult rat ATII cells (Barquin et al. 1997). In the ovine lung, treatment with cortisol during FD120-130 does not increase α 1-Na-K-ATPase mRNA at 130 days (Jesse et al. 2009). However, cortisol concentrations remained well below values reported at birth (Jesse et al. 2009). Discrepancies in the effects of GCs may be related to differential responses between fetuses and adult animals, as well as to the duration of the GC treatment itself (Chalaka et al. 1999).

In rat FDLE, T3 increases Na-K-ATPase activity and the presence of the α 1-subunit in the plasma membrane of FD19 cells, but not earlier (Lei et al. 2007). Similar results have been obtained in adult rat alveolar epithelial cells and ATII cells (Lei et al. 2003). In contrast, administering TRH and dexamethasone to pregnant rat dams alone or combined has no effect on α 1- or β 1-Na-K-ATPase mRNA levels in the lungs of their fetuses (Tchepichev et al. 1995).

In heterozygous adult mice, a 50% decrease in both α 1- and β 1-Na-K-ATPase lung protein expression impairs their alveolar liquid clearance response to cAMP (Looney et al. 2005). In alveolar epithelial cells from adult male rats, the synthetic cell-permeable cAMP analog dibutyryl cAMP (DBcAMP) increases α 1-Na-K-ATPase mRNA (Dagenais et al. 2001). Dexamethasone potentiates the effect of DBcAMP on α 1-Na-K-ATPase mRNA (Dagenais et al. 2001). In rat FDLE, although it has no effect on the gene transcription level, the long-acting beta-agonist terbutaline increases α 1-Na-K-ATPase protein amounts (Rahman et al. 2010). In both rat FDLE and adult ATII cells, terbutaline increases the activity of Na-K-ATPase (Rahman et al. 2010, Minakata et al. 1998).

2.5 Serum- and glucocorticoid-inducible kinase 1 (SGK1)

Serum- and glucocorticoid-inducible kinase 1 (SGK1) is a Ser/Thr kinase (Loffing et al. 2006). In mice, SGK1 is expressed in the lung beginning on day 12.5, corresponding to late embryonic lung development (Lee et al. 2001). High levels of SGK1 are found in the distal epithelium and terminal bronchi/bronchioles at gestational day 14.5 (Lee et al. 2001). In the ovine fetal lung, SGK1 mRNA and protein are present by 80 days of gestation, corresponding to the end of the pseudoglandular or the early canalicular stage, with gene expression peaking at 145 days (i.e. 2-3 days before term) (Keller-Wood et al. 2009). SGK1 mRNA is present in the lungs of human stillborn fetuses (GA 21-41 weeks) as early as week 21 of gestation (Wirbelauer et al. 2007).

In animal models, an increase in SGK1 mRNA and protein during late gestation is associated with increased lung liquid absorption (Li et al. 2009). In a model of lipopolysaccharide-induced lung injury, the activation of SGK1 tends to attenuate the increase in lung edema caused by the injury (Zhu et al. 2012).

2.5.1 Regulation of SGK1

The function of SGK1 may be augmented via increased transcription, activation by phosphorylation and regulation of subcellular localization (Loffing et al. 2006). Factors inducing SGK1 include glucocorticoids, mineralocorticoids and insulin, but also e.g. oxidative stress and TGF- β (Webster et al. 1993, Loffing et al. 2006, Zhu et al. 2012). Some of the stimuli are cell-specific, but the effects of GCs are ubiquitous (Loffing et al. 2006).

In human H441 and A549 cell lines, dexamethasone induces the transcription and activation of SGK1, with a maximal transcriptional increase as early as 1 hour (Itani et al. 2002a, Itani et al. 2002b, Thomas et al. 2004, McTavish et al. 2009). Thus, SGK1 is an immediate-early response gene. The promoter of the SGK1 gene contains a GRE (Webster et al. 1993, Itani et al. 2002b), with additional transcription sites including a cAMP responsive element-binding site (Loffing et al. 2006). Accordingly, in submandibular gland epithelial cells, cAMP increases SGK1 mRNA and protein (Vasquez et al. 2008). In contrast, in the bronchial epithelial cell line H441, DBcAMP does not increase SGK1 mRNA or protein (Thomas et al. 2004). SGK1 may be activated by phosphorylation, with maximal activation occurring at 10-40 minutes (Loffing et al. 2006). SGK1 activity is, in addition to GCs, augmented by e.g. cAMP, insulin and growth factors (Thomas et al. 2004, Loffing et al. 2006, McTavish et al. 2009, Watt et al. 2012, Zhu et al. 2012).

2.5.2 SGK1 in the regulation of ENaC and Na-K-ATPase

SGK1 induces transcription of the α - and β ENaC genes (Boyd et al. 2005, Zhang et al. 2007, McTavish et al. 2009, Boyd et al. 2005), increases the abundance of

α -, β -, and γ ENaC at the cell membrane, and amiloride-sensitive sodium currents (Debonneville et al. 2001, Snyder 2002, Friedrich et al. 2003, Watt et al. 2012). SGK1 increases the activity of ENaC and Na-K-ATPase by increasing the number of channels in the plasma membrane and by activating the channels present in the membrane (Loffing et al. 2006). The augmentation of ENaC cell-surface expression and activity results from the binding to and phosphorylation of the neural precursor cell expressed developmentally downregulated protein 4-2 (Nedd4-2) by SGK1 (Snyder 2002, Debonneville et al. 2001). The phosphorylation of Nedd4-2 reduces its binding to ENaC, and thus the subsequent ubiquitination, internalization and degradation of ENaC (Loffing et al. 2006, Debonneville et al. 2001, Snyder et al. 2001, McTavish et al. 2009). In newborn rats, silencing of Nedd4-2 is associated with increases in α - and β ENaC mRNA and protein as well as with a decrease in extravascular lung water (Li et al. 2007). In addition to inhibiting Nedd4-2, SGK1 may recruit silent ENaC channels in the plasma membrane and/or promote channel insertion (Diakov et al. 2004). Some researchers have suggested that SGK1 also phosphorylates and, thus, activates ENaC directly (Diakov et al. 2004), whereas others (Friedrich et al. 2003) have suggested that direct phosphorylation is unlikely. In *Xenopus* oocytes, SGK1 increases Na-K-ATPase cell surface expression and activity (Setiawan et al. 2002, Zecevic et al. 2004).

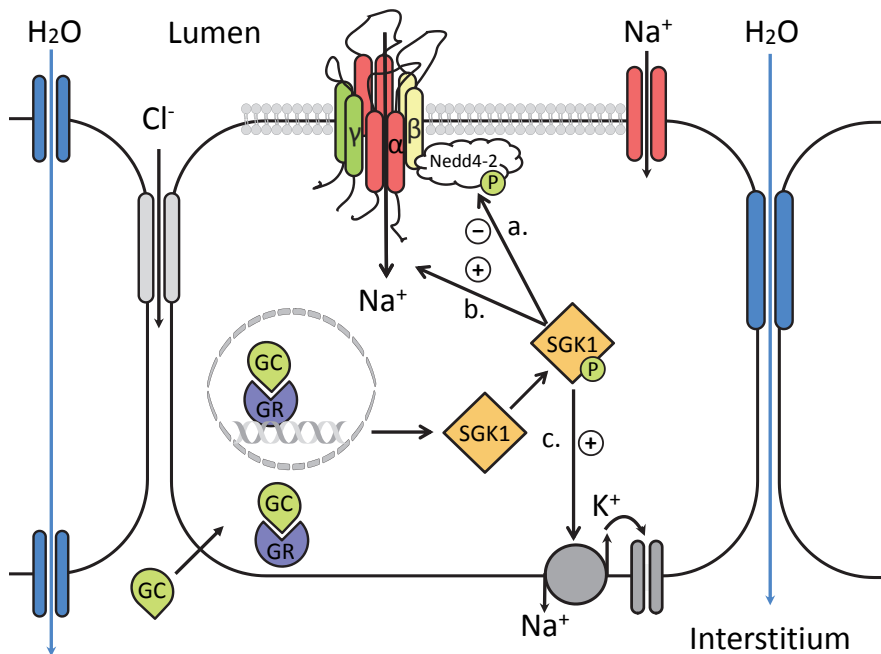


Figure 4. SGK1 (a) inhibits Nedd4-2, thereby reducing the ubiquitination and degradation of ENaC, and increasing activities of (b) ENaC and (c) Na-K-ATPase.

The effects of GCs on ENaC show both an early and a late phase, with the early phase supposedly depending on activation via the SGK1-Nedd4-2 pathway (Husted et al. 2007). The increased expression levels of the ENaC subunits then mediate later (after four hours) and sustained effects of GCs (Husted et al. 2007, Diakov et al. 2004). However, the SGK1-Nedd4-2 pathway may also be involved in later effects of GCs (Li et al. 2009).

2.6 Respiratory morbidity in newborn infants

2.6.1 Respiratory distress syndrome (RDS)

RDS results from surfactant deficiency resulting in alveolar collapse and subsequent decreased functional residual capacity as well as increased dead space (Agrons et al. 2005) in addition to structural immaturity of the lung (Sweet et al. 2013). Even with only mild respiratory distress, the amount of lung water is higher in preterm than in term infants (Adams et al. 2002), and deficient lung liquid absorption may contribute to RDS (O’Brodivich et al. 1996).

Clinical signs of RDS, in its natural course beginning at or soon after birth and increasing in severity during the first two days include cyanosis, grunting, intercostal retractions, and tachypnea (Sweet et al. 2013). Requirements for the classical definition of RDS include low arterial oxygen pressure, or the need for supplemental oxygen to achieve adequate oxygen tension levels, and classical radiographic features (Sweet et al. 2013). RDS can be diagnosed based on the extent of granularity, air bronchograms, effacement of cardiac and diaphragmatic silhouettes and aeration in chest radiographs (Edwards et al. 1985). However, surfactant treatment alleviates these findings (Edwards et al. 1985). With early treatment, including early CPAP and prophylactic surfactant administration, the definition and diagnosis of RDS is more complicated (Sweet et al. 2013). Moreover, until recent years, the clinical management of very immature infants with immediate intubation and prophylactic surfactant may have overestimated the incidence of RDS (Jobe 2012, Sweet et al. 2013). That said, the incidence of RDS in very low birth weight infants (i.e. infants weighing below 1500 grams at birth) ranges from 98% at week 23 to 86% at week 28 (Stoll et al. 2010). At 30-32 weeks GA, the incidence of RDS is 23.2%, and at weeks 33-34, 6.3% (Altman et al. 2013). Even at term gestation (i.e. 37 weeks), in CS-delivered infants the risk for RDS decreases until a GA of 39 weeks (Tita et al. 2009).

2.6.2 Transient tachypnea of the newborn (TTN)

TTN is an important cause of respiratory morbidity in both late preterm (GA 34^{0/7} to 36^{6/7} weeks) and term infants. The reported incidence of TTN ranges from 6.4 to 14.7% in infants delivered at week 34 (Hibbard et al. 2011, Melamed et al. 2009) to 0.2% at 40 weeks GA (Hibbard et al. 2011). The risk for TTN in infants delivered by elective CS is elevated, ranging from 4.8 to 10% at week 37 to 2.1 to 2.5% at week 39 of gestation (Hansen et al. 2008, Tita et al. 2009). In a population of late preterm infants from singleton, low-risk pregnancies (excluding e.g. infants of mothers with diabetes or hypertension), even one of five infants delivered by CS at 34 weeks had TTN (Melamed et al. 2009). In addition to CS and low GA, risk factors for TTN include male sex and low Apgar scores (Altman et al. 2013).

The etiology of TTN is considered the retention of lung liquid, which results in respiratory distress (O’Brodivich et al. 1996). Some researchers have also proposed a role for modest surfactant deficiency (Machado et al. 2011). The clinical symptoms, observed within hours after delivery, include tachypnea, grunting, retractions, and nasal flaring (Guglani et al. 2008). Chest radiographs show perihilar streaking, liquid in the interlobar fissure, and congestion (Guglani et al. 2008). However, such radiographic findings may be minimal or absent. Symptoms and radiographic findings are transient, usually resolving within three days (Guglani et al. 2008). Consequently, diagnosis requires the exclusion of other causes of tachypnea. Although TTN is often mild, the condition can be complicated by air leaks or it may result in severe maladaptation and persistent pulmonary hypertension (Kezslser et al. 1992, Jain and Eaton 2006).

2.6.3 Radiographic methods in diagnosing TTN, RDS and lung edema

Chest x-rays have been essential in the diagnosis of RDS and contribute to diagnosing TTN. However, surfactant treatment alleviates findings related to RDS (Edwards et al. 1985). More recently, ultrasound has served as a tool for diagnosing RDS and TTN (Copetti et al. 2008, Copetti et al. 2007).

In ultrasound, the normal lung is invisible below the interface between the pleura and the lung. However, the pleura-lung interface causes horizontal artifacts that appear as parallel lines (i.e. A-lines) below the pleural line (Copetti et al. 2007). While A-lines appear in the normal lung, vertically oriented B-lines, or comet-tail artifacts, are related to lung edema and represent sub-pleural interlobular septa thickened by edema (Lichtenstein et al. 1997). In infants with TTN, the lower lung fields show very dense B-lines, with clearly lesser density in the superior lung fields (Copetti et al. 2007). This phenomenon, which the authors call the double lung point sign, predicted TTN with both a sensitivity and specificity of 100% (Copetti et al. 2007). In preterm infants with RDS, the ultrasound reveals generalized alveolar-interstitial syndrome, with dense B-lines visualized as white lung fields, pleural line abnormalities including thickening, coarseness, and irregularity, as well as subpleural hypoechogenic areas representing lung consolidations (Copetti et al. 2008).

2.6.4 Glucocorticoids in the prophylaxis of respiratory distress

Since the first trial of antenatal GCs in the prophylaxis of respiratory distress in preterm infants published in 1972 (Liggins and Howie 1972), evidence for the beneficial effects of antenatal GC treatment in reducing the incidence of RDS in preterm infants is abundant.

The fetal adrenal gland synthesizes cortisol from week 7 to 10 of gestation, but this function appears to be suppressed until mid- or late gestation when fetal cortisol production increases substantially (Ishimoto and Jaffe 2011). As a result, in combination with other factors such as changes in the amounts and activities of 11 β -hydroxysteroid dehydrogenase types 1 (which converts inactive cortisone to cortisol) and 2 (which converts cortisol to cortisone) during late pregnancy, fetal cortisol concentrations increase from mid-gestation, surging rapidly during the last weeks of gestation (Murphy BE 1982, Murphy BE 1983, Murphy VE et al. 2003, Kajantie et al. 2003, Oh et al. 2006, Provost et al. 2013).

The genomic effects of GCs are mediated via the glucocorticoid receptor (GR) complex (Bolt et al. 2001, Czock et al. 2005). GRs are found in all parts of the human adult lung as well as in the developing lung of the human fetus (Condon et al. 1998, Bolt et al. 2001). After binding to the GC, the intracellular GR forms dimers and translocates to the nucleus. The GR functions as a ligand-activated transcriptional regulator by binding to DNA upstream of the promoter regions of genes (i.e. to the GREs), followed by either the activation or repression of gene transcription. The post-transcriptional, translational and post-translational effects of GCs include the modification of mRNA stability and translational efficacy, as well as protein processing and secretion (Bolt et al. 2001, Czock et al. 2005, Provost et al. 2013).

The effects of exogenous GCs are similar to both the structural and functional effects of increasing cortisol concentrations during lung maturation. Importantly, GCs upregulate components of surfactant (surfactant proteins and phospholipid) at both the transcriptional and post-transcriptional level (Bolt et al. 2001). However, the earliest changes in the lungs are structural: the volume fraction of lung parenchyma and the numerical density of alveoli increase, whereas the interstitial tissue and alveolar walls thin (Polglase et al. 2007). These changes are associated with improved lung function two days after the administration of antenatal GCs (Polglase et al. 2007). Antenatal GCs also enhance the maturation of pulmonary epithelial cells and the differentiation of ATII cells, induce changes in the connective tissue matrix, and augment antioxidant enzyme levels (Bolt et al. 2001, Provost et al. 2013).

A large part of the data on the effects of antenatal GCs on preterm newborn lung function comes from studies in sheep. Effects include increases in total lung gas volume, lung compliance, and an improved ventilatory efficiency index, possibly as early as 15 hours after fetal injection (Stein et al. 1994, Polk et al. 1995, Ikegami et al. 1996a, Ikegami et al. 1996b, Polglase et al. 2007, Jobe et al. 2009). In preterm human infants, a single course of antenatal GCs one to seven days before delivery increases respiratory system compliance (McEvoy et al. 2001).

Antenatal glucocorticoids in preterm infants

According to a Cochrane Review (Roberts and Dalziel 2006), antenatal GCs reduce the total RDS incidence to about two thirds (relative risk 0.66, 95% confidence interval 0.59-0.73). Similar reductions are found by evaluating their efficacy in different GA groups < 34 weeks of gestation, but not in infants born > 34 weeks (Roberts and Dalziel 2006). In a large cohort study, antenatal GCs reduced the risk for RDS, but not for TTN, in moderately preterm infants (GA 30^{0/7} to 34^{0/7} weeks) (Altman et al. 2013). The two most commonly used antenatal GC drugs, betamethasone and dexamethasone, do not differ with regard to reducing the risk for RDS (Brownfoot et al. 2013).

The optimal timing for antenatal GC-treatment is 24 hours to seven days before delivery (Roberts and Dalziel 2006). Therefore, researchers have further investigated the effects of repeating the treatment if, more than seven days after the initial treatment, the infant remains undelivered and the risk for preterm birth remains.

Antenatal glucocorticoids in late preterm and term infants

Few prospective, randomized studies have examined the effects of antenatal GCs on respiratory morbidity in late preterm (GA 34^{0/7} to 36^{6/7} weeks) infants. One prospective randomized, placebo-controlled and blinded study found no reduction in TTN or RDS (Porto et al. 2011). A retrospective study aimed to determine the effect of antenatal GCs on neonatal outcome in cases of determined fetal lung immaturity at 34-37 weeks (Yinon et al. 2012). Here, composite respiratory morbidity (RDS, TTN, and the need for ventilatory support) was lower in infants exposed to antenatal GCs (Yinon et al. 2012). Trials performing secondary analyses to estimate the possible benefits of antenatal GCs have found no reduction, or even an increase, in the incidence of respiratory morbidity (Gyamfi-Bannerman et al. 2012, Kamath-Rayne et al. 2012).

In infants delivered at term by elective CS, antenatal GCs reduced the likelihood of being admitted to special baby units due to respiratory distress (Stutchfield et al. 2005). This trial was randomized, but not placebo-controlled.

3 RESEARCH RATIONALE AND OBJECTIVES

A significant part of the data on lung liquid clearance, its molecular mechanisms and its regulation, in newborns originates from animal studies. In humans, preterm or even early term delivery as well as elective CS predispose infants to respiratory morbidity. We hypothesized that this elevated risk for respiratory morbidity is related to inadequate perinatal sodium and, thus, liquid absorption. Also, in light of the evidence of the importance of GCs in lung development, pulmonary adaptation and in the prophylaxis of respiratory morbidity, as well as experimental data of their effects on the molecular mechanisms of lung liquid clearance, we hypothesized that airway ENaC, Na-K-ATPase, and SGK1 gene expression is associated with endogenous GC concentrations and can be augmented by exogenous GCs. Specifically, the aims were to study:

- whether the amounts of lung liquid in newborn infants, as quantified with ultrasound, differ measurably depending on the mode of delivery.
- whether ENaC, Na-K-ATPase and SGK1 gene expression in the airway epithelium of infants at higher risk for respiratory distress (i.e. late preterm and early term infants as well as infants delivered by elective CS) differ from those in vaginally delivered and term infants.
- whether the airway epithelial gene expression of ENaC, Na-K-ATPase, and SGK1 is associated with endogenous cortisol levels.
- whether exogenous GCs, administered as a repeat dose of antenatal betamethasone, affect airway ENaC gene expression and thereby associate with morbidity in preterm infants.

4 STUDY SUBJECTS AND METHODS

4.1 Study population

The ethics committee of the Helsinki University Central Hospital approved the studies, as did the ethics committee of the Oulu University Hospital for Study IV. The parents of the study subjects provided their written, informed consent for all studies.

4.1.1 Study I

The study population (Table 1) comprised 42 term singleton infants to healthy mothers, 22 delivered vaginally and 20 by elective CS. Indications for CS delivery were breech presentation (n = 6), fear of delivery (n = 7), previous CS (n = 4), placenta previa (n = 2), and pelvic malformation (n = 1). Transthoracic ultrasound of the lungs was performed at 1, 3 and 24 hours after delivery (Table 1).

Table 1. Study populations and sampling in Studies I-III.

	Study I	Study II	Study III
	n = 42	n = 69	n = 87
Ultrasound, hours			
1 st ultrasound	1.1 (0.9-1.5)		
2 nd ultrasound	3.3 (2.9-3.9)		
3 rd ultrasound	24.4 (23.0-27.3)		
Epithelial cell sampling, hours			
Day 0		1.3 (0-2.9)	1.3 (0-2.9)
Day 1		24.7 (22.1-29.1)	24.9 (17.9-29.4)
Day 2		48.3 (40.4-53.8)	
Saliva sampling, hours			
Day 0		1.4 (0.2-1.9)	
Day 1		24.9 (22.6-28.8)	
Day 2		48.5 (45.6-51.5)	

All values are median (range)

4.1.2 Study II

We recruited 71 term (GA \geq 37 weeks) singleton infants to mothers without significant morbidity (i.e. pregestational or gestational diabetes mellitus, pre-eclampsia or chorioamnionitis) to the study. Two vaginal deliveries resulted in emergency CS, so we excluded these infants from further analysis. The study therefore included 69 infants delivered vaginally (n = 22) or by elective CS (n = 47). The majority of elective CS was performed due to breech presentation (n = 22). Other common indications included previous CS (n = 10), and fear of delivery (n = 9).

We obtained umbilical cord samples at birth for cortisol and free triiodothyronine (FT3) analyses. Airway epithelial cell samples for quantitating ENaC gene expression were obtained within 3 hours, as well as at 25 and 48 hours postnatally (Table 1). In a subset of infants delivered by elective CS (n = 28), the first sample was obtained < 30 minutes after delivery. In the other infants, the first epithelial cell sample was gathered 1.5 (1.0-2.9) hours (median [range]) postnatally. Saliva samples for determination of cortisol concentrations were collected at < 2 h, at one day, and at two days postnatally (Table 1).

4.1.3 Study III

The study population comprised 87 term and late preterm infants, including 51 term infants from Study II. Exclusion criteria were pregestational or gestational diabetes mellitus of the mother, chorioamnionitis, antenatal glucocorticoid treatment < 14 days before delivery, or peroral glucocorticoid treatment of the mother. Of twin pregnancies (n = 3) we included only either of the twins. Two late preterm infants were delivered by CS in labor, and one term infant by emergency CS. The indications for elective CS (n = 50) included breech presentation (n = 21), previous CS (n = 6), and fear of delivery (n = 9).

Umbilical cord blood for analysis of cortisol was obtained at delivery. Airway epithelial samples from nasal epithelium for measuring ENaC, Na-K-ATPase (Janér et al, unpublished data) and SGK1 gene expression were obtained within 3 hours of birth and at one day of age (Table 1). In a subpopulation of term infants delivered by elective CS (n = 28) the first sampling was performed at a median 5 min after delivery to minimize the effects of delivery and postnatal stress. In the other infants, epithelial cell samples were obtained at 1.5 (0.3-2.9) hours (median[range]) postnatally.

4.1.4 Study IV

The study included 22 preterm infants born between August 2002 and January 2005 (Table 2). All infants had been enrolled in a randomized, blinded, placebo-controlled clinical trial evaluating the effect of a repeat dose of antenatal betamethasone on infant morbidity (Peltoniemi et al. 2007). Of the infants 18

were born at the Women’s Hospital, Helsinki University Central Hospital, and 4 infants at Oulu University Hospital. Their pregnant mothers were randomly assigned to either a repeat single dose of 12 mg betamethasone or saline as a placebo intramuscularly if preterm birth was imminent and the standard course of 12 mg betamethasone repeated once had been completed more than seven days previously. The mothers of eight infants, including one pair of twins, received the repeat dose of 12 mg betamethasone. The placebo group comprised 14 infants of ten mothers, including one pair of twins and two sets of triplets (Table 2).

Two mothers in the betamethasone group and one in the placebo group had pre-eclampsia, and two mothers in each group were diagnosed with chorioamnionitis. Cell samples from the nasal airway epithelium were obtained twice during the first two days of life (Table 2).

Table 2. *Study population and sampling time points in Study IV*

	Repeat betamethasone	Placebo
	<i>n = 8</i>	<i>n = 14</i>
Sets of twins, n	1	1
Sets of triplets, n	0	2
First epithelial cell sampling, median (range), hours	2.8 (1.3-4.5)	1.8 (1.0-5.0)
Epithelial cell sampling day 1, median (range), hours	24.3 (20.0-27.0)	26.2 (13.9-28.6)

4.2 Methods

4.2.1 Radiological analyses

Ultrasound estimation of lung liquid content

The technique for estimating lung liquid content in Study I was adapted from that of Copetti (Copetti et al. 2007). We used a portable device (Esaote MyLab Sat) with a 6-13 MHz linear transducer (Copetti et al. 2008) to obtain images for estimating the lung liquid content. At each examination, six video clips were recorded: scans were performed along the mid-axillary lines, and along anterolateral intercostal spaces, and were repeated at both the right and left upper and lower hemithorax. The abundance of B-lines on the video clips was scored on a five-step scale – 1) no artifacts, 2) B-lines in < 25 % of the surface area, 3) < 50%, 4) < 75%, and 5) > 75% – independently by two radiologists blinded to the clinical characteristics

of the infants (including mode of delivery). If visual analysis was indefinite, we measured the width of the B-line on a frozen image, selecting the view with the most abundant B-lines, and divided it by the width of the transducer, thus obtaining a percentage. We then calculated the mean score of six views.

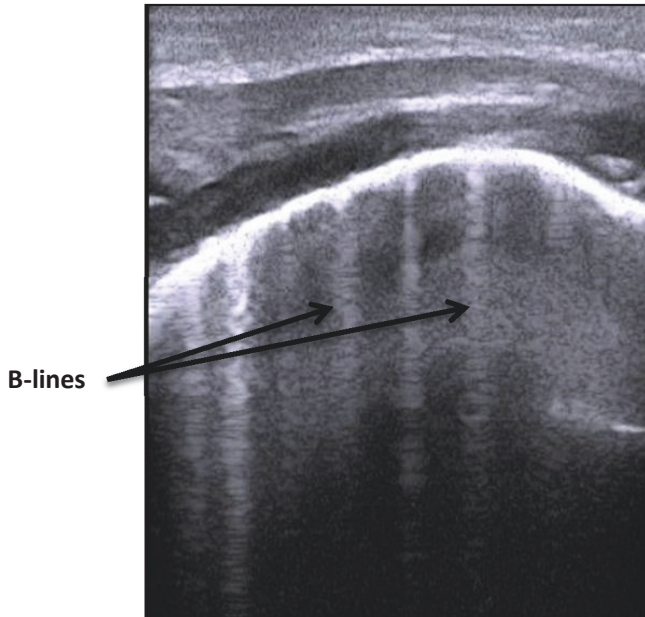


Figure 5. *Ultrasound view of the lung showing B-lines. Image by L. Martelius.*

Radiological assessment of RDS severity

In Study IV, we assessed the severity of RDS based on chest X-rays as described by Edwards (Edwards et al. 1985). The degree of granularity, air bronchograms, cardiac and diaphragm silhouettes and aeration in the x-rays were evaluated and scored from 1 to 5, with 1 signifying mild changes, and 5, the most severe. Thus, the assessment resulted in a total severity score of 4 to 20. For each preterm infant, we evaluated chest x-rays from 1 to 5 and 19 to 32 hours postnatally. In case of multiple x-rays during this time period, we scored the one closest in time to the nasal epithelial sampling. All x-rays were obtained solely on clinical grounds. A senior neonatologist (AK) scored the x-rays.

4.2.2 Sampling

Umbilical cord blood

In Studies II and III, umbilical cord blood samples were obtained after clamping and cutting the umbilical cord and collected into sterile EDTA-tubes. The cord blood obtained represented venous or mixed artero-venous blood. Samples were centrifuged at 1000 x g for 15 minutes to separate the plasma, which was aliquoted and stored at -80 °C until analysis.

Saliva

In Study II, saliva was obtained by allowing the infant to suck on a cotton swab (Salivette®, Sarstedt AG & Co, Nümbrecht, Germany) for five minutes. The swabs were stored at 8°C until centrifugation at 4°C at 4000 x g no more than 24 hours after collection. The saliva was then stored at -80 °C.

Airway cells

We used a sterile plastic probe for airway cell sampling in Studies II, III, and IV (RhinoPro®, Arlington Scientific Inc., Springville, UT, USA). Cells were obtained by gentle abrasion of the inferolateral wall of the nasal cavity, repeated in both nostrils. If a nasogastric feeding tube or a nasotracheal intubation tube was present, we preferably repeated the abrasion twice on their contralateral side. Samples were immediately lysed in a lysis buffer (RNeasy kit, Qiagen, Valencia, CA, USA) containing 10 µL of beta-mercaptoethanol per 1 mL of buffer with a sterile insulin syringe (BD Micro-Fine™, Becton, Dickinson and company, NJ, USA). The lysed cell samples were stored at -80 °C prior to the RNA isolation.

4.2.3 Laboratory analyses

ELISAs

Cord plasma cortisol concentrations were determined with a commercial ELISA kit (RE52061, IBL, Hamburg, Germany). The range of the assay was 0-800 ng/mL. Intra-assay variation reported by the manufacturer was 3.2-8.1%, and the inter-assay variation, 6.5-7.7%. All samples were analyzed undiluted in duplicate, the optical density at 450 nm read with a Thermo Labsystems Multiskan® EX microplate reader (Thermo Fisher Scientific, Vantaa, Finland) and the mean concentration calculated.

The ELISA kit for quantitation of cortisol in saliva was manufactured by R&D (KGE008, R&D Systems Inc., Minneapolis, MN, USA). The sensitivity of the assay, expressed as the minimum detectable dose of cortisol, was 0.071 ng/mL. Inter-assay and intra-assay variations, as reported by the manufacturer, were 9.3-21.1%

and 5.4-9.2%, respectively. Saliva samples were diluted to 1:20 before analysis and all samples were analyzed as duplicates. If necessary (i.e. if the concentration of cortisol in the sample fell outside the standard range), the analysis was repeated with the sample diluted to 1:10 or 1:50. Concentration values were based on the optical density at 450 nm with the Thermo Labsystems Multiskan® EX microplate reader (Thermo Fisher Scientific).

Liquid chromatography-tandem mass spectrometry

In Study III, cord blood cortisol concentrations were analyzed with liquid chromatography-tandem mass spectrometry. The method has been described earlier (Turpeinen et al. 2003), and analysis was carried out with minor modifications. Prior to analysis, an internal standard of 40 µl of 0.25 µM D₃-cortisol (Cambridge Isotope Laboratories, Andover, MA) in 50% (vol/vol) methanol was added to 50 µl of plasma and extracted with 3 ml of dichloromethane. After mixing for 3 min, the organic phase was collected and evaporated to dryness under nitrogen. The residue was dissolved in 2 ml of 40% methanol. Calibrators contained 10-1000 nmol/l of cortisol (Sigma, St.Louis, MO, USA) and were prepared in 40% methanol. Sample extracts and calibrators were analyzed in a volume of 25 µl with an API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, Canada). Peripherals included an Agilent series 1200 HPLC system with a binary pump (Santa Clara, CA, USA). Separation was performed on a SunFire C18 column (3.5 µm, 2.1 x 50 mm; Waters, Milford, MA, USA) with a linear methanol (B) gradient. The flow rate was 300 µl/min. The gradient was 0 min 40% B, 1.5 min 90% B, 2.5 min 90% B, and 3-10 min 40% B. Cortisol was detected using electrospray ionization in the negative mode with the following transitions: m/z 361.2 to m/z 331.2 and the internal standard, m/z 364.2 to m/z 334.1.

Chemiluminescent Microparticle Immunoassays

Plasma FT3 in Study II was analyzed with an ARCHITECT® Free T3 Chemiluminescent Microparticle Immunoassay (Chemiflex®, Abbott Laboratories, Abbott Park, IL, USA) using an Abbott Architect i2000 analyzer (Abbott). TSH values were obtained from standard clinical hypothyreosis screening data. The laboratory of the Helsinki University Central Hospital (HUSLAB) performed both FT3 and TSH analyses.

Isolation of total RNA

RNA isolation was performed with the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA concentrations were measured with spectrofluorometry for Study I and, partly, for Study II. For spectrofluorometric RNA quantitation, we utilized a commercially available kit (RiboGreen RNA Quantitation Kit, Molecular Probes, Oregon, USA). The content of RNA in the sample was obtained from a plot of the standard, which was based on the emission at 520

nm following excitation at 480 nm with the spectrofluorometer (LS50B, Perkin Elmer, Shelton, CT, USA). For the remaining part in Studies II and III, we used a spectrophotometer (NanoDrop® ND-1000 [ThermoScientific, Wilmington, DE, USA]) to determine the RNA concentrations, including an assessment of RNA purity (absorbance at 260/230 nM).

RT-PCR

The reverse transcription reaction of 120 ng of RNA was performed in a volume of 50 μ L as duplicates using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The cDNA was subsequently used for singleplex real-time PCR in a volume of 25 μ L/well on 96-well plates with an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). All samples were analyzed in duplicate. The PCR-reactions were performed with the Universal Master Mix (containing a ROX™ Passive Reference to correct for fluorescent fluctuations due to changes in concentration or volume) from Applied Biosystems according to the manufacturer's instructions.

We used pre-developed, exon-exon spanning TaqMan® assays for quantitation of α -, β -, and γ ENaC, α 1-Na-K-ATPase, and SGK1 (Hs00168906_m1, Hs00165722_m1, Hs00168918_m1, Hs00167556_m1 and Hs00178612_m1, Applied Biosystems). The assay for the epithelial cell marker cytokeratin 18 (CK18) used for normalization, had been designed with Primer Express® Software (Applied Biosystems) (Helve et al. 2006). The sequence of the CK18 probe was CTT CAC CAC TCG CTC C, that of the forward primer was TCT CCC CGG ACA GCA TGA, and that of the reverse primer was GGA CCG GTA GTT GGT GGA GAA. The concentration of the probe in the PCR reaction was 240 nM. In Studies II and IV, absolute quantitation was performed based on a standard curve generated as a dilution series of a sample with known quantities of the respective mRNAs. This sample consisted of RNA from the epithelial cells of a healthy nasal turbinate obtained during rhinoplasty (Study IV) or of RNA from pooled adult nasal epithelium abrasion samples (Study II). The amounts of α -, β -, and γ ENaC and CK18 mRNAs in the sample from the nasal turbinate had been previously quantitated with quantitative competitive RT-PCR (Helve et al. 2006). Their amounts in the pooled adult epithelial abrasion samples were determined with real-time RT-PCR. For absolute quantitation, the normalized amounts of the respective ENaC mRNAs appear as amol/fmol CK18. In Study III, we used relative mRNA quantitation. Calculations were performed according to the comparative $\Delta\Delta$ Ct-method ($2^{-\Delta\Delta C_t}$) including an interplate calibrator sample. Because the sample for the standard curve for absolute quantitation in Studies II and IV differed, and Study III used relative quantitation, the numerical PCR results in the individual studies are incomparable.

4.2.4 Assessment of mean oxygen requirement

In Study IV, we calculated the mean fraction of inspired oxygen (FiO₂) for 2-72 hours postnatally. The mean FiO₂ was obtained by multiplying the FiO₂ at 2, 6, 12, 24, 36, 48 and 72 hours of age with the duration of the preceding time interval, adding the products and then dividing the sum by the total duration.

4.2.5 Statistical methods

In Studies I-III, we used SPSS versions 17.0 and 20.0 (IBM SPSS, Chicago, IL, USA) for statistical analyses. In Study IV, analyses were performed with GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS 13.0 (IBM SPSS, Chicago, IL, USA).

When comparing two independent groups, we used the Student's t-test or Mann-Whitney U-test for normally or non-parametrically distributed continuous variables. We compared categorical data with Fisher's exact and Pearson χ^2 -tests and used the Wilcoxon-Mann-Whitney test to analyze ordinal data. Depending on the distribution of the data, we used the paired t-test or Wilcoxon's signed rank test to analyze continuous variables within groups. We used Friedman's test for non-parametrical data followed by Dunn's post-hoc test to compare more than two time points.

We analyzed correlations according to Pearson's product-moment correlation for normally distributed data and Spearman's rank correlation for non-parametrical data. We performed standard linear multiple regression analysis to assess the significance of continuous and categorical variables in predicting the amounts of ENaC, Na-K-ATPase and SGK1 mRNA. SGK1 was log-transformed to comply with assumptions of multiple linear regression. The unstandardized Beta signifies the estimated unit change in the outcome variable associated with one unit change of the independent variable. The infants delivered by CS in labor were included in the regression analyses as VD or CS based on the intended mode of delivery. For all analyses, a two-sided p-value of < 0.05 was considered statistically significant.

5 RESULTS

5.1 Clinical outcomes

In Study I, none of the infants showed signs of respiratory distress. Cord arterial pH was lower in VD infants (Table 3).

Table 3. *Clinical characteristics in Study I*

	VD	CS	P
	<i>n = 22</i>	<i>n = 20</i>	
GA, median (range), weeks	40.3 (37.1-41.7)	39.4 (39.0-42.0)	0.05
Birth weight, mean \pm SD, grams	3490 \pm 433	3410 \pm 306	0.56
Gender (M/F), n	10/12	9/11	0.54
Apgar 1 min, median (range)	9 (8-10)	9 (8-10)	0.95
Cord arterial pH, mean \pm SD	7.24 \pm 0.07	7.29 \pm 0.05	0.010

In Study II, two male infants had TTN after elective CS; another 11 infants received supplemental oxygen treatment following delivery by CS. The group of vaginally delivered infants differed from those delivered by elective CS with regard to GA and cord pH and base excess (BE) (Table 4).

Table 4. *Clinical characteristics and umbilical cord hormone concentrations in Study II*

	VD	CS	P
	<i>n = 22</i>	<i>n = 47</i>	
GA, median (range), weeks	40.0 (37.9-41.9)	39.3 (38.1-40.9)	0.001
Birth weight, mean \pm SD, grams	3535 \pm 360	3470 \pm 422	0.27
Gender (Male/Female), n	14/8	21/26	0.14
Apgar 1 min, median (range)	9 (8-10)	9 (6-10)	0.71
Cord arterial pH, mean \pm SD	7.25 \pm 0.09	7.30 \pm 0.05	0.026
Cord arterial BE, mean \pm SD	-4.72 \pm 2.82	-1.82 \pm 1.79	< 0.001
Cord blood TSH, median (range), mU/L	6.2 (3.3-27.0) <i>n = 20</i>	5.3 (1.9-8.3) <i>n = 44</i>	0.016
Cord blood FT3, mean \pm SD, pmol/L	2.26 \pm 0.38 <i>n = 20</i>	2.22 \pm 0.27 <i>n = 41</i>	0.93
Cord blood cortisol, median (range), ng/mL	259 (139-378) <i>n = 19</i>	113 (41-200) <i>n = 40</i>	< 0.001

Three of the infants in Study III had TTN, and ten infants without TTN received supplemental oxygen treatment, usually shortly after delivery and for only a few minutes. Umbilical cord arterial BE was lower in VD infants (Table 5).

Table 5. Clinical characteristics and umbilical cord hormone concentrations in Study III

	Late preterm	Term VD	Term CS	P
	<i>n</i> = 16	<i>n</i> = 21	<i>n</i> = 50	
GA, median (range), weeks	36.0 (35.0-36.5)	39.7 (37.0-41.9)	39.2 (37.9-40.9)	0.20
Birth weight, mean \pm SD, grams	2715 \pm 223	3340 \pm 378	3511 \pm 467	0.14
Gender (Male/Female), n	14/2	13/8	27/23	0.13
Apgar 1 min, median (range)	9 (7-9)	9 (8-10)	9 (6-10)	0.54
Cord arterial pH, mean \pm SD	7.25 \pm 0.08	7.26 \pm 0.09	7.29 \pm 0.05	0.20
Cord arterial BE, mean \pm SD	-3.61 \pm 3.41	-4.45 \pm 2.65	-1.85 \pm 1.90	<0.001
Cord blood TSH, mean \pm SD, mU/L	6.54 \pm 5.97	9.34 \pm 6.55	5.47 \pm 2.10	0.021
Cord blood cortisol, median (range), nmol/L	62 (28-135)	114 (36-215)	76 (33-170)	0.004

P values are for term VD vs. CS (according to the intended mode of delivery).

The mothers in Study IV had completed the initial standard course of betamethasone 15.5 (6.4-61.8) and 19.6 (8.7-46) (median [range]) days antenatally in the repeat betamethasone and the placebo groups, respectively ($p = 0.48$). We found no differences with regard to the timing of the respective study drugs to delivery or first sampling (4.8 [0.9-50.6] vs. 6.1 [0.9-22.7] hours; $p = 0.76$ and 7.1 [4.1-54.0] vs. 7.8 [3.0-27.2]; $p = 0.92$ for repeat betamethasone and placebo groups). Cord arterial pH was lower in the repeat betamethasone group (Table 6).

In the group of infants whose mothers had received a repeat dose of betamethasone, 6 of 8 infants had RDS, as did 7 of 14 infants in the placebo group ($p = 0.38$). The betamethasone and the placebo groups showed no differences in the duration of ventilator dependency, days on nCPAP or the need for supplemental oxygen (Table 6). The groups also showed no differences in the proportions of prenatal factors (including the preterm premature rupture of membranes, chorioamnionitis, and pre-eclampsia) or neonatal morbidity (including persistent ductus arteriosus, bronchopulmonary dysplasia, and sepsis) (all $p > 0.58$).

Table 6. *Clinical characteristics in Study IV*

	Repeat betamethasone	Placebo	<i>P</i>
	<i>n = 8</i>	<i>n = 14</i>	
GA, median (range), weeks	30.4 (28.0-34.6)	31.5 (27.0-33.4)	0.76
Birth weight, mean \pm SD, grams	1402 \pm 338	1508 \pm 590	1.00
Mode of Delivery (VD/CS), n	2/6	2/12	0.60
Gender (Male/Female), n	5/3	6/8	0.66
Apgar 1 min, median (range)	7 (1-9)	8 (1-10)	0.34
Cord arterial pH, mean \pm SD	7.23 \pm 0.1	7.32 \pm 0.04	< 0.001
Cord arterial BE, mean \pm SD	-3.90 \pm 2.73	-3.21 \pm 2.92	0.53
Days on ventilator, median (range), days	2 (1-24)	7 (0-19)	0.33
Days on nCPAP, median (range), days	4 (0.5-27)	0.5 (0.5-29)	0.53
Duration of oxygen supplementation, median (range), days	3 (1-37)	3.5 (1-64)	0.17

5.2 Lung liquid content

Based on the abundance of B-lines on the ultrasound, the amount of lung liquid decreased between 1 and 24 hours postnatally in the VD as well as in the CS groups (both $p < 0.001$). The amount of lung liquid at 3 hours was higher in infants delivered by CS than in those delivered by VD ($p = 0.012$, Figure 6), but not at 1 or 24 hours after delivery ($p = 0.24$ and $p = 0.06$, respectively).

The interobserver correlation between the independent analyses performed by two radiologists was high ($r^2 = 0.86$).

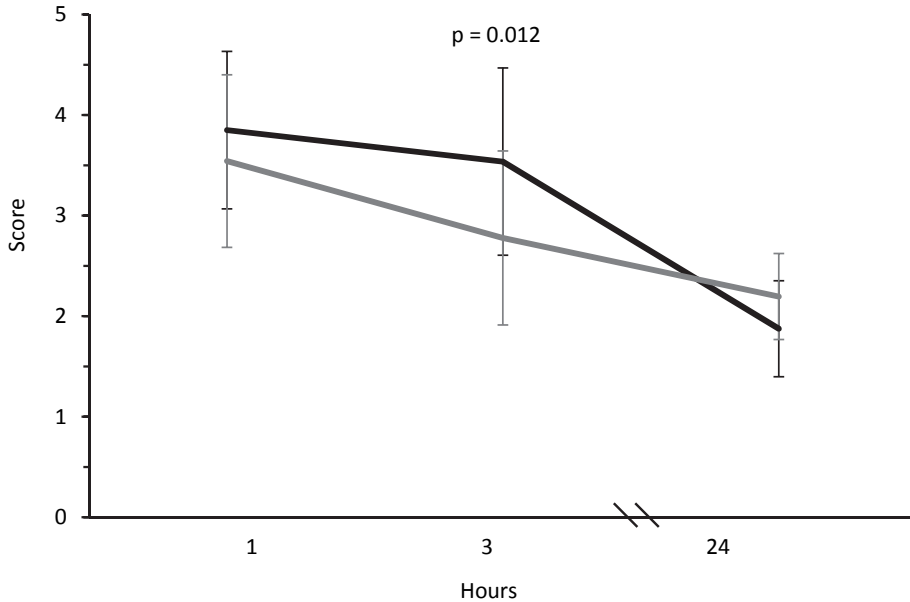


Figure 6. Mean score for abundance of B-lines in the lungs of VD (gray line) and CS (black line) infants. Error bars depict standard deviation from the mean.

5.3 Hormone concentrations

The umbilical cord plasma cortisol concentration was higher in infants delivered vaginally than in those delivered by CS (Tables 4 and 5). Cord cortisol concentrations correlated positively with GA in the population of late preterm and term infants (Spearman $r = 0.267$, $p = 0.021$; $n = 74$) as well as in the population of term infants only (Spearman $r = 0.532$, $p < 0.001$; $n = 59$). We detected no differences in cortisol concentrations in saliva of VD and CS infants (data not shown). The concentrations of cortisol in saliva showed no significant changes between the sampling time points at a median of 1.4 hours ($n = 27$), 25 hours ($n = 16$) and 49 hours ($n = 12$) (39.3 [7.9-196.3], 50.1 [6.5-174.0], and 12.8 [1.7-62.6] ng/mL, respectively; $p = 0.11$).

Cord blood TSH, but not FT3, was lower in infants delivered by CS (Tables 4 and 5).

5.4 Expression of ENaC

5.4.1 In late preterm and term infants

α - or β ENaC expression showed no differences between CS infants sampled at < 30 min and those sampled at a median of 1.5 hours postnatally (all $p > 0.069$, data not shown). Thus, these two time points were combined in the further analysis

of ENaC expression. At first sampling in the first study of term infants, α ENaC mRNA was lower in infants delivered by elective CS than in vaginally delivered infants (Table 7). In the latter population of term infants, we detected no difference (Figure 7A). The amounts of β ENaC mRNA showed no differences between VD and CS infants at any time point of sampling (all $p > 0.078$; Table 7 and Figure 7B).

In infants delivered by CS, α ENaC mRNA was higher at one day of life than it was only a few hours after delivery (Figure 7A), with peak expression occurring at 22-29 hours postnatally (Table 7). In both CS and VD infants, β ENaC expression decreased during the first day of life (Table 7 and Figure 7B).

Table 7. ENaC expression in term infants delivered by VD and CS (Study II)

		VD	CS
		<i>n</i> = 22	<i>n</i> = 47
α ENaC mRNA	0-3 h	55.9 (28.1 - 81.9) <i>n</i> = 21	45.6 (20.3 - 70.2) <i>n</i> = 42
	22-29 h	37.4 (8.4 - 123.3) <i>n</i> = 15	51.2 (12.4 - 81.2) <i>n</i> = 30
	46-52 h	47.4 (17.0 - 89.9) <i>n</i> = 13	47.5 (15.6 - 67.6) <i>n</i> = 24
β ENaC mRNA	0-3 h	24.6 (10.9 - 41.0) <i>n</i> = 15	25.3 (5.9 - 46.1) <i>n</i> = 40
	22-29 h	7.2 (1.0 - 20.6) <i>n</i> = 13	7.7 (1.7 - 18.9) <i>n</i> = 28
	46-52 h	6.3 (0.8 - 51.5) <i>n</i> = 13	6.6 (1.3 - 15.3) <i>n</i> = 19

α ENaC mRNA was higher in VD than in CS infants at 0-3 h; $p = 0.022$. In CS infants, α ENaC expression peaked at 22-29 h postnatally; $p = 0.033$. β ENaC expression decreased from 0-3 to 22-29 h in VD infants ($p = 0.008$) and CS infants ($p < 0.001$). All values are amol/fmol CK18, median (range).

In the study population of term infants, we detected no correlation between α - or β ENaC mRNA at 0-3 hours and GA ($p = 0.16$ and $p = 0.20$). However, in the term infants delivered by elective CS sampled within 30 min of delivery, α ENaC mRNA correlated positively with GA (Pearson $r = 0.407$, $p = 0.043$; $n = 25$). Furthermore, in these infants, α ENaC mRNA at first sampling also correlated with cord cortisol (Pearson $r = 0.635$, $p = 0.001$; $n = 22$). In a partial correlation analysis after controlling for GA, the correlation between α ENaC mRNA and cord cortisol remained statistically significant ($p = 0.005$). In the total term study population, at first sampling α ENaC mRNA correlated positively with salivary cortisol concentrations (Spearman $r = 0.479$, $p = 0.015$; $n = 25$).

α - and β ENaC expression showed no correlation with cord blood TSH or FT3 (all $p > 0.23$).

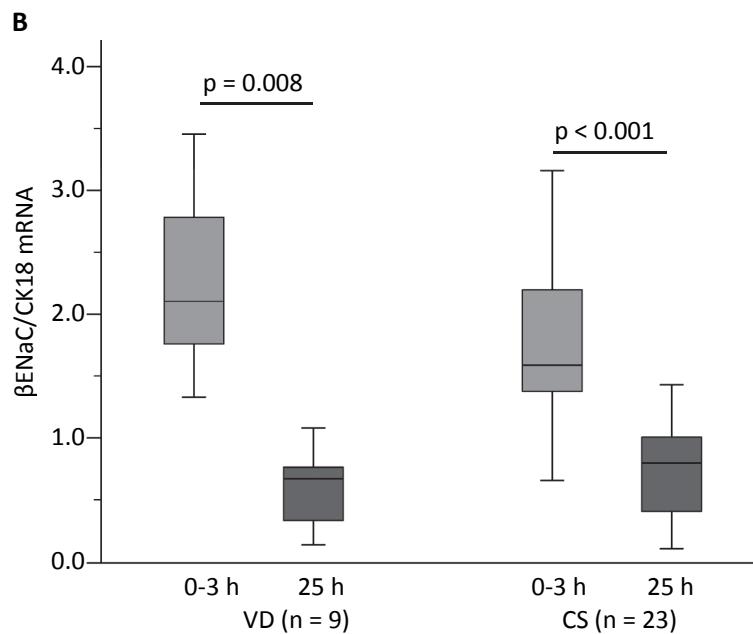
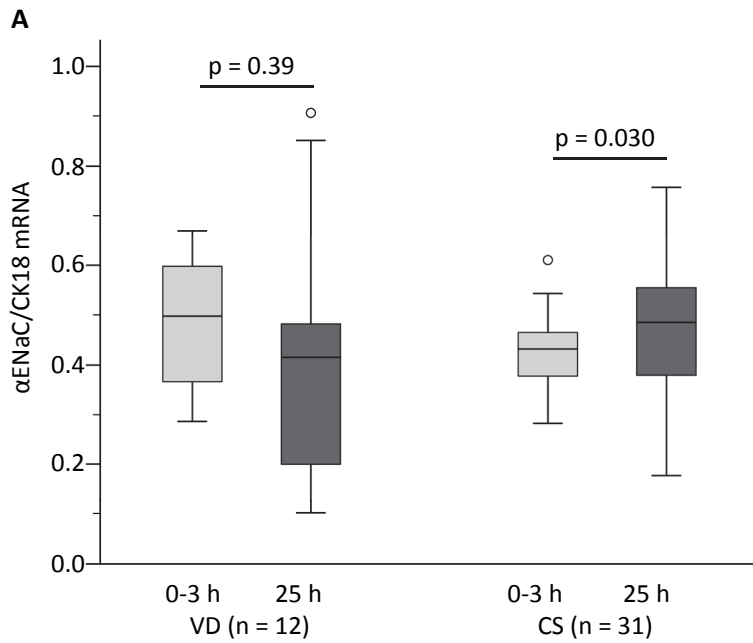


Figure 7A) αENaC and **B)** βENaC mRNA at 0-3 h (gray boxes) and 25 h (dark gray boxes) in term infants. Boxes are medians and quartiles; a circle represents an extreme value.

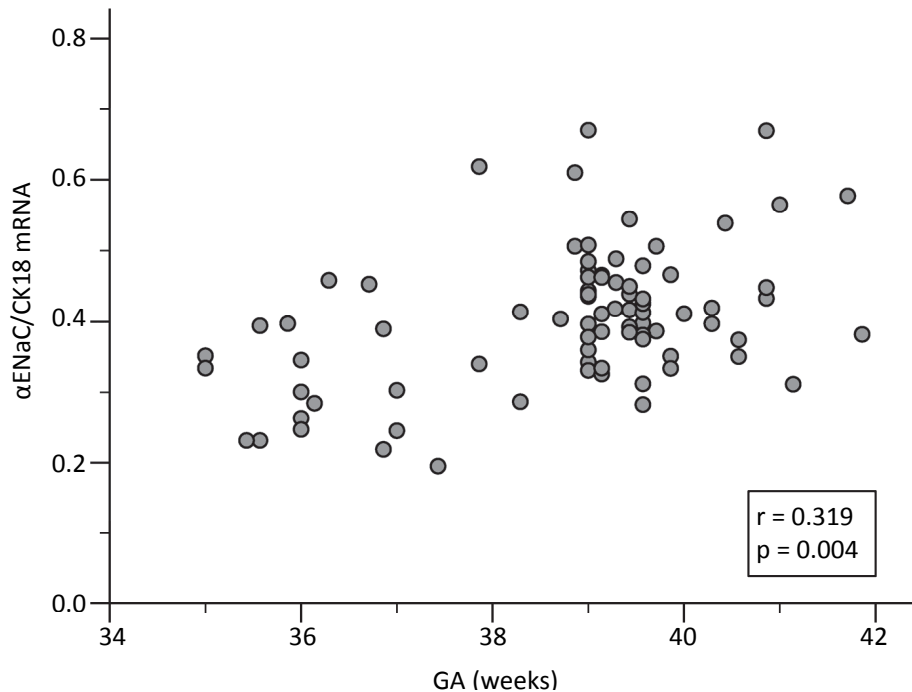


Figure 8. Correlation of α ENaC mRNA at 0-3 h with GA ($n = 81$). α ENaC mRNA was lower in late preterm than in term infants ($p = 0.006$), and lower in late preterm and early term infants (GA 35^{0/7}-38^{6/7}) than in infants delivered ≥ 39 weeks of gestation ($p < 0.001$).

In the population of late preterm and term infants, α ENaC expression at 0-3 hours correlated positively with GA and cord cortisol (Spearman $r = 0.319$, $p = 0.004$; $n = 81$ [Figure 8] and Spearman $r = 0.345$, $p = 0.003$; $n = 71$, respectively). β ENaC expression at 0-3 hours correlated positively with cord cortisol (Spearman $r = 0.407$, $p = 0.001$; $n = 59$. Figure 9), whereas its correlation with GA was not statistically significant (Spearman $r = 0.225$, $p = 0.07$; $n = 68$).

With multiple linear regression analysis, we determined the possible associations of α - and β ENaC mRNA at 0-3 h with GA, cortisol, mode of delivery, gender, cord arterial pH and BE, as well as sample timing. The analysis showed that only GA (unstandardized Beta = 0.027 [95% CI: 0.015, 0.039, $p < 0.001$]) predicted α ENaC mRNA at 0-3 hours ($R^2 = 0.204$, $p < 0.001$; $n = 80$). The model with the highest explanatory power for β ENaC expression at 0-3 h ($R^2 = 0.224$; $p = 0.001$; $n = 59$) included cord cortisol (unstandardized Beta 0.005 [95% CI: 0.002, 0.008], $p = 0.005$) and gender (unstandardized Beta -0.316 [95% CI: -0.663, -0.096]; $p = 0.010$), where male gender was associated with lower β ENaC expression. Further analysis included cord blood TSH as an independent variable and found that a model that includes cord cortisol (unstandardized Beta = 0.006 [95% CI: 0.003, 0.009, $p < 0.001$], gender (unstandardized Beta = -0.304 [95% CI: -0.585, -0.022, $p = 0.035$]) and TSH (unstandardized Beta = -0.041 [95% CI: -0.071, 0.010,

$p = 0.010$) predicted β ENaC mRNA ($R^2 = 0.321$, $p < 0.001$; $n = 57$) (Janér et al., unpublished data).

At 1.5 hours postnatally, α ENaC mRNA was lower in late preterm than in term infants ($p = 0.006$, Figure 8), whereas the β ENaC mRNA amounts were similar at both 1.5 and 25 hours postnatally ($p > 0.09$, data not shown). Because the incidence of respiratory morbidity remains elevated even at 38 weeks GA (Hansen et al. 2008), we set the cut-off at 39 weeks. Here, both α - and β ENaC mRNA amounts at 1.5 hours were lower in late preterm and early term infants (GA 35^{0/7} to 38^{6/7}) than in infants delivered at or after 39 weeks of gestation (α ENaC [Fig 8] and β ENaC/CK18 mRNA: 1.55 [1.34 - 1.77] vs. 1.90 [1.50 - 2.30], $p = 0.007$; $n = 19$ and $n = 29$).

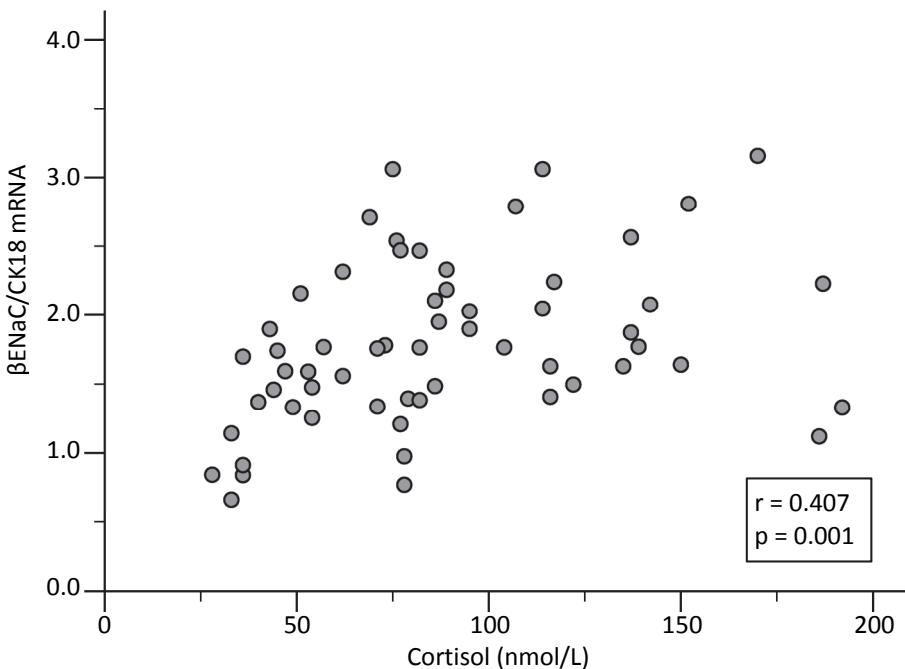


Figure 9. Correlation of β ENaC mRNA at 0-3 h with cord blood cortisol ($n = 59$).

5.4.2 In preterm infants

We found no differences between the repeat betamethasone and placebo groups in α -, β -, or γ ENaC mRNA levels at 1-5 or 20-29 hours after delivery (Table 8). Although the expression of the α ENaC-subunit in the total population at 1-5 hours correlated with GA (Spearman $r = 0.497$, $p = 0.019$; $n = 22$), linear regression analysis revealed an association between α ENaC and GA only in infants without RDS (1.5 units/week of gestation, $p = 0.004$). RDS infants showed no gestation-

dependent increase in α ENaC mRNA, and the regression coefficients for infants with and without RDS differed significantly ($p = 0.023$). No associations of β - or γ ENaC with GA occurred. In the total study population, the amounts of α - and γ ENaC mRNA were similar at 1-5 and 20-29 hours postnatally (both $p > 0.36$, Table 8). However, a decrease in β ENaC mRNA was evident ($p = 0.039$, Table 8).

Table 8. ENaC expression in preterm infants

		Repeat betamethasone	Placebo	P
		<i>n</i> = 8	<i>n</i> = 14	
α ENaC mRNA	1-5 h	2.88 (0.02-9.11) <i>n</i> = 8	3.10 (0.30-10.47) <i>n</i> = 14	0.92
	20-29 h	0.83 (0.36-8.50) <i>n</i> = 7	0.76 (0.01-14.77) <i>n</i> = 10	0.74
β ENaC mRNA	1-5 h	5.90 (0.01-12.17) <i>n</i> = 8	6.87 (0.38-20.42) <i>n</i> = 14	0.53
	20-29 h	2.07 (0.08-9.92) <i>n</i> = 6	2.14 (0.07-11.62) <i>n</i> = 10	0.71
γ ENaC mRNA	1-5 h	0.71 (0.00-3.85) <i>n</i> = 6	0.83 (0.17-5.48) <i>n</i> = 10	0.49
	20-29 h	0.14 (0.01-2.00) <i>n</i> = 6	0.38 (0.03-3.63) <i>n</i> = 7	0.53

All values are amol/fmol CK18, median (range). P-values are for the repeat betamethasone vs. the placebo group.

We then analyzed chest x-rays to determine whether the expression of ENaC was related to the severity of respiratory distress. We found a trend towards a negative correlation between the expression of α ENaC at 1-5 hours and the respiratory distress-severity score in radiographs obtained the closest in time to the epithelial cell sampling (Spearman $r = -0.441$, $p = 0.067$; $n = 18$). Analyses of ENaC expression and mean supplemental oxygen administration showed a negative correlation between β ENaC mRNA and mean FiO₂ at both 1-5 hours and 20-29 hours (Spearman $r = -0.472$, $p = 0.027$; $n = 22$ and Spearman $r = -0.603$, $p = 0.013$; $n = 16$, respectively).

5.5 Expression of Na-K-ATPase

We also analyzed the airway expression of α 1-Na-K-ATPase in the population of late preterm and term infants (Janér et al., unpublished data) and found a trend towards a positive correlation between α 1-Na-K-ATPase mRNA and cord cortisol (Spearman $r = 0.237$, $p = 0.068$; $n = 60$). However, α 1-Na-K-ATPase mRNA showed no correlation with GA ($p = 0.18$; $n = 69$). Similarly, linear regression analysis

showed no association between $\alpha 1$ -Na-K-ATPase at 0-3 hours and GA, cortisol, mode of delivery, gender, cord arterial pH and BE, or sample timing.

Term infants born VD and CS showed no differences in $\alpha 1$ -Na-K-ATPase mRNA (both $p > 0.22$, data not shown). In both VD and CS infants, $\alpha 1$ -Na-K-ATPase mRNA decreased between 0-3 and 25 h ($p = 0.017$ and $p < 0.001$, respectively; data not shown).

At 1.5 hours postnatally, late preterm and term infants showed no differences in $\alpha 1$ -Na-K-ATPase mRNA ($p = 0.11$, data not shown). However, setting the cut-off at 39 weeks' gestation showed lower $\alpha 1$ -Na-K-ATPase mRNA at 1.5 hours postnatally in the population of late preterm and early term infants than in infants delivered at or after 39 weeks (Figure 10). At 25 hours, no difference remained (Figure 10). Between 1.5 and 25 hours, $\alpha 1$ -Na-K-ATPase mRNA decreased both in late preterm and early term and in infants delivered after 39 weeks ($p = 0.047$ and $p = 0.008$, respectively; Figure 10).

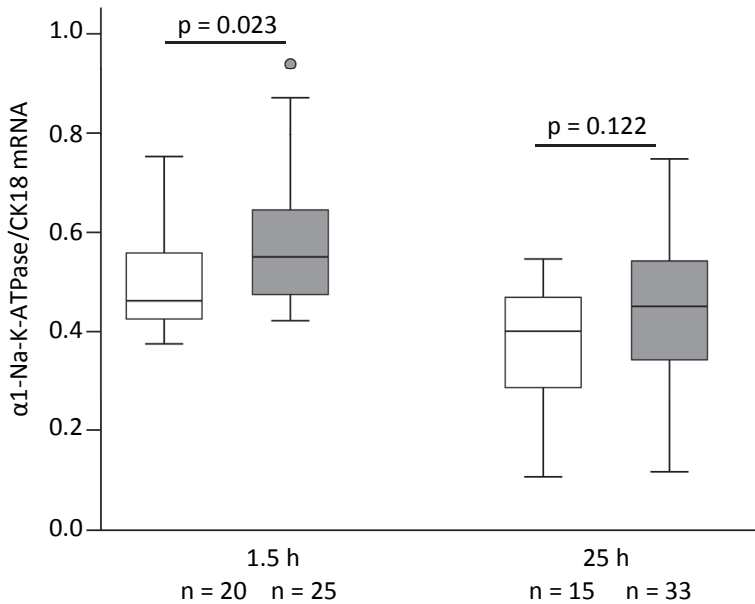


Figure 10. $\alpha 1$ -Na-K-ATPase mRNA in late preterm and early term infants (open boxes) vs. infants delivered ≥ 39 weeks (gray boxes) at 1.5 h and 25 h postnatally. Boxes are medians and quartiles; a circle represents an extreme value.

5.6 Expression of SGK1

In multiple regression analysis, we determined the possible association between SGK1 mRNA at 1.5 hours postnatally and GA, cortisol, mode of delivery, gender, cord arterial pH and BE, and sample timing. GA (unstandardized Beta = 0.129 [95% CI: 0.070, 0.189], $p < 0.001$) and mode of delivery (unstandardized Beta = -0.484 [95% CI: -0.723, -0.246], $p < 0.001$) predicted SGK1 mRNA at 1.5 hours ($R^2 = 0.395$, $p < 0.001$; $n = 50$). VD and higher GA were associated with higher expression of SGK1.

SGK1 mRNA was higher in VD than in CS infants at 1.5 hours, whereas no difference was evident at 25 hours (Figure 11, and $p = 0.54$, data not shown). In infants delivered by CS and sampled at 1.5 hours, SGK1 mRNA was higher than in CS infants sampled at 5 min after delivery (Figure 11).

In VD infants, the expression of SGK1 at 1.5 hours was lower not only in late preterm than term infants (0.21 [0.12-0.30] vs. 0.31 [0.22-0.41] SGK1/CK18 mRNA; $p = 0.042$, $n = 11$ and $n = 19$), but also in late preterm and early term infants than in infants delivered at or after 39 weeks (0.26 [0.18-0.34] and 0.40 [0.29-0.50] SGK1/CK18 mRNA; $p = 0.020$, $n = 17$ and $n = 13$).

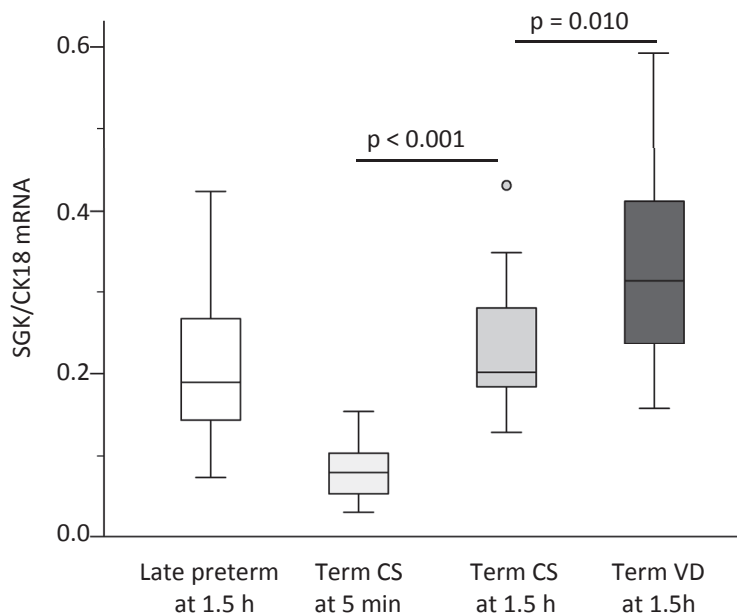


Figure 11. SGK1 expression at 5 min and 1.5 h in late preterm and term infants. Late preterm (open boxes; $n = 15$), term CS at 5 min (light gray boxes; $n = 25$), term CS at 1.5 h (gray boxes; $n = 15$), and term VD (dark gray boxes; $n = 19$). Boxes are medians and quartiles; a circle represents an extreme value.

6 DISCUSSION

This thesis aimed to study and measure the absorption of lung liquid at birth and to study the mechanisms related to the perinatal removal of liquid from the airways and lungs. The studies were performed in healthy term infants delivered vaginally and in clinical situations associated with delayed lung liquid absorption, namely elective cesarean section and delivery at preterm, late preterm or early term gestation. In these clinical models, we studied the amount of lung liquid and its changes, as well as the gene expression of important players in the process of transepithelial sodium transport in airway epithelium during early postnatal life. The main focus was on ENaC, the rate-limiting step in lung liquid absorption. We evaluated the effects of maturity and mode of delivery, with the concentration of cortisol serving as an indicator of maturation as well as of physiological birth stress, on the expression of ENaC. We determined the effect of the exogenous GC betamethasone on ENaC expression in preterm infants.

6.1 Mode of delivery and lung liquid clearance in term infants

In term infants delivered vaginally or by elective CS, the amount of lung liquid, quantified with ultrasound, decreased during the first day after delivery. In CS-delivered infants, in the absence of signs of respiratory distress, the lung liquid content at 3 hours after birth was significantly higher than in vaginally delivered infants. Our findings are in agreement with previous data (Copetti et al. 2007) showing that in healthy term newborn infants, B-lines are present but decrease during the first day of life. Furthermore, B-lines are more frequent in infants delivered by CS (Copetti et al. 2007). This finding corroborates the findings of animal studies and emphasizes the importance of labor in the transition to air-breathing. In fact, this finding may indicate that lung liquid clearance during the first hours after delivery differs depending on the presence or absence of the physiological stress of labor. Although some lung liquid is removed before birth in VD infants (Bland et al. 1979, Bland et al. 1980), the lack of a difference in lung liquid content at 1 hour after delivery may indicate that substantial absorption of liquid takes place after birth. In line with this, the amount of extravascular water in newborn rabbits begins to decrease after 30-60 minutes postnatally (Bland et al. 1980). Importantly, though, the first breaths significantly improve the aeration of the lungs, as shown in CS-delivered animals (Siew et al. 2009).

The α ENaC subunit is indispensable in the formation of the ENaC channel (Canessa et al. 1994), and in its absence, newborn rodents die due to pulmonary edema and respiratory distress (Hummler et al. 1996, Li et al. 2007). *In vitro* experiments indicate that β ENaC contributes to the maximal activity of ENaC (Canessa et al. 1994, McDonald et al. 1995) as well as to the formation of highly sodium-selective channels (Jain et al. 2001). Sufficient amounts of β ENaC could

also affect trafficking of the ENaC-complex from the endoplasmatic reticulum to the plasma membrane, since efficient trafficking requires all three subunits (Snyder 2005). Although no overt respiratory distress is evident in newborn mice lacking the β ENaC subunit, studies have reported an increase in the lung wet:dry ratio (McDonald et al. 1999). In addition, in adult mice, a lack of β ENaC may lead to reduced alveolar liquid clearance (Randrianarison et al. 2008). Importantly, β ENaC is vital to lung liquid absorption induced by β -agonists (Randrianarison et al. 2008).

In our first gene expression study of term infants, we found that the airway expression of α ENaC within hours after birth is lower in CS than in VD infants, whereas the latter study showed no differences. This may have occurred because in the previous study, GA was lower in CS than in VD infants. No differences in β ENaC between VD and CS infants were evident at either time point of sampling. Whereas β ENaC mRNA decreased in both groups during the first postnatal day in accordance with our previous studies (Helve et al. 2006, Helve et al. 2007) and animal data (Jesse et al. 2009), the pattern of α ENaC expression differed depending on the mode of delivery. In VD infants, the amount of α ENaC mRNA showed no change, whereas in CS infants, α ENaC mRNA increased significantly from 0-3 hours to one day of age. The results are in line with those of our previous studies, in which a decrease in α ENaC expression during the first postnatal day was evident in term VD, but not in CS, infants (Helve et al. 2007). Furthermore, another study showed that α ENaC mRNA at one day of age was higher in CS than in VD infants (Helve et al. 2006). Similarly, α ENaC mRNA in animal lungs increases after CS, paralleling a rise in cortisol and subsequently resulting in increased lung liquid clearance (Baines et al. 2000). In line with this finding, nasal PD during the first postnatal day, predominantly a reflection of sodium absorption, is higher in infants delivered by CS than in term vaginally delivered infants (Gowen et al. 1988). This finding, as well as our finding that α ENaC mRNA increases during the first postnatal day after elective CS, seems to suggest the operation of compensatory or adaptive mechanisms (Gaillard et al. 2005).

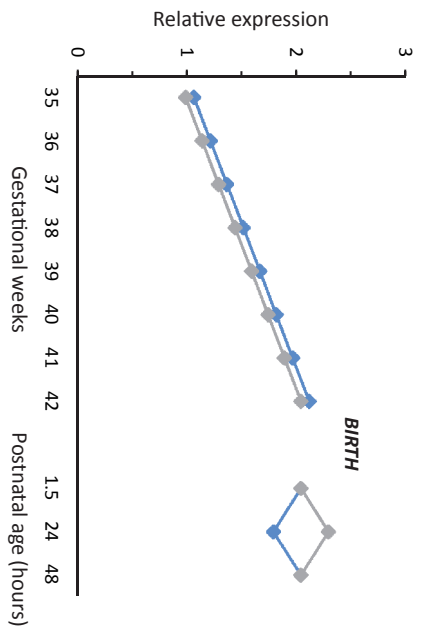
In our population of term infants, SGK1 expression at 1.5 hours after delivery was higher after VD than after CS, and regression analysis revealed that CS is associated with lower SGK1. In addition to lower cortisol concentrations in CS than in VD infants, the lack of a surge in catecholamines due to the absence of labor could contribute to insufficient transcription and activation of SGK1 in CS infants (Itani et al. 2002a, Itani et al. 2002b, Thomas et al. 2004, Vasquez et al. 2008). Since SGK1 increases the gene and cell-surface expression as well as the activity of ENaC (Boyd et al. 2005, Zhang et al. 2007, McTavish et al. 2009, Debonneville et al. 2001, Friedrich et al. 2003, Watt et al. 2012), low SGK1 expression could be associated with insufficient sodium absorption via ENaC, resulting in inadequate lung liquid clearance and respiratory distress.

6.2 Effect of gestational age on ENaC, Na-K-ATPase, and SGK1 expression

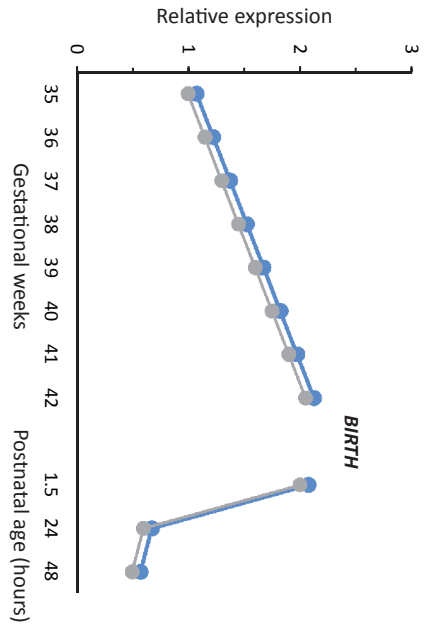
Epidemiological data show that the incidence of respiratory morbidity, including TTN and RDS as well as composite respiratory morbidity, remains elevated not only in late preterm, but even beyond term gestation (i.e. 37 weeks) (Morrison et al. 1995, Hansen et al. 2008, Melamed et al. 2010, Tita et al. 2009, Hibbard et al. 2010). In accordance with animal data and our previous findings (O’Brodivich et al. 1993, Tchepichev et al. 1995, Jesse et al. 2009, Helve et al. 2007), α ENaC expression correlated with GA within hours after delivery in this population of late preterm and term infants. Not only was α ENaC mRNA within hours after birth lower in late preterm than in term infants but, importantly, with a cut-off of 39 weeks, both α - and β ENaC expression, as well as α 1-Na-K-ATPase and SGK1 expression was lower before than after this point. Worth noting is that in population of small preterm infants, α ENaC mRNA associated with GA only in infants without RDS. Figure 12 summarizes ENaC, Na-K-ATPase, and SGK1 expression patterns, depending on GA and mode of delivery.

In light of the data on the roles of α - and β ENaC in sodium transport and lung liquid clearance, our finding of lower expression of these subunits in infants delivered preterm or at early term suggest that this population is predisposed to respiratory morbidity due to retained lung liquid. This finding is in accordance with the epidemiological data showing that the higher incidence of respiratory morbidity persists until 39 gestational weeks (Morrison et al. 1995, Hansen et al. 2008, Melamed et al. 2010, Tita et al. 2009, Hibbard et al. 2010). Likewise, the finding that SGK1 expression within hours after delivery is lower in CS than in VD infants may provide a physiological rationale for the higher incidence of respiratory morbidity following CS and our finding of higher lung liquid content in CS infants. Thus, birth by CS at late-preterm or early-term gestation constitutes a double-hit, further raising the risk for respiratory distress over that of infants delivered vaginally at term. In the absence of the need for immediate interventions, these physiological data support the practice of postponing elective CS to 39 or more weeks.

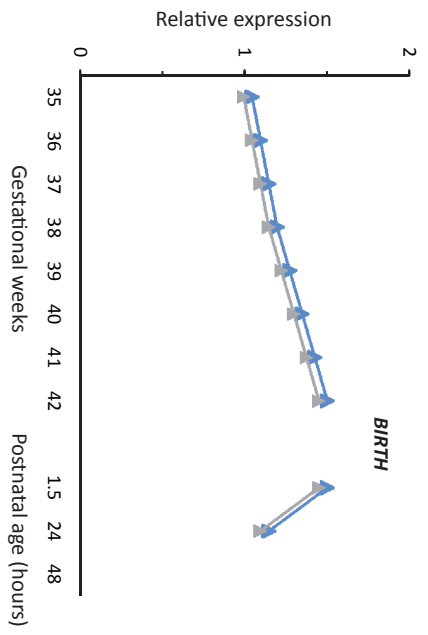
a. α ENaC



b. β ENaC



c. α 1-Na-K-ATPase



d. SGK1

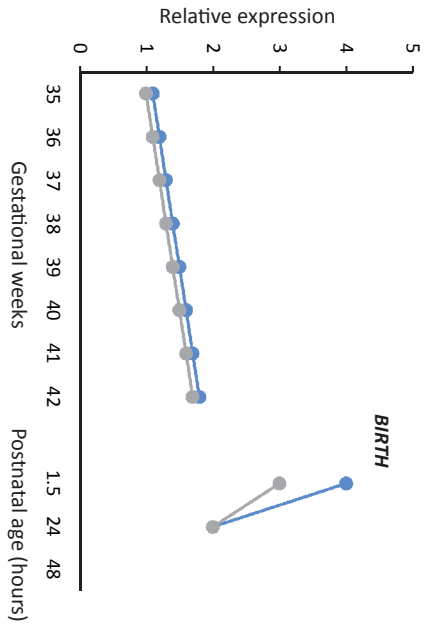


Figure 12. Schematic summaries of relative a) α ENaC, b) β ENaC, c) α 1-Na-K-ATPase, and d) SGK1 expression during late gestation and early postnatal life. The blue line indicates VD, and the gray line, CS infants. The figures are not to scale and do not represent values in the original datasets.

6.3 Endogenous glucocorticoids in regulation of ENaC and Na-K-ATPase

Glucocorticoids are essential in pulmonary development and adaptation. Their effects on pulmonary adaptation are partially related to the priming of catecholamine-induced lung liquid absorption (Wallace et al. 1995, Wallace et al. 1996). In both preterm delivery and delivery by elective CS, the hormonal milieu of the infant differs from that of the physiological situation in term, vaginally delivered infants (Murphy et al. 1983, Oh et al. 2006, Mears et al. 2004).

In the present studies, α ENaC mRNA correlated with cord blood and salivary cortisol concentrations in term infants. However, in the population of late preterm and term infants, the amounts of α ENaC mRNA showed no significant association with cord cortisol concentrations. In contrast, β ENaC mRNA correlated with cord cortisol concentrations. α 1-Na-K-ATPase mRNA showed no association with cord cortisol. In animals and *in vitro* experiments, the effects of GCs on ion transport vary depending on the species, target tissue, age, and other environmental factors, such as oxygen tension levels (Otulakowski et al. 2006). While GCs induce the *in vitro* gene expression of all three ENaC subunits as well as that of α 1- and β 1-Na-K-ATPase subunit mRNAs (Venkatesh et al. 1997, Chalaka et al. 1999, Sayegh et al. 1999, Lazrak et al. 2000, Itani et al. 2002a, Ramminger et al. 2004) in animal fetuses, GC treatment reportedly upregulates the lung gene expression only of α ENaC, with no effect on β -, γ ENaC or α 1-Na-K-ATPase (Tchepichev et al. 1995, Jesse et al. 2009). In adult animals, however, GCs increase β ENaC mRNA and the alveolar liquid clearance mediated by ENaC (Noda et al. 2003). In the present studies, while the association of α ENaC mRNA with cortisol concentrations remains unclear, the association of β ENaC mRNA and cortisol seems evident.

In humans, besides reducing RDS in preterm infants, antenatal GCs reduce admissions to special care units due to respiratory morbidity in term infants delivered with elective CS (Stutchfield et al. 2005). We show that β ENaC, which plays a regulatory role in increasing ENaC activity (Jain et al. 2001, Canessa et al. 2004) and is central to beta-agonist-induced lung liquid absorption (Randrianarison et al. 2008), associates with endogenous cortisol concentrations in a population of late preterm and term infants. Thus, part of the effect of exogenous GCs in the prophylaxis of respiratory distress could be mediated through the induction of ENaC. Importantly, the effects of GCs are not limited to the transcriptional level; GCs also promote protein translation, cell surface expression, and ENaC activity (Itani et al. 2002a and 2002b, Otulakowski et al. 2006, Watt et al. 2012). Therefore, in pulmonary adaptation where catecholamines activate ENaC and lung liquid clearance, the induction of β ENaC by exogenous GCs could be the focus of interventions aiming to prevent or treat respiratory distress due to insufficient lung liquid clearance. Other researchers (Ramachandappa and Jain 2008) have proposed that the duration of GC exposure required for an effect on ENaC makes strategies that include antenatal GC administration preferable to postnatal interventions in order to facilitate pulmonary adaptation. Here, augmenting the

availability of ENaC via antenatal GC treatment could serve as a platform for the subsequent postnatal activation of lung liquid clearance via the administration of, for example, beta-agonists (Ramachandrapra and Jain 2008, Armangil et al. 2011).

6.4 Repeat antenatal glucocorticoids in regulation of ENaC

In late preterm and term infants, we found evidence of the physiological regulation of airway ENaC expression and lung liquid clearance by GA, cortisol, and mode of delivery. Considering the effects of GCs on ENaC, lung liquid clearance, and in the prophylaxis of RDS, we hypothesized that a repeat dose of antenatal betamethasone administered to the mother would increase airway ENaC expression in preterm infants. However, we found that a repeat dose of antenatal betamethasone had no effect on ENaC expression in the preterm infants. Potential causes for this finding could include inappropriate treatment timing, insufficient dosing, or the presence of multiple gestations.

The range of timing for administering the repeat dose of betamethasone was 0.9 to 50.6 (median 4.8) hours before delivery and 4.1 to 54.0 (median 7.1) hours before the first sampling. Experimental data *in vitro* and in animal models indicate that ENaC subunit mRNA increases within two to eight hours of GC-exposure (Tchepichev et al. 1995, Itani et al. 2002, Lazrak et al. 2000). Thus, in our study, the timing with regard to influencing gene expression seems adequate. However, the clinical benefits of antenatal GCs are observed only 24 hours after administration (Roberts and Dalziel 2006).

The maximal increase in the amounts of α -, and β -, and γ ENaC mRNA in human fetal lung explants occurs at concentrations of 30-100 nM, and half-maximal stimulation at 3-10 nM dexamethasone (Venkatesh et al. 1997). In adult humans, circulating concentrations of 50-100 nM dexamethasone are obtained via the intravenous administration of a dose of 5 mg of dexamethasone (McTavish et al. 2009), which is equivalent to 4 mg of betamethasone. The repeat maternal dose of betamethasone used in our study in preterm infants was 12 mg. It therefore seems unlikely that the lack of effect of the repeat dose of betamethasone in our study is related to GC concentrations insufficient to induce ENaC expression.

In multiple gestations, the effect of antenatal GCs on respiratory morbidity may be absent (Roberts and Dalziel 2006). Since almost half of our preterm study population consisted of infants from multiple gestations, this could have resulted in the lack of any difference in ENaC expression with the repeat dose of antenatal betamethasone.

The number of preterm infants in the intervention study was low, and due to its experimental nature, we conducted no power analysis. While infants for similar intervention studies are difficult to obtain, this low number could have affected our results.

Several trials on the possible benefits of administering repeat doses of antenatal GCs have been published since the initiation of our intervention study. One large

randomized, placebo-controlled, blinded trial reported a reduction in RDS with a once-weekly repeated dose of betamethasone (Crowther et al. 2006). Also, a single repeat course of two doses of betamethasone reduced RDS in infants delivered at less than 34 weeks GA (Garite et al. 2009). In contrast, a trial by Wapner and colleagues found no reduction in RDS (Wapner et al. 2006). However, this study was terminated prematurely due to concerns about lower birth weights in the repeat betamethasone group, as well as to recruitment difficulties (Wapner et al. 2006). According to a meta-analysis of randomized trials, repeat antenatal GCs significantly decrease the incidence of RDS (risk ratio 0.83, 95% confidence interval 0.75-0.91) (McKinlay et al. 2012).

The reason for the ineffectiveness of a repeat dose of antenatal betamethasone on ENaC expression remains uncertain. Nevertheless, our result was in line with that of the larger clinical study, which found no beneficial effect of a repeat dose of betamethasone on RDS (Peltoniemi et al. 2007). In that study, administering the repeat dose of betamethasone less than 24 hours before delivery actually increased the incidence of RDS. A higher ENaC expression in the repeat betamethasone group of our study would have contrasted with this clinical finding, since the administration of betamethasone preceded the birth by a median of less than five hours.

6.5 The role of thyroid hormones

Animal studies indicate that thyroid hormones increase lung liquid absorption and improve lung function (Stein et al. 1994, Polk et al. 1995). These hormones may augment the effect of GCs on ENaC transcription and activity *in vitro* (Champigny et al. 1994, Otulakowski et al. 1999). *In vivo*, however, data on the effect of TRH on α ENaC expression are contradictory (O’Brodivich et al. 1993, Tchepichev et al. 1995), and in human fetal lung explants, T3 does not increase ENaC mRNA amounts beyond those already augmented by dexamethasone (Tchepichev et al. 1997). In addition, thyroid hormones show no beneficial effect on respiratory morbidity in preterm infants (Osborn and Hunt 2007, Biswas et al. 2003). In the population of term infants, we found no correlation between the ENaC expression and cord blood TSH or T3 concentrations. However, in further analysis of the late preterm and term population data, TSH emerged as a statistically significant variable contributing to a model that predicted β ENaC mRNA. Thus, the role of thyroid hormones in regulating ENaC transcription in human infants remains uncertain. Although thyroid hormones may boost ENaC activity (Richard et al. 2003, Champigny et al. 1994), our and most previous data suggest that any significant, independent effect of thyroid hormones on perinatal lung liquid clearance related to ENaC expression would be unlikely.

6.6 Methodological aspects and future perspectives

Our first study shows that bedside lung ultrasound is useful in identifying the physiological changes in lung liquid amounts during pulmonary adaptation. Ultrasound may also be useful in diagnosing TTN and RDS (Copetti et al. 2007, Copetti et al. 2008). In infants with TTN, ultrasound can detect improvement in the disease via the progressive disappearance of the compact white pattern in the basal regions of the lungs (Copetti et al. 2007). The disappearance of the double lung point sign, pathognomonic to TTN, coincides with clinical improvement (Copetti et al. 2007), and ultrasound findings can serve to predict the need for respiratory support (Raimondi et al. 2012). The learning curve in performing ultrasound to evaluate lung edema is short (Lichtenstein et al. 1997). Importantly, the concordance between separate assessments of ultrasound scans is strong in both our and previous studies (Raimondi et al. 2012). Ultrasound is also useful in differential diagnostics, since it permits the detection of, for example, pneumothorax, pulmonary hemorrhage, and pneumonia (Copetti et al. 2007). Thus, the use of ultrasound in identifying causes of respiratory distress and evaluating treatment strategies seems worth considering in the clinical setting.

In Study II, we used ELISA to analyze cortisol concentrations in both cord blood and saliva. For Study III, liquid chromatography tandem-massspectrometry became available, so the results for cortisol concentrations in these two studies are incomparable.

The number of infants with results for salivary cortisol concentrations is low due mainly to inadequate amounts of saliva obtained at sampling. Consequently, the power of this study in particular to detect differences between several time points was reduced.

We obtained epithelial cell samples from the nasal epithelium because the ion transport over the proximal airway respiratory epithelium correlates with lung function (Fajac et al. 1998, Helve et al. 2006) and altered nasal PD reflects both TTN and RDS (Gowen et al. 1988, Barker et al. 1997). Importantly, obtaining the samples from easily available parts of the respiratory system is, from the infants' perspective, preferable to more invasive methods. Even though nasal PD in newborn infants may offer information on ion channel activity, the method is challenging to perform in term newborn infants (own observations). Also, epithelial cell sampling is much faster and less invasive than PD measurements. With regard to human *in vivo* data on ENaC, Na-K-ATPase and SGK1 in the respiratory epithelium of the newborn, our gene expression data are unique.

The focus of Ultrasound is also useful in differential diagnostics, since e.g. pneumothorax, the right side (Copetti et al. 2007). this thesis was to study the gene expression of ENaC, Na-K-ATPase, and SGK1. Although post-transcriptional mechanisms clearly play a significant role in their regulation, recent results in infants indicate that a correlation exists between the expression of α ENaC and airway transepithelial sodium transport (Kaskinen et al. 2016). A limitation of this thesis is that the expression data and the data on the end-points at the organ

level (i.e. lung liquid content) are unavailable from the same individuals. Thus, to further investigate the regulation of ion transport and lung liquid clearance during pulmonary adaptation, a combination of several methods – including the determination of hormone concentrations, the measurement of ion transport activity and the study of end-organ function (e.g. lung compliance and the quantitation of lung liquid amounts) – would be appropriate.

7 CONCLUSIONS

The results of this thesis suggest the following conclusions:

In term, healthy infants the amount of lung liquid measured a few hours after delivery is higher in CS-delivered infants than in VD infants, a difference that is detectable with ultrasound. Lung liquid decreases during the first day of life, and at one day postnatally, CS and VD infants show no significant differences in lung liquid amounts.

In term infants, SGK1 expression is lower after CS than after VD. Low SGK1 could predispose newborn infants to insufficient lung liquid clearance and respiratory distress.

The airway gene expression of α - and β ENaC, α 1-Na-K-ATPase, and SGK1 is lower in infants delivered at late preterm or at early term gestation than in infants delivered at or after 39 weeks of gestation. This may contribute to the higher incidence of respiratory morbidity in infants delivered before 39 weeks of gestation and offers a physiological rationale for the practice of postponing elective CS until completing 39 weeks of gestation.

In late preterm and term infants, the expression of β ENaC in the airway epithelium correlates with cord cortisol concentrations. Independently of GA, β ENaC mRNA decreases during the first postnatal day, which is in line with active regulation perinatally. The induction of β ENaC expression by GCs may contribute to more efficient lung liquid absorption.

In small preterm infants, a repeat dose of antenatal betamethasone does not seem to augment ENaC gene expression. This is in line with the results of a larger related clinical study showing no benefit from the repeated treatment in reducing RDS.

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Mikko, I am amazed by your compassion, durability, and never-ending efforts for our family. I am eternally grateful for having you at my side. My son Leo Samuel, you are immensely precious to me.

Helsinki, June 2016

A handwritten signature in black ink that reads "Cecilia Janér". The signature is written in a cursive, flowing style.

Cecilia Janér

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