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ROLE OF WATER CHANNELS IN KIDNEY AND LUNG

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Stockholm 2009

Published by Karolinska Institutet. Printed by [Universitetsservice US-AB]

ISBN 978-91-7409-714-6

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ABSTRACT

Aquaporins (AQPs) are membrane proteins that function as water channels facilitating a rapid and regulated transport of water across cell membranes. AQPs in kidney and lung play pivotal roles for the regulation of water homeostasis. In this thesis, I addressed questions concerning functional significance of AQPs in the kidney and lung, with particular emphasis on the role of AQPs in pathophysiological conditions related to an impaired water balance during postnatal adaptation.

We assessed urinary AQP2 level in preterm and term infants during the postnatal period. We could demonstrate that urinary AQP2 correlates with the overall maturity of tubular function in human neonates. Urinary AQP2 may reflect AQP2 expression level associated with different physiological and pathophysiological conditions.

We further investigated whether AQP2 plays a role in water balance disturbances in preterm infants treated with prostaglandin inhibitors for patent ductus arteriosus closure. Urinary AQP2 level, along with urinary osmolality, was significantly decreased during both ibuprofen and indomethacin treatment. The overall low urinary osmolality and the dissociation between urinary osmolality and urinary AQP2 level indicate that AQP2 may not be a factor contributing to the fluid retention sometimes observed in infants with patent ductus arteriosus treated with prostaglandin inhibitors.

To investigate whether AQP4 may play a role in renal K^+ transport, we performed a series of experimental studies and developed a mathematical model, indicating that AQP4, Na^+, K^+-ATPase and inwardly rectifying K^+ channel Kir7.1 form a transporting microdomain, where AQP4 may play a modulating role for K^+ transport across the basolateral membrane of the principal cell by maintaining a local extracellular K^+ concentration that is favorable for K^+ recirculation across the basal membrane, and thus for the activity of Na^+, K^+-ATPase and K^+ secretion.

We also study the role of AQPs and ion transporters in pathophysiological conditions related to delayed clearance of fetal lung fluid. We have found that the neonatal respiratory distress is associated with changes in β-ENaC and AQP5 expression. The lower β-ENaC expression may be one of the factors that predispose to the development of respiratory distress syndrome in the newborn infants. The higher AQP5 expression may provide the possibility for reabsorption of postnatal lung liquid, which contributes to quick recovery of infants with transient tachypnea of the newborn.

In conclusion, this thesis presents novel data about AQPs in the immature kidney and lung during the early postnatal period and demonstrates the crucial role of AQPs for postnatal adaptation in infants, especially in premature infants. Alterations in the expression of water and ion transportors in infants is associated with pathophysiological conditions related to impaired renal regulation of fluid and electrolyte balance, as well as delayed clearance of lung fluid. Furthermore, AQP4 expressed in the kidney may not only be important for water transport, but also be involved in renal K^+ transport.

LIST OF PUBLICATIONS

I. Zelenina M, **Li Y**, Glorieux I, Arnaud C, Cristini C, Decramer S, Aperia A, Casper C.

Urinary aquaporin-2 excretion during early human development. *Pediatric Nephrol*ogy 21: 947–952, 2006.

II. **LI Y**, Plat-Willson G, Marcoux MO, Zelenina M, Aperia A, Casper C.

Urinary aquaporin-2 excretion during ibuprofen or indomethacin treatment in preterm infants with patent ductus arteriosus. *Manuscript*.

III. Zelenina M, **LI Y**, Kamali-Zare P, Sakuraba S, MacAulay N, Zelenin S, Bondar A, Brismar H, Aperia A.

A role for AQP4 in renal K^+ transport. *Manuscript*.

IV. **Li Y**, Marcoux MO, Gineste M, Vanpee M, Zelenina M, Casper C.

Expression of water and ion transporters in tracheal aspirates from neonates with respiratory distress. *Acta Paediatrica*, 98 (11): 1729-37, 2009.

CONTENTS

LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
AECs	Alveolar epithelial cells
AGA	Adequate for gestational age
AKI	Acute kidney injury
AQPs	Aquaporins
AVP	Arginine vasopressin
BAL	Bronchoalveolar lavage
BPD	Bronchopulmonary dysplasia
cAMP	Cyclic adenosine 3´, 5´-monophosphate
CKD	Chronic kidney disease
CLD	Chronic lung disease
COX	cyclooxygenase
Cr	creatinine
CRE	cAMP-response element
CREB	cAMP-response element binding protein
DLS	Diffusion limited space
ECS	Extracellular space
ENaC	Epithelial sodium channel
FENa	The fractional excretion of sodium
GFR	Glomerular filtration rate
Kir	Inwardly rectifying K^+ channel
IVH	Intraventricular hemorrhage
MAP	Mean arterial pressure
NDI	Nephrogenic diabetes insipidus
NEC	Necrotizing enterocolitis
N-PD	Nasal potential difference
NSAIDs	Non-steroidal anti-inflammatory drugs
OAPs	Orthogonal arrays of particles
PDA	Patent ductus arteriosus
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2 or prostacyclin
PGs	Prostaglandins

PHA	Pseudohypoaldosteronism
PKA	Protein kinase A
RDS	Respiratory distress syndrome
ROMK	Kir1.1
SGA	Small for gestational age
TA	Tracheal aspirate
TTN	Transient tachypnea of the newborn
V_2R	V_2 receptor

1 INTRODUCTION

1.1 AQUAPORIN WATER CHANNEL FAMILY

Aquaporins (AQPs) are a family of membrane proteins that function as water channels facilitating a rapid and regulated transport of water across biological cell membranes. The first AQP was identified in 1991 by Agre and co-workers (Preston *et al.*, 1992), leading to the identification of the whole family. For the discovery, Professor Peter Agre was awarded the 2003 Nobel Prize in chemistry. The discovery elucidated the mechanism of water movement across cell membranes, providing insight into the physiology and pathophysiology of water balance at the molecular level (Nielsen *et al.*, 2002).

At least 13 AQPs have been identified in mammals to date, which are divided into three subfamilies: (a) aquaporins, which specifically transport water; (b) aquaglyceroporins, which are permeable to water and some other small non-polar molecules, such as glycerol and urea; and (c) unorthodox aquaporins, which either have a low homology with conventional aquaporins or differ functionally from both aquaporins and aquaglyceroporins (Chen *et al.*, 2005; Rojek *et al.*, 2008; Benga, 2009).

The AQP family proteins form homotetramers in the cell membrane, containing four functional channel pores. AQP monomer has six transmembrane domains, five connecting loops and intracellular NH_2- and COOH-termini. Two highly conserved Asn-Pro-Ala (NPA) signature motifs in the intramembrane loops (B and E) form the channel pore (Laski, 2006). Water transport *via* AQPs occurs bidirectionally and is driven by osmotic gradients (King *et al.*, 2004).

AQPs are present in multiple tissues involved in fluid transport, as well as in non-fluid-transporting tissues, where they are involved in a variety of physiological processes, such as neuronal activity, cell migration and cell adhesion (Verkman, 2005).

1.2 AQPs IN KIDNEY COLLECTING DUCT

In the kidney, at least eight AQPs have been identified at distinct sites along the nephron. Three AQP isoforms (AQP2, AQP3 and AQP4) are present in the kidney collecting duct, which is the major site for vasopressin (AVP)-mediated regulation of water reabsorption. AQP2 is located in intracellular vesicles and in the apical plasma membrane in principal cells of the collecting duct. In the process of reabsorption, water

enters the cell via AQP2 in the apical plasma membrane and exits via AQP3 and AQP4, which are present in the basolateral membrane (Figure 1). The transport of water from the luminal fluid across the tubular epithelium is driven by the hypertonicity of medullary interstitium (Nielsen *et al.*, 2002).

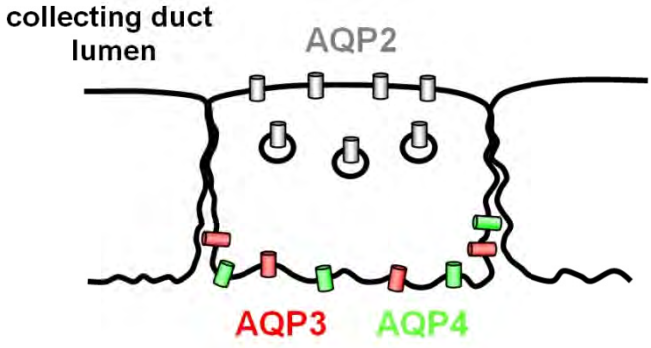

Figure1. AQPs in kidney collecting duct principal cells (image by Marina Zelenina).

AQP2 and urinary concentrating ability

AQP2 is the main target for AVP in collecting duct principal cells and plays a critical role in urinary concentrating ability. It is well documented that there is a direct correlation between the collecting duct osmotic water permeability and AQP2 levels in the apical plasma membrane (Nielsen *et al.*, 1995; Yamamoto *et al.*, 1995). AQP2 abundance in the apical membrane is regulated by AVP by both short-term and long-term mechanisms.

In the short-term regulation, AVP activates intracellular trafficking of AQP2 from the vesicular pool to the apical plasma membrane. The insertion of AQP2 into the membrane occurs by exocytotic fusion of AQP2-bearing vesicles with the membrane (Figure 2), which increases the capacity of the membrane to transport water from the luminal fluid into the cells (Takata *et al.*, 2008; Brown *et al.*, 2009).

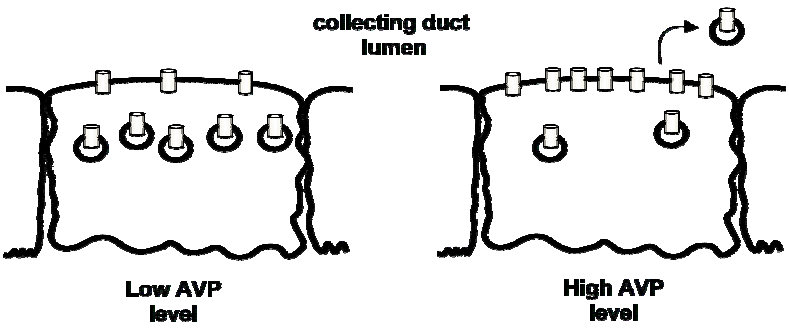

Figure 2. AQP2 trafficking (image by Marina Zelenina).

The mechanisms of AQP2 trafficking have been extensively investigated. AVP binds to V_2 receptor (V_2R), a guanine nucleotide-binding protein (G-protein)-coupled receptor, which stimulates adenylyl cyclase (AC) and increases intracellular cyclic adenosine 3´, 5´-monophosphate (cAMP) levels. This results in activation of protein kinase A (PKA), which phosphorylates AQP2 at serine 256 in the COOH-terminus. This phosphorylation triggers the trafficking of AQP2-bearing vesicles to the plasma membrane (van Balkom *et al.*, 2002). Once the AVP stimulus is removed, AQP2 is retrieved from the apical surface back to intracellular vesicles by clathrin mediated endocytosis (Sun *et al.*, 2002; Bouley *et al.*, 2006). The cellular redistribution of AQP2 in response to AVP is very prompt, taking seconds or minutes to occur (Nielsen *et al.*, 1995; Knepper *et al.*, 1997; Lorenz *et al.*, 2003).

In contrast to AQP2, both AQP3 and AQP4 are constitutively present at the basolateral membrane.

In addition to the short-term regulation of trafficking, AQP2 is also regulated at the level of protein expression, which is essential for long-term renal regulation of body water balance. AVP stimulation of V_2R activates phosphorylation of transcription factor CREB (cAMP-response element binding protein), which then binds to the CRE (cAMP-response element) in the promoter of the AQP2 gene to increase gene transcription (DiGiovanni *et al.*, 1994; Matsumura *et al.*, 1997; Yasui *et al.*, 1997).

There is no evidence for long-term regulation of AQP4 expression in the kidney by AVP, but the expression of AQP3 has been shown to be regulated (Terris *et al.*, 1996).

In addition to AVP, both short- and long-term regulation of AQP2 can also occur by AVP-independent mechanisms. For example, prostaglandin E_2 (PGE_2) and dopamine are shown to decrease collecting duct water permeability by counteracting AVP-induced AQP2 translocation to the apical plasma membrane (Hebert *et al.*, 1990; Nadler *et al.*, 1992; Sun *et al.*, 1996; Nejsum *et al.*, 2005). A previous study in our group has shown that action of PGE_2 is associated with retrieval of AQP2 from the plasma membrane, which appears to be independent of AQP2 phosphorylation (Zelenina *et al.*, 2000).

1.2.1 AQP2 and disorders of water balance

Many studies have demonstrated that altered expression and apical targeting of AQP2 play a critical role in water balance disorders. Mutations in human gene encoding

AQP2 have been demonstrated in patients with congenital NDI, an inability to concentrate urine in response to AVP (Deen *et al.*, 1994). The mutated AQP2 seems to be retained in the intracellular compartments and fails to traffic efficiently to the cell surface, losing water channel function (Sohara *et al.*, 2006). Acquired NDI has also been observed to be associated with downregulation of AQP2 expression. This defect can be a side effect of drug treatment, such as lithium therapy, and is seen in multiple pathophysiological conditions, including hypokalemia, hypercalcemia, ureteral post-obstruction, and acute or chronic renal failure (Marples *et al.*, 1995; Marples *et al.*, 1996; Nielsen *et al.*, 2002; Chen *et al.*, 2005). In contrast, increased expression of AQP2 has been described in several conditions associated with expanded extracellular fluid volume, such as congestive heart failure, liver cirrhosis and pregnancy (Nielsen *et al.*, 1997; Jonassen *et al.*, 1998; King *et al.*, 2004).

1.2.2 Urinary AQP2 excretion

During AQP2 recycling, approximately 3% of AQP2 in the kidney is excreted into the urine (Rai *et al.*, 1997). AQP2 is present in both soluble and membrane-bound forms in the urine, which is probably derived from membrane fractions or membrane vesicles released by the fusion of multivesicular bodies to the apical membrane (Wen *et al.*, 1999; Pisitkun *et al.*, 2004). Evidence from animal studies demonstrate that AQP2 excretion is proportional to AQP2 level in the apical plasma membrane of kidney collecting duct, rather than to overall expression of AQP2 in the kidney (Rai *et al.*, 1997). In both animal models and human subjects, water loading is associated with a suppressed urinary AQP2 excretion, while an increased level of endogenous or exogenous AVP results in an increased urinary AQP2 excretion (Kanno *et al.*, 1995; Rai *et al.*, 1997; Wen *et al.*, 1999). A positive correlation is also found between urinary AQP2 excretion and plasma AVP levels in healthy adult human. Urinary level of AQP2 has therefore been used as a reliable marker of AVP action in various physiological and pathological conditions in adult human (Kanno *et al.*, 1995; Knoers *et al.*, 1995).

1.3 NEONATAL RENAL FUNCTION AND WATER/ELECTROLYTE HOMEOSTASIS

The newborn infant is largely water. Water comprises approximately 75% of body weight at full term and about 80–85% in preterm infants. It is shown that healthy term infants lose approximately 5-10% of total body weight, and premature infants lose

approximately 10-15% during the first one week of life. This reflects body water loss, which occurs largely from the extracellular compartment, and mainly via the kidney. During this period, the infant has an increased diuresis and associated natriuresis, resulting in a decrease of the extracellular fluid volume (Bauer *et al.*, 1991; Chow *et al.*, 2008).

The kidney plays a central role in the physiologic transition from intrauterine to extrauterine life. Adaptation to the extrauterine environment requires that the kidney assumes responsibility for the regulation of water and electrolyte balance. Normal nephronogenesis appears at 5 weeks of gestation and continues until 34-36 weeks of gestation. When birth occurs before 34 weeks of conceptional age, the extrauterine environment is not optimal for glomerular development. Premature infants may therefore have an impaired glomerulogenesis and reduced nephron number. Increasing evidence shows that low birth weight infants are at greater risk of developing CKD, hypertension and metabolic syndrome later in life, which is associated with low nephron number (Brenner *et al.*, 1996; Luyckx *et al.*, 2005; Barker, 2006).

Although nephron formation is complete by 34 to 36 weeks of gestation, the structures of nephron do not mature simultaneously. Under normal conditions, the early neonatal period is characterized by a maturation of renal function.

1.3.1 Evaluation of renal function in neonates

Renal glomerular function and glomerular filtration rate (GFR)

The main physiological limitation of the newborn kidney is its very low GFR, which limits the postnatal renal functional adaptation to endogenous and exogenous stress. This becomes even more problematic in premature infants who have significantly lower GFR than the term infants, primarily due to incomplete nephrogenesis (Gallini *et al.*, 2000). The GFR increases during the first week of life following increased blood pressure and decreased renal vascular resistance. The rise in GFR is slower in the premature infants with low gestational age. Moreover, premature infants are affected by multiple factors that influence the kidney function and predispose to the development of acute kidney injury (AKI). Risk factors for the development of AKI are a low Apgar score at 5 min, disease states (such as respiratory distress syndrome RDS, and Patent ductus arteriosus PDA), antenatal and postnatal use of non-steroidal anti-inflammatory

drugs (NSAIDs) and therapeutic interventions (such as mechanical ventilation) (Toth-Heyn *et al.*, 2000; Andreoli, 2004; Cuzzolin *et al.*, 2006; Askenazi *et al.*, 2009).

Creatinine (Cr) clearance, as an assessment of GFR, in neonates is unreliable, unless a bladder catheterization is used to assure an accurate timed urine collection. Such an invasive procedure is not indicated for routine use. Instead, serum Cr is used widely to evaluate renal glomerular function in neonatal units.

When serum Cr is used as an indicator of renal insufficiency, gestational and postnatal age and maternal factors need to be taken into account. Serum Cr reflects maternal levels during the first few days of life, and is therefore not a reliable index of renal function. It has been shown that glomerular function is positively correlated with gestational and postnatal age (Gallini *et al.*, 2000). In full-term healthy newborns, the GFR rapidly increases and the serum Cr declines progressively with increasing postnatal age to reach stable neonatal level by two weeks of life. In preterm infants, serum Cr does not fall steadily from birth, but increases in the first days of life, reaching a peak on day 4, followed by a progressive decline that stabilizes at 3 to 4 weeks of life (Gallini *et al.*, 2000; Drukker *et al.*, 2002). The transient increase in serum Cr is probably caused by the tubular reabsorption of Cr due to passive backflow of Cr across leaky immature tubular epithelium (Matos *et al.*, 1998; Drukker *et al.*, 2002). In the very premature infants, the decrease in serum Cr is much slower, perhaps reflecting a retarded progression in glomerular function and/or a greater Cr backflow. Therefore, it is suggested that serum Cr levels must be interpreted in the context of the clinical renal status of the newborn. Repeated determination of serum Cr should be performed to evaluate renal glomerular function.

Tubular function

Although tubular function is quite adequate in healthy term infants, immature or compromised infants are at increasing risk for water and electrolyte balance disorders. The fractional excretion of sodium (FENa), which is the amount of urinary sodium excretion as a percent of the filtered sodium, is an indicator of renal tubular function. FENa is high in neonates. The premature infants can have FENa as high as approximately 5% after birth and even higher (15%) in preterm infants of less than 30 weeks' gestational age (Siegel *et al.*, 1976). In these infants, excessive urinary sodium losses exceeding dietary sodium intake create negative sodium balance.

Renal tubular handling of sodium undergoes maturational change during postnatal development (Gallini *et al.*, 2000; Delgado *et al.*, 2003). The high FENa decreases, at least in the term neonate, followed by renal sodium conservation during the next two to three postnatal weeks. However, the preterm infants of less than 34-36 weeks' gestational age, with incomplete nephrogenesis, have difficulty conserving sodium, which frequently leads to a prolonged negative sodium balance and hyponatremia during the early postnatal life (Drukker *et al.*, 2002).

1.3.2 Urinary concentrating ability in neonates

The newborn infant has a limited ability to concentrate urine. This can lead to severe water and electrolyte disorders, especially in preterm infants, which manifest as hyponatraemia, hypernatraemia, polyuria, dehydration or edema. The concentration of urine depends on the presence of the medullary osmotic gradient, maintained by NaCl and urea in the medullary interstitium, and the ability of the collecting duct to respond to the action of AVP. Thus, limitation in any of these mechanisms would be predicted to contribute to the low capacity to concentrate urine in the newborn.

It is well known that the collecting duct in the immature kidney has limited ability to increase its permeability to water in response to AVP. The ability to secrete AVP is intact in the newborn, but the response of kidney to AVP is low (Bonilla-Felix, 2004). The mechanisms contributing to the limited AVP response are complex. Factors, which are suggested to contribute to the relative resistance to AVP in the immature kidney, including V_2R, AC, PKA, cAMP, phosphodiesterase and AQP2 in the collecting duct principal cells, have been studied (Baum *et al.*, 2003).

The expression of AQP2 is significantly lower in immature kidney than in adult (Yasui *et al.*, 1996). In rat kidney, AQP2 is detectable at a fetal age of 18 days, which is followed by a progressive increase of expression with postnatal age, with adult levels reached at the age of about 10 weeks (Bonilla-Felix *et al.*, 1997; Baum *et al.*, 1998). A similar development has been observed in ovine kidney (Butkus *et al.*, 1999). Bonilla-Felix *et al.* demonstrated a direct correlation between urine osmolality and AQP2 expression at all developmental stages in infant rats on *ad libitum* fluid intake. In ovine kidney, the increasing sensitivity of the fetal kidney to AVP was also shown to be largely due to an increasing AQP2 expression (Butkus *et al.*, 1999).

However, AQP2 expression is not the only limiting factor for urinary concentrating capacity. In immature rats subjected to water deprivation and exogenous AVP, the increase in AQP2 expression is not accompanied by a proportional increase in urine osmolality (Bonilla-Felix *et al.*, 1997).

1.3.3 Urinary AQP2 excretion in neonates

Urinary AQP2 excretion reflects the changes in plasma AVP level in adults (Kanno *et al.*, 1995). However, the immature kidney is resistant to AVP. In human, AQP2 is expressed early in gestation followed by a progressive increase into the adult level (Devuyst *et al.*, 1996). AQP2 can be detected in low concentration in neonatal urine samples (Tsukahara *et al.*, 1998). However, the available data concerning the postnatal changes in urinary AQP2 excretion in human newborns are limited, reporting conflicting results.

Tsukahara *et al.* reported a significant postnatal decrease in urinary AQP2 excretion in both preterm and full-term neonates between day 1 and day 4 of life, which was associated with a parallel decrease in urine osmolality and AVP excretion. They also found a significant positive correlation of AQP2 excretion with urine osmolality (Tsukahara *et al.*, 1998).

Nyul *et al.* also reported a decrease in urine osmolality during the first week of life in full-term infants. However, they found no consistent changes in urinary AQP2 level during this period. In preterm infants, urinary AQP2 level remained unchanged during the first 4 weeks followed by an abrupt increase thereafter. Urine osmolality did not follow the developmental pattern of AQP2 excretion, suggesting that during the postnatal period, urinary AQP2 excretion does not serve as a direct marker of the renal action of AVP and the renal capacity to concentrate urine (Nyul *et al.*, 2002).

1.4 RENAL EFFECTS OF NSAIDs IN NEONATES

1.4.1 Patent ductus arteriosus

Ductus arteriosus connects the pulmonary artery to the descending aorta in fetus. The ductus is functionally closed in 90% of healthy term infants by 72 hours of life (Reller *et al.*, 1988). Failure of the ductus to close after birth may cause significant clinical problems. The incidence of PDA is inversely related to gestational age. In a large

network of neonatal intensive care units, the frequency of PDA in infants weighting 501 to 1500g was 31% (Network, 1993).

A hemodynamically significant PDA, depending on the degree of left-to-right shunting through patent ductus, can cause pulmonary overcirculation and/or systemic hypoperfusion. The impairment of the peripheral perfusion in the pulmonary, gastrointestinal, cerebral and renal organ systems increases the risk for RDS, chronic lung disease (CLD), necrotizing enterocolitis (NEC), intraventricular hemorrhage (IVH), impaired neurodevelopment, and decreased renal function. The pulmonary overcirculation also increases the risk of pulmonary edema and RDS. Therefore, treatment of PDA to close the ductus in preterm infants is indicated. Pharmacotherapy is often the first treatment option (Sekar *et al.*, 2008).

The conventional pharmacological treatment to promote the closure of the ductus in preterm infants is indomethacin, a NSAID, inhibiting cyclooxygenase (COX), which is the rate-limiting enzyme for the synthesis of prostaglandins (PGs). Ibuprofen, another NSAID, has been shown to be as effective as indomethacin in closing the PDA. However, concern remains regarding their safety, since it has been shown that indoemthacin and ibuprofen may reduce blood flow to the brain, gastrointestinal tract and kidney, leading to complications such as IVH, NEC, gastrointestinal hemorrhage or perforation, and transient or permanent renal dysfunction (Clyman, 1996; Ohlsson *et al.*, 2008).

1.4.2 Comparison of ibuprofen and indomethacin

Over the last decade, a number of randomized controlled clinical trials and meta-analyses comparing the effects of ibuprofen and indomethacin in the preterm infants with PDA were performed. However, the issue of whether or not ibuprofen has fewer adverse effects has not been fully settled (Van Overmeire *et al.*, 2000; Gournay *et al.*, 2004; Thomas *et al.*, 2005; Shah *et al.*, 2006; Ohlsson *et al.*, 2008).

In the kidney, the adverse effects of ibuprofen appear to be less pronounced compared to indomethacin. Two meta-analyses indicate that ibuprofen reduces the risk of oliguria and is associated with lower serum Cr levels following treatment as compared to indomethacin (Thomas *et al.*, 2005; Ohlsson *et al.*, 2008). It is also shown that indomethacin, but not ibuprofen, significantly reduced renal blood flow velocity in the preterm infants with PDA. Even though both drugs caused a significant increase in

relative vascular resistance, the increase returned to pre-treatment levels at 60 min after administration of ibuprofen, but it remained increased even at 120 min after treatment with indomethacin (Pezzati *et al.*, 1999).

In the lung, however, ibuprofen may increase the risk for CLD. The need for oxygen therapy at 28 days postnatally was found to be significantly more likely to occur in infants treated with ibuprofen than in infants receiving indomethacin (Thomas *et al.*, 2005). Pulmonary hypertension has been observed in infants after the use of ibuprofen for prophylaxis or for PDA treatment (Gournay *et al.*, 2004; Ohlsson *et al.*, 2008).

In the brain, indomethacin significantly decreases cerebral blood flow and may therefore perturb neuronal function in human newborns. In contrast, ibuprofen may exert no effect on cerebral blood flow, and probably has little effect on cerebral perfusion (Mosca *et al.*, 1997; Patel *et al.*, 2000). However, the observations did not translate into benefits for outcomes. Administration of prophylactic ibuprofen did not prevent IVH (Gournay *et al.*, 2004; Van Overmeire *et al.*, 2004). In addition, even though the treatment with indomethacin resulted in a substantial reduction in IVH, there was no evidence of improved neurological outcomes at 18 to 21 months follow-up and up to school age (Ment *et al.*, 1996; Ment *et al.*, 2000). Whether ibuprofen has a positive impact on long-term neurological outcome remains to be determined.

In summary, ibuprofen does not appear to confer any net benefits over indomethacin for the treatment of PDA. It is suggested that, as both drugs are equally effective in closing PDA, the clinician needs to weight the potential side effects versus the benefits for an individual infant when choosing which drug to use.

1.4.3 Renal function of PGs

NSAIDs exert their action by inhibiting PGs synthesis. COX, which is the key enzyme in PG synthesis, exists in two isoforms, COX-1 and COX-2. Both isoforms are constitutively expressed in the kidney and are affected by NSAIDs.

It is well known that PGs contribute to normal renal function. PGs, particularly PGE_2 and PGI_2, the major PGs synthesized by COX in the kidney, increase afferent blood flow and decrease vascular resistance, playing an important role in the maintenance of renal perfusion and GFR. PGs, particularly PGE_2, also contribute to maintain an increased sodium and water excretion. In the absence of AVP, PGE_2 stimulates water permeability, whereas PGE_2 inhibits cAMP generation and water reabsorption in the

presence of AVP. The complex effects of PGE_2 might be due to the presence of four subtypes of E-prostanoid receptors (EP1, EP2, EP3 and EP4), activating diverse signaling pathways (Narumiya *et al.*, 1999; Breyer *et al.*, 2000). The increase in water permeability in the collecting ducts caused by PGE_2 itself might be mediated by EP4 receptor (Sakairi *et al.*, 1995). The inhibitory effects of PGE_2 on water reabsorption, in the presence of AVP, might be mediated by EP1 or/and EP3 receptors, which are highly expressed in the collecting duct (Hebert, 1994). PGE_2 is shown to inhibit AVP-induced water permeability by increasing intracellular Ca^{2+} and by activating PKC in the collecting duct principal cells (Hebert *et al.*, 1990; Hebert *et al.*, 1991). The previous study from our group shows that PGE_2 counteracts AVP action by retrieving AQP2 from the plasma membrane. The inhibition of PGE_2 production by indomethacin results in reduced endocytosis of AQP2 and therefore increases abundance of AQP2 in plasma membrane in rats (Zelenina *et al.*, 2000).

1.4.4 Mechanisms of renal side effects of NSAIDs in neonates

Varying degrees of renal insufficiency, characterized by oliguria and increased serum Cr, have been reported in both ibuprofen and indomethacin treated preterm infants (Clyman, 1996; Van Overmeire *et al.*, 2000; Thomas *et al.*, 2005; Shah *et al.*, 2006; Ohlsson *et al.*, 2008). The exact mechanisms underlying the reduced urine output and water retention have not been fully understood.

The increased risk for water retention in the premature infants may be due to a decrease in renal perfusion and GFR, which strongly depends on the vasodilatatory PGs in early neonatal life (Gleason, 1987; Toth-Heyn *et al.*, 2000). PGs mediate the vasodilating effect on afferent arterioles to protect the immature kidney from high levels of vasoconstricting angiotensin II (Antonucci *et al.*, 2007), and the PG inhibitors may influence this delicate balance between vasoconstrictory and vasodilatory forces. The effects of NSAIDs on water homeostasis may also be due to a relief of the inhibiting effect of PGE_2 on AVP in the collecting duct, which is anticipated to increase the presence of AQP2 in the apical membrane of the collecting duct (Zelenina *et al.*, 2000). Study in healthy adults demonstrated that ibuprofen increased the urinary excretion of AQP2 (Pedersen *et al.*, 2001).

However, renal side effects associated with the use of ibuprofen and indomethacin in the premature infants may differ from those in adults, because the immature kidney

appears to be more sensitive to PGs. Animal studies show that there is an increased affinity for prostanoid receptor and high concentrations of PGs, particularly PGE_2 in the immature kidney (Moel *et al.*, 1985; Reyes *et al.*, 1990; Antonucci *et al.*, 2007). Whether or not ibuprofen and indomethacin may relieve the inhibiting effect of PGE_2 on AVP and increase the presence of AQP2 in the apical membrane of the collecting duct in immature kidney is an interesting open question.

1.5 K^+ HOMEOSTASIS: ROLE OF WATER AND K^+ TRANSPORTERS

1.5.1 K^+ diet and K^+ homeostasis

K^+ is the principal intracellular cation. It exists predominantly in the intracellular fluid at concentrations of 140 to 150 mmol/l and in the extracellular fluid at concentrations of 3.5 to 5 mmol/L. A high intracellular K^+ concentration is critical for cell growth, whereas maintenance of extracellular K^+ within the normal narrow range is essential for the function of many body cells, especially excitable cells. Membrane resting potential depends on the intracellular/extracellular K^+ concentration ratio. Minute changes in the smaller extracellular concentration can result in large changes in the electrical properties of cells. The maintenance of extracellular K^+ concentration and K^+ gradient is dependent on several processes: dietary K^+ load, the distribution of K^+ between the intracellular and extracellular compartment, and excretion of K^+.

The kidney collecting duct is responsible for the final excretion of K^+ (Muto, 2001). The role of kidney in maintaining K^+ homeostasis during varying dietary K^+ intake has been well studied. Under physiological conditions, plasma K^+ concentration is not significantly affected by daily K^+ intake. This is achieved by both extrarenal and renal K^+ handling. An increase in dietary K^+ intake is accompanied by shifting K^+ from the extracellular fluid to the intracellular volume. Renal K^+ excretion increases by stimulating K^+ secretion in the collecting duct principal cells. When the K^+ intake is restricted, K^+ is shifted from intracellular volume to extracellular fluid. Renal K^+ excretion decreases by both suppressing K^+ secretion in the cortical collecting duct and stimulating K^+ absorption in the inner medullary collecting duct.

Factors affecting renal K^+ excretion include distal Na^+ delivery, renin-angiotensin-aldosterone system, AVP, dietary K^+ intake, acid-base status, urine flow, and serum K^+ concentration (Evans *et al.*, 2005).

However, if K^+-deficient or high K^+ diet persists, which may disrupt the ability of the body to adapt efficiently to varying dietary K^+ intake, hypokalemia or hyperkalemia may occur.

Hypokalemia is a common, and sometimes life-threatening, electrolyte abnormality. It has been known that prolonged hypokalemia causes renal injury consisting of renal hypertrophy and tubulointerstitial fibrosis, which may lead to renal failure (Fervenza *et al.*, 2001). Functional abnormalities in prolonged K^+ deficiency include decreased renal blood flow and GFR, polydipsia and AVP- resistant decrease in urinary concentrating ability, which is due to a downregulation of AQP2 expression and reduced production of cAMP (Marples *et al.*, 1996).

Hyperkalemia can be a life-threatening and emergency condition. The toxicity of hyperkalemia is a result of its effect to depolarize cell membranes, including cardiac and skeletal muscle cells. The potentially deadly effect of hyperkalemia on cardiac muscle is of greatest concern.

1.5.2 AQP4 and K^+ transport

The water channel AQP4 is expressed in several cell types, including astrocytes and collecting duct principal cells.

AQP4 exists in two isoforms, M1 and M23 (Rash and Yasumura, 1999; Furman et al., 2003). Both isoforms function as water channels and form stable tetramers. Only the shorter isform M23 forms higher order assemblies within the plasma cell membrane, known as orthogonal arrays of particles (OAPs). The relative ratio of M1: M23 isoforms, which may determine the size of OAPs within the plasma membrane, is tissue specific (Jung *et al.*, 1994). The presence of OAPs may increase the water permeability of AQP4 (Silberstein et al., 2004) and facilitate AQP4 intracellular anchoring (Tait et al., 2008). New AQP4 isoforms have been suggested recently; however the functional significance is unclear.

In the brain, AQP4 is implicated in etiology of brain edema (Saadoun *et al.*, 2009). AQP4 is also suggested to be involved in the maintenance of neuronal activity. Recovery from neuronal activation requires rapid clearance of K^+ ions from

extracellular space (ECS), and AQP4 has been shown to be important for this process. AQP4 deficiency results in a reduced seizure threshold and prolonged seizure duration (Eid *et al.*, 2005; Binder *et al.*, 2006). Delayed K^+ uptake from ECS was found in α-syntrophin knockout mice, which exhibit the loss of AQP4 expression in the end-foot domain of astrocytes (Amiry-Moghaddam *et al.*, 2003). Colocalization of AQP4 with the inward rectifying potassium channel Kir4.1 suggests that AQP4 may be involved in the functional coupling between water and K^+ transport in response to neuronal activity in the brain (Amiry-Moghaddam *et al.*, 2003). We hypothesized that AQP4 may also play a role for K^+ transport in the kidney.

In the kidney, AQP4 is present in the basolateral plasma membrane of collecting duct principal cells, primarily in the inner medullary collecting duct. AQP4, together with AQP3, provides basolateral exit pathway for water entering the cells via AQP2 at the apical membrane (Nielsen *et al.*, 2002).

Except for the concentration of urine, collecting duct principal cells also play a key role in the regulated secretion of K^+ and thus in the maintenance of K^+ homeostasis. K^+ secretion is a two-step process in the kidney (Figure 3). Na^+, K^+-ATPase transport K^+ into the cell, and the intracellular K^+ passively diffuses into the urine via apical K^+-selective channels (ROMK). Na^+, K^+-ATPase is the key element, since it pumps K^+ into the cell, and transports Na^+ out of the cell, which drives Na^+ entry from the lumen of the collecting duct to the cell through apical epithelial Na^+ channel (ENaC). Na^+ entry creates lumen-negative voltage. Both high intracellular K^+ concentration and lumen-negative voltage create a favorable electrochemical gradient for intracellular K^+ to diffuse into the urine (Giebisch *et al.*, 2007).

A fraction of the K^+ that is taken up into the cell via Na^+, K^+-ATPase recycle back into the extracellular space via low conductance K^+ channels in the basolateral membrane (Figure 3), which is important for the maintenance of the membrane potential and the efficiency of the Na^+, K^+-ATPase (Huang, 1998; Muto, 2001). The low conductance K^+ channel expressed in basolateral membrane of the collecting duct principal cells is the inwardly rectifying K^+ channel Kir7.1.

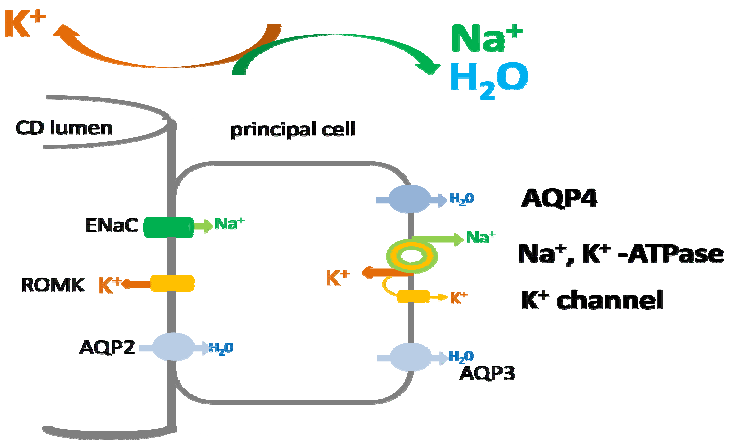

Figure 3. Renal K^+ secretion in collecting duct principal cells.

1.5.3 Inwardly rectifying K^+ channels

Inwardly rectifying K^+ channel family (Kir channels) is a large group of K^+-selective channels that shares the property of supporting larger inward than outward K^+ current (Bichet *et al.*, 2003). There are currently seven known subfamilies of Kir channels (Kir1-7). Kir channels are widely present in different tissues of the human body and regulate many important cellular processes, including resting membrane potential, cell excitability, maintenance of extracellular and intracellular K^+ homeostasis and renal K^+ transport (Huang, 1998). Kir7.1, which is recently identified member of Kir channel family, exhibits a very weak inward rectification and very low single channel conductance. The permeability of Kir7.1 is almost independent of external K^+ (Doring *et al.*, 1998; Krapivinsky *et al.*, 1998; Partiseti *et al.*, 1998).

In the kidney, Kir7.1 is expressed predominantly in the basolateral membrane of a number of epithelial cells, including proximal tubule, the thick ascending limb and the collecting duct principal cells. It has been suggested that Kir7.1 regulates membrane potential by providing a steady background K^+ current and that it functions together with Na^+, K^+-ATPase to contribute to tubular K^+ homeostasis and renal K^+ secretion (Ookata *et al.*, 2000).

1.6 WATER HOMEOSTASIS: ROLE OF WATER AND ION TRANSPORTERS

1.6.1 AQPs in the lung

At least four AQP isoforms, AQP1, AQP3, AQP4, and AQP5, are expressed in the lung tissue. AQP1 is expressed in pleura and capillary endothelium; the other AQPs are localized in the airway epithelial cells. AQP3 is expressed in the basolateral membrane of basal cells of the tracheal epithelium. AQP4 is present in the basolateral membrane of ciliated columnar cells in bronchi and trachea. AQP5 is localized in the apical membrane of type-I alveolar epithelial cells (AECs) and the apical plasma membranes of the secretory epithelium in upper airway and salivary glands. The distinct patterns of AQPs expression in the lung provide indirect evidence for their involvement in lung fluid transport (Verkman, 2007).

Gene knockout studies in mice showed that alveolar fluid clearance is not affected by AQP deletion, suggesting that AQPs in the lung have little or no physiological role (Song *et al.*, 2000). However, the growth and development of lung and lung epithelial function in rodents is different from that in long-gestation mammals and human. The expression and distribution of AQPs in the lung is species-specific. In contrast to mice, in which little AQPs is expressed in fetal life, the lung of sheep, a long-gestation species, expresses all four AQPs, beginning well before birth (Liu *et al.*, 2005).

AQP5 is responsible for the majority of water transport across the apical membrane of alveolar epithelium. Decreased expression of AQP5 in the lung was observed in pathological conditions, such as viral infection (Towne *et al.*, 2000), lung inflammation (Towne *et al.*, 2001) and acute lung injury (Jiao *et al.*, 2002). This suggests that the altered expression of AQP5 may play a significant role in alveolar fluid clearance and pulmonary edema. In the ovine lung, AQP5 is developmentally modulated by factors regulating lung growth and development, such as corticosteroids and keratinocyte growth factor, suggesting that alveolar AQP5 may play a role in rapid clearance of perinatal lung liquid (Liu *et al.*, 2003). Studies on AQP5 expression in human neonates are lacking.

1.6.2 Lung liquid clearance in perinatal period

In lung development, there is a switch of the lung epithelium from net secretion to net absorption, to prepare for the transition from intrauterine to extrauterine life. The secretion of fetal lung fluid as a result of active Cl^- transport into the lumen of the airways is essential for the growth and differentiation of the lung (Figure 4). Absorption of lung liquid from the airway must start just before birth in preparation for normal gas exchange (Wilson *et al.*, 2007). Ineffective clearance of lung liquid in the perinatal period may contribute to respiratory distress in the newborn (O'Brodovich, 2005).

It is well demonstrated that active Na^+ transport is critical for clearance of fetal lung liquid. The Na^+ absorption occurs via apical amiloride-sensitive ENaC and basolateral Na^+, K^+-ATPase, which drives water absorption via AQP5 form lung lumen to the interstitium (Mutlu *et al.*, 2005).

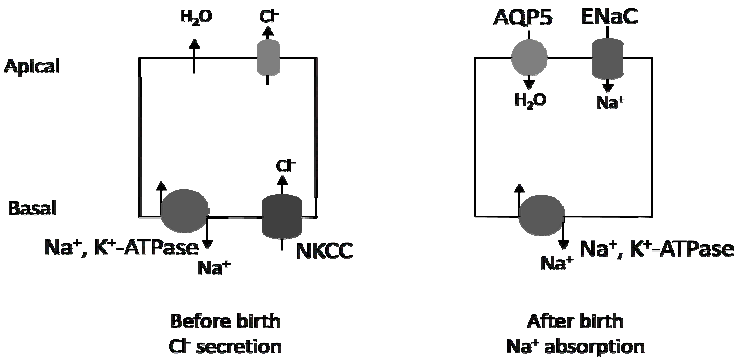

Figure 4. Lung fluid secretion before birth and reabsorption after birth in alveolar epithelium (NKCC: Na-K-2Cl cotransporter).

Several endogenous mediators, such as catecholamines, glucocorticoids and oxygen, have been proposed to trigger the switch of the lung epithelium from secretion to absorption, regulating the clearance of the fetal lung liquid (Barker *et al.*, 2002).

Spontaneous labor appears to trigger release of catecholamines. The high circulating concentration of catecholamines stimulates fetal lung liquid clearance. The stimulation is mediated by β2-adrenergic receptors and increases transepithelial Na^+ transport (Walters *et al.*, 1978; Olver *et al.*, 1986). Glucocorticoids are also shown to stimulate liquid reabsorption in the late gestation fetal lung. Glucocorticoids increase the activity of existing ENaC channels and the number of available channels in the plasma membrane by inhibiting ENaC internalization, and also enhance the expression of

ENaC by stimulating the gene transcription in the alveolar epithelia (Eaton *et al.*, 2009; Pitkanen, 2009).

Other factors, which could be involved in the absorption of fetal lung liquid, include thyroid hormones, dopamine and AVP.

1.6.3 Amiloride-sensitive epithelial sodium channel

ENaC, which is expressed in the apical membrane in many epithelial cells, including principal cells in the distal nephron of the kidney and epithelial cells in the lung airway, is highly selective Na^+ channel sensitive to amiloride. ENaC is a heteromeric protein composed of three structurally related subunits (α, β and γ). Each subunit shares approximately 30 to 40% sequence identity with the others and has two presumed membrane-spanning domains, a large extracellular loop, and intracellular NH_2- and COOH-termini. It has been proposed that different combinations of the various subunits comprising the channel could produce channels with varying biophysical properties and regulatory mechanisms (Matalon *et al.*, 2002). α-ENaC alone may possess some activity, whereas a tetramer (often two α, one β and one γ) has been reported to have maximal activity.

It has been shown that ENaC is present in fetal lung and the expression of lung ENaC is developmentally regulated. All three subunits of ENaC can be detected at 17- to 18-day gestational age in the rat fetal lung and the expression of ENaC subunits (mainly α- and γ-ENaC) sharply increases in the late prenatal and early postnatal period, when the lung switches from a secretory to an absorptive epithelium (O'Brodovich *et al.*, 1993; Talbot *et al.*, 1999).

ENaC is the rate-limiting step for the active Na^+ absorption. It is shown that instillation of amiloride into the trachea of newborn guinea pigs results in the delay of lung liquid clearance (O'Brodovich *et al.*, 1990). The physiological role of ENaC in lung liquid balance was clearly demonstrated in knockout mice and naturally occurring ENaC mutations in human. ENaC α subunit knockout mice are unable to clear fetal liquid from their air spaces and die from respiratory distress soon after birth (Hummler *et al.*, 1996). ENaC β or γ subunit deficient mice clear lung liquid more slowly compared to the wild type mice, suggesting that β- or γ-ENaC facilitates lung liquid clearance (Barker *et al.*, 1998; McDonald *et al.*, 1999).

In human, the loss-of-function mutation in each of the three ENaC genes results in severe salt-wasting type I pseudohypoaldosteronism (PHA-1). Patients with systemic PHA-1 have been shown to fail to absorb liquid from airway surfaces, resulting in an increased volume of liquid in the airways, which suggests that the recurrent episodes of respiratory dysfunction in young patients with PHA-1 may be caused by the excessive amount of liquid in the airways due to pulmonary ENaC dysfunction (Kerem *et al.*, 1999). The loss-of-function mutations in PHA-1 are also shown to be associated with neonatal RDS. Case reports have described patients with RDS who survived the neonatal period and later developed PHA-1 (Malagon-Rogers, 1999).

Evidence from human neonatal studies also suggests that inadequate Na^+ absorption via ENaC may play a role in the development of neonatal respiratory distress.

The first important evidence for the role of decreased sodium channel activity in the pathogenesis of transient tachypnea of the newborn (TTN) and RDS came from the study measuring nasal potential difference (nasal PD, N-PD) which correlates with lung compliance in human neonates. The maximal and amiloride sensitive NPD were significantly lower in the preterm infants with RDS. These observations suggest that impairment of Na^+ absorption across the respiratory epithelia of premature infants may contribute to the pathogenesis of TTN and RDS (Barker *et al.*, 1997; Helve *et al.*, 2005).

Using nasal epithelial samples, Helve *et al.* measured the mRNA expression of ENaC in 7 healthy term infants and 5 preterm infants with RDS within 5 hours of life. The preterm infants with RDS had markedly lower levels of α-, β- and γ-ENaC mRNA compared to healthy term infants (Helve *et al.*, 2004; Helve *et al.*, 2007).

In a later study from the same group, it was found that at 1 to 5 hours of life, mRNA expression of α-, β- and γ-subunits of ENaC was lower in preterm than in term infants. Significant correlation was found between gestational age and expression of α- and β-ENaC, but not γ-ENaC. By 22 to 28 hours after birth, the expression of β-ENaC in the preterm infants and all three subunits of ENaC in the term infants decreased. However, the expression of all three subunits was similar in the preterm and term infants during this period. There was no significant correlation between gestational age and expression of α-, β- and γ-subunits of ENaC (Helve *et al.*, 2004; Helve *et al.*, 2007).

1.6.4 Neonatal respiratory distress (TTN and RDS)

The most common causes of neonatal respiratory distress are TTN and RDS (Hermansen *et al.*, 2007). Both TTN and RDS are characterized by ineffective clearance of fetal lung fluid.

TTN, also known as wet lung, is believed to result from incomplete resorption of lung liquid from the newborn. It is responsible for more than 40% of all cases of neonatal respiratory distress, and has been reported to occur frequently in near-term or term infants (3.6 to 5.7 per 1,000 term infants). Risk factors for TTN include birth by cesarean delivery, male gender, lower gestational age, macrosomia, maternal asthma and maternal diabetes (Guglani *et al.*, 2008).

TTN is characterized by tachypnea shortly after birth and typical radiographic presentation suggestive of retained lung fluid. TTN is generally considered as a benign and self-limited disorder and most of the infants recover quickly, within 2 to 5 days. Some infants, however, become seriously ill and require significant intervention, including mechanical ventilation, associated with morbidity and mortality. TTN may be complicated by hypoxemia, air leak, and persistent pulmonary hypertension. TTN is not believed to increase the risk for respiratory disease or other long-term sequelae after recovery. However, there is an increasing evidence of linking TTN with childhood asthma (Birnkrant *et al.*, 2006).

RDS is a condition of pulmonary insufficiency due to a lack of alveolar surfactant, along with structural and functional immaturity of the lung. In addition, RDS is associated with a delayed absorption of fetal lung liquid due to defective Na^+ transport mechanisms (Barker *et al.*, 2002; O'Brodovich, 2005). The greatest risk factor for RDS is prematurity, additional factors include male gender, perinatal asphyxia, and maternal diabetes. The incidence and severity of RDS generally increase with decreasing gestational age and birth weight. Lemons *et al.* reported that 26% of infants with birth weight of 1251 to 1500 g were diagnosed with RDS, increasing to 78% in infants with birth weight of 501 to 750 g (Lemons *et al.*, 2001). Typically, the preterm infants with RDS present mild respiratory distress immediately after birth, and worsen within the first few hours of life. The classic features of RDS include tachypnea, grunting, retraction, and cyanosis accompanied by increasing oxygen requirements. Respiratory failure may develop. The diagnosis can be confirmed on chest X-ray with a classical ‘‘ground glass’’ appearance and air bronchograms. The clinical course depends on the

severity of RDS and the size and maturity of the infant at birth. The most premature infants are at greatest risk to develop complications, including IVH, PDA, air leak, and infection, which contribute to prolonged requirements for oxygen and ventilator support.

2 AIMS OF THE STUDY

The overall aim of this thesis was to elucidate the role of AQPs in kidney and lung, with emphasis on the role of AQPs in postnatal adaptation and in pathological conditions related to an impaired water homeostasis. I have examined whether information obtained from animal studies can be applied to human and whether results obtained from studies on adults can be applied to infants

The main aims of the theses were:

- To study the expression of renal AQP2 in human neonates during postnatal adaptation under physiological and pathological conditions.
- To investigate whether disturbances in water homeostasis, which are reported in infants with patent ductus arteriosus treated with prostaglandin inhibitors, can be attributed to activation of AQP2.
- To investigate whether AQP4 may play a role in renal K^+ transport.
- To determine the role of AQPs and ion transporters in pathological conditions related to an impaired neonatal lung fluid balance during postnatal human lung maturation.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

Sprague-Dawley rats were obtained from Scanbur (Sollentuna, Sweden) and treated according to the Karolinska Institutet regulations concerning care and use of laboratory animals. The experiments were approved by the Stockholm North ethical evaluation board for animal research. All rats were kept under standard housing conditions. To collect the tissues, the rats were anesthetized with thiobutabarbital (8 mg/100 g body wt).

In immunoblotting analysis (Paper III and IV) and co-immunoprecipitation and GST pull down studies (Paper III), the rats were fed standard rat chow with a free access to water. In Paper III study, we also investigated the expression of AQPs in kidney collecting duct from rats fed with various K^+ diet. Rats were fed normal, K^+-deficient or high K^+ diet (TD.88238, TD.88239 and TD.94121 respectively; Harlan Teklad, Madison, WI) for one week. The rats had free access to water.

Xenopus laevis were obtained from Centre de Recherces de Biochimie Macromoléculaire (CNRS, Montpellier, France). To remove oocytes, female frogs were anaesthetized with tricaine (2 g/L, 3-aminobenzoic acid ethyl ester, Sigma-Aldrich Danmark, Brøndby, Denmark). The surgical procedures complied with Danish legislation and were approved by the controlling body under the Ministry of Justice.

3.1.2 Cells

LLC-PK_1 cells, an epithelial cell line derived from pig kidney (European Collection of Cell Cultures, Center for Applied Microbiology & Research, Salisbury, Wiltshire, UK; subpassages 6–16), were cultured on coverslips (Bioptechs, Butler, PA, USA) in medium 199 (Sigma-Aldrich, Tyresö, Sweden) containing 50 U/ml penicillin and 50 µg/ml streptomycin supplemented with 10% FBS and 2 mM L-glutamine. On the second day of culture, the cells were transfected with AQP4 or AQP3 cDNA constructs using CLONfectin (Clontech Laboratories, Takara Bio, Mountain View, USA) according to the manufacturer's protocol. Experiments were performed at the fourth day of culture.

Xenopus laevis oocytes were cultured overnight at 19 °C in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Hepes-Tris, pH 7.4) before injection with mRNA of rat AQP4 M1 (~50 ng) using a Nanoject microinjector (Drummond). Injected and non-injected oocytes were kept in Kulori medium at 19 °C for 3-7 days before water permeability measurements were performed.

3.1.3 cDNA Constructs

In water permeability measurements (Paper III), we used cDNA encoding AQP4 and AQP3.

For water permeability measurements in LLC-PK1 cells, the coding region of the long AQP4 isoform, AQP4.M1 (GenBank AF469168), was cloned as a GFP fusion protein. AQP4.M1 cDNA was amplified using reverse transcription-PCR with a mouse brain total RNA as template and following primers: 5'-GCCACATGGTGCAGAATCTTTC-3' (sense), 5'-CCTCTAGTCATACGGAAGACAATACCTCTC-3' (antisense). The cDNA was cloned into pEGFP-C1 vector for expression of AQP4.M1 fused with GFP at NH_2-terminus (GFP-AQP4.M1) in mammalian cells.

For water permeability measurements in *Xenopus* oocytes, a short version of rat AQP4 M1 obtained from Dr. Søren Nielsen (University of Aarhus, Denmark) was converted into the full-length AQP4.M1 using PCR and subcloned into an oocyte expression vector pXOOM (Jespersen *et al.*, 2002). The construct was linearized downstream of the poly(A)-segment, *in vitro* transcribed with T7 mMESSAGE mMACHINE (Ambion, Applied Biosystems, Naerum, Denmark) and the resulting mRNA was purified using MEGAclear (Ambion).

For GST pull-down assay, a construct encoding the fusion protein containing GST and 32 first amino acids of rat AQP4.M1 (GenBank accession number U14007), was prepared using Gateway Technology (Invitrogen). cDNA fragment encoding amino acids 1-32 of rat AQP4.M1 was amplified by AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) using the following primers: 5´-CACCATGAGTGACGGAGCTGCAGCG-3´ and 5´-TTGAGTCCAGACGCCTTTGAAAGC-3´. The PCR product was cloned in pENTR/D-TOPO vector using pENTR Directional TOPO Cloning Kit (Invitrogen) and subcloned into pDEST-15 using Gateway Technology.

cDNA fragments encoding full-length human AQP3 were obtained by amplification from the human lung QUICK-Clone cDNA library (Clontech). The construct for expression of human AQP3 fused with GFP at NH_2-terminus (GFP-hAQP3) was subcloned in-frame into the pEGFP-N2 vector.

3.1.4 Antibodies

Affinity-purified rabbit polyclonal antibodies against AQP2 (1:1,000, H7661), AQP3 (1:6,000, # 4500AP) and AQP5 (1:3,000, LL220), β-ENaC (1:100, # 5161) and γ-ENaC (1:200, # 5163) were kindly provided by Prof. S. Nielsen (Institute of Anatomy, Aarhus University, Denmark). Others included rabbit anti-AQP4 polyclonal antibody (1:2,000; Chemicon, AB3594), rabbit anti-α-ENaC polyclonal antibody (1:2,000; Chemicon, AB3530P), mouse anti-Na^+, K^+-ATPase α1 monoclonal antibody (1:10,000; Upstate Biotechnology, 05-369), and mouse anti-human serum albumin monoclonal antibody (1:1,000; Abcam, ab10241).

3.2 PATIENT POPULATIONS

Infants admitted to the neonatal intensive care unit of the Children's Hospital of Toulouse (France) were eligible for the studies. The studies were approved by the local ethics committee of the Hospital of Toulouse. Informed parental consent was obtained at enrollment of each infant. Gestational age of the infants was calculated from the mother's menstrual history and was confirmed by ultrasonography.

Paper I involved 123 newborn infants (gestational age 26.4–39 weeks) during period of seven months from March 1 to September 30, 2003. Exclusion criteria were: severe congenital anomalies, hydrops fetalis, or treatment with dopamine, indomethacin, diuretics or nephrotoxic antibiotics within 10 days before sampling.

Paper II included 53 preterm infants (gestational age 24-33 weeks) with symptomatic PDA. Twenty-three infants received indomethacin from May to August in 2003 and 30 received ibuprofen from June 2006 to June 2007. Exclusion criteria were: congenital anomalies, hydrops fetalis, IVH, clinical bleeding or bleeding tendency (platelet count $\leq 100,000/\mu L$).

Paper IV involved 32 newborn infants (gestational age 26-41 weeks) requiring intubation and mechanical ventilation during the period of May 1 to August 19, 2004.

Exclusion criteria were parental refusal to participate, hydrops fetalis or lung hypoplasia. One infant was excluded due to late postnatal age at sampling (411 h after birth).

3.2.1 Patient groups

In Paper IV study, all infants (*n* = 32) were first grouped according to the lung X-ray appearance. Infants with normal lung X-ray appearance (*n* = 6) were assigned to a control group, which included infants with no pulmonary disease, having apnea of prematurity (*n* = 2), oesophagal atresia before intervention (*n* = 2), hypoventilation (*n* = 1), and asphyxia (*n* = 1). Infants with abnormal lung X-ray appearance (*n* = 26) were further divided into three groups, based on the clinical diagnosis. Eight infants fulfilled the RDS criteria. Eight infants fulfilled the criteria for TTN. All other infants (*n* = 10) were retrospectively assigned to a mixed group. The cause for the intubation included sepsis (*n* = 4), meconium aspiration syndrome (MAS; *n* = 2), pulmonary atresia with intact ventricular septum (*n* = 1), choanal atresia (*n* = 1), hemorrhagic shock (*n* = 1), and thoracic fibrosarcoma with bilateral chylothorax (*n* = 1). All infants showed abnormal lung X-ray appearance, including edema, pneumonia, MAS or opacity.

3.2.2 Diagnostic criteria

Diagnosis of PDA was based on echocardiogram and color Doppler test. PDA was considered as significant (symptomatic) when the following criteria were met: (a) a ductal diameter > 1.5 mm (b) a left ventricular output index of more than 300 ml/kg/min (c) left atrium to aortic root ratio greater than 1.5; (d) retrograde or absent diastolic flow in the cerebral anterior artery or in the descending thoracic aorta (Kluckow *et al.*, 1995).

RDS criteria were defined as follows: PaO_2 < 50 mm Hg (< 6.6 kPa) in room air, central cyanosis in room air or need for supplemental oxygen to maintain PaO_2 > 50 mm Hg (> 6.6 kPa) as well as classical chest X-ray appearances: “ground glass” appearance and air bronchograms (Sweet *et al.*, 2007).

TTN was diagnosed based on clinical and radiologic findings. The infants had tachypnea (respiratory rates greater than 80 breaths /min), grunting and retractions within a few hours of birth. Chest X-rays showed prominent perihilar streaking and

fluid in the interlobar fissures. Other conditions such as pneumonia, RDS and pneumothorax were excluded.

3.2.3 Administration of ibuprofen and indomethacin

Ibuprofen was given intravenously in initial dose of 10 mg/kg, followed by two doses of 5 mg/kg each, after 24 and 48 hours. Indomethacin was given intravenously in three equal doses 0.2 mg/kg with 12 hours intervals. Echocardiographic assessment was performed after each dose of the drugs to evaluate the patency of the ductus. Treatment was discontinued if the ductus had closed after administration of one or two doses.

3.2.4 Urine samples

Spot urine samples were collected during 3 to 6 hours period. After collection, the urine was immediately frozen and stored at -20°C.

In Paper I, urine samples were collected during the first week (early sample) and the third week (late sample) of postnatal life. Totally the study involved 123 newborn infants. Both early and late urine samples were obtained from 22 infants. A single early urine sample was obtained from 49 infants, and a single late urine sample was obtained from 52 infants. The reasons for the missing samples were: admission to the unit after the time of early sampling, or discharge from the hospital/transfer to another neonatal unit before the time of later sampling.

In Paper II, urine samples were collected before treatment and after each dose administration of ibuprofen and indomethacin. From 30 infants who received the 1st dose of ibuprofen, 25 urine samples were collected before treatment and 28 samples after the 1st dose. One infant closed the ductus after the 1st dose. From 29 infants who received the 2nd dose, 28 urine samples were collected after the dose. From 24 infants who received the 3rd dose, 16 urine samples were collected after the dose. The reasons to discontinue ibuprofen treatment after the 2nd dose were: PDA closure (3 infants), sepsis (1 infant), and renal failure with high serum Cr (165 μmol/L, 1 infant). From 23 infants who received the 1st dose of indomethacin, 10 urine samples were obtained before treatment and 14 samples after the 1st dose. From 21 infants who received the 2nd dose, 15 urine samples were collected after the dose. From 14 infants who received the 3rd dose, 6 urine samples were collected after the dose. The reason to discontinue the treatment was successful PDA closure, except in one infant, in which

discontinuation after the 1st dose was due to severe hyponatremia (104 mmol/L). The missing samples were due to a failure in collecting urine.

3.2.5 Tracheal aspirate (TA) samples

TA samples were collected during routine endotracheal suctioning. The median sampling time was 31.5 (range 3.5-228) hours after birth and 22 (range 3.5-227.75) hours after intubation. One TA sample was collected from each patient. To collect TA, sterile normal saline (0.5 mL) was instilled into endotracheal tube and airway secretions were aspirated using a suction catheter. The catheter was then flushed with additional 0.5 mL of sterile saline to rinse aspirated secretions from the catheter wall. After collection, TA samples were immediately frozen and stored at -80°C for protein expression analysis.

3.3 METHODS

3.3.1 Dot immunoblotting

To determine the relative AQP2 level in the urine (Paper I and II), urine samples were spotted on a nitrocellulose membrane using Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Sweden). AQP2 was detected using rabbit polyclonal antibodies (H7661), goat anti-rabbit horseradish peroxidase conjugated IgG and ECL Plus Western blotting analysis system (Amersham, Sweden). The obtained X-ray films were scanned, and the images were analyzed using ImageJ software. AQP2 signals were linearly dependent on the amount of urine applied on the membrane. The antibody signals from different experiments were compared using six “standard” urine samples that were run together with every set of patient samples. To calculate urinary AQP2 level, the optical density of AQP2 signals was normalized to creatinine level in the urine (a.u./mmol urinary Cr). To calculate urinary AQP2 excretion, the optical density of AQP2-specific signals was multiplied by urinary flow rate (a.u./kg/h).

3.3.2 Semiquantitative immunoblotting

For protein expression analysis (Paper III and IV), membrane fraction from rat lung and kidney was prepared. To obtain the total membrane fraction of kidney medulla and lung, the tissue from an adult rat was homogenized (300 mM sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 20 µg/ml protease inhibitor cocktail,

Complete, Roche Applied Science, Basel, Switzerland). The homogenate was centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was then centrifuged at 100,000 g for 1 h at 4°C. The resultant pellet, containing both plasma membrane and intracellular vesicles, was resuspended in 1 x SDS-PAGE sample buffer, heated at 65°C for 40 min.

For protein expression analysis (Paper IV), the TA samples were solubilized in 1 x SDS-PAGE sample buffer and heated at 100°C for 5 min.

After protein concentration measurement (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA), prepared samples were resolved using SDS/PAGE electrophoresis (7.5-12% gel, 20 µg protein/lane). The proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Proteins were detected using specific antibodies (see materials) and ECL Plus Western blotting analysis system. After immunodetection, the PVDF membranes were stained with amido black (staining: amido black 0.1% wt/vol, methanol 10% vol/vol, and acetic acid 2% vol/vol, 5 min; destaining; methanol 45% vol/vol and acetic acid 7% vol/vol, 3-4 times for 5 min). The X-ray films and stained PVDF membranes were scanned, and the obtained images were analyzed using ImageJ or Quantity One software. The antibody signal was normalized for total protein amount calculated from the amido black staining in each lane.

3.3.3 Co-immunoprecipitation

Rat renal medullae were homogenized in RIPA buffer: 10 mM Tris, 150 mM NaCl, 1mM EDTA, 0.5% NP-40 (Nonidet P 40 Substitute, Fluka Chemie, Buchs, Switzerland), pH 7.4, containing 20 µg/mL protease inhibitor cocktail. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was precleared with a species-specific immunoprecipitation (IP) matrix (ExactaCruz detection system; Santa Cruz Biotechnology, Heidelberg, Germany) for 1-2 h at 4°C with rotation. IP antibody (5-10 µg) or equal amount of nonspecific immunoglobulin was incubated with another portion of the IP matrix overnight at 4°C with rotation. IP antibody - IP matrix complexes washed two times with PBS were added with the precleared lysate (1.75 mg protein) and incubated for 3 h at 4°C with rotation. The antibody - protein - IP matrix complex was washed three times with RIPA buffer and ones with PBS. After the final wash, the proteins were eluted by boiling (100°C for 3 min) or heating (37°C for 15

min) in 50 µl of 2 x SDS-PAGE sample buffer and analyzed by Western immunoblotting.

3.3.4 GST pull-down assay

GST and GST-fused NH_2-terminus of AQP4 were expressed in BL21 strain of *E. coli*. Overnight cultures from single colonies were used to inoculate 100 mL of NZYM Broth medium (Fluka Biochimica, Sigma-Aldrich Chemie, Germany) containing 100 µg/mL ampicillin. Protein expression was induced at bacteria density A_{600} = 0.6 o.u. by adding 0.2% w/v L-arabinose. After 4-h incubation at 37°C, the cultures were chilled on ice and harvested by a 10 min centrifugation at 2,800 g at 4°C. All following procedures were performed at 4°C. The cultures were resuspended in 4 ml of lysis buffer 1 (20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 0.25 % sodium deoxycholate, 0.5 % Triton X-100, 0.5 % NP-40, 20 µg/mL protease inhibitor cocktail, pH 7.5) and sonicated 9 times for 5 sec with 90 sec intervals. The lysates were centrifuged for 5 min at 4,000 g, the supernatants were then centrifuged for 15 min at 21,000 g. The resulting supernatants were added to 40 µl of a 75% slurry of Glutathione-Sepharose 4B beads (Amersham Biosciences, Sweden) and incubated for 1 h. The beads were washed three times with the lysis buffer 1. Renal medullary tissue (55-65 mg) was homogenized by Potter-Elvehjem homogenizer at 4°C in 1 ml of lysis buffer 2 (50 mM Tris, 150 mM NaCl, 1% NP-40, 20 µg/mL protease inhibitor cocktail, pH 7.5). After centrifugation for 10 min at 5,000 g at 4°C, protein concentration in the supernatant was measured by Lowry method (Bio-Rad Laboratories, Hercules, CA) and adjusted to 1 mg/mL with the lysis buffer 2. The beads with GST or GST-fused NH_2-terminus of AQP4 were incubated with 1 ml of the lysate overnight at 4°C and washed three times with the lysis buffer 2. The proteins were solubilized by boiling in 2 x SDS-PAGE loading buffer and analyzed using Western immunoblotting.

3.3.5 Water permeability (P_f) measurements

Measurements of the P_f in LLC-PK_1 cells

The P_f in LLC-PK_1 cells was measured using a method, which allows us to determine the P_f in individual cells within a cell monolayer and to compare the P_f of the cells that do and do not express GFP-tagged proteins.

The coverslips with the cells were mounted in a closed perfusion chamber (Focht Live Cell Chamber System, Bioptechs) on the stage of a Zeiss 410 invert laser scanning microscope, and the transfected cells were identified by the presence of GFP signal in the plasma membrane. The cells were loaded with calcein (Invitrogen, Eugene, Oregon, USA) by incubation for 5 min in solution containing 20 μM calcein-AM (acetoxymethyl ester of calcein). The cells were superfused with isotonic solution (300 mosM) and scanned every 2 s to monitor changes in calcein fluorescence after the solution was switched to hypoosmotic (200 mosM).

Isotonic solutions contained: 1) 137 mM NaCl, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (total $[K^+]$ = 4.2 mM); 2) 139.7 mM NaCl, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 1 mM KH_2PO_4, 0.5 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 (total $[K^+]$ = 1 mM); 3) 133.2 mM NaCl, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 6.5 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (total $[K^+]$ = 8 mM). To obtain hypoosmotic solutions, concentration of NaCl was reduced by 50 mM. The cells were exposed to the isotonic solutions with an increased or decreased $[K^+]$ for 1-1.5 min before the switch of osmolarity.

The series of images were analyzed off-line by measuring the time course of the calcein fluorescence in cytoplasmic regions of individual cells. The initial slope of the fluorescence curve (about 10 s after the switch of osmolarity) was used in the P_f calculations. During this period, cell swelling is believed to be proportional to the permeability of the cellular membranes to water and is not influenced by mechanisms aimed at regulatory volume decrease.

P_f was calculated using the following equation. $P_f = \tau\ (1- b/V_0)\ [\gamma\ (A/V)_0\ )\ V_w\ \Delta\varphi_0]^{-1}$, where τ is the time constant calculated for every cell from the curve showing changes in fluorescence intensity inside the cell during osmotic swelling. $(1- b/V_0)$ represents the osmotically active portion of the cell volume. This portion is calculated for each cell line in separate experiments using direct volume measurements performed before and after osmotic swelling. The constant γ is calculated as the slope of the relative fluorescence *vs* the relative osmolarity calibration curve. $(A/V)_0$ is the initial cell surface-to-volume ratio. V_w is the partial molar volume of water (18 cm^3/mol), and $\Delta\varphi_0$ is the initial osmotic gradient (outside- inside).

To measure the P_f in *Xenopus* oocytes, the oocytes were impaled by two microelectrodes filled with 1 mM KCl, one providing the clamp current and the other

measuring the membrane potential. The images of the oocytes circumference were recorded at a rate of 25 points per second using a low magnification objective. During the experiments, the oocytes were perfused by control isoosmotic solution (100 mM NaCl, 4.2 mM KCl, 3.8 mM choline chloride, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) or a solution where KCl was added or substituted by equimolar choline chloride to obtain an increased or decreased K^+ concentration. To measure P_f, the oocytes were challenged by switching the isoosmotic solution to a corresponding hypertonic solution obtained by adding 20 mM mannitol. The osmotic P_f was derived from the rate of the oocyte shrinkage induced by the hypertonicity.

P_f was calculated as $L_p = -J_v [A \Delta\pi V_w]^{-1}$, where J_v is the water flux during the osmotic challenge, A is the true membrane surface area (about 9 times the apparent area due to membrane foldings, around 0.53 cm^2), $\Delta\pi$ is the osmotic challenge, and V_w is the partial molal volume of water, 18 cm^3/mol. The contribution from the water permeability of the lipid membrane, which was less than 10% of the total L_p, was subtracted from the obtained values.

3.3.6 Mathematical modeling

Modeling was performed using the Virtual Cell modeling and simulation framework software (The National Resource for Cell Analysis and Modeling (NRCAM), www.vcell.org).

The model is based on a simplified geometric description of a diffusion-limited space (DLS) formed by the narrow infoldings in the basal membrane of collecting duct principal cells. Parameters that are modeled are membrane potential (V_m) and the concentrations of [K^+] and [H_2O]. Initial [K^+] in DLS was set to 4 mM, which also was defined as the equilibrium [K^+]. As a boundary condition for the model, [K^+] at the outer edges of the extracellular space (ECS) is set to a fixed level. The V_m at steady state is defined by the Nernst potential for K^+. The model takes into account that [K^+] in DLS is controlled by three mechanisms: diffusion from/to ECS, active transport by Na^+, K^+-ATPase, and electro-diffusion via K^+-channels. Water transport via AQPs is modeled as an osmotically driven flux, where [K^+] and [H_2O] are the factors contributing to the osmotic pressure.

3.4 STATISTICAL ANALYSIS OF THE DATA

In all studies, assumptions of normality and homogeneity of variance were checked. For variables with a normal distribution, the *t*-test for unpaired samples was used to analyze the difference between each two groups. Linear regression analysis was used to examine correlation between the variables. For variables with a skewed distribution, the Mann–Whitney U-test was used to analyze the difference between each two groups. Spearman correlation between two variables was examined. In Paper I, regression analysis was performed on log-transformed data. Stepwise multiple linear regression procedure was used to investigate the independent contribution of factors. In Paper II, Friedman's one way analysis of variance was used to analyze the difference between two or more paired samples. In Paper IV, analysis of covariance was performed to compare groups after adjustment for gestational age at birth or postnatal age at sampling. The difference with *P* values less than 0.05 was considered to be statistically significant. All probability values are two-sided.

4 RESULTS AND DISCUSSION

4.1 PAPER I

To investigate the role of AQP2 in immature kidney for postnatal adaptation, we measured urinary AQP2 level in newborn infants during the first and the third week of life.

Urinary AQP2 level and urinary osmolality

Urinary AQP2 level at the third week of life was not changed as compared to the first week. At both the first and the third week of life, urinary AQP2 level did not correlate with urinary osmolality, which was overall very low in all infants, indicating that urinary concentration and urinary AQP2 excretion are dissociated in infants. Our results support the notion that immature kidney is resistant to AVP, suggesting that in infants urinary AQP2 cannot serve as a direct marker of the renal action of AVP.

Correlation of urinary AQP2 level and gestational age at birth

At the third week of life, we found a significant positive correlation between urinary AQP2 level and gestational age at birth. Newborn infants, especially premature infants, have limited tubular function, which is related to gestational age (Gallini *et al.*, 2000). The increased urinary AQP2 level in full-term neonates seems to reflect a higher overall maturity of distal tubular function in these infants.

Correlation of urinary AQP2 level and serum Cr level

Urinary AQP2 level was inversely correlated with serum Cr level during the third week of life. Serum Cr is used to assess renal glomerular function. The decreased urinary AQP2 level in infants with high serum Cr concentration indicates a decreased AQP2 expression in conditions of renal function impairment.

Correlation of urinary AQP2 level and serum bicarbonate concentration

Urinary AQP2 level was positively correlated with serum bicarbonate concentration during the third week of life, suggesting that the level of urinary AQP2 is decreased in conditions of acidosis. The concentration of serum bicarbonate is determined predominately by the renal bicarbonate threshold which is low in term and even lower in preterm infants due to the immaturity (Drukker *et al.*, 2002). Infants with lower

serum bicarbonate levels, who may have more immature kidneys, have lower urinary AQP2 level.

Correlation of urinary AQP2 level and urinary Na^+/K^+ ratio

At the first week of life, we observed a positive correlation between urinary AQP2 level and urinary Na^+/K^+ ratio. The urinary Na^+/K^+ ratio is considered to reflect the responsiveness of kidney tubules to aldosterone action. High urinary Na^+/K^+ ratio may indicate low aldosterone activity and reduced Na^+ reabsorption in renal tubule. We speculate that the higher urinary AQP2 level, at higher urinary Na^+/K^+ ratio, could reflect a compensatory increase in AQP2 expression that occurs to minimize water losses caused by decreased Na^+ reabsorption.

4.2 PAPER II

To investigate whether AQP2 plays a role in disturbances of water balance, which are reported in preterm infants treated with PG inhibitors for PDA closure, we determined urinary AQP2 level during ibuprofen or indomethacin treatment (before treatment, after the 1st dose, after the 2nd dose and after the 3rd dose).

Effect of ibuprofen treatment

In infants that have no missing values at all four time points of the study, urinary AQP2 level was significantly decreased during ibuprofen treatment. Urinary osmolality was also significantly decreased during the treatment, while fluid intake was significantly increased.

Effects of indomethacin treatment

Urinary AQP2 level was decreased after the 3rd dose of indomethacin as compared to the level after the 2nd dose (n = 5). Urinary osmolality decreased after the 2nd dose of indomethacin compared to the time point after the 1st dose (n = 4). There was a significant increase in fluid intake after the 1st dose of indomethacin as compared to the pretreatment value (n = 14).

During both ibuprofen and indomethacin treatment, we observed a significant decrease in urinary AQP2 level and urinary osmolality in preterm infants. These findings were unexpected, suggesting that PGs in the immature kidney may have effects that are different from those that are observed in the mature kidney.

Correlation analysis

In infants that did not have any missing data in the first three time points of ibuprofen treatment, urinary AQP2 level did not significantly correlate with urinary osmolality before treatment, after the 1st dose or after the 2nd dose of ibuprofen. There was a significant inverse correlation between urinary osmolality and fluid intake before ibuprofen treatment (R^2 = 0.41), but not after the 1st dose or after the 2nd dose of ibuprofen.

Both urinary AQP2 level and urinary osmolality were decreased during ibuprofen and indomethacin treatment. Nevertheless, there was no significant correlation between the two parameters, neither before ibuprofen treatment, nor during the treatment. Our results suggest that AQP2 may not be a factor contributing to the water retention sometimes observed in PDA infants treated with PG inhibitors.

Discussion (Paper I and Paper II)

Our studies indicate that urinary AQP2 correlates with the overall maturity of tubular function in human neonates, reflecting maturation of the kidney collecting ducts at a molecular level. In infants at this early age, urinary AQP2 cannot serve as a direct marker of the renal action of AVP, but may reflect AQP2 expression level associated with different pathophysiological conditions.

It is well documented that immature kidney is resistant to AVP. There was no correlation between plasma AVP and plasma or urine osmolality in infants during the first 3 weeks of life (Rees *et al.*, 1980). Urinary AVP, reflecting its plasma concentration, also did not correlate with urinary osmolality (Wiriyathian *et al.*, 1986). Urinary AVP level in 1-day-old term and preterm infants were almost comparable to the adult values followed by gradual decrease during the first few days after birth. Urinary osmolality, however, did not follow this pattern. We found that both urinary AQP2 and urinary osmolality, which was overall low, remained unchanged during the first 3 weeks of life. Urinary AQP2 level did not correlate with urinary osmolality during this period. There was also no significant correlation between the two parameters in ibuprofen treated infants, neither before, nor during the treatment. Our results indicate urinary AQP2 cannot serve as a direct marker of the renal action of AVP in the immature kidney.

Inhibition of PG production was expected to increase urinary AQP2 excretion, since the relief of inhibitory action of PGs on AVP effect was anticipated to increase the

presence of AQP2 in the apical membranes of the collecting duct principal cells (Zelenina *et al.*, 2000). In line with this, ibuprofen was shown to increase urinary AQP2 excretion in healthy human adults (Pedersen *et al.*, 2001). However, we observed a significant decrease in urinary AQP2 level and urinary osmolality in preterm infants, suggesting that the knowledge about the renal physiology of the adult can not always be transferred to the infant kidney.

The overall low urinary osmolality and the dissociation between urinary osmolality and urinary AQP2 level indicate that the fluid retention sometimes observed in PDA infants treated with PG inhibitors is not caused by increased levels of functional AQP2.

4.3 PAPER III

In the brain, AQP4 has been shown to play an important role for K^+ transport (Saadoun *et al.*, 2009). We hypothesized that AQP4 may also be important for K^+ handing in kidney collecting ducts. In particular, we tested a hypothesis that AQP4, Na^+, K^+-ATPase and Kir7.1 form a K^+-transporting microdomain, where AQP4 might serve to maintain a favorable gradient for K^+ recirculation and to stabilize membrane potential.

Water permeability of AQP4, but not AQP3 is modulated by extracellular K^+ concentration

We first tested whether extracellular K^+ concentration ($[K^+]_e$) modulates AQP4 water permeability using LLC-PK_1 cells transiently transfected with AQP4. At normal $[K^+]_e$, 4.2 mM, AQP4-transfected cells had a significantly, 2-fold, higher water permeability than non-transfected cells. When the cells were exposed to a higher $[K^+]_e$, 8 mM, the water permeability of AQP4-transfected cells was significantly increased. Conversely, the water permeability of AQP4-transfected cells was significantly decreased at $[K^+]_e$ of 1 mM. Notably, these variations in $[K^+]_e$ had no effect on the water permeability of non-transfected cells.

To test whether the effects of extracellular K^+ were specific for AQP4, we also measured water permeability in LLC-PK_1 cells transfected with AQP3. Variation in $[K^+]_e$ had no effect on water permeability in AQP3-transfected cells.

Water permeability of AQP4 is independent of extracellular K^+ concentration and membrane potential in Xenopus oocytes

To investigate the mechanism underlying the effect of $[K^+]_e$ on AQP4 water permeability, we employed the *Xenopus laevis* oocyte expression system. We examined whether variations in $[K^+]$ and/or its effect on membrane potential can be sensed by AQP4 molecule.

The water permeability of AQP4-expressing oocytes was first examined at 1, 4.2, and 8 mM K^+ in the extracellular medium with the membrane potential clamped to –50 mV. In these conditions, the water permeability of AQP4-expressing oocytes was not affected by the changes in $[K^+]_e$. We next examined the water permeability of AQP4-expressing oocytes at normal $[K^+]_e$, with the membrane potential clamped to -25, -50, -75, or -100 mV. The water permeability of AQP4-expressing oocytes was not dependent on the membrane potential. Our results indicate that AQP4 molecule does not possess a voltage or K^+ sensor.

AQP4, but not AQP3, assembles with Kir7.1 and Na^+, K^+-ATPase α1 subunit in rat renal medullary tissue

To examine the possibility that AQP4 interacts with Kir7.1 and Na^+, K^+-ATPase, we first immunoprecipitated Kir7.1 from renal medullary lysate, and examined the precipitate for the presence of AQP4 and AQP3. Kir7.1 was found to co-immunoprecipitate with AQP4, but not with AQP3. When Na^+, K^+-ATPase α1 subunit was immunoprecipitated from rat renal medulla, AQP4, but not AQP3, was detected in the precipitate. When AQP4 was immunoprecipitated, the Na^+, K^+-ATPase α1 subunit was detected in the precipitate.

To further examine the interaction between Na^+, K^+-ATPase and AQP4, we also performed GST pull-down study using a peptide corresponding to the full-length NH_2-terminus of the long form of AQP4. We found that the NH_2-terminal peptide, but not the control GST, precipitated Na^+, K^+-ATPase α1 subunit from rat renal medullary lysate. Our results suggest that the interaction between AQP4 and the Na^+, K^+-ATPase α1 subunit is mediated by the NH_2-terminus of AQP4.

Mathematical model showing a link between AQP-mediated water transport and K^+ transport in a diffusion limited space

To test the possibility that water flow via AQP4 may be coupled to K^+ transport in the kidney, we developed a model describing the dynamic distribution of K^+ in the extracellular space (ECS) adjacent to the basal membrane of the collecting duct

principal cells. The narrow interstitial compartment between the cells constitutes a diffusion-limited space (DLS), which is in free diffusion contact with a wider ECS. We tested how changes in $[K^+]$ in ECS influenced $[K^+]$ in DLS in different three conditions: 1) no AQPs are present in the plasma membrane; 2) AQPs are present, but are not regulated; 3) AQPs are present and regulated by changes in $[K^+]$ in the DLS.

When no AQPs are present in the plasma membrane around DLS, the simulation shows a rapid change of $[K^+]$ in DLS following changes of $[K^+]$ in the ECS. The final $[K^+]$ in DLS equilibrates with $[K^+]$ in ECS.

In the simulations where AQPs are expressed in the plasma membrane, the water flux balances the K^+ diffusion from ECS to DLS. The resulting $[K^+]$ in DLS follows the increase or the decrease of $[K^+]$ in ECS, but it is closer to the initial equilibrium $[K^+]$ than in simulations with no AQPs present in the membrane.

In the model where AQPs in the plasma membrane are regulated by extracellular K^+, the increase in $[K^+]$ in DLS, caused by the rise of $[K^+]$ in ECS, is compensated faster than in the model with non-regulated AQPs due to both an osmotic effect and an increase in the channel water permeability.

Expression of AQP4 in kidney medulla is, in contrast to AQP2 and AQP3, not affected by K^+-deficient and high K^+ diet

In rats kept on the diets for one week, K^+-deficient diet has lead to severe hypokalemia, and high K^+ diet to hyperkalemia. Serum Cr concentration was significantly increased in the rats fed K^+-deficient diet compared to that in control rats.

There was no change in the protein expression of AQP4 in rats kept on K^+-deficient or high K^+ diet as compared to control rats. In contrast, AQP3 was significantly downregulated in rats on K^+-deficient diet; and AQP2 was significantly downregulated in both K^+-deficient and high K^+ diet.

Discussion (Paper III)

Our study suggests that AQP4 expressed in renal epithelial cells might not only be important for bulk water transport, but also for the ion composition of the microenvironment adjacent to the plasma membrane.

We found that extracellular K^+ modulates AQP4 water permeability, but AQP4 itself does not possess a voltage or K^+ sensor. It is well established that the water

permeability of AQP4 can be regulated by phosphorylation/dephoshorylation signaling pathways (Zelenina *et al.*, 2002). Changes in $[K^+]_e$ may influence intracellular ion content and pH. It is an intriguing possibility that the effects of extracellular K^+ concentration on AQP4 may be mediated by changes in intracellular pH and/or ionic content that have activated one or more signaling pathways.

Another possibility is that changes in $[K^+]_e$ influence AQP4 by an effect on one of the proteins that interact with AQP4, such as Na^+, K^+-ATPase or K^+ channels. We found that AQP4 assembles with Na^+, K^+-ATPase and Kir7.1 in the renal medulla, indicating that AQP4, Na^+, K^+-ATPase and Kir7.1 are associated in a transporting microdomain. In the transporting microdomain, AQP4 may play a modulating role for K^+ transport across the basolateral membrane of the principal cell by maintaining a local extracellular K^+ concentration that is favorable for K^+ recirculation across the basal membrane, and thus for the activity of Na^+, K^+-ATPase and K^+ secretion.

4.4 PAPER IV

To determine whether neonatal respiratory distress is related to changes in water and ion transporter expression in lung epithelium, we examined the protein abundance of water channels (AQP3, AQP4 and AQP5) and ion transporters (ENaC and Na^+, K^+-ATPase) in TA from neonates. Immunoblot analysis demonstrated the presence of AQP5, all three subunits of ENaC and Na^+, K^+-ATPase α1 in the TA samples. Other water channels, AQP1, AQP3 and AQP4, were not detected in TA.

AQP5 level in TA

AQP5 expression in TA was significantly higher in TTN infants as compared to RDS infants, or compared to non-TTN infants with abnormal lung X-rays, which included infants from both RDS group and mixed group. The differences in AQP5 levels remained statistically significant after controlling for postnatal age at sampling. AQP5 level in TTN infants had a tendency to be higher when compared to control infants who had normal lung X-rays. There was no significant difference in AQP5 expression between infants with RDS and control infants.

ENaC level in TA

The level of β-ENaC in TA was significantly lower in infants with RDS as compared to TTN infants, and as compared to infants in the control group. The difference in β-ENaC levels remained statistically significant after controlling for postnatal age at

sampling. There was no significant difference in β -ENaC level between TTN and control infants.

There was also no significant difference in α- and γ-ENaC level between TTN and RDS infants, between TTN and control infants, and between RDS and control infants.

Na^+, K^+-ATPase α1 level in TA

The protein level of Na^+, K^+-ATPase α1 did not differ between TTN and RDS infants, between TTN and control infants and between RDS and control infants.

Correlation analysis

AQP5 level had no significant correlation with gestational age at birth or postnatal age at sampling in each of the four individual groups or in the whole study population.

In all samples taken together, β-ENaC level did not correlate with the level of α- and γ-ENaC. However, α-ENaC level had a significant correlation with γ-ENaC ($R^2 = 0.69$). The level of α-, β- and γ-ENaC in TA had no significant correlation with gestational age at birth or postnatal age at sampling in each of the four individual groups or in the whole study population.

In the whole study population, the level of Na^+, K^+-ATPase α1 had a significant correlation with gestational age at birth ($R^2 = 0.14$). No correlation was observed between Na^+, K^+- ATPase α1 expression and postnatal age at sampling.

Discussion (Paper IV)

The present study is a step forward in investigation of the role of ion and water transporters in postnatal human lung maturation. Two interesting clinical studies have been performed in humans during the neonatal period using nasal epithelium samples to measure mRNA expression of ENaC (Helve *et al.*, 2004; Helve *et al.*, 2007). We use TA that has been suggested to be more suitable substitute for bronchoalveolar lavage, which is the standard technique of collecting epithelial lining fluid from the lungs of humans, for the studies of newborn lung fluid (Dargaville *et al.*, 1999; D'Angio *et al.*, 2002). In addition, we perform protein level measurements that give complementary, and functionally more relevant, information about the role of ion and water transporters than measurements of mRNA expression. Our finding of a lower level of β-ENaC in RDS infants is in agrrement with what has been reported from the nasal scrape mRNA analysis in infants with RDS (Helve *et al.*, 2007).

We found that β-ENaC level was not correlated with the level of α-ENaC or γ-ENaC, but there was a significant correlation between ENaC α- and γ- subunits. β-ENaC was also the only ENaC subunit changed in RDS infants in this study. Our data suggest that ENaC subunits are differentially regulated in human lung during perinatal period and that β-ENaC may have a specific role for the development of human lung function.

Infants with RDS had a lower gestational age compared to the other groups. The level of β-ENaC did, however, not have any significant correlation with this parameter. Therefore, the decreased β-ENaC expression can not simply be due to the lower gestational age in RDS infants. This lower β-ENaC expression in infants fulfilling the criteria for RDS is likely to contribute to the pathogenesis of the disorder. Interestingly, the expression of AQP5, which is the major AQP in the lung, was similar in RDS and control infants, indicating a more selective maturity-dependent effect on the epithelial sodium channel.

Another finding in our study was that AQP5 level in TA was significantly higher in TTN than in RDS infants and overall compared to infants with abnormal lung X-rays. Decreased expression of AQP5 in the lung was observed in pathological conditions, such as lung infection (Towne *et al.*, 2000), lung inflammation (Towne *et al.*, 2001), and acute lung injury (Jiao *et al.*, 2002), which suggests that the altered expression of AQP5 may play a significant role in pulmonary edema . TTN results from a delay in clearance of fetal lung liquid and is generally considered as a self-limited disorder. We therefore speculate that the increased AQP5 expression could represent a compensatory change in TTN, enhancing lung fluid clearance.

5 CONCLUSIONS

Urinary AQP2 correlates with the overall maturity of tubular function in human neonates. During the early postnatal period, changes in excretion of AQP2 will indicate maturation of the kidney collecting ducts at a molecular level. Urinary AQP2 cannot serve as a direct marker of the renal action of AVP in neonates, but may reflect changes in AQP2 expression level associated with different physiological and pathological conditions.

Both ibuprofen and indomethacin decreased urinary AQP2 level and urinary osmolality. The overall low urinary osmolality and the dissociation between urinary osmolality and urinary AQP2 level indicate that the fluid retention sometimes observed in infants with patent ductus arteriosus treated with prostaglandin inhibitors is not caused by increased levels of functional AQP2. Thus, knowledge about the renal physiology of the adult can not always be transferred to the infant kidney.

In kidney, AQP4 may assemble with Na^+, K^+-ATPase and Kir7.1 in a K^+ transporting microdomain, helping to maintain a favorable gradient for K^+ efflux and to stabilize membrane potential. This assembly may be important for the K^+ secretion by the collecting duct principal cells. Our results suggest that AQPs expressed in epithelial cells might not only be important for bulk water transport, but also for the ion composition of the microenvironment adjacent to the plasma membrane.

Neonatal respiratory distress is associated with changes in β-ENaC and AQP5 expression. The lower β-ENaC expression may be one of the factors that predispose to the development of respiratory distress syndrome in the newborn infants. The higher AQP5 expression may provide the possibility for reabsorption of postnatal lung liquid, which contributes to quick recovery of infants with transient tachypnea of the newborn.

6 CLINICAL IMPLICATIONS AND FURTHER PERSPECTIVES

Water and electrolyte management is one of the most challenging aspects of neonatal care of infants, particularly premature infants. Our final goal is to identify novel preventive and therapeutic strategies for disorders associated with abnormal water balance in the newborn infants. The presented studies lead to a better understanding of the role of water channels and ion transporters in postnatal adaptation and in pathological conditions related to an impaired water homeostasis, but also raise new issues for future studies.

Early recognition is of fundamental importance for providing effective treatment and minimizing complications related to disorders of water balance in newborn infants. However, the assessment of water balance in the newborn infants is particularly challenging. The presented studies demonstrated that urinary AQP2 correlates with the overall maturity of tubular function in human neonates and may reflect AQP2 expression level associated with different physiological and pathological conditions. Further research is needed to assess whether urinary AQP2 measurement could be used as a reliable non-invasive biomarker to foresee disorders of water homeostasis in the newborn.

Both ibuprofen and indomethacin are used to promote the closure of the ductus in preterm infants. However, concern remains regarding their safety. We report novel data about the effects of ibuprofen and indomethacin on urinary AQP2 and water balance in the immature kidney, contributing to the understanding the mechanism of water retention observed in infants with PDA treated with PG inhibitors. Our studies indicate that it is not an excessive AQP2 function that is the major cause of water retention reported in infants treated with prostaglandin inhibitors. This suggests that improving glomerular function might, in those cases, be more relevant for prevention and management of the water retention. More studies will be needed to elucidate how to counteract the undesirable effect of prostaglandin inhibitors on glomerular filtration in the immature neonates.

It has been shown that multiple factors affecting water balance often occur in combination in preterm infants. Most infants with PDA in this study experienced RDS with mechanical ventilation. A few infants also received gentamicin. RDS, mechanical

ventilation and gentamicin, as risk factors for acute renal injury (Toth-Heyn *et al.*, 2000; Andreoli, 2004), are shown to have an effect on the PGs system and water balance. Further studies are needed to investigate the combined effect of multiple risk factors on the water balance in order to optimize the clinical use of pharmacological agents and help to prevent iatrogenic damage during early postnatal life.

Nephrogenesis is not completed in infants with PDA who are often born at less than 34 weeks' gestation and receive ibuprofen or indomethacin during active nephrogenesis. Therefore, the question whether PG inhibitors have a direct effect on nephron number needs to be answered. It is also necessary to evaluate the long-term outcomes associated with the use of ibuprofen and indomethacin in preterm infants.

Our studies indicate that AQP4 may be involved in renal K^+ transport. The role of the functional microdomain containing AQP4, Na^+, K^+-ATPase and Kir7.1 for K^+ secretion in the kidney collecting duct principal cells needs to be further elucidated. One of the nearest tasks will be to investigate how changes in dietary K^+ intake will influence K^+ balance in AQP4 knockout mice model. The mechanism by which increased extracellular K^+ concentration enhances AQP4 water permeability also needs to be further explored. In the future experiments, we will use epithelial cell lines to examine how transepithelial K^+ transport is modified by inhibitors of interaction between AQP4 and Na^+, K^+-ATPase and between AQP4 and K^+ channels. For this, we will use information obtained in the present studies indicating that it is NH_2-terminus of AQP4 that is involved in the interaction of AQP4 with Na^+, K^+-ATPase. A peptide corresponding to this part of AQP4 molecule might therefore be used as an inhibitor of AQP4 and Na^+, K^+-ATPase interaction in the cells. To be able to use similar approach to modulate AQP4 - K^+ channel interaction, we will first need to identify the part of AQP4 involved in this interaction.

Neonatal care has changed dramatically over the past decades, and the improvement in antenatal corticosteroid treatment, surfactant replacement therapy and ventilator support greatly reduced the severity and mortality from RDS (Rodriguez, 2003). Despite that, RDS continues to be a major cause of neonatal mortality, and the incidence of bronchopulmonary dysplasia (BPD) remains high. The overall aim of the clinical research is to identify novel preventive and therapeutic strategies for RDS and BPD. Our study provides evidence supporting the important role of AQP5 and ENaC in neonatal respiratory distress. New therapeutic strategies targeting AQP5 and ENaC

may enhance lung fluid clearance and thereby facilitate the recovery of infants with respiratory distress and improve the long-term outcomes.

Further studies need to address the question of how antenatal corticosteroid treatment, surfactant replacement therapy and ventilator support, which have been proved to greatly reduce the severity of RDS and mortality, influence the expression of ENaC and AQP5. Moreover, little is known about the lung-kidney interaction in premature infants. Therefore, the role of water balance disorders and renal insufficiency on the development of RDS and BPD in premature infants needs to be explored.

7 ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to all the people who have supported and helped me during my doctoral education and the formation of this thesis, especially:

PhD Marina Zelenina, my main supervisor, for sharing your knowledge and experience with me, and for your guidance, support, kind patience and always being available throughout this study.

Professor Anita Aperia, my supervisor, for providing me the opportunity to work in your lab, introducing me to the world of research and sharing your scientific knowledge with me.

Dr Charlotte Casper, for your supervision of clinical projects, for interesting discussion and advice, and for your encouragement and pleasant collaboration.

All my co-authors: Alexander Bondar, Catherine Arnaud, Christelle Cristini, Geneviève Plat-Willson, Hjalmar Brismar, Isabelle Glorieux, Marie-Odile Marcoux, Martine Gineste, Mireille Vanpee, Nanna MacAulay, Padideh Kamali-Zare, Sergey Zelenin, Shigeki Sakuraba, Stéphane Decramer, for your contribution and fruitful collaboration.

I also want to thank all my colleagues for discussion and help: Carolina, Evgeniya, Eivor, Eli, Georgiy, Haiyan, Jacob, Josefin, Juan, Lena, Linda, Lollo, Markus, Nermin, Nina, Rachel, Siobhan, Susanne, Tomas, Tong, Ulla, Xiaoli, Yutong, Zuzana. To Ann-Christine for encouragement and helping me with all kind of problems, Raija for helping me with the paper work, Lill-Britt for teaching me basic techniques.

My friends in Sweden: Jinghong, Didier, Wang Di, Shen Jinfeng, Zong Mei, Xie Hailian, Zhang Songbai, Meng Chunxia, Hu zhe, Guan Jikuan, for friendship and help.

My friends in China: Wu Qun, Teng Jie, Cheng Yanjie, Guan Jingfan, Zhou Jin, Chen Hong, Liu Yan, Yu Wenying, Wang Tie, for being such good friends and having wonderful time together.

My family: To my mother and father and my brother and sister, for never ending love and unconditional support; and to my nephew and niece for bringing a smile to my face.

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