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Long-term Outcome and Extraintestinal Manifestations in Congenital Chloride Diarrhea

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ACADEMIC DISSERTATION

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List of original publications

This thesis is based on the following publications:


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The publications are referred to in the text by their roman numerals.
Abstract

The rare inherited disease congenital chloride diarrhea (CLD) is caused by over 30 different mutations in the solute carrier family 26 member 3 (SLC26A3 alias DRA) gene on chromosome 7q22.3-31.1. SLC26A3 encodes for an apical epithelial Cl/HCO₃⁻ exchanger, the intestinal loss of which leads to profuse Cl⁻-rich diarrhea, and a tendency to hypochloremic and hypokalemic metabolic alkalosis. Although untreated CLD is usually lethal in early infancy, the development of salt substitution therapy with NaCl and KCl in the late 1960s made the disease treatable. While the salt substitution allows normal childhood growth and development in CLD, data on long-term outcome have remained unclarified.

One of the world’s highest incidences of CLD—1:30 000 to 1:40 000—occurs in Finland, and CLD is part of the Finnish disease heritage. We utilized a unique sample of Finnish patients with the homozygous V317del genotype for CLD to characterize the long-term outcome of the disease. Another purpose of this study was to search for novel manifestations of CLD based on the expression of SLC26A3 in the sweat gland and male seminal vesicle.

Clinical analysis of long-term outcome was performed on a sample of 36 patients (ages 10-38; median 21), comprising 80% of the Finnish patients aged over seven years. Of them, eight adult males participated in the clinical assessment of fertility as well. Additionally, the data on one deceased Swedish patient with end-stage renal disease (ESRD) and renal transplantation were included in the analysis of CLD-associated renal injury. We searched for expression of the SLC26A3 protein, in relation to other ion transporters, in the tissues of the human male reproductive tract and in the human kidney. Moreover, we performed a pilot trial with oral butyrate in five patients to find whether butyrate is effective therapy for the diarrheal symptoms of CLD.

This study shows that the long-term outcome of treated CLD is favorable. Almost all patients rated their general state of health as excellent or good, and few found the diarrheal symptoms of CLD disturbing. In untreated or poorly treated cases, however, chronic contraction and metabolic imbalance may lead to renal injury, and even to ESRD. The relatively high incidence (28%) of chronic kidney disease in our series underlines the importance of early diagnostics, sufficient salt substitution, and regular follow-up of CLD. Our results demonstrate, for the first time, a low-level expression of SLC26A3 mRNA and protein in the human kidney. Although SLC26A3 may play a minor role in homeostasis, post-transplant recurrence of renal changes undoubtedly shows the unlikelihood of direct transporter modulation in the pathogenesis of CLD-related renal injury.

This study reveals novel manifestations of CLD. These include an increased risk for hyperuricemia, inguinal hernias, and probably for intestinal inflammation. The most notable finding of this study is CLD-associated male subfertility. This involves a low concentration of poorly motile spermatozoa with abnormal morphology, high seminal plasma Cl⁻ with a low pH, and a tendency to form spermatoceles. Together with a finding of high sweat Cl⁻ in CLD, this study provides novel data on extraintestinal actions of the SLC26A3 gene, both in the male reproductive tract and the sweat gland.
SLC26A3 immunoexpression appeared at multiple sites of the male reproductive tract: in elongating spermatids, efferent ducts of the testis, ductus epididymis, and seminal vesicle. That the expression of SLC26A3 arose in part together with the main interacting proteins—cystic fibrosis transmembrane conductance regulator (CFTR) and Na⁺-H⁺-exchanger 3 (NHE3)—suggests novel sites for the cooperation of these proteins. SLC26A3 is therefore likely to promote electroneutral absorption of NaCl together with NHE3 and to modulate HCO₃⁻ secretion together with CFTR in the human male reproductive tract. As evidence of the importance of these actions, defects occurring in any of these transporters are associated with reduced male fertility.

Options to resolve the diarrheal symptoms of CLD have been limited. Unfortunately, our pilot trial indicated the inefficacy of oral butyrate as well. As the responses to oral butyrate were variable, however, factors other than the SLC26A3 genotype only are likely to account for butyrate’s ability to modulate intestinal electrolyte transport in CLD.

In conclusion, early diagnosis and sufficient salt substitution therapy with NaCl and KCl provide a favorable long-term outcome in CLD. In non-optimally treated disease, however, risk for renal involvement arises. Of the novel manifestations, the major issues in clinical management are male subfertility and spermatoceles, resulting from the defective Cl⁻/HCO₃⁻ exchange in the male reproductive tract. Fortunately, normal spermatogenesis is likely to make artificial reproductive technologies to treat infertility—and even make unassisted reproduction—possible.
Abbreviations

A adenine
aa amino acid
AGE acute gastroenteritis
AEI anion exchanger 1
AMRC apical mitochondria-rich cell
AQP aquaporin
AR androgen receptor
ATPase adenosine triphosphatase
AZF azoospermia factor
BMI body mass index
bp base pair
C cytosine
Ca$^{2+}$ calcium ion
CAII carbonic anhydrase II
cAMP cyclic adenosine monophosphate
CBAVD congenital bilateral absence of the vas deferens
CCD cortical collecting duct of the kidney
cDNA complementary deoxyribonucleic acid
CF cystic fibrosis
CFTR cystic fibrosis transmembrane conductance regulator
CKD chronic kidney disease
Cl$^{-}$ chloride ion
CLD congenital chloride diarrhea
CNF congenital nephrotic syndrome
CNT connecting tubule of the kidney
CPITN community periodontal index of treatment needs (dentistry)
Cr-EDTA chrome ethylenediaminetetraacetic acid
DAB diaminobenzidine
DMFS decayed, missed, filled surfaces of teeth index (dentistry)
DNA deoxyribonucleic acid
DRA down-regulated in adenoma
DTDST diastrophic dysplasia sulphate transporter
E3KARP NHE3 kinase A regulatory protein
ECG electrocardiography
ED efferent ducts of the testis
ENaC epithelial sodium channel
ER estrogen receptor
ESRD end-stage renal disease
FSH follicle stimulating hormone
G guanine
GAPDH glyceraldehyde 3-phosphate dehydrogenase, a housekeeping gene
GFR glomerular filtration rate
H⁺  hydrogen ion
HCO₃⁻  bicarbonate ion
IgG  immunoglobulin G
IVF  in vitro fertilization
IVS8-T  intron 8 polythymidine tract
K⁺  potassium ion
KCl  potassium chloride
Mg²⁺  magnesium ion
mRNA  messenger ribonucleic acid
LH  luteinizing hormone
Na⁺  sodium ion
NaCl  sodium chloride
NH₄Cl  ammonium chloride
NHE  Na⁺/H⁺ exchanger
NHERF  Na⁺/H⁺ exchanger regulatory factor
NKCC1  Na⁺-K⁺-2Cl⁻ cotransporter
NMIGG  normal mouse IgG fraction
NRIGG  normal rabbit IgG fraction
OMIM  online Mendelian inheritance in man
p  short arm of a chromosome
PAT1  putative anion transporter 1
PCR  polymerase chain reaction
PDZ  postsynaptic density protein PSD95, the Drosophila homologue Disc-large, and the tight junction protein ZO-1
PDZK1  PDZ domain protein kidney 1
Pi  phosphate ion
PKA  protein kinase A
q  long arm of a chromosome
R domain  regulatory domain
RNA  ribonucleic acid
RT-PCR  reverse transcriptase polymerase chain reaction
SCFA  short-chain fatty acid
SD  standard deviation
SLC26  solute carrier family 26
SLC26A  solute carrier family 26 member
SPSS  Statistical Package for Social Sciences
STAS  sulphate transporter and anti-sigma antagonist
T  thymidine
TBST  tris-buffered saline with Tween
UTI  urinary tract infection
V-ATPase  vacuolar proton-transporting ATPase
WHO  World Health Organization

The gene names are in italics and protein names capitalized.
Introduction

One of the top ten causes of death worldwide is diarrhea, claiming millions of lives annually. In the developing countries, diarrhea is associated with poverty and contaminated water supplies. In the more affluent countries, diarrhea is rarely lethal if treated with adequate fluid replacement therapy. Diarrhea still is, however, one of the main causes for pediatric emergency visits worldwide and is responsible for one-fourth of the deaths in children under five years (Kosek et al. 2003).

Although congenital chronic diarrheas are rare and often difficult to diagnose and manage, they provide important models for understanding the basic pathophysiological mechanisms of diarrhea. Among these diarrheal disorders are congenital sodium diarrhea (Booth et al. 1985, Holmberg and Perheentupa 1985), congenital sucrase-isomaltase deficiency (Ouwendijk et al. 1996), congenital glucose-galactose malabsorption (Martin et al. 1996), and congenital lactase deficiency (Kuokkanen et al. 2006).

In 1945, two simultaneous case reports described a novel disease called congenital alkalosis with diarrhea (Darrow 1945, Gamble et al. 1945). In this disorder with alkalosis, the other major feature was a high concentration of fecal Cl. Two decades later, alkalosis turned out actually to be a feature secondary to the Cl-rich diarrhea (Perheentupa et al. 1965, Launiala et al. 1968). Familial enrichment of the cases in Finland resulted in establishment of the inherited nature of the disease, which was called familial chloride diarrhea (Perheentupa et al. 1965). This disease was added, with the name “congenital chloride diarrhea” (CCD or CLD), as the ninth rare disorder to the entity of the Finnish disease heritage (Norio et al. 1973).

In Finland, finding patients with CLD provided the basis for both the clinical characterization of the disease and therapeutic trials. The life-saving therapy, salt substitution with NaCl and KCl, was introduced in Finland in the late 1960s. Thereafter, it has been used worldwide to prevent metabolic imbalance and to allow normal childhood growth and development in CLD (Holmberg et al. 1977a).

Although cases with CLD emerge worldwide, most of the clinical reports, and especially those among larger series of patients, originate in Finland. As CLD is such a rare disease, Finnish clinical experience has provided valuable data for physicians and patients worldwide. Due to the lethality of CLD until the late 1960s, the long-term outcome of CLD has remained unclarified. Meanwhile, the identification of the genetic defect behind CLD (Hoglund et al. 1996b) and the discovery of over 30 different mutations in this solute carrier family 26 member 3 (SLC26A3) gene (Makela et al. 2002) have elucidated the underlying pathophysiological mechanisms of CLD. Interestingly, expression of SLC26A3 messenger RNA (mRNA) or protein appears not only in the intestine but also in the sweat gland, testis, and male seminal vesicle (Hoglund et al. 1996b, Haila et al. 2000). When combined with the preliminary clinical evidence of male subfertility in CLD, these data prompted us to hypothesize that CLD involves extraintestinal manifestations. The main purpose of this study was to characterize the long-term prognosis of CLD and to reveal whether the extraintestinal expression of SLC26A3 is associated with any pathological features when it is disrupted in CLD.
Review of the literature

1 Clinical picture of CLD

1.1 Clinical symptoms

The only prenatal sign of CLD is polyhydramnios due to intrauterine onset of watery diarrhea. Other clear hallmarks of CLD include preterm birth, lack of meconium, and abdominal distention resembling that of intestinal obstruction (Perheentupa et al. 1965, Launiala et al. 1968, Holmberg et al. 1977a). The main feature of CLD, acidic Cl\(^-\)-rich diarrhea, leads to excessive loss of fluid and salts immediately after birth, and further to weight loss, dehydration, and jaundice (Holmberg et al. 1977a). The first signs of salt depletion in CLD, hyponatremia and hypochloremia, are soon after accompanied by hypokalemic metabolic alkalosis. If untreated, this metabolic imbalance, together with severe dehydration, is lethal even during the first weeks or months of life (Holmberg 1986).

1.2 Diagnosis of CLD

CLD diagnosis is based on its typical clinical picture and a high concentration of fecal Cl\(^-\), exceeding 90 mmol/l after correction of the fluid and electrolyte depletion (Holmberg 1986). Although genetic testing for CLD is possible (See Section 2), the simple measurement of fecal Cl\(^-\) is still sufficient to confirm CLD diagnosis.

Antenatal suspicion of CLD arises due to polyhydramnios. Even at the end of the second trimester, ultrasonic investigation of a fetus with CLD reveals dilated intestinal loops (Kirkinen and Jouppila 1984, Groli et al. 1986, Poggiani et al. 1992). Despite the intrauterine onset of diarrhea, concentrations of electrolytes in the amniotic fluid are normal (Holmberg et al. 1977a). Some other abnormalities, e.g., increased concentrations of alpha-fetoprotein, may be measurable, but without clinical significance (Hartikainen-Sorri et al. 1980).

Neonatally, the fluid-filled intestinal loops in the abdominal X-ray may result in a suspicion of intestinal obstruction and unnecessary surgery (Langer et al. 1991). In addition, differential diagnosis of CLD includes other inherited diarrheas such as congenital sodium diarrhea (Booth et al. 1985, Holmberg and Perheentupa 1985), glucose-galactose malabsorption (Martin et al. 1996), and congenital sucrase-isomaltase deficiency (Ouwendijk et al. 1996). In these diseases and in other secretory diarrheas, alkaline feces exclude the possibility of CLD (Holmberg 1986).
1.3 Therapeutic approaches

The early findings of the intestinal Cl/base exchange defect provided the basis for therapeutic trials (Darrow 1945, Gamble et al. 1945, Perheentupa et al. 1965). Replacement of the intestinal loss of fluid and salts was tested with different combinations of salts such as KCl, NaCl, or NH₄Cl (Perheentupa et al. 1965). Of these, KCl therapy maintained the normal concentrations of serum electrolytes, but slight alkalosis and Cl⁻-free urine remained (Pasternack et al. 1967). Moreover, the metabolic imbalance during the KCl therapy was associated with multiple renal changes: juxtaglomerular hyperplasia, hyalinized glomeruli, nephrocalcinosis, or vascular changes (Pasternack and Perheentupa 1966, Pasternack et al. 1967). Combination therapy with NaCl and KCl, however, reversed those renal changes partially and offered protection from the renal involvement during childhood (Holmberg et al. 1977b).

The oral substitution therapy recommended is a solution of 1.8% NaCl and 1.9% KCl. After intravenous administration in early infancy, the salt substitution is peroral and involves 2 to 5 daily doses. The salts are diluted either in a large amount of water for several days of use or diluted one dose at a time in a glass of water (Holmberg 1978, Holmberg 1986). The optimal dosage of Cl⁻ ranges from 6 to 8 mmol/kg/day in infants, and from 3 to 4 mmol/kg/day in older patients. Adequate excretion of Cl⁻ into the urine, in addition to normal electrolyte and acid-base status, confirms the sufficiency of salt substitution (Holmberg et al. 1977a, Holmberg 1986).

Although the salt substitution inhibits development of hypochloremic and hypokalemic metabolic alkalosis in CLD, diarrhea is persistent. Due to the watery stools, a common problem in these children is soiling (Holmberg 1986). High-dose therapy with glucocorticoids reduces the diarrhea but is inapplicable for long-term use because of adverse effects (Perheentupa et al. 1965). Several more easily applicable agents—including anion exchange resins (Pearson et al. 1973), prostaglandins (Minford and Barr 1980, Holmberg and Perheentupa 1982), and spironolactone (Asano et al. 1994)—have failed to resolve the diarrheal symptoms.

One case report proposed that an effective diarrhea-reducing therapy is oral administration of the proton-pump inhibitor omeprazole (Aichbichler et al. 1997). Another study in patients with optimal salt substitution disproved the efficacy of omeprazole, however (Hoglund et al. 2001a). During this study, a case report showed that oral administration of a short-chain fatty acid (SCFA) butyrate normalized stool volume and consistency in an 11-year-old Italian patient (Canani et al. 2004). Thus far, the only agent with evidence for a moderate but temporary diarrhea-reducing effect, up to 30 to 40%, is cholestyramine. It binds bile acids, reducing intestinal secretion not only in bile-acid induced diarrhea but also, by some unclarified mechanism, in CLD (Brocklehurst and Walker 1978, Holmberg et al. 1982).
1.4 Complications of CLD

During childhood, combination therapy with NaCl and KCl protects from the common complications of untreated CLD: constant dehydration and hypoelectrolytemia, retarded growth and development, activation of the renin-aldosterone system, and renal involvement (Pasternack and Perheentupa 1966, Pasternack et al. 1967, Holmberg et al. 1977a, Holmberg 1986). Rare CLD complications include volvulus, mental subnormality, non-specified colitis, and hyperuricemia (Holmberg 1986, Nuki et al. 1991, Kagalwalla 1994). Moreover, CLD involves teeth anomalies (deciduous teeth hypoplasia and other enamel defects) and an increased resistance to dental caries, the latter probably being due to the oral salt solutions (Mylarntaniemi and Holmgren 1975, Kerebel et al. 1984). Except for one study suggesting that the mutations for CLD predispose to intestinal cancer (Hemminki et al. 1998), reports of CLD-associated malignancies are non-existent.

Although untreated CLD is usually lethal in early infancy, and the known complications are associated mostly with untreated disease, several patients have survived for months or even years without a correct diagnosis. One such patient with recurrent episodes of ileus survived undiagnosed for 21 years (Pearson et al. 1973). Even in Finland, a patient born in the 1990s had his CLD diagnosed at age 5, due to misinterpretation of the diarrheal symptoms as food allergy (Leskinen 1996). In these few patients, a potential contributor to survival is increased consumption of dietary salt. On the other hand, survivors with untreated CLD seem to develop a less severe, chronic course of the disease, the identification of which may be highly challenging (Holmberg 1986). Their persistent diarrhea is, however, often associated with a special vulnerability to dehydration and severe hypoelectrolytemia both during the episodes of gastroenteritis or during other common infections of childhood (Holmberg et al. 1977a).

1.5 Early data on CLD pathophysiology

The very first reports on CLD suggested that the underlying cause of the acidic Cl⁻-rich diarrhea is a defect in intestinal Cl⁻/base transport (Darrow 1945, Gamble et al. 1945). First, what was unclear was whether the primary defect in CLD was increased secretion or reduced absorption of Cl⁻. After localization of the defect in the terminal ileum and colon (Launiala et al. 1968, Turnberg 1971), perfusion studies demonstrated the basic defect in the Cl⁻/HCO₃⁻ exchange (Turnberg 1971, Bieberdorf et al. 1972, Pearson et al. 1973). Moreover, the low fecal HCO₃⁻ and reduced colonic absorption of Na⁺ in CLD provided initial data on coupled Na⁺/H⁺ exchange in the colon. Further perfusion studies revealed that inhibition of NaCl absorption and fecal acidity induce the osmotic diarrhea in CLD (Holmberg et al. 1975).
2 Genetic basis of CLD

2.1 Geographical distribution of CLD

2.1.1 The Finnish disease heritage and CLD

The very first report on CLD in Finland revealed that the disease is inheritable (Perheentupa et al. 1965). The familial occurrence of cases allowed identification of both the autosomal recessive inheritance trait and uneven geographical distribution of CLD (Norio et al. 1971). These families originated almost exclusively in eastern Finland. In this region, the population history involves a small number of settlers who inhabited northeastern Finland in the 16th century. Thereafter, genetic isolation with local founder effects resulted in the enrichment of many rare disease-causing alleles in this subpopulation. In this unique genetic pool, not only the major mutation for CLD but also for many other rare diseases enriched, forming the basis for the entity called the Finnish disease heritage (Nevanlinna 1972, Norio et al. 1973). This series of disorders involves almost 40 Mendelian diseases which are very rare elsewhere and which have expanded in the Finnish population through single founder mutations (de la Chapelle 1993). That the Finnish gene pool is less variable than those of the general European or Baltic populations provides possibilities for genetic studies in both monogenic and polygenic disorders (de la Chapelle 1993, Service et al. 2006).

2.1.2 Other populations with CLD

The first diagnosed cases with CLD were Americans partly of Italian descent (Darrow 1945, Gamble et al. 1945). Later, more than 250 cases with CLD have been reported worldwide. Although single cases arise from all continents, CLD geographical distribution is uneven (Figure 1).

One-fifth of all reported CLD patients originate from Finland, where incidence is around 1:30 000 to 1:40 000 (Hoglund et al. 1998a). Other populations with a higher than average incidence are Polish (1:200 000), Kuwaiti, and Saudi Arabian. In the countries around the Persian Gulf, and especially in Kuwait, local estimates of incidence total as high as 1:3 200 to 1:5 000 due to consanguineous marriages (Tomaszewski et al. 1987, Lubani et al. 1989, Shaltout et al. 1989, Abdullah et al. 1990, Kagalwalla 1994, Kierkus et al. 1995, Badawi et al. 1998, Kere et al. 1999). The exact figures for incidence at population level remain, however, unascertainable.
2.2 Identification of the CLD gene

The CLD locus was originally mapped to 7q31, close to the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Kerem et al. 1989, Bear et al. 1992), with candidate-gene-based linkage analysis (Kere et al. 1993). Further mapping of the CLD locus allowed the identification of four regional candidate genes, the most interesting of which was down-regulated in adenoma (DRA) (Hoglund et al. 1995, Hoglund et al. 1996a). Previously, this gene had been cloned as a potential tumor suppressor gene based on its abundant expression in the colon but its absence from colonic adenomas and adenocarcinomas (Schweinfest et al. 1993, Taguchi et al. 1994). Support for the role of DRA in CLD came from the fact that the protein encoded by this gene was an intestine-specific sulfate transporter (Silberg et al. 1995).

Identification of the homozygous V317del mutation in the DRA gene among Finnish patients, and H124L and 344delT mutations in the Polish patients showed that DRA mutations are responsible for CLD (Hoglund et al. 1996b).

2.3 CLD gene in the SLC26 transporter family

The DRA gene comprises 21 exons and spans 37.7 kb on the minus strand of chromosome 7q22.3-31.1 (Haila et al. 1998, Kent et al. 2002). The translation initiation codon is located in exon 2 and the termination codon in exon 21. The protein product of DRA is an apical transmembranic protein with 764 amino acids, involving 14 probable hydrophobic
membrane-spanning domains and a sulphate transporter and anti-sigma antagonist (STAS) domain covering amino acids 525 to 720 in the cytoplasmic COOH-terminal end (Figure 2) (Byeon et al. 1996, Bairoch et al. 2005). The DRA protein is a Cl/HCO$_3^-$ exchanger which acts in conjunction with other epithelial ion transporters, modulating net transport in the epithelial cells (See Section 3) (Melvin et al. 1999, Moseley et al. 1999, Jacob et al. 2002, Lamprecht et al. 2006).

The very first studies of DRA showed notable protein homology with the human diastrophic dysplasia sulphate transporter (DTDST) gene (Hastbacka et al. 1994) and with a number of genes of the sulfate permease family in other species. Later, the DRA gene was included as a third member in the novel human solute carrier family 26 (SLC26), which involves 11 structurally homologous anion exchangers (Table 1) (Mount and Romero 2004, Kere 2006). Of these, several are associated in a homozygous form with rare human diseases. SLC26A2 mutations cause autosomal recessive bone and cartilage disorders: diastrophic dysplasia (OMIM 222600), achondrogenesis type IB (OMIM 600972), atelosteogenesis type II (OMIM 256050), and multiple epiphyseal dysplasia (OMIM 226900) (Hastbacka et al. 1994, Hastbacka et al. 1996, Superti-Furga et al. 1996, McKusick 2007). SLC26A3 (alias DRA) mutations cause CLD (OMIM 214700) (Hoglund et al. 1996b, McKusick 2007). SLC26A4 mutations cause Pendred syndrome (OMIM 274600), accounting for around 10% of the cases with hereditary deafness, and enlarged vestibular aqueduct syndrome (OMIM 600791) (Everett et al. 1997, Li et al. 1998, McKusick 2007). SLC26A5 mutations are associated with autosomal recessive non-syndromic neurosensory deafness (Zheng et al. 2000, Liu et al. 2003). Both the SLC26A2-associated diastrophic dysplasia and SLC26A3-associated CLD are, through single founder mutations, part of the Finnish disease heritage (Hoglund et al. 1996b, Hastbacka et al. 1999, Norio 2003).

**Figure 2** Predicted structure of the human DRA (alias SLC26A3) protein and localization of the Finnish founder mutation V317del for CLD. The COOH-terminal cytoplasmic tail includes two protein interaction motifs (See Section 3.3): the STAS domain comprising amino acids 525 to 720, and the PDZ interaction motif involving four terminal amino acids.
Table 1  Characteristics of SLC26 family members.

<table>
<thead>
<tr>
<th>Gene (alias)</th>
<th>Genbank</th>
<th>Chromosome</th>
<th>Expression</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC26A1 (Sat-1)</td>
<td>NM_022042</td>
<td>4p16.3</td>
<td>Liver, kidney, brain, muscle</td>
<td>-</td>
<td>(Bissig et al. 1994)</td>
</tr>
<tr>
<td>SLC26A2 (DTDST)</td>
<td>NM_000112</td>
<td>5q31-34</td>
<td>Widespread</td>
<td>Chondrodysplasias</td>
<td>(Hastbacka et al. 1994)</td>
</tr>
<tr>
<td>SLC26A3 (DRA, CLD)</td>
<td>NM_000111</td>
<td>7q22.3-31.1</td>
<td>Intestine, testis, seminal vesicle, sweat gland</td>
<td>Congenital chloride diarrhea</td>
<td>(Schweinfest et al. 1993, Hoglund et al. 1996b, Haila et al. 2000)</td>
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<tr>
<td>SLC26A4 (Pendrin)</td>
<td>NM_000441</td>
<td>7q31</td>
<td>Thyroid, kidney, cochlea</td>
<td>Pendred syndrome and enlarged vestibular aqueduct syndrome</td>
<td>(Everett et al. 1997, Li et al. 1998)</td>
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<tr>
<td>SLC26A7</td>
<td>NM_052832</td>
<td>8q22.2</td>
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<td>-</td>
<td>(Lohi et al. 2002a, Petrovic et al. 2003)</td>
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<tr>
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<td>NM_052961</td>
<td>6p21.3</td>
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<td>(Lohi et al. 2002a, Vincourt et al. 2003)</td>
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<td>1q32</td>
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<td>SLC26A10</td>
<td>NM_133489</td>
<td>12q13</td>
<td>Pseudogene</td>
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<td>(Lohi et al. 2002a)</td>
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<tr>
<td>SLC26A11</td>
<td>NM_173626</td>
<td>17q25</td>
<td>Widespread</td>
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<td>(Lohi et al. 2002a, Vincourt et al. 2003)</td>
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2.4 Expression pattern and mutation spectrum of the SLC26A3 gene

In both humans and rodents, expression of SLC26A3 mRNA and protein emerges in the absorptive surface epithelium of the small intestine, especially in the ileum but also in the duodenum, and in the surface epithelium of the colon (Figure 3). In the intestinal crypts, the signal is lacking (Hoglund et al. 1996b, Jacob et al. 2002). In the human male reproductive tract, a very weak signal for SLC26A3 mRNA appears in the testis (Hoglund et al. 1996b). Although the first study showed abundant expression of SLC26A3 mRNA in the prostate, future studies found the SLC26A3 protein actually in the epithelium of the human male seminal vesicle (Hoglund et al. 1996b, Haila et al. 2000). Moreover, SLC26A3 is expressed in the epithelium of the human sweat gland (Haila et al. 2000). In cell cultures, expression of SLC26A3 appears in epithelial cells derived from the pancreatic duct (Greeley et al. 2001) and the trachea (Wheat et al. 2000).

Regarding diseases, expression of SLC26A3 mRNA often is decreased in ulcerative colitis, but data on protein expression are inconsistent (Yang et al. 1998, Haila et al. 2000, Lohi et al. 2002b). In intestinal adenomas and adenocarcinomas, expression of SLC26A3 is significantly down-regulated or even undetectable (Schweinfest et al. 1993, Antalis et al. 1998).

Over 30 different mutations—including the founder mutations of Finland, Poland, and Arabic countries—cause CLD (Table 2) (Hoglund et al. 1998a, Makela et al. 2002). The Finnish founder mutation V317del (Figure 2), in-frame deletion of a valine at codon 317, originates from a small northeastern subpopulation from 400 to 500 years ago, presumably through only one ancestor (Norio et al. 1973, de la Chapelle 1993, Hoglund et al. 1996b). The carrier frequency of this Finnish major mutation is around 1% in eastern Finland, but significantly lower in southwestern parts of the country (Hoglund et al. 1996b).

The wide spectrum of mutations suggests that novel mutations in the CLD gene are relatively common. At least codon 120, found to harbor the mutation G120S in three independent families, is one of the mutational hot-spots. Despite the various types of mutations and their wide distribution in different regions of the SLC26A3 gene, evidence of genotype–phenotype differences in CLD is non-existent (Makela et al. 2002). Even the identical genetic background of CLD may, however, result in variable types of clinical courses if the diagnosis is delayed or salt substitution insufficient (Holmberg et al. 1977a, Hoglund et al. 2001a).
<table>
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<th>Mutation</th>
<th>Exon</th>
<th>Nucleotide change</th>
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**Polymorphisms**

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3 Pathophysiology of CLD

3.1 Basic structure of the intestine

The intestine comprises two parts, the small and large intestine, with different structural and functional properties. The small intestine involves the duodenum, jejunum, and ileum. The colon, or the large intestine, comprises the cecum and ascending, transverse, descending, and sigmoid colon (Gartner and Hiatt 1997). The parts of the four-layered intestinal wall are the mucosa, submucosa, muscularis externa, and serosa. The mucosa is further divided into the epithelium, a connective tissue layer called the lamina propria, and a smooth muscle layer called the muscularis mucosa. In the inter villar spaces of the small intestine and in the basal foldings of the colon, the lamina propria forms the core of the crypts of Lieberkühn (Figure 3) (Gartner and Hiatt 1997).

The main structural difference between the small intestine and colon is the folded epithelium of the small intestine, involving villi and microvilli to increase the absorptive surface of the epithelium. Enterocytes, the major cell population of the intestinal epithelium, form a simple columnar layer of cells which are responsible for the absorptive actions of the intestine. As the life-span of enterocytes is short, around 3 to 5 days, continuous maturation of the regenerative cells in the basal crypts and migration of these cells into the surface epithelium occurs. In addition to regenerative cells, the proliferation zone of the crypts of Lieberkühn involves undifferentiated Cl secreting epithelial cells, and enteroendocrine cells which secrete hormones such as cholecystokinin, secretin, and gastric inhibitory peptide into the intestinal lumen. Mucus-secreting goblet cells appear throughout the intestine, mostly in the surface epithelium. In the basal crypts of the small intestine, Paneth cells act as an essential part of the antimicrobial barrier by releasing antimicrobial peptides into the luminal space (Figure 3) (Gartner and Hiatt 1997).

![Figure 3](image-url) Absorptive surface epithelium of the small intestine (left) involves finger-like villi, whereas the surface epithelium of colon (right) forms foldings into the lamina propria. Intestinal absorption, including the SLC26A3-mediated transport, occurs mostly through the surface epithelium, and intestinal secretion arises from crypts.
3.2 Main principles of intestinal absorption and secretion

Both the small and large intestine possess several essential mechanisms for absorption and secretion of electrolytes and fluid (Turnberg 1970, Turnberg et al. 1970). These mechanisms vary depending on the segment of the intestine. A simplified model is that small-intestinal secretion arises from crypts and absorption from villi. In the colon, crypts exhibit both secretory and absorptive actions, whereas the surface epithelium is absorptive only (Figure 3) (Field 2003). Additionally, the epithelium of the colon is less permeable than that of the small intestine, making active transport mechanisms crucial.

Intestinal fluid absorption is secondary to Na\(^+\) absorption, which is regulated by several ion transporters of the epithelial brush border, each depending on the segment of intestine (Field 2003).

In the upper small intestine, Na\(^+\) absorption is coupled with absorption of organic solutes, i.e., amino acids, oligopeptides, and sugars (Schultz et al. 1966). Absorption of organic solutes and salts and their further transport from enterocytes to the lateral intercellular space forms the osmotic gradient for paracellular water absorption. This coupled absorption of Na\(^+\) together with amino acids and sugars forms the basis for management of acute diarrheas with oral rehydration therapy (Schultz et al. 1966).

In the jejunum, Cl\(^-\) movement is passive, and electroneutral Na\(^+\) absorption occurs through the Na\(^+\)/H\(^+\) exchangers (NHEs). Of these, the isoforms NHE2 and NHE3 are expressed in the apical epithelium of the small intestine and colon (Hoogerwerf et al. 1996, Zachos et al. 2005). NHE3 seems, however, to be the most relevant transporter, since the knock-out mouse exhibits chronic diarrhea (Schultheis et al. 1998). In contrast, the NHE2 knock-out mouse suffers from gastric dysfunction only, without intestinal defects (Miller et al. 2002). The third NHE of the intestine, NHE1, is expressed on the basolateral membrane of the enterocytes. It regulates homeostasis by removing H\(^+\) ions from the cells, thereby affecting the apical secretion of HCO\(_3\)\(^-\) as well (Tyagi et al. 2000).

In the ileum and proximal colon, absorption of NaCl occurs predominantly via the coupled action of NHE3 and Cl\(^-\)/HCO\(_3\)\(^-\) exchangers (Field 2003). The two main Cl\(^-\)/HCO\(_3\)\(^-\) transporters involved in the anion exchange of the small-intestinal and colonic brush border are CLD-related SLC26A3 (Hoglund et al. 1996b) and the putative anion transporter (PAT1) alias SLC26A6 (Table 1) (Lohi et al. 2000). In the small intestine, the predominant anion transporter is SLC26A6, whereas the major transporter in the ileum and colon is SLC26A3 (Wang et al. 2002). In addition, several basolateral exchangers such as the Na\(^+\)/K\(^+\)-ATPase and Cl\(^-\) and K\(^+\) channels are necessary for development of the electrochemical gradient for intestinal absorption. In the distal colon, however, absorption of Na\(^+\) is electrogenic, occurring via an aldosterone-sensitive epithelial Na\(^+\) channel (ENaC) (Canessa et al. 1994).

Short-chain fatty acids (SCFA) produced from the nonabsorbed carbohydrates by colonic bacteria are involved in the intestinal absorption through several mechanisms such as apical SCFA/HCO\(_3\)\(^-\) and Cl\(^-\)/SCFA exchange. As the apical SCFA/HCO\(_3\)\(^-\) exchange is linked to the parallel Na\(^+\)/H\(^+\) exchange, absorption of SCFAs and especially that of butyrate promotes electroneutral NaCl and fluid absorption in the colon (Binder et al. 2005).
Intestinal secretion arises from the epithelial crypts and is secondary to anion secretion, especially that of the Cl\(^{-}\) channel CFTR, i.e., the cystic fibrosis transmembrane conductance regulator (Welsh et al. 1982, Riordan et al. 1989, Schwiebert et al. 1995). Cyclic AMP (cAMP) stimulates, via protein kinase A (PKA), intestinal secretion by opening the apical Cl\(^{-}\) channel CFTR and the basolateral K\(^{+}\) channel, which together promote the activity of the basolateral Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (Geck and Heinz 1986, Greger 2000, Kunzelmann and Mall 2002, Field 2003). CFTR directly mediates not only Cl\(^{-}\) but also HCO\(_3\)\(^{-}\) secretion throughout the intestine (Trezise and Buchwald 1991, Strong et al. 1994). At least in the small intestine, the CFTR channel is able to modulate intestinal HCO\(_3\)\(^{-}\) secretion by acting in conjunction with the apical Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchangers of the SLC26 family (See Section 3.3.2) (Geibel et al. 2000, Field 2003, Lamprecht and Seidler 2006).

In most cases with diarrhea, more than one of the driving forces—osmosis, active secretion, exudation, or altered motility of the intestine—contribute (Field 2003). In ulcerative colitis, both the absorption of Na\(^{+}\) and that of Cl\(^{-}\) and water are defective. The underlying cause seems to be the decreased electrogenic Na\(^{+}\) absorption through ENaC in the distal colon, although the colonic electroneutral NaCl transport, occurring mostly through NHE3 and SLC26A3, is diminished as well (Sandle 2005).

### 3.3 Pathophysiological basis of CLD

Although intestinal absorption and secretion form separate entities, they constitute a complex network of transporters, the regulation of which is essential for both the absorptive and secretory actions of the intestinal epithelium. Regarding CLD, even early studies revealed inhibited Na\(^{+}\) absorption, despite the primary defect of the epithelial Cl/base exchange (Holmberg et al. 1975). Later studies characterized the basic defect of CLD as loss of the apical Cl/HCO\(_3\)\(^{-}\) exchange activity in the ileum and colon (Moseley et al. 1999). Secondarily, accumulation of Cl\(^{-}\) and H\(^{+}\) into the luminal space disrupts epithelial Na\(^{+}\)/H\(^{+}\) transport, leading to intestinal loss of both NaCl and fluid, and to the profuse diarrhea of CLD. In untreated disease, hypochloremia, hyponatremia, and dehydration result in activation of the renin-angiotensin system. The resultant hyperaldosteronism induces Na\(^{+}\) reabsorption in the distal colon and especially in the distal tubule of the kidney, resulting in the secondary K\(^{+}\) depletion which leads to an increase in both the hypokalemia and metabolic alkalosis in untreated CLD (Holmberg 1978).

The SLC26A3 protein is the colon’s most essential apical anion transporter. Evidence for this is the life-threatening diarrheal phenotype of CLD, and recently, existence of SLC26A3-deficient mice with a diarrheal phenotype closely similar to that of CLD (Schweinfest et al. 2006). SLC26A3-deficient mice exhibit, however, unique features uncharacteristic of human CLD. These include significant growth retardation, occasionally development of prolapsed rectums, and most interestingly, an aberrant growth pattern of the colonic mucosa with an expanded proliferative zone of colonic crypts and hyperplasia of the surface epithelium (Schweinfest et al. 2006).
PDZ domains are the most common protein interaction modules of the human genome. The abbreviation “PDZ” comes from the names of the first three proteins found to comprise this domain: postsynaptic density protein PSD95, the Drosophila homologue Disc-large, and the tight junction protein ZO-1. The basic structure of PDZ domains comprises 90 to 100 amino acids involving two α-helices and six β-sheets which form a hydrophobic binding site for PDZ motifs (Harris and Lim 2001). In most of the PDZ adapter proteins, several PDZ domains with variable binding abilities exist with other common protein interaction domains. Binding of several proteins to one PDZ adapter protein is therefore likely to allow formation of larger interactive protein networks (Brone and Eggermont 2005, Lamprecht and Seidler 2006).

In the SLC26A3 protein, the PDZ motif comprises four C-terminal amino acids, glutamate-threonine-lysine-phenylalanine (ETKF) (Figure 2) (Lamprecht et al. 2002). This domain is able to bind to several PDZ adapter proteins: NHE3 kinase A regulatory protein (E3KARP), NHE3 regulatory factor (NHERF), and PDZ domain protein kidney 1 (PDZK1) (Lamprecht and Seidler 2006).

In the ileum and proximal colon, electroneutral NaCl absorption occurs through the coupled activity of NHE3 and SLC26A3 (Figure 4) (Melvin et al. 1999). Although NHE3-related human diseases remain non-existent, severe diarrhea in both NHE3 knock-out mice and in SLC26A3-deficient humans and mice reveal the crucial role of these proteins. To underline the importance of the coupled transport, NHE3 knock-out mice have increased levels of intestinal SLC26A3 mRNA, and SLC26A3-deficient mice show massive upregulation of intestinal NHE3 mRNA and protein. In addition, SLC26A3-deficient mice exhibit significant upregulation of the other Na+ channel, aldosterone-sensitive ENaC, in the distal colon (Melvin et al. 1999, Schweinfest et al. 2006).

Although an exact model for the interaction between SLC26A3 and NHE3 is lacking, these proteins are coupled at least via E3KARP, NHERF, and PDZK1. Most of the knock-out models for these PDZ adapter proteins show only minor intestinal defects, suggesting that intestinal networks of transporters rather than single regulators are crucial (Lamprecht and Seidler 2006). In many diarrheal conditions, the electroneutral NaCl absorption of the intestine is affected (Kunzelmann and McMorran 2004). It remains unclear, however, whether endogeneous substances, drugs, or bacterial and viral enterotoxins affect directly or through PDZ-interacting proteins the activity of NHE3.

In addition to NHE3, other PDZ-interacting proteins may also be of importance for the function of SLC26A3. For instance, cytosolic carbonic anhydrase II (CAII) is required for the full SLC26A3-mediated bicarbonate transport in cell cultures. This direct interaction of SLC26A3 and CAII is, however, unable to stimulate the transport activity of SLC26A3 (Sterling et al. 2002). In the small intestine, SLC26A3 is expressed together with CFTR, and both these proteins are capable of binding to the PDZ domains of PDZK1. SLC26A3 and CFTR may thus regulate HCO₃⁻ secretion in the small intestine, but most probably, not in the colon (See Section 3.3.2) (Rossmann et al. 2005).
3.3.2 STAS domain coupling SLC26A3 with CFTR

The activation of SLC26A3 is increased in the presence of CFTR, and vice versa. The basis for this interaction is phosphorylation of the cytosolic regulatory domain, R domain, of CFTR and its binding to the STAS domain of SLC26A3 (Figure 2) (Aravind and Koonin 2000, Ko et al. 2002). In addition to this domain interaction, binding of these proteins to the PDZ adapter proteins facilitates the interaction by some unspecified mechanism (Ko et al. 2004).

The early studies showed that the primary defect in cystic fibrosis (CF) is reduced activity of the Cl$^{-}$ channel CFTR (Kerem et al. 1989, Riordan et al. 1989, Bear et al. 1992). Later studies illustrated, however, that the defective Cl$^{-}$ transport actually results from the inability of the mutant forms of CFTR to activate the HCO$_3$- transport through Cl$^-$/HCO$_3$- exchangers (Lee et al. 1999a, Lee et al. 1999b). That mild phenotypes of CF are associated with normal Cl$^{-}$ channel activity, but with aberrant HCO$_3$- secretion only, was a notable finding (Sheppard et al. 1993, Choi et al. 2001a). Further studies showed that CFTR specifically up-regulates Cl$^-$/HCO$_3$- exchange activity of the SLC26 gene family members SLC26A3, SLC26A4, and SLC26A6 (Greeley et al. 2001, Ko et al. 2002). The interaction of these transporters is based on the binding of the R domain of CFTR into the STAS domains of those SLC26 family members. In addition, this interaction is enhanced when CFTR is activated by phosphorylation or by some PDZ-interacting proteins (Ko et al. 2004).

The present model suggests that in the pancreatic duct, for instance, the interaction between CFTR and SLC26A6 takes place in the proximal part of the duct, whereas in more distal parts, interaction of CFTR and SLC26A3 occurs (Ko et al. 2004). On the other hand, the CFTR channel alone is able of secreting both Cl$^{-}$ and HCO$_3$- anions, making a more complex model than this one probable (Linsdell et al. 1997, Spiegel et al. 2003).
regulatory action between CFTR and SLC26A3 (Figure 5) seems to be essential for epithelial HCO$_3^-$ secretion in the tissues where both the transporters are expressed, meaning at least the small intestine (Moseley et al. 1999, Greger 2000, Jacob et al. 2002, Rossman et al. 2005), pancreatic duct (Greeley et al. 2001, Ko et al. 2004), and tracheal epithelium (Wheat et al. 2000). The essential physical association between CFTR and SLC26 transporters requires intact STAS and R domains. As at least two CLD-causing mutations on the STAS domain of SLC26A3 prevent the activation of CFTR as well, CFTR may also play a role in the pathogenesis of CLD (Ko et al. 2004).

**Figure 5**  
SLC26A3 is coupled with CFTR in epithelial cells of the pancreatic duct.

### 3.3.3 Mutation-associated pathophysiology in CLD

Functional data on mutant forms of SLC26A3 are limited. Regarding the Finnish founder mutation V317del (Figure 2), the anion exchange activity is lacking despite the normal levels of SLC26A3 mRNA and protein in the CLD colon (Hoglund et al. 1996b, Melvin et al. 1999, Moseley et al. 1999). An associated feature of the mutation V317del is a substitution of tryptophan for cysteine ten codons upstream (C307W), the frequency of which alone is around 14% in the healthy Finnish population. This C307W variant is, as expected, compatible with normal anion exchange activity and is therefore considered a meaningless SLC26A3 polymorphism (Hoglund et al. 1996b, Moseley et al. 1999).

The mouse V317del-homologue mutant V310del is retained in the endoplasmic reticulum, failing to show any expression on the apical plasma membrane (Ko et al. 2002). Similar to the human V317del, some other CLD-associated mouse mutants, e.g., L489R and I668-669ins, are expressed on the plasma membrane. None of the mutants show, however, any Cl$^-$/HCO$_3^-$ exchange activity in cell cultures (Ko et al. 2002, Chernova et al. 2003). These findings are in line with the fact that data on genotype-phenotype differences in CLD are lacking (Makela et al. 2002).
4 Molecular genetics of male infertility

4.1 Basic structure and electrolyte economy of the male reproductive tract

Mammalian sperm is produced in the seminiferous epithelium of the testis. Testicular tissue, surrounded by a connective tissue capsule, is divided into hundreds of lobules with seminiferous tubules. Between these tubules appear septal walls and androgen-secreting Leydig cells (Holstein et al. 2003).

The seminiferous epithelium (Figure 6) comprises different developmental stages of spermatogonia, primary and secondary spermatocytes, and spermatids. Between these maturing cells appear Sertoli cells. They form invaginations and provide both nutrition and supportive functions for the germ cells. Moreover, Sertoli cells organize the delivery of mature spermatids to the testicular lumen and secrete various endocrine and paracrine substances to regulate spermatogenesis (Holstein et al. 2003).

In addition to producing spermatozoa, the seminiferous epithelium of the testis secretes fluid to the male reproductive tract (Yeung et al. 1991, Holstein et al. 2003). Of this fluid, as much as 95% is already reabsorbed in the initial zone of the efferent ducts (ED) of the testis before spermatids pass into the epididymis (Figure 6). Fluid reabsorption in the ED occurs via a paracellular route and through the water channels of the non-ciliated cells, whereas the ciliated cells take up other substances by endocytosis (Clulow et al. 1998). Fluid reabsorption is essential for both sperm concentration and maintenance of the optimal microenvironment for sperm maturation. During transport through the epididymal ducts, the composition of sperm is gradually changed by both reabsorptive and secretory actions before storage in the distal epididymis and vas deferens. The underlying mechanisms for this fluid reabsorption in both the ED and epididymis are poorly understood. Regulation of this reabsorption is an unclear process as well, although at least flow, luminal conditions, and sex steroids contribute (Clulow et al. 1998).

As the fluid reabsorption is coupled to active solute transport, a striking change in the luminal environment of the ED is the decline in concentrations of Na\(^+\), K\(^+\), and Cl\(^-\). In rodents, concentrations of Na\(^+\) and Cl\(^-\) decrease from 100 mmol/l in the testis to 25 mmol/l in the distal epididymis (Jenkins et al. 1980). The subsequent increase in osmolality allows the shrinkage of epididymal sperm. This sperm volume regulation is a crucial feature for both sperm morphology and motility. For instance, incubation of sperm with K\(^+\) raises sperm volume and, at the same time, reduces the efficiency of forward progression (Yeung et al. 2003).

One of the most well-established transporters in the ED is the apical Na\(^+\)/H\(^+\) exchanger NHE3. In rodents, it is expressed on the apical membrane of the non-ciliated cells responsible for fluid reabsorption (Bagnis et al. 2001, Kaunisto and Rajaniemi 2002). Other apical transporters in the mouse non-ciliated cells include the water channel aquaporin-1 (AQP1) and CFTR (Fisher et al. 1998, Leung et al. 2001a). Although NHE3 is the major transporter for reabsorption, uptake of luminal fluid in the ED is dependent on movements of both Na\(^+\) and Cl\(^-\). In this regulated process, a possible apical Cl\(^-\)/HCO\(_3^-\) exchanger may be important, acting in parallel with NHE3 (Hansen et al. 2004). Of the
SLC26 anion exchangers, SLC26A6 appears on the apical membrane of the non-ciliated cells of the human ED (Kujala et al. 2007). In addition, expression of SLC26A3 mRNA emerges in the mouse ED (Lee et al. 2001). The role of these SLC26 family members in the ED remains, however, undetermined.

Spermatozoa reach their competence in motility during their passage through the ducts of the epididymis, in which reabsorption occurs at a much lower rate than in the ED. Whereas Na$^+$-dependent fluid reabsorption in the epididymis is low, anion secretion is crucial for the balance of the luminal microenvironment. Both NHE3 and CFTR are expressed in the apical membrane of the epididymal ducts in rodents. The data on the precise cell types involved are inconsistent (Bagnis et al. 2001, Leung et al. 2001b). Of the SLC26 family members, SLC26A6 appears on the apical mitochondria-rich cells (AMRCs) of the epididymis (Palacios et al. 1991) and SLC26A7 on the basolateral membrane of the basal cells (Kujala et al. 2007), suggesting their role in the epididymal anion exchange.

An essential feature in both the ED and epididymal transport is sperm acidification by proton pumping through the apical vacuolar proton-transporting ATPase (V-ATPase). This acts to promote an acidic microenvironment for the storage of sperm in the cauda epididymis and vas deferens. The acidic luminal environment keeps spermatozoa immotile during storage, and allows motility only after neutralization by the alkaline fluids from the prostate and seminal vesicles (Breton et al. 1996, Herak-Kramberger et al. 2001). HCO$_3^-$ ions alone are able to activate sperm motility during ejaculation (Okamura et al. 1985). Furthermore, CFTR-mediated HCO$_3^-$ secretion from the endometrial epithelium is required for sperm capacitation and egg-fertilizing ability (Wang et al. 2003).

Figure 6  Cross-section of a seminiferous tubule from a patient with CLD (left), and arrangement of the tubules of the testis and epididymis (right).
4.2 Genetic causes of male infertility in humans

4.2.1 Chromosomal abnormalities

Primary failure of spermatogenesis, resulting in azoospermia (no sperm in the semen) or oligozoospermia (sperm count <5 million/ml), is often caused by numerical or structural chromosomal abnormalities (Gianotten et al. 2004). In addition to various karyotype changes, these defects include Y-chromosomal deletions. Both partial and complete deletions in the *azoospermia factor* (AZF) locus of the Y chromosome result in a failure of spermatogenesis and in various abnormalities of testicular histology (Pryor et al. 1997). The most common consequence of Y-chromosomal deletions is azoospermia (Tiepolo and Zuffardi 1976). Among Finnish azoospermic/oligozoospermic men, their 9% incidence of Y deletions equals figures from other countries (Aho et al. 2001).

4.2.2 Cystic fibrosis and CBAVD

A hallmark of cystic fibrosis (CF) is congenital bilateral absence of the vas deferens (CBAVD) (Kaplan et al. 1968). In addition, CF involves various anomalies of the seminal vesicles (Olson and Weaver 1969). Despite the obstructive azoospermia and infertility in CF, spermatogenesis is active, but spermatozoa morphology may be abnormal (Denning et al. 1968). CFTR may thus play a primary role in anion secretion not only in the epididymis and vas deferens (Tizzano et al. 1994), the sites of the anatomical defect, but also in the seminiferous epithelium (Gong et al. 2001).

CBAVD, together with anomalies of the seminal vesicles, emerges also as a distinct entity in otherwise healthy men (Meschede et al. 1997). In these subjects with CBAVD and infertility only, mutations in the CFTR gene are common. As the frequency of at least one mutated CFTR allele totals as much as 70 to 80% (Anguiano et al. 1992), CBAVD can be considered a mild reproductive form of CF. Many of the patients with CBAVD only are heterozygous for CFTR mutations, or even homozygous for two mild mutations (De Braekeleer and Ferec 1996). The most common cause of CBAVD is, however, heterozygosity for both one CFTR mutation and one 5T allele in the intron-8 polymorphic polythymidine tract (IVS8-T; alleles 5T, 7T, or 9T) of CFTR (Chillon et al. 1995). The 5T allele in the intron 8/exon 9 splice-acceptor site results in alternative splicing of mRNA, and in skipping of exon 9 (Chu et al. 1993). As a low number of normal CFTR transcripts leads to defective activity of the CFTR channel, the 5T allele is regarded as a mild mutation. When present in *trans* with another CFTR mutation, 5T can result in male infertility, non-classic CF, or even in the normal phenotype (Chillon et al. 1995, Meschede et al. 1997). The underlying cause of various 5T-associated phenotypes seems to be the number of the adjacent TG repeats, ranging from 10 to 13. Long TG repeats together with the 5T allele are more likely to result in aberrant CFTR splicing and in increased disease penetrance (Groman et al. 2004).
The presence of only one 5T allele or CFTR mutation in some males with CBAVD, or even in those with idiopathic oligozoospermia, suggests that some other genetic defects may contribute (van der Ven et al. 1996, Meschede et al. 1997, Larriba et al. 1998, Schulz et al. 2006). As the first evidence of compound genetic defects, heterozygosity for Y-chromosomal deletions and CFTR mutations may lead to idiopathic male infertility (Karpman et al. 2007).

In Finland, CF is a very rare entity with an incidence of 1:25 000, being one of the lowest among Caucasian populations (Kere et al. 1994). The Finnish mutation spectrum of CFTR is unique, as well (Kinnunen et al. 2005). Among Finns, the role of CFTR mutations or the 5T allele in male infertility remains unclarified.

4.2.3 Other causes

Mutations or expanded CAG repeats in the androgen receptor (AR) gene are associated with male infertility (Gianotten et al. 2004). Among Finns, some rare AR variants may account for male infertility, whereas CAG repeats seem to be uncommon (Lund et al. 2003a, Lund et al. 2003b). In addition to AR, the estrogogenic pathway and especially variants in the estrogen receptor α (ERα) gene may play a role in male infertility (Galan et al. 2005). As for other candidate genes for male infertility, functional evidence is limited or lacking (Gianotten et al. 2004).

4.3 Genetic causes of male infertility in mice

4.3.1 Estrogen receptor α and NHE3

In mice, an essential gene for male fertility is estrogen receptor α (ERα). Estrogen promotes, through ERα, the NHE3-mediated fluid reabsorption in the ED of the testis (Zhou et al. 2001, Hess 2003). Targeted disruption of ERα results in downregulation of NHE3, and inhibition of the Na⁺-dependent fluid reabsorption. The resulting accumulation of fluid in the ED, dilation of the tubules of the ED, and dilution of sperm lead to infertility. Moreover, disrupted epithelial cytoarchitecture in the ED causes obstruction and reflux of the accumulating fluids, inducing testicular atrophy (Hess 2003).

Similar to that seen in ERα-deficient mice, accumulation of fluid into the ED and infertility emerge also in NHE3 knock-out mice, making NHE3 probably the most critical protein for fluid reabsorption in the ED (Zhou et al. 2001).

In addition to NHE3, estrogen regulates the mRNA levels of several other transporters in the mouse ED. Of these, CFTR and SLC26A3 are, unlike NHE3, regulated by estrogen receptor β (ERβ) (Lee et al. 2001). Interestingly, the reproductive capacity of ERβ knockout mice is totally normal (Ogawa et al. 1999). Similarly, CFTR knock-out mice exhibit normal reproductive function (Souwaert et al. 1992), although CFTR mutations cause male infertility in humans.
4.3.2 Defective ion transport during spermatogenesis

Defects in ion transport during spermatogenesis seem to play a role in mouse male infertility. Targeted disruption of the Cl-/HCO$_3^-$ exchanger AE2, which is expressed in developing spermatozoa, causes infertility by interrupting spermatogenesis (Medina et al. 2003). Similarly, disruption of the Na$^+$-K$^+$-2Cl$^-$ cotransporter (NKCC1), which shows a wide pattern of expression in the seminiferous epithelium, causes defective spermatogenesis (Pace et al. 2000). In addition, defects in sperm volume regulation are associated with poor motility of sperm and mouse male infertility (Yeung et al. 2000).

5 Chloride transport in the kidney cortical collecting duct

5.1 Cell types and transporters

The cortical collecting duct (CCD) of the kidney exhibits several mechanisms for Cl$^-$ transport. These include voltage-driven paracellular absorption, the driving force of which is Na$^+$ reabsorption through the aldosterone-sensitive epithelial Na$^+$ channel ENaC of the principal cells. The majority of the Cl$^-$ transport is transcellular, however, occurring through the intercalated cells of CCD (Schuster and Stokes 1987).

Intercalated cells are either H$^+$-secreting type A or HCO$_3^-$-secreting type B cells. The third type is non-A non-B cells (Kim et al. 1999). Between the intercalated cells are located principal cells which reabsorb Na$^+$ through apical ENaC, and luminal water through the apical water channel aquaporin 2 (AQP2) (Figure 7) (Nielsen et al. 1993).

In the type A intercalated cells, apical V-ATPase excretes protons into the urine (Wagner et al. 2004). At the same time, HCO$_3^-$ ions are transported into the blood through a basolateral Cl-/HCO$_3^-$ exchanger called the anion exchanger 1 (AE1) (Kim et al. 1999). In the type B intercalated cells, transporter processes are opposed by an apical Cl-/HCO$_3^-$ exchanger SLC26A4, excreting HCO$_3^-$ into the urine (Royaux et al. 2001), and basolateral V-ATPase, transporting H$^+$ into the blood (Kim et al. 1999). The transport processes of the non-A non-B intercalated cells remain undetermined. In these cells, V-ATPase is present on the basolateral membrane, and both V-ATPase and SLC26A4 are present on the apical membrane (Figure 7) (Kim et al. 1999).
5.2 Regulation of chloride transport

As Cl⁻ is part of the transport processes in both the apical and basolateral membrane of the intercalated cells, Cl⁻ is needed for both the acidification and alkalinization of the urine, meaning that Cl⁻ transport in the CCD is essential for the final regulation of acid-base balance (Schuster and Stokes 1987).

The apical Cl⁻/HCO₃⁻ exchanger SLC26A4 is regulated in response to changes in Cl⁻ balance. Cl⁻ depletion specifically upregulates SLC26A4, increasing both the reabsorption of Cl⁻ and excretion of HCO₃⁻ (Quentin et al. 2004, Vallet et al. 2006). Thus, upregulation of SLC26A4 may attenuate the development of metabolic alkalosis during salt restriction. Accordingly, during salt depletion, SLC26A4-deficient mice are more prone to the development of metabolic alkalosis (Royaux et al. 2001).

In addition to the regulation of acid-base status, SLC26A4 is essential for the maintenance of blood pressure and for fluid and electrolyte balance. Aldosterone specifically upregulates SLC26A4 on the type B intercalated cells of the CCD, resulting in increased NaCl uptake, volume expansion, and hypertension (Royaux et al. 2001). To support the central role of SLC26A4 in the regulation of blood pressure, SLC26A4-deficient mice fail to undergo an increase in blood pressure in response to aldosterone analogues (Verlander et al. 2003).
Aims of the study

The aims of this study were

1) to characterize the long-term outcome of CLD in a unique sample of Finnish patients

2) to search for novel manifestations of CLD, based on extraintestinal expression of the *SLC26A3* gene

3) to study the effectiveness of oral butyrate in resolving the diarrheal symptoms of CLD
Materials and methods

1 Study subjects and samples

1.1 Patients with CLD (I, II, IV, V)

By connecting genetic studies (Hoglund et al. 1996b) and collaboration with the pediatric gastroenterologists of the Finnish university hospitals, we ascertained that altogether 46 patients aged over seven years had had a diagnosis of CLD. Of these, one had died accidentally, two had been lost to follow-up, and seven refused to participate. The remaining 36 patients (ages 10-38; median 21), comprising 20 females (56%) and 16 males (44%), were enrolled. They belonged to 27 families, of which four had two affected, and three had three affected children. All patients presented a typical clinical picture of CLD, with a high concentration of fecal Cl (>90 mmol/l) (Holmberg 1986). Mutational analysis of the SLC26A3 gene was available for 31 (86%) patients, and showed a homozygous V317del genotype (n=30) or a heterozygous V317del/344delT genotype (n=1).

For the first part of the study (I), the patients were divided into subgroups based on their decade of birth: 1960s (n=8; ages 31-38), 1970s (n=7; ages 22-30), and 1980s (n=21; ages 10-21). The 1980s group comprised three patients actually born in 1990 or 1991.

In the second part of the study (II), the fertility potential was assessed in eight Finnish males (ages 18-38; median 29.5) with CLD. All of them had participated in the first part of the study, as well. All but one of the men had an established genetic background of CLD with the homozygous V317del genotype.

In the fourth part of the study (IV), the influence of butyrate was under study in a series of five patients with CLD. Of these, three belonged to the original study population of 36 patients (I), but the two youngest were enrolled as new participants in the butyrate trial. Peripheral blood DNA samples were available from all these patients and were later sequenced for all SLC26A3 exons (patient 1) and analyzed for CFTR mutations.

In the fifth part of the study (V), renal outcome was assessed among the original study sample of 36 patients (I). Data on one male patient (homozygous V317del genotype) with renal transplantation due to end-stage renal disease (ESRD) were assessed as a separate case (case 1). Material for another case (case 2) came from a deceased Swedish girl (homozygous H124L genotype) with a CLD-associated ESRD and renal transplantation.
1.2 Tissue samples (II, III, V)

Testis biopsy specimens (n=2), one containing also the ED region of the testis, were obtained during operations for large spermatoceles from two males (aged 29 and 30) with the homozygous V317del genotype for CLD (II). Specimens were sectioned, fixed with formaldehyde, and embedded in paraffin. Sections with a thickness of ~5 µm were stained with hematoxylin and eosin for histological analyses (II) or underwent immunohistochemical procedures for expression studies (III). Control samples were archival specimens from adult men (aged 22-65) obtained from the Department of Pathology, HUSLAB, Helsinki University Central Hospital. The specimens involving both testis and epididymis (n=11) were from patients orchiectomized for seminoma, cysta dermoides, cystadenoma, carcinoma embryonale, or teratoma. The seminal vesicles (n=9) and prostates (n=4) were from patients with radical prostatectomies due to focal prostatic carcinoma. Original reports of the pathologists were reviewed before use of histologically healthy samples with normal spermatogenesis (III). Stages of spermatogenesis were assessed (Holstein et al. 2003), and specimens of the ED and epididymis were divided histologically into initial and terminal zones according to their anatomical positions on the slides and based on their cell morphology and location (Robaire and Hinton 2002). In the seminal vesicles and prostates, higher glandular and lower unreactive ductal epithelium were histologically recognized (Gartner and Hiatt 1997).

One kidney biopsy specimen from case 1, taken before transplantation at the age of 19, was available for immunohistochemistry (V). In addition, kidney biopsy slides after transplantation, stained with hematoxylin and eosin or von Kossa stain for calcium deposits, were available from both case 1 and case 2. Archival human kidney samples came from the Department of Pathology, HUSLAB, Helsinki University Central Hospital. The nephrectomy specimens (n=5) were from patients with focal nephroblastoma or renal cell carcinoma, and kidney biopsy samples (n=5) from healthy kidney donors. Histologically healthy areas of the samples were used, after review of the original reports of the pathologists (V).

Frozen cortical samples from normal human kidneys (n=3) not needed for transplantation use and cortical samples from patients nephrectomized for congenital nephrotic syndrome (CNF) (n=9) were the gift of a collaborator (H. Jalanko) for reverse transcriptase polymerase chain reaction (RT-PCR) (V). Non-glomerular cortical portions of healthy human kidneys (n=4) not needed for transplantation use were obtained correspondingly for western blotting.

Control complementary DNA (cDNA) samples (V) were from human multiple tissue cDNA panels (kidney and skeletal muscle: Clontech, Mountain View, CA, USA), and from Gene Pool cDNA (colon and kidney: Invitrogen, Carlsbad, CA, USA).

Commercial human tissue lysates from kidney, colon (positive control), and liver (negative control) were used in western blotting (V) (Zyagen Laboratories, San Diego, CA, USA).
2 Methods

2.1 Clinical analysis of long-term outcome (I, V)

Participants’ medical data were reviewed from birth. Clinical studies were performed at the pediatric units of the university hospitals in Helsinki (patients, n=17), Oulu (n=12), or Kuopio (n=7). From current heights and weights, height standard deviation (SD) scores were compared with the Finnish reference values (Sorva et al. 1990). In adults, body mass index (BMI) 19 to 25 kg/m² was regarded as normal. In children, BMI cut-off points for overweight were based on age and sex (Lindgren et al. 1995).

Laboratory studies involved 24-h blood pressure recording, chest X-ray, electrocardiography (ECG), and renal ultrasound with an assessment of measured renal lengths as SD scores (Rosenbaum et al. 1984, Miletic et al. 1998). Laboratory tests were total blood count, erythrocyte sedimentation rate, venous blood gas analysis (Astrup), serum K⁺, Na⁺, Cl⁻, Ca²⁺, Mg²⁺, Pi, uric acid, alkaline phosphatase, creatinine, urea nitrogen, osmolality, plasma renin activity, and aldosterone. Standardization of the two different radioimmunoassay techniques for aldosterone measurements (DiaSorin, Inc., Stillwater, MN, USA; DPC, Los Angeles, CA, USA) was performed in the laboratory of HUSLAB, Helsinki University Central Hospital. Concentrations of fecal K⁺, Na⁺, and Cl⁻ were measured from single specimens. Urine analyses (24-h collection) were for pH and for concentrations of K⁺, Na⁺, Cl⁻, Ca²⁺, Mg²⁺, Pi, and protein. Chrome 51 ethylenediaminetetraacetic acid (⁵¹Cr-EDTA) clearances allowed estimation of the glomerular filtration rates (GFR). Quantitative sweat tests with stimulation by pilocarpine iontophoresis furnished a record of sweat Cl⁻ excretion. The most of the laboratory data was assessed in study I, but investigations for renal function were reported separately (V).

The oral and dental examinations involved 28 patients. The decayed, missing, filled surfaces of teeth (DMFS) index allowed assessment of dental health, and the community periodontal index of treatment needs (CPITN) index of periodontal health. The quality of the dental enamel was visually evaluated, followed by panoramic radiographs of the dentition.

The patients completed a questionnaire including details of therapy, compliance, dietary factors, general state of health (five-step scale: excellent, good, satisfactory, relatively poor, poor), marital status, children, education, and employment.

Numerical data were analyzed by the Statistical Package for Social Sciences (SPSS) software versions 10.0 or 15.0. Significance was set at p<0.05. Fisher’s exact test compared differences in mortality between groups (I). Differences between laboratory data were determined two groups at a time by the Mann-Whitney U test (I), a test also used to compare relative renal lengths with patients’ relative heights (V). Spearman’s correlation coefficients (rₛ) were used to study relations between measurements of renal function and other laboratory data (V).
2.2 Clinical analysis of male fertility (II)

Eight males with CLD underwent physical examinations with venous blood samples taken to measure levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and inhibin B. Single semen samples were provided by masturbation (after an abstinence of 3-7 days). Sperm analyses were performed according to the WHO guidelines (Menkveld et al. 2001). Sperm motility classes were: A with rapid forward progressive spermatozoa, B with slowly forward progressive, C with non-progressive, and D with immotile spermatozoa. Normal values for sperm motility were ≥50% with forward progressive (categories A and B) or ≥25% with rapid progressive (category A) spermatozoa within 60 minutes of ejaculation. For sperm morphology, the reference for normal spermatozoa of ≥30% was used.

Patency of the vas deferens was assured by measurement of levels of neutral alpha-glucosidase (Lund University, Sweden). Secretory potential of the seminal vesicles was studied by measurement of seminal plasma fructose, and of the prostate by zinc measurement (Lund University). In addition, pH values and levels of seminal plasma K⁺, Na⁺, and Cl⁻ were assessed. To obtain reference values, seminal plasma pH, electrolytes, and fructose were measured in eight healthy males with pregnancy-proven fertility.

2.3 Butyrate trial (IV)

During the entire study, the five study patients received their salt substitution, followed a normal diet, and completed a daily diary to record their dosage of salt substitution and butyrate, possible adverse events, and diarrheal symptoms including number and consistency of stools (watery, semiformal, formed), soiling, abdominal distention, and stomach cramps.

Initially, these patients kept their diaries for five days during their normal salt substitution therapy, providing baseline data on their diarrheal symptoms. Thereafter, they received two daily doses of capsules with calcium and magnesium butyrate salts (Butyric acid complex; BioCare, UK) at a butyrate dose of 100 mg/kg/day for 10 days. After clinical evaluation, blood, urine, and fecal samples were obtained at the butyrate trial entry, immediately after the 10-day butyrate trial, and 20 days after halting the butyrate. Laboratory tests comprised total blood count, venous blood gas analysis, plasma concentrations for ammonium ion, unconjugated and conjugated bilirubin, and parathyroid hormone, plasma renin activity, serum K⁺, Na⁺, Cl⁻, Ca²⁺, Mg²⁺, Pi, osmolality, creatinine, aldosterone, urate, urea nitrogen, protein, albumin, glucose, alanine transaminase, aspartate transaminase, alkaline phosphatase, and gamma glutamyl transferase. Fecal K⁺, Na⁺, and Cl⁻, concentrations of the fecal inflammatory markers α1-antitrypsin and calprotectin, and concentrations of urine K⁺, Na⁺, Cl⁻, Ca²⁺, Mg²⁺, and Pi were measured from single specimens.
2.4 Antibodies (III, V)

Antiserum for SLC26A3, raised in rabbits against the synthetic peptide FNPSQEKDGDFT, corresponding to amino acids 732-745 of the published cDNA sequence, had been purchased from Research Genetics (Huntsville, AL, USA) (Haila et al. 2000). This antiserum was diluted to 1:350, and preimmune serum served as a negative control in consecutive sections (III, V).

Mouse monoclonal antibody against a synthetic peptide from the N-terminus of the human CFTR protein (CFTR Ab-2, MM13-4; NeoMarkers, Fremont, CA, USA) was used at 1-2 µg/ml, and normal mouse serum IgG fraction (NMIGG; Labvision, Fremont, CA, USA) served as a negative control in consecutive sections (III).

Rabbit polyclonal, affinity-purified antibody for the 22 C-terminal amino acids of the rat NHE3 (NHE-31A; Alpha Diagnostics International, San Antonio, TX, USA) was used at 1 µg/ml, and normal rabbit serum IgG fraction (NRIgG; DAKO, Hamburg, Germany) as a negative control in consecutive sections (III).

The following affinity-purified polyclonal antibodies were used in immunohistochemistry on kidney samples (V): rabbit anti-human vacuolar type H+-ATPase (V-ATPase) B1/2 (V-ATPase-B, sc-20943; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Kujala et al. 2005), rabbit anti-rat aquaporin 2 (AQP2) (AQP21-A; Alpha Diagnostics International, San Antonio, TX, USA), and mouse anti-human Pendrin (alias SLC26A4) (MBL, Woburn, MA, USA) (Royaux et al. 2001). NRIgG and NMIGG served as negative controls.

2.5 Immunohistochemistry (III, V)

Formalin-fixed and paraffin-embedded specimens were cut into ~5-µm sections on a microtome and mounted on glass slides. Antigens were retrieved by boiling in a 10-mM citrate buffer (pH 6.0) for 10 min (III, NHE3), 15 min (III, SLC26A3), or 20 min (III, CFTR; V). Immunohistochemistry was performed with EnVision kits (DakoCytomation, Carpinteria, CA, USA): EnVision+ System K4010 (SLC26A3, AQP2, and NHE3), EnVision+ System K4006 (SLC26A4 and CFTR), and EnVision Doublestain System K1395 (SLC26A3 and V-ATPase) according to manufacturer’s instructions. The sections were incubated with the primary antibodies SLC26A3 (1:350), SLC26A4 (0.2 µg/ml), CFTR (1-2 µg/ml), NHE3 (1 µg/ml), AQP2 (1 µg/ml) for 60 min, or at 4°C overnight (III, SLC26A3). The slides were stained with diaminobenzidine (DAB) for 8 min, and counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany). Finally, the slides were dehydrated, and cover slips were placed on top.

In doublestained slides, DAB staining was followed by a blocking agent provided by the kit supplier, and incubation with the V-ATPase antibody (1:1000) for 30 min at room temperature. The slides were stained with Fast Red for 4 min, counterstained with hematoxylin, treated with 0.037 mmol/l ammonia water, rinsed, and covered.
2.6 **CFTR mutation analysis (IV)**

To clarify the potential contributors to the diverse butyrate responses, we searched for possible butyrate-responsive variants of the *CFTR* gene (Kerem 2006). *CFTR* fragments were prepared by a multiplex amplification kit (Amplification *CFTR*; Innogenetics, Gent, Belgium) from DNA samples extracted from patients’ peripheral blood samples. INNO-LiPA CFTR17+Tn and CFTR19 test strips (Innogenetics) were used. These assays recognize IVS8-T polymorphism on intron 8, and the following 36 mutations: 621+1G>T, 3849+10kbC>T, 2183AA>G, 394delTT, 2789+5G>A, R1162X, 3659delC, R117H, R334W, R347P, G85E, 1078delT, A455E, 2143delT, E60X, 2184delA, 711+5G>A, F508del, G542X, N1303K, W1282X, G551D, 1717-1G>A, R553X, CFTRdel2,3(21kb), 1507del, 711+1G>T, 3272-26A>G, 3905insT, R560T, 1898+1G>A, S1251N, L148T, 3199delE, 3120+1G>A, and Q552X. These *CFTR* assays cover, in total, 87% of the Finnish *CFTR* mutations (Kinnunen et al. 2005).

2.7 Sequencing (IV)

In the butyrate-responsive subject (patient 1), exons (n=21) and exon/intron boundaries of the *SLC26A3* gene were amplified by PCR with intronic primers (Haila et al. 1998). PCR amplifications (10 µl/reaction) involved 20 ng of genomic DNA, 200 µM of each nucleotide, 2 mM MgCl\(_2\), 9 pmol of each primer, and 0.25U AmpliTaq Gold DNA polymerase with 1X buffer (Applied Biosystems, Foster City, CA, USA). The PCR protocol involved 1 cycle of 10 min at 94°C, 30 cycles of 94°C for 45 seconds, 57°C for 45 seconds, 72°C for 45 seconds, 72°C for 45 seconds, and 1 cycle of 72°C for 10 min.

For all subjects in the butyrate trial, the polymorphic IVS8-T locus of intron 8 of the *CFTR* gene and the number of adjacent TG repeats were analyzed with intronic primers: 5’-TAATGGATCATGGGCCATGT-3’ and 3’-ACAGTGTTGAATGTGGTCGA-5’ (Zielenski et al. 1991). PCR amplifications were performed as described for *SLC26A3*, except for the annealing temperature of 54°C for *CFTR*.

The fragments were characterized by direct sequencing (ABI 3730xl DNA Analyzer; Applied Biosystems) in one direction (*SLC26A3*) or two directions (*CFTR*) with the BigDye Terminator 3.1 chemistry (Applied Biosystems). SeqScape Software 2.5 (Applied Biosystems) was used for the analysis of *SLC26A3* sequences with the complete human *SLC26A3* DNA (GenBank accession NM_000111) as a reference sequence. Sequences of the polymorphic IVS8-T locus of *CFTR* were assessed manually.

2.8 RT-PCR (V)

Frozen cortical samples from human kidney were homogenized and used for total RNA extraction with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), after which cDNA was synthesized with the SuperScript III First Strand Synthesis System (Invitrogen,
Carlsbad, CA, USA) using random hexamers. The quality of cDNA was ensured by PCR for a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, Foster City, CA, USA).

SLC26A3 primers, 5’-AAACACCCGTAGGAGATTGCTTC-3’ and 3’-ATCAGCATTCCTTTAAGTTTCC-5’, were designed into exonic junctions to avoid amplification of genomic DNA. PCR assays were prepared in 25-µl volumes involving 4 µl cDNA template, 200 µM of each nucleotide, 0.4 µM of both primers, 4% dimethyl sulfoxide, 1 x Phusion GC Buffer, and 0.02 U/µl Phusion High-Fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland). The touch-down PCR protocol was: 98°C for 30 s, 35 cycles of 98°C for 10 s, 60°C minus 0.5°C per cycle for 30 s, and 72°C for 1 min 30 s, without final extension. PCR products of 348 bp, corresponding to nucleotides 1010-1357 of the published SLC26A3 cDNA, were subjected to electrophoresis on a 2% agarose gel. The products were purified with ExoSAP-IT (USB, Cleveland, Ohio, USA) and sequenced in both directions (ABI 3730xl DNA Analyzer; Applied Biosystems, Foster City, CA, USA).

2.9 Western blotting (V)

Kidney samples (n=7) were homogenized in Laemmli sample buffer with 5% β-mercaptoethanol. Electrophoresis was performed with 30 µg of each sample in a 9% polyacrylamide gel. The proteins were transferred onto a Hybond-C extra nitrocellulose membrane (Amersham Pharmacia Biotech, Bucks, UK) by standard protocols. After blocking with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST), pH 7.6, the membranes were incubated with the SLC26A3 antiserum (1:350), or with the preimmune serum (1:350), followed by incubation with peroxidase-conjugated goat anti-rabbit IgG (1:2000) (Roche Diagnostics, Mannheim, Germany). Chemiluminescence served for visualization of the protein bands (GE Healthcare, Princeton, NJ, USA).

3 Ethics

The study protocols involving all parts of the study were approved by the Ethics Committee of the Hospital for Children and Adolescents, University of Helsinki. Informed consent was obtained from each patient or, when appropriate, from parents.
Results

1 Long-term outcome in CLD

1.1 Survival, diagnosis, growth, and general state of health

After 1972, no CLD-associated deaths occurred (I, Figure 1). Mean age of patients at diagnosis of CLD was 2.7 months (range, 0-59) with a tendency towards earlier diagnoses during the most recent decades (I, Figure 2). Most patients (97%) had attended annual follow-up visits at the pediatric units until the age of 20. Later in life, ten patients (28%) made irregular follow-up visits for CLD.

The patients showed normal growth with a mean relative height of +0.07 SD, mean expected height being +0.17 SD. Among the oldest patients, early growth retardation was evident, but normalization of mean growth was observable by age three years (I, Figure 3). At the time of evaluation, ten patients (28%) were slightly overweight, and five (14%) underweight. An 18-year-old female with unspecified colitis and severe malnutrition had a BMI of only 11.7 kg/m$^2$.

In addition to CLD, a variety of other diseases emerged in this series of patients (I, Table 3), but in the questionnaires, 31% regarded their health as excellent, and 61% as good. Two patients defined their health as satisfactory, and only one as poor.

Most patients (89%) had had standard schooling; indications for some special education at comprehensive school were moderate learning difficulties (n=3) and mild sensorineural hearing loss (n=1).

1.2 Diarrhea and salt substitution

All patients reported watery diarrhea from two to seven (mean 3.6) times per day. Most patients had adjusted to their diarrhea, experiencing only minimal social disadvantages. None of the patients had marked abdominal distention, but soiling was a common finding; 53% of the patients aged under 20, and 32% over 20 reported occasional soiling during the night-time or during physical exertion. Three young adults (8%) with frequent soiling problems found the diarrheal symptoms of CLD disturbing.

To temporarily reduce the diarrhea, four patients had used short courses of cholestyramine (dose 2 g x 2/day), resulting in a moderate reduction in diarrhea for two to four weeks. In addition, 14% of the patients reported that dietary bulk reduced their diarrheal symptoms.

According to the past clinical guidelines, patients born in the 1960s had received salt substitution with KCl during their first four to ten years of life (Holmberg 1986), whereas the younger patients were already receiving increasing doses of NaCl and KCl from the time of their diagnoses (I, Table 1). Current daily doses of Cl$^-$ ranged from 0.9 to 5.3
(mean 2.8) mmol/kg per day. The substitution was taken as 2 to 5 doses per day as a ready-made solution (26 patients) of NaCl 18 g/l (308 mmol/l) and KCl 19 g/l (255 mmol/l), or as one dose at a time diluted in a glass of water (10 patients). Substitution therapy was well tolerated, but after ingestion of the salts, two patients (6%) experienced occasional stomach cramps.

Regular salt substitution was the rule for most patients (81%), but six patients had frequent breaks (>2 weeks), often after beginning to take responsibility for their own therapy (mean age, 12.9 years).

The youngest patients (group 1980s) achieved the most nearly optimal electrolyte and acid-base balance, with no hypokalemia, hypochloremia, or alkalosis, and adequate secretion of Cl\(^-\) into the urine in all subjects (I, Table 2). Of other laboratory tests, elevated erythrocyte sedimentation rates (>30 mm/h in four patients) were evident in 10 patients (28%). Serum creatinine, urea nitrogen, and blood pressure were normal in all but the single patient with a renal transplant, who showed a creatinine concentration of 455 \(\mu\)mol/l, urea nitrogen of 45 mmol/l, and blood pressure of 124/89 mm Hg. Chest X-rays and ECGs showed no abnormalities.

### 1.3 Major extraintestinal findings

#### 1.3.1 Intestinal inflammation

Solitary cases of intestinal inflammation emerged. A 37-year-old woman had a diagnosis of CLD at 2.5 months and Crohn’s disease at 18 years. In addition, a 18-year-old woman exhibited frequent watery diarrhea (6-7 times/day), soiling problems, enuresis, and weight deficit (BMI 11.7 kg/m\(^2\)) requiring intermittent parenteral nutrition. Despite extensive investigations, no other disease than CLD and unspecified colitis had been diagnosed in this patient with a homozygous V317del genotype for CLD. In her sister, a totally uneventful course of CLD occurred.

#### 1.3.2 Renal impairment

In our series, 10 patients (28%) fulfilled the criteria for chronic kidney disease (CKD) (V, Table 1). In both the one Finnish patient with CLD and ESRD (V, case 1) and in the Swedish patient with CLD, agenesis of the left kidney, and ESRD (V, case 2), the most striking renal feature was abundant nephrocalcinosis. It appeared in the patients’ own kidneys, and later, in their transplants as well (V, Figure 1). Ultrasonic signs of nephrocalcinosis were also common in other patients, although the decline in their GFR levels was only mild (V, Table 1).

Electrolytes, acid-base and fluid balance, and calcium-phosphate balance showed slight abnormalities only in individual patients, but urine was alkaline (pH>7) in 13 (37%) (V, Table 2).
Relative renal lengths, showing a positive correlation with GFR values, fell significantly below the patients’ relative heights (V, Figure 2).

The subgroup of patients with lower GFR levels (<110 ml/min/1.73m$^2$) had significantly lower excretion of urinary NaCl, and lower blood pressure, but higher erythrocyte sedimentation rates, as a non-specific measure of inflammation (V, Figure 3A-D). Regarding urinary measurements, a positive correlation appeared between urinary excretion of Ca$^{2+}$ and NaCl (V, Figure 3E-F). Inadequate urinary Cl$^-$ excretion (<83 mmol/day) was associated with lower Na$^+$ excretion and with higher urinary pH, but with no differences in venous blood HCO$_3^-$ or aldosterone (V, Figure 4).

1.3.3 Hyperuricemia

The patient with a history of ESRD and renal transplantation had gouty arthritis and an elevated serum urate level of 548 µmol/l despite allopurinol medication. Three additional patients in the 1960s group were hyperuricemic (364, 484, and 576 µmol/l) without gout. In renal ultrasound, two had calcifications. The mean concentration of serum urate was significantly lower in the 1980s group when compared with the 1960s (p<0.05) and the 1970s group (p<0.05).

1.3.4 Elevated sweat chloride

Mean sweat Cl$^-$ concentration was 36 (range, 8-70) mmol/l; 64% had a concentration below 40, 24% between 40 and 60, and 12% above 60 mmol/l. For screening cystic fibrosis with the sweat test, a concentration <40 mmol/l is regarded as negative, and >60 mmol/l as consistent with the disease (LeGrys 1996).

1.3.5 Inguinal hernias, spermatoceles, and male subfertility

Of 16 men, four (25%) had undergone surgery for inguinal hernia, three of them in infancy. Additionally, one adult had undergone surgery for spermatocele. As a novel finding, bilateral spermatoceles appeared in two adults.

Of the four men who lived in a permanent relationship with a woman, three had experienced infertility. None had naturally conceived children, but one had fathered a child through in vitro fertilization (IVF). By contrast, seven women with CLD had a total of 14 healthy children after uneventful pregnancies.

1.3.6 Infections

All but one patient had been hospitalized for acute gastroenteritis (AGE). Such hospitalization was most common in early childhood with a mean 3.6 (SD=2.7) episodes
during the first five years of life. Later in life, the incidence was 2.1 (SD=2.6) hospitalizations between ages six and ten, and 1.5 (SD=2.0) between 11 and 20. In adulthood, only four patients had been hospitalized due to AGE. Five patients (14%) had experienced at least one AGE with a dehydration-associated faint or convulsion. Of these, the most severe hypokalemic (K⁺ 2.7 mmol/l) episode had resulted in an out-patient resuscitation.

Urinary tract infections (UTIs) (range, 1-16 per patient) were documented in 13 patients (36%). The mean number of UTIs per patient during the first 10 years of life was significantly higher in the 1960s group than for the 1980s (p<0.01). Occasional enuresis occurred in 33% at school-age, 28% by the age of 11, and 8% by the age of 15.

1.3.7 Teeth

The low mean DMFS index (10.1) and low CPITN figures indicated good dental and oral health, and good periodontal state. Moderate enamel defects emerged in 43%, minor forms including opacities in 25%, and faultiness of dental enamel in 32%. Three patients had a permanent tooth congenitally missing, and a 37-year-old female had a sialolith in her right submandibular duct.

2 Male subfertility in CLD

All eight men had normal masculinization, testes sizes, and sexual function. Testis biopsy samples, taken from two patients during the operations for spermatoceles (Tables 3 and 4: patients 4 and 5), showed normal testicular histology.

Hormonal measurements were normal, but semen analyses revealed a low concentration of poorly motile spermatozoa with abnormal morphology, indicative for oligoasthenoteratozoospermia (sperm concentration <20 x 10⁶/ml and motility or normal forms or both <20%) in all but one patient with azoospermia (no sperm in semen) (Tables 3 and 4: patient 5). Any correlation between age and sperm parameters of the patients was non-existent.

Concentrations of seminal plasma Cl⁻ (Table 4) were significantly higher in the men with CLD (mean 66 mmol/l) than in their healthy controls (mean 39 mmol/l) (p<0.001), whereas concentrations of Na⁺ and K⁺ were equal. An abnormally low pH of seminal plasma emerged in three patients (Table 4), the difference in mean pH values between patients (pH=7.1) and pregnancy-proven healthy controls (pH=7.5) remaining insignificant (p=0.06). The levels of seminal plasma alpha-glucosidase were normal in CLD, proving the patency of the vas deferens. The levels of seminal plasma fructose in CLD and controls were equal, reflecting otherwise unaffected vesicular absorptive and secretory functions. The levels of seminal plasma zinc were normal, as well (unpublished data), indicating normal secretion of prostatic fluids.
Table 3  
*Sperm volume, count, and motility (WHO classification) in eight men with CLD (modified from II).*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Volume (&gt;2 ml)</th>
<th>Count (&gt;20x10^6/ml)</th>
<th>Total count (&gt;40x10^6)</th>
<th>Motility A (≥25%)</th>
<th>Motility A+B (≥50%)</th>
<th>Clinical data</th>
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<td>5</td>
<td>0</td>
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<td>11</td>
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</tr>
<tr>
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<td>36</td>
<td>4</td>
<td>3</td>
<td>12</td>
<td>0</td>
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<td>Infertility</td>
</tr>
<tr>
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<td>8.5</td>
<td>1.2</td>
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Table 4  
*Seminal plasma pH, electrolytes (mmol/l), and sperm morphology in eight men with CLD (modified from II).*

<table>
<thead>
<tr>
<th>Patient</th>
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<th>pH (&gt;7.2)</th>
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<th>K⁺</th>
<th>Cl⁻</th>
<th>Abnormal morphology (%)</th>
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<th>body (%)</th>
<th>tail (%)</th>
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</table>
3 Expression studies in the human male reproductive tract

3.1 Expression profiles of SLC26A3, CFTR, and NHE3

3.1.1 Seminiferous tubules of the testis

Positive staining for SLC26A3 appeared in the head of the elongating spermatids (Sb-Sc) of stages III to VI, but not in the fully developed spermatozoa. Immunoreactivity emerged at the cell periphery, first as a polarized labeling (stage III), secondly as a hood on the head of the elongating spermatid (stage IV), and finally as a band surrounding the center of the head of the spermatids (stages V-VI). The staining patterns in the males with CLD and the controls were equivalent (III, Figure 1).

Labeling of CFTR appeared in the pachytene (stages III-V) and diplotene spermatocytes (stage VI). The round staining patterns suggested the presence of CFTR on the cell membrane. In the elongating spermatids (Sb), a cranially polarized staining pattern (stage III) and a hood-like expression profile (stage IV) of CFTR were identical to those of SLC26A3. In the later stages of spermatogenesis and in the fully developed spermatozoa, expression of CFTR was absent, similarly to that seen for SLC26A3. The expression profiles of the males with CLD and the controls were similar (III, Figure 1).

In the seminiferous epithelium of the testis, expression of NHE3 was absent (data not shown).

3.1.2 Efferent ducts of the testis

In the initial zone of the ED, apical membranes of the non-ciliated cells showed immunoreactivity for SLC26A3, CFTR, and NHE3, whereas the ciliated cells remained negative. In the terminal zone of the ED, no specific immunostaining for the transporters was observable.

The ED of the patient with CLD was histologically the proximal type, but showed no staining for SLC26A3 or CFTR. Instead, the expression of NHE3 in the non-ciliated cells was present, similar to that seen in control samples (III, Figure 2).

3.1.3 Epididymis

In the epididymides of the control patients, apical expression of SLC26A3 and CFTR appeared on the luminal side of the apical mitochondria-rich cells (AMRCs) (Palacios et al. 1991). Other cell types remained negative for the proteins studied (III, Figure 2).
3.1.4 Seminal vesicle

Both the higher glandular epithelium and lower unreactive ductal epithelium of the seminal vesicles of the controls showed intense apical immunoreactivity for SLC26A3. In the case of CFTR, no staining appeared in the higher glandular epithelium, but strong labeling was present, together with SLC26A3, on the luminal side of the lower ductal epithelium (III, Figure 2).

3.1.5 Prostate

In the controls’ prostate samples, the apical border of the prostatic duct epithelium showed intense immunoreactivity for CFTR (III, Figure 2), whereas the higher glandular epithelium remained negative (data not shown). Stainings for SLC26A3 and NHE3 remained negative (data not shown).

4 Clinical trial with oral butyrate in CLD

Butyrate slightly reduced the diarrhea in only one of the five patients (IV, Table 1: patient 1). Other patients reported no effect (patient 2) or even an increased diarrhea (patients 3-5). Accordingly, butyrate reduced the mean number of stools only in patient 1 (from 4.2 to 3.4 stools/day). Butyrate had no effect on soiling episodes or watery consistency of stools in any of the patients. It was well tolerated, although three patients (patients 3-5) reported slight abdominal distention and increased bowel movements (unpublished data).

The majority of the laboratory measurements remained unaltered during the butyrate trial. In all patients, butyrate reduced fecal K\(^+\) secretion, but urine and serum concentrations of K\(^+\) showed more individual variation. In butyrate-responsive patient 1, the fecal concentration of Cl\(^-\) showed a substantial 1.4-fold and Na\(^+\) a 1.9-fold decrease at the end of the butyrate trial. In contrast, a total inhibition of urine Cl\(^-\) excretion, a mean 1.7-fold decrease in urine Na\(^+\) excretion, and a tendency toward metabolic alkalosis appeared in the non-responsive patients (n=4) (IV, Figure 1). Although butyrate reduced concentrations of serum urea-N (4.2 to 3.6 mmol/l) and urate (198 to 189 µmol/l) in patient 1, the other patients showed increased mean concentrations of both urea-N (4.4 to 6.1 mmol/l) and urate (255 to 281 µmol/l) (unpublished data).

In butyrate-responsive patient 1, fecal calprotectin and α1-antitrypsin levels showed a 17-fold and 6-fold increase 20 days after stopping butyrate (IV, Figure 1), without signs of intestinal inflammation. Spontaneous normalization of the values was recorded three months later.

None of the patients had CFTR mutations or the 5T allele at the polymorphic locus IVS8-T of CFTR. In addition, sequencing of the coding regions (n=21) and exon-intron boundaries of SLC26A3 (Haila et al. 1998) from the DNA sample of butyrate-responsive patient 1 revealed no other variants than the Finnish founder mutation V317del and the associated homozygous C307W variant 10 codons upstream (Hoglund et al. 1996b).
5 Expression of SLC26A3 in the human kidney

Variable levels of expression of renal SLC26A3 mRNA and protein were confirmed with RT-PCR and western blotting (V, Figure 5). SLC26A3 immunolabeling appeared on the apical membrane of the distinct cells of the connecting tubule (CNT) and cortical collecting duct (CCD) (V, Figure 5). As both these ducts have an apical AQP2 transporter in the principal cells (Nielsen et al. 1993), expression of SLC26A3, occurring in a cell type different from that of AQP2, was localized in the intercalated cells. Doublestaining suggested SLC26A3 expression in the cell type with mainly basal and cytoplasmic staining for V-ATPase as being characteristic of the type B intercalated cells (Kim et al. 1999). The apical signal for SLC26A4 in at least some of the SLC26A3-positive tubules suggested that the cell type was either the type B or nonA-nonB intercalated cell (Kim et al. 1999). In the biopsy sample of the patient with CLD, expression of SLC26A3 was lacking, although staining profiles for other transporters were comparable to those of controls.
Discussion

1 Long-term outcome

The first single cases with congenital chloride diarrhea (CLD) were described back in 1945, but not until 20 years later did understanding of the underlying intestinal defect result in finding a life-saving therapy. Salt substitution with NaCl and KCl is, even today, the only effective therapy (Holmberg et al. 1977a). After this treatment was introduced in Finland, with one of the world’s highest CLD incidences, our country has had the world’s largest series of adolescent and adult patients. This set of patients made identification of the gene for CLD possible (Hoglund et al. 1996b). In this study, we used this unique sample of Finnish patients to provide data on the long-term outcome of CLD.

This study series involved 80% of all Finnish patients aged over 7 years (ages 10-38; median 21). High infant mortality (45%) among patients born in the 1960s, and refusal to participate (23%) among those born in the 1970s were the most substantial reasons for non-participation. The final sample of 36 subjects comprised 40%, 54%, and 95% of the patients born in the 1960s, 1970s, and 1980-1991 (I, Figure 1).

After the introduction of salt substitution in the late 1960s, only two infants have died of probable CLD in Finland. CLD cases are usually recognized during fetal development, based on ultrasonic findings showing polyhydramnios and fluid-filled intestinal loops of a fetus (Kirkinen and Jouppila 1984). To the best of our knowledge, only two Finnish patients have lived with undiagnosed CLD for one year or more. One of these is a boy who received his diagnosis at the age of five—as late as in 1995 (Leskinen 1996). Diagnostic pitfalls—food allergies in that case—are worth noticing, even in high-incidence areas such as Finland. Single cases with novel mutations for CLD appear worldwide, both in developing and more affluent countries (Table 2). In these low-incidence countries, such CLD infrequency makes misdiagnoses highly probable.

Childhood dosing with salt substitution has increased during the last decades, but later in life, therapy seems easily to remain insufficient. Even in our series of patients, minor abnormalities in acid-base and electrolyte balance or in growth were present in a respective 60% and 22% (I, Table 2). Accordingly, the current mean dose taken of Cl⁻, 2.8 mmol/kg/day, fell below the recommended adult dosage of 3 to 4 mmol/kg/day (Holmberg 1986). One-fourth of our patients reported consumption of salty food, reflecting the insufficient dosage with salt substitution. To overcome signs of inadequate treatment—low urinary Cl⁻, low serum K⁺, and metabolic alkalosis—we increased the mean daily dose of Cl⁻ in 24 (67%) patients to at least a minimum of 3.0 mmol/kg/day.

This study is, to our knowledge, the first to show that salt substitution therapy with NaCl and KCl, proved to enable normal childhood growth and development (Holmberg et al. 1977a), allows favorable long-term outcome in CLD. In agreement with this finding, 92% of our patients regarded their general state of health as excellent or good. Although the diarrheal symptoms of CLD were persistent, only minor soiling problems remained in adulthood. The patients had adjusted to their diarrheal symptoms, and only four (11%)
reported occasional use of cholestyramine to temporarily reduce their diarrhea and prevent soiling.

Combination therapy with NaCl and KCl seems to allow normal growth and development in CLD even if the diagnosis is delayed and early therapy inadequate. To support this, therapy with NaCl and KCl normalized early growth retardation in the patients born in the 1960s, despite late-diagnosed CLD or pure KCl therapy in early childhood or both (I, Figure 3). A common feature in those 28% of the patients with chronic kidney disease (CKD) was, however, late-diagnosed CLD, indicating that early contraction affects renal outcome in CLD (V, Table 1). As evidence of the role of early changes, onset of CLD-related renal injury in the Finnish case with ESRD was neonatal. A probable contributor to the progression of renal injury in this patient was inadequate salt substitution. In other patients, however, sufficient salt substitution seems to have offered protection from major renal changes. In contrast, the Swedish case with ESRD exhibited a critical condition with the CLD diagnosis coming only after the second transplantation. Although the chronic tendency to hypovolemia seems to affect renal growth in CLD (V, Figure 2), the unique finding of a solitary kidney in that Swedish case was probably unrelated to CLD.

Our results in a series of patients suggest that the pathophysiological basis of nephrocalcinosis, the most striking renal feature of CLD, includes NaCl and volume depletion. Along the nephron, Ca\(^{2+}\) reabsorption occurs mostly through a passive paracellular route in parallel with sodium reabsorption, Cl\(^-\) movement acting as an additional driving force for reabsorption (Friedman 1998, Stanton and Bruce 1998, Sayer et al. 2004). Unlike in CLD, an almost universal feature in tubulopathies with nephrocalcinosis is hypercalciuria (Sayer et al. 2004). Despite the absence of hypercalciuria in CLD, the diarrhea-related NaCl and volume depletion (Holmberg 1986, Melvin et al. 1999) may play a role in the pathogenesis of nephrocalcinosis. In CLD, NaCl and fluid depletion is always accompanied by reduced GFR and even by hypotension (V, Figure 3). As the primary determinants of saturation are excretion of calcium salts and water (Sayer et al. 2004), in CLD, hypovolemia together with low NaCl delivery to the thick ascending limb of Henle and distal tubule of the kidney could fail to provoke an electrochemical gradient for Ca\(^{2+}\) reabsorption, allowing urine supersaturation and the formation of luminal crystals. This hypothesis could also provide an explanation of the early findings of nephrocalcinosis in KCl-treated CLD, despite the systemic balance (Pasternack and Perheentupa 1966, Pasternack et al. 1967, Holmberg et al. 1977b). To further support the role of hypovolemia, urine volume predicts urinary supersaturation in children with idiopathic urolithiasis (Lande et al. 2005), and increased water load offers protection from crystallization in adult idiopathic stone-formers (Borghi et al. 1999).

Rapidly progressive calcification and renal failure in CLD seem to develop only in the presence of long-lasting hypochloremic and hypokalemic metabolic alkalosis, as seen in our patients’ own kidneys and in their transplants. The different mutations in these two cases with ESRD suggest that renal impairment is independent of genotype. Most probably, complex compensatory actions modulate both the onset and clinical course of the kidney disease. A striking example of these potential compensatory mechanisms is the Finnish boy with his diagnosis of CLD at age five. Despite his late-diagnosed disease, this
currently 11-year-old patient has totally normal renal function. Although some effects on renal function may emerge with age, along with episodes of metabolic imbalance, some unidentified mechanisms are likely to modulate the individual susceptibility to CLD-related renal impairment.

In addition to diarrhea and renal involvement, other CLD-related manifestations arose in this study (Figure 8). CLD seems to be associated with an age-dependent increasing risk for hyperuricemia. As in the case of renal involvement, the probable pathogenesis of hyperuricemia involves the tendency toward chronic dehydration (Choi et al. 2005). However, only our patient with a renal transplant (V, case 1) had developed gouty arthritis.

As one patient had Crohn’s disease and another had a diagnosis of unspecified colitis, CLD may be associated with an increased risk for intestinal inflammation. Whether the elevated erythrocyte sedimentation rates in ten of our patients (28%) were related to intestinal inflammation, or even to decline in renal function, remains unclear. As downregulation of SLC26A3 emerges in the inflamed colonic mucosa (Yang et al. 1998, Lohi et al. 2002b), a link between intestinal inflammation and the primary genetic defect of CLD is possible. Despite the slightly increased risk for gastrointestinal malignancies in one study (Hemminki et al. 1998), in this series of patients, no cancers arose.

Acute gastroenteritis (AGE) may lead to life-threatening complications in CLD due to the associated susceptibility to rapid dehydration, hypokalemia, and alkalosis. Although this tendency persisted into adulthood, the severe episodes became less frequent. To prevent AGE-related dehydration, most adult patients had learned to take an extra dose of salt substitution.

Similarly, adding salt substitution during excessive sweating may be important, as reported by some of our patients (unpublished data). The increased concentrations of sweat Cl in 12% of the patients suggest a minor role for SLC26A3 in the sweat gland. As the major protein mediating thermoregulation and salt retention through the sweat glands is CFTR (Sato and Sato 2000), the sweat gland may be an additional site for the interaction of SLC26A3 and CFTR (Wheat et al. 2000, Jacob et al. 2002, Ko et al. 2004, Rossmann et al. 2005).

Of the minor medical conditions in our patients, allergic diseases including hay fever, asthma, and atopic eczema were common but showed a prevalence comparable with that of the general Finnish population (Huurre et al. 2004). Dental and oral health was good, in concordance with a report of caries-protecting influence of oral salt substitution (Mylarniemi and Holmerg 1975).

Of 16 males, four had been operated on for inguinal hernia, three of them in infancy. That is ten times as high as the average prevalence of hernias (Maisonet 2003) and suggests a role for elevated intra-abdominal pressure in the pathogenesis. Additionally, spermatoceles were more common than in the general population (Gutman et al. 1986). Later, this study showed that formation of spermatoceles in CLD is associated with male subfertility. Although 38% of the adult males reported infertility in the first part of the study, no abnormalities in female reproductive capacity in CLD emerged.
2 SLC26A3 and male subfertility

The molecular basis of male infertility is heterogeneous and poorly understood. Here, we demonstrate that CLD is associated with a unique phenotype showing accumulation of water into the male reproductive tract, formation of spermatoceles, and subfertility. Despite the normal spermatogenesis in CLD, male subfertility involves a low concentration of poorly motile sperm with abnormal morphology. A striking finding of high seminal plasma Cl with a low pH supports a role for a primary Cl/HCO3 exchange defect in the male reproductive tract. In agreement with this hypothesis, we detected SLC26A3 immunolocalization at multiple sites of the male reproductive tract: elongating spermatids, non-ciliated cells of ED, AMRCs of the ductus epididymis, and as described (Haila et al. 2000), of the epithelium of the seminal vesicle.

NHE3, the main SLC26A3-interacting protein of the intestine, is crucial for normal mouse male fertility (Zhou et al. 2001). As both SLC26A3 and NHE3 were localized on the luminal side of the non-ciliated cells of the human ED, their cooperation in reabsorbing NaCl at this site is putative. In particular, the accumulation of fluid into the male reproductive tract in both males with CLD and in NHE3 knock-out mice (Zhou et al. 2001) suggests that not only the action of NHE3 but also that of SLC26A3 is essential for the fluid reabsorption in the ED (Clulow et al. 1998, Hansen et al. 2004). A lack of function of SLC26A3 may, similarly to that seen in the CLD intestine (Holmberg et al. 1975, Melvin et al. 1999, Lamprecht et al. 2002), lead to secondary inhibition of the Na+/H+ exchange through NHE3. Thus, defective NHE3 function may further enhance the accumulation of fluid into the ED, the luminal dilation, and the dilution of the epididymal sperm in CLD (Zhou et al. 2001).
The unique finding of large bilateral spermatoceles in CLD suggests that defective SLC26A3-mediated anion transport may be an important contributor to the formation of spermatoceles. The pathophysiology of spermatoceles, including aging and inflammatory changes, remains mostly undetermined (Rubenstein et al. 2004). As spermatoceles are, however, cystic dilations of the ED region, defective fluid reabsorption and increased intraluminal pressure at this site may play a role in the pathogenesis.

Concerning human male infertility, the gene of interest is CFTR. Its variants are associated with various infertility-related phenotypes from CF-associated CBAVD to idiopathic oligozoospermia even in carrier males (Chillon et al. 1995, van der Ven et al. 1996, Meschede et al. 1997, Schulte et al. 2006). The CFTR channel has distinct abilities involving Cl− channel activity and HCO₃− secretion, the latter probably being mediated by the anion transporters of the SLC26 family (Ko et al. 2002, Ko et al. 2004). Interestingly, the ability to activate HCO₃− transport is even more sensitive to CFTR mutations (Choi et al. 2001b). In the milder forms of CF with abnormal HCO₃− secretion but normal Cl− channel activity, the only manifestation can be male infertility (Meschede et al. 1997). This suggests that rather than Cl−, the crucial ion for normal male fertility is HCO₃−.

Both SLC26A3 and CFTR were detected in the non-ciliated cells of the ED, the AMRCs of the epididymis, and the ductal epithelium of the seminal vesicle. This suggests that SLC26A3 may act together with CFTR as a ductal HCO₃− secretor in the male reproductive tract, similarly to that function proposed for the pancreatic duct and small intestine (Greeley et al. 2001, Ko et al. 2002, Ko et al. 2004). Although data on the transporter actions in different cell types of the male reproductive tract are lacking, HCO₃− directly affects sperm motility, and after ejaculation, sperm capacitation (Okamura et al. 1985, Wang et al. 2003). Therefore, some features in CFTR-associated male infertility may actually implicate the loss or reduced activity of epithelial HCO₃− secretion through SLC26A3.

In men with classical CF, the primary cause of their low seminal plasma pH (Kaplan et al. 1968) is the absence of or anomalies of the seminal vesicles, which normally secrete HCO₃−-rich fluids to allow the viability and motility of spermatozoa after ejaculation (Anguiano et al. 1992). Although low seminal plasma pH is a rare finding in conditions other than CF, decreased levels of HCO₃− may be associated with male infertility (Okamura et al. 1985). Our results, showing a low pH for seminal plasma in CLD, suggest accordingly that decline in pH might be the most important contributor to the poor sperm motility in CLD.

To make the puzzle more complex, even the normal seminal plasma pH values in our series were associated with equally poor motility of sperm. This suggests that factors other than pH only, for instance high seminal plasma Cl− and dilution of epididymal sperm, disrupt the sperm motility in CLD, as well. Most probably, diverse actions of epithelial transporters—such as those of SLC26A3, NHE3, and CFTR—induce complex regulatory networks and produce distinct micro-environments in the male reproductive tract. These local circumstances may further regulate the net reabsorption and secretion, and affect sperm maturation and motility.

In the ED of the single patient with CLD, SLC26A3 and CFTR immunoeexpression was absent, despite the expression of NHE3. The testicular tissues from two patients with
CLD showed, however, expression profiles identical to those of controls for both SLC26A3 and CFTR. In agreement with this finding, the V317del-mutated SLC26A3 shows some apical and strong cytoplasmic signals in the intestinal epithelium for both the SLC26A3 mRNA and protein (Hoglund et al. 1996b, Haila et al. 2000). Therefore, the lack of SLC26A3 and CFTR expression in the ED of our CLD patient may be secondary to his luminal dilation and spermatocyte formation, rather than being a primary finding. As no CLD samples were available for expression studies in other tissues, such as ductus epididymis and seminal vesicle, any final assessment of CLD-related expression profiles remained impossible.

The role of the expression of SLC26A3 and CFTR observed in the elongating spermatids remains unclear. In CF, inconsistent histological findings (hypospermatogenesis, increased number of dysmorphic spermatozoa) of spermatogenesis are probably secondary to CBAVD (Denning et al. 1968, Kaplan et al. 1968, Mak et al. 2000). Limited data support, however, the presence of CFTR in both the human and rat testis (Tizzano et al. 1994, Larriba et al. 1998, Gong et al. 2001). Although SLC26A3—even together with CFTR—may play a primary role in developing spermatozoa, histologically normal spermatogenesis in CLD allows assisted reproductive technologies to treat infertility. Limited clinical experience in CLD males shows, however, that fertilizations in vitro might be highly challenging (unpublished data).

An interesting prospect is whether some heterozygous SLC26A3 variants might, similarly to that seen for CFTR mutations (van der Ven et al. 1996, Schulz et al. 2006), account for male subfertility without causing the intestinal phenotype of CLD. This seems to be improbable in the case of the Finnish founder mutation V317del, however, as no evidence of any fertility-related problems has arisen among the fathers of our patients. Despite this fact, our initial results suggest that some rare variants of the SLC26A3 gene may be more common in men with idiopathic infertility than in their normozoospermic or fertile controls (unpublished data).

Finally, the unpublished observations of reduced fertility in SLC26A3-deficient mice (Schweinfest et al. 2006) establish the crucial role of SLC26A3 in both human and mouse male fertility (personal communication). That CFTR knock-out mice exhibit normal reproductive function (Snouwaert et al. 1992), but knock-out mice for SLC26A3 or NHE3 are subfertile or infertile (Zhou et al. 2001, Schweinfest et al. 2006), supports a crucial role for the functional SLC26A3 in the male reproductive tract.

### 3 Variable effects of oral butyrate in CLD

Because the SLC26A3 gene responsible for CLD encodes for the major apical anion transporter of the colon (Moseley et al. 1999, Schweinfest et al. 2006), options to resolve the diarrheal symptoms of CLD have been limited. An Italian case report showed, however, that administration of oral butyrate normalized the stool pattern in an 11-year-old male patient with CLD (Canani et al. 2004). That study prompted us to study the influence of butyrate on diarrhea among Finnish patients with CLD.
The short-chain fatty acid butyrate is the most essential end-product of bacterial carbohydrate fermentation in the colon and is the principal nutrient for colonocytes. It maintains the normal cell population and inhibits tumorigenesis through multiple mechanisms (Topping and Clifton 2001). Regarding CLD, the most important function of luminal butyrate is its potential to modulate colonic epithelial ion transport. The net effect of increased electroneutral absorption of NaCl and fluid occurs mostly through activation of the Na\(^+-\)H\(^+\) exchangers NHE2 and NHE3, whereas the direct effect of butyrate on the apical membrane Cl\(^-\)/HCO\(_3\)^- exchange is actually inhibitory (Yun et al. 1993, Vidyasagar et al. 2005).

That only one patient in our pilot study showed a modest response to oral butyrate was surprising. Even more strikingly, the distinct pattern of laboratory measurements in this butyrate-responsive patient consistently differed from that of the other patients—despite the comparable baseline concentrations of electrolytes (IV, Figure 1). Actually, the clinically non-responsive patients (n=4) demonstrated signs of salt depletion in CLD, including decreased renal excretion of NaCl and a tendency to alkalosis. These changes supported, despite the slightly inconsistent fecal measurements, butyrate-induced loss of intestinal NaCl and fluid.

This study shows that responses to oral butyrate in CLD are variable, ranging from beneficial to slightly harmful. Adding our data to the Italian butyrate-responsive case with two different SLC26A3 mutations, Q495H and A547E (Canani et al. 2004, Cardillo 2005), suggests for the first time that genotype-phenotype differences may affect responses to diarrhea-reducing therapies in CLD. In the case of butyrate, diverse responses seem to arise even within the same genotype for CLD, as seen in our patients with the homozygous V317del mutation. Lack of an oral slow-release form of butyrate is likely to allow partial absorption even in the small intestine, potentially resulting in variable colonic concentrations of butyrate, and in part explaining the diversity of responses (Topping and Clifton 2001).

In the case of CFTR mutations, the ability of butyrate to partially restore the function of the mutated CFTR channel depends on the genotype (Kerem 2006). Although CFTR mutations and the 5T allele were excluded from this study, the modulatory action of CFTR may contribute to the dramatic butyrate response in the one Italian case. It is possible that the A547E-mutated STAS domain in that patient fails to interact with the R domain of CFTR (Lamprecht and Seidler 2006). This could result in reduced baseline activity of CFTR (Ko et al. 2004), explaining the atypically severe diarrhea in that patient. Accordingly, the improved action of CFTR caused by butyrate may account for the dramatic butyrate response in that single case. As the sequencing of the whole coding region of SLC26A3 in our butyrate-responsive patient revealed an intact STAS domain, however, other genes are likely to modulate the actions of butyrate.

This study uncovered important safety issues. The slight butyrate-induced hyperuricemia in our four butyrate-nonresponsive patients (unpublished data) was probably related to volume depletion (Choi et al. 2005), but butyrate-induced hyperuricemia (Gore et al. 2001) and the increased risk for hyperuricemia in CLD are worth noticing. More important, the increased fecal concentrations of calprotectin and α1-antitrypsin after butyrate deprivation in the butyrate-responsive patient suggest that the
dosage of butyrate should be gradually tapered before being halted. As downregulation of SLC26A3 mRNA and upregulation of CFTR are characteristic of the inflamed colonic mucosa (Yang et al. 1998, Lohi et al. 2002b), and the increased risk for intestinal inflammation appears both in CLD and CF (Bruzese et al. 2004), the CLD colon may be especially vulnerable to modulatory actions of butyrate, and to butyrate deprivation. Finally, the slightly increased risk of colon carcinoma in CLD (Hemminki et al. 1998) makes monitoring of intestinal inflammation in any future trials with potential diarrhea-reducing agents of CLD even more important.

4 SLC26A3 in the human kidney

Several of the SLC26 family members mediate renal anion exchange (Table 1). Of them, SLC26A1 is located in the basolateral and SLC26A6 on the apical epithelium of the proximal tubule of the kidney. At least SLC26A6 possesses essential mechanisms for anion transport in the proximal tubule, as its defective function causes mouse urolithiasis (Jiang et al. 2006, Sindic et al. 2007). In the intercalated cells of the cortical and outer medullary collecting duct, respectively, are located apical SLC26A4 and basolateral SLC26A7. These probably contribute to the regulation of both acid-base homeostasis and blood pressure. Of the other SLC26 family members, SLC26A2, SLC26A9, and SLC26A11 mRNA are expressed in the kidney (Table 1).

Adding the data on variable levels of SLC26A3 mRNA and protein expression in the kidney, our results suggest a minor role for SLC26A3 in renal physiology. Unlike this study, many previous studies have failed to show SLC26A3 expression in the kidney, either in humans or in mice (Hoglund et al. 1996b, Haila et al. 2000, Wang et al. 2005). Similarly, microarray data on SLC26A3 expression are inconsistent, with only a few human kidney samples showing expression of SLC26A3 RNA (http://www.ncbi.nlm.nih.gov/geo) (Edgar et al. 2002).

One possibility for the discrepancy observed in renal SLC26A3 expression is its highly limited expression on the apical membrane of the distinct intercalated cells of the CNT and CCD. Another possibility is that compensatory up- or downregulation of the expression occurs in response to metabolic imbalance, as shown for mouse SLC26A4 and SLC26A7. Systemic Cl⁻ depletion upregulates renal SLC26A4 in the type B intercalated cells of mice (Quentin et al. 2004, Vallet et al. 2006), whereas hypokalemic or hypertonic medium in cell cultures drives SLC26A7 from endosomes to the basolateral membrane of the type A intercalated cells (Xu et al. 2006), promoting anion exchange. If similar upregulation of SLC26A3 occurred in the presence of some common metabolic imbalance, it could in many respects explain the inconsistent data on renal SLC26A3 expression. To support this hypothesis, our results exemplified highly variable levels of both renal SLC26A3 mRNA and protein. Although the one paraffin-embedded CLD-kidney sample showed no staining for SLC26A3 in immunohistochemistry, the lack of other CLD samples made overall assessment of this finding difficult.

An attractive and likely candidate for the regulation of renal SLC26A3 expression and function may be the Cl⁻/HCO₃⁻ exchanger SLC26A4. This protein shares the closest
structural similarity (60%) with SLC26A3 (Mount and Romero 2004), and has a similar site of expression in the kidney CNT and CCD (Royaux et al. 2001), where the final regulation of acid-base and NaCl balance occurs. Although SLC26A4 is the major Cl⁻/HCO₃⁻ exchanger in both the type B and nonA-nonB intercalated cells (Schuster and Stokes 1987, Soleimani et al. 2001), renal manifestations or metabolic alkalosis are neither features of SLC26A4 deficiency in human disease pendred syndrome (Everett et al. 1997), nor are they characteristic of SLC26A4-deficient mice (Royaux et al. 2001). Similarly, the loss of functional SLC26A3 in CLD is not associated with renal Cl⁻ wasting. These disease-associated features and the similar expression patterns of SLC26A3 and SLC26A4 raise the question as to whether a lack of the function of one transporter could be compensated for by the corresponding function of the other. To support this hypothesis, treated CLD was associated with normal electrolyte and acid-base balance, but strikingly, with alkaline urine in 37% of the patients. In CLD, upregulation of renal Cl⁻/HCO₃⁻ exchange, possibly that of SLC26A4, is therefore likely to occur. As upregulation of renal SLC26A4 in mice occurs merely in response to Cl⁻ depletion (Quentin et al. 2004, Vallet et al. 2006), the potential contributor in CLD may be the intestinal loss of Cl⁻. Thus, in the presence of minor Cl⁻ depletion in CLD, compensatory activation of SLC26A4 may inhibit the development of hypochloremia and metabolic alkalosis.

To further support our hypothesis, corresponding reciprocal upregulation of SLC26A3 occurs in the pancreatic duct of SLC26A6-knockout mice, enabling normal HCO₃⁻ and fluid secretion (Ishiguro et al. 2007). In the presence of the major anion exchanger SLC26A6, expression of SLC26A3 is low if not absent, however (Hoglund et al. 1996b, Ishiguro et al. 2007). In agreement with this finding, in CLD, pancreatic manifestations are non-existent.

Our study shows that CLD-related renal injury affects both the patients’ own kidneys and the patients’ transplants. Therefore, renal SLC26A3 may play only a minor role in homeostasis. More studies are necessary to assess the complex renal process and regulation of the Cl⁻/HCO₃⁻ exchange, the entity to which several SLC26 transporters contribute.
Conclusions and future prospects

This study reveals that salt substitution therapy with NaCl and KCl will allow a favorable long-term outcome in CLD. In untreated or poorly treated cases, the most severe complication is renal involvement, which can ultimately lead to ESRD and renal transplantation. The main contributors to the renal injury, and to small size kidneys, are chronic hypovolemia and metabolic imbalance. Although this study, to our knowledge, demonstrates for the first time expression of SLC26A3 in the human kidney, the post-transplant recurrence of renal injury in CLD demonstrates the role of metabolic changes, rather than direct transporter modulation, in the pathogenesis of renal involvement.

Despite the salt substitution therapy, diarrhea in CLD is persistent. Based on our pilot study, modulation of the intestinal ion transport by oral butyrate is unlikely to replace the lost Cl⁻/HCO₃⁻ exchange activity characteristic of CLD. Our results propose, however, that responses to diarrhea-reducing therapies in CLD vary, even within the same genotype. In the series of Finnish patients with CLD, a slight response to oral butyrate in only one patient supports no further trials. The dramatic response in the single Italian case indicates, however, that butyrate action may be beneficial in some cases with CLD. Therefore, only studies among patients with several different genotypes for CLD can reveal whether butyrate has, similar to that seen in CF, efficacy for patients with certain genotypes only.

The most interesting novel finding of this study is CLD-associated male subfertility. Based on our data, the underlying cause may be the defective SLC26A3-mediated Cl⁻/HCO₃⁻ exchange in the male reproductive tract. In the ED of the testis, disruption of SLC26A3, probably resulting in inhibition of NHE3 and modulation of CFTR, may lead to poor reabsorption of water and NaCl, aberrant secretion of HCO₃⁻, and further to the tendency to form spermatoceles. Acidic, hypo-osmotic, Cl⁻-rich seminal plasma is likely to further inhibit normal sperm maturation and motility. Disruption of SLC26A3 in the epididymis and seminal vesicle, perhaps together with CFTR, may similarly affect sperm maturation and motility.

Normal testicular histology provides possibilities for assisted reproductive technologies to treat male subfertility in CLD. Clinical experience suggests, however, that difficulties may arise even in the case of in vitro fertilization. Fortunately, some Finnish men with CLD have succeeded in producing a child, even without assisted reproduction. Further studies should elucidate the role of SLC26A3 during spermatogenesis, and the success of infertility treatments in CLD. In addition, a probable association of rare SLC26A3 variants with the complex trait of idiopathic male infertility needs to be clarified.

It is worth remembering that our results stem from a highly homogenous population of Finnish patients with an identical genetic background for CLD. Therefore, future work is necessary to assess phenotypical differences, if any, in CLD. At least in the knock-out mouse model, profuse diarrhea and male subfertility seem to emerge similarly to that seen in the human disease. It is therefore likely that patients with CLD share, independent of genotype, the main clinical features that we describe in this study.
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